Original Article
Up-regulation of Bromodomain 4 promotes cell proliferation and predicts poor prognosis in gastric cancer

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Abstract: Background: Aberrant Bromodomain 4 (BRD4) expression and its oncogenic function have been reported in several human malignancies. The aim of the present study was to investigate the expression and function of BRD4 in human gastric cancer (GC). Methods: Immunohistochemistry (IHC) was performed to evaluate BRD4 expression in 85 pairs of GC specimens and adjacent non-tumor tissues. Then, the associations of BRD4 expression with clinicopathological features and patient's overall survival were determined. Furthermore, invitro assays were used to determined the function of BRD4 on GC cells. Results: IHC revealed that BRD4 was upregulated in GC tissues compared to the corresponding non-tumor tissues. Increased BRD4 expression was associated with poor histological differentiation, advanced TNM stage, lymph nodes metastasis and shorter overall survival. Furthermore, by respectively upregulateing or depleting BRD4 in well-differentiated MKN-28 cells or poor-differenitated MKN-45 cells, we found that BRD4 could act as a promotor of GC cell proliferation and cell apoptosis resistance. Conclusions: Our study suggested that BRD4 was upregulated in GC and might serve as a novel prognostic biomarker and therapeutic target for GC patients.

Keywords: BRD4, gastric cancer, biomarker, progression

Introduction

Gastric cancer (GC) is the second most common carcinoma of human in the world, which contributes to 10% newly diagnosed cases every year and high mortality worldwide [1]. Despite recent advances in clinical and experimental oncology, patients are commonly diagnosed at the advanced stage [2]. In China, the long-term prognosis of GC patients with advanced stage is poor, with a 5-year survival rate of 25% [3]. Therefore, there is an urgent need to explore the mechanisms underlying GC at the molecular level and develop novel biomarkers for its early diagnosis, targeted therapy and prognosis evaluation.

The bromodomain and extra terminal domain (BET) family is composed of the three ubiquitously expressed BRD4, BRD3, and BRD2, and the testis-specific BRDT form [4]. Bromodomain 4 (BRD4) is one of the best studied member of

the BET that bind to acetylated lysine residues in histones, which function as important reader molecules that associate with acetylated histones and participate in the assembly of chromatin complexes and transcription activators at specific promoter sites [5, 6]. Recent research showed that BRD4 plays an important role in the regulation of cell growth that lead to the genesis and development of many diseases including cancer [7]. For example, Yan et al showed overexpression of BRD4 contributed to progressive features and poor prognosis of human urothelial carcinoma of the bladder [8]. Hu et al showed that BRD4 was increased in colorectal cancer and BRD4 inhibitor could inhibited colorectal cancer growth and metastasis [9]. Hong et al suggested that epigenetic reader BRD4 inhibition as a therapeutic strategy to suppress E2F2-cell cycle regulation circuit in liver cancer [10]. Zhou et al found that down-regulation of miR-329 promoted cell invasion by

Table 1. The correlation between expression characteristic of BRD4 in GC specimens and GC clinicopathologic features

Clinicopathologic features	BRD4 expression		
	Low (n=39)	High (n=46)	P*
Age (years)			
≤60	17	20	0.992
>60	22	26	
Gender			
Male	21	29	0.391
Female	18	17	
Diameter (cm)			
≤5	22	19	0.165
>5	17	27	
Location			
Distal third	20	21	0.605
Middle or proximal third	19	25	
Histological differentiation			
Poorly differrntion	17	31	0.027
Middle/well differrntion	22	15	
Lymph nodes metastasis			
No	28	10	0.000
Yes	11	35	
TNM stage			
I, II	26	16	0.003
III, IV	13	30	

regulating BRD4 and predicted poor prognosis in hepatocellular carcinoma [11]. Although there has been demonstrated that BRD4 acted as a potential therapeutic target for several cancers, it has never been investigated in GC and the role of BRD4 in GC is still unclear.

In the present study, we explored the expression of BRD4 in both GC tissues and three GC cell lines presenting different cell differentiation (MKN-45, SGC-7901 and MKN-28). In addition, the relationship between BRD4 and GC patients' clinicopathologic features and prognosis was analyzed. Furthermore, we regulated the expression of BRD4 in GC cells to study the effect of BRD4 on GC cell proliferation, apoptosis and cell cycle distribution. Our findings suggested that BRD4 could act as a probable prognostic biomarker and a potential therapeutic target for the treatment of GC.

Materials and methods

Patients and clinical specimens

Matched fresh GC specimens and adjacent non-tumor tissues were collected from 85 patients who underwent gastrectomy at The First People's Hospital of Shangqiu between January 2009 and December 2011. All specimens were snap frozen in liquid nitrogen immediately following collection and stored at -80°C until use. None of the patients had received chemotherapy or radiotherapy before surgery. Clinicopathologic information is summarized in **Table 1**. Overall survival was calculated from the date of initial surgical operation to death or last follow-up. The Research Ethics Committee of The First People's Hospital of Shangqiu approved this study and all patients provided written informed consent.

Immunohistochemistry analysis

The tissues were fixed in 10% neutral formalin and then embedded in paraffin. Paraffin sections (4 μm thick) were deparaffinized in xylene and rehydrated in grade alcohol, followed by boiling in 10 mmol/L of citrate buffer for antigen retrieval. The sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4°C with primary BRD4 antibody (1:200; Abcam) after the inhibition of endogenous peroxidase activities for 30 min with methanol containing 0.3% H_2O_2. Washing the slides thrice with PBS, it was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min according to the instruction of the UltraSensitive S-P (Maixin).

Evaluation of immunohistochemical staining

Two pathological doctors who knew nothing about the clinicopathological information of the patients independently conducted the evaluation of the immunohistochemical staining. The immunoreactive scores besides BRB4 were determined by the sum of extension and intensity as literature reported previously [12]. The scores of the intensity of the staining was followed as: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining, and +++, marked staining. The extension was evaluated and recorded as the percentage of the area of staining as following: 0, less than 5%; +, 5%-25%; ++, 26%-50%; +++, 51%-75% and

++++, more than 75%. Afterwards, we combine the scores of the intensity and extension of the immunohistochemical staining as: -, 0; +, 1-2; ++, 3-5; +++, 6-7. Meanwhile, the cases with scored - or + were determined as BRD4 low expression group; and the ++ or +++ were determined as the high expression group.

Cell culture

Three GC cell lines (MKN-45, SGC-7901 and MKN-28) and the immortalized gastric epithelium cell line (GES-1) were purchased from American Type Culture Collection (ATCC), from which MKN-45, SGC-7901 and MKN-28 cells are respectively present poorly, moderately- and well-differentiation stage. All cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 ug/ml streptomycin and 100 U/ml Penicillin in a humidified cell incubator at 37°C with an atmosphere of 5% CO_2.

Cell transfection

Recombinant adenovirus Ad5/F35 (Ad5/F35-BRD4) was constructed for over expressing BRD4 and Ad5/F35-Null was used as negative control (GenePhrma). Well-differentiated MKN-28 cells were transfected with Ad5/F35-BRD4 or Ad5/F35-Null.

Poorly-differentiated MKN-45 cells were transfected with pGPU6/GFP/Neo vectors (Gene-Phrma) containing shRNA against BRD4 (sh-BRD4) by using Lipofectamine 2000 (Invitrogen), and non-containing ones were used as negative control (sh-NC). Cell were cultured and maintained in medium containing 200 μg/ml G418.

Cell proliferation assay

Cell proliferation was assayed using CCK-8 (Dojindo) according to the manufacturer's instruction. Briefly, a total of approximately 10000 GC cells were plated in 96-well plates, treated with 10 ml/well of CCK-8 solution during the last 4 h of culture, and the cell proliferation curves were plotted using the 450 nm absorbance at each time point. All experiments were performed in triplicate.

Flow cytometric analysis of cell cycle and apoptosis

Transfected GC cells were harvested and stained with propidium iodide according to the

Cell Cycle Analysis Kit (Biyuntian), and then assessed by flow cytometer. The percentage of the cells in different phases was counted. For apoptosis analysis, FITC Annexin V Apoptosis Detection Kit (BD Biosciences) was used. The transfected cells were re-suspended in binding buffer containing Annexin V-FITC and propidium iodide, and assessed by flow cytometer according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIzol reagents (Life Technologies) according to the manufacturer's instructions. The first-strand cDNAs were synthesized through High-Capacity cDNA Reverse Transcription Kit (ABI). RT-primers of BRD4 was synthesized by Sangon Biotech Company (Shanghai) as follow: 5'-GTCAAACT-GGGTCTACCGGATT-3' (forward) and 5'-CTTTTC-CAGCGTTTGTGCCA-3' (reverse). Quantitative Real-time PCR (qRT-PCR) was carried out according to TagMan Gene Expression Assays protocol (ABI).

Western blot

Cells were lysed in lysis buffer containing protease inhibit. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred to PVDF membranes (Bio-Rad). After being blocked in Tris buffered saline (TBS) containing 5% nonfat milk, the membranes were incubated with specific primary antibodies (Abcam) at 4°C for 12 h and then with horseradish peroxidase-conjugated anti-mouse antibody for 2 h at room temperature. ECL detection reagent (Amersham LifeScience) was used to demonstrate the results.

Statistical analysis

Statistical analysis was carried out by using SPSS 18.0. Differences among the groups were assessed by Wilcoxon signed-rank test, Pearson chi-square test, Log-rank test or Student's *t* test as indicated. *P* values less than 0.05 were considered statistically significant.

Results

BRD4 is highly expressed in GC tumors and cell lines

In order to explore the expression of BRD4 in human GC, 85 GC tissues were confirmed by
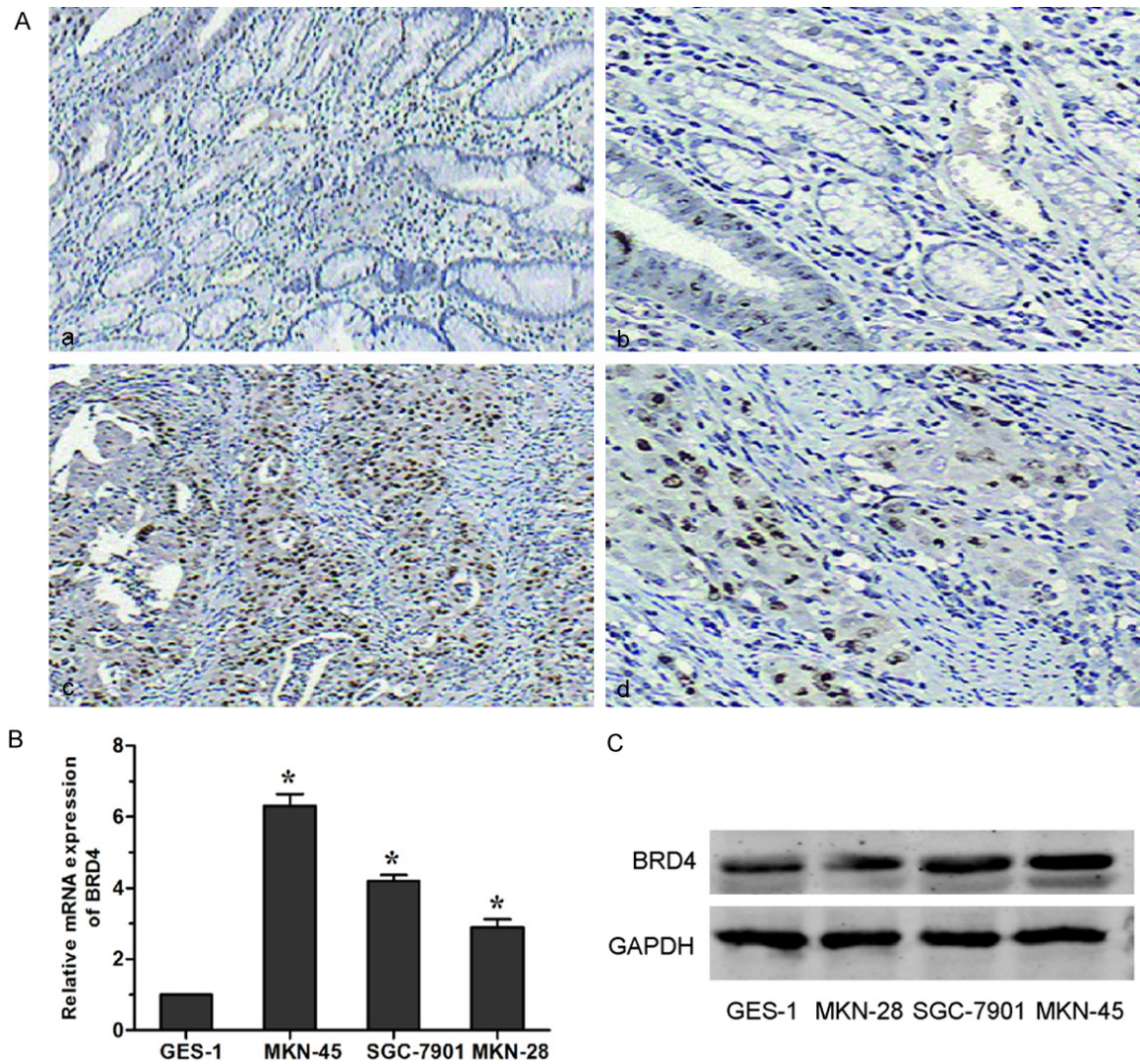
Figure 1. The BRD4 expression levels in GC tissues and cell lines. (A) ICH analysis of BRD4 protein expression in GC tissues. (Aa) IHC expression of BRD4 in adjacent non-tumor tissue (×40). (Ab) IHC expression of BRD4 in adjacent non-tumor tissue (×100). (Ac) IHC expression of BRD4 in GC tissue (×40). (Ad) IHC expression of BRD4 in GC tissue (×100). (B, C) The expression of BRD4 mRNA and protein in GC cell lines (MKN-45, SGC-7901 and MKN-28) and GES-1 cells were examined by qRT-PCR (B) and Western blot (C). *$P<0.05$.

surgical operation and pathology were selected and examined by IHC. As shown in **Figure 1A**, ICH staining indicated that BRD4 protein was mainly accumulated in the nuclei of the GC cells. Besides, the expression of BRD4 in GC tissues was significantly higher than in adjacent non-tumor tissues ($P<0.05$; **Figure 1A**). Next we evaluate the expression levels of BRD4 mRNA and protein in three GC cell lines, our data showed that BRD4 was upregulated in MKN-45, SGC-7901 and MKN-28 cells both on mRNA and protein levels compared to GES-1 cells ($P<0.05$; **Figure 1B** and **1C**). Interestingly, we found a degressive expression characteris-

tic of BRD4 from poorly-differentiated MKN-45 cells to mediated-diferentiated SGC-7901 cells and well-differentiated MKN-28 cells. As this gradient expression characteristic of BRD4, we suggested that BRD4 were probably an aberrantly highly expressed molecule potentially concerning with GC cell dedifferentiation.

Relationship between BRD4 expression and GC patients' clinicopathologic features

As BRD4 presented a higher expression in poorly-differentiated GC cell than the mediated- and well-differentiated ones, we speculated
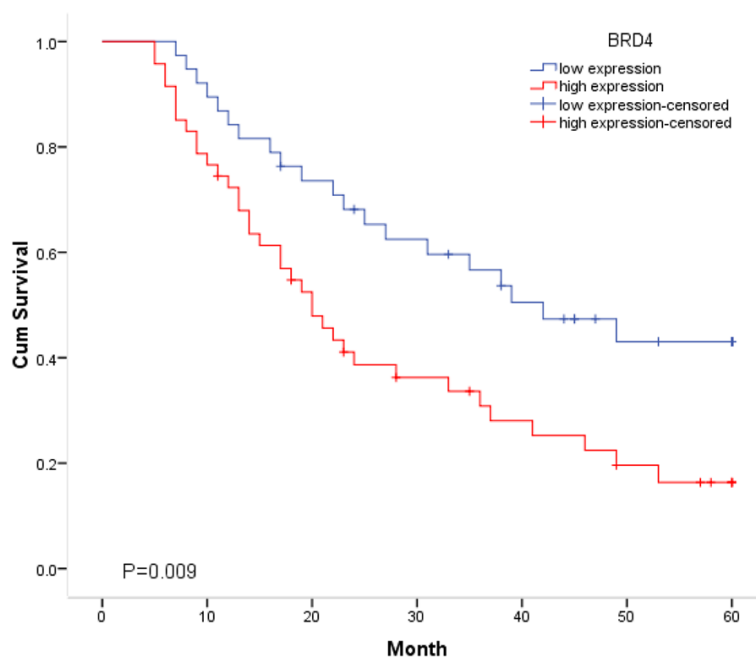
Figure 2. High BRD4 expression predicts poor prognosis of GC patients. Kaplan-Meier analyses showed that patients with high BRD4 expression had a poor overall survival than those in low BRD4 expression. (P=0.009, log-rank test).

that expression characteristic of BRD4 could be a factor associate with some of the GC clinicopathologic features. In the the study, the relationship between BRD4 expression and the patients' clinicopathologic features was shown in **Table 1**. We found that high expression of BRD4 was associated with GC patients with poor histological differentiation, advanced TNM stage and lymph nodes metastasis (P<0.05).

High BRD4 expression predicts poor prognosis of GC patients

We further examined the correlation between BRD4 expression and GC patients' prognosis. Kaplan-Meier analysis showed that patients with high BRD4 expression had a significantly shorter overall survival than those with low BRD4 expression (P<0.05; **Figure 2**). These data suggested that high BRD4 expression predicts poor prognosis in GC, and BRD4 might function as an oncogene in GC tumorigenesis.

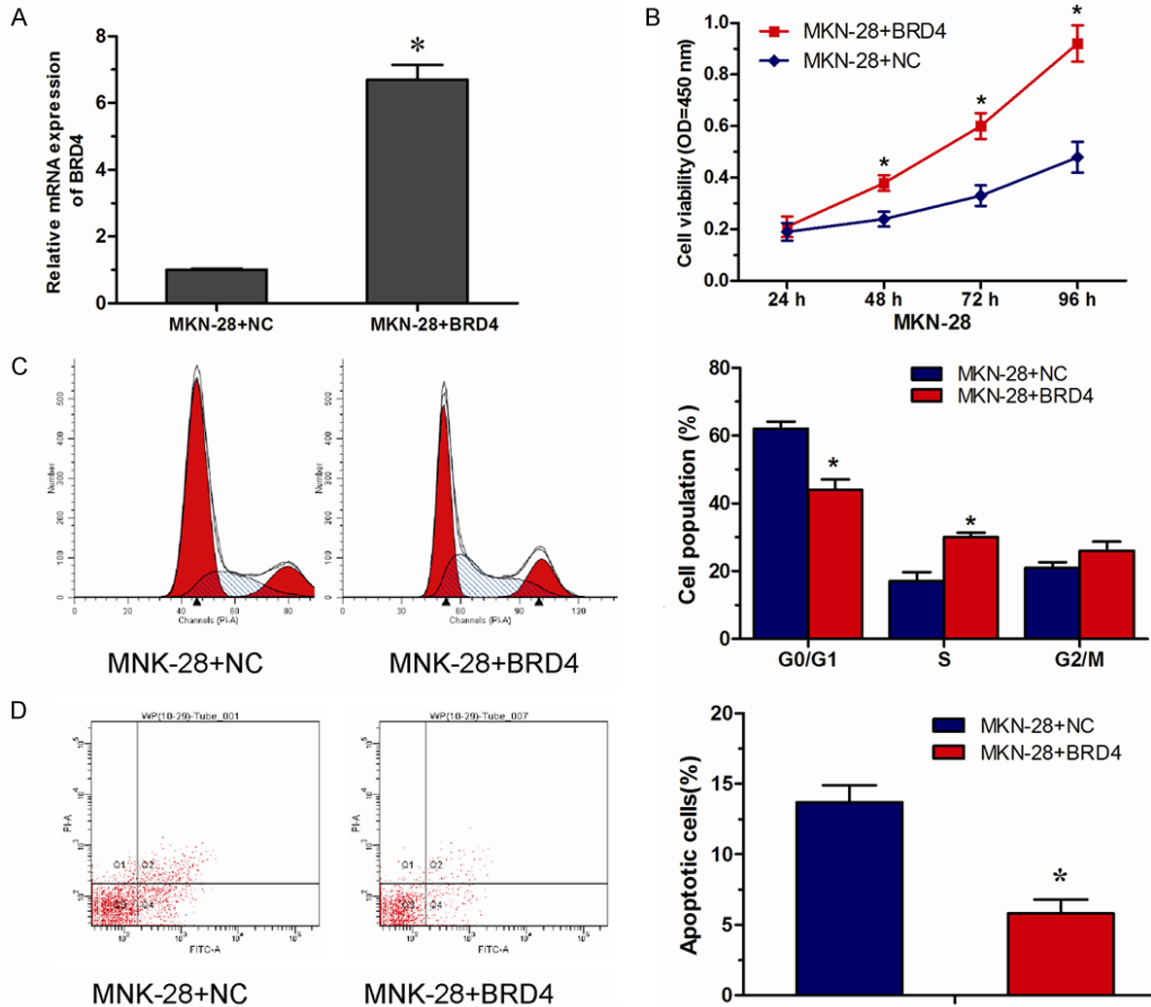*Overexpression of BRD4 promotes MKN-28 cell proliferation*

We applied recombinant adenovirus Ad5/F35 as a vector to over-express BRD4 in well-differ-

entiated MKN-28 cells, which presented the lowest BRD4 expression among the three GC cell lines (P<0.05; **Figure 3A**). CCK-8 assay showed that the proliferation ability of BRD4 overexpressing MKN-28 cells was significantly accelerated compared with that of the controls (P<0.05; **Figure 3B**). Next, flow cytometric analysis was used to explore whether the effect of BRD4 on cell proliferation was by altering cell apoptosis or cell cycle progression. Cell apoptosis analysis showed that overepression of BRD4 in MKN-28 cells inhibited the cell apoptosis rate compared to the control (P<0.05; **Figure 3C**). Cell cycle distribution analysis of MKN-28 cells showed that overepression of BRD4 resulted in a significant decrease in the cellular population in G0/G1 phase but a sharp increase in S phase (P<0.05; **Figure 3D**).

Knockdown of BRD4 suppresses MKN-45 cell proliferation

MKN-45 cells were poorly-differentiated GC cells presented highest BRD4 expression among the three GC cell lines. By transfecting MKN-45 cells with sh-BRD4, we successfully decreased the expression of BRD4 in MKN-45 cells (P<0.05; **Figure 4A**). According to the result that BRD4 induced promotion of well-differentiated MKN-28 cell proliferation, we presumed that depletion of BRD4 would inhibit the cell proliferation of MKN-45 cells. CCK-8 assay showed that decreased expression of BRD4 significantly inhibited MKN-45 cells proliferation compared to control group (sh-NC) (P<0.05; **Figure 4B**). Cell apoptosis analysis showed that down-regulated expression of BRD4 promoted the apoptosis rate of MKN-45 cells compared to sh-NC group (P<0.05; **Figure 4C**). Cell cycle distribution analysis showed that reduced expression of BRD4 in MKN-45 cells could arrest cells in G1 phase compare to sh-NC group (P<0.05; **Figure 4D**). These results indicated that BRD4 function as a promoter in

Figure 3. Overexpression of BRD4 on MKN-28 cells proliferation, cell apoptosis and cell cycle. A. The relative expression of BRD4 in MKN-28 cells transfected with Ad5/F35-BRD4 expressing vector or Ad5/F35-Null vector. B. CCK-8 assays showed that overexpression of BRD4 promoted MKN-28 cells proliferation. C. Cell apoptosis showed that overexpression of BRD4 inhibit MKN-28 cells apoptosis. D. Flow cytometric analysis showed that overexpression of BRD4 decreased the MKN-28 cells population in G0/G1 phase. *P<0.05.

GC cell proliferation, and the functions of BRD4 were probably associated with GC cells dedifferentiation.

Discussion

GC is one of the most common malignancies worldwide contributes to a second cancer causing mortality next to lung cancer, and high mortality in GC is mainly due to relapse and metastasis [13]. Despite the research progress has gradually reveal some of the key elements of GC initiation and procession, the mechanism of the biological and molecular basis in GC yet leaves us lots of unknown.

The bromodomain (BrD)-containing proteins have been shown to exert a great role in the interface between chromatin remoding and transcriptional regulation [14]. The epigenetic regulators in cancer biology has become focus research in recent years. BRD4 is one of the best studied member of the BET that bind to acetylated lysine residues in histones, which function as important reader molecules that associate with acetylated histones and participate in the assembly of chromatin complexes and transcription activators at specific promoter sites [15, 16]. Histone acetylation is one of the medication of the histones undergo post-translational, which relaxes chromatin struc-
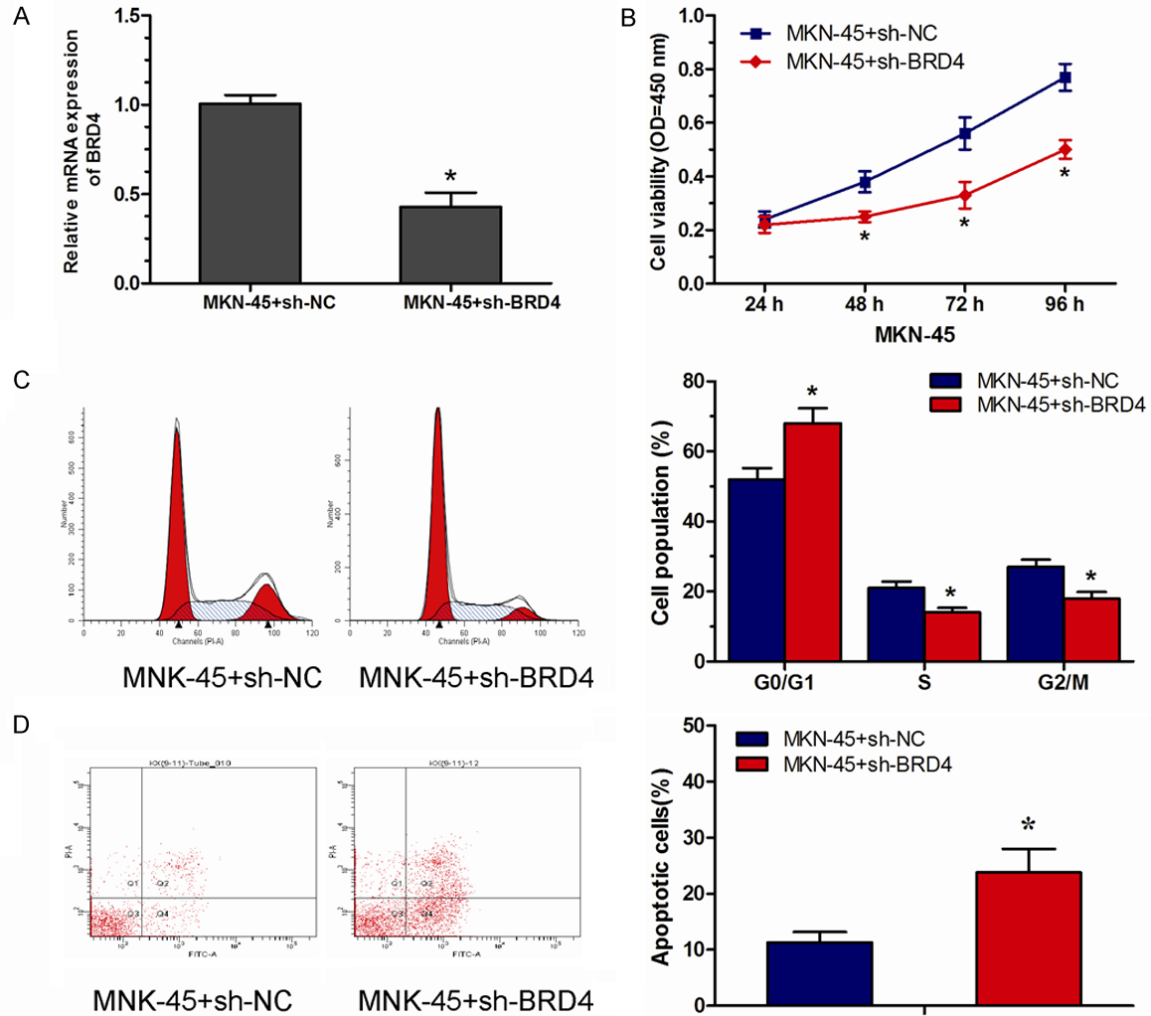
Figure 4. The effects of inhibition of BRD4 on MKN-45 cells proliferation, cell apoptosis and cell cycle. A. The relative expression of BRD4 in MKN-45 cells transfected with sh-BRD4 or sh-NC. B. CCK-8 assays showed that depletion of BRD4 suppress MKN-45 cells proliferation. C. Cell apoptosis showed that depletion of BRD4 promote MKN-28 cells apoptosis. D. Flow cytometric analysis showed that depletion of BRD4 promoted MKN-28 cell cycle arrest in G0/G1 phase. *P<0.05.

ture through loosening the interaction with DNA [17]. Many studies had reported the link between aberrant histone acetylation and cancer [18]. Recent studies showed that BRD4 played important roles in the development, inflammation and certain types of cancer [19, 20]. For example, increased expression of BRD4 had been found in bladder cancer, liver cancer and colorectal cancer [8-11]. On the other side, previous studies have demonstrated that suppression of BRD4 either using shRNAs or the small-molecule inhibitor have achieved a great role in anti-cancer effects [20, 21]. However, the clinical prognosis and potential functions of BRD4 in GC is still unclear.

In the present study, we explored the expression of BRD4 in three GC cell lines presenting different differentiation stages. Our data showed that BRD4 expression was increased in GC cell lines compared to GES-1 cells. Moreover, the expression of BRD4 was degressive from poorly-differentiated MKN-45 cells to well-differentiated MKN-28 cells. Those findings suggested that aberrant expressed of BRD4 play important roles in GC progression.

As our data revealed, we investigated the expression of BRD4 in GC tissues. IHC showed that the expression of BRD4 in GC tissues was significantly higher than in adjacent non-tumor

tissue. In addition, we found that increased BRD4 expression was significantly associated with poor histological differentiation, advanced TNM stage and lymph nodes metastasis. Kaplan-Meier survival analysis revealed that patients with high BRD4 expression had a significantly poor overall survival than those with low BRD4 expression. Our findings indicated that BRD4 could be a target correlative with GC prognosis and progression. The relationship between BRD4 and GC clinicopathologic features might be an evidence that high expression of BRD4 related with GC cell dedifferentiation.

We further selectively depleted BRD4 in poorly-differentiated MKN-45 cells and up-regulated BRD4 in well-differentiated MKN-28 cells to explore the role of BRD4 in GC progression. As we expected, when BRD4 was downregulated in MKN-45 cells, cell proliferation was suppressed with a significant increased in cell apoptosis and an arrest of cell cycle in G0/G1 phase. On contrast, the proliferation ability was accelerated in MKN-28 cells with BRD4 overexpression, in addition, the cell apoptosis rate was reduced and cell cycle was decreased in G0/G1 phase. These data suggested that BRD4 play critical roles in the GC cell generation.

In conclusion, we found that BRD4 acts as a tumor oncogene in GC by promoting cell proliferation, regulating cell cycle and inhibiting cell apoptosis. Our data demonstrated that BRD4 could serve as a novel target for treatment or prognostic marker of GC.

Disclosure of conflict of interest

None.

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