Prognostic Value of BTNL9 Combined with Genes of Proteasome and Ubiquitin System in Pancreatic Cancer

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Research

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Abstract

Background: An accurate prognostic prediction can improve the individualized management of patients with pancreatic cancer (PC), and the exploration of biomarkers with prognostic value for clinical practice is the prerequisite of the work. Butyrophilin-Like 9 (BTNL9) has recently been found to function as a tumor suppressor gene in a variety of malignancies and has the potency to serve as a prognostic biomarker. Our aim was to explore the relationship between BTNL9 expression and the prognosis of PC, and to unearth its upstream and downstream molecular mechanisms.

Methods: The RNA expression of BTNL9 was analyzed in 5 datasets from Gene Expression Omnibus (GEO) database. The protein expression of BTNL9 was detected by immunohistochemistry in a cohort including 42 PC patients. The relationship between BTNL9 expression and prognosis was analyzed by survival and prognostic factors analysis. Online database and Gene Set Enrichment Analysis (GSEA) were used to explore the upstream and downstream molecular mechanisms of BTNL9. Correlation analysis and CIBERSORT were applied to investigate the relationship between BTNL9 and tumor immunology.

Results: In multiple datasets and our cohort, BTNL9 expression was decreased in PC tissues. Patients with high expression of BTNL9 had a better prognosis. BTNL9, age and N stage were identified as the independent prognostic factors of PC. BTNL9 was predicted to be down-regulated by hsa-miR-1910-5p, and it may be involved in the proteasome and PC signaling pathway. Interestingly, genes of proteasome (PSMD2, PSMD7 and PMSD14) and deubiquitin system (USP20, USP27X and USP30) combined BTNL9 could improve the prognostic prediction of PC. In addition, the expression of BTNL9 correlates with the expression of immune checkpoints and influences the infiltration of tumor immune cells.

Conclusions: BTNL9 can serve as a prognostic marker of PC, and high expression of BTNL9 was generally associated with better prognosis. Combined the expression of BTNL9 and the expression of PSMD2, PSMD7, PMSD14, USP20, USP27X and USP30 can more accurately analyze the prognosis of patients with PC.

Background

Pancreatic cancer (PC) is the seventh leading cause of cancer death in the world.¹ Since 2000, the mortality of PC in men has continued to creep up by 0.3% a year, and the incidence has gradually increased by about 1% a year.² Because of its poor prognosis, PC is predicted to overtake breast cancer as the third leading cause of cancer death in European countries by 2025.¹ An accurate prognosis prediction can provide an important reference for patients with PC to receive accurate and individualized treatment. Therefore, looking for prognostic biomarkers of PC has been the focus of the research.

The Butyrophilin-Like (BTNL) protein family is a member of the immunoglobulin superfamily, and has significant homology and similar structural characteristics with B7-like molecules that regulate the immune response of T cells. Thus, the BTNL family may play a role in inflammation and cancer by

regulating T cells.^{3,4} $\gamma\delta$ T cell is considered to have the tumor suppressive effect in a variety of cancers, including colon cancer, melanoma and breast cancer. It functions by their production of cytokines and chemokines, antigen-presenting, antigen-regulation, and direct toxic effects on tumor cells.⁵⁻⁷ Previous studies have demonstrated that the biological role of the BTNL family in cancer is mainly realized through the activation of $\gamma\delta$ T cells.^{8,9}

The research on the relationship between BTNL family and cancer mainly focuses on its member BTNL9. Previous studies have reported that BTNL9 is downregulated in tissues including breast cancer, colon cancer, lung adenocarcinoma and uveal melanoma, and it inhibits malignant phenotypes of breast cancer and uveal melanoma cells.¹⁰⁻¹⁴ In addition, high expression of BTNL9 is associated with better prognosis in patients with breast cancer and uveal melanoma.^{12,14} These results clue that BTNL9 may act as a tumor suppressor gene in many types of cancer. Whether the expression of BTNL9 is also a prognostic marker in PC, whether it is related to the occurrence and development of PC, and what is the molecular mechanism, all these questions need to be explored in this study.

Results

1. The expression of BTNL9 increases in PC tissues

In GSE62165, GSE62452 and GSE71729, the expression of BTNL9 was found to be down-regulated in PC tissues compared to the adjacent normal tissues (Figure 1A). In GSE28735 and GSE15471, the expression of BTNL9 was also found to be decreased in PC tissues by comparison of paired-samples (Figure 1A). Using Gene Expression Profiling Interactive Analysis (GEPIA) online analysis tool, the decreased expression of BTNL9 in PC was shown in the comparison between PC samples from The Cancer Genome Atlas (TCGA) database and normal pancreas samples from Genotype Tissue Expression Project (GTEx) project (Figure 1B).

To further confirm the results of online high-throughput data, PC and adjacent normal tissues from a cohort of 42 patients with PC were used to detect BTNL9 expression. Based on immunohistochemical detection and quantitative analysis by H-score, it was found that the expression of BTNL9 in PC tissues was lower than that in normal tissues (Figure 1D, E). In adjacent normal tissues, BTNL9 was highly expressed in pancreatic ductal epithelial cells and low in pancreatic acinar cells, mainly distributed in cell membrane and cytoplasm (Figure 1D).

2. BTNL9 expression is associated with prognosis of PC

High expression of BTNL9 in both the TCGA database and our cohort was associated with a longer overall survival (OS) (Figure 2A, B). In both cohorts, patients with BTNL9 high-expression had a median OS of more than 20 months. BTNL9 expression was used to construct the time-dependent Receiver Operating Characteristic (ROC) of TCGA PC patients at 1, 2 and 3 years, and the Area Under the Curve (AUC) was 0.633, 0.622 and 0.614, respectively (Figure 2C). In terms of prognosis analysis, univariate and

multivariate analysis showed that BTNL9, age and N stage were independent factors for survival of PC (Figure 2D).

3. Prediction of the upstream miRNA of BTNL9

178 miRNAs could be found simultaneously in ≥ 2 databases, and 64 miRNAs were shown negatively correlated with BTNL9 expression. After intersection, a total of 9 candidate miRNAs were obtained as follows: Hsa-miR-222-5p, hsa-miR-1910-5p, hsa-miR-2355-3p, hsa-miR-2355-5p, hsa-let-7b-3p, hsa-miR-1304-3p, hsa-miR-1304-5p, hsa-miR-1304-5p, Hsa-miR-3922-3p, hsa-miR-106b-3p, and hsa-miR-3129-3p (Figure 3A). Among the 9 candidate miRNAs, only hsa-miR-1910-5p, hsa-miR-1304-5p, and hsa-miR-3922-3p were showed to be associated to the survival of the patients in the TCGA database (Figure 3B, D). GSE163031 was used to verify the expression of miRNA in PC and adjacent normal tissues, and only hsa-miR-1910-5p was expressed significantly higher in PC tissues (Figure 3C). Based on the above results, hsa-miR-1910-5p is predicted to act as an upstream non-coding RNA molecule targeting BTNL9 in PC.

4. The signaling pathway involved in BTNL9 and effects of BTNL9 combined with proteasome and deubiquitin family genes on prognosis

In TCGA database, GSEA pathway enrichment analysis showed that proteasome and pancreatic cancer signaling pathway were significantly enriched in PC tissues with low expression of BTNL9 (Figure 4A). The expression of proteasome family genes PSMD2, PSMD7, PMSD14 and deubiquitination family genes USP20, USP27X and USP30 were significantly different in PC tissues with high and low expression of BTNL9 (Figure 4B). The expression of BTNL9 was significantly correlated with the expression of PSMD2, PSMD7, PMSD14, USP20, USP27X and USP30 (Figure 4C). Simultaneously, their expression can influence the survival of patients with different BTNL9 expression. Specifically, patients with high BTNL9 expression and low PSMD2/PSMD7/PMSD14 expression tended to have the best OS, while patients with low BTNL9 expression and high PSMD2/PSMD7/PMSD14 expression tended to have the worst prognosis. In contrast, patients with high BTNL9 expression, accompanied by high USP20/ USP27X/ USP30 expression had the best OS. Conversely, the prognosis was the worst (Figure 5).

5. Relationship between BTNL9 and immune checkpoint expression and tumor immune cells infiltration

BTNL9 has previously been found to act as a costimulatory molecule involved in the activation of T cells.⁸ Furthermore, we analyzed the effect of BTNL9 on immune checkpoint expression and immune cells infiltration in PC. In terms of the expression of immune checkpoints, the expression of BTLA, CD40LG, IDO2, CD160 was higher in PC tissues with high expression of BTNL9, while the expression of CD86, HAVCR2, PDCD1LG2 was decreased (Figure 6A, B). Through the TISIDB database, it was found that a negative correlation between the expression of BTNL9 and the expression of PD-L1 in PC (Figure 6C). In previous clinical trial (IMvigor210),¹⁵ the response to atezolizumab was found to be worse in high BTNL9 expression patients with urothelial cancer (Figure 6D). In terms of immune cell infiltration, the

proportion of naïve B cell, CD8 T cells, Tregs was higher in patients with high expression of BTNL9, while the proportion of Mast cells resting, Eosinophils was lower (Figure 7A, B). Single cell sequencing data of PC tissue was analyzed by Tumor Immune Single-cell Hub (TISCH) online tool (PAAD-CRA001160), showing that BTNL9 expressed in a variety of immune infiltrating cells, including CD8Tex, DC, B cells, and monocytes/macrophages, but the most abundant expression was in endothelial cells (Figure 7C).

Discussion

In the present study, we analyzed the data of TCGA- Pancreatic adenocarcinoma (PAAD) and five GEO datasets (GSE62452, GSE71729, GSE15471, GSE28735, and GSE62165) to explore the expression of BTNL9 in PC. It was found that the expression of BTNL9 was downregulated in PC tissues. This differential expression scenario was verified by immunohistochemistry. Subsequently, we found that patients with high expression of BTNL9 had a better OS in the TCGA-PAAD dataset. BTNL9 expression, which could also stably predict the 1 - , 2-and 3-year survival of PC, and was an independent prognostic factor. These findings suggested that BTNL9 may serve as a prognostic marker for PC. To explore the molecular mechanism of the effect of BTNL9 on PC, we analyzed and identified the possible upstream miRNA and the enrichment signaling pathways of BTNL9. We found that BTNL9 may be involved in the proteasome signaling pathway in PC. Since previous studies have shown that the expression of proteasome family genes has prognostic value in patients with PC and BTNL9 expression was associated with the expression of several genes in ubiquitin-proteasome systems, we investigated the effect of the expression of BTNL9 combined with proteasome or deubiquitination family genes on the survival of patients with PC. It was found that both the expression of BTNL9 and the ubiquitination proteasome system genes effected on the survival, and there is a correlation between their effects. These results suggested that BTNL9 combined with the ubiquitin proteasome system genes may improve the capability of the prognostic prediction in PC. From this perspective, the relationship between BTNL9 and ubiquitin-proteasome system genes, and the specific molecular mechanism need to be further studied.

Ubiquitin proteasome system (UPS) is a key regulator of many molecular pathways. Protein oxidation mainly depends on proteasome and participates in the development of cancer. Proteasomes not only remove abnormal proteins that may be misfolded, aged or destroyed by oxidation, but also regulate the half-life of short-lived regulatory proteins such as cyclins and transcriptional regulators. Protein degradation is further complicated by the role of proteasomes in the degradation of a large number of proteins, such as oncogenes, tumor suppressor proteins, transcription factors, and signaling molecules.¹⁶ Many studies have shown that cancer cells have higher levels of proteasomes than normal cells in relation to high levels of oxidative stress.^{17,18} The proteasomal pathway also regulates the processing of MHC-I class antigens¹⁹ and metabolic enzymes such as tyrosine aminotransferase and copper/zinc superoxide dismutase,^{20,21} which may be considerable in cancer development. The differences in proteasome levels and activity between tumor cells and normal cells suggest that proteasomes may be a promising target for cancer therapy.²² There is growing evidence that abnormal expression of certain UPS associated members can cause UPS dysfunction, thereby altering proteolysis of multiple tumor

promoters and/or suppressors in human cancers.^{23,24} For example, PSMD2, PSMD7, and PSMD14 are upregulated in a variety of cancers^{25–33} and play a pro-cancer role by reducing the stability of P21 and P27;^{25,32} USP20, USP27X and USP30 can promote the malignant characteristics of some kinds of tumor cells.^{25,34–39} In this study, it was found that there was a certain relationship between the expression of BTNL9 and PSMD2, PSMD7 and PSMD14. Whether BTNL9 is also related to the stability of P21 and P27 needs further exploration.

Since BTNL9 has been formerly reported to be involved in the activation of T cells, we investigated the immune-related effects of BTNL9 on PC. In the present study, it was found that the expression of CD40LG was upregulated while HAVCR2 and PDCD1LG2 were downregulated in PC tissues with high expression of BTNL9. CD40LG was considered as an immune-stimulating factor, HAVCR2 and PDCD1LG2 were immune-inhibiting factors. This implied the role of BTNL9 in promoting tumor immunity in PC. Meanwhile, high expression of BTNL9 was accompanied by decreased expression of PDCD1LG2 and PD-L1. In the Phase II clinical study of Imvigor210,¹⁵ BTNL9 expression was found higher in patients with no respond to anti-PD-L1 treatment, which suggests that elevated BTNL9 expression may indicate a poor response to PD1/PD-L1 immunotherapy. In the aspect of tumor immune infiltration, the proportion of the naive B cell, CD8 T cells, and Tregs increased in patients with high expression of BTNL9, while the resting mast cell and Eosinophils decreased. From the perspective of tumor immune microenvironment, the influence on immune cell infiltration does not directly reflect whether the expression of BTNL9 was related to the immune escape of tumor cells, which may be different from the process of promoting the activation of T cells under normal immune scenario. In general, it can be seen that BTNL9 may have a complex and multiple relationship with the occurrence of tumor immunity in PC, which is worthy of further exploration.

Conclusion

BTNL9 expression is lower in PC tissues than in the adjacent normal tissues, and its elevated expression is closely associated with better survival. Furthermore, the expression of PSMD2, PSMD7, PSMD14, USP20, USP27X, and USP30 in PC patients with high expression of BTNL9 would lead to different survival conditions. The combined expression of BTNL9 and above genes can get a more accurate prognostic prediction in patients with PC.

Methods

1. Access to public data

Data sets containing RNA expression profiles of PC tissues and adjacent normal tissues were searched and selected from GEO for analysis of BTNL9 expression. The downloaded datasets included GSE62165, GSE62452, GSE71729, GSE28735 and GSE15471. The RNA sequencing data, clinical characteristics and survival data of PC in TCGA were downloaded for survival analysis and prognosis analysis of BTNL9.

2. Patient selection and cohort establishment

Patients with PC were retrospectively included in the First Affiliated Hospital of Chengdu Medical College from January 2014 to December 2020. Inclusion criteria: 1. Postoperative PC, 2. Paraffin or pathological sections of PC tissue and its adjacent normal tissue were preserved, 3. Complete clinical information is available (including age, gender TNM stage, and survival data). Exclusion: patients of their families refused to participate in the study. Finally, a total of 42 PC patients meeting the conditions were selected, and the clinical data of the patients were shown in Table 1. The study was approved by the ethics committee of The First Affiliated Hospital of Chengdu Medical College (2020CYFYIRB-BA-1200) and all subjects or their family member had signed the informed consent of participation.

Table 1
Clinical information of the study population

	TCGA	Our cohort
Sample size	177	42
Age (mean $\pm$ SD)	64.74 (10.80)	55.17 (10.59)
Gender (%)		
Female	79 (44.6)	17 (40.5)
Male	98 (55.4)	25 (59.5)
Grade (%)		
G1+G2	124 (70.1)	36 (85.7)
G3+G4	53 (29.9)	6 (14.3)
NA	-	-
T Stage (%)		
T1+T2	29 (16.4)	17 (40.5)
T3+T4	148 (83.6)	25 (59.5)
NA	-	-
N Stage (%)		
N0	48 (27.1)	20 (47.6)
N+	126 (71.2)	22 (52.4)
NA	3 (1.7)	-
TNM Stage_AJCC(%)		
I+II	168 (94.9)	29 (69.0)
III+IV	9 (5.1)	13 (31.0)
NA	-	-
Event (%)		
Alive	79 (44.6)	14 (33.3)
Dead	98 (55.4)	28 (66.7)
Median OS (month)	18.20	12.50

3. Immunohistochemistry

Rabbit anti-human BTNL9 antibody (bs-8434R, Bioss) and Goat anti-rabbit IgG (kit-5030, MXB Biotechnologies) were used for immunohistochemistry. The specific experimental operation process was described as our previous research.⁴⁰ The H value was used to quantify the expression of BTNL9, and it was calculated according to the following formula:

The H score = (percentage of cells of strong intensity × 3) + (percentage of cells of moderate intensity × 2) + (percentage of cells of weak intensity × 1). The mean value of the 10 randomly visual fields was taken as the H value of each section.

3. Survival analysis and prognostic analysis

The RNA sequencing data and survival data of TCGA-PAAD were utilized for survival analysis, and the patients with PC were divided into high and low BTNL9 expression groups by using the method of calculating the best cutoff using X-tile (3.6.1), and the survival of the two groups was compared. AUC of 1-, 2-, and 3-year survival prediction for BTNL9 was generated by using the SurvivalROC package. Prognostic factors were analyzed by using the RNA sequencing data of TCGA-PAAD, clinical characteristics and survival data. The variables included in the prognostic analysis included age, gender, differentiation, T stage, N stage and AJCC stage.

4. Prediction of the upstream miRNA of BTNL9

BTNL9 was input as the target to query in PITA, RNA22, miRMAP, MICROT, MIRANDA, PICTAR, and TargetSCAN databases, respectively. The miRNAs that could be found simultaneously in ≥ 2 databases were identified. The relationship between BTNL9 expression and the miRNAs expression in TCGA-PAAD was analyzed by LinkOmics, and the miRNA with $R < 0$ and $p < 0.05$ was selected. The two groups of miRNAs obtained above were intersected to obtain candidate miRNAs, and the candidate miRNAs were put into ENCORI to calculate the expression of miRNAs in PC tissues and normal tissues, and the effect of miRNAs on survival of patients with PC.

5. Signal pathway enrichment analysis of BTNL9 and survival analysis of combined markers

According to the median value of BTNL9, TCGA-PAAD patients were divided into two groups with high expression and low expression, and the KEGG signaling pathway enrichment analysis of BTNL9 was performed by GSEA 4.0.3. The TCGA-PAAD data were used to analyze the expression of proteasome and deubiquitination family genes in patients with high or low expression of BTNL9. The survival of patients with PC was analyzed by combining BTNL9 with proteasome and deubiquitination family genes.

6. Relationship between BTNL9 and immune checkpoints and tumor immune cells infiltration

The 47 immune checkpoints were summarized through literature review, and the relationship between BTNL9 and the 47 immune checkpoints was calculated by using the RNA sequencing data of TCGA-PAAD. TCGA-PAAD RNA sequencing data were input into CIBERSORT to calculate the proportion of 22

immune cells in tissue samples. According to the median value of BTNL9, patients in TCGA-PAAD were divided into two groups with high expression and low expression, and the difference of immune cells infiltration between the two groups was calculated. TISCH is an online tool for analyzing the tumor microenvironment using single-cell sequencing data. It is used to investigate the expression of BTNL9 in different immune cells.

7. Statistics

R software V4.0.0 and GraphPad Prism 8 were applied in the present study. The Mann-Whitney U test was used for the non-parametric test of two independent groups, and the Wilcoxon rank-sum test was used for the non-parametric test of two paired groups. Survival analysis was performed by Kaplan-Meier method and Log-rank test. Prognostic factors were analyzed by univariate and multivariate Cox analysis. Two-sided test was used in all statistics, and $p < 0.05$ was considered statistically significant.

Abbreviations

PC, Pancreatic cancer;

Butyrophilin-Like 9, BTNL9;

GEO, Gene Expression Omnibus;

GSEA, Gene Set Enrichment Analysis;

BTNL, Butyrophilin-Like;

GEPIA, Gene Expression Profiling Interactive Analysis;

GTEx, Genotype Tissue Expression Project;

TCGA, The Cancer Genome Atlas;

OS, Overall survival;

ROC, Receiver Operating Characteristic;

AUC, Area under the curve;

TISCH, Tumor Immune Single-cell Hub;

PAAD, Pancreatic adenocarcinoma;

UPS, Ubiquitin proteasome system.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of The First Affiliated Hospital of Chengdu Medical College (2020CYFYIRB-BA-1200) and all subjects or their family member had signed the informed consent of participation.

Consent for publication

Not applicable.

Availability of data and material

The following information was supplied regarding data availability: the clinical information of the 42 pancreatic adenocarcinoma patients included in our cohort are available in a Supplemental File.

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Competing interests

The authors declare there are no competing interests.

Authors' contributions

Ke Xu: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Methodology (equal); Writing-original draft (lead). **Qingfan Mo:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Methodology (equal); Writing-original draft (equal). **Bo Liu:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Methodology (equal); Writing-original draft (supporting). **Rongfei Huang:** Formal analysis (equal); Methodology (equal); Writing-original draft (supporting). **Wei Zhou:** Methodology (equal); Writing-original draft (supporting). **Tao Ren:** Supervision (lead); Writing-review & editing (lead).

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Figures

Figure 1

BTNL9 differently expresses in PC and normal tissues. (A) RNA expression level of BTNL9 in PC tissues and adjacent normal tissues from 5 GEO data sets. (B) Differential expression of BTNL9 in PC samples (n =179) and normal samples (n =171) from TCGA and GTEx databases. (C) and (D) Representative tumor and normal tissues sections from PC patients were subjected to IHC staining using BTNL9 antibodies in

our cohort (n = 42). (E) Comparison of BTNL9 expression between PC tissues and matched adjacent normal tissues in our cohort.

Figure 2

BTNL9 is significantly correlated with prognosis of PC. (A) and (B) Comparison of the OS between BTNL9 high expression and low expression patients in TCGA database and our cohort, respectively. (C) ROC of TCGA PC patients at 1, 2 and 3 years survival rate. (D) The univariate and multivariate Cox regression analysis for prognosis of PC.

Figure 3

Prediction of the upstream miRNA of BTNL9. (A) and (B) 9 candidate miRNAs were identified from at least 2 databases and negatively correlated with BTNL9 expression. (C) Comparison of hsa-miR-1910-5p expression between PC and adjacent normal tissues in GSE163031. (D) Comparison of OS between high hsa-miR-1910-5p expression and low expression patients with PAAD in TCGA database.

A **B** **C** **Figure 4**

The signaling pathway BTNL9 is involved in. (A) Significantly enriched KEGG pathways in the low BTNL9 expression group through GSEA analysis. (B) The expression of proteasome family genes and deubiquitination family genes in PC tissues with high and low expression of BTNL9. (C) The relationship between BTNL9 expression and proteasome family genes and deubiquitination family genes expression.

Figure 5

Combination of BTNL9 and proteasome or ubiquitin system genes expression in the OS analysis of patients with PC.

Figure 6

Relationship between BTNL9 and immune checkpoint genes expression. (A) The heatmap of 32 immune checkpoint genes expression in low and high BTNL9 expression patients. (B) The significantly differential expression of immune checkpoint genes in low and high BTNL9 expression groups. (C) On the basis of the TISIDB database, the expression of BTNL9 had a negative relationship with the expression of PD-L1

in PAAD tissues. (D) BTNL9 expression influences the response to atezolizumab in patients with urothelial cancer.

Figure 7

The Relationship between the BTNL9 expression and the immune cells infiltration. (A) The panorama of the immune cells infiltration in patients with PAAD of TCGA database. (B) Comparison of the proportion

of immune cell infiltration between patients with BTNL9 high expression and low expression. (C)
Expression of BTNL9 in different immune infiltrating cells (dataset of PAAD-CRA001160).

Supplementary Files

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