

BTG2 Serves as a Potential Prognostic Marker and Correlates with Immune Infiltration in Lung Adenocarcinoma

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Background: B-cell translocation gene 2 (*BTG2*) has been revealed to be involved in the occurrence and development of multiple cancers. However, the role of *BTG2* in lung adenocarcinoma (LUAD) is still ambiguous. Thus, this study aims to investigate the prognostic value of *BTG2* and its correlation with immune infiltration in LUAD.

Methods: The expression of *BTG2* in LUAD was analyzed using the TIMER and UALCAN databases. The correlations between *BTG2* expression and clinicopathological factors were investigated using the UALCAN databases. The Kaplan–Meier plotter, GEPIA, and TCGA databases were employed to assess the prognostic value of *BTG2*. The STRING database and Cytoscape software were used to construct an interaction network and mine co-expression genes. The TISIDB database was examined for a correlation between *BTG2* and driver genes in LUAD. Enrichment analysis of co-expressed genes and *BTG2* was performed using the LinkedOmics database. Finally, the correlations between *BTG2* and immune infiltrates were investigated using the TIMER, GEO, and TISIDB database.

Results: *BTG2* was significantly downregulated in LUAD. The decreased expression of *BTG2* in LUAD was significantly correlated with higher cancer stages and shorter duration of overall survival. The expressions of *BTG2*-related co-expression genes were associated with the prognosis in LUAD. The expression of *BTG2* was closely associated with the mutations of *TP53* and *ROS1*. Enrichment analysis revealed that *BTG2* was significantly correlated with immune-associated signaling pathways and function. In addition, the expression of *BTG2* was found to be closely related to immune infiltration, multiple gene markers of immune cells, chemokines, and chemokine receptors.

Conclusion: Our findings have effectively demonstrated that *BTG2* expression was downregulated in LUAD, indicating poor prognosis. Closely relating to immune cell infiltration, *BTG2* may be a promising immune-related biomarker and molecular target for patients with LUAD.

Keywords: *BTG2*, lung adenocarcinoma, prognosis biomarker, gene mutation, immune infiltration

Introduction

Lung cancer is one of the most common malignant tumors, ranking first in terms of cancer-related mortality worldwide.^{1–3} Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer.⁴ Lung adenocarcinoma (LUAD) is the major histological subtype of NSCLC.^{4,5} Owing to the high proportion of patients diagnosed with advanced and metastatic disease, the 5-year survival rate of LUAD remains extremely low.⁶ The treatment of LUAD remains a major challenge for clinicians. With the introduction of immune checkpoint inhibitors into clinical practice, the treatment outcome of LUAD has been remarkably improved. PD-L1 and tumor mutation burden (TMB) are indicated as predictive biomarkers

for immunotherapy. Numerous patients with high PD-L1 expressions or high TMB show limited benefit from immunotherapy, while negative PD-L1 expression or low TMB demonstrated response to PD-1/PD-L1 inhibitors. Cumulative evidence has indicated that immune infiltration plays a vital role in tumorigenesis, and affects the efficacy of immunotherapy, and prognosis of patients with cancers including LUAD.^{7,8} Therefore, it is crucial to clarify the potential regulating mechanisms of immune cell infiltration and further identify sensitive prognostic immune-related biomarkers and molecular targets to identify more effective therapeutic strategies for patients with LUAD.

B-cell translocation gene 2 (*BTG2*), a member of the anti-proliferative BTG/TOB gene family,⁹ maps within a chromosomal segment (1q32)¹⁰ and contains two regions named box A and box B.¹¹ *BTG2* is closely involved in a variety of biological processes, such as cell cycle,¹² cell senescence,¹³ cell differentiation,¹⁴ hematopoiesis,¹⁵ oxidative damage,¹⁶ gene transcription,¹⁷ and DNA damage repair.¹⁸ Noteworthy, accumulating evidence has revealed that down-regulated *BTG2* expression plays a vital role in tumor occurrence and development.^{19,20} Several studies reported that the downregulation of *BTG2* was associated with the poor prognosis of cancers including NSCLC, breast cancer, hepatocellular carcinoma and bladder cancer.^{21–24} Zhang and colleagues reported that circular RNA circCRIM1 was able to promote *BTG2* expression via down-regulating miR-125b-5p expression, thereby suppressing the migration, invasion, epithelial-mesenchymal transformation (EMT), and glycolysis in lung adenocarcinoma cell.²⁵ These studies suggested that *BTG2* was a potential prognostic marker for cancer. Recent experimental data demonstrated that *BTG2* was closely involved in the regulation of immune response, including the regulation of B cell development, and T cell activation, and the induction of apoptosis in monocyte, which played key roles in anti-tumor immunity.^{26–30} However, the prognostic value of *BTG2* and its correlation with immune infiltrates in LUAD remain unclarified.

Given the crucial role of *BTG2* in tumorigenesis and progression, and the potential effect of *BTG2* on immune cell infiltration, the aim of this study is to investigate the prognostic value of *BTG2* and its correlation with immune infiltration in LUAD.

Materials and Methods

TIMER Database

TIMER (<https://cistrome.shinyapps.io/timer/>) is an online analysis tool for immune infiltrating cells across diverse cancer types.³¹ The *BTG2* expression for pan-cancer was analyzed using the TIMER database, and we also performed a detailed evaluation on the correlations between *BTG2* expression and immune cell infiltrations as well as multiple immune markers. Further investigation was conducted to analyze the relationships between *BTG2* expression and key driver genes (*TP53* (*p53*), *ROS1*, *ALK*, *KRAS*, *EGFR*, *BRAF*, *ERBB2* (*HER-2*), *NRAS*).

UALCAN Database

UALCAN (<http://ualcan.path.uab.edu/>) is a comprehensive web resource for analyzing cancer OMICS data.³² We compared the *BTG2* expression between LUAD and normal samples using this database. Besides, we assessed the correlations between *BTG2* expression and clinicopathological parameters in this database.

Kaplan–Meier Plotter Database

The Kaplan–Meier plotter (<https://kmplot.com/analysis/>) is capable of assessing the effect of 54k genes (mRNA, miRNA, protein) on survival in 21 cancer types.³³ In Kaplan–Meier plotter database, the survplot function of the survival Bioconductor package in R software was used for univariate and multivariate Cox regression analysis.³⁴ We used Kaplan–Meier Plotter database to determine the prognostic role of *BTG2* in overall survival (OS). Based on the upper quartile of *BTG2* expression, LUAD patients were divided into high and low groups. The prognostic value of *BTG2*-co-expressed genes for LUAD was also analyzed using this database.

GEPIA Database

GEPIA (<http://gepia.cancer-pku.cn>) is an open database for analyzing RNA sequencing expression of tumors and normal samples based on TCGA and GTEx.³⁵ Survival analysis was performed using the python package lifelines.³⁵ We chose

quartiles as cut-off point for *BTG2* grouping and obtained the correlations between OS and *BTG2* expression using the GEPIA database.

TCGA Database

The Cancer Genome Atlas (TCGA) contained the clinical characteristic information available in public databases.³⁶ UCSC Xenaweb site (<http://xena.ucsc.edu/>) is a handy site to help quickly download TCGA database, which was used to perform univariate and multivariate Cox regression analysis using “survminer” and “survival” packages in R (V4.1.1) software. Consistently, we chose the upper quarter as the cut-off value for the *BTG2* grouping.

STRING Database

STRING (<https://www.string-db.org/>) provides integrated and consolidated protein–protein interaction data.³⁷ We constructed a protein–protein interaction (PPI) network information, and co-expressed genes were identified using the Cytoscape 3.8.2 version software.³⁸

TISIDB Database

TISIDB (<http://cis.hku.hk/TISIDB>) integrates multiple types of data resources in oncoimmunology, including 988 genes related to anti-tumor immunity and the association between any gene and immune features for 30 TCGA cancer types.³⁹ The correlations between *BTG2* expression and chemokines as well as chemokine receptors were comprehensively investigated through this platform.

Single-Cell RNA Sequencing Datasets Acquisition and Processing

The LUAD single cell validation dataset was downloaded from the Gene Expression Omnibus Database (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) with the accession number *GSE189357*. The LUAD dataset was processed through a set of process steps including data filtering, quality control, principal component analysis (PCA), reducing the dimension, and t-distributed stochastic neighbor embedding (tSNE) clustering. The clustering results were annotated using SingleR of R software and previous literature reports.

LinkedOmics Database

LinkedOmics (<http://linkedomics.org/login.php>) contains multiple sets of data from 32 types of cancer within the TCGA.⁴⁰ The site has three modules: LinkFinder, LinkInterpreter and LinkCompare. The LinkInterpreter module was used to perform GO analysis CC (cellular component), BP (biological process), and MF (molecular function), as well as analysis of KEGG pathway, panther pathway, reactome pathway, and WikiPathway.

Results

The Expression Level of *BTG2* in LUAD Tissues

We compared the *BTG2* expression between malignant cancer and normal tissues using the TIMER database. Compared to normal tissue, the *BTG2* expression significantly decreased in many malignancies, including LUAD (Figure 1A). These results were further confirmed based on the analysis using UALCAN database, with decreased *BTG2* expression in LUAD (Figure 1B).

Relationship Between *BTG2* Expression and Clinicopathological Factors in LUAD

Next, we evaluated the correlations between *BTG2* expression and the clinicopathological factors (cancer stages, status of lymph node metastasis (LNM), age, gender, smoking habits, race). Results from the UALCAN database showed that the *BTG2* expression was significantly associated with cancer stages and LNM in LUAD (Figure 2A and B), while the relationship with age, gender, patient’s smoking habits, and race was of no significance (Figure 2C–F).

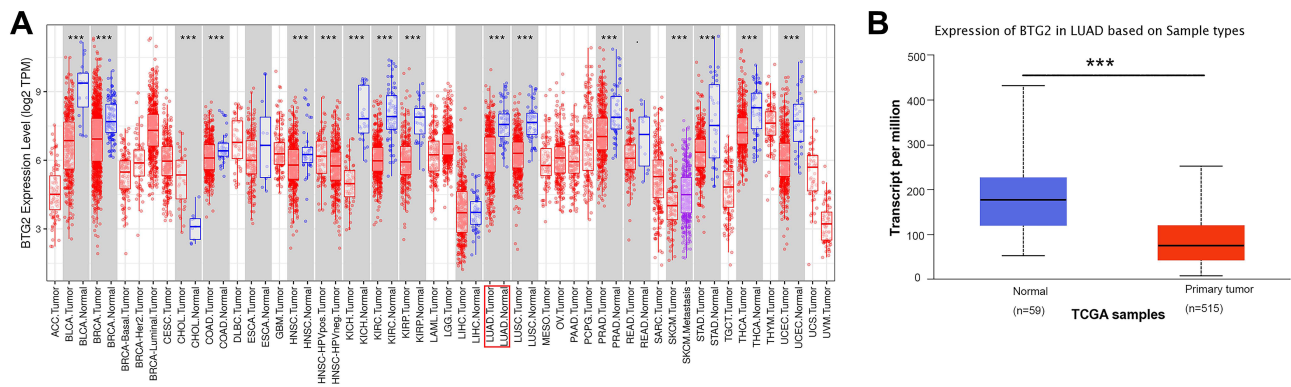


Figure 1 The expression levels of *BTG2* in LUAD tissues. **(A)** The TIMER database showed that *BTG2* expression levels were reduced in most of the different tumor types, especially in LUAD, compared with corresponding normal tissues. **(B)** *BTG2* expression in LUAD was lower than normal tissues in the UALCAN database (** $p < 0.001$). **Abbreviation:** LUAD, lung adenocarcinoma.

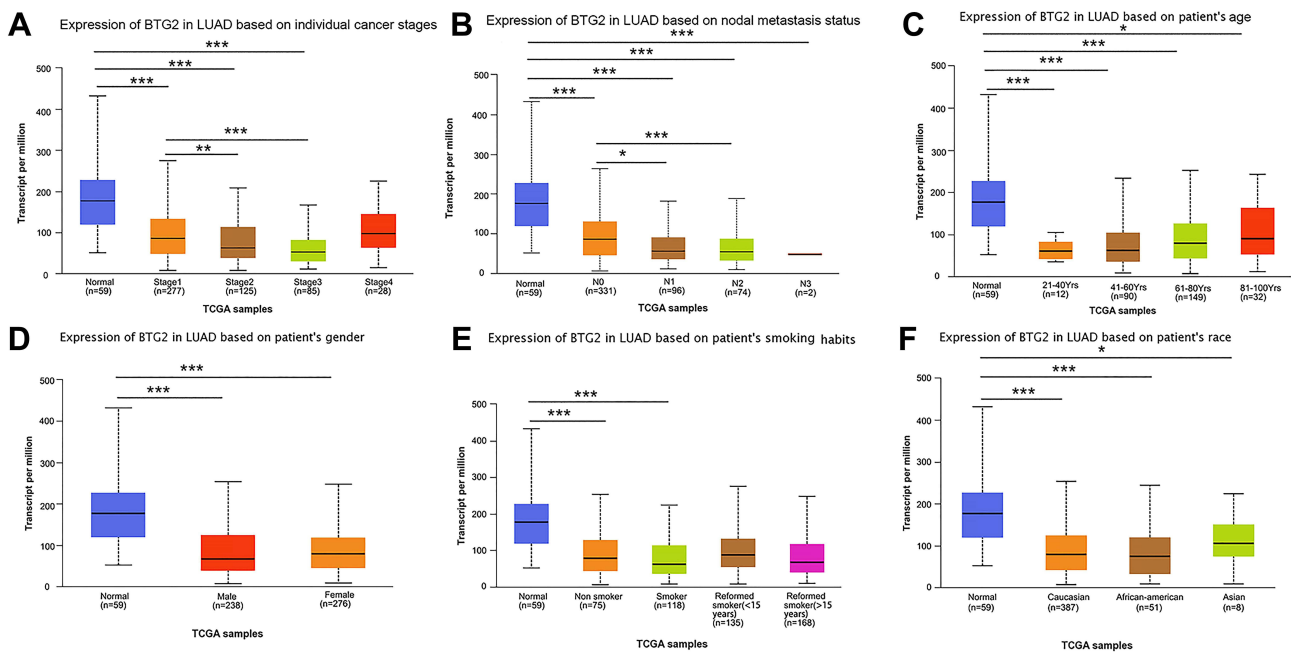


Figure 2 The expressions of *BTG2* in LUAD based on clinicopathological parameters in UALCAN database. **(A–F)** The correlation between *BTG2* expression and cancer stages, nodal metastasis status, age, gender, smoking habits, and race (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Prognostic Analysis of *BTG2* in LUAD

Subsequently, the Kaplan–Meier plotter database was used to assess the prognostic significance of *BTG2* expression in LUAD patients. Kaplan–Meier curves showed that the down-regulated *BTG2* expression in LUAD was significantly associated with short OS duration [HR: 0.44, 95% CI: 0.31–0.61, logrank $P=7.6e-07$] (Figure 3A). In addition, given the correlation between *BTG2* expression and some clinical features, we also further discussed the effect of the interaction between *BTG2* expression and clinical characteristics of LUAD patients on prognosis. Low *BTG2* expression was associated with shorter OS duration in either females or males, in ever-smokers, and in patients with stage I, but the opposite was true in patients receiving chemotherapy (Table 1; $P < 0.05$). The results of Log rank test in GEPIA database also support the negative effect of low *BTG2* expression on OS [HR: 0.4, logrank $P=2.2e-05$] (Figure 3B). To further verify the prognostic value of *BTG2* in LUAD, TCGA database with

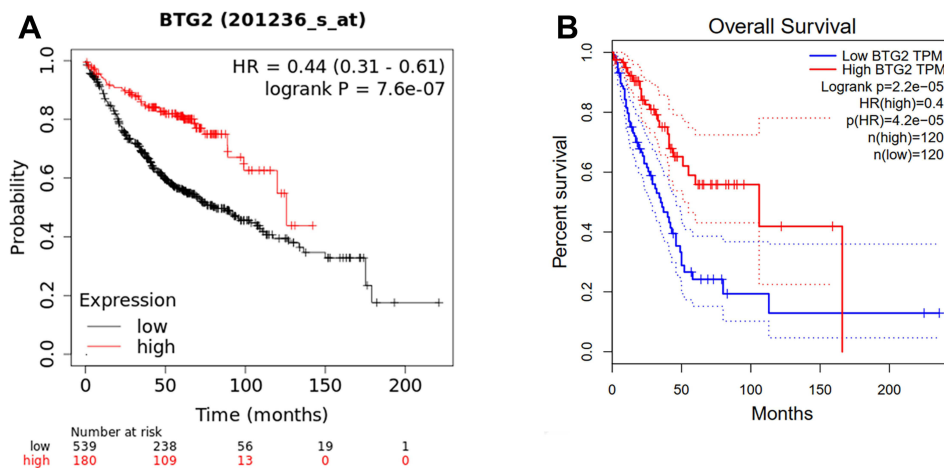


Figure 3 Down-regulated *BTG2* in LUAD was associated with poor prognosis of overall survival in Kaplan–Meier plotter database (A) and GEPIA database (B).

468 LUAD patients was used to perform univariate and multivariate Cox regression analysis. Our results confirmed that *BTG2* was still an independent prognostic factor of OS after adjusting for potential confounders (Table 2). Baseline characteristics of TCGA LUAD patients were presented in Table S1.

Table I The Kaplan–Meier Plotter Analysis of Predictive Effect of *BTG2* Expression on Prognosis in LUAD Based on Clinicopathological Factors

Clinicopathological Factors	OS (n=719)		
	N	HR (95%CI)	p
Gender			
Female	317	0.53(0.31-0.9)	1.7e-02
Male	344	0.38(0.24-0.62)	5.7e-05
Smoking history			
Ever	246	0.29(0.14-0.61)	4.6e-04
Never	143	0.61(0.21-1.8)	0.37
Stage			
1	370	0.27 (0.13-0.53)	4.6e-05
2	136	0.73(0.42-1.29)	0.28
3	24	1.74 (0.58-5.16)	0.31
4	4	–	–
T stage			
T1	123	0.66(0.31-1.39)	0.27
T2	105	0.66(0.33-1.32)	0.24
T3	4	–	–
T4	0	–	–
N stage			
N0	184	0.61 (0.33-1.15)	0.12
N1	44	0.79 (0.33-1.88)	0.59
N2	3	–	–
M stage			
M0	231	0.67 (0.41-1.1)	0.11
M1	1	–	–
Chemotherapy			
No	21	1.3 (0.27-6.33)	0.74
Yes	36	10.16(1.94-53.37)	7.9e-04

Note: *, p<0.05, ***, p<0.001, ****, p<0.0001. Consistent P-value notations are used throughout the paper.
Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval; p, p values.

Table 2 Univariate and Multivariate COX Regression Analysis of OS for *BTG2* from TCGA Cohort (n = 468)

	Characteristics	Univariate Analysis		Multivariate Analysis	
		HR (95%CI)	P	HR (95%CI)	P
Gender	FEMALE VS. MALE	0.91 (0.67-1.2)	0.53	1 (0.69-1.5)	0.91
Age	≥ 65 VS. < 65	1.2 (0.9-1.7)	0.19	1.2 (0.83-1.8)	0.32
Smoking_history	YES VS. NO	0.97 (0.64-1.5)	0.9	1.1 (0.64-1.9)	0.75
T stage	T3-T4 VS. T1-T2	2.1 (1.3-3.2)	0.001	2.3 (1.3-4)	0.0027
N stage	N2-N3 VS. N0-N1	2.2 (1.5-3.2)	<0.001	2.1 (0.91-4.8)	0.082
M stage	M1 VS. M0	2.1 (1.3-3.6)	0.0049	2 (0.83-4.7)	0.12
Pathologic_stage	III-IV VS. I-II	2.4 (1.7-3.3)	<0.001	1 (0.44-2.4)	0.96
Targeted_molecular_therapy	YES VS. NO	1.1 (0.83-1.6)	0.42	0.63 (0.4-0.99)	0.046
History_of_neoadjuvant_treatment	YES VS. NO	14 (5.1-40)	<0.001	9.8 (3.2-31)	<0.001
Radiation_therapy	YES VS. NO	2.4 (1.7-3.5)	<0.001	2.4 (1.5-4)	<0.001
<i>BTG2</i> expression	High VS. Low	0.63 (0.45-0.87)	0.005	0.66 (0.45-0.98)	0.041

Abbreviations: HR, hazard ratio; CI, confidence interval; *p*, *p* values.

Protein–Protein Interaction (PPI) and *BTG2*-Co-Expressed Genes Analysis

In order to elucidate the potential interactions between proteins, the protein–protein interaction (PPI) network was conducted based on the STRING database, and the top 10 *BTG2*-correlated genes were screened by the MCC algorithm in the cytoHubba plug-in of Cytoscape software. The interactions are plotted in **Figure 4A**, and the top 10 *BTG2*-correlated genes were *TP53* (*p53*), *CCND1*, *MDM2*, *HSP90AA1* (*HSP90N*), *CDK2*, *CDK4*, *ATM*, *SIRT1*, *CDK6*, and *BCL2L1* (**Figure 4B**). Further analysis from the Kaplan–Meier plotter database using Log rank test showed that all but *CCND1* [HR : 0.92, 95%=0.73–1.15, logrank *p*=0.45], *HSP90AA1* [HR: 1.02, 95%=0.81–1.29, logrank *p*=0.85], and *CDK6* [HR : 0.94, 95%=0.74–1.2, logrank *p*=0.62] were closely associated with the duration of OS in LUAD. (**Figure 5A–J**).

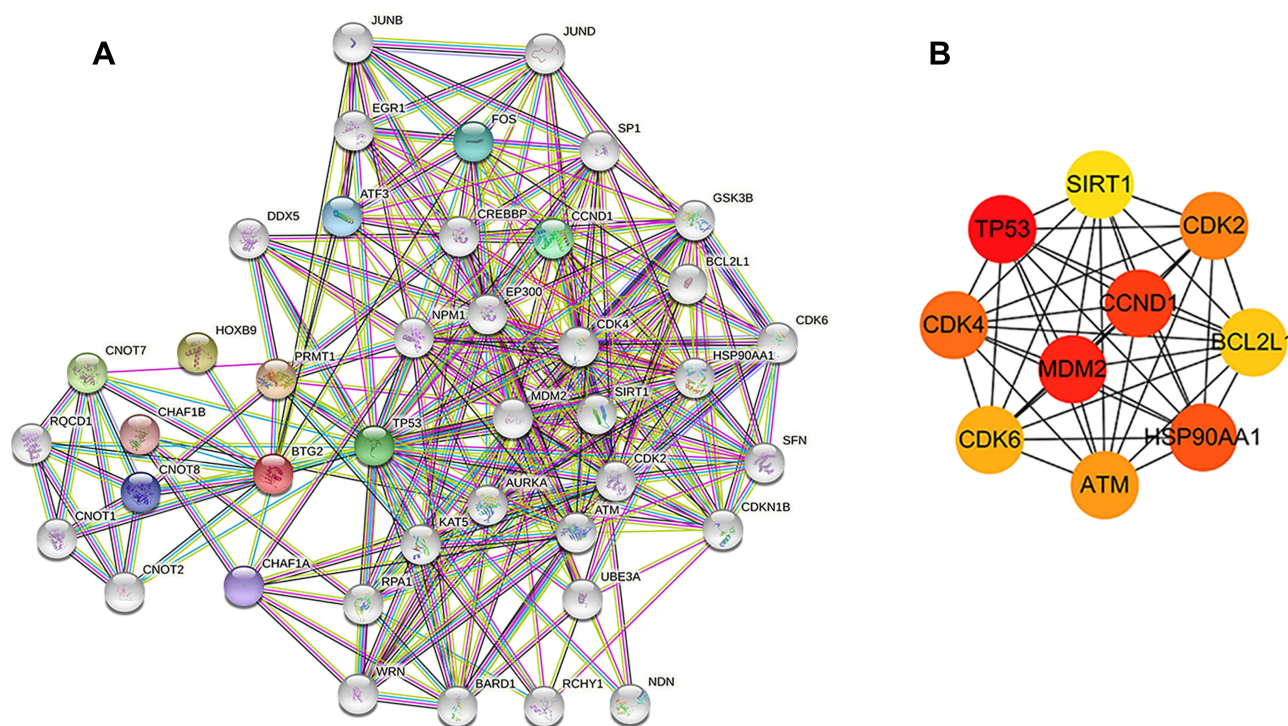


Figure 4 Protein–protein interaction network (PPI). **(A)** The PPI network of *BTG2* was constructed in the STRING database. **(B)** The top ten genes with high degrees were obtained by the MCC algorithm in the cytoHubba plug-in of Cytoscape software.

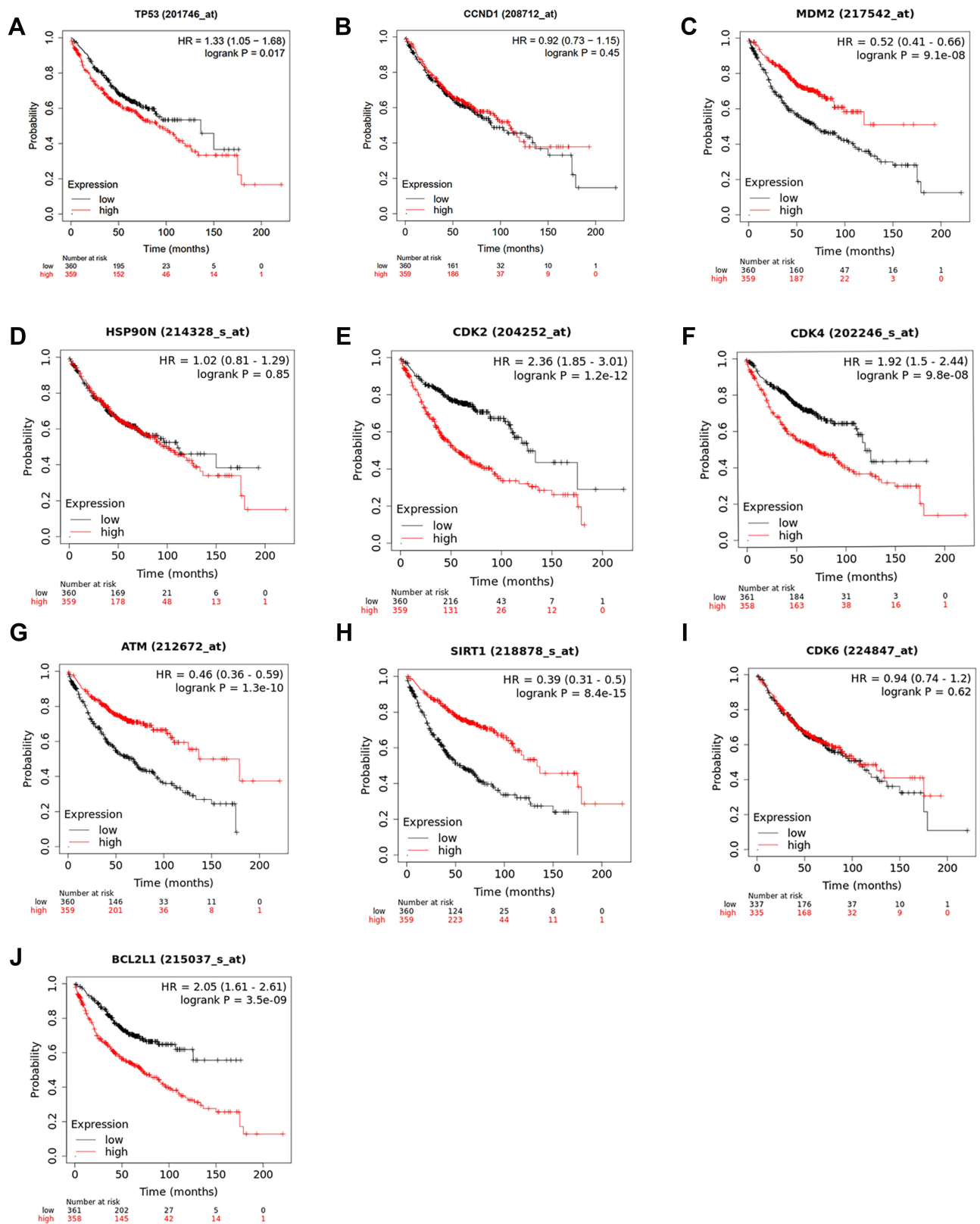


Figure 5 Relationship between co-expression genes and prognosis. (A–J) Overall survival of the top ten co-expression genes was analyzed in the Kaplan–Meier plotter database.

Abbreviation: PPI, protein–protein interaction.

Relationship Between *BTG2* Expression and Key Driver Gene Mutations in LUAD

Key driver gene mutations were closely related to the treatment outcome and prognosis of LUAD.^{41–44} We therefore used the TISIDB database to analyze the correlations between *BTG2* expression and key driver genes in LUAD, including *TP53* (*p53*), *ROS1*, *ALK*, *KRAS*, *EGFR*, *BRAF*, *ERBB2* (*HER-2*), *NRAS* (Figure 6A–H). Our results showed that the down-regulated *BTG2* expression was significantly associated with *TP53* ($p < 0.001$) and *ROS1* ($p < 0.05$) mutations (Figure 6A and B).

Enrichment Analysis of *BTG2* in LUAD

To explore the potential functions and signaling pathways of target genes, GO analysis of BP (biological process), MF (molecular function), and CC (cellular component), KEGG pathway (Kyoto Encyclopedia of Genes and Genomes), Reactome pathway, Panther pathway, and WikiPathway were assessed by using the LinkedOmics database. The biological processes were mainly enriched in transmembrane transport and cell differentiation (Figure 7A). For molecular function, the *BTG2* was enriched primarily in cytokine binding and protein activity (Figure 7B). The cell component enrichment analysis indicated that the *BTG2* was correlated with membranes and endosome lumen (Figure 7C). It is worth noting that the analysis of KEGG pathway, Reactome pathway, Panther pathway, WikiPathway, BP, and MF revealed that *BTG2* was significantly correlated with immune responses and immune signaling pathways, including the production of molecular mediator involved in inflammatory response, interleukin-17 production, leukocyte differentiation, cytokine binding, Th17 cell differentiation, cytokine–cytokine receptor interaction, B cell activation, TGF- β signaling pathway, and B cell receptor signaling pathway (Figures 7A–D and 8A–C).

Relationship of *BTG2* Expression With the Immune Infiltration Levels, Markers of Different Subsets of Immune Cells, and Chemokines

Since *BTG2* was significantly correlated with immune responses and immune signaling pathways, we further analyzed the correlation between *BTG2* expression and the immune infiltration levels using the TIMER database. Our results showed that *BTG2* expression significantly correlated with tumor purity, and the infiltration levels of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophil cells, and dendritic cells in LUAD (Figure 9A). With these promising

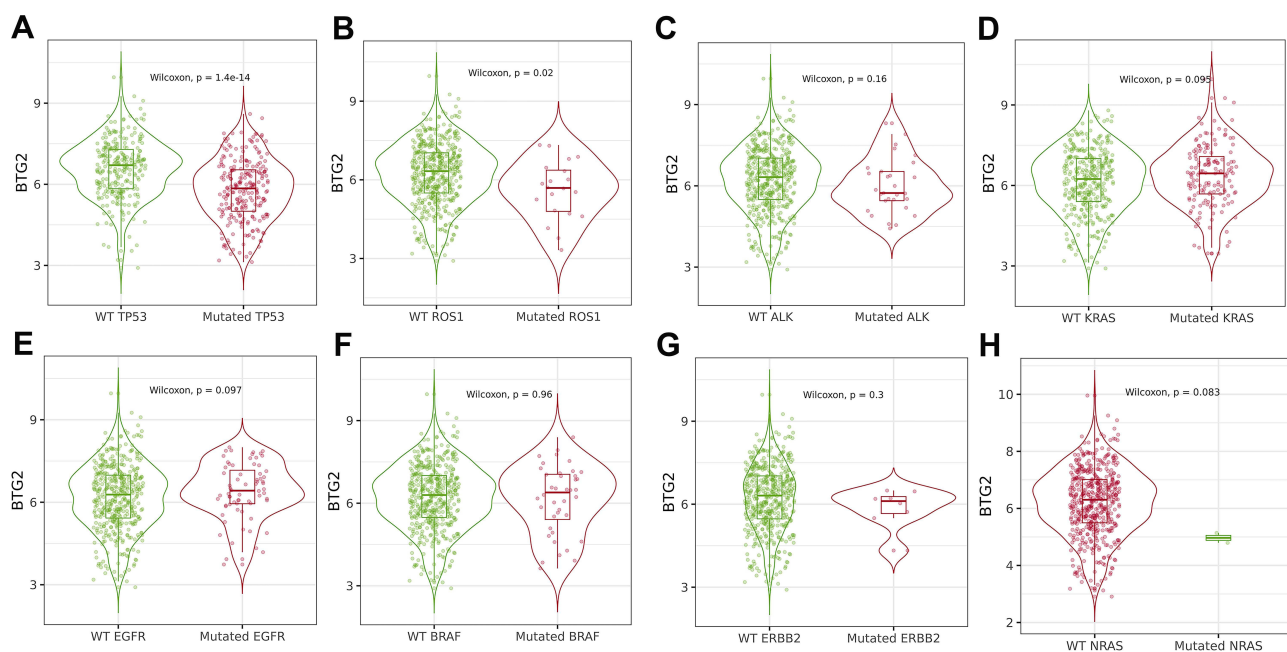


Figure 6 The association between *BTG2* expression and driver genes mutation status in TISIDB database. (A) *TP53* status. (B) *ROS1* status. (C) *ALK* status. (D) *KRAS* status. (E) *EGFR* status. (F) *BRAF* status. (G) *ERBB2* status. (H) *NRAS* status.

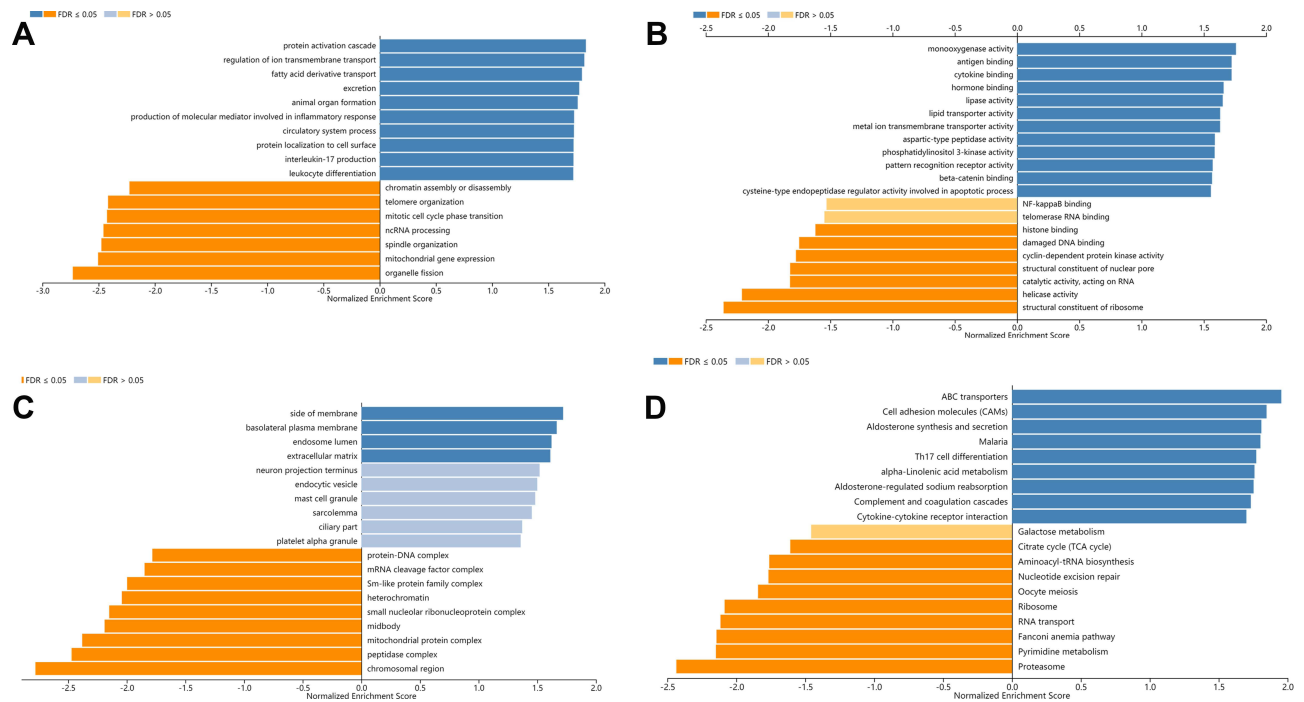


Figure 7 GO and KEGG enrichment analysis of *BTG2* co-expressed genes in LinkedOmics database. **(A)** GO biological process. **(B)** GO molecular function. **(C)** GO cellular components. **(D)** KEGG pathway. Dark blue and orange indicate $FDR \leq 0.05$, light blue and orange indicate $FDR > 0.05$. **Abbreviations:** GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

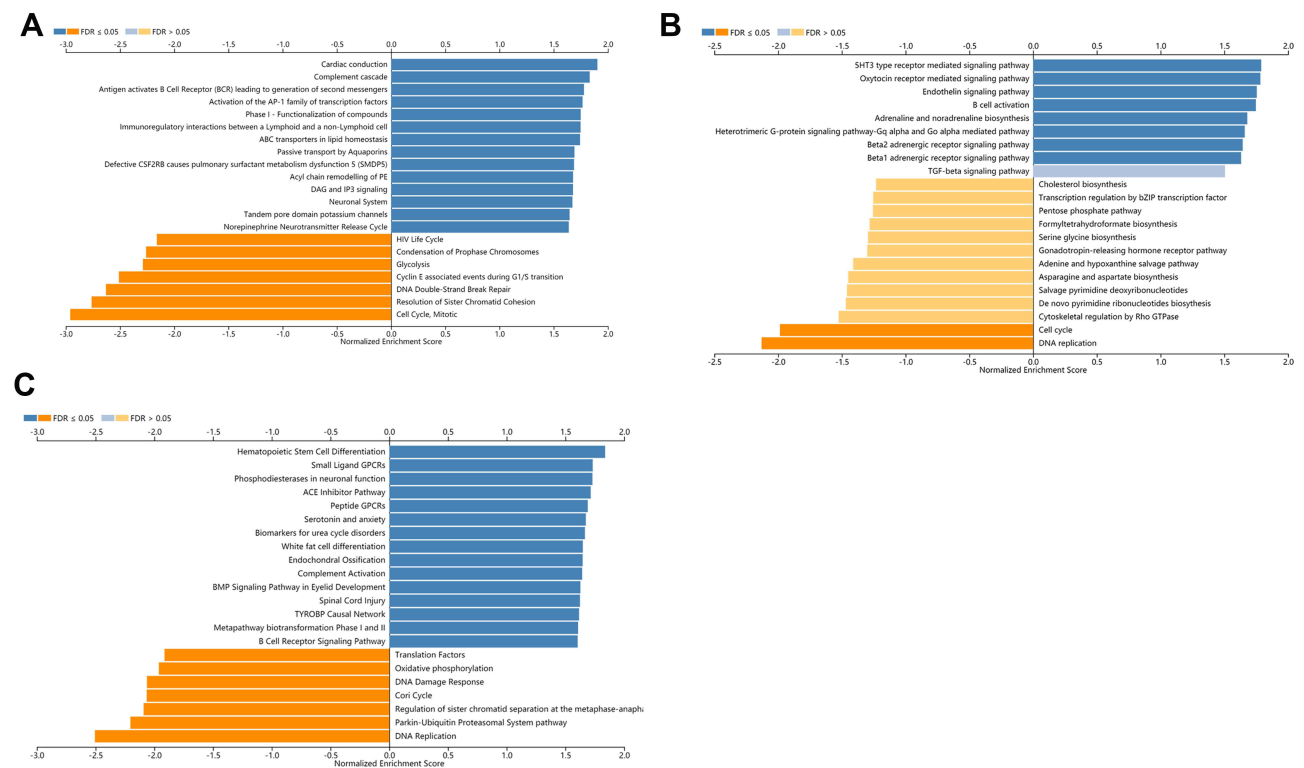


Figure 8 Pathway enrichment analysis of *BTG2* co-expressed genes in LinkedOmics database. **(A)** Reactome pathway. **(B)** Panther pathway. **(C)** WikiPathway. Dark blue and orange indicate $FDR \leq 0.05$, light blue and orange indicate $FDR > 0.05$. **Abbreviation:** FDR, false discovery rate.

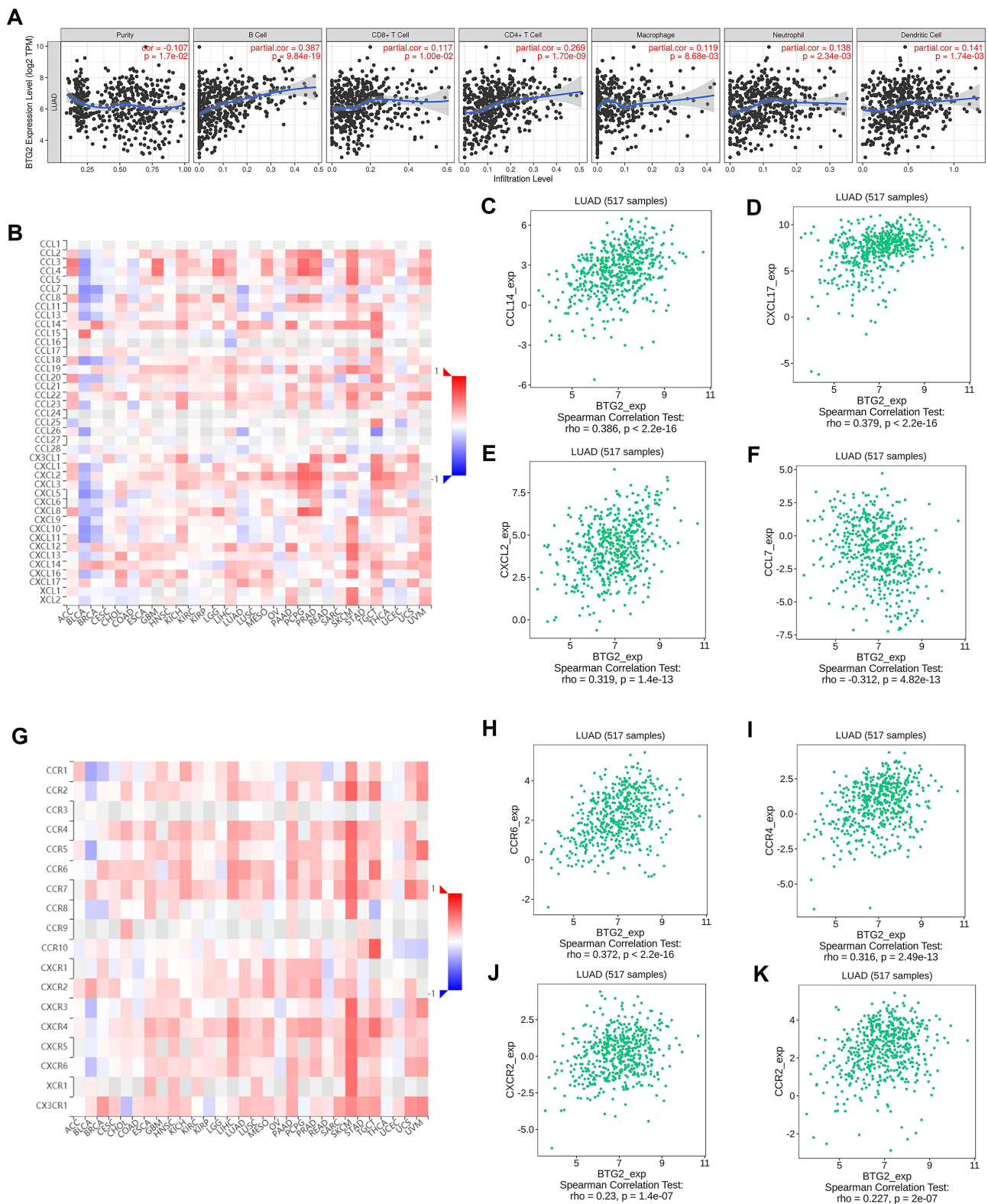


Figure 9 The association between *BTG2* and the immune infiltration levels and chemokines in LUAD. **(A)** Relationship of *BTG2* with tumor purity and infiltrating levels of B cell, CD8+T cell, CD4+T cell, macrophage, neutrophil, and dendritic cell in TIMER database. **(B–F)** The correlation between *BTG2* and chemokines in TISIDB database. **(G–K)** The correlation between *BTG2* and chemokine receptors in TISIDB database.

results, we next focused on the correlation between *BTG2* expression and various immunocytochemical markers of different subsets of immune cells in LUAD, including CD8+T cells, T cells (general), B cells, monocytes, TAMs, M1 and M2 macrophages, neutrophils, natural killer cells, dendritic cells, functional T cells (Th1 cells, Th2 cells, Tfh cells, Th17 cells) and Tregs, as well as exhausted T cells. We found that *BTG2* expression had a significant correlation with most markers of different subsets of immune cells, which mainly concentrated on T cells (general) (CD3D, CD3E and CD2), B cells (CD19 and CD79A), neutrophils (CD66b, CD11b and CCR7), natural killer cells (KIR2DL1 and KIR2DL4), dendritic cells (HLA-DPB1, HLA-DQB1, HLA-DRA, HLA-DPA1, CD1C and CD11c), Th1 cells (TBX21, STAT4, STAT1, and TNF), Th2 cells (STAT6, STAT5A and IL-13), Th17 cells (STAT3 and IL-17A), Tregs (FOXP3, CCR8, STAT5B and TGFB1) (Table 3). Strikingly, we found that immune escape markers such as CTLA4 and GZMB were significantly correlated with *BTG2* expression in LUAD (Table 3). Additionally, we further analyzed the correlation between *BTG2* expression and multiple chemokines and chemokine receptors in LUAD using the TISIDB database (Figure 9B–K). It is worth noting that the chemokines CCL14, CXCL17, CXCL2 and CCL7 (Figure 9C–F), as well as chemokines receptors CCR6, CCR4, CXCR2 and CCR2, were the most relevant adjustment factors to *BTG2* expression in LUAD (Figure 9H–K).

Validation of Single Cell Sequencing

Considering the heterogeneity of different cells, we further validated the expression of *BTG2* in LUAD tissue and different immune cells by single cell sequencing. As shown in Figure 10A, we filtered single cells of LUAD that expressed too many and too few genes. According to standard deviation, 15 PC with relatively gentle dimensions were selected for dimensionality reduction (Figure 10B). Based on PCA results, we mapped the expression of *BTG2* gene in LUAD patients and immune cell infiltration, which showed *BTG2* expression was highly expressed in NK cells, T cells, B cells, monocytes, macrophages, and neutrophils (Figure 10C and D).

Discussion

BTG2 maps within the chromosomal segment (1q32),¹⁰ and belongs to the BTG/TOB gene family that regulates cell cycle, cell proliferation, differentiation, and apoptosis.^{9,45,46} *BTG2* is described as an immediate early gene^{47,48} and regulated by p53 activation.¹² Previous studies have reported low expression of *BTG2* in a variety of malignant tumors, including NSCLC,²¹ breast cancer,⁴⁹ laryngeal carcinoma,⁵⁰ prostate cancer,⁵¹ renal cell carcinoma,^{21,49–52} and closely associated with poor outcomes.^{21–23} Down-regulated *BTG2* expression can promote tumorigenesis and progression through activating proliferation, migration, and invasion of tumor cells, while upregulated *BTG2* expression generates the opposite effect.^{53–55} For example, MiR-25-3p was found to promote the proliferation of triple negative breast cancer⁵⁶ by targeting *BTG2*. Li et al⁵⁷ found that miR-365 promoted pancreatic ductal adenocarcinoma development by suppressing *BTG2* expression. Lin et al⁵⁸ found that miR-1278 inhibited metastasis of colorectal cancer via upregulation of *BTG2*. Tsui et al⁵⁹ reported that *BTG2* overexpression inhibited the growth of bladder cancer cells. Huang and colleagues found that down-regulated *BTG2* expression promoted progression through inhibiting cancer stem cell-like features of side population cells in hepatocellular carcinoma, and low expression of *BTG2* was associated with shorter OS and disease-free survival.⁶⁰ Wang et al⁶¹ reported that *BTG2* was abnormally decreased in esophageal squamous cell carcinoma (ESCC) patients, and a more pronounced decrease was found in radiotherapy-resistant patients as compared with radiotherapy-sensitive patients, suggesting that downregulation of *BTG2* was able to predict the progression and radioresistance in ESCC. Recent investigations also reported that *BTG2* overexpression could inhibit the proliferation and invasiveness of LUAD cells,⁶² while circular RNA circCRIM1 was able to promote *BTG2* expression to suppress the migration, invasion, EMT, and glycolysis of LUAD cell.²⁵ Wang et al further confirmed that hypomethylation and high expression of *BTG2* significantly correlated with improved survival outcome in patients with LUAD.⁶³ Additionally, some studies have found that BTG2 played an important role in tumor immune regulation.^{26–30} These studies suggested that BTG2 might be a potential prognostic immune-related biomarker for LUAD, but the role of BTG2 in LUAD has not been reported. In this study, we observed that *BTG2* was significantly downregulated in LUAD. Decreased expression of *BTG2* in LUAD was significantly correlated with advanced cancer stages and nodal metastasis. Further results showed that low BTG2 expression was associated with worse OS in LUAD. Besides, results based on

Table 3 Correlation Analysis Between BTG2 and Markers of Immune Cells in Lung Adenocarcinoma from the TIMER Database

Description	Gene Markers	None		Purity	
		Cor	p	Cor	p
CD8 ⁺ T cell	CD8A	0.094	*	0.068	0.133
	CD8B	0.09	*	0.067	0.138
T cell (general)	CD3D	0.142	**	0.111	*
	CD3E	0.232	****	0.222	****
	CD2	0.209	****	0.188	****
B cell	CD19	0.288	****	0.283	****
	CD79A	0.306	****	0.308	****
Monocyte	CD86	0.098	*	0.06	0.186
	CD115 (CSF1R)	0.16	***	0.13	**
TAM	CCL2	0.062	0.157	0.022	0.627
	CD68	0.068	0.121	0.04	0.375
	IL-10	0.179	****	0.15	***
M1 Macrophage	INOS (NOS2)	0.121	**	0.105	*
	IRF5	-0.017	0.704	-0.049	0.278
	COX2 (PTGS2)	0.053	0.233	0.069	0.125
M2 Macrophage	CD163	0.103	*	0.071	0.117
	VSIG4	0.06	0.175	0.027	0.553
	MS4A4A	0.118	**	0.08	0.0742
Neutrophils	CD66b (CEACAM8)	0.258	****	0.253	****
	CD11b (ITGAM)	0.139	**	0.107	*
	CCR7	0.295	****	0.284	****
Natural killer cell	KIR2DL1	0.121	**	0.109	*
	KIR2DL3	0.041	0.352	0.018	0.687
	KIR2DL4	-0.117	**	-0.127	**
	KIR3DL1	0.079	0.073	0.061	0.179
	KIR3DL2	0.044	0.319	0.027	0.548
	KIR3DL3	-0.028	0.525	-0.033	0.466
	KIR2DS4	0.085	0.053	0.071	0.115
Dendritic cell	HLA-DPBI	0.301	****	0.287	****
	HLA-DQBI	0.188	****	0.152	***
	HLA-DRA	0.226	****	0.202	****
	HLA-DPAI	0.268	****	0.249	****
	BCDA-1 (CD1C)	0.323	****	0.299	****
	BDCA-4 (NRPI)	0.095	*	0.084	0.0632
	CD11c (ITGAX)	0.198	****	0.185	****
Th1	T-bet (TBX21)	0.176	****	0.157	***
	STAT4	0.17	***	0.144	**
	STAT1	-0.074	0.0934	-0.098	*
	IFN- γ (IFNG)	-0.036	0.418	-0.062	0.173
	TNF- α (TNF)	0.182	****	0.149	***
Th2	GATA3	0.053	0.228	0.009	0.844
	STAT6	0.311	****	0.318	****
	STAT5A	0.241	****	0.223	****
	IL-13	0.137	**	0.115	*
Tfh	BCL-6	0.225	****	0.238	****
Th17	IL-21	0.07	0.11	0.055	0.226
	STAT3	0.299	****	0.307	****
	IL-17A	0.108	*	0.104	*

(Continued)

Table 3 (Continued).

Description	Gene Markers	None		Purity	
		Cor	p	Cor	p
Treg	FOXP3	0.15	***	0.118	**
	CCR8	0.212	****	0.183	****
	STAT5B	0.34	****	0.331	****
	TGF- β 1 (TGFB1)	0.113	*	0.096	*
Exhausted T cells	PD-1 (PDCDI)	0.067	0.13	0.044	0.329
	CTLA4	0.192	****	0.172	***
	LAG3	0.051	0.25	0.033	0.467
	TIM-3 (HAVCR2)	0.062	0.159	0.017	0.706
	GZMB	-0.075	0.0882	-0.109	*

Notes: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Abbreviations: Cor, R value of Spearman correlation; p, p values; TAM, tumor-correlated macrophage; Th1, T helper 1 cell; Th2, T helper 2 cell; Tfh, follicular helper T cell; Th17, T helper type 17 cell; Treg, regulatory T cell.

a PPI network reveal that the top 10 *BTG2*-correlated genes were *TP53* (*p53*), *CCND1*, *MDM2*, *HSP90AA1* (*HSP90N*), *CDK2*, *CDK4*, *ATM*, *SIRT1*, *CDK6*, and *BCL2L1*. Previous studies demonstrated that these *BTG2*-related co-expressed genes were closely involved in tumorigenesis and progression.^{64–70} Consistently, our further study showed that all *BTG2*-related co-expressed genes in LUAD but *CCND1*, *HSP90AA1*, and *CDK6* were closely associated with the duration of overall survival. Collectively, all the findings above suggested that *BTG2* played a key role as a tumor suppressor and might become a promising prognostic biomarker as well as a molecular target for patients with LUAD.

It is well-known that classic driver gene mutations largely determine the therapeutic approaches and prognosis in patients with LUAD. Previous studies have reported that *EGFR* and *ALK* mutations were associated with prognostic markers of LUAD.⁷¹ *EGFR* has been shown to be associated with immune cell infiltration and overall survival in LUAD.⁷² We thus did correlation analysis between *BTG2* expression and key driver gene mutations, including *TP53* (*p53*), *EGFR*, *ROS1*, *ALK*, *KRAS*, *BRAF*, *ERBB2* (*HER-2*), and *NRAS*. The analysis showed that down-regulated *BTG2* was significantly associated with *TP53* and *ROS1* mutations. It has been confirmed that *TP53* mutation is one of the most frequently mutated genes in LUAD.^{73,74} *TP53* mutation acquires oncogenic features, which may trigger chromosomal/genomic instability and further leads to a high tumor mutation burden, eventually resulting in more aggressive malignancy and worse clinical outcome in LUAD.^{74–78} *ROS1* rearrangement, the major type of *ROS1* gene alteration, has been confirmed as a vital biomarker for targeted therapy in advanced LUAD.^{79–81} Strikingly, recent studies highlighted that both *TP53* mutation and *ROS1* rearrangement but not *EGFR* mutation frequently overlapped with high PD-L1 expression in LUAD. Moreover, a study has reported that *TP53* mutation was associated with immune-related prognostic markers of LUAD.⁸² Ge et al⁸³ found that *TP53* mutation significantly varied with immune scores. In this study, the *BTG2* was correlated to *TP53* and *ROS1* mutations, but not *EGFR*, indicating that *BTG2* might be involved in the effect of *TP53*- or *ROS1*-mutations on immune activity, and closely associated with immunity.^{44,77,78,84,85}

We next conducted the enrichment analyses, and the results revealed that *BTG2* was closely related to multiple immune-related processes and pathways including interleukin-17 production, leukocyte differentiation, cytokine binding, Th17 cell differentiation, cytokine–cytokine receptor interaction, B cell activation, TGF- β signaling pathway, B cell receptor signaling pathway. Based on these findings, we further analysed the associations between *BTG2* expression and immune infiltrates in LUAD. We observed an obvious positive correlation between *BTG2* expression and the infiltrated level of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and DCs in LUAD, which suggested that high *BTG2* expression might up-regulate the abundance of immune-infiltrating cells. Consistently, relevant studies have shown that *BTG2* was directly able to regulate the proliferation, development, and differentiation of immune infiltrating cells. For example, the *BTG2*-PRMT1 protein complex was found to antagonize the proliferation of pre-B cells to promote the development of B cells.^{27,28} In a MO5 melanoma transplantation model, *BTG2* was found to transiently regulate anti-tumor immunity by affecting the

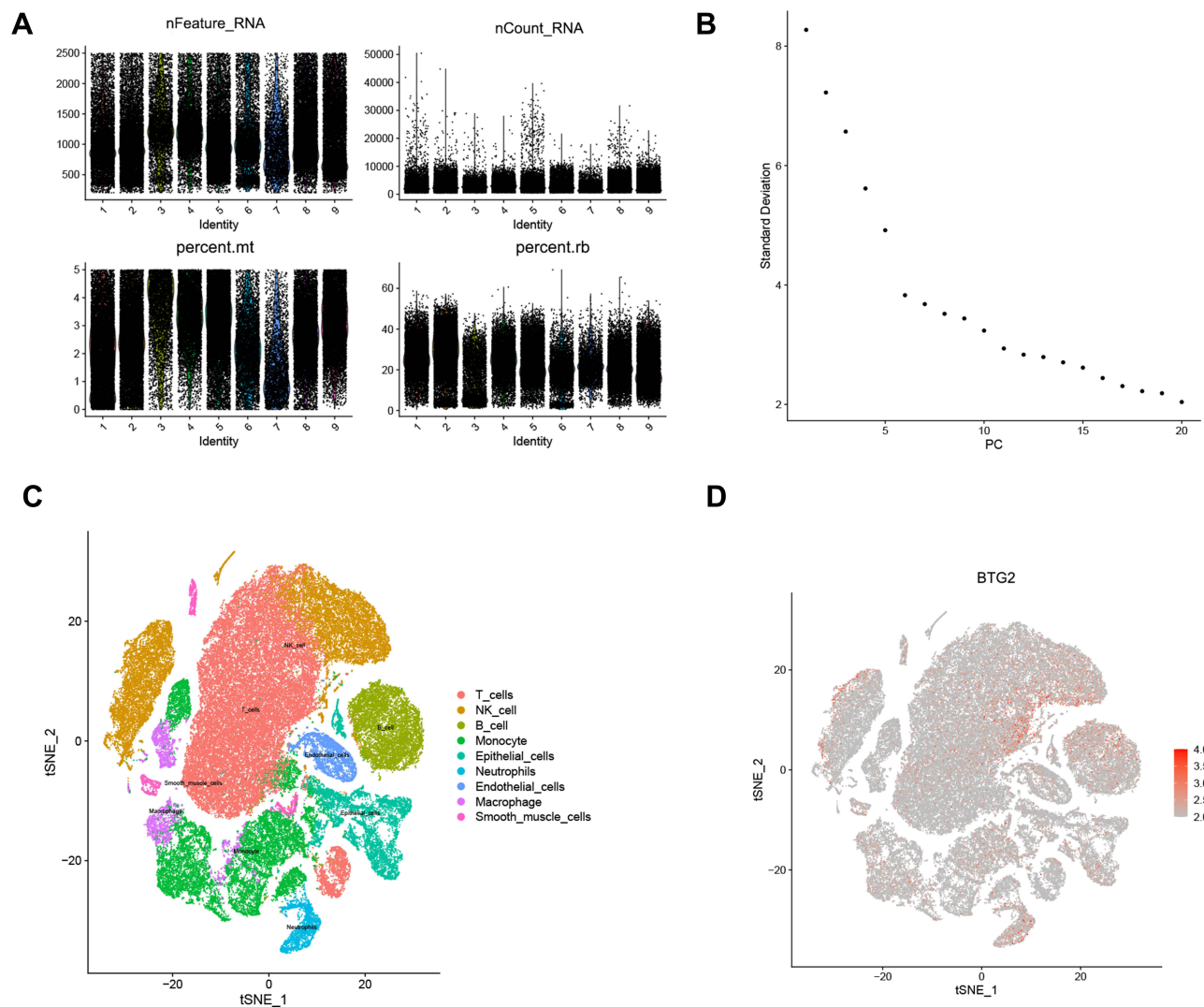


Figure 10 Validation of the expression by single cell RNA-seq. **(A)** Quality control of LUAD data. **(B)** Standard deviation diagram of principal components. **(C)** Cell annotation of tSNE cluster graph (color-coded by clusters, cell subsets). **(D)** tSNE plot of *BTG2* expression (red represents high gene expression).
Abbreviations: mt, mitochondria; rb, ribosome; tSNE, t-distributed stochastic neighbor embedding.

frequencies of granzyme B+ CD8+ T-cells and CD107a+ CD8+ T-cells in vivo.³⁰ Research from Passeri et al⁸⁶ identified *BTG2* as a regulator of differentiations of myelocytic leukemia cells and CD34+ hematopoietic precursor cells. Considering the heterogeneity of different cells, we further validated the expression of *BTG2* in different immune cells by single cell sequencing, finding that *BTG2* expression was highly expressed in NK cells, T cells, B cells, monocytes, macrophages, and neutrophils in the LUAD immune microenvironment. Similarly, Terra et al⁸⁷ also found that *BTG2* was highly expressed in immune organs such as thymus and spleen. These findings further enhanced the reliability of *BTG2* regulation of immune cell infiltration in LUAD. Based on the close relationship between *BTG2* and immune cell infiltration, we further demonstrated that the *BTG2* expression had strong correlations with various marker sets, including T cells (general) (CD3D, CD3E and CD2), B cells (CD19 and CD79A), neutrophils (CD66b, CD11b and CCR7), dendritic cells (HLA-DPB1, HLA-DQB1, HLA-DRA, HLA-DPA1, CD1C and CD11c), Th1 cells (TBX21, STAT4, STAT1, and TNF), Th2 cells (STAT6, STAT5A and IL-13), Th17 cells (STAT3 and IL17A), Tregs (FOXP3, CCR8, STAT5B and TGFB1). Immune checkpoint inhibitors, including cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and PD-1/PD-L1 inhibitors, have been shown to have dramatic anti-tumor activity in a variety of solid tumors, immune-related biomarkers therefore attract intense interest.^{88–91} In our study, significant correlations were found between *BTG2* expression and T cell exhaustion markers including CTLA-4

and GZMB. Further exploration of the potential role of *BTG2* in immune marker may benefit patients treated with immune checkpoint inhibitors. Altogether, these data reveal a critical role of *BTG2* on immune infiltration in LUAD.

As we know, different immune infiltrating cells are recruited by different chemokines and chemokine receptors in the tumor microenvironment.^{92–95} Liu et al⁹⁶ found that CC chemokine receptors might be immunotherapeutic targets and inflammation-related prognostic biomarkers in LUAD. Yu et al⁹⁷ also reported that CXC chemokines were related to prognostic value. Based on these findings, we analyzed and demonstrated that the *BTG2* expression was closely related to the levels of many chemokines (CCL14, CXCL17, CXCL2, and CCL7, etc) and receptors (CCR6, CCR4, CXCR2, and CCR2, etc). Evidently, CCL14 was reported to induce macrophage bone marrow homing, proliferation, and polarization in multiple myeloma.⁹⁸ CXCL17 was able to recruit CD11b+Gr1 high F4/80-cells and accelerated tumor development.⁹⁹ Xu et al¹⁰⁰ found that ILC2s promoted hepatocellular carcinoma progression via CXCL2-neutrophil induced immunosuppression. Zhang et al¹⁰¹ reported that CCL7 recruited cDC1 to promote antitumor immunity in NSCLC. Lian and colleagues found that Eomes recruited Treg cells through the CCL20-CCR6 pathway, thereby promoting esophageal carcinoma progression.¹⁰² CCR4 has been shown to recruit Th2 cells which could induce the further expression of CCR4 ligands.¹⁰³ Nywening et al¹⁰⁴ revealed that myeloid recruitment in pancreatic ductal adenocarcinoma was disrupted by targeting both tumour-associated CXCR2+ neutrophils and CCR2+ macrophages. Based on our results and these previous researches, it is conceivable to hypothesize that *BTG2* plays a vital role in the regulation of chemokine-mediated immune infiltration in LUAD.

Inevitably, there are some limitations in our studies. First, due to this research based on the analysis of multiple public databases, more prospective clinical trials should verify the prognostic value of *BTG2*. Second, further effective external experiments through LUAD cell lines and molecular biological methods are needed to clarify the mechanism of *BTG2* in LUAD immunity. Thirdly, considering the limited predictive value of single gene, further studies on *BTG2* single gene combined with multi-gene or multi-group analysis are needed.

Conclusion

In conclusion, our findings have effectively demonstrated that *BTG2* expression is downregulated in LUAD, which is associated with higher cancer stage, and predicts poor prognosis. With close relationship with immune cell infiltration and tumor immune regulation, *BTG2* may be a promising immune-related biomarker and molecular target for patients with LUAD.

Data Acquisition

The databases used in our study include the following: TIMER (<https://cistrome.shinyapps.io/timer/>), UALCAN (<http://ualcan.path.uab.edu/>), The Kaplan–Meier plotter (<https://kmplot.com/analysis/>), GEPIA (<http://gepia.cancer-pku.cn>), STRING (<https://www.string-db.org/>), TISIDB (<http://cis.hku.hk/TISIDB>), LinkedOmics (<http://linkedomics.org/login.php>) databases.

Statement of Ethics

This study was exempted from ethics by the Ethics Committee of Guangxi Medical University Cancer Hospital.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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