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Butyrophilin-Like 9 (BTNL9) Suppresses Invasion and Correlates with Favorable Prognosis of Uveal Melanoma

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Uveal melanoma (UM) is the most common intraocular malignancy, and the prognosis of patients with advanced stage of UM is very dismal. The T cell receptor ectopic expression of butyrophilin-like 9 (BTNL9) has been observed in several types of cancers, but the expression and clinical significance of BTNL9 in UM is unclear.

Material/Methods: In our study, we detected the expression of BTNL9 in 6 pairs of UM tissues and adjacent tissues using quantitative real-time polymerase chain reaction (qRT-PCR), and further investigated BTNL9 expression with immunohistochemistry (IHC) in a retrospective cohort consisted of 62 UM patients. The correlations between BNTL9 expression and clinicopathological factors were analyzed with Fisher's test, and the prognostic significance of BTNL9 was evaluated with univariate analysis and multivariate analysis. Using experiments *in vitro*, we investigated the function of BTNL9 in UM proliferation and invasion.

Results: BTNL9 mRNAs in adjacent tissues were remarkably higher than in UM tissues. The percentages of BTNL9 low expression and high expression were 56.45% and 43.55%, respectively. High expression of BTNL9 was significantly associated with favorable prognosis of UM. BTNL9 expression was identified as a prognostic biomarker predicting better outcome of UM patients. Moreover, BTNL9 could suppress invasion instead of proliferation in melanoma cell line.

Conclusions: BTNL9 was a favorable prognostic factor of UM and it could suppress invasion of UM, suggesting that BTNL9 detection could help stratify high-risk patients with UM after operation and guide more precise surveillance and treatment.

MeSH Keywords: **Genes, T-Cell Receptor delta • Melanoma • Neoplasm Invasiveness • Prognosis**

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Background

Though uveal melanoma (UM) is a rare subset of all melanomas, it is the most common primary cancer of the eyes [1]. UM only accounts for 5% of all melanoma cases, but it makes up approximately 85–95% of all ocular malignancy in adults [2]. The biological features and prognoses are different between UM and cutaneous melanoma [3]. Historically, no systemic therapy has been demonstrated to improve the survival time of patients with UM in an advanced stage, and the outcome of these patients is considered dismal [4]. More approaches are being discovered thanks to new understanding of phenotype and gene expression profiling of UMs. For example, UMs can be divided into 2 classes with low and high metastatic risk according to gene profiling [5]. Activating mutations in genes encoding the G-protein-alpha subunits *GNAQ* or *GNA11* and ectopic stimulation of downstream signaling, including MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, were observed in about 85–95% UM patients [6,7]. This initiated the interest in UM drugs, and several drugs blocking MAPK or PI3K/AKT signaling have been in clinical trials [1]. However, the treatment options for UM are still very limited, which requires us to explore more biomarkers and drug targets.

Butyrophilin (BTN) and btn-like (BTNL) families could modulate the T cell response and further influence inflammatory disorders and cancers [8]. BTN and BTNL genes are members of the immunoglobulin superfamily. Seven human BTN genes and 5 BTNL genes have been identified in the human genome [9,10]. The BTNL family consisted of BTNL2, BTNL3, BTNL8, BTNL9, and SKINTL [11]. Previous studies demonstrated the essential role of BTN and BTNL family in the activation of γδT cells [12]. The γδT cell is one special kind of T cell because of the T cell receptors (TCRs) composed of γ and δ chain, and it is considered to play a tumor suppressor role in many kinds of tumors, such as melanoma, breast cancer, ovarian cancer, and colon cancer [13–15].

Although it has been generally accepted that the ectopic function of T cells was associated with proliferative disorders especially cancer, the role of most BTN and BTNL family members in tumorigenesis and cancer progression is little understood. As a member of BTNL family, the downregulation of BTNL9 has been reported in colon cancer compared with normal tissues [16]. However, the clinical significance of BTNL9 in melanoma is still unclear. In our study, we detected the expression of BTNL9 in 6 pairs of UM tissues and adjacent tissues with quantitative real-time polymerase chain reaction (qRT-PCR), and further investigated BTNL9 expression with immunohistochemistry (IHC) in a retrospective cohort consisted of 62 UM patients. The correlations between BTNL9 expression and clinicopathological factors were analyzed, and the prognostic significance of BTNL9 was evaluated with univariate analysis and multivariate analysis. Using experiments *in vitro*, we investigated the function of BTNL9 in UM proliferation and invasion.

Material and Methods

Cells and agents

Human melanoma MeWo cells were cultured in RPMI medium 1640 (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 7% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR primers, small interfering RNA (siRNA) of BTNL9, and a scrambled sequence was purchased from Biosune Cooperation (Shanghai, China), and the transfection were realized by lipofectamine RNAiMax (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Antibody of BTNL9 (Cat. No. orb2153) was from Biorbyt Company (Cambridge, UK) and antibody of β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Patients and follow-ups

From 2000 to 2015, a total of 88 patients were diagnosed with primary UM and underwent enucleation in Linyi Central Hospital and Jining No.1 People's Hospital. Sixty-two patients were selected for the final validation cohort with available specimens for IHC detection and valid follow-ups. Six pairs of fresh UM tissues and tumor adjacent tissues were obtained from surgery and preserved in liquid nitrogen immediately for qRT-PCR. The study was approved by the Ethics Committee of Linyi Central Hospital and Jining No.1 People's Hospital. All the specimens were obtained with prior content of patients.

Immunohistochemistry and evaluation

The expression of BTNL9 was detected with IHC in Streptavidin peroxidase complex method as described previously [17]. In brief, after deparaffinization and rehydration, the antigen retrieval was achieved with 0.01 M boiled citrate buffer (pH=6.0). Endogenous activity was inactivated by 3% H_2O_2 and unspecific binding was blocked by 1% bovine serum albumin. Specimens were incubated in primary antibody of BNTL9 at 1: 100 concentration overnight at 4°C, and then in secondary antibody (Beyotime Biotechnology, Shanghai, China) for 1 hour at room temperature. 3'-diaminobenzidine solution was applied for antigen visualization. Finally, the slides were counterstained with hematoxylin.

The results of IHC were evaluated by 2 senior pathologists unaware of clinical data. The IHC results of representative IHC area were quantified by the software ImageJ Pro (National Institutes of Health, MD, USA) by analyzing the staining intensity and

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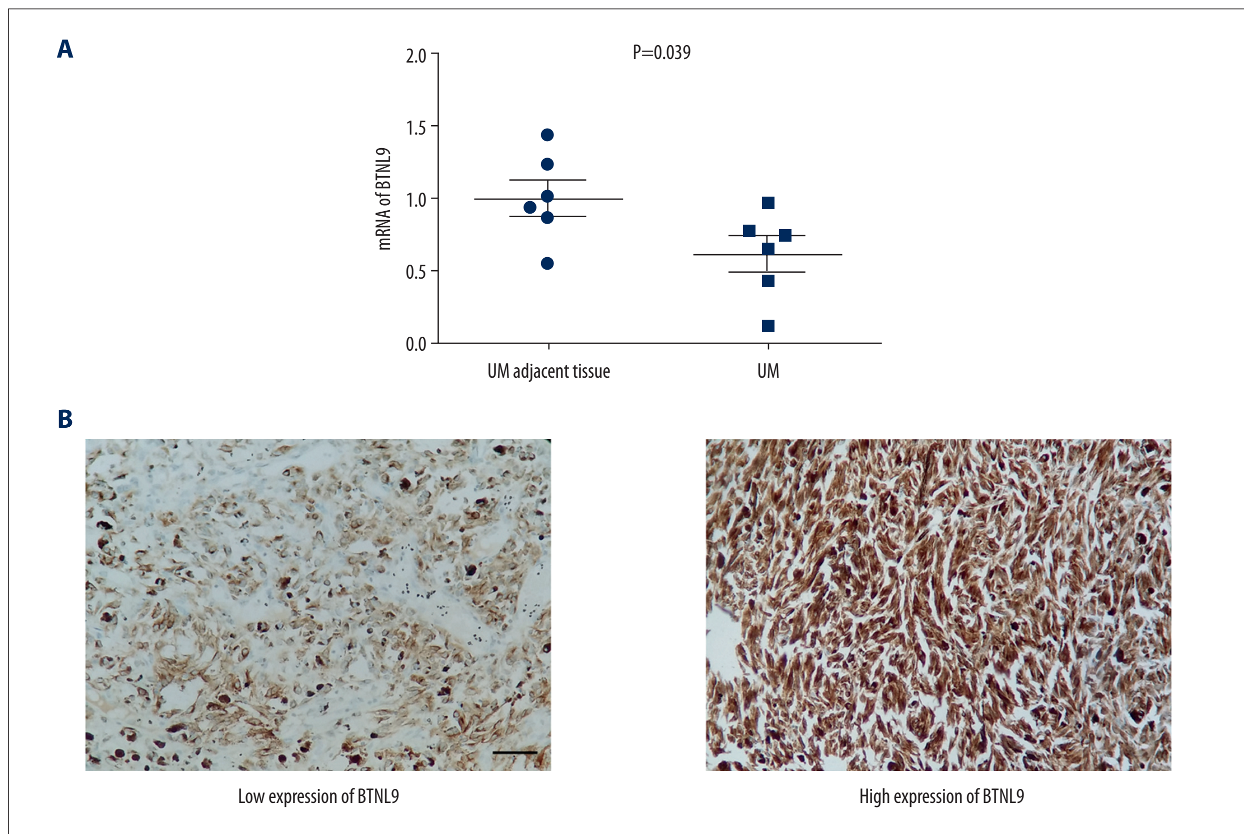

Figure 1. Expression of BTNL9 in uveal melanoma (UM) tissues and tumor adjacent tissues. (**A**) The expression of BTNL9 mRNA in UM was significantly lower than that in adjacent tissues. BTNL9 mRNA was detected with qRT-PCR in 6 pairs of UM tissues and adjacent tissues. (**B**) Representative images of low expression and high expression of BTNL9. BTNL9 expression was detected with IHC in 62 cases of UM. Scale bar: 50 μm. BTNL9 – butyrophilin-like 9; qRT-PCR – quantitative real-time polymerase chain reaction; IHC – immunohistochemistry.

area of staining cells according to previous study [17]. Receiver operating characteristic (ROC) curve of IHC results was drawn to define the cutoff of our validation cohort according to previous study [18]. The point with the highest sum of specificity and sensitivity in ROC curve was set as the cutoff, dividing the cohort into subgroups with high and low BTNL9 expression.

Reverse transcription and quantitative PCR

The mRNA levels of BTNL9 in UM tissues and the tumor adjacent tissues were estimated with qRT-PCR. The mRNA of tissues was extracted with TRIzol and RNAeasy miniprep kit (Qiagen, Valencia, CA, USA) according to the manual. Reverse transcription was applied in SYBR green method with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers for qRT-PCR were designed as follows:
BTNL9: forward ATGGTGGACCTCTCAGTCTCC,
Reverse GCCAGGATGGGATACTCAGG;
β-actin: forward ACCATGTACCCTGGCATTGC,
reverse CGGACTCGTCATACTCCTGC. Relative quantification of mRNA was calculated by $2^{-\delta\delta CT}$ method, and data were standardized with the average mRNA level of UM adjacent tissue as baseline.

MTT assay

Proliferation of melanoma cell line was estimated with MTT assay. In brief, 24 hours after the transfection of BTNL9 siRNA or scrambled RNA, MeWo cells were seeded into 96-well plates at equal cell number. After starving cells for 6 hours with serum free medium, normal medium was added, which was set as time 0. Cells were cultured for 12, 24, 36, 48, and 60 hours. At each indicated time, MTT at concentration of 5 mg/mL was added 10 μL per well. The 4 hours later, the supernatant was removed, and the crystals were dissolved in DMSO. The optical density (OD) at 570 nm was measured with spectrophotometer (Molecular Devices Company, USA). The average OD 570 of control group was defined as the baseline. The proliferation index of other groups was calculated as their ratio to baseline. Every group had at least 6 parallel wells and data of our experiments were from at least 3 independent experiments.

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Table 1. Basic information of UM patients.

Factor	Number	Petcentage
Age		
<50	47	75.81%
≥50	15	24.19%
Sex		
Male	28	45.16%
Female	34	54.84%
Histologic type		
Spindle	42	67.74%
nonspindle	20	32.26%
Largest tumor diameter		
<15 mm	25	40.32%
≥15 mm	37	59.68%
Pigment		
<1/3	16	25.81%
<1/3–2/3	41	66.13%
>2/3	5	8.06%
Scleral invasion		
Superfacial	46	74.19%
Medial+deep	16	25.81%
Ciliary body involvement		
No	54	87.10%
Yes	8	12.90%
Lymphatic infiltration		
No	54	87.10%
Yes	8	12.90%
BTNL9		
Low	35	56.45%
High	27	43.55%

Table 2. Correlation between BTNL9 expression and clinicopathologic factors.

Characters	BTNL9		*P**
	Low	High	
Age (yrs)			
<50	25	22	0.39
≥50	10	5	
Sex			
Male	18	10	0.309
Female	17	17	
Histologic type			
Spindle	25	17	0.586
Non-spindle	10	10	
Largest tumor diameter			
<15 mm	11	14	0.124
≥15 mm	24	13	
Pigment			
<1/3	8	8	0.559
<1/3–2/3	25	16	
>2/3	2	3	
Scleral invasion			
Superficial	24	22	0.381
Medial+deep	11	5	
Ciliary body involvement			
No	28	26	0.123
Yes	7	1	
Lymphatic infiltration			
No	31	23	0.719
Yes	4	4	

* Calculated by Fisher's test. BTNL9 – butyrophilin-like 9.

Transwell assay

Invasion of melanoma cell line was estimated with Transwell assay with pre-coated 8-μm pore chamber (BD Biosciences Company). In brief, 10^5 cells per well were seeded into the upper compartment in medium with 1% FBS, with 10% FBS as a chemoattractant in lower compartment. 12 hours later, cells of upper compartment were swabbed out carefully and the invaded cells at bottom were fixed with formalin. Invaded cells were stained with crystal violet and counted at 200 magnifications. At least 6 random visual fields under microscopy were selected to count cells. Data of our experiments were from at least 3 independent experiments.

Statistical analysis

All data were analyzed with software SPSS 22.0 (IBM Corporation, New York, USA). Fisher's test was applied to analyze the correlation between BTNL9 expression and the clinicopathological factors. Survival curves were presented with Kaplan-Meier method, and statistical difference was analyzed with log-rank test. Cox-regression hazard model was applied to confirm independent prognostic factors. In experiments *in vitro*, data were presented as means ±SEM, and statistical significance was assessed with the Student's *t*-test. *P* values <0.05 was considered as significant.

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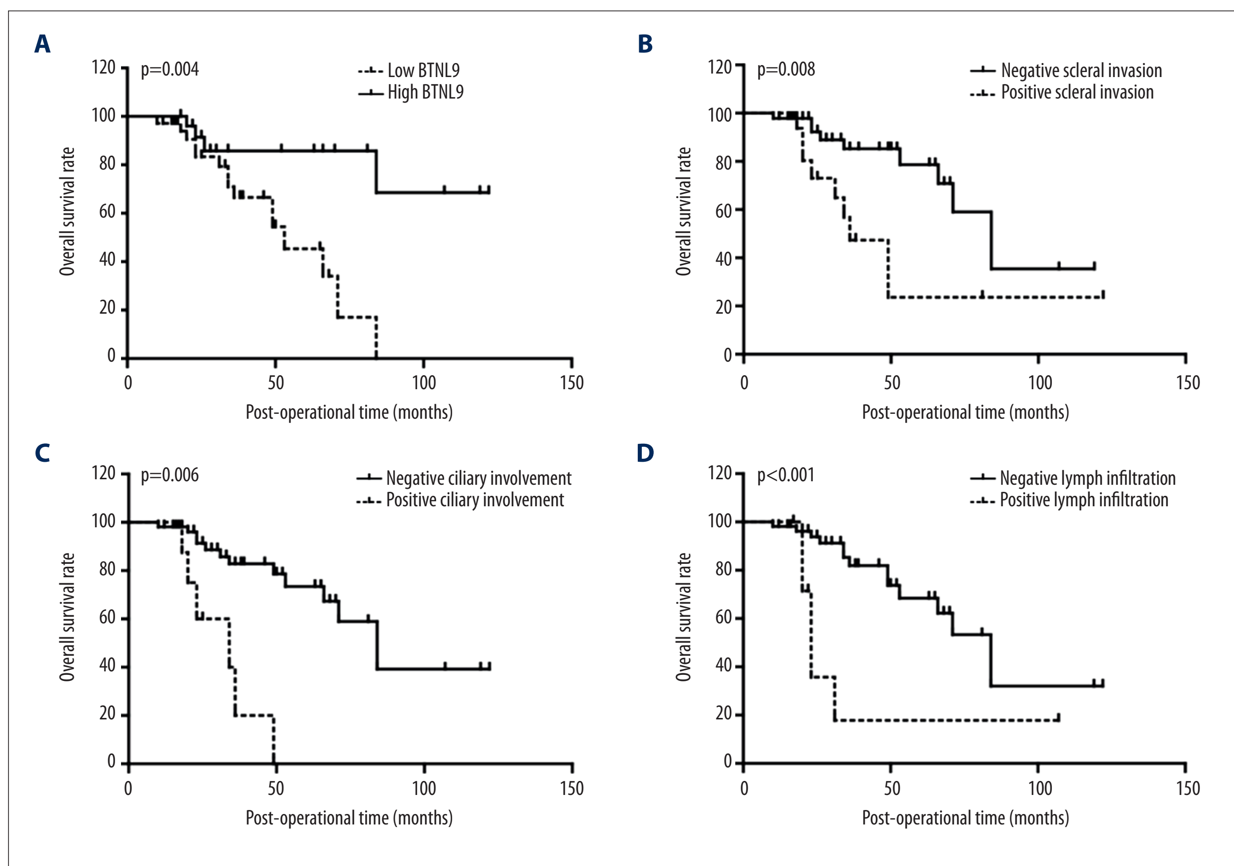

Figure 2. Survival curves of uveal melanoma (UM) patients were stratified with different factors. Patients with low BTNL9 expression (**A**), positive scleral invasion (**B**), positive ciliary involvement (**C**), and positive lymphatic invasion (**D**) had poorer prognosis. BTNL9 – butyrophilin-like 9.

Results

Expression of BTNL9 in UM tissues and adjacent tissues

Previous study pointed that BTNL9 had lower expression in colon cancer compared with normal colon epithelium [16], so we first evaluated the BTNL9 expression in UM tissues and their adjacent tissues with (qRT-PCR). The mRNA level of BTNL9 in 6 pairs of UM tissues and their adjacent tissues were compared. It turned out that BTNL9 mRNAs in adjacent tissues were remarkably higher than those in UM tissues, suggesting the potential role of BTNL9 in tumorigenesis of UM (Figure 1A). Moreover, we investigated the expression of BTNL9 in 62 cases of UM, and divided them into BTNL9 high expression and low expression subgroups according to the cutoff defined with ROC curves (Figure 1B). In our study, the percentages of BTNL9 low expression and high expression were 56.45% and 43.55%, respectively (Table 1).

Correlation between BTNL9 and clinicopathological factors

Many clinicopathological factors of UM, including patients' sex, age, histologic type, the largest tumor diameter, tumor pigment, scleral invasion, ciliary body involvement, and lymphatic infiltration were enrolled into our study. The correlations between these clinicopathological factors and BTNL9 expression were analyzed with the Fisher's test (Table 2). In our study, no parameter had notable correlation with BTNL9 expression.

Prognostic value of BTNL9

The prognostic significance of BTNL9 in tumors has never been investigated before, so here we evaluated its prognostic value by univariate analysis first (Table 3). With Kaplan-Meier method and log-rank test, we demonstrated that BTNL9 expression was remarkably associated with better prognosis of patients with UM (P=0.002). The 5-year overall survival rates of patients with low or high BTNL9 expression were 45.5% and 85.7%, respectively (Figure 2A). Moreover, several other clinicopathological factors, such as scleral invasion (P=0.008), ciliary body involvement (P<0.001) and lymphatic infiltration (P=0.006) were all identified as prognostic variables in our study (Figure 2B–2D).

The prognostic factors in univariate analysis were further enrolled into multivariate analysis to confirm the independent

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Table 3. Prognostic value of BTNL9 and clinicopathological factors of UM.

Characters	5-year OS rate	*P**	HR	95%CI	*P#*
Age (yrs)					
<50	62.6		1		
≥50	49.8	0.484	0.42	0.12–1.44	0.169
Sex					
Male	65.7		1		
Female	60.9	0.683	0.78	0.24–2.56	0.687
Histologic type					
Spindle	56.3				
Non-spindle	76.1	0.891			
Largest tumor diameter					
<15 mm	75.9				
≥1 5mm	54.3	0.268			
Pigment					
<1/3	92.3				
<1/3–2/3	54.8				
>2/3	50.0	0.293			
Scleral invasion					
Superficial	78.7		1		
Medial+deep	23.7	0.008	2.56	0.80–8.15	0.113
Ciliary body involvement					
No	73.4		1		
Yes	0.0	<0.001	4.42	1.33–14.7	0.015
Lymphatic infiltration					
No	68.5		1		
Yes	17.9	0.006	7.07	1.74–28.72	0.006
BTNL9					
Low	45.5		1		
High	85.7	0.004	0.23	0.06–0.89	0.033

* Calculated with log-rank test, # calculated by Cox-regression model. HR – hazard radio; CI – confidence interval; BTNL9 – butyrophilin-like 9; UM – uveal melanoma.

prognostic factors with Cox-regression hazard model (Table 3). Patient sex and age were considered essential clinicopathological factors. In our study, the high expression of BTNL9 was identified as an independent favorable prognostic factor in our study (*P*=0.033). Post-operational risk of UM-related death of patients with high expression of BTNL9 was 0.33-fold ratio compared with those with low expression of BTNL9 (95% CI=0.06–0.89). In addition, ciliary body involvement (*P*=0.015, HR=4.42, 95% CI=1.33–14.7) and lymphatic infiltration (*P*=0.006, HR=7.07, 95% CI=1.74–28.72) were also confirmed as independent prognostic factors in our study.

BTNL9 could suppress invasion of UM

We observed that BTNL9 expression was significantly associated with favorable prognosis of UM, suggesting the tumor suppressor role of BTNL9 in UM. So, we further investigated the function of BTNL9 in UM cells. The expression of BTNL9 of human melanoma MeWo cell line was first knocked down with siRNA, and successful BTNL9 silencing was validated with immunoblotting and qRT-PCR (Figure 3A, 3B). After knocking down BTNL9 expression, we detected the influence of BTNL9 on melanoma proliferation and invasion, with MTT assay and Transwell assay.

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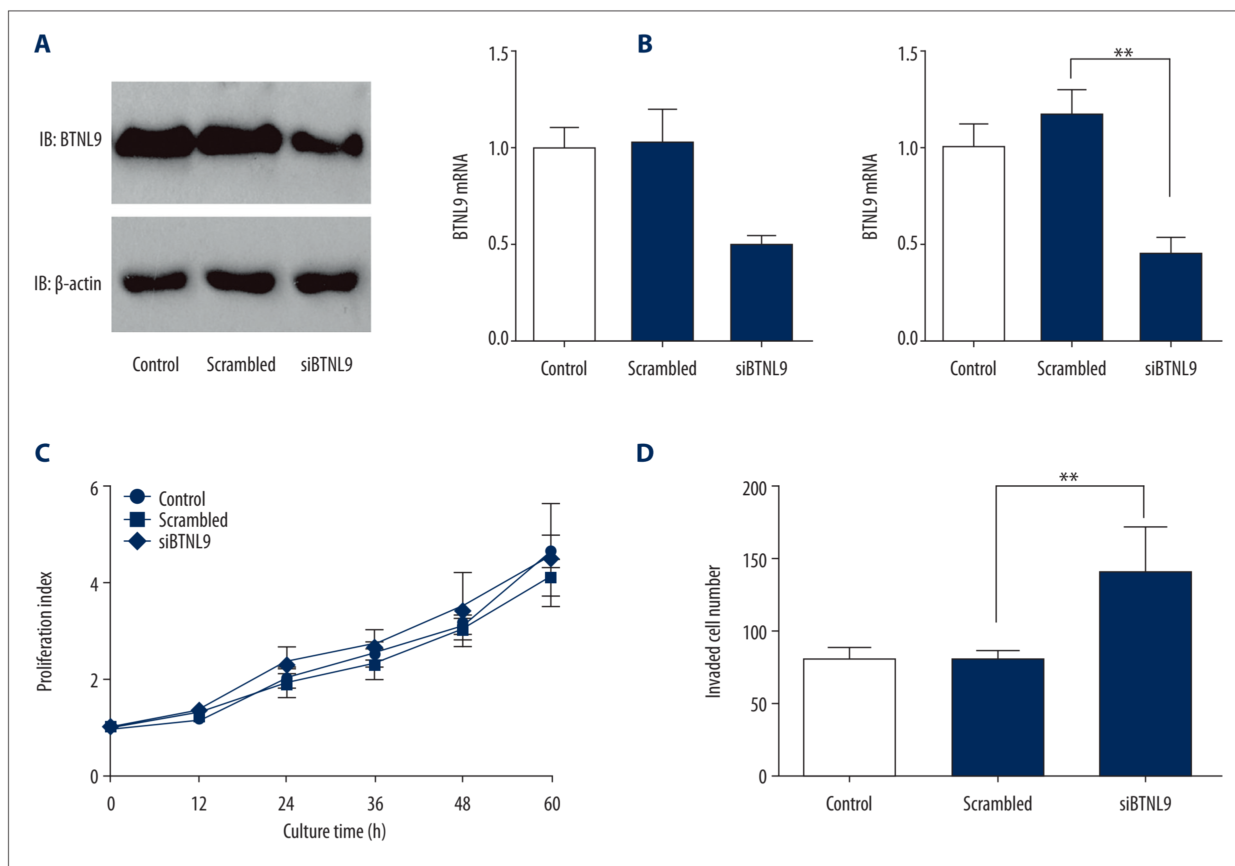

Figure 3. BTNL9 could suppress invasion of MeWo cells. (**A**) **Left panel:** successful knockdown of BTNL9 was verified with western blotting. **Right panel:** data of western blotting from 3 independent experiments were analyzed with ImageJ software. (**B**) Successful knockdown of BTNL9 was verified with qRT-PCR. (**C**) Silencing BTNL9 had no significant influence on proliferation of melanoma cell line MeWo. (**D**) BTNL9 knockdown increased the invasion of MeWo cells. BTNL9 – butyrophilin-like 9; qRT-PCR – quantitative real-time polymerase chain reaction.

As a result, there was no significant difference of proliferation between BTNL9 knockdown and control group (Figure 3C), whereas silencing BTNL9 substantially promoted the invasion of MeWo cells (Figure 3D). These results suggested that BTNL9 could suppress melanoma invasion instead of proliferation.

Discussion

The genic mapping of UM has achieved a serious of progresses and expanded the understanding of UM. One biological feature of UM is the mutations in guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) and guanine nucleotide-binding protein subunit alpha-11 (GNA11), which constitutively stimulates downstream signaling pathway including MAPK and PI3K/AKT signaling pathway [19,20]. Several other genetic alterations were also unveiled in the development of UM such as BRCA1-associated protein 1 (*BAP1*) or splicing factor 3B subunit 1 (*SF3B1*), which were both associated with phenotype and prognosis of UM [21]. These findings dramatically improved the interest on clinical trial of targeted agents against the MAPK pathway. However, there is still no FDA-approved target drug for metastatic UM, and the response rates to MAPK antagonists are generally less than 10% [22].

Besides targeted therapy, immunotherapy, especially therapy based on immune checkpoint inhibitors targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1), dramatically improved survival rates of patients with cutaneous melanoma [23]. However, the response of UM to immunotherapy is much less significant compared with cutaneous melanoma, and the underlying mechanism has not been well elucidated [24]. Fortunately, other immune-based therapies exhibit promising signs to treat UM like IMCgp100, which could recognize melanocyte-associated antigen glycoprotein 100 (gp100) and redirect a potent T cell-mediated immune response toward gp100 positive melanoma cells [25].

In any immunotherapy, TCRs are essential components because of their functions to recognize antigens, pathogens

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or cellular stress. TCRs are composed of heterodimer of an α and β chain in most T cells, while a few TCRs are comprised of γ and δ chain, which have different gene *loci* and abundance from α and β chain [26]. Unlike the αβT cells most expressed in peripheral blood, the γδT cells are predominate in tissues such as the skin, intestine and reproductive tract [27]. The γδT cells are involved in the infiltration of several types of tumors including melanoma, breast, ovarian, colon, lung, pancreatic and prostate, and are considered to have potent antitumor activity [13–15]. The BTN and BTNL family have been demonstrated to be essential in the activation of γδT cells [12], which have critical immunological functions in infectious diseases, tumors, and homeostasis [27]. In our study, our conclusion corresponded with previous studies that showed that BTNL9 could suppress UM invasion and was correlated with favorable prognosis, indicating therapy targeting BTNL9 might be a promising approach to suppress invasion and treat UM.

BTN and BTNL family are homologous to B7 protein family. Many B7 homologous proteins have been demonstrated to regulate T lymphocytes response. Some have co-stimulatory effect to T cell response like B7-1, ICOS, etc., while some have co-inhibitory functions including PD-L1, PD-L2, B7-H3, etc. [9,10]. Many of them are well-studied molecules and have helped generate great breakthroughs in cancer treatment like anti-PD-1 immunotherapy. Recent studies have demonstrated the emergent role of BTN/BTNL in modulating response of γδ T cells [28], but whether BTN/BTNL family could influence immune-response of UM needs further research. In the BTNL family, BTNL9 has attracted less attention compared with other members. We hope our findings will initiate more interest on BTNL9 function in UM and help improve new therapeutic approaches, especially immune-based therapy for UM.

In our study, we demonstrated that BTNL9 expression was significantly downregulated in 62 cases of UM. The sample size was relatively small because of the low incidence of UM. Here the tumor suppressor role of BNTL9 was unveiled by clinical observation and experiments *in vitro*, but the underlying mechanism of how BTNL9 suppresses UM needs further investigation. Our study expanded the clinical significance of BTNL9 in UM and points to its tumor suppressor role. Detecting BTNL9 could help identify patients with low risk and high risk, facilitating post-operational surveillance. BTNL9 might be a potential drug target and therapies stimulating BTNL9 might help in the treatment of UM patients. We hope our findings can initiate more attempts to explore the potency of BTNL9 as a drug target in UM.

Conclusions

We, for the first time, identified BTNL9 as a prognostic factor of UM indicating favorable prognosis, and demonstrated that BTNL9 could suppress invasion of UM. Our results suggest that BTNL9 detection could help stratify high-risk patients after UM operation and guide more precise surveillance and treatment.

Conflicts of interest

None.

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