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Original Article

Prognostic significance of BZW2 expression in lung adenocarcinoma patients

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Abstract: Lung adenocarcinoma (LUAD) is the most common pathologic subgroup of lung cancer. The role of basic leucine zipper and W2 domains 2 (BZW2) in tumorigenesis has been investigated, while the functions and molecular mechanisms of BZW2 in LUAD remain undetermined. Our study aimed to investigate the effect of BZW2 in LUAD tumorigenesis and prognostic prediction. LUAD patients who underwent complete resection with tumor available for histologic evaluation were collected, and immunohistochemistry (IHC) was performed and scored for intensity of BZW2 expression. Overall survival (OS) and disease free survival (DFS) were estimated and compared between groups. Hazard ratios (HRs) for death were estimated using univariable and multivariable Cox proportional hazards models. BZW2 was considerably raised in the archival tissue samples from LUAD patients relative to those in healthy controls. High BZW2 expression was associated with unfavorable OS and DFS in LUAD patients. Coincidently, the up-regulated BZW2 was related to tumorigenesis, including tumor size, stage, and lymphatic invasion. In addition, we also found a positive correlation between *BZW2* and *EIF5* expression. BZW2 may be a clinical molecular biomarker for the prognosis of LUAD patients.

Keywords: BZW2, lung adenocarcinoma, immunohistochemistry, biomarker

Introduction

Lung cancer is a globally leading cause of cancer-associated mortality, accounting for millions of cancer deaths in China [1]. In clinical practice, adenocarcinoma is the most frequent histologic subtype [2]. Despite the current standard of care and emerging targeted therapies for LUAD, 5-year overall survival rates still vary from 4-17%, and most LUAD patients are diagnosed at advanced stages with poor prognosis [3]. Identification of potential molecular biomarkers with prognostic and clinicopathological significance is an urgent need for the early diagnosis of LUAD. Up to now, microarray, RNA-seq, and other technologies led to the detection of clinicopathologic markers that drive LUAD genesis [4-6]. Nonetheless, these markers are not adequately specific to enhance the diagnostic accuracy of LUAD, so there is a notable demand for the discovery of novel molecular markers for the prediction of cancer-associated metastasis and recurrence [7].

BZW2 is a member of the basic-region leucine zipper (bZIP) superfamily of transcription factors [8]. There are few studies reported on the effect of bZIP superfamily in cancer [9]. A previous study revealed that BZW2 is up-regulated in hepatocellular carcinoma and its overexpression induces hepatocellular carcinoma cells proliferation and drug resistance by inactivating the PI3K/AKT/mTOR signaling pathway, suggesting BZW2 plays vital roles in the regulation of hepatocellular carcinoma [10], while knockdown of *BZW2* gene in bladder cancer cell consistently resulted in the suppression of cell growth, cell arrest at G1-phase, and reduction in viable cell numbers [11, 12]. However, the role of BZW2 in LUAD is uncertain.

In this study, we investigated the BZW2 expression by immunohistochemistry in primary LUAD patients, and evaluated its correlation with clinical and pathological features, especially with overall survival (OS) and disease free survival

(DFS). Our data showed that BZW2 is a potential prognostic predictor for LUAD.

Materials and methods

Microarray analysis

Public microarray datasets were used to evaluate our hypothesis and results. Survival analysis of large cohort microarray dataset was performed in The Cancer Genome Atlas (TCGA) cohort and Gene Expression Omnibus (GEO) dataset. The correlation between the expression of *BZW2* and *EIF5* in LUAD was investigated by GSE31210 database [13].

Patients and clinical features

Primary LUAD tissue samples were from 48 patients who underwent radical surgery at the Xiangshui People's Hospital (Xiangshui, China) from February 2015 to November 2018. None of the LUAD patients received any forms of treatments (immunotherapy, radiation therapy, chemotherapy or other cancer-associated treatments) before radical surgery. The demographic and clinical information, including gender, age, tumor size, smoking history, degree of differentiation, and lymph node metastasis, were provided from pathology and clinical records.

Immunohistochemical analysis and pathologic evaluation

Immunohistochemistry of BZW2 was performed on 3 μm-thick sections of the LUAD tumor, using anti-BZW2 antibody (Bioss, Beijing). In brief, the primary antibody was diluted at 1:200 and incubated with the slices at 4°C overnight. The secondary antibody was applied for 30 min, followed by incubation with the avidin-biotin and streptavidin complex. The images were visualized with microscope (Olympus, Tokyo).

Two pathologists who were blinded to clinical outcome independently performed the IHC scoring. BZW2 expression was initially evaluated according to the percentage and intensity of BZW2-positive cell. In brief, immunostaining intensity of BZW2 was categorized as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. Immunostaining percentage of BZW2 was also scored as 4 levels: 0, 0-25%; 1+, 26-50%; 2+, 51-75%; 3+, 75-100%. The components of the percentage and intensity scores were employed as the ultimate IHC scoring. Low BZW2 expression was defined as a score of 0 and + and high expression as a score of ++ and +++.

Statistical analysis

Various statistical analyses were performed to assess the role of BZW2 expression on clinical features and outcome in LUAD patients. Two-tailed Student's t-test and one-way analysis of variance were adopted to compare two or multiple experimental groups. Chi-square test was adopted to compare clinical and pathological features between the BZW2 high and BZW2 low groups. Survival curves were made according to the Kaplan-Meier method, and the log-rank test was utilized to analyze significance between these curves. The role of BZW2 on survival was analyzed by univariate and multivariate Cox proportional hazard models. For all analyses, the SPSS v23 and GraphPad Prism 6 were employed, and significance was defined as $^{*}P \leq 0.05$.

Results

BZW2 expression in the TCGA database

To evaluate the possibility that BZW2 is important for LUAD, we examined *BZW2* expression in corresponding noncancerous tissues and LUAD using the Gene Expression Profiling Interactive Analysis (GEPIA) website based on a TCGA database [14]. Notably, *BZW2* expression in LUAD samples was significantly higher compared to normal lung samples ($P<0.05$, **Figure 1A**). In detail, we further found that *BZW2* expression in stage III & IV, the worst sub-group in LUAD patients, was dramatically higher than expression in the other seven groups ($P<0.05$, **Figure 1B**). In addition, LUAD patients bearing high *BZW2* expression suffered poor clinical outcomes relative to low *BZW2* expressing patients in the TCGA cohort. As shown in **Figure 1C** and **1D**, elevated *BZW2* expression is linked to a significantly shorter response duration of OS and DFS respectively. Thus we may hypothesize that *BZW2* acts as an oncogene in LUAD as well and apply IHC for further investigation.

Association between BZW2 expression and clinicopathologic characteristics

We tried to characterize BZW2 protein expression in normal samples and in LUAD samples

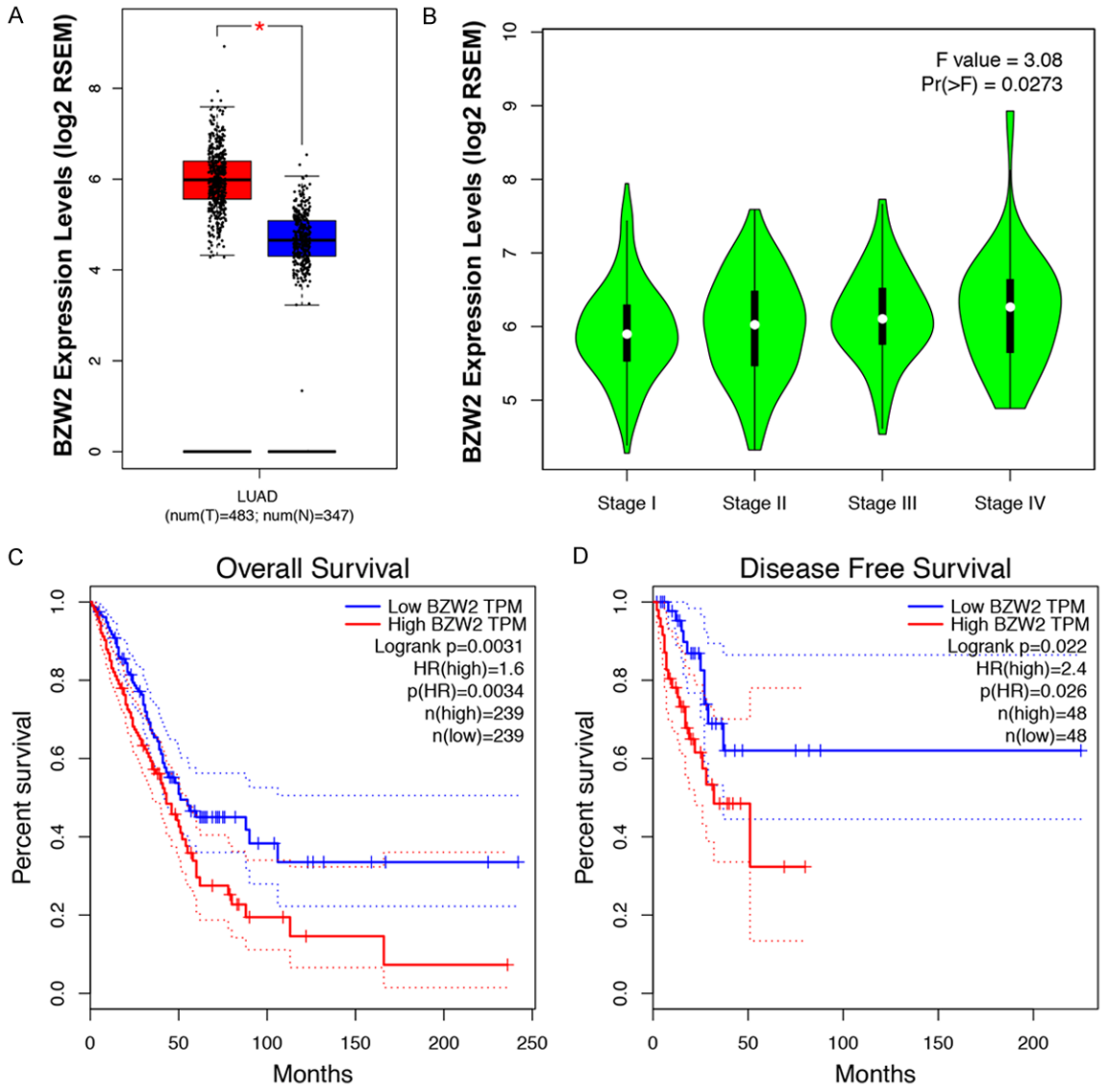

Figure 1. Expression level of *BZW2* in LUAD. A. *BZW2* messenger RNA expression was higher in LUAD tissues than in normal lung tissues. B. *BZW2* expression in different LUAD TMN stage cells. C, D. Kaplan-Meier analysis on the overall survival and disease-free survival of LUAD patients in TCGA database based on the *BZW2* expression.

by analyzing immunohistochemical staining images from HPA database [15] and found that normal tissues had no BZW2 staining. Conversely, LUAD had moderate-to-strong BZW2 staining (**Figure 2A**). To further assess the BZW2 expression in LUAD patients, we performed the staining in 48 cases of LUAD patients and divided these patients into different groups. Twenty-four patients (50%) were classified into the high expression group, depending on the immunostaining intensity (**Figure 2B**). BZW2 expression levels relative to clinicopathologic characteristics were then investigated (**Table 1**). More high BZW expression tumors were stage III (66% *vs.* 29%), and high BZW2 expression tumors were larger (58% *vs.* 25%). We then explored other histopathologic markers related to tumor invasion, aggressiveness, and metastases. Lymphatic invasion was more common in the high BZW2 expression group (66% *vs.* 25%); moderate and high histologic architectural tumor grade was more common in the high BZW2 expression group (62% *vs.* 33%).

Higher BZW2 expression predicts poor prognosis in LUAD

To assess the biological outcomes of elevated BZW2 in MM, we first compered the survival of two groups. In the high BZW2 expression group,

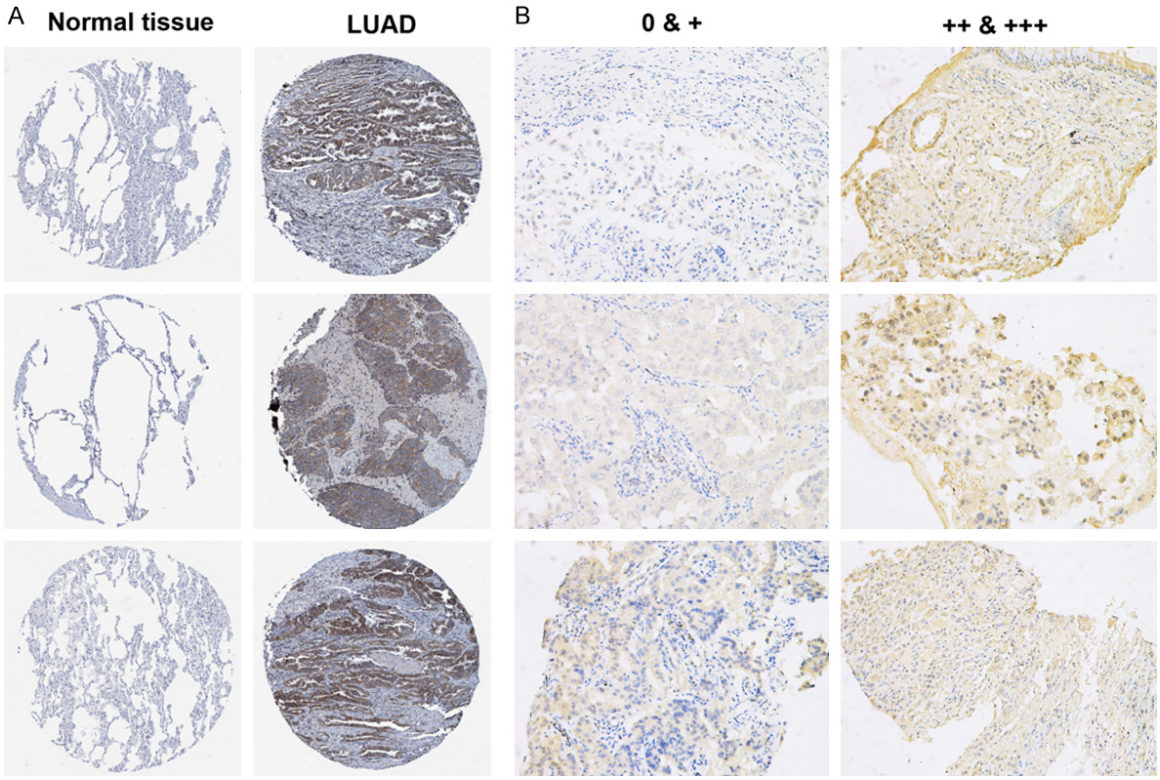

Figure 2. Representative types of BZW2 protein expression in tissue samples. A. BZW2 protein expression was significantly higher in LUAD tissues (right row) in comparison with normal respiratory epithelial tissues (left row). Images were downloaded from the Human Protein Atlas (HPA). B. BZW2 expression in LUAD patients. Representative case with a lack of BZW2 expression (left row); Representative case with stable BZW2 expression (right row).

Table 1. Relation of the characteristics in 48 LUAD patients

Characteristic	No. of Patient/ Total No. (%)	0 & + (n=24)	++ & +++ (n=24)	*p* value
Age (≥65 yr)	28/48 (58)	16/24 (66)	12/24 (50)	0.241†
Male sex	33/48 (68)	14/24 (50)	19/24 (79)	0.119†
Smoking history	19/48 (39)	9/24 (37)	10/24 (41)	0.767†
Degree of differentiation (moderate or high)	23/48 (47)	8/24 (33)	15/24 (62)	0.043†
Tumor size (≥5 cm)	20/48 (41)	6/24 (25)	14/24 (58)	0.019†
Lymph node metastasis (positive)	22/48 (45)	6/24 (25)	16/24 (66)	0.003†
Tumor stage (III)	23/48 (47)	7/24 (29)	16/24 (66)	0.009†

†The chi-square test was used.

17 (70%) patients died from any cause, with 7 (30%) alive at the end of this study. Comparatively, in the low BZW2 expression group, 4 patients (16%) died from any cause, with 20 (84%) alive at the end of the study. As shown in **Figure 3A**, LUAD patients with high BZW2 expression had an inferior OS ($P<0.001$). Furthermore, **Table 2** shows the impact of BZW2 expression and clinicopathologic characteristics on outcomes. Univariate analysis showed that BZW2 (HR=2.230, 95% CI: 1.395-3.566, $P=0.001$), tumor size (HR=2.441, 95% CI: 1.022-5.831, $P=0.045$), and smoking history (HR=3.135, 95% CI: 1.271-7.736, $P=0.013$) were significant predictors related to OS in LUAD. Furthermore, in the multivariate Cox analysis, BZW2 expression (HR=3.050, 95% CI: 1.219-7.633, $P=0.017$) was still an independent prognostic indicator of OS in LUAD.

In the high BZW2 expression group, 21 (87%) patients died from any cause or distant recur-

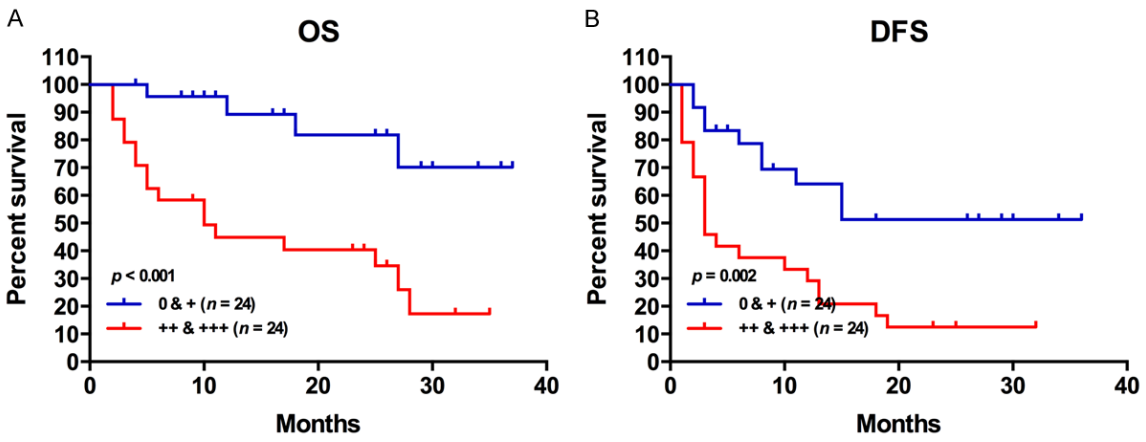

Figure 3. High BZW2 expression is associated with reduced overall survival and disease-free survival.

Table 2. Univariate and multivariate Cox regression analyses for OS in 48 LUAD patients

Variable	Univariate model			Multivariate model		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ≥65 yr	0.688	0.291-1.625	0.394			
Male sex	1.895	0.631-5.688	0.254			
Smoking history	3.135	1.271-7.736	0.013	1.923	0.783-4.769	0.153
Tumor size	2.441	1.022-5.831	0.045	2.264	0.943-5.439	0.068
Lymph node	2.528	0.813-7.863	0.109			
Tumor stage	1.780	0.651-4.866	0.261			
Differentiation	1.689	0.674-4.327	0.264			
BZW2 ++ & +++	2.230	1.395-3.566	0.001	3.050	1.219-7.633	0.017

Table 3. Univariate and multivariate Cox regression analyses for DFS in 48 LUAD patients

Variable	Univariate model			Multivariate model		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ≥65 yr	0.799	0.384-1.661	0.548			
Male sex	1.159	0.528-2.542	0.714			
Smoking history	2.331	1.115-4.876	0.025	2.556	1.220-5.357	0.013
Tumor size	2.011	0.965-4.190	0.062			
Lymph node	2.359	0.966-5.756	0.059			
Tumor stage	2.210	0.978-4.990	0.056			
Differentiation	1.246	0.600-2.587	0.556			
BZW2 ++ & +++	1.685	1.208-2.350	0.002	1.659	1.194-2.305	0.003

rence, with 3 (13%) alive at the end of this study. Comparatively, in the low BZW2 expression group, 10 patients (41%) had recurrence or died of disease, with 14 (59%) alive without disease at the end of our study. As shown in **Figure 3B**, LUAD patients with high BZW2 expression had an inferior DFS (*P*=0.002). Furthermore, **Table 3** also shows the impact of BZW2 expression and clinicopathologic characteristics on outcome. Univariate analysis showed that BZW2 (HR=1.685, 95% CI: 1.208-2.350, *P*=0.002) and smoking history (HR=2.331, 95% CI: 1.115-4.876, *P*=0.025) were significant predictors related to DFS in LUAD. In the multivariate Cox analysis, BZW2 expression (HR=1.659, 95% CI: 1.194-2.305, *P*=0.003) was still an independent prognostic indicator of DFS in LUAD.

BZW2 positively correlates with the EIF5 in LUAD

BZW2, also known as EIF5-mimic protein, is a paralogous human protein containing a C-terminal HEAT domain that resemble the HEAT domain of EIF5 [16]. Using STRING tools, the protein-protein interaction analysis also showed that BZW2 and EIF5 interact or co-express in the human Homo sapiens protein interaction network (**Figure 4A**). Given that BZW2 is involved in EIF5 mediated-LUAD

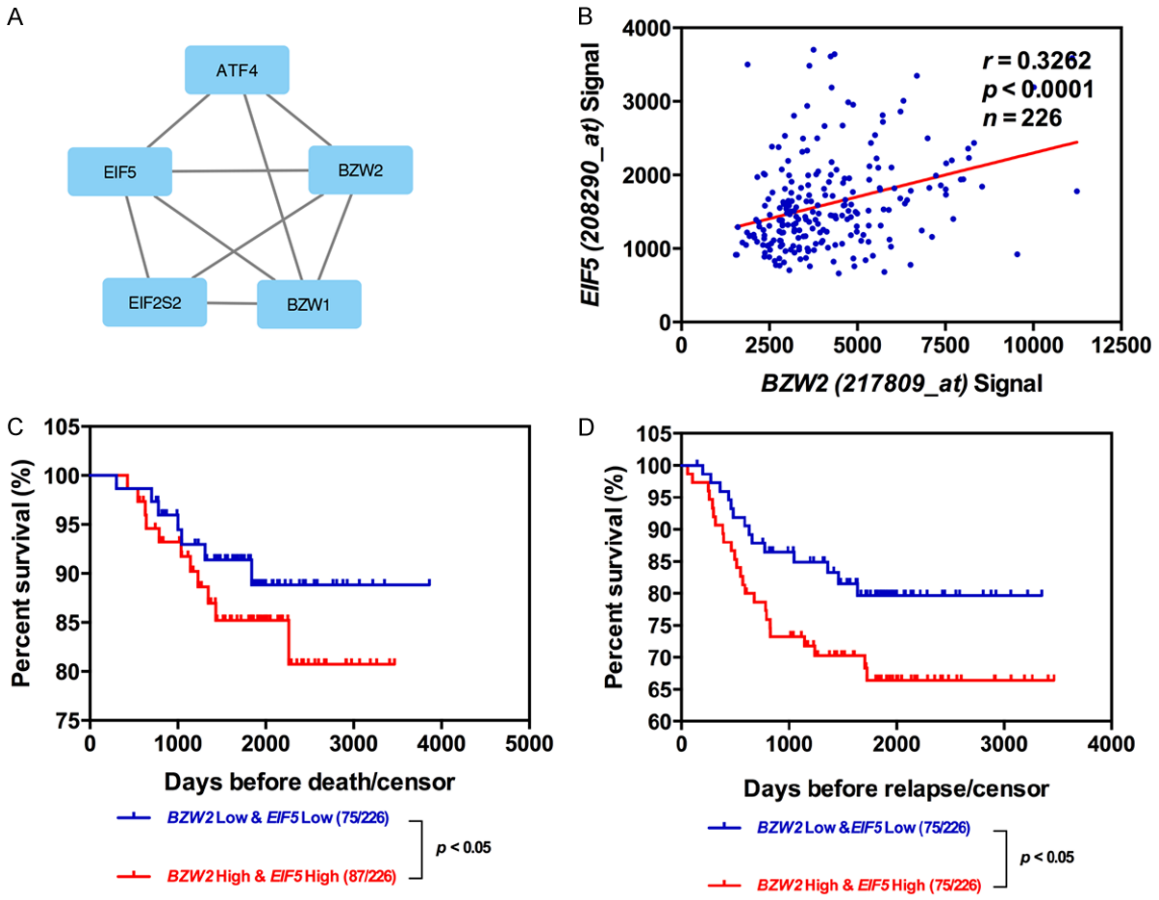

Figure 4. Relationship between the expression of *BZW2* and *EIF5* in LUAD. A. The Protein network was constructed by online software string. B. Pearson correlation analysis between *BZW2* levels and *EIF5* levels from GSE31210 microarray dataset. C, D. Kaplan-Meier analyses of OS and DFS among LUAD patients with different expression levels of *BZW2* and *EIF5*.

progression, we investigated the correlation between *BZW2* and *EIF5* expression in GSE31210. *EIF5* expression was significantly correlated with *BZW2* expression, with *R* value of 0.3262, respectively (**Figure 4B**). To further investigate whether *BZW2* and *EIF5* have synergistic or additive effects in patient outcome, the LUAD patients were classified into 2 groups including *BZW2 low/EIF5 low* and *BZW2 high/EIF5 high*, and Kaplan-Meier analyses showed clearly that the *BZW2 high/EIF5 high* group had the worst outcome in OS and DFS ($P<0.05$, **Figure 4C** and **4D**). These data strongly support findings that BZW2 interacts with EIF5 and promotes its oncogenic function in LUAD.

Discussion

Risk factors to identify LUAD patients who have high risk for prognosis and distant recurrence are still poorly defined [7]. Currently, the most applied risk stratification for LUAD patients is TNM stage [17]. Nevertheless, the long-term survival of LUAD patients remains poor [18, 19]. It is important to identify a novel biomarker to improve the outcome of LUAD. BZW2 was initially identified in osteosarcoma, and further research showed BZW2 was overexpressed in multiple tumors. What is more, high expression of BZW2 is associated with tumor aggressiveness [10, 11, 20]. To our knowledge, there was no study about the expression and clinical significance of BZW2 in LUAD. Therefore, we investigated the BZW2 expression, collected demographic and clinical information, and explored the correlation between the BZW2 expression and clinicopathologic characteristics. Coincidentally, our study showed that the BZW2 was overexpressed in LUAD patients. We further explored the correlations of elevated BZW2

expression and clinicopathologic characteristics in LUAD. As shown in **Table 1**, the elevated BZW2 expression is related to aggressive features (including lymph node metastasis, poor histopathologic differentiation, and advanced clinical stage).

In survival analysis, BZW2 expression, tumor size, and smoking history were screened as independent factors that were significantly related to OS and DFS of LUAD patients by using univariate modes. Furthermore, multivariate analysis also identified that BZW2 acts as the independently prognostic indicator for LUAD patients. Kaplan-Meier curves also depict that the lifespan of LUAD patients with high BZW2 expression suffered a poor overall survival (OS) and disease free survival (DFS). Currently, this is the first study indicating a correlation between the expression of BZW2 and prognostic significance in LUAD patients. As BZW2 expression was easily evaluated by immunohistochemistry on formalin-fixed and paraffin-embedded sections, immunohistochemistry of BZW2 would identify LUAD with poor prognosis.

It has been established that EIF5 plays essential roles in promoting the ribosome pre-initiation complex assembly and accurate translation initiation [21]. Previous research has shown EIF5A2 was overexpressed of in various human tumors [22-24]. EIF5A2 has been shown to play an important role in malignant cell proliferation, transformation, and metastasis [25]. It can bind to the cap structure located at the 5' end of mRNA and is fundamental for mRNA translation initiation. It is a major factor in the initial process of protein synthesis. By screening BZW2-interaction proteins reported by STRING database, we found that *BZW2* and *EIF5* mRNA expression is positively correlated in GSE31210 database. Additionally, the high expression of *BZW2* and *EIF5* group showed the worst prognosis in several groups of LUAD patients. These clinical results strongly support that BZW2 up-regulates EIF5 expression and promotes its oncogenic function.

To sum up, our study identified that BZW2 was overexpressed in LUAD. The elevated BZW2 expression was related to aggressive clinicopathologic features and poor prognosis, suggesting that BZW2 could be applied as a novel prognostic biomarker in LUAD.

Disclosure of conflict of interest

None.

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