Butyrophilin-Like 9 Regulates Immune Infiltration and Serves as a Prognostic Marker in Lung Adenocarcinoma

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Abstract

Background: Butyrophilin (BTN) and butyrophilin-like (BTLN) belong to immunoglobulin superfamily, and also pertain the B7 co-stimulatory molecules family, which has multiple roles in immune modulation. Whether the BTNL9 expression in lung adenocarcinoma (LUAD) correlates with outcome has not been evaluated.

Methods: Oncomine and GEPIA were used to analyze the BTNL9, while its mRNA expression in LUAD and corresponding adjacent tissues was investigated using TIMER. The clinical prognosis of BTNL9 was assessed in the GEPIA, Kaplan-Meier plotter, and OncoLnc. Besides, the correlation between BTNL9 and tumor-infiltrating immune cells (TILs) was analyzed using TIMER and GEPIA. The correlation between BTNL9 expression and drug response was analyzed using CARE.

Results: BNL9 expression was significantly low in LUAD. Low BTNL9 expression was associated with poor OS, and its expression was found to be regulated by both epigenetic regulation and post-transcriptional modification. BTNL9 expression was significant positively correlated with ImmuneScore and ESTIMATEScore. Moreover, BTNL9 expression was positively correlated with infiltrating levels of B cells, CD4+T, and macrophages, but Kaplan-Meier analysis showed that BTNL9 expression in B cells and DCs was significantly associated with OS. Furthermore, BTNL9 expression has significant positive CARE scores.

Conclusions: Low BTNL9 expression can prevent the infiltration of naïve B cells and DCs in the tumor microenvironment and worsen the outcome in LUAD patients. Besides, these findings also suggest the potential role of BTNL9 as a prognostic biomarker and a new immuno-target.

Background

GLOBOCAN 2018 estimated the number of new lung cancer cases and mortality in lung cancer to be 2,093,876 and 1,761,007, respectively[1]. Besides, lung cancer accounts for 11.6% of the total cancer cases and 18.4% of the total cancer deaths, hence it is considered the most frequently diagnosed cancer[1]. In China, lung cancer is also the most commonly diagnosed and a leading cause of cancer-related deaths [2]. LUAD and lung squamous cell carcinoma (LUSC) are the most common histological subtypes of non-small cell lung cancer (NSCLC), accounting for approximately 85% of all the subtypes[3]. LUAD has been considered the most prevalent histopathological subtype since 1985[4]. The development of immunotherapy has significantly improved the prognosis of LUAD patients. However, the 5-year survival rate of LUAD remains disappointing[4]. Therefore, new immuno-targets and treatment strategies require to be urgently developed.

Butyrophilin (BTN) and butyrophilin-like (BTLN) belong to the immunoglobulin superfamily and are also related to the B7 family of co-stimulatory molecules, which play a significant role in the T cell suppression, regulation of epithelial cell and T cell interplays[5]. The human BTN genes are located in the MHC class II domain of the short arm of chromosome 6 (6p22.1) and include three subfamilies, which form phylogenetically related groups: BTN1, BTN2, and BTN3. BTN1A1 belongs to BTN1, BTN2A1, BTN2A2, and BTN2A3 (BTN2A3P) belong to BTN2, and BTN3A1, BTN3A2, and BTN3A3 belong to BTN3 subfamilies. Moreover, butyrophilin-like proteins (BTLN: BTLN2, BTLN3, BTLN8, BTLN9, and BTLN10) and SKINT-like (SKINTL) are classified in the family of butyrophilins[5–7].

The functions of BTN and BTLN family members have not yet been elucidated, especially in tumor microenvironment (TME). BTN1A1, BTN2A2, and BTLN2 proteins through cell cycle arrest have been reported to show an inhibitory effect on CD4 + T and CD8 + T cell proliferation[6]. BTN3 family exerts an immunosuppressive effect, which inhibits T cell activation. However, the functions of BTLN3, BTLN8, and BTLN9 remain unknown[5].

In this study, we investigated the relationship between the expression of BTN and BTLN and prognosis in LUAD patients[8]. Gene Expression Omnibus[9], TIMER[10], KM plotter[11], UALCAN[12], OncoLnc[13], Oncomine[14], TisGDB[15] among other databases, has been used to screen for BTN or BTLN molecules, and to further explore their signal networks and tumor-immune interactions, providing a theoretical basis for precise prognosis and treatment of LUAD.

Methods

BTNL9 mRNA expression in LUAD

The "Tidyverse"[16] and "Table 1" packages of R software[17] (version 4.0.1) were used to extract the clinical parameters of the LUAD dataset in TCGA, including age, gender, race, TNM staging, ECOG score, EGFR mutation, KRAS mutation, and radiotherapy information.

Table 1
BTNL9 mRNA expression and characteristics in TCGA-LUAD

Characteristics	BTNL9 mRNA		
	High (N = 291)	Low (N = 294)	Overall
Age (years)			
< 40	1 (0.3%)	3 (1.0%)	4 (0.7%)
40–60	77 (26.4%)	101 (34.5%)	178 (30.4%)
> 60	208 (71.2%)	176 (60.1%)	384 (65.6%)
Sex			
Female	165 (56.5%)	151 (51.5%)	316 (54.0%)
Male	127 (43.5%)	142 (48.5%)	269 (46.0%)
Race			
Asian	5 (1.7%)	2 (0.7%)	7 (1.2%)
Black	30 (10.3%)	28 (9.6%)	58 (9.9%)
White	232 (79.5%)	221 (75.4%)	453 (77.4%)
T stage			
T1	115 (39.4%)	76 (25.9%)	191 (32.6%)
T2	145 (49.7%)	176 (60.1%)	321 (54.9%)
T3	20 (6.8%)	30 (10.2%)	50 (8.5%)
T4	10 (3.4%)	10 (3.4%)	20 (3.4%)
N stage			
N0	199 (68.2%)	172 (58.7%)	371 (63.4%)
N1	46 (15.8%)	61 (20.8%)	107 (18.3%)
N2-N3	35 (12.0%)	54 (18.4%)	89 (15.2%)
M stage			
M0	192 (65.8%)	202 (68.9%)	394 (67.4%)
M1	12 (4.1%)	15 (5.1%)	27 (4.6%)
ECOG			
0–1	60 (20.5%)	54 (18.4%)	114 (19.5%)
2–3	15 (5.1%)	10 (3.4%)	25 (4.3%)
4	1 (0.3%)	1 (0.3%)	2 (0.3%)
EGFR mutation			
No	113 (38.7%)	99 (33.8%)	
Yes	47 (16.1%)	39 (13.3%)	
KRAS mutation			
No	21 (7.2%)	23 (7.8%)	44 (7.5%)
Yes	17 (5.8%)	9 (3.1%)	26 (4.4%)
Radiation therapy			

Characteristics	BTNL9 mRNA		
	High (N = 291)	Low (N = 294)	Overall
No	45 (15.4%)	46 (15.7%)	91 (15.6%)
Yes	32 (11.0%)	38 (13.0%)	70 (12.0%)

Gepia Database Analysis

Search the expression of BTN and BTNL family genes in LUAD in the GEPIA database (<http://gepia2.cancer-pku.cn/#index>)[18]. The conditions were set as follows: |Log2FC| Cutoff = 1, p-value Cutoff = 0.01, log2(TPM + 1) for log-scale, Jitter Size = 0.4 and Match TCGA normal and GTEx data. The overall Survival setting conditions were: Group Cutoff selects median, Cutoff-High = 50%, and Cutoff-Low = 50%. BTN or BTNL molecules that meet the significant difference between LUAD and adjacent tissue were only BTNL8 and BTNL9. Then survival analysis and select the molecule with significant differences, and the result is only BTNL9 in LUAD. BTNL9 was confirmed through the survival analysis of the three databases KM plotter (<http://www.kmplot.com>)[11], UALCAN (<http://ualcan.path.uab.edu/index.html>)[12] and OncoLnc (<http://www.oncolnc.org/>)[13], and subsequent network analysis including immune infiltrates were presented. GEPIA was used to analyze the correlation between BTNL9 and methyltransferase expression, B, and DC cell immune infiltration.

Oncomine Database Analysis

The mRNA expression of BTNL9 in different tumors was identified using the Oncomine database (<https://www.oncomine.org/>)[14]. The default setting parameters of the database were used, with the threshold parameters set as a P-value of 0.01, a fold change of 1.5, and a top 10% gene ranking.

Tissgdb Database Analysis

TissGDB is the tissue-specific gene annotation database in cancer (<https://bioinfo.uth.edu/TissGDB/index.html?cst=17567836353851237153>)[15]. Forest plot of Cox proportional hazard ratio (HR) and 95% CI of overall survival (OS) and relapse-free survival (RFS) were performed in 28 cancer types .

Timer Database Analysis

TIMER is a comprehensive resource for systematical analysis of immune infiltrates across diverse cancer types (<http://timer.comp-genomics.org/>)[10, 19]. Using Gene_DE module, BTNL9 mRNA expression was calculated in cross-carcinoma(*: P-value < 0.05; **: P-value < 0.01; ***: P-value < 0.001). The expression of BTNL9 in LUAD and its correlation with the six immune infiltration cells, namely B cells, CD4 + T cells, CD8 + T cells, macrophages, neutrophils, and dendritic cells, used as the Gene and Survival module. Gene_Corr module was used to analyze the correlation between BTNL9 expression and B and DC cells[20].

Starbase Database Analysis

We used three databases: miRMap (<https://mirmap.ezlab.org/>)[21], TargetScan (http://www.targetscan.org/vert_72/)[22], and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>)[23] to predict the miRNAs that could bind to BTNL9, consider the intersection, verify in the Starbase database (<http://starbase.sysu.edu.cn/panCancer.php>)[24], and select miRNAs with significant differences between co-expression and overall survival.

Sangerbox Database Analysis

Sangerbox (<http://sangerbox.com/Index>) was developed by Hangzhou Mugu Technology Co., Ltd. It mainly uses public data or existing data for immune-related analysis and directly issues a set of charts and analysis reports. Sangerbox uses the R software ESTIMATE package to analyze the immune score and stromal score of each TCGA tumor sample, and GSEA to analyze the enrichment for the KEGG

and HALLMARK pathways in the BTNL9 high and low expression groups. Analysis of the relationship between BTNL9 and TMB was determined using Spearman correlation analysis.

Statistical Analysis

The boxplots of BTNL9 expression were extracted from GEPIA. Overall survival was determined from GEPIA, KM plotter, UALCAN, and OncoLnc databases. The expression of BTNL9 across cancer species was extracted in TIMER. RFS and OS HR were extracted from TisGDB database. The correlation between the miRNA and BTNL9 expression was determined in Starbase, as well as the expression of miRNA on the survival of LUAD. BTNL9 expression and single cancer cell biological behavior of LUAD was performed by Pearson correlation analysis. The correlation between BTNL9 expression, the 6 immune cells, and survival analysis was assessed in TIMER. In all the above analyses, $P < 0.05$ was considered statistically significant.

Results

BTNL9 mRNA expression in LUAD

The "Tidyverse" and "Table 1" packages of R software (version 4.0.1) was used to extract the clinical parameters of the LUAD data set in TCGA. Further, we analyzed the age, gender, race, TNM staging, ECOG score, EGFR mutations, KRAS mutations, and radiotherapy information of the two groups of patients with high and low expression of BTNL9. There is no significant difference in two groups (data not shown), and we organized them into a table for descriptive analysis (Table 1).

Btl9 Is The Key Gene Of Butyrophilins In Luad

The expression of BTNL8 and BTNL9 were significantly different in LUAD, and both tumor tissues were significantly lower than normal tissues (Fig. 1A). BTNL9 significantly correlated with the clinical stage, N stage and P53 mutation, while BTNL8 significantly correlated with the clinical stage and N stage (Fig. 1B). However, survival analysis revealed that BTNL8 was not significantly correlated with OS, while BTNL9 was significantly correlated with OS in LUAD (Fig. 1C). Prognoscan[25] analysis of the NSCLC dataset found that BTNL9 expression was significantly correlated with RFS and OS in the GSE31210 study, and BTNL9 expression was significantly correlated with OS in GSE3141 study (Supplementary Table 1). KM plotter, UALCAN, and OncoLnc were used to validate the findings, and the results showed that high expression of BTNL9 significantly improved the OS of LUAD (Fig. 1D). Therefore, we regarded BTNL9 as the key gene of BTN and BTNL family in LUAD.

Expression Of Btl9 In Pan-cancers And Its Prognostic Correlation

We analyzed the expression of BTNL9 in pan-cancer, and analysis of pan-cancers from the Oncomine database showed that BTNL9 expression was significantly reduced in breast cancer, one dataset of colon cancer, lung cancer, kidney cancer, and crabtree uterus cancer compared with the normal tissue. However, BTNL9 expression significantly increased in brain and CNS cancer, four datasets of colorectal cancer, esophageal cancer, leukemia, and lymphoma (Fig. 2A and Supplementary Table 2). We further examined BTNL9 expression using TCGA RNA sequencing data (TIMER). As shown in Fig. 2B, BTNL9 expression was significantly reduced in Bladder Urothelial Carcinoma (BLCA), Breast invasive carcinoma (BRCA), Cholangio carcinoma (CHOL), Esophageal carcinoma (ESCA), Glioblastoma multiforme (GBM), Head and Neck squamous cell carcinoma (HNSC), Kidney renal papillary cell carcinoma (KIRP2), LUAD, and LUSC, but significantly increased in Colon adenocarcinoma (COAD), Kidney Chromophobe (KICH), and Kidney renal clear cell carcinoma (KIRC). The TisGDB database analysis revealed that the correlation of BTNL9 expression with LUAD's RFS HR was 0.87 [95%CI (0.79, 0.96)], and the OS HR was 0.87 [95%CI (0.8, 0.96)] (Fig. 2C, 2D).

Upstream And Downstream Regulatory Network Of Btl9

In previous study, we found that the expression of BTNL9 in LUAD was significantly lower compared with the normal tissues. DNA methylation is related to gene expression. Methyltransferases (DNMTs) including DNMT1, DNMT2, DNMT3A, and DNMT3B. Analysis of the GEPIA database revealed that the expression of BTNL9 in normal lung tissue was significantly positively correlated with DNMTs ($r = 0.35, P = 0.059$), but there was no correlation in LUAD ($r = -0.019, P = 0.67$) (Fig. 3A, 3B), suggesting that DNA methylation may be involved in the pathogenesis of LUAD. We further analyzed the upstream regulation of BTNL9, predicted miRNAs that bind to BTNL9 through

miRMap[21], TargetScan[26], and miRWalk[23], and considered the intersection of the 3 databases to obtain 248 miRNAs (Fig. 3C). The miRNAs were verified in Starbase[24] database and found that, hsa-miR-30b-3p, hsa-miR-4709-3p and hsa-miR-6514-39 were significantly positively correlated with BTNL9 expression ($r = 0.312$, $P = 5.25E-13$, $r = 0.277$, $P = 1.74E-10$, and $r = 0.103$, $P = 0.02$, respectively), and the 3 miRNAs were highly expressed and significantly prolonged the OS of LUAD (HR = 0.66, $P = 0.0058$, HR = 0.63, $P = 0.0023$, and HR = 0.73, $P = 0.036$, respectively) (Fig. 3D). Furthermore, we predicted the lncRNA binding to BTNL9 through LncMap[27] database and obtained 18 lncRNAs (Supplementary Table 3), which were verified and predicted the OS correlation in these lncRNAs through LncACTdb2.0[28] database. lncRNA AP001462.6 was found to bind to BTNL9, and the high expression of AP001462.6 significantly prolonged the OS in LUAD ($P = 0.049$) (Fig. 3E).

Moreover, we analyzed the protein network that binds to BTNL9 through the STRING[29] database and found that there were 7 binding proteins (Fig. 3F). The top 2 binding proteins were predicted using the cytoHubba module of Cytoscape[30] (Fig. 3G). The proteins that bind to BTNL9 were predicted to be HTRA4 and TM4SF19. HTRA4 gene encodes a member of the HtrA family of proteases, which may function as a secreted oligomeric chaperone protease to degrade misfolded secretory proteins[18]. We hypothesized that the low expression of BTNL9 in LUAD may be related to ubiquitination degradation. Analysis of the Ubibrowser[31] database revealed that E3 (MARCH8) ligases can bind the substrate BTNL9 (Confidence level is high, and score = 0.805), and there is one potential E3 recognizing domain and two potential E3 recognizing motif (Fig. 3H, 3I).

Low BTNL9 expression significantly enriches proteasome and increases cancer malignancy

To determine the biological function of BTNL9 expression on LUAD, the Sangerbox tool was used to divide the TCGA samples into high and low BTNL9 expression groups. Gene set enrichment analysis (GSEA) for KEGG and HALLMARK pathways were performed. The results revealed that the top 3 KEGGs pathways with high expression of BTNL9 were significantly enriched in vascular_smooth_muscle_contraction, phosphatidylinositol_signaling_system, and abc_transporters (Fig. 4A). The top 4 KEGGs pathways associated with low expression of BTNL9 were parkinsons_disease, oxidative_phosphorylation, DNA replication, and proteasome (Fig. 4B). GSEA for the HALLMARK pathway revealed that the top 3 pathways associated with high BTNL9 expression were bile_acid_metabolism, heme_metabolism, and wnt_beta_catenin_signaling, while the top 4 pathways associated with low BTNL9 expression were E2F_targets, glycolysis, myc_targets_v1, and mTORC1_signaling (Fig. 4C, 4D).

Metabolic reprogramming is a hallmark of cancer, and intrinsic and extrinsic factors contribute to metabolic phenotypes in tumors. As cancer develops from pre-tumor lesions to local, clinically obvious malignant tumors to metastatic cancer, the phenotype and dependence of metabolism also develop[32]. Through single-cell RNA (scRNA) analysis of LUAD in CancerSEA[33] database (Fig. 4E), BTNL9 expression was found to be significantly negatively correlated with tumor malignant features including invasion ($r = -0.53$, $P < 0.0001$), metastasis ($r = -0.35$, $P = 0.011$), EMT ($r = -0.47$, $P = 0.0006$), proliferation ($r = -0.37$, $P = 0.0086$), Hypoxia ($r = -0.36$, $P = 0.011$), and DNA damage ($r = -0.34$, $P = 0.017$) (Fig. 4F). Therefore, the low expression of BTNL9 promotes the malignant features of LUAD.

Correlation between BTNL9 and markers of infiltrating immune cells and its prognosis

Tumor mutation burden (TMB) is measured by the number of somatic mutations that occur at an average of 1 Mb in the coding region (exon region) of the tumor cell genome (non-synonymous mutations). The total number of synonymous mutations indicates that the mutation types mainly include single nucleotide mutations (SNV) and small fragments of insertion/deletion (Indel) and other forms of mutations. Spearman's correlation test analyzed the correlation between BTNL9 and TMB in TCGA-LUAD and found that BTNL9 was significantly negatively correlated with TMB ($P = 1.4E-9$) (Fig. 5A). Further, analysis of the somatic mutation pattern of BTNL9 in LUAD (Frame_Shift_Del, Missense, and Splice), revealed that the mutation frequency of BTNL9 in LUAD was 1.14% (Fig. 5B). Genetic mutations shape TME[34]. SangerBox tool used the R software package ESTIMATE to analyze the correlation between BTNL9 and stromal score. The results showed that BTNL9 was significantly positively correlated with ImmuneScore ($r = 0.129$, $P = 0.003$) and ESTIMATEScore ($r = 0.106$, $P = 0.016$). However, StromalScore was not significantly different (Fig. 5C, 5D, 5E).

The correlation between BTNL9 and TME infiltrating immune cells was analyzed using TIMER[10] database and found that BTNL9 was negatively correlated with tumor purity ($r = -0.124$, $P = 5.6E-03$), and significantly positively correlated with B cells ($r = 0.24$, $P = 8.88E-8$), CD4 + T ($r = 0.283$, $P = 2.24E-10$) and macrophages ($r = 0.209$, $P = 3.35E-6$) (Fig. 5F). Moreover, survival analysis showed that high expression of BTNL9 in B cells ($P = 0$) and DC cells ($P = 0.048$) significantly prolonged the OS of LUAD (Fig. 5G).

DC also have a trend of difference, and high DC expression of BTNL9 significantly prolonged OS in LUAD (Fig. 5F, 5G). We conducted a detailed analysis of TME infiltrated DC and B cells using the TIMER database, which revealed that DC and its subtypes cDCs1 and cDCs2[35] were all associated with BTNL9 expression before and after purity adjustment. GEPIA database analysis found that normal

lung tissue was not correlated with DC and its subtypes cDCs1 and cDCs2, but was significantly positively correlated with LUAD (Table 2), indicating that all DCs regulated by BTNL9 may participate in the LUAD immune response. B cells are heterogeneous, and include two subtypes: naïve B cells and plasma B cells[36]. TIMER analysis found that total B cells and naïve B cells were significantly related to BTNL9 expression before and after purity adjustment, but plasma B cells were not correlated to BTNL9 expression before and after purity adjustment. GEPIA analysis found that total B cells and naïve B cells were not correlated to BTNL9 expression in normal lung tissues, but significantly positively correlated in LUAD. Plasma B cells showed no correlation with BTNL9 in both normal tissues and LUAD (Table 2), indicating that BTNL9 may promote naïve B cell anti-tumor immune response.

Table 2

Correlation analysis between BTNL9 and relate gene set markers of significant innate and adaptive immunity cells in TIMER and GEPIA database

Immune cell	Marker	TIMER				GEPIA			
		None		purity		Normal		Cancer	
		Spearman's ρ	P Value	Spearman's ρ	P Value	Spearman correlation coefficient	P Value	Spearman correlation coefficient	P Value
DC	CD1C	0.36	1.65E-17	0.34	1.03E-14	-0.022	0.87	0.33	1.5E-13
	HLA-DPA1	0.23	1.64E-07	0.20	8.47E-06				
	HLA-DPB1	0.29	8.39E-12	0.27	8.03E-10				
	HLA-DQB1	0.21	1.31E-06	0.18	8.16E-05				
	HLA-DRA	0.18	4.54E-05	0.14	0.0020				
	ITGAX	0.24	1.63E-08	0.22	1.10E-06				
	NCR1	0.04	0.35980601	0.01	0.8450				
	NRP1	0.03	0.41981037	0.03	0.4973				
cDC1s	CD8A	0.03	0.55316497	-0.03	0.5613	-0.028	0.83	0.22	1.20E-06
	CLEC9A	0.40	1.00E-21	0.38	1.16E-18				
	XCR1	0.41	2.70E-22	0.39	7.37E-20				
cDC2s	CLEC12A	0.22	3.50E-07	0.18	3.61E-05	0.18	0.16	0.45	7.1E-25
	ESAM	0.51	1.01E-35	0.50	2.89E-33				
B cell	CD19	0.23	1.93E-07	0.20	5.30E-06	0.017	9.00E-01	0.37	8.30E-17
	FCER2	0.40	7.97E-21	0.38	2.18E-18				
	MS4A1	0.34	6.45E-16	0.33	2.59E-14				
	SDC1	0.07	0.0917	0.09	0.0523				
Naïve B cell	CD19	0.23	1.93E-07	0.20	5.30E-06	0.082	0.54	0.43	8.60E-23
	CD22	0.50	2.44E-34	0.51	1.10E-34				
	CD83	0.33	6.56E-15	0.31	2.09E-12				
	MS4A1	0.34	6.45E-16	0.33	2.59E-14				
	TCL1A	0.25	1.57E-08	0.21	1.31E-06				

		TIMER			GEPIA				
Plasma B cell	CD38	0.04	0.3699	0.08	0.0746	-0.19	0.15	0.059	0.19
	TNFRSF17	0.04	0.4053	0.08	0.0814				

Btl9 Expression Is To Be Associated With Drug Response

Computational Analysis of Resistance (CARE) is a software that uses compound screening data to identify genome-scale biomarkers of targeted therapeutic response. According to the Pearson correlation between the gene expression profile of the cancer sample and the CARE scoring vector, the patient is predicted as a responder or a non-responder[37]. BTNL9 expression has significant positive CARE scores for many compounds in Cancer Cell Line Encyclopedia (CCLE), Genomics of Drug Sensitivity in Cancer (GDSC, previously named CGP), and The Cancer Therapeutics Response Portal (CTRP) cohorts, suggesting that loss of BTNL9 expression may promote drug resistance toward many targeted therapies (Fig. 6, and Table 3).

Table 3

Loss of *BTNL9* expression may promote drug resistance toward many targeted therapies in CGP, CCLE, and CTRP cohorts

	Drug	Target	t-value	p-value
	IOX2	EGLN1	5.91996	4.56E-09
	PHA-793887	CDK9	4.92172	1.02E-06
	OSI-027	MTOR	4.55428	5.99E-06
	Ispinesib	KIF11	4.42265	1.09E-05
	Nilotinib	ABL1	4.3886	1.30E-05
	Axitinib	PDGFRA	4.33456	1.64E-05
	NG25	MAP4K2	4.29746	1.92E-05
	Nilotinib	KIT	4.26379	2.26E-05
	BMS345541	IKBKB	4.12048	4.13E-05
	GSK525762A	BRD2	3.92075	9.50E-05
	CAY10603	HDAC6	3.91591	9.69E-05
	TubastatinA	HDAC6	3.89552	0.000105
	GSK525762A	BRD4	3.83187	0.000136
CGP dataset	PHA-793887	CDK1	3.4184	0.000658
	Fluorouracil	TYMS	3.09815	0.002008
	TPCA-1	IKBKB	3.09042	0.002061
	1256580-46-7	ALK	3.01459	0.002646
	CAL-101	PIK3CD	2.99145	0.002852
	Belinostat	HDAC6	2.82403	0.004852
	AT7519	CDK9	2.81016	0.00506
	CP-466722	ATM	2.78047	0.005542
	Enzastaurin	PRKCB	2.76951	0.00573
	SB590885	BRAF_V600E.Mutation	2.6446	0.008337
	Vorinostat	HDAC6	2.54112	0.011233
	Nutlin-3	MDM2	-2.94731	0.003295
	Dasatinib	EPHA2	-3.23912	0.001305
	Quizartinib	FLT3	-3.33865	0.000877
	1173900-33-8	PIK3CB	-3.55986	0.00039
	Linifanib	FLT3	-5.1822	2.71E-07
	870483-87-7	CSF1R	-9.60837	7.14E-21
	Panobinostat	HDAC1	3.29253	0.001066
	abraxane	TUBB	2.83794	0.00473
CCLE dataset	Palbociclib	RB1	2.64989	0.008358
	Topotecan	TOP1	2.45344	0.014498
	Sorafenib	FLT3	-4.25026	2.56E-05

	Drug	Target	t-value	p-value
	9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	NR3C1	5.30047	1.51E-07
	abraxane	TUBB	4.50938	7.51E-06
	Alisertib	AURKB	4.50069	7.83E-06
	PAC-1	CASP3	4.27611	2.16E-05
	660868-91-7	PLK1	4.21764	2.79E-05
	Gossypol	BCL2	4.19093	3.12E-05
	Decitabine	DNMT1	4.18042	3.24E-05
	722544-51-6	AURKB	4.14645	3.74E-05
	Etoposide	TOP2B	4.0066	6.76E-05
	3,5-bis(4-methylbenzylidene)piperidin-4-one	USP13	3.92444	9.47E-05
	180002-83-9	CNR2	3.91953	9.68E-05
	CICLOPIROX	RRM1	3.90668	0.000102
	Dabrafenib	BRAF_V600E.Mutation	3.84863	0.00014
	BRD-K62801835-001-01-0	EZH2	3.80929	0.000151
	BI2536	PLK1	3.69725	0.000233
	Nilotinib	ABL1	3.67464	0.000255
	Cerulenin	HMGCS1	3.66163	0.000268
	TW-37	BCL2	3.585	0.000358
	zebularine	DNMT1	3.57912	0.000366
	Pevonedistat	NAE1	3.55057	0.000409
	BAS02002358	GPB1	3.52883	0.000442
	KU-60019	ATM	3.52842	0.000443
	narciclasine	RHOA	3.47702	0.000536
	Ki8751	PDGFRA	3.43765	0.000619
	4ly1	HDAC1	3.39584	0.000719
CTRP dataset	SCHEMBL12182311	EIF4E	3.35831	0.000822
	Belinostat	HDAC1	3.35122	0.000886
	Masitinib	PDGFRA	3.32101	0.000941
	BIBR1532	TERT	3.31812	0.000949
	UNII-UZ77T1VFBM	BIRC5	3.30878	0.000985
	Nutlin-3	MDM2	3.2982	0.001018
	CHEMBL2058177	EIF4E	3.29052	0.001045
	NSC373989	MDM2	3.24218	0.001239
	Imatinib	ABL1	3.22213	0.001327
	Tacrolimus	PPP3CB	3.13945	0.001761
	Olaparib	PARP1	3.07789	0.002159

Drug	Target	t-value	p-value
SMR001317659	PDE4A	3.03729	0.002467
Axitinib	PDGFRA	3.02572	0.002563
Pubchem_92131101	KIF11	3.01909	0.002617
abraxane	TUBB1	3.01218	0.002678
GSK461364	PLK1	2.97879	0.002987
Sorafenib	PDGFRA	2.93438	0.003443
Nutlin-3	TP53	2.88952	0.003968
SMR000068650	S1PR2	2.88871	0.003992
MK-1775	WEE1	2.88827	0.003985
BRD-K53855319-001-01-2	SIRT1	2.87063	0.004217
Apicidin	HDAC1	2.7849	0.005485
TelomeraseInhibitorIX	TERT	2.7825	0.005527
Pluripotin	MAPK1	2.76988	0.005741
PHA-793887	CDK1	2.75543	0.005999
Vorinostat	HDAC1	2.74595	0.006174
I-BET151	BRD4	2.73756	0.006332
SCHEMBL12182311	EIF4A2	2.68306	0.007448
112522-64-2	HDAC2	2.66828	0.007855
RG108	DNMT1	2.65008	0.008212
PF184	IKBKB	2.63052	0.008701
Pazopanib	PDGFRA	2.63026	0.008704
JQ-1	BRD4	2.59578	0.009615
SCHEMBL13833318	HDAC1	2.56094	0.010633
CAY10603	HDAC6	2.55261	0.010887
GSK525762A	BRD4	2.51599	0.012071
4CA-0620	PLK1	2.43094	0.01529
BCP9000801	MDM2	2.41963	0.015783
Belinostat	HDAC6	2.37335	0.018128
Tipifarnib	FNTA	2.36404	0.018536
prima-1	TP53	2.35318	0.018879
BMS345541	IKBKB	2.35317	0.018872
Gemcitabine	RRM1	2.3315	0.019984
SMR001317659	PDE4B	2.28061	0.022841
Doxorubicin	TOP2B	2.26369	0.023866
Rigosertib	PIK3CA	2.22431	0.026433
3,5-di-tert-butylchalcone	RARA	-2.40858	0.016249
Dasatinib	EPHA2	-2.95126	0.003261

Discussion

This study systematically analyzed the correlation between human BTNL9 and LUAD and normal adjacent tissues and revealed that BTNL9 can be considered the key gene of butyrophilins in LUAD. Through multiple database survival analysis, this study demonstrated that high BTNL9 expression significantly prolonged OS of LUAD. BTNL9 is significantly related to B cells, CD4 + T, and macrophages in the TME of LUAD, while high expression of BTNL9 in B and DC cells significantly prolongs the OS of LUAD. GEPIA correlation analysis shows that BTNL9 is related to DC cells and naïve B cells but not plasma B cells in LUAD. Moreover, BTNL9 expression is associated with many targeted therapy response.

The low expression of BTNL9 in LUAD is regulated by unknown mechanisms. DNA methylation is the most common form of DNA modification, which plays an important role in normal cell physiology, increased DNA methylation, and loss of demethylation, which frequent in cancer. DNMTs play an important role during abnormal DNA methylation. Gene body hypermethylation may activate oncogenes, and promoter hypermethylation often causes the suppression of tumors[38]. GEPIA analysis revealed that BTNL9 and DNMTs were significantly related to normal lung tissues, but not to LUAD (Fig. 3A, 3B). However, the molecular mechanism of DNMTs regulating BTNL9 in LUAD is unclear. miRNA and lncRNA are common non-coding RNAs, involved in tumor-promoting and suppressing, depending on the type of tumor[38]. We predicted that the 3 miRNAs (hsa-miR-30b-3p, hsa-miR- 4709-3p, and hsa-miR-6514-39) were significantly positively correlated with BTNL9 in LUAD, and their high expression significantly prolongs OS (Fig. 3C, 3D), by exerting anti-cancer activity in LUAD. Hsu Y-L et al. reported that BTNL9 may act as a tumor suppressor in LUAD and may be regulated by hsa-miR-183-5p, but the specific regulatory network was not explained[39]. We further predicted that lncRNA AP001462.6 could bind to BTNL9, and the high expression significantly prolonged the OS of LUAD (Fig. 3E). Moreover, we analyzed the protein interaction network of BTNL9 and found that the interacting proteins played a role in immune regulation, protease hydrolysis, serine/threonine kinase regulation, etc. (Fig. 3F, 3G), and protease hydrolysis is related to ubiquitination degradation. The analysis also revealed that BTNL9 has a potential E3 recognizing domainbinding site (Fig. 3H, 3I). These indicates that BTNL9 in LUAD may be regulated by DNA methylation and non-coding RNA, and the protein may also be regulated by ubiquitination degradation after translation.

To understand the biological function of BTNL9 in LUAD, GSEA analysis was performed and revealed that low expression of BTNL9 was enriched in energy metabolism (oxidative_phosphorylation, glycolysis, myc_targets v1[40], and mTORC1_signaling[41]), DNA replication, and protease hydrolysis (Fig. 4A-4D). Metabolic reprogramming triggers selective gene amplification and a large gene family which drives cellular functions to promote cancer cell growth and proliferation[40]. The above functions were subsequently verified from a single cell perspective and found that BTNL9 expression was significantly negatively correlated with cancer cell malignant behaviors such as proliferation, invasion, EMT, metastasis, and hypoxia, etc. in LUAD (Fig. 4E, 4F). This indicates that BTNL9 may play an important and extensive tumor suppressor effect in LUAD.

We further analyzed the relationship between BTNL9 and TME and found that the mutation frequency of BTNL9 in LUAD was about 1.14%, and BTNL9 was significantly negatively correlated with TMB (Fig. 5A, 5B). BTNL9 was significantly positively correlated with ImmuneScore and ESTIMATEScore (Fig. 5C, 5D, 5E). Previous studies have shown that high ImmuneScore and ESTIMATEScore significantly improve the prognosis of LUAD[42]. This shows that BTNL9 plays an important role in TME immune regulation. Moreover, we analyzed the correlation between BTNL9 and TILs and found that BTNL9 was significantly negatively correlated with tumor purity, while previous studies have found that low tumor purity is associated with poor prognosis[43]. Although BTNL9 is significantly related to B, CD4 + T, and macrophages, survival analysis showed that BTNL9 was only significantly related to B and DC cells (Fig. 5F, 5G).

DCs play a pivotal role in shaping the innate and adaptive immune response because they have the unique ability to initiate T cell responses and differentiate them into effector lineages[35], while B cells have antigen presentation, cytotoxicity, and antibody production functions, which are an important part of adaptive immunity[44]. TIMER combined with GEPIA database analysis showed that the expression of BTNL9 on DCs (cDC1s and cDC2s) was not correlated with normal adjacent tissues, but was significantly correlated with LUAD tissues (Table 2). cDC1s can migrate to tumor-draining lymph nodes, activate and attract T cells, secrete cytokines, and present antigens in TME, thereby enhancing the function of local cytotoxic T cells[45]. cDC2s present antigens to MHCII, activate CD4 + T cells, and effectively polarize TILs into anti-tumor T helper cell 1 (Th1) or Th17 phenotype[46]. BTNL9 on B cells (naïve B cells) was not correlated with normal adjacent tissues but was significantly correlated with LUAD tissues, except for plasma B cells, suggesting that BTNL9 regulates the function of naïve B cells in TME. Previous studies have also shown that naïve B cells are down-regulated in advanced NSCLC and are related to poor prognosis[36]. Furthermore, CARE database analysis showed that BTNL9 expression is to be associated with effective target therapy response (Fig. 6, and Table 3).

Conclusions

In conclusion, this study shows that BTNL9 is lowly expressed in LUAD, and the low expression is associated with poor prognosis. BTNL9 may be regulated by both epigenetic regulation and post-transcriptional modification. These regulatory mechanisms may change the TME and result in a poor prognostic phenotype and drug resistance. Therefore BTNL9 may play a pivotal role in anti-immune suppression by affecting the TME and can be considered as a prognostic marker and new immuno-target for LUAD.

Abbreviations

LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; NSCLC: non-small cell lung cancer; BTN: Butyrophilin; BTLN: butyrophilin-like; GEPIA: gene expression profiling interactive analysis; TIMER: tumor immune estimation resource; CARE: Computational Analysis of REsistance; GEO: gene expression omnibus; ECOG: Eastern Cooperative Oncology Group; DNMTs: methyltransferases; GSEA: gene set enrichment analysis; scRNA: single-cell RNA; SNV: single nucleotide mutations; TMB: tumor mutation burden; CCLE: Cancer Cell Line Encyclopedia; GDSC: Genomics of Drug Sensitivity in Cancer; CTRP: The Cancer Therapeutics Response Portal; TILs: tumor-infiltrating immune cells; TME: tumor microenvironment.

Declarations

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Author contributions

ZC, TD contributed to the conception and design of the study. WM, JL, ZC, NH, and SZ contributed to data collection. ZC, WM, and TD analyzed and interpreted the data. ZC drafted the manuscript. All authors approved the final version of the report.

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Availability of data and materials

All data in this study comes from public and open source databases.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

. BTNL9 mRNA Expression and prognosis in LUAD. (A) BTN and BTNL family expression panel in LUAD compared to adjacent tissues in the GEPIA database. (B) Correlation between BTNL8 and BTNL9 expression and LUAD clinical stages, N stage, and P53 mutation in the UALCAN database (BTNL8 comparison in clinical stage (Normal-vs-Stage1, $P=1.01E-04$; Normal-vs-Stage2, $P=7.08E-03$; Normal-vs-Stage3, $P=1.36E-04$; Normal-vs-Stage4, $P=1.78E-08$), N stage (Normal-vs-N0, $P=6.56E-04$; Normal-vs-N1, $P=8.27E-10$; Normal-vs-N2, $P=5.96E-04$; Normal-vs-N3, $P=1.97E-10$; N0-vs-N1, $P=3.27E-03$; N0-vs-N3, $P=6.79E-04$), and P53 status (Normal-vs-TP53-Mutant, $P=1.27E-06$; Normal-vs-TP53-NonMutant, $P=1.80E-03$). BTNL9 comparison in clinical stage (Normal-vs-Stage1, $P=1.21E-12$; Normal-vs-Stage2, $P=3.60E-13$; Normal-vs-Stage3, $P=2.40E-12$; Normal-vs-Stage4, $P=2.25E-12$), N stage (Normal-vs-N0, $P=1.08E-12$; Normal-vs-N1, $P=1.85E-12$; Normal-vs-N2, $P=1.02E-12$; Normal-vs-N3, $P=1.67E-12$; N0-vs-N1, $P=1.02E-03$; N2-vs-N3, $P=3.01E-02$), and P53 status (Normal-vs-TP53-Mutant, $P=1.85E-12$; Normal-vs-TP53-NonMutant, $P=4.10E-12$; TP53-Mutant-vs-TP53-NonMutant, $P=1.77E-04$)). (C) The correlation between BTNL8 and BTNL9 expression with overall survival of LUAD in GEPIA. (D) Correlation between BTNL9 expression and LUAD overall survival in KM plotter, OncoLnc, and UALCAN databases.*: P-value < 0.05; **: P-value < 0.01; ***: P-value < 0.001.

Figure 2

mRNA Expression of BTNL9 in pan-cancer and hazard ratio of relapse-free survival and overall survival, in LUAD. (A) Increased or decreased expression of BTNL9 in various tumors compared to adjacent tissues in the Oncomine database. (B) BTNL9 expression in pan-cancer in the TCGA database analyzed by TIMER (*P < 0.05, **P < 0.01, ***P < 0.001). (C) BTNL9 expression and hazard ratio of relapse-free survival, and (D) overall survival.

Figure 4

Low expression of BTNL9 significantly enriches proteasome and promotes tumor malignancy in LUAD. (A, B, C, D) High and low BTNL9 expression groups presented using the Sangerbox tool and GSEA for KEGG and HALLMARK pathways. (E) single-cell RNA (scRNA) analysis of the BTNL9 expression is shown by the t-SNE plot, and (F) molecular function of BTNL9 in LUAD using the CancerSEA database demonstrates that BTNL9 is significantly negatively correlated with tumor invasion, metastasis, EMT, proliferation, hypoxia, and DNA damage in LUAD.

Figure 5

Correlation between BTNL9 and tumor-infiltrating immune cells and its OS prognosis. (A) Sangerbox tools analysis of the correlation between BTNL9 and TMB, and (B) the somatic mutation pattern of BTNL9 in LUAD, and (C, D, E) ESTIMATE scores. (F) Correlation between BTNL9 and TME infiltrating immune cells analyzed using TIMER database, and (G) overall survival.

Figure 6

BTNL9 expression is associated with target therapy response BTNL9 expression has significant positive CARE scores for many compounds in Cancer Cell Line Encyclopedia (CCLE), Genomics of Drug Sensitivity in Cancer (GDSC, previously named CGP), and The Cancer Therapeutics Response Portal (CTRP) cohorts.

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