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.⁵⁻⁶ 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.\textsuperscript{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 (\textit{BTG2}), a member of the anti-proliferative BTG/TOB gene family,\textsuperscript{9} maps within a chromosomal segment (1q32)\textsuperscript{10} and contains two regions named box A and box B.\textsuperscript{11} \textit{BTG2} is closely involved in a variety of biological processes, such as cell cycle,\textsuperscript{12} cell senescence,\textsuperscript{13} cell differentiation,\textsuperscript{14} hematopoiesis,\textsuperscript{15} oxidative damage,\textsuperscript{16} gene transcription,\textsuperscript{17} and DNA damage repair.\textsuperscript{18} Noteworthily, accumulating evidence has revealed that down-regulated \textit{BTG2} expression plays a vital role in tumor occurrence and development.\textsuperscript{19,20} Several studies reported that the downregulation of \textit{BTG2} was associated with the poor prognosis of cancers including NSCLC, breast cancer, hepatocellular carcinoma and bladder cancer.\textsuperscript{21–24} Zhang and colleagues reported that circular RNA circCRIM1 was able to promote \textit{BTG2} expression via down-regulating miR-125b-5p expression, thereby suppressing the migration, invasion, epithelial-mesenchymal transformation (EMT), and glycolysis in lung adenocarcinoma cell.\textsuperscript{25} These studies suggested that \textit{BTG2} was a potential prognostic marker for cancer. Recent experimental data demonstrated that \textit{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.\textsuperscript{26–30} However, the prognostic value of \textit{BTG2} and its correlation with immune infiltrates in LUAD remain unclarified.

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

Materials and Methods

\textbf{TIMER Database}

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

\textbf{UALCAN Database}

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

\textbf{Kaplan–Meier Plotter Database}

The Kaplan–Meier plotter (\textit{https://kmplot.com/analysis/}) is capable of assessing the effect of 54k genes (mRNA, miRNA, protein) on survival in 21 cancer types.\textsuperscript{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.\textsuperscript{34} We used Kaplan–Meier Plotter database to determine the prognostic role of \textit{BTG2} in overall survival (OS). Based on the upper quartile of \textit{BTG2} expression, LUAD patients were divided into high and low groups. The prognostic value of \textit{BTG2}-co-expressed genes for LUAD was also analyzed using this database.

\textbf{GEPIA Database}

\textit{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.\textsuperscript{35} Survival analysis was performed using the python package lifelines.\textsuperscript{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. 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. We constructed a protein–protein interaction (PPI) network information, and co-expressed genes were identified using the Cytoscape 3.8.2 version software.

**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. 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. 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).
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 \( P = 7.6 \times 10^{-7} \)] (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 \( P = 2.2 \times 10^{-5} \)] (Figure 3B). To further verify the prognostic value of BTG2 in LUAD, TCGA database with...
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.

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

### Table 1: 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.
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 ten genes with high degrees were obtained by the MCC algorithm in the cytoHubba plug-in of Cytoscape software.

**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 |
| BTG2 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.

**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 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 ≤ 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 ≤ 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 chemokine 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), and belongs to the BTG/TOB gene family that regulates cell cycle, cell proliferation, differentiation, and apoptosis. BTG2 is described as an immediate early gene and regulated by p53 activation. Previous studies have reported low expression of BTG2 in a variety of malignant tumors, including NSCLC, breast cancer, laryngeal carcinoma, prostate cancer, renal cell carcinoma, and belongs to the BTG/TOB gene family that regulates cell cycle, cell proliferation, differentiation, and apoptosis. Additionally, some studies have found that BTG2 played an important role in tumor immune regulation. 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

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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 | *     | 0.069 | 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 | ****  |
|                           | CD11 b (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-DPB1| 0.301 | ****  | 0.287 | ****  |
|                           | HLA-DQB1| 0.188 | ****  | 0.152 | ****  |
|                           | HLA-DRA | 0.226 | ****  | 0.202 | ****  |
|                           | HLA-DPA1| 0.268 | ****  | 0.249 | ****  |
|                           | BCDA-1 (CD1C)  | 0.323 | ****  | 0.299 | ****  |
|                           | BDCA-4 (NRPI1) | 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 | *     |
| Th17                      | BCL-6  | 0.225 | ****  | 0.238 | ****  |
|                           | IL-21  | 0.07  | 0.11  | 0.055 | 0.226 |
|                           | STAT3  | 0.299 | ****  | 0.307 | ****  |
|                           | IL-17A | 0.108 | *     | 0.104 | *     |

(Continued)
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. 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. EGFR has been shown to be associated with immune cell infiltration and overall survival in LUAD. 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 ROSI mutations. It has been confirmed that TP53 mutation is one of the most frequently mutated genes in LUAD. 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. ROSI rearrangement, the major type of ROSI gene alteration, has been confirmed as a vital biomarker for targeted therapy in advanced LUAD. Strikingly, recent studies highlighted that both TP53 mutation and ROSI 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. Ge et al found that TP53 mutation significantly varied with immune scores. In this study, the BTG2 was correlated to TP53 and ROSI mutations, but not EGFR, indicating that BTG2 might be involved in the effect of TP53- or ROSI-mutations on immune activity, and closely associated with immunity.

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. In a MO5 melanoma transplantation model, BTG2 was found to transiently regulate anti-tumor immunity by affecting the

| Description | Gene Markers | None | Cor | p | Purity | Cor | p |
|-------------|-------------|------|-----|---|-------|-----|---|
| Treg        | FOXP3       | 0.15 | *** | 0.118 | **   | 0.118 | **   |
|             | CCR8        | 0.212 | **** | 0.183 | **** | 0.183 | **** |
|             | STAT5B      | 0.34  | **** | 0.331 | **** | 0.331 | **** |
|             | TGF-βI (TGFB1) | 0.113 | * | 0.096 | * | 0.096 | * |
|             | PD-1 (PDCD1) | 0.067 | 0.13 | 0.044 | 0.329 | 0.044 | 0.329 |
|             | CTLA4       | 0.192 | **** | 0.172 | ***   | 0.172 | ***   |
|             | LAG3        | 0.051 | 0.25 | 0.033 | 0.467 | 0.033 | 0.467 |
|             | TIM-3 (HAVCR2) | 0.062 | 0.159 | 0.017 | 0.706 | 0.017 | 0.706 |
|             | GZMB        | -0.075 | 0.0882 | -0.109 | * | -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.
frequencies of granzyme B+ CD8+ T-cells and CD107a+ CD8+ T-cells in vivo.\textsuperscript{30} Research from Passeri et al\textsuperscript{86} 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 al\textsuperscript{87} 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 TGFβ1). 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.\textsuperscript{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. Liu et al. found that CC chemokine receptors might be immunotherapeutic targets and inflammation-related prognostic biomarkers in LUAD. Yu et al. 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, CXCIR2, and CCR2, etc). Evidently, CCL14 was reported to induce macrophage bone marrow homing, proliferation, and polarization in multiple myeloma. CXCL17 was able to recruit CD11b+Gr1 high F4/80-cells and accelerated tumor development. Xu et al. found that ILC2s promoted hepatocellular carcinoma progression via CXCL2-neutrophil induced immunosuppression. Zhang et al. 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. CCR4 has been shown to recruit Th2 cells which could induce the further expression of CCR4 ligands. Nywening et al. 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.
The authors report no conflicts of interest in this work.

Disclosure

The authors report no conflicts of interest in this work.

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