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Original Article

JARID1B modulates breast cancer cell apoptosis by regulating p53 expression

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Abstract: Jumonji AT-rich interactive domain 1B (JARID1B) has been implicated in breast cancer progression, but its role in apoptosis has not been explored. The present study was designed to investigate the effect of JARID1B on breast cancer cell apoptosis. Apoptosis was assessed by TUNEL, flow cytometry and caspase-3 activity. JARID1B and p53 expression were examined by Western blot. Cell viability was measured by an MTT assay. We found that JARID1B is overexpressed in the breast cancer cell line and in breast cancer tissues. Upregulated expression of JARID1B in breast cancer tissues correlates with poor patient prognosis. The apoptosis of breast cancer cells is significantly increased by RNA interference targeting JARID1B. Moreover, the expression of p53 is modulated by JARID1B; the silencing of JARID1B exhibits greatly increased p53 expression at the protein level. The inhibition of p53 by small interfering RNA (siRNA) reverses the JARID1B siRNA-induced increase of apoptosis. Our results collectively suggest that JARID1B plays a key role in breast cancer cell apoptosis, and it may partially achieve this role through p53.

Keywords: Breast cancer, JARID1B, p53, apoptosis

Introduction

Breast cancer is one of the most frequent carcinomas and the second leading cause of cancer-related mortality in women, with an estimated 1.5 million new cases per year [1, 2]. The pathological progression of breast cancer is multistage and complicated, consisting of oncogenesis, proliferation, apoptosis, invasion, and metastasis [3]. Apoptosis, or programmed cell death, is an important control mechanism of normal cell physiology [4]. A deficiency in apoptosis is one of the key features of cancer cells; restoring and activating apoptosis in cancer cells is a major target of cancer treatment [5-7]. Consequently, targeting the induction of apoptosis might be a good therapeutic strategy to combat breast cancer.

JARID1B specifically removes the trimethyl modification of H3K4, inhibiting gene transcription [8]. Previous studies have suggested that JARID1B plays a vital role in the development of breast cancer, and it is therefore considered to be an important drug target protein [9-12]. Some studies have demonstrated that JARID1B is a critical player in the regulation of apoptosis in cancer progression [13, 14]. For instance, JARID1B knockdown results in G1 arrest and early apoptosis by suppressing Bcl-2 family members in head and neck squamous carcinoma cells [13]. The down-regulation of JARID1B expression inhibits cell proliferation, induces apoptosis and blocks the cell cycle in human acute lymphoblastic leukemia cells [14]. Although JARID1B expression has been studied in breast cancer, little is known about the function and mechanism of JARID1B in breast cancer cell apoptosis.

p53 is a master regulatory tumor suppressor gene that suppresses the expression of numerous target genes; this suppression functions to block cell proliferation or activate cell death programs, and this ultimately prevents tumor development and growth [15]. It has been reported that JARID1B modulates lung cancer cell proliferation and invasion by regulating p53

expression [16]. Thus, we speculated that JARID1B might regulate p53 in breast cancer cell apoptosis. We aimed to determine whether JARID1B inhibition could induce apoptosis in breast cancer cells, and if so, to elucidate the mechanisms involved.

Materials and methods

Patients and tissue samples

A total of 100 breast cancer tissue samples, along with matched adjacent normal tissues, were used in this study. All of the samples were obtained from patients who were diagnosed with stage IA to IIIA breast cancer and who underwent breast surgery at the Second Affiliated Hospital of Harbin Medical University between 2015 and 2017. The patients ranged in age from 21 to 73 years old, with a mean of 42 years. None of the patients received adjuvant chemotherapy, radiotherapy or immunotherapy before surgery. Written informed consent was obtained from all of the patients who participated in this study, which was approved by the Ethical Committee of Harbin Medical University.

Immunohistochemistry

5-μm thick specimen sections were embedded in paraffin. The sections were deparaffinized in xylene, rehydrated with graded alcohol, and endogenous peroxide activity was blocked with 3% H_2O_2 for 15 min. Tissue sections were incubated with JARID1B mouse polyclonal antibody (1:100 dilution, Abcam, USA) at 4°C overnight in a humid chamber. A secondary biotinylated antibody was used for 30 min at room temperature. Antigen-antibody complexes were detected using the streptavidin-peroxidase method (15 min exposure) with diaminobenzidine (DAB) as the chromogen substrate (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, United States). The peroxidase signal was visualized by treatment with a DAB substrate-chromogen system for 8 min. Finally, the sections were stained lightly with hematoxylin, and PBS was used in place of the primary antibody as a negative control. Five views were examined per slide, and 100 cells were observed per view at 200× magnification. Positive reactions were defined as those showing brown immunostaining in the cell cytoplasm, nucleus, and membrane. The intensity of staining was determined as: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining. Tumor cell area: 0 = positive staining in less than 5% of tumor cells; 1 = positive staining in 5-25% of tumor cells; 2 = positive staining in 26-50% of tumor cells; 3 = positive staining in 51-75% of tumor cells.

Cell culture and transfection of JARID1B siRNA

The human breast carcinoma cell line MCF-7 and the normal breast epithelial cell line (MCF-10A) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics in a 5% CO_2 incubator at 37°C.

JARID1B siRNA and p53 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA negative control (NC) was synthesized by Shanghai GenePharma Co., Ltd. The siRNA NC sequence was: forward (5'-UUCUCCGAACGUGUCACGUTT-3') and reverse (5'-ACGUGACACGUUCGGAGAATT-3'). MCF-7 cells (1×10^5 per well) were starved in serum-free medium for 24 h before transfection with the X-treme GENE siRNA transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. The final concentration of JARID1B siRNA and p53 siRNA was 200 nM.

Cell viability assay

Cells (2×10^4 cells/well) were seeded in a 96-well culture plate. Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. The absorbance was measured at 490 nm. The cell viability was expressed as a percentage of relative viable cells to the control cells.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Apoptosis of MCF-7 was detected with the in situ Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche, USA) according to the manufacturer's instructions. After TUNEL staining, the MCF-7 cells were stained using DAPI (Sigma-Aldrich) and observed using a laser scanning confocal microscope (Olympus, Fluoview 1000, Tokyo, Japan). The number of

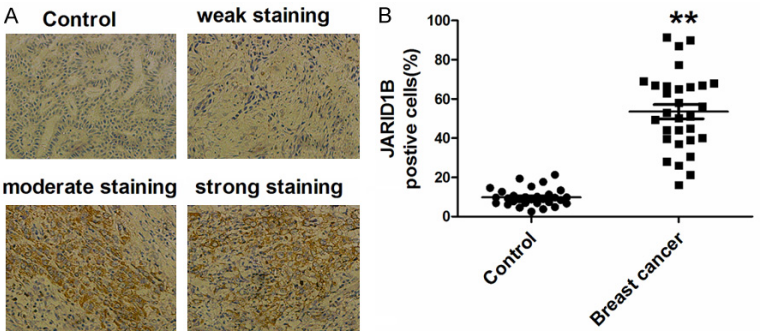

Figure 1. The expression of JARID1B was upregulated in breast cancer tissues, and increased JARID1B expression correlates with poor patient prognosis. A. Representative images of JARID1B immunohistochemical staining in normal breast tissue and breast cancers. 200×. B. The numbers of JARID1B-positive cells in normal and tumor tissues were analyzed. $^{**}P < 0.01$ vs. control group, n = 30 each group.

Table 1. Demographic characteristics of patients with breast cancer

Characteristics	JARID1B expression		Total	*P*
	Low	High		
Age (Years)				
≤ 45	10	36	46	0.64
> 45	14	40	54	0.38
Residual tumor size (cm)				
< 1	8	4	12	0.46
1-3	16	42	58	0.023
> 3	0	30	30	0.001
Histological grade				
G1	7	20	27	0.047
G2	5	27	32	0.013
G3	2	39	41	0.004
Differentiation				
Well	3	34	37	0.016
Moderate	14	10	24	0.56
Poor	39	0	39	0.003
Depth of Invasion				
T1	14	12	26	0.97
T2	10	50	60	0.029
T3	0	14	14	0.018
Lymph node metastasis				
N0	18	36	54	0.51
N1/N2/N3	6	40	46	0.026

Statistical analysis of tumor characteristics correlated with clinicopathological parameters. (n = 100, G = Grade, T1 ≤ 1 cm, T2 ≤ 2 cm, T3 ≤ 3 cm, N0 = 0 lymph nodes, N1-N3 = 1-3+ lymph nodes), Differences were considered to be statistically significant when $P < 0.05$.

apoptotic cells is presented as a percentage of the total cells counted.

Flow cytometry

The apoptotic cells were analyzed by flow cytometry using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Camarillo, CA, USA). The MCF-7 cells were treated with JARID1B siRNA for 48 hours. The cells were harvested and washed with cold PBS. After centrifugation, the supernatant was discarded, and the cell pellets were resuspended in 1 X annexin-binding buffer to a final concentration of 1×10^6 cells/ml. After adding 1 μL of Alexa Fluor 488 annexin V and 1 μL of 100 μg/ml PI working solution into each 100 μL cell, the suspension cells were incubated at room temperature for 15 minutes. Stained cells were detected by flow cytometry measuring the fluorescence emission at 530 nm and 575 nm.

Western blot analysis

Total protein samples were extracted from MCF-7 and MCF-10A cells for immunoblotting analysis. Protein samples (70 μg) were fractionated by SDS-PAGE (10% polyacrylamide gels) and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk PBS for 2 h and then incubated at 4°C overnight with the following primary antibodies: JARID1B (1:100, Abcam, Cambridge, MA, USA), p53 (1:1000, Abcam, Cambridge, MA, USA), and GAPDH (1:2000, ZSJZ-Bio, Beijing, China). After washing, the membranes were incubated with a secondary antibody for 1 h. Images were captured on the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Western blot bands were quantified using Odyssey CLx v2.1 software. The data were normalized to GAPDH as an internal control.

Caspase-3 activity assay

Caspase-3 activity in MCF-7 cells was determined with colorimetric assay kits (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. MCF-7 cells were lysed in an ice-cold cell lysis buffer for 15 min, and then centrifuged at 20,000× g for 10 min at 4°C. 30 μl of the supernatant was incubated with 10 μl of substrate (2 mM Ac-DEVD-

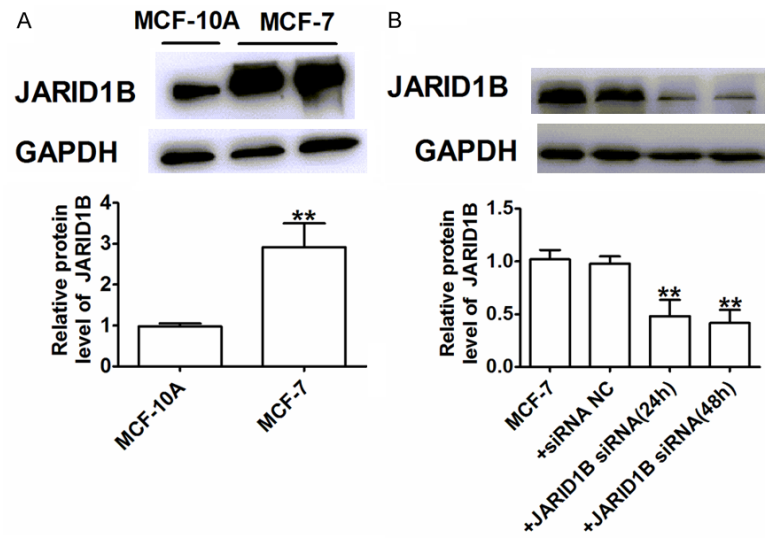

Figure 2. The establishment of stable JARID1B transfection in breast cancer cell lines. A, B. Representative western blot bands of JARID1B protein. Values given were normalized to the band intensity of GAPDH as an internal control. A: $^{**}P < 0.01$ vs. MCF-10A; B: $^{**}P < 0.01$ vs. MCF-7 and siRNA NC groups. n = 4 each group.

pNA) in 60 μl of assay buffer at 37°C for 2 h. The absorbance was measured at 405 nm.

Data analysis

Group data were expressed as the mean ± SEM. and analyzed by SPSS17.0 software. Student's t-test was performed for two-group comparisons. One-way ANOVA followed by Dunnet's t-test was used for multiple-group comparisons. Differences were considered to be statistically significant when $P < 0.05$. Figures were constructed by GraphPad Prism 5.0 software.

Results

Expression of JARID1B was upregulated in breast cancer tissues and increased JARID1B expression correlates with poor patient prognosis

To examine JARID1B expression in breast cancer, we first compared the expression of JARID1B in 30 breast cancer tissue samples to the expression in the adjacent normal tissues using immunohistochemistry. Human normal breast tissues did not show the JARID1B protein by immunostaining, but the ratio of JARID1B-positive cells in the breast cancer samples was higher than in the normal breast tissue samples (**Figure 1A**, **1B**). These results indicate significant overexpression of JARID1B in the breast cancer cells. To evaluate whether increased JARID1B staining in malignant breast cancer correlates with a worse prognosis, further analyses were conducted to discern the correlation of JARID1B expression with a series of clinicopathological parameters in 100 breast cancer cases. We demonstrated that JARID1B expression had significant correlations with the depth of invasion, lymph node metastasis and tumor size (**Table 1**). However, there was no statistically significant connection between JARID1B expression and other clinicopathological parameters, such as patient age or gender. Thus, the upregulation of JARID1B might play an important role in the tumorigenesis of breast cancer.

Establishment of stable JARID1B transfection in breast cancer cell lines

The JARID1B expression levels in MCF-7 cells and one normal breast cell line, MCF-10A cells, were measured by Western blot (**Figure 2A**). The results show that JARID1B was highly expressed in MCF-7 cells compared to MCF-10A cells. The efficiency of JARID1B knockdown by siRNA was verified at the protein level (24, 48 h) relative to MCF-7 and siRNA-NC (**Figure 2B**). These results show that we could use siRNA to generate a stable JARID1B knockdown in MCF-7 cells.

The inhibition of JARID1B promoted apoptosis in breast cancer cells

To investigate the possible effects and mechanisms of JARID1B in breast cancer cells, we used siRNA to silence JARID1B and then determined caspase-3 activity and cell apoptosis. As illustrated in **Figure 3A**, cell viability was reduced in MCF-7 cells by the transfection of JARID1B siRNA for 24, 48, and 72 h in a time dependent manner. So JARID1B siRNA for 72 h in MCF-7 cells was used for subsequent experiments. JARID1B siRNA-induced apoptosis was

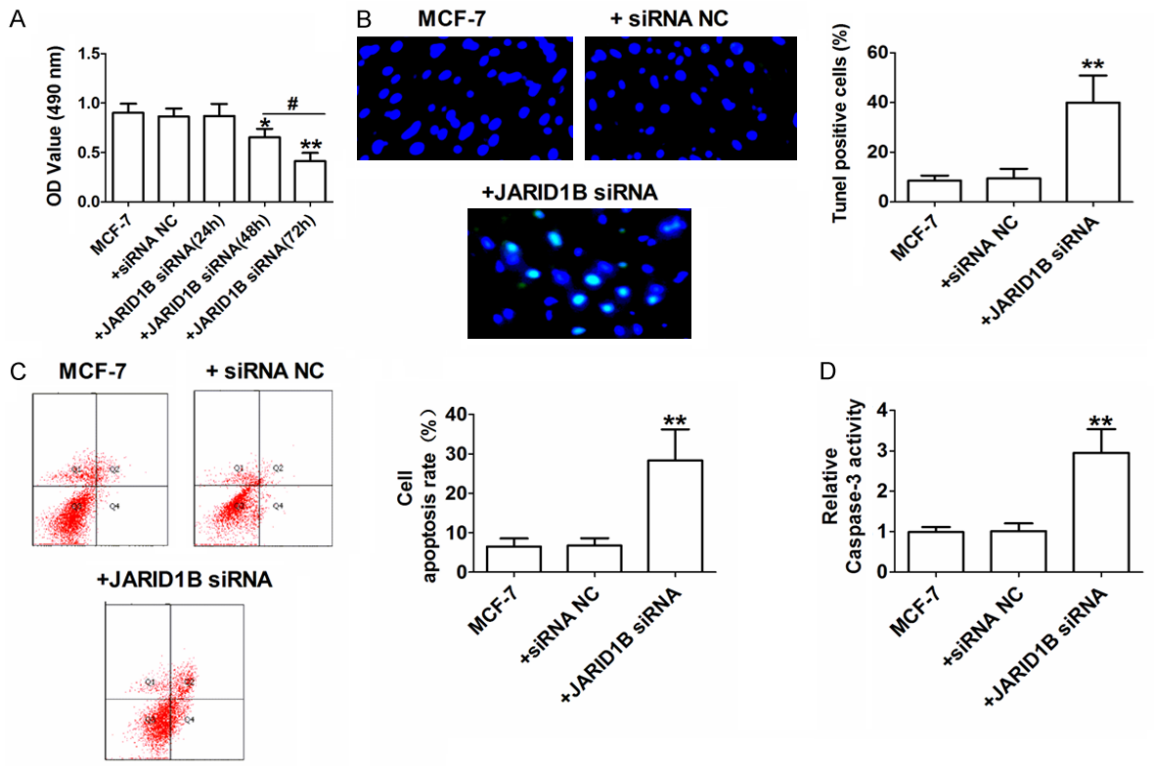

Figure 3. The inhibition of JARID1B promoted apoptosis in breast cancer cells. A. Cell viability was determined by the MTT assay. B. Representative images were taken with nuclear stain DAPI (blue) and apoptosis stain TUNEL (green) and depicts the percentage of TUNEL positive nuclei of MCF-7 cells after JARID1B siRNA administration. 40x magnification. C. Representative images of flow cytometry using Annexin V-FITC and PI staining. Right upper quadrant: the cells that produced late apoptosis; right lower quadrant: the cells that produced early apoptosis; left lower quadrant: viable cells. Bar graph showing increased proportion of early and late apoptotic cells after JARID1B siRNA administration in MCF-7 cells. D. Caspase-3 activity. A: $*P < 0.05$, $**P < 0.01$ vs. MCF-7, $^{\#\#}P < 0.01$ JARID1B siRNA (48 h) vs. JARID1B siRNA (72 h). B-D: $**P < 0.01$ vs. MCF-7 and siRNA NC groups. n = 4 each group.

further confirmed using a TUNEL assay and flow cytometry; the number of apoptotic cells was increased by 48 h after JARID1B siRNA treatment (**Figure 3B**, **3C**). Finally, we measured the changes in caspase-3 activity. Caspase-3 is known to be a key downstream protease that executes the breast cancer apoptotic cascade; activation of caspase-3 is considered to be the last step in caspase dependent apoptosis [7, 17, 18]. As illustrated in **Figure 3D**, JARID1B siRNA increased the level of caspase-3 activity in MCF-7 cells. On the basis of our data analysis, we conclude that JARID1B siRNA triggers a potential apoptotic effect in breast cancer cells.

JARID1B siRNA promoted MCF-7 cells apoptosis partly via p53

p53 has important roles in the apoptosis of various cancer types, including breast cancer [15, 18, 19]. Thus, we examined whether p53 was involved in JARID1B-mediated tumor apoptosis. First, the efficiency of p53 knock-down by siRNA was verified at the protein level (**Figure 4A**). Next, we evaluated the effects of JARID1B siRNA on p53 expression in MCF-7 cells by Western blot. As shown in **Figure 4B**, knockdown of JARID1B dramatically upregulated the expression of p53 in MCF-7 cells. After we transfected the MCF-7 cells with JARID1B siRNA and p53 siRNA, p53 expression was significantly inhibited compared with JARID1B siRNA alone. These results indicate that JARID1B can mediate p53 in breast cancer cells. We further tested the role of p53 in JARID1B siRNA-induced apoptosis by knocking down p53 expression using siRNA. As shown in **Figure 4C** and **4D**, JARID1B siRNA reduced cell viability and increased caspase-3 activity in MCF-7 cells. These effects were reversed following p53 knockdown using siRNA. Thus, JARID1B siRNA promoted the apoptosis of breast cancer cells at least in part through p53 induction.

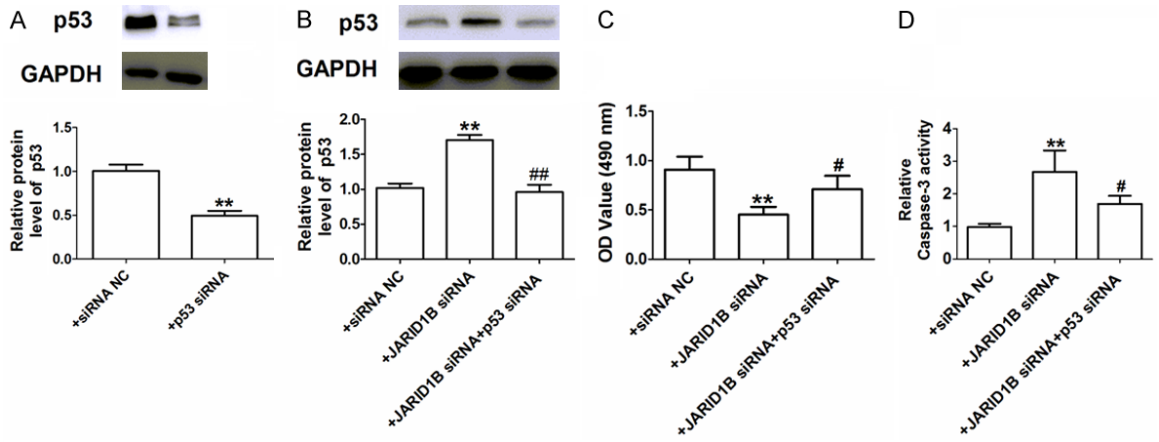

Figure 4. JARID1B siRNA promoted MCF-7 cell apoptosis partly via p53. A, B. Representative Western blot bands of the p53 protein. Values given are normalized to the band intensity of GAPDH as an internal control. C. Cell viability determined by MTT assay. D. Caspase-3 activity. $^{**}P < 0.01$ vs. +siRNA NC, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. JARID1B siRNA. n = 4 each group.

Discussion

In the present study, we found that JARID1B was overexpressed in both breast cancer cell lines and breast cancer tissues but not in normal breast tissues. The apoptosis of breast cancer cells was significantly increased by the interference of JARID1B by siRNA. Moreover, we also found that JARID1B siRNA dramatically increased p53 expression at the protein level. The inhibition of p53 reversed the JARID1B siRNA-induced apoptosis. Our results collectively suggest that JARID1B is overexpressed in breast cancer where it plays an important role in breast cancer cell apoptosis, perhaps partially by mediating p53 expression. Our study has thus clarified a novel effect and mechanism of JARID1B in response to breast cancer cell apoptosis.

Breast cancer is one of the most frequent malignancies and the second leading cause of cancer-related mortality in women. It is a complex disease with multiple deregulated signaling pathways, including apoptosis [3, 7]. Targeting apoptotic pathways has emerged as an attractive approach for cancer treatment. So far, numerous natural and synthetic compounds have been reported to possess anticancer activities through the induction of different apoptotic pathways [7, 20, 21]. A better understanding of the function of apoptosis in breast cancer may provide new therapeutic pathways for disease prevention or control.

The JARID1 family of proteins can promote transcriptional activation, thus affecting important processes such as hormone response, stem cell renewal, germ cell development, and cellular proliferation and differentiation [16, 22]. Recent studies have shown that JARID1B is correlated with invasive ductal carcinoma of the breast [8-12]. For instance, high luminal JARID1B activity is associated with poor outcomes in patients with hormone receptor positive breast tumors [8]. The histone demethylase JMJD2B promotes hormonally responsive breast carcinogenesis [9-11]. JARID1B plays a key role in early embryonic development, in the development and differentiation of the normal mammary gland, and in estrogen induced growth of estrogen receptor positive (ER+) breast cancer [12]. These results suggest that JARID1B may be involved in breast tumorigenesis. Some studies have shown that the depletion of JARID1B could induce apoptosis in head and neck squamous carcinoma cells and human acute lymphoblastic leukemia cells [13, 14]. However, the possible effect and mechanism of JARID1B in breast cancer cell apoptosis has remained scarcely investigated.

To unravel the function of JARID1B in breast cancer cell apoptosis, we first examined the levels of JARID1B in breast cancer samples and matched normal breast tissue samples. The results showed that JARID1B was significantly increased in cancers, but not in the normal breast tissues, which suggested that JARID1B was a candidate tumor oncogene in breast cancer. To obtain a better understanding of the clinical significance of JARID1B expression, further analyses were conducted to discern the

correlation of JARID1B expression with a series of clinicopathological parameters in 100 cases of breast cancer patients. We demonstrated that JARID1B expression had significant correlations with the depth of invasion, lymph node metastasis, and tumor size. Next, we transfected JARID1B siRNA into MCF-7 breast cancer cells to inhibit its expression. Our in vitro experiments demonstrated that the inhibition of JARID1B significantly enhanced the apoptosis of breast cancer cells and inhibited cell viability. These results indicate that JARID1B siRNA effectively promotes breast cancer cells apoptosis.

The p53 protein is a well-known tumor suppressor and has been demonstrated to have an essential role in breast cancer proliferation, apoptosis, and invasion [19, 22]. Previous research has reported that JARID1B promotes the proliferation, migration, and invasion activities of lung cancer cells partially via downregulated p53 [16]. Therefore, we studied the connection between JARID1B and p53 in breast cancer cell apoptosis. Our results indicated that the level of p53 was significantly increased in JARID1B knockdown cells. The Inhibition of p53 by siRNA reversed the JARID1B siRNA induced promotion of apoptosis. All of these results demonstrated that JARID1B siRNA promotes the apoptosis of breast cancer cells partially via upregulated p53.

As a whole, our study reveals that JARID1B plays an important role in regulating breast cancer cell apoptosis and that this role may be mediated by p53. Thus, we propose that the candidate tumor oncogene JARID1B may be an effective novel therapeutic target in the treatment of breast cancer.

Disclosure of conflict of interest

None.

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