

CBX7, a Potential Prognostic Biomarker in Lung Adenocarcinoma

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Background: Lung adenocarcinoma (LUAD) is a major type of NSCLC and has high morbidity and mortality. The identification of useful prognostic biomarkers for LUAD is important. CBX7 has been reported in various cancers yet its expression level and potential roles have not been fully understood.

Methods: GEPIA, Oncomine, TCGA, KM plotter and OSLuca databases were used to explore the expression profile and prognostic effects of CBX7 mRNA expression in patients with LUAD. TIMER was used to explore the relationship between CBX7 and immune infiltrating cells. GSEA was used to further explore the potential biological process and pathways regulated by CBX7 in LUAD. Lastly, IHC detection of CBX7 in 95 samples was used to validate the result.

Results: We found CBX7 was downregulated in LUAD in GEPIA, Oncomine and TCGA databases. TCGA, KM plotter and OSLuca databases suggested that CBX7 was associated with poor clinical outcomes and low survival rate. Using TIMER, we found that CBX7 might be associated with immune infiltration. Via gene set enrichment analysis, we found that tumor-associated biological processes and signaling pathways were enriched in the CBX7 downregulated group. Using clinical samples, we found that CBX7 protein has low expression in LUAD and was associated with poor survival.

Conclusion: CBX7 might serve as a promising biomarker and potential molecular target in LUAD.

Keywords: CBX7, LUAD, prognosis, biomarker, immune infiltration

Introduction

Lung cancer has the highest morbidity and mortality not only in China but also worldwide. About 80% of lung cancers are non-small-cell lung cancers.¹ NSCLC consisted of 3 subtypes, including lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and large cell carcinoma.² Among these, LUAD has been widely accepted as the dominant subtype, especially among Asian women.³ Though various efforts have been made, the prognosis of lung cancer remains poor. In addition, clinical outcomes may vary even when similar treatments are applied to patients with the same histology and TNM stages. Such instability could be caused by genetic intra-tumor heterogeneity, which has been a major obstacle for lung cancer treatment.⁴ As considerable progress is being made regarding molecular biological techniques, such as next-generation sequencing, it is now possible to further understand the molecular features in lung cancer.

Polycomb group (PcG) complexes are epigenetic regulatory complexes that conduct transcriptional repression of target genes via chromatin modification. Polycomb

Table 1 Comparison of CBX7 in LUAD and Normal Lung Tissues Across 5 Datasets in Oncomine

Dataset	ADC/Normal Lung	Fold Change	t-test	P value
Hou et al,2010	45/65	-2.539	-12.164	1.54E-19
Su et al,2007	27/30	-2.786	-8.358	3.36E-11
Selamat et al,2012	58/58	-2.374	-13.417	1.53E-22
Landi et al,2008	58/49	-2.344	-13.103	7.50E-24
Okayama et al,2012	226/20	-1.749	-9.735	9.93E-11

repressive complexes 1 and 2 (PRC1 and PRC2) are the two major forms of PcG, which are required for maintaining the stemness of embryonic stem cells and numerous types of adult stem cells. Abnormally increased expression of either PRC1 or PRC2 triggers aberrant silencing of corresponding target genes, which leads to the appearance of a broad spectrum of carcinomas.^{5,6} Chromobox7 (CBX7) belongs to the CBX protein family, which is involved in the organization of polycomb repressive complex 1 (PRC1). All of these proteins are involved in the regulation of heterochromatin, gene expression, and developmental progress.⁵ Recent studies have reported that CBX7 expression is decreased in many cancers, such as cervical cancer,⁷ pancreatic cancer,⁸ thyroid, colorectal, and breast cancer.⁵ However, the expression profile and prognostic value of CBX7 in LUAD samples remains unclear at present. This study aims to explore the differential expression and potential role of CBX7 in LUAD.

Therefore, in the current research, we investigated the expression profile and prognostic effects of CBX7 mRNA

expression in patients with LUAD using various databases, including TCGA, Oncomine, GEPIA, OSLuca and Kaplan–Meier plotter. TIMER was used to explore the relationship between CBX7 and immune infiltrating cells. GSEA was used to further explore the potential biological processes and pathways regulated by CBX7 in LUAD. Lastly, IHC detection of CBX7 in 95 samples was used to validate the result. Overall, our research preliminarily yet systematically characterizes the expression profiles of CBX7 in LUAD and reveals that the detection of the CBX7 expression status in LUAD patients is instructive and may be applied as potential biomarkers for prognostic assessment.

Materials and Methods

Data Mining in TCGA

The UCSC Xena browser (<https://xenabrowser.net/>) was used to download the Cancer Genome Atlas (TCGA) dataset of lung adenocarcinoma (LUAD). The expression level of CBX7 mRNA expression

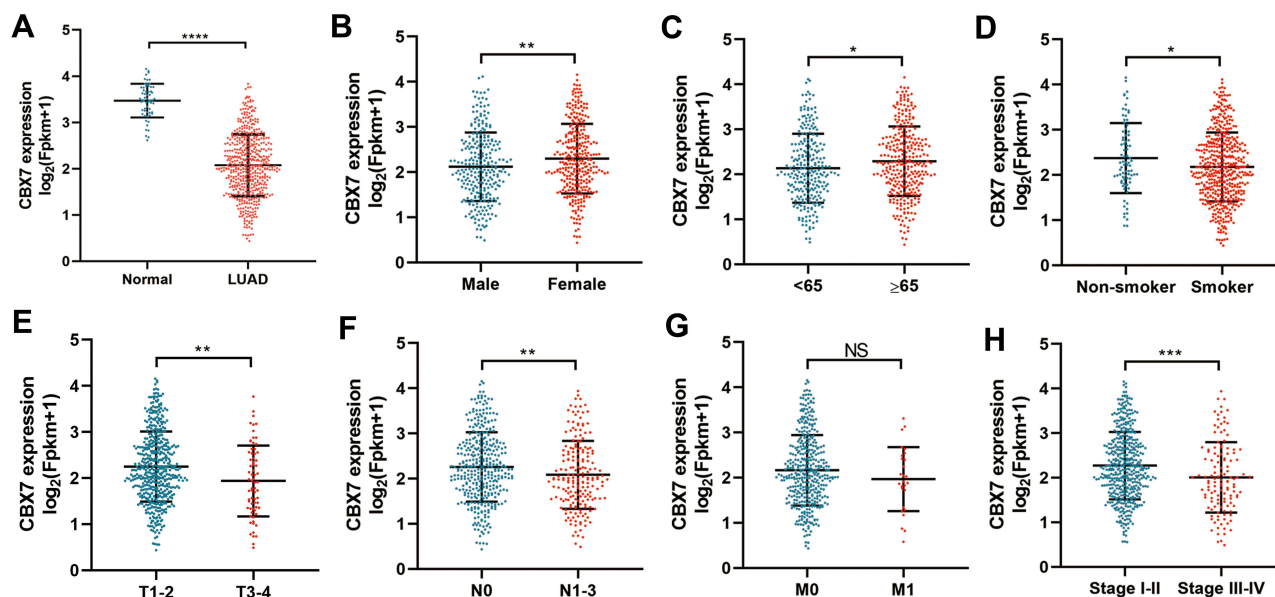


Figure 1 The expression level of CBX7 in LUAD and its relationship with clinicopathological characteristics in TCGA. (A) CBX7 was downregulated in LUAD when compared with normal tissues. (B–H) the relationship between CBX7 expression and sex (B), age (C), smoking status (D), T (E), N (F), M (G) and stage (H). *, ** and *** represented P <0.05, 0.01 and 0.001 respectively. NS represented not significance.

Table 2 The Relationship Between CBX7 and the Clinicopathological Characteristics of Patients with LUAD in TCGA Dataset

Parameters	Low (N =246)	High (N =249)	P
Age	63.72±10.4	66.8±9.5	0.001
Sex			
Female	120(48.8%)	156(58.4%)	0.03
Male	126(51.2%)	111(41.6%)	
Smoking History			
2/3/4/5	209(85.0%)	215(80.5%)	0.33
I	30(12.2%)	45(16.9%)	
No data	7(2.8%)	7(2.7%)	
T			
T1-T2	206(83.7%)	240(89.9)	0.047
T3-T4	39(15.9%)	25(9.4%)	
Tx	1(0.4%)	2(0.7%)	
N			
N0	141(57.6%)	189(70.8%)	<0.001
N1-3	102(41.6%)	69(25.8%)	
Nx	2(0.8%)	9(3.4%)	
M			
M0	232(94.3%)	253(95.5%)	0.688
M1	14(5.7%)	12(4.5%)	
Pathologic Stage			
I-II	176(71.5%)	220(82.4%)	<0.001
III-IV	69(28.0%)	41(15.4%)	
No data	1(0.4%)	6(1.4%)	
Recurrence status			
Living	118(48.0%)	158(59.2%)	0.039
Dead	81(32.9%)	69(25.8%)	
No data	47(19.1%)	40(15.0%)	
Living Status			
Living	136(55.3%)	183(68.5%)	0.003
Dead	105(42.7%)	76(28.5%)	
No data	5(2%)	8(2.5%)	

level in tumor and normal lung tissues was collected. In addition, the clinicopathological characters and survival status were collected.

Oncomine and GEPIA Database Analysis

538 samples from 5 Oncomine datasets (www.oncomine.org)⁹ were used to analyze the CBX7 expression in LUAD vs normal tissues. Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>)¹⁰ was used to explore the expression profile in pan-cancers.

Meta-Analyses of CBX7 Expression in GEO Dataset in OSLuca Online Database

The OSLuca online database (<http://bioinfo.henu.edu.cn/LUCA/LUCAList.jsp>)¹¹ contains multiple lung cancer datasets, which can perform hazard ratio analysis on the impact of CBX7 in LUAD. The hazard ratio (HR) and its 95% confidence interval (CI) in each eligible dataset were extracted for meta-analysis. The combined HRs were estimated graphically by Forest plots. Heterogeneity between studies was detected by the Q test and the I² metric (no heterogeneity: I² = 0–25%; moderate heterogeneity: 25–50%; large heterogeneity: 50–75%; and extreme heterogeneity: 75–100%). If P_Q ≥ 0.10 in the Q test or I² < 50%, the fixed-effect model (the Mantel Haenszel method)¹² was used. Otherwise, a random effect model analysis was conducted.¹³ Subgroup analyses based on sex, age, smoking status and stage were conducted. In addition, publication bias was assessed by the method reported by Begg and Mazumdar.¹⁴ The meta-analysis was conducted by STATA v11.2 (StataCorp, College Station, Texas, USA).

Data Mining in Kaplan-Meier Plotter

Kaplan-Meier plotter (www.kmplot.com)¹⁵ was used to analyze the relationship between CBX7 mRNA expression and overall survival (OS) as well as recurrence-free survival (RFS). The hazard ratio (HR) with 95% confidence intervals (CI) and log-rank P value were calculated

The Relationship Between CBX7 and Immune Microenvironment by TIMER

TIMER(<https://cistrome.shinyapps.io/timer/>)¹⁶ is a web resource for systematical evaluations of the clinical impact of different immune cells among diverse cancer types. The level of abundance regarding six immune cell types: B cell, CD4 T cell, CD8 T cell, neutrophil, macrophage and dendritic cell in the tumor microenvironment can be estimated. Gene markers of tumor-infiltrating immune cells included markers of CD8+T cells, T cells (general), B cells, monocytes, TAMs, M1macrophages, M2 macrophages, neutrophils, natural killer (NK)cells, dendritic cells (DCs), T-helper 1 (Th1) cells, T-helper 2(Th2) cells, follicular helper T (Tfh) cells, T-helper 17 (Th17) cells, Tregs, and exhausted T cells. These gene markers have been referred in prior studies. In current study, we used TIMER to explore the relationship between CBX7 and gene markers of immune infiltrating cells in LUAD.

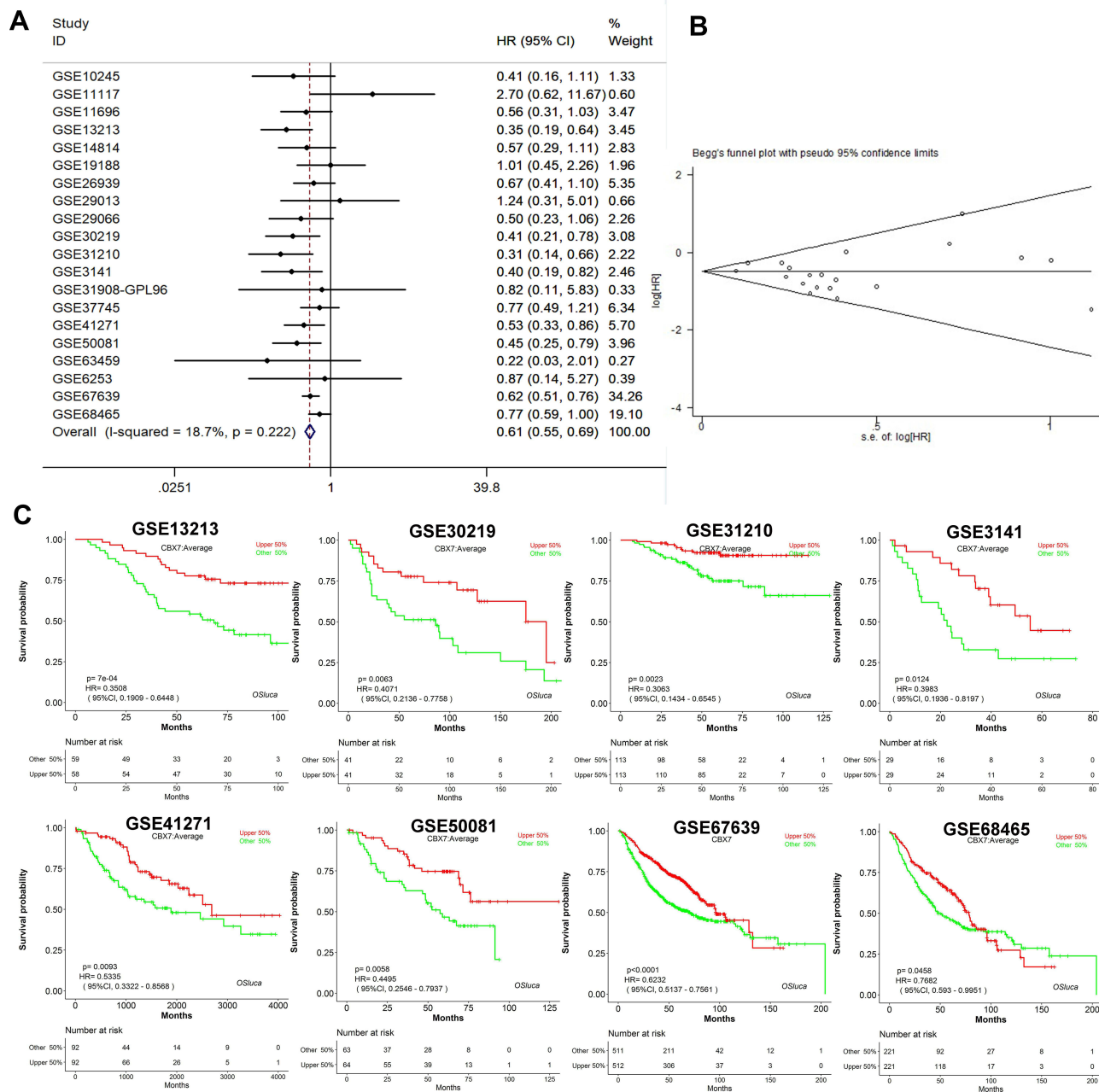


Figure 2 Meta-analyses of CBX7 expression in GEO dataset in OSLuca online database. **(A)**, Forrest plot of hazard ratio (HR) for the association of CBX7 expression with overall survival (OS) in LUAD. **(B)**, The funnel plot of the meta-analysis of the impact of CBX7 expression on overall survival (OS) in LUAD. **(C)**, Kaplan-Meier curves of OS for CBX7 expression in LUAD in GSE13213, GSE30219, GSE31210, GSE3141, GSE41271, GSE50081, GSE67639 and GSE68465.

Gene Set Enrichment Analysis (GSEA)

GSEA was used to enrich the mRNAs predicted to be correlated with pathways. $P < 0.05$ was considered to indicate statistical significance, with reference to gene sets from the Molecular Signatures Database (MSigDB) of GO gene sets and KEGG gene sets, respectively. The number of permutations was set at 1000. Enrichment results satisfying a numerical P-value < 0.05 and a false

discovery rate (FDR) < 0.25 were considered to be statistically significant.

Immunohistochemistry (IHC) Staining

95 LUAD tissues and corresponding adjacent normal lung tissues were collected from patients receiving surgery at the Department of Thoracic Surgery, the First Affiliated Hospital of Kunming Medical University from Jan 2015

Table 3 Summarized Meta-Analysis Results of the Impact of CBX7 Expression on Overall Survival in Patients with LUAD

Analysis	No. of Studies (Patients)	HR (95% CI)	P	Heterogeneity		Publication Bias	
				I ² (%)	Phet	Begg's p	Egger's p
Overall	20 (2864)	0.612 (0.546,0.685)	<0.001	18.7	0.222	0.871	0.456
Gender							
Male	17(1419)	0.681(0.585,0.794)	<0.001	14.8	0.28	0.387	0.31
Female	18(1412)	0.614(0.516,0.730)	<0.001	0	0.723	0.88	0.643
Age							
<60	17(1080)	0.592(0.481,0.729)	<0.001	14.8	0.281	0.484	0.235
>60	17(1693)	0.627(0.545,0.721)	<0.001	3	0.42	0.484	0.504
Smoking							
Yes	8(830)	0.628(0.516,0.765)	<0.001	0	0.509	0.536	0.509
No	6(233)	0.830(0.483,1.424)	0.498	25.4	0.244	0.707	0.631
Stage							
I	11(1414)	0.569(0.470,0.689)	<0.001	0	0.832	0.213	0.167
II	11(415)	0.661(0.504,0.866)	0.003	0	0.94	1	0.647
III	7(265)	0.734(0.557,0.993)	0.044	0	0.746	0.368	0.689

Abbreviation: Phet, P for Heterogeneity.

and Dec 2016. Anti-CBX7 (1:100 dilution, Cat. 14876-1-AP, ProteinTech, Wuhan, China) were used for IHC staining. Antibody staining was visualized with DAB and hematoxylin counterstain (ZSGB-Bio). The percentage of positively stained cells was scored on a scale of 0 to 4 as follows: 0: < 1%, 1: 1–25%, 2: 25–50%, 3: 50–75% and 4: > 75%. The staining intensity was scored from 0 to 3 as follows: 0: negative, 1: weak, 2: moderate, and 3: strong. The immunoreactivity score (IRS) for each case was generated by multiplying percentages of positive cells and staining intensities. Immuno-stained sections were photographed using a microscope (Olympus Corporation, Tokyo, Japan).

Statistical Analysis

Kaplan-Meier survival analysis using log rank test was used to calculate the median survival time (MST). Univariate and multivariate Cox proportional hazards models were used to calculate the HR and 95% CI. Statistical analyses were performed using R software v3.5.0 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism v7.00 (GraphPad Software Inc., USA). $P < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) analysis for death and recurrence detection was performed to identify the Youden index for CBX7 expression (the cutoff to separate patients) in Kaplan Meier curves of OS and RFS.

Result

Expression Profile and Clinicopathological Characteristics of CBX7 in LUAD in Oncomine and TCGA Datasets

Five datasets^{17–21} from Oncomine were used to evaluate the CBX7 expression in LUAD and normal lung tissue, all of which demonstrated CBX7 was downregulated in LUAD compared with normal lung tissues. Fold change (FC) of LUAD exceeded 2 in 4 studies (Table 1). Furthermore, data of 59 normal lung tissues and 526 LUAD tissues were obtained from TCGA dataset to evaluate the expression and clinicopathological characteristics of CBX7 in LUAD. Expression level of CBX7 was found to be significantly downregulated in LUAD compared with normal lung tissues ($P < 0.001$) (Figure 1A).

In addition, the expression profile of CBX7 in pan-cancer was also investigated by Oncomine and GEPIA databases. We found CBX7 was not only downregulated in LUAD but also downregulated in most cancers (Supplementary Figure S1), indicated CBX7 may play a tumor suppressor gene in many cancers.

495 LUAD patients with full clinicopathological characteristics were further analyzed. Low CBX7 expression was found to be more common in male, age <65 years, as well as smokers. Low CBX7 expression seemed to be associated with increased tumor size,

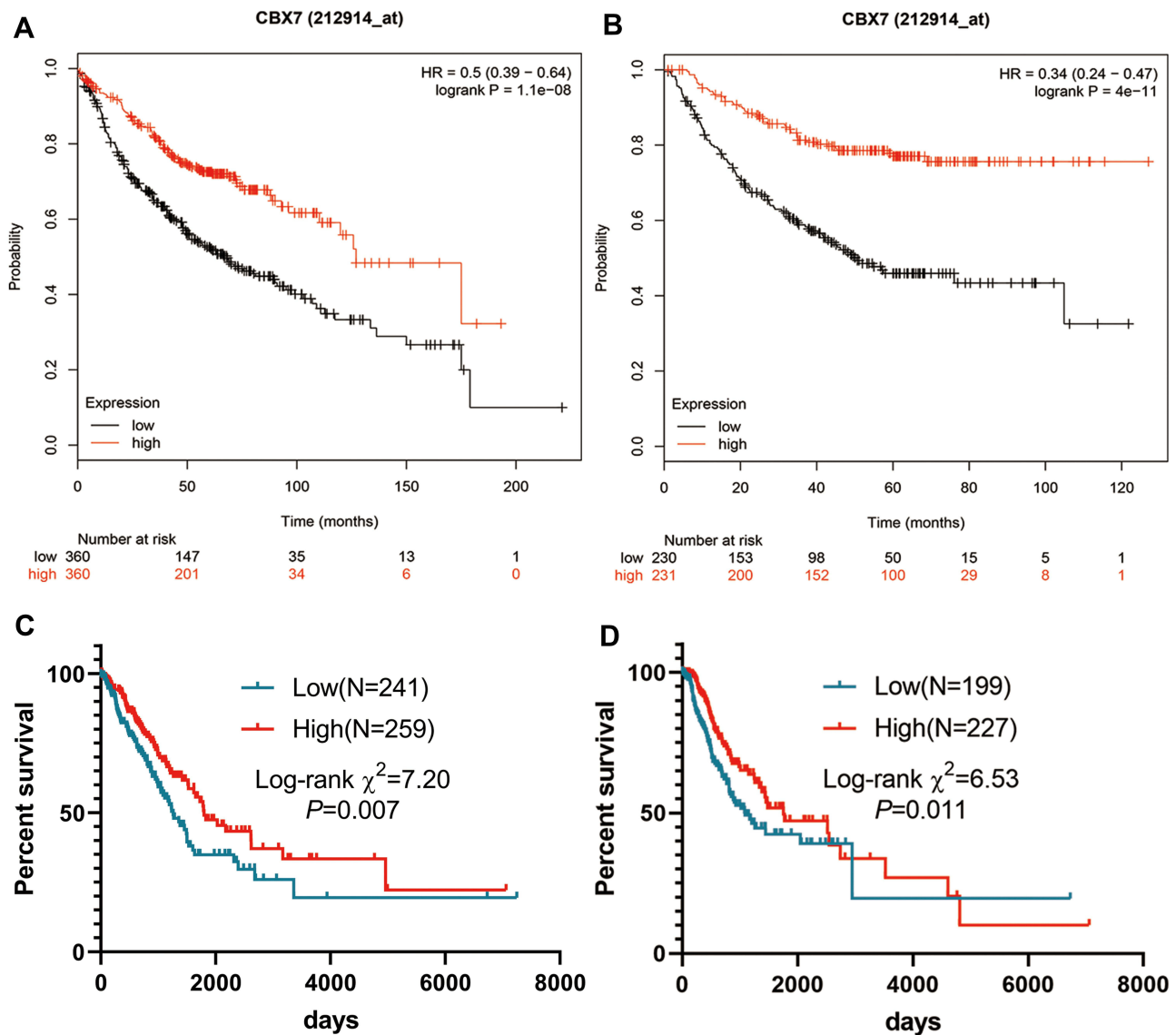


Figure 3 Kaplan-Meier curves of overall survival (OS) and recurrence-free survival (RFS) for CBX7 expression in LUAD. (A and B) Kaplan-Meier curves of OS (A) and RFS (B) in KM plotters. (C and D) Kaplan-Meier curves of OS (C) and RFS (D) in TCGA.

lymph node metastasis, distant metastasis and advanced TNM stage ($P < 0.05$) (Figure 1). ROC analysis yielded the cutoff value for CBX7 as 2.0119. According to this cut-off value, 495 LUAD patients were divided into two groups, low CBX7 group ($n=246$) and high CBX7 group ($n=249$). Similar results were obtained when continuous data was transformed into binary data. CBX7 expression level was found to be associated with age, sex, tumor size (T), lymph node metastasis (N), distant metastasis (M) and TNM stage. In addition, CBX7 expression was associated with recurrence and living status (Table 2).

CBX7 Expression and OS in LUAD by Meta-Analysis of OSluca Online Database

A total of 20 GEO datasets including 2864 LUAD patients from OSluca were included for analysis. The pooled analysis suggested reduced CBX7 was associated with worse OS (HR=0.612, 95% CI = 1.39–1.80, $p < 0.001$), with no heterogeneity between studies ($I^2=18.7\%$, $P=0.222$) (Figure 2A and Table 3). The shape of the funnel plot was symmetrical for all comparisons suggesting no publication bias detected (Figure 2B). In subgroup analysis, most subgroups such as sex, age, smoking status and stage all suggested a significant association between low CBX7

Table 4 Univariate and Multivariate Analysis of OS and RFS in LUAD Patients in TCGA Dataset

	OS				RFS			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
	HR	P	HR	P	HR	P	HR	P
Age (Continuous)	1.008(0.993–1.024)	0.282			1.010(0.993–1.026)	0.257		
Gender Male vs female	1.041(0.777–1.395)	0.787			0.892(0.645–1.233)	0.49		
Smoking history Yes vs no	0.896(0.593–1.354)	0.603			1.191(0.741–1.913)	0.471		
T T3-4 vs T1-2	2.367(1.615–3.468)	<0.001	1.735(1.133–2.658)	0.011	2.023(1.244–3.287)	0.004	1.721(1.032–2.870)	0.048
N N1-3 vs N0	2.773(2.066–3.722)	<0.001	2.323(1.632–3.306)	<0.001	1.730(1.248–2.396)	0.001	1.503(1.004–2.252)	0.048
M M1 vs M0	2.315(1.382–3.877)	0.001	1.480(0.806–2.718)	0.206	1.388(0.648–2.974)	0.399		
Pathologic Stage III+IV vs I+II	2.695(1.975–3.675)	<0.001	1.179(0.760–1.832)	0.462	1.717(1.172–2.516)	0.006	1.110(0.681–1.811)	0.675
CBX7 expression (Continuous)	0.673(0.533–0.848)	0.001	0.749(0.584–0.959)	0.022	0.693(0.545–0.882)	0.003	0.712(0.553–0.917)	0.009

and poor OS with no heterogeneity (Table 3). Eight representative KM plots from OSLuca online database (GSE13213, GSE30219, GSE31210, GSE3141, GSE41271, GSE50081, GSE67639 and GSE68465) are showed in Figure 2C.

Prognostic Role of CBX7 in LUAD in KM Plotter and TCGA

Firstly, KM plotter was used to assess the prognostic role of CBX7 in LUAD. Low CBX7 expression was found to be associated with poor OS (HR=0.50, 95% CI=0.39–0.64, $P=1.1 \times 10^{-8}$) (Figure 3A) and RFS (HR=0.34, 95% CI=0.24–0.47, $P=4 \times 10^{-8}$) (Figure 3B). TCGA dataset analysis showed that patients with low CBX7 expression tend to have worse OS (Figure 3C) and RFS (Figure 3D). Moreover, univariate and multivariate Cox regression

analyses were conducted. In univariate analysis, T, N, M pathologic stage and low CBX7 protein level were correlated with worse OS, while T, N pathologic stage and low CBX7 protein level were correlated with worse RFS (Table 4). Further multivariate showed that only T, N and CBX7 were associated with poor OS and RFS (Table 4).

CBX7 Expression Was Associated with Tumor-Infiltrating Immune Cells in LUAD

The relationship between CBX7 and immune infiltration in LUAD was evaluated by TIMER. CBX7 was found to be negatively correlated with tumor purity ($r=-0.246$, $P=3.2 \times 10^{-8}$). There was a positive correlation between CBX7 expression and B cell ($r=0.492$, $P=7.52 \times 10^{-31}$), CD8+ T cell ($r=0.16$, $P=2.97 \times 10^{-4}$), CD4+ T cell ($r=0.49$, $P=1.33 \times 10^{-33}$), macrophage ($r=0.265$, $P=3 \times 10^{-9}$), neutrophil

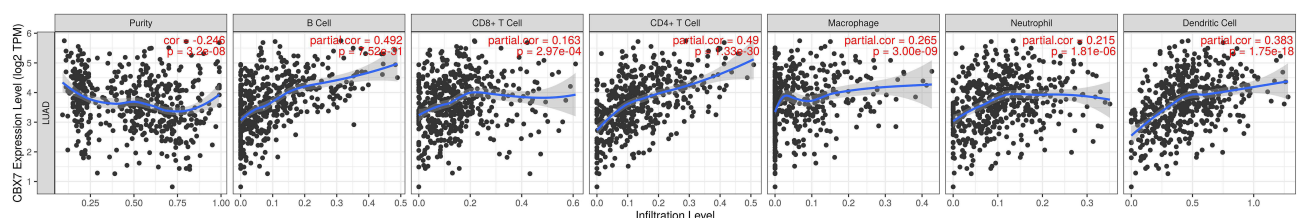
**Figure 4** CBX7 expression level has significant negative correlations with infiltrating levels of B cell, CD4+ T cells, Macrophages, Neutrophils and DCs in LUAD.

Table 5 The Relationship Between CBX7 and Relate Genes and Markers of Immune Cells in TIMER

		None		Purity	
		Cor	P	Cor	P
CD8+ T cell	CD8A	0.183	0.0000303	0.092	0.0403
	CD8B	0.12	0.00632	0.047	0.299
T cell (general)	CD3D	0.226	2.29E-7	0.119	0.0083
	CD3E	0.373	1.78E-18	0.299	1.22E-11
	CD2	0.354	1.18E-16	0.274	6.26E-10
B cell	CD19	0.413	1.15E-22	0.36	1.60E-16
	CD79A	0.352	1.68E-16	0.292	3.66E-11
Monocyte	CD86	0.262	1.58E-09	0.179	6.19E-05
	CD115 (CSF1R)	0.401	2.42E-21	0.341	7.14E-15
TAM	CCL2	0.185	2.36E-05	0.108	1.04E-03
	CD68	0.281	6.86E-11	0.22	8.47E-07
	IL10	0.284	4.91E-11	0.206	3.86E-06
M1 Macrophage	INOS (NOS2)	0.126	4.15E-03	0.092	4.12E-02
	IRF5	0.378	6.47E-19	0.325	1.34E-13
	COX2(PTGS2)	-0.066	0.134	-0.06	0.184
M2 Macrophage	CD163	0.266	8.85E-10	0.201	6.71E-06
	VSIG4	0.229	1.53E-07	0.164	2.61E-04
	MS4A4A	0.261	1.90E-09	0.187	2.98E-05
Neutrophils	CD66b (CEACAM8)	0.37	4.12E-18	0.366	4.79E-17
	CD11b (ITGAM)	0.437	1.18E-25	0.381	1.89E-18
	CCR7	0.507	5.20E-35	0.457	7.81E-27
Natural killer cell	KIR2DL1	0.039	0.377	0.002	0.967
	KIR2DL3	0.017	0.698	-0.04	0.371
	KIR2DL4	-0.14	1.41E-03	-0.208	3.10E-06
	KIR3DL1	0.048	0.279	-0.006	0.889
	KIR3DL2	0.046	0.3	-0.015	0.748
	KIR3DL3	0.088	4.59E-02	-0.11	1.47E-02
	KIR2DS4	0.059	0.181	0	0.998
Dendritic cell	HLA-DPB1	0.547	1.47E-41	0.509	8.03E-34
	HLA-DQB1	0.441	6.08E-26	0.393	1.29E-19
	HLA-DRA	0.434	5.16E-25	0.381	1.60E-18
	HLA-DPA1	0.507	6.11E-35	0.465	7.76E-28
	BDCA-1(CD1C)	0.513	5.91E-36	0.472	9.16E-29
	BDCA-4(NRP1)	0.117	8.09E-03	0.089	4.77E-02
	CD11c(ITGAX)	0.443	3.37E-26	0.391	1.87E-19
Th1	T-bet(TBX21)	0.359	4.34E-17	0.292	3.76E-11
	STAT4	0.33	1.57E-14	0.258	6.24E-09
	STAT1	0.0042	0.341	-0.034	0.447
	IFN-γ(IFNG)	-0.006	0.889	-0.096	3.36E-02
	TNF-α(TNF)	0.314	3.17E-13	0.24	6.64E-08
Th2	GATA3	0.324	4.53E-14	0.258	6.06E-09
	STAT6	0.394	1.36E-20	0.427	2.57E-23
	STAT5A	0.544	5.22E-41	0.5	1.75E-32
	IL13	0.158	3.08E-04	0.119	8.28E-03

(Continued)

Table 5 (Continued).

		None		Purity		
		Cor	P	Cor	P	
Tfh	BCL6		0.308	8.63E-13	0.315	7.01E-13
	IL21		0.077	8.23E-02	0.021	0.64
Th17	STAT3		0.268	6.77E-10	0.284	1.28E-10
	IL17A		-0.013	0.761	-0.068	0.133
Treg	FOXP3		0.323	6.13E-14	0.251	1.55E-08
	CCR8		0.319	1.15E-13	0.246	3.11E-08
	STAT5B		0.479	5.75E-31	0.473	8.06E-29
	TGFβ (TGFB1)		0.414	9.10E-23	0.37	2.11E-17
T cell exhaustion	PD-1 (PDCD1)		0.218	5.56E-07	0.127	4.66E-03
	CTLA4		0.27	4.41E-10	0.178	7.02E-05
	LAG3		0.117	7.95E-03	0.035	0.441
	TIM-3 (HAVCR2)		0.236	6.00E-08	0.148	9.75E-04
	GZMB		-0.12	6.53E-03	-0.237	9.54E-08

($r=0.215$, $P=1.81e-6$), and dendritic cell ($r=0.383$, $P=1.75e-18$) (Figure 4). In addition, various immune marker genes corresponding to different tumor-infiltrating immune cells (CD8+ T cells, T cells (general), B cells, monocytes, TAMs, M1 and M2 macrophages, neutrophils, NK cells, Th1, Th2, Tfh, Th17, Treg and T cell exhaustion) were also investigated by TIMER and CBX7 were

found to be associated with most of these marker genes. Generally, CBX7 expression were positively correlated with the majority of gene markers of different functional T cells (CD8+ T, Th1, Th2, Tfh, Th17, Treg and T cell exhaustion), B cells, dendritic cells, neutrophils and monocyte after purifying adjustments. CBX7 was related to tumor-associated macrophage (TAM, M1 and M2

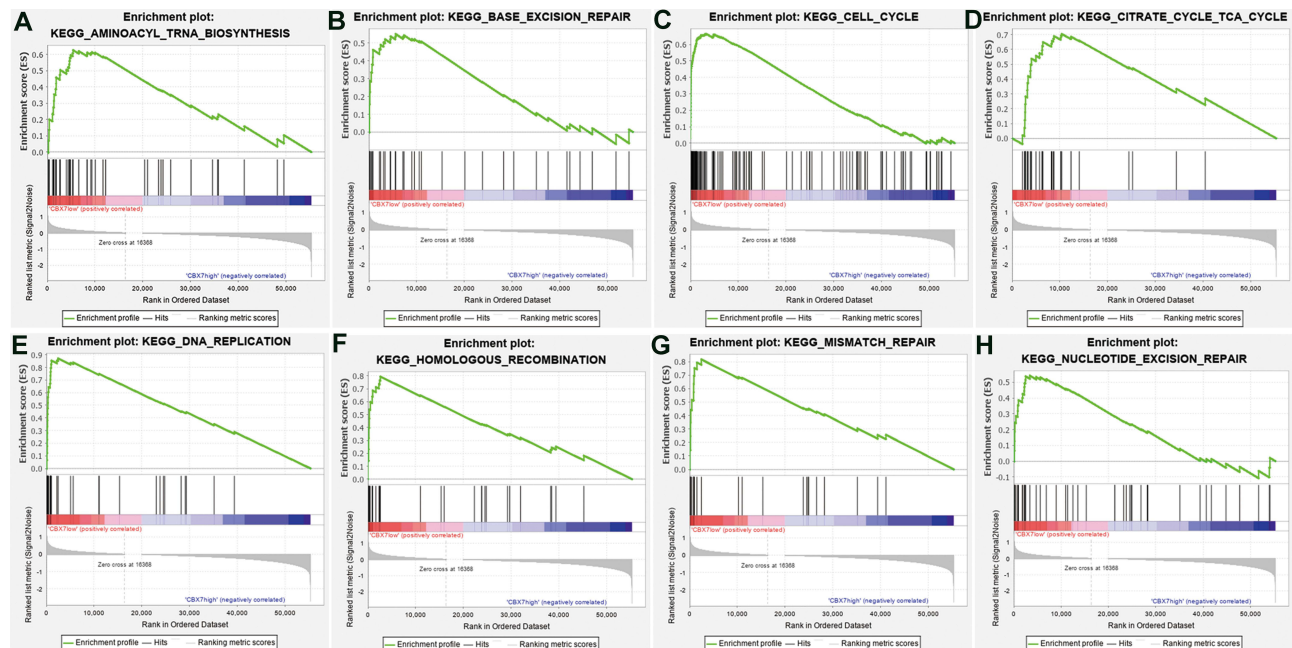


Figure 5 Gene Set Enrichment Analysis (GESA) results for tumors with low CBX7 expression in LUAD.

Table 6 Gene Set Enrichment Analysis Result of CBX7 Low Expression Group

	GS Details	Size	ES	NES	NOM P-val	FDR q-val
1	KEGG_PYRIMIDINE_METABOLISM	98	0.5940191	2.08511	0	0.022382332
2	KEGG_CITRATE_CYCLE_TCA_CYCLE	31	0.7048713	2.0597153	0.010351967	0.0156989
3	KEGG_MISMATCH_REPAIR	23	0.8160027	2.0580044	0	0.010465934
4	KEGG_PROTEASOME	46	0.8175272	2.055871	0	0.008497239
5	KEGG_HOMOLOGOUS_RECOMBINATION	28	0.79443586	2.0513682	0	0.007408555
6	KEGG_DNA_REPLICATION	36	0.87027	2.0502183	0	0.006319825
7	KEGG_CELL_CYCLE	124	0.66358805	2.0225832	0	0.007860474
8	KEGG_PROTEIN_EXPORT	24	0.6990288	2.0219886	0	0.006925017
9	KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	41	0.62645996	1.9365052	0.01778656	0.01809742
10	KEGG_OXIDATIVE_PHOSPHORYLATION	131	0.65311223	1.8973855	0.018108651	0.026013676
11	KEGG_PARKINSONS_DISEASE	127	0.6037786	1.8604995	0.024048096	0.032946516
12	KEGG_RNA_POLYMERASE	29	0.59177357	1.8334113	0.017509727	0.038483705
13	KEGG_OOCYTE_MEIOSIS	112	0.45340592	1.8099531	0.013565891	0.043382503
14	KEGG_ALZHEIMERS_DISEASE	165	0.4686439	1.8079304	0.016359918	0.041234728
15	KEGG_HUNTINGTONS_DISEASE	180	0.46163434	1.7813784	0.026262626	0.04682553
16	KEGG_NUCLEOTIDE_EXCISION_REPAIR	44	0.54154724	1.7526313	0.038387716	0.053722583
17	KEGG_RNA_DEGRADATION	59	0.47982225	1.7003796	0.035928145	0.07108344
18	KEGG_ONE_CARBON_POOL_BY_FOLATE	17	0.61857325	1.6639311	0.02745098	0.08360174
19	KEGG_BASE_EXCISION_REPAIR	35	0.5514795	1.6436532	0.04357143	0.08530487
20	KEGG_RIBOFLAVIN_METABOLISM	16	0.55264455	1.634612	0.022222223	0.08583631
21	KEGG_RIBOSOME	87	0.7805994	1.60399	0.035196688	0.097854406
22	KEGG_GLYOXYLATE_AND_DICARBOXYLATE_METABOLISM	16	0.5471389	1.5795733	0.044624746	0.1067692
23	KEGG_PURINE_METABOLISM	158	0.37173504	1.5757207	0.0327553	0.104687735
24	KEGG_P53_SIGNALING_PATHWAY	68	0.4271343	1.501431	0.043032788	0.13903996

macrophage) infiltration (Table 5), which suggested that CBX7 play critical roles in immune microenvironment in LUAD.

GSEA Result of CBX7

Potential mechanism of CBX7 was explored and analyzed by GSEA. Pathways that were observed to be significantly enriched between CBX7 high 10% group and low 10% groups were analyzed. The result suggested that CBX7 may participate in Aminoacyl tRNA biosynthesis, base excision repair, cell cycle, citrate cycle TCA cycle, DNA replication, homologous recombination, mismatch repair, and nucleotide excision repair pathways (Figure 5 and Table 6).

IHC Validation of CBX7 in LUAD

95 paraffin-embedded LUAD and corresponding adjacent normal lung tissues were included for analysis. The IHC scores of paired LUAD tissues and their adjacent normal tissues showed that protein level of CBX7 is down-regulated in 95 LUAD patients ($P < 0.001$) (Figure 6 and Figure 7A). Low CBX7 protein level was found to be correlated with differentiation degree, tumor size and lymph node metastasis (N). No significant association was found in region, sex,

smoking status and TNM stage (Figure 7). The cutoff value yielded by ROC analysis for CBX7 IRS was 7.5, according to which patients were divided into low CBX7 ($n=46$) and high CBX7 groups ($n=49$). CBX7 expression was associated with living status, no significant association was found in age, region, sex, smoking history, differentiation degree, T, N, M, TNM stage and recurrence status (Table 7). The prognostic role of CBX7 in LUAD was evaluated by univariate and multivariate Cox regression analyses. In univariate analysis, T, N, M, pathologic stage and low CBX7 protein level were correlated with the worst OS, while differentiation degree, N, M, pathologic stage and low CBX7 protein level were correlated with unfavorable RFS (Table 8 and Figure 8). In multivariate analysis, only pathologic M and CBX7 protein expression were associated with poor OS and RFS (Table 8).

Discussion

Our current study comprehensively investigated the prognostic role of CBX7 in LUAD. Through online database and our individual dataset, we demonstrated that CBX7 was down-regulated in LUAD tissues and might serve as an independent prognostic biomarker in LUAD.

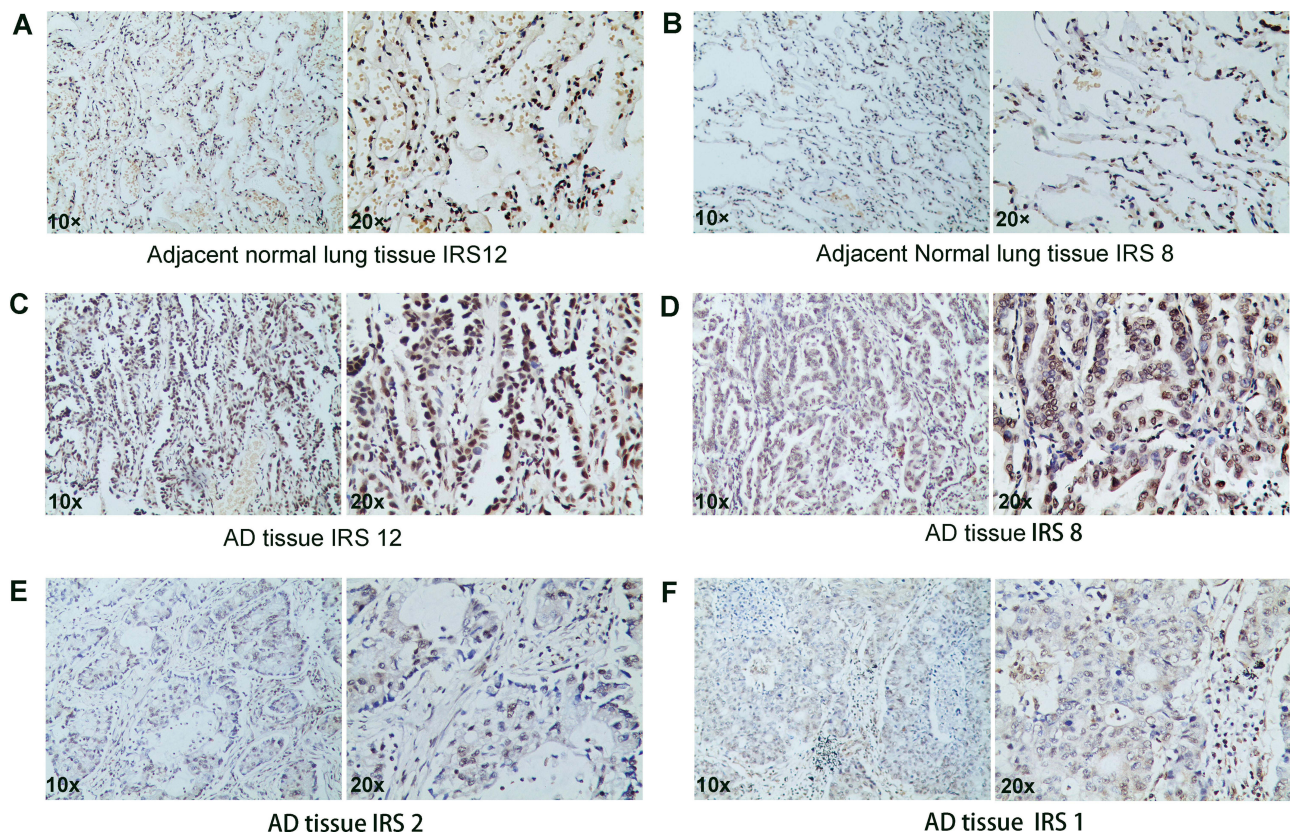


Figure 6 Immunostaining of CBX7 in normal lung tissues and LUAD.

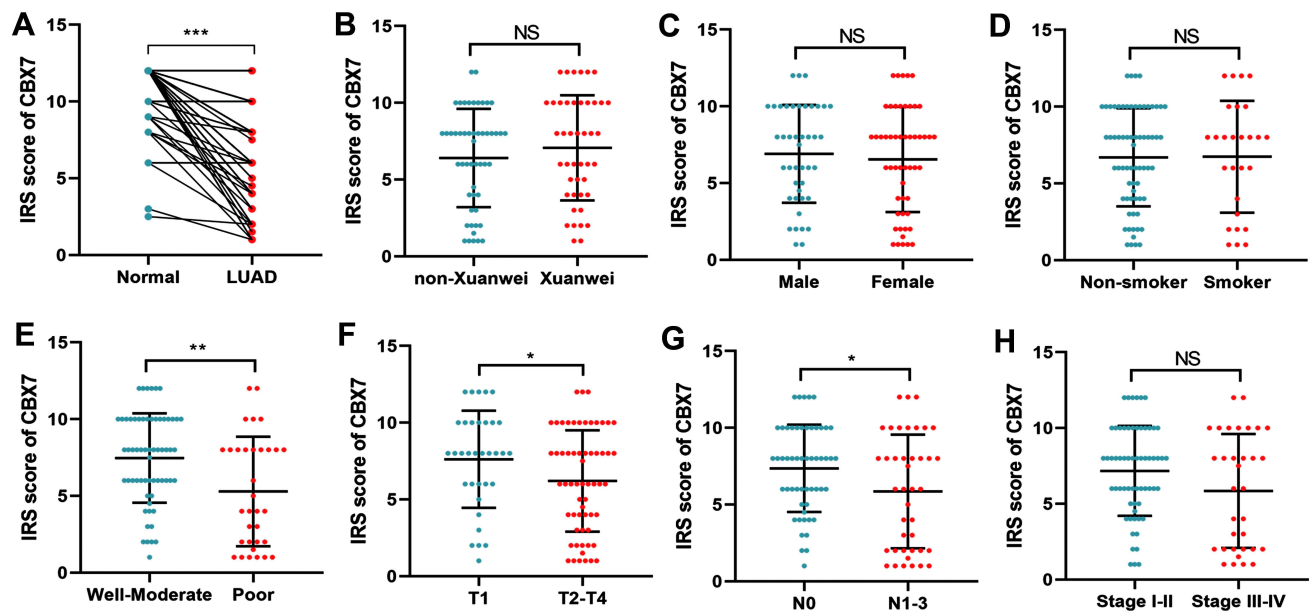


Figure 7 The expression level of CBX7 and its relationship with clinicopathological characteristics in 95 patients with LUAD. **(A)** CBX7 was downregulated in LUAD when compared with normal tissues. **(B–H)** The relationship between CBX7 expression and region **(B)**, sex **(C)**, smoking status **(D)**, differentiation **(E)**, T **(F)**, N **(G)** and stage **(H)**. *, ** and *** represented $P < 0.05$, 0.01 and 0.001 respectively. NS represented not significance.

Table 7 The Relationship Between CBX7 and the Clinicopathological Characteristics in 95 patients with LUAD

Parameters	Low (N =46)	High (N =49)	P
Age	55.3±11.1	54.5±9.1	0.001
Region			0.9
Xuanwei	21(45.7%)	23(46.9%)	
No-Xuanwei	25(54.3%)	26(53.1%)	
Sex			0.941
Female	21(45.7%)	22(44.9%)	
Male	25(54.3%)	27(55.1%)	
Smoking History			0.625
Yes	12(26.1%)	15(30.6%)	
No	34(73.9%)	34(69.4%)	
Differation			0.193
Well+moderate	27(58.7%)	35(71.4%)	
Poor	19(41.3%)	14(28.6%)	
T			0.056
T1	12(26.1%)	22(44.9%)	
T2-T4	34(73.9%)	27(55.1%)	
N			0.192
N0	23(50.0%)	31(63.3%)	
N1-3	23(50.0%)	18(36.7%)	
M			0.25
M0	45(97.8%)	44(89.8%)	
M1	1(2.2%)	5(10.2%)	
Pathologic Stage			0.399
I-II	28(60.9%)	34(69.4%)	
III-IV	18(39.1%)	15(30.6%)	
Recurrence status			0.192
Living	12(26.1%)	20(40.8%)	
Dead	34(79.3%)	29(59.2%)	
Living Status			0.032
Living	11(23.9%)	22(44.9%)	
Dead	35(76.1%)	27(55.1%)	

LUAD and LUSC were the major types for NSCLC. With increasing prevalence and its dominant histology, LUAD has been selected as the focus of this study. Another important reason stands as to avoid bias that could possibly arise due to different biological processes and molecular profiles between LUAD and LUSC. Further studies are necessary to investigate the roles of CBX7 in LUSC and other subtypes in order to confirm whether CBX7 could serve as a potential biomarker in lung cancer treatment. As previous studies reported that CBX7 was downregulated in many cancers including hepatocellular carcinoma, glioma,

pancreatic cancer, colon, urothelial and thyroid cancer, such characteristic was also associated with poor prognosis in hepatocellular, glioma, pancreatic cancer and colon cancer. As far as human lung carcinoma is concerned, no detectable level of CBX7 has been detected in the samples mentioned, whereas CBX7 is abundantly expressed in normal lung tissue. Interestingly, LOH was reported in 50% of informative cases. Additionally, the expression of CBX7 was lost in the “normal” area surrounding carcinomas (non-pathological at the morphological evaluation) in 50% of the analyzed cases. Intriguingly, these cases also involved LOH, suggesting that the decreased expression of CBX7 could be involved in the transition of epithelial lung cells toward a fully transformed and malignant phenotype. In this study, the expression profile and the prognostic role of CBX7 in LUAD have been comprehensively investigated using both online database and private database. Experimental results suggested that low CBX7 was associated with aggressive phenotypes, such as stage and definition, which is consistent with TCGA and private database analysis. Hence, this study is presented with rounded and scientifically sound evidence.

Reduced CBX7 expression was associated with aggressive phenotypes and poor survival status among lung cancer and other cancers, suggesting that CBX7 serves as a novel tumor-suppressive gene. Pallante et al stated that the loss of CBX7 promoted cancer development in mice, and CBX7-knockout (CBX7-KO) mice were more likely to develop liver and lung neoplasia compared with wide-type. Mouse embryonic fibroblasts (MEFs) derived from CBX7-KO mice was used to explore the effect of CBX7 regarding cellular proliferation and CBX7^{+/-} and CBX7^{-/-} MEFs were found to grow faster than CBX7^{+/+} MEF. In addition, the percentage of S-phase cell in CBX7-KO MEF was higher than wild-type MEF.²² Several studies have shed light on the mechanism of reduced CBX7 promoting cancer development. It has been reported that CBX7 was able to sustain the expression of the E-cadherin gene (CDH1) through binding to its promoter, as well as counteracting with anti-transcriptional effect of histone deacetylase 2 (HDAC2) and Protein Arginine Methyltransferase 1(PRMT1).^{23,24} As a result, the reduced expression of CBX7 decreases E-cadherin expression, which then leads to the critical event epithelial-to-mesenchymal transition (EMT).^{23,24} CBX7 binds the E-box to inhibit TWIST-1 function, tumorigenicity, and metastatic potential. Deletion of CBX7 in sEOC can sufficiently reactivate TWIST-1-induced transcription, prompt mesenchymal transformation, and enhanced tumorigenicity in vivo.²⁵ CBX7 interacts with HMGA1b, which binds to and regulates

Table 8 Univariate and Multivariate Analysis of OS and RFS in 95 Patients with LUAD

	OS				RFS			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age (Continuous)	1.007(0.981–1.033)	0.621			1.010(0.985–1.035)	0.452		
Gender Male vs female	1.174(0.708–1.947)	0.534			1.161(0.706–1.910)	0.557		
Region Xuanwei vs Non-Xuanwei	0.656(0.393–1.095)	0.107			0.691(0.418–1.144)	0.151		
Smoking history Yes vs no	0.895(0.506–1.584)	0.703			0.816(0.462–1.439)	0.482		
Differentiation Poor vs well+moderate	1.526(0.908–2.566)	0.111			1.790(1.081–2.965)	0.024	1.242(0.719–2.146)	0.436
T T2-4 vs T1	1.822(1.039–3.197)	0.036	1.278(0.700–2.333)	0.425	1.720(0.994–2.977)	0.053		
N N1-3 vs N0	2.840(1.691–4.796)	<0.001	1.276(0.508–3.208)	0.604	2.541(1.533–4.209)	<0.001	1.346(0.554–3.271)	0.511
M M1 vs M0	4.621(1.959–10.900)	<0.001	4.075(1.513–10.972)	0.005	4.014(1.705–9.449)	0.001	3.055(1.165–8.012)	0.023
Pathologic Stage III+IV vs I+II	2.969(1.763–5.000)	<0.001	1.665(0.665–4.168)	0.276	2.656(1.596–4.420)	<0.001	1.545(0.622–3.841)	0.349
CBX7 expression (Continuous)	0.869(0.802–0.942)	0.001	0.877(0.805–0.955)	0.003	0.892(0.824–0.965)	0.004	0.902(0.829–0.982)	0.018

the acetylation of CCNE1 promoter. As a coding gene for cyclin E, CCNE1 regulates cell cycle that is crucial for G1/S transition. Another mechanism through which CBX7 involves with cancer progression is that CBX7 negatively modulates the expression of SPP1 gene that encodes the osteopontin protein, a chemokine promoting cell migration.^{23,26}

This study also investigated the potential role of CBX7 in LUAD via GSEA analysis. Several cancer-related pathways were identified including aminoacyl tRNA biosynthesis, base excision repair, cell cycle, citrate cycle TCA cycle, DNA replication, homologous recombination, mismatch repair, and nucleotide excision repair pathways. All of these pathways have been proved to be closely related to cancer development, and some, for example, cell cycle pathways, have been reported and investigated by previous studies.^{22,23} How CBX7 participate in DNA damage repair pathways (base excision repair, DNA replication, homologous recombination, mismatch repair, and nucleotide excision repair pathways)? PcG proteins participate in DNA damage response (DDR). The majority of PcG proteins, including EZH2 and the CBXs, localize to regions of damaged chromatin in order to

recruit DNA damage repair machinery.^{27,28} Inhibiting CBXs may enhance chemotherapeutic response by preventing DNA damage repair, allowing massive accumulation of DNA damage.²⁹ However, the role of CBX7 in the process is still unknown. Further studies need to be conducted for verification.

Tumor microenvironments, especially tumor-infiltrating immune cells, play an important role in the prognosis of lung cancer. This study demonstrated that CBX7 affects and mediates immune infiltration level in LUAD. CBX7 expression was positively related to the level of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and DCs infiltration. CBX7 expression was also positively related to most gene markers of T cells, B cells, tumor-associated macrophages (TAM), M1, M2, DC cell, and T helper cells (Th1, Th2, Tfh, and Th17). High level of these tumor-infiltrating immune was associated with better survival in lung cancer. CBX7 could regulate immune infiltration level by mediating gene expression in these cells. Also, in the pathway enriched analysis, we found that CBX may participate in DNA damage repair pathways (base excision repair, DNA replication, homologous recombination, mismatch repair, and nucleotide excision repair

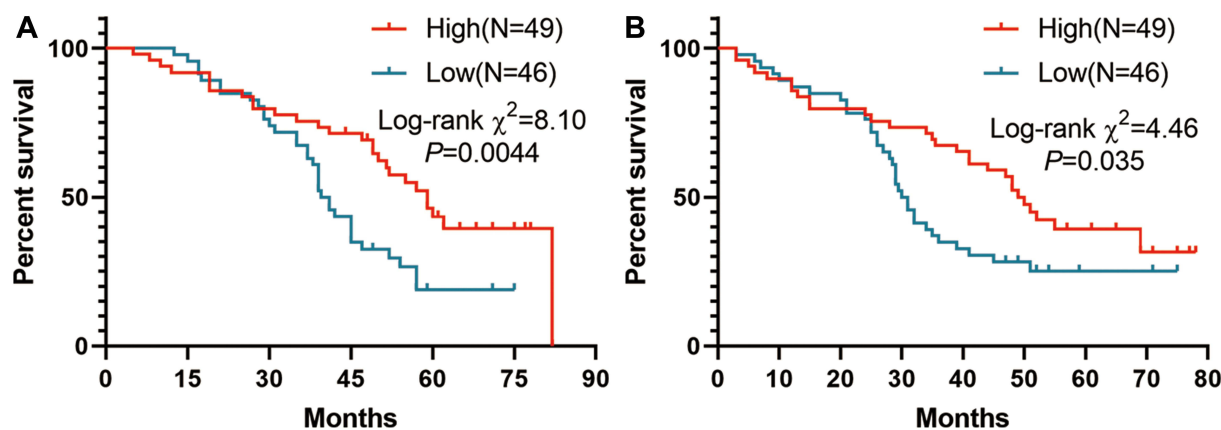


Figure 8 Kaplan-Meier curves of overall survival (OS)(A) and recurrence-free survival(RFS) (B) for CBX7 expression in 95 patients with LUAD.

pathways). The deficiency of DNA damage repair pathways may result in the high tumor mutation burden (TMB), which may be associated with immune infiltration. However, the precise role of CBX7 in tumor immune microenvironment and tumor progression still needs exploration through future studies.

To validate the potential role of CBX7, 95 paraffin-embedded LUAD and corresponding adjacent normal lung tissue samples were included for analysis. CBX7 was found to be downregulated in LUAD tissues and was associated with malignant phenotypes such as T/N/M. In addition, multiple cox analysis showed that CBX7 is an independent prognostic factor, indicating that CBX7 might serve as a useful prognostic biomarker in LUAD.

In conclusion, this study demonstrated that CBX7 is downregulated in LUAD, which is associated with unappealing clinicopathological features and poor prognosis proved by both online datasets and our clinical samples. CBX7 stands as a crucial mediating factor regarding LUAD tumorigenesis due to its correlation with several cancer-related pathways and its regulating effects on immune infiltration level. Hence, CBX7 might serve as a promising biomarker and potential molecular target regarding clinical treatments of LUAD. Future large cohort and mechanism studies are necessary to validate and further reveal the molecular mechanisms and pathways of CBX7 in LUAD.

Ethical Approval and Consent to Participate

95 surgical LUAD samples used in this manuscript have been conducted in accordance with the principles

of the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Our study was also approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University. All patients were obtained with informed consent.

Author Contributions

Yunping Zhao and Xiaobo Chen: conception and design of the work. Yanlong Yang and Zaoxiu Hu: conception, design of the work and have drafted the work. Linzhu Yang: the acquisition, analysis. Hongwen Sun and Qinghe Yu: substantively revised. Fang Yin, Yongmeng Sun, Xinming Zhu, Shen Li, Zaoxiu Hu and Lisha Pu collected data and analyzed. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This study was supported by Scientific Research Foundation of Yunnan Education Department (2019J1234 and 2019J1282), Yunnan Provincial Department of Science and Technology-Kunming Medical University Joint Foundation (202001AY070001-144 and 202101AY070001-172), Doctoral Research Foundation of the First Affiliated Hospital of Kunming Medical University (2018BS012) and Science and Technology Personnel Training Program of High-level Health Technicians in Yunnan Province (H-2017014).

Disclosure

The authors have no conflicts of interest to declare.

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