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Original Article

Overexpression of histone demethylase JMJD5 promotes metastasis and indicates a poor prognosis in breast cancer

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Abstract: In this study, we showed the expression of JMJD5 was increased in breast cancer tissues and breast adenocarcinoma cell lines MCF-7 as well as triple negative breast cancer cell lines MDA-MB-231 compared with paired adjacent normal mammary tissues and normal mammary epithelial cell lines MCF-10A. The higher expression of JMJD5 was significantly corresponded with clinical stage, histological grade and lymph node metastasis. Overexpression of JMJD5 promoted cell invasion and induce EMT, while JMJD5 siRNA inhibits MDA-MB-231 cells invasion in vitro. Moreover, qChIP analysis revealed the Snail family proteins Snai1 was the direct target of JMJD5 in breast cancer cells. Luciferase reporter assays suggested that the overexpression of JMJD5 resulted in the activation of Snail1 promoter-driven luciferase reporter. The changes in the level of RNA and protein implied that the activation of Snail was the important mechanisms by which JMJD5 triggers metastasis. We also detected the higher expression of JMJD5 protein was an independent unfavorable biomarker for worse overall survival in breast cancer patients. Therefore, our results identified an important role for JMJD5 in breast cancer through the regulation of snail1.

Keywords: JMJD5, snail, biomarker, metastasis, breast cancer

Introduction

JMJD5 was a member of the Jumonji C domain-containing dioxygenase family. It was firstly reported as a demethylase to demethylate H3K36me2 [1] and was required for cell cycle progression. The function of JMJD5 was usually as a transcriptional activator [2], JMJD5$^{-/-}$ embryos showed severe growth retardation, resulting in embryonic lethality [3]. Further, JMJD5 was reported to interact directly with pyruvate kinase muscle isozyme (PKM) 2, had a positive function in activating hypoxia-inducible factor (HIF)-1α-mediated transactivation to modulate cancer cell metabolic reprogram [4]. The JMJD5 loss-of-function studies in breast cancer cell line MCF7 indicated cell cycle arrest [2]. However, the clinical significance of JMJD5 in breast cancer remains unclear. We are interested in whether JMJD5 could influence cell migration, invasion and other malignant status in breast cancer.

As reported, breast cancer is the most common tumor and the second leading reason of cancer-related mortality in women [5]. Epithelial-to-mesenchymal transition (EMT) is the initiation step of metastasis [6, 7]. During EMT, epithelial cells lost epithelial markers, dissolve cell-cell junctions, and acquire invasive traits of mesenchymal cells characteristics [8]. Downregulation of E-cadherin is considered to be the hallmark of EMT [9]. The Snail family proteins Snai1 (also known as Snail) and Snai2 (also known as Slug) are well known transcriptional repressors to repress E-cadherin and key inducers of EMT [10]. To have a role in the regulation of Snail, such as Dyrk2, by degrading snail, could control the epithelial-mesenchymal transition in breast cancer [11]. GSK-3beta could bind to and phos-

phorylates Snail to dually regulate the function of it [12]. We are interested in whether there is more regulator of snail to be discovered which contribute to the breast cancer metastasis.

Materials and methods

Antibodies and reagents

Antibodies used were: JMJD5, Snail, H3, H3K36me1, H3K36me2, and H3K36me3 from Abcam; β-actin from Santa Cruz Biotechnology. The small interfering RNAs (siRNA) targeting human JMJD5, control siRNA are synthesized from (Sigma Aldrich). The sequence for control siRNA was: 5'-UUCUCCGAACGUGUCACGU-3'.

Cell culture and reporter assay

The cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were maintained in DMEM supplemented with 10% FBS. MDA-MB-231 cells were maintained in L15 supplemented with 10% FBS without CO_2. MCF-10A were cultured in MEGM Bullet Kit. Transfections were carried out using Lipofectamine 2000 or Lipofectamine RNA imax (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Luciferase activity was measured using a dual luciferase kit (Promega, Madison, WI) according to the manufacturer's protocol. The JMJD5 plasmid and Snail luciferase reporters together with Renilla plasmid, using Lipofectamine LTX-Plus (Invitrogen). 48 hours after transfection, the firefly and Renilla luciferases were assayed, the firefly luciferase activity was normalized to that of Renilla luciferase. Each experiment was performed in triplicate and repeated at least three times. The amount of DNA was kept invariability by addition of empty vector in each transfection.

Real-time RT-PCR (qPCR)

Total cellular RNAs were isolated with the TRIzol reagent (Invitrogen) and used for first strand cDNA synthesis with the Reverse Transcription System (Promega) according to the manufacturer's protocol. Quantitation of all gene transcripts was done by qPCR using SYBR Premix ExTaq kit (Takara) and an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA), with GAPDH as a normalizing control. The primer pairs used were as follows: JMJD5 forward primer: 5'-GAAGTGGAGTTTGGAGTATATCC-3', *JMJD5* reverse primer: 5'-GATCTGGTCAAAGAGCTGGT-3'; *snail* forward primer: 5'-GGACTCTAATCCAGAGTTTACC-3', *snail* reverse primer: 5'-AGGAAGAGACTGAAGTAGAGG-3'; *β-actin* forward primer: 5'-CGTGGACATCCGCAAAGAC-3', *β-actin* reverse primer: 5'-CTCGTCATACTCCTGCTTGC-3'; *E-cadherin* forward primer: 5'-ACCTGGTTCAGATCAAATCCA-3', *E-cadherin* reverse primer: 5'-CTATCCAGAGGCTCTGTCAC-3'; *N-cadherin* forward primer: 5'-TCTGACAATGGAATTCCTCC-3', *N-cadherin* reverse primer: 5'-CAAATGGTCCAGCATTTGGA-3'; *Vimentin* forward primer: 5'-TTAAAGGAACCAATGAGTCCCT-3', *Vimentin* reverse primer: 5'-CCAGATTAGTTTCCCTCAGG-3'.

Each reaction was performed in triplicate, and the 2^-ΔΔ method was used to determine the relative gene expression levels.

Patients and tissue specimens

All patients gave written informed consent. 110 Breast carcinoma tissues were obtained from Shandong University Qilu Hospital between January 2002 and December 2012. The pathological information was acquired from the Pathology Department of Qilu Hospital. Patients had received tumor-specific therapy before diagnosis was excluded. Samples were frozen in liquid nitrogen immediately after surgical removal and maintained at -80°C until used for real-time PCR and Western blot. All human tissue was collected using protocols approved by the Ethics Committee of the Shandong University. In the 110 breast cancer cases, there were all females with age ranging from 22 years to 75 years (the median is 54.23 years). The overall survival time was calculated from the diagnosis date to breast cancer-related death.

Lentiviral production and infection

Recombinant lentiviruses were constructed by subcloning human JMJD5 into the iDuet101 shuttle vector. The recombinant construct as well as two assistant vectors, pUOG and pCMV, were then transiently transfected into HEK 293T cells. Viral supernatants were collected 48 hours later, clarified by filtration, and concentrated by ultracentrifugation. The concentrated virus was used to infect 5×10^5 cells in a 60-mm dish with 8 μg/ml polybrene. Infected

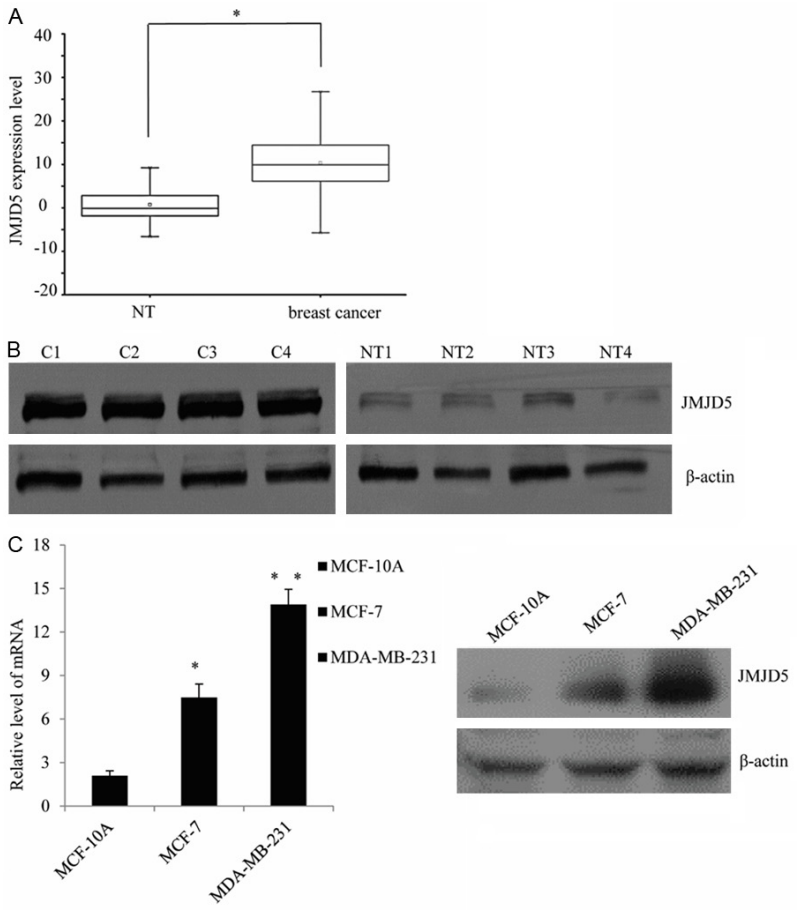


Figure 1. The expression of JMJD5 was increased in the breast cancer tissues and cell lines. A. qRT-PCR analysis of JMJD5 expression in 30 pair's breast cancer tissues and their corresponding adjacent normal tissues (NT). B. Western blotting analysis of JMJD5 expression in the relative 4 breast cancer tissues and adjacent normal mammary tissues. C. The expression of JMJD5 was detected in breast cancer cell lines (MCF-7 and MDA-MB-231) and MCF-10A cell line using qRT-PCR analysis and western blotting.

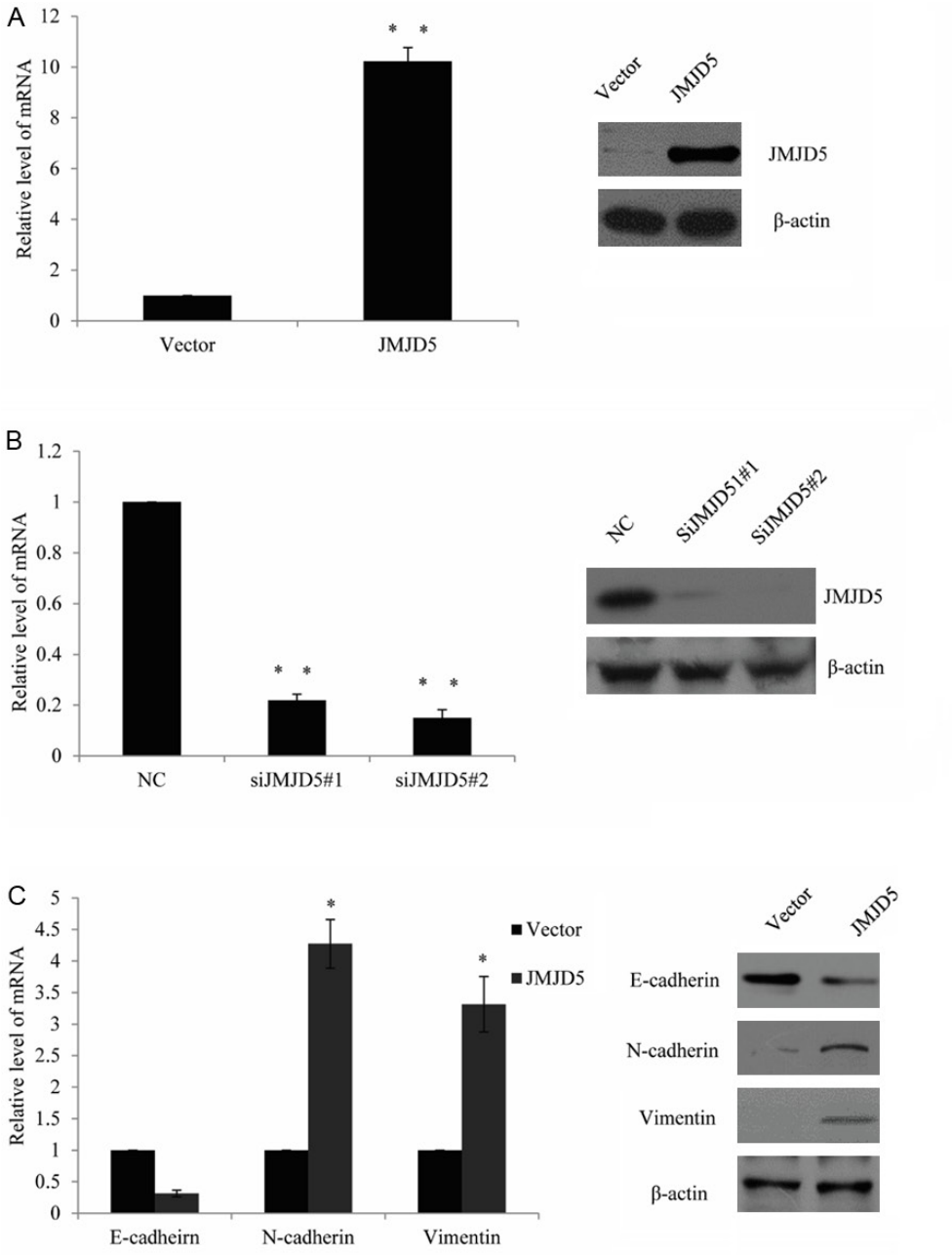
MDA-MB-231 cells were selected with 2 μg/ml hydromycin (Merck). siRNA sequences targeting JMJD5 were designed and cloned into the pLL3.7 shuttle vector. The recombinant construct as well as three assistant vectors, pREE, VSUG, and RSU/REU, were then transiently transfected into HEK 293T cells. Viral supernatants were collected 48 hours later, clarified by filtration, and concentrated by ultracentrifugation. The concentrated virus was used to infect 5×10^5 cells in a 60-mm dish with 8 μg/ml polybrene.

Western blotting

Seventy-two hours after the infection, cellular lysates were prepared by incubating the cells in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5-1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 40 min at 4°C. This was followed by centrifugation at 14,000 g for 15 min at 4°C. The supernatant proteins were eluted in 5 × SDS-PAGE loading buffer and boiling for 10 min. The resultant materials of cell lysates were resolved using 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. For Western blot analysis, membranes were incubated with appropriate antibodies overnight at 4°C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using Western blotting Luminol reagent (Santa Cruz Biotechnology) according to the manufacturer's recommendation.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to the procedure described previously [13-16]. ChIP lysis buffer (2% SDS, 5 mM EDTA, 50 mM Tris-HCl). Dilution buffer (1% Triton X-100, 1 mM EDTA, 250 mM NaCl, 20 mM Tris-HCl-Cl) TSE I (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 250 mM NaCl, 20 mM Tris-HCl); TSE II (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 250 mM $NaCl_2$0 mM, Tris-HCl); buffer III (0.25 M LiCl, 2% NP-40, 2% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl); TE buffer (10 mM Tris-HCl, 1 mM EDTA); Elution buffer (1% SDS, 0.1 M $NaHCO_3$). Briefly, cross-linked chromatin was sonicated into 200-bp to 500-bp fragments. The chromatin was immunoprecipitated using anti-JMJD5 antibody, H3K36me1, H3K36me2, and H3K36me3 antibody, Normal rabbit immunoglobulin G (IgG) was used as a negative control. Quantitative polymerase chain reaction (PCR) was then conducted using SYBR Green Mix (Takara Bio, Otsu, Japan). The following primer pairs were used for *snail* gene


A
Relative level of mRNA
Vector
JMJD5
JMJD5
β-actin
B
Relative level of mRNA
NC
siJMJD5#1
siJMJD5#2
NC
SiJMJD51#1
SiJMJD5#2
JMJD5
β-actin
C
Relative level of mRNA
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Vimentin
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N-cadherin
Vimentin
β-actin

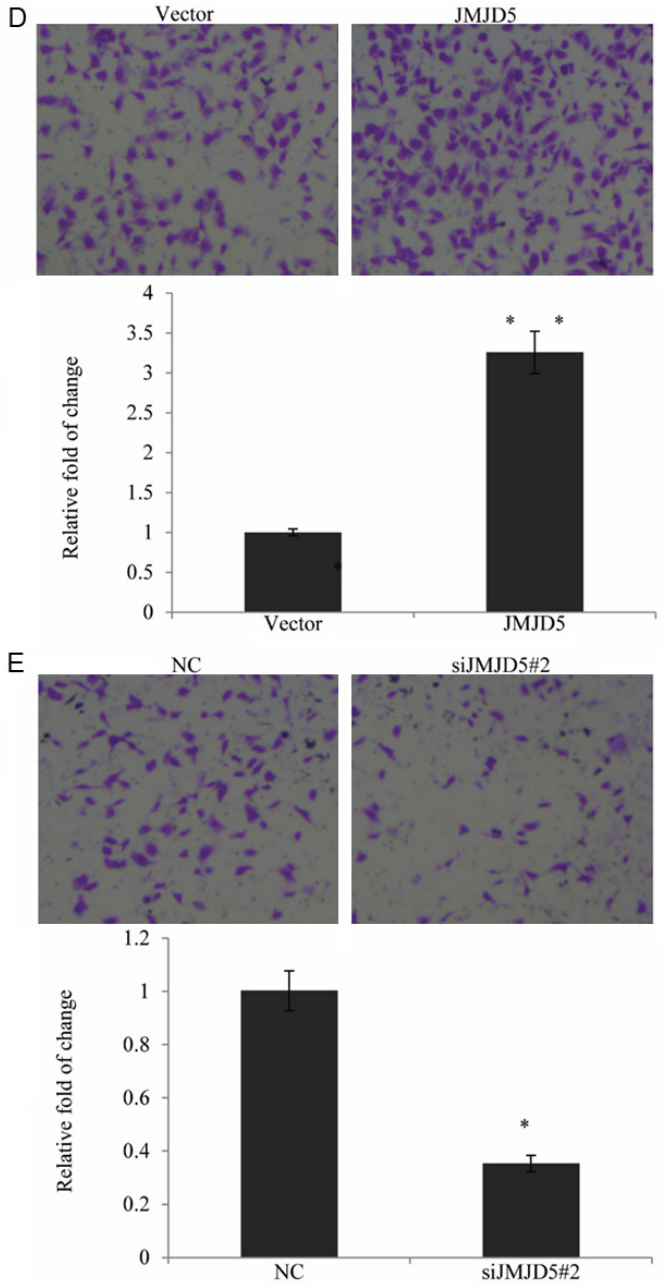
D
Vector
JMJD5
Relative fold of change
Vector
JMJD5
E
NC
siJMJD5#2
Relative fold of change
NC
siJMJD5#2

Figure 2. Overexpression of JMJD5 induces EMT and breast cancer metastasis. A. qRT-PCR and western blotting analysis of JMJD5 expression after MCF-7 cells were transfected with lentivirus carrying JMJD5 constructs. GAPDH was used as a normalization control. B. The knockdown efficiencies of siJMJD5#1, siJMJD5#2 in MDA-MB-231 cells were confirmed by qRT-PCR and western blotting analysis. C. qRT-PCR and western blotting analysis showed that after ectopic expression of JMJD5 in MCF-7 cells, the expression of E-cadherin N-cadherin, Vimentin were detected. D. Overexpression of JMJD5 promoted the MCF-7 cells invasion. The relative invasive cells were shown in the right. $^{*}P < 0.05$, and $^{**}P < 0.01$. E. Knockdown of JMJD5 inhibited the MDA-MB-231 cells invasion. Transwell assay were carried out. Representative photos were shown in each group. $^{*}P < 0.05$, and $^{**}P < 0.01$.

sequence: 5'-CCCCTATG-GAGCCGTGTT-3' (forward) and 5'-GGACCTGGTTAGAGTTTCGTT-3' (reverse).

Invasion analysis

Invasion assays were performed using Transwell invasion chambers coated with Matrigel (BD, USA) according to manufacturer's instruction. Cells were transfected with Vector, JMJD5, control siRNA or siJMJD5 and transferred on the top of Matrigel-coated invasion chambers in a serum-free DMEM. The lower chambers were added with 10% fetal calf serum. After 24 hours, MDA-MB-231 cells that remained on the top of the filter were wiping off and cells that migrated to the lower surface were stained with 0.2% crystal violet solution (Sigma) and counted. For MCF-7 cells, the incubation time was 72 hours.

Statistical analysis

SPSS 17.0 software was used for the Statistical analysis. The difference expression of JMJD5 messenger RNA (mRNA) between breast cancer samples and paired adjacent normal mammary tissues was detected by the Wilcoxon signed rank test. Statistical significance was assessed by comparing mean values (± SD) using a Student's t-test for independent groups. In the survival analysis, Kaplan-Meier analyses were used to analyze 110 breast cancer patients. Univariate and multivariate Cox regression models were used to calculate hazard ratios in the breast cancer patients. *P* values less than 0.05 were considered significant.

Results

Expression of JMJD5 was increased in the breast cancer tissues and cell lines

In order to identify the role of JMJD5 in breast cancer, we collected 110 breast cancer tissues and adjacent normal mammary tissues. First, we performed real-time PCR to measure the mRNA expression of JMJD5 in 30 breast cancer tissues and adjacent normal mammary tissues. The breast cancer tissues showed higher JMJD5 mRNA expression levels of compared with adjacent normal mammary tissues ($P < 0.001$, **Figure 1A**). Furthermore, we detected the protein level of JMJD5 in four pairs of breast cancer tissues by western blotting. Consistently with the mRNA, the JMJD5 protein was obviously overexpressed in breast cancer tissues (C1, C2, C3, C4) compared with adjacent normal mammary tissues (NT1, NT2, NT3, NT4) (**Figure 1B**). qRT-PCR and western blotting showed the level of JMJD5 in breast adenocarcinoma cell lines MCF-7 and triple negative breast cancer cell lines MDA-MB-231 were significantly higher, compared that with the normal breast cell lines MCF-10A (**Figure 1C**).

Overexpression of JMJD5 induces EMT and breast cancer metastasis

In order to understand the role of JMJD5 contributes to breast cancer, recombinant lentivirus expressing JMJD5 constructs were developed and respectively transfected into MCF-7 cells. qRT-PCR and Western blotting was used to detect the transfection efficiency (**Figure 2A**), to expect, the JMJD5 lentivirus was successfully expressed. For loss of function analysis, two relative siRNAs of JMJD5 were separately transfected into the MDA-MB-231 cells, the knockdown efficiencies of siJMJD5#1, siJMJD5#2 were measured at the mRNA level and protein level (**Figure 2B**). Of the three siRNAs, siJMJD5#2 seemed the best knockdown efficiency. qRT-PCR and Western blotting analysis showed that ectopic expression of JMJD5 in MCF-7 cells can inhibit the expression of epithelial marker E-cadherin and induce the mesenchymal marker N-cadherin, vimentin expression (**Figure 2C**). Further, when MCF-7 cells were transferred with JMJD5 lentivirus, there was an increase in the invasive potential of MCF-7 cells (**Figure 2D**), as measured by the

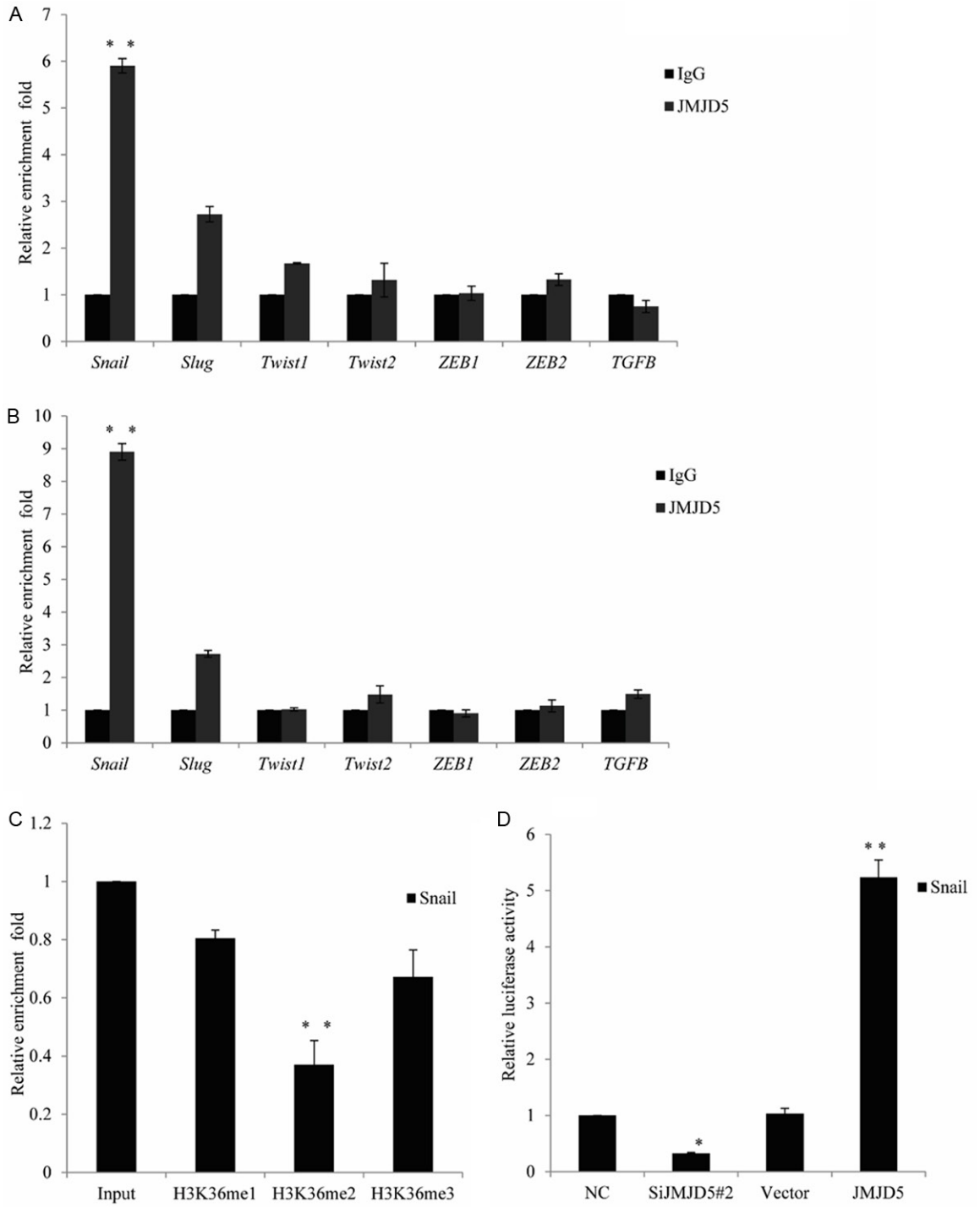

Figure 3. Snail is a direct target of JMJD5 in breast cancer cells. A. Recruitment of JMJD5 on Snail promoter. qChIP experiments were performed in MCF-7 cells with JMJD5 antibody and mouse normal IgG. B. qChIP experiments were performed in MDA-MB-231 cells with JMJD5 antibody and mouse normal IgG. C. qChIP assays were performed with antibody against H3K36me1, H3K36me2, and H3K36me3 on the promoter of snail, normal mouse IgG was used as negative control, total DNA (Input was normalized to 1) as positive control. D. JMJD5 regulates Snail promoter-driven luciferase activity. MCF-7 cells were transfected with snail promoter luciferase, JMJD5 overexpression constructs or JMJD5 relative siRNAs, luciferase activities were measured. Normalized to those of Renilla. Each bar indicates mean ± S.D, experiments were repeated three times. $^{*}P < 0.05$; $^{**}P < 0.01$.

transwell analysis. To the contrary, whereas know down of JMJD5 (siJMJD5#2) resulted in a decrease in the invasive potential of MDA-MB-231 cells (**Figure 2E**).

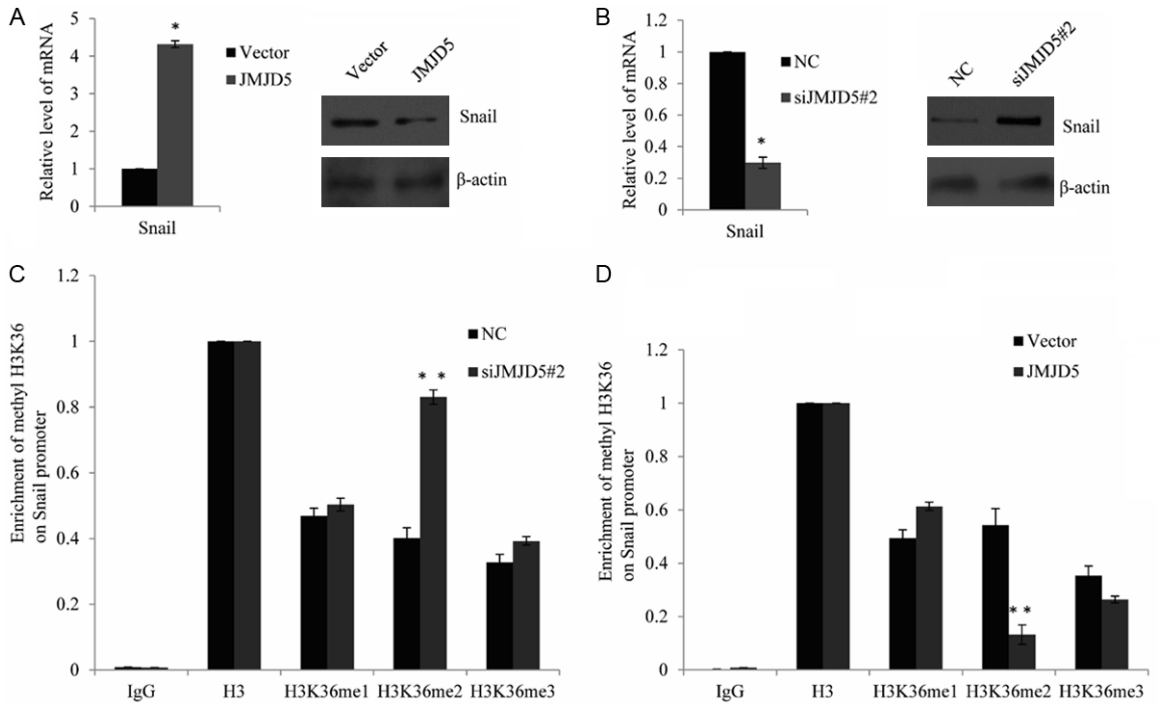

Figure 4. The activation of Snail is the mechanism for JMJD5 triggering metastasis. A. The mRNA level of Snail was detected 48 hours after MCF-7 cells were transfected with JMJD5 constructs, GAPDH was used as a normalization control. The protein level of Snail was detected using western blotting, β-actin was as control. B. The Snail mRNA was confirmed by Quantitative real-time PCR after MDA-MB-231 cells were transfected with JMJD5 siRNAs, as protein level was measured by western blotting, (right panel). Experiments were repeated three times, Error bars represent mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$. C. In JMJD5 depletion MDA-MB-231 cells, on the promoter of Snail, qChIP assays were performed with JMJD5, or mono-, di-, and tri-methylated H3K36-specific antibodies. H3 was used as normalized control. D. JMJD5 was overexpressed in MCF-7 cells, qChIP were performed with antibodies against JMJD5, H3K36me1, H3K36me2, and H3K36me3 on the snail promoter, with anti-H3 normalized as 100%.

Snail1 is a direct target of JMJD5 in breast cancer cells

In order to investigate the mechanism of JMJD5 involved in breast cancer EMT and metastasis, we carried out qChIP analysis in MCF-7 cells with JMJD5 antibody and mouse normal IgG as negative control. Different genes promoters were detected such as EMT inducer Snail, Snail2 (Slug), Twist1, Twist2, ZEB1, ZEB2 and TGFB. We found that Snail was a putative target gene of JMJD5 (**Figure 3A**). The similar results were further proved in the MDA-MB-231 cells, when using Snail promoter primer, there were obviously increases in the anti-JMJD5 groups compared with the normal IgG (**Figure 3B**). Considering JMJD5 as a demethylase to demethylate H3K36me2, to explore whether H3K36me2 demethylation was catalyzed by JMJD5 on the promoter of Snail, qChIP assays were performed with antibody against H3K36me1, H3K36me2, and H3K36me3 in MCF-7 cells (**Figure 3C** left panel) and MDA-MB-231 cells (Figure 3C right panel), we found there was obviously decrease of H3K36me2 on the Snail promoter (**Figure 3C**). To further support Snail is a direct target, luciferase reporter activity assays were carried out, in MCF-7 cells, under JMJD5 overexpression or depletion, leading to a significant effect on the activation of Snail promoter-driven luciferase reporter (**Figure 3D**).

Activation of Snail is the mechanism for JMJD5 triggering metastasis

Consistent with the promoter occupancy, MCF-7 cells were transfected with JMJD5 constructs, the mRNA level and protein expression of Snail increased (**Figure 4A**). To the contrary, in MDA-MB-231 cells, the knockdown of JMJD5 leading to the inhibition of Snail, measured by qRT-PCR and Western blotting analysis (**Figure 4B**). Further, qChIP experiments indicated that

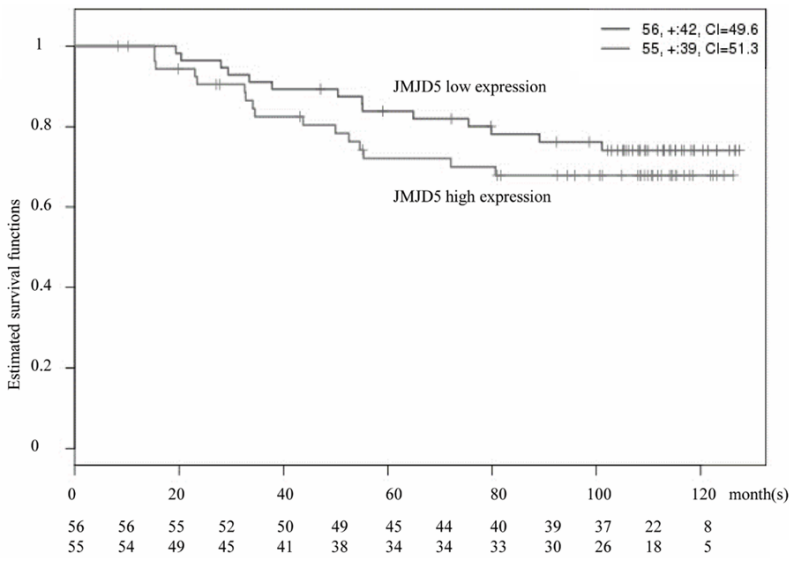

Figure 5. Increased JMJD5 expression was an independent unfavorable biomarker for worse overall survival in breast cancer patients. The living time of the 110 breast cancer were plotted using Kaplan-Meier curves, the y-axis shown the survival probability, the x-axis shown the survival times and the overall survival rate was compared using the Cox proportional hazards regression, and $P < 0.05$ was considered significant.

Table 1. Clinicopathologic variables in 110 breast cancer patients

Variables	No. (n = 110)	JMJD5 protein expression		*P* value
		Low	High	
Clinical stage				
I-II	57	32	25	0.020
III-IV	53	18	35	
Age				
< 55	67	30	37	0.858
≥ 55	43	20	23	
Triple-negative cancer				
No	59	37	22	< 0.001
Yes	61	13	38	
Histological grade				
G1	49	31	18	0.001
G2-G3	61	19	42	
Estrogen receptor				
Positive	47	34	13	< 0.001
Negative	63	16	47	
HER2				
Positive	73	33	40	0.941
Negative	37	17	20	
Lymph node metastasis				
No	43	27	16	0.003
Yes	67	23	44	

while depletion of JMJD5 resulted in a marked reduction of the recruitment of JMJD5 at the promoter of the target gene snail, as well as the increase of H3K36me2 (**Figure 4C**). In MCF-7 cells with JMJD5 overexpression, the binding on the snail promoter increased, and the H3K36me2 was obviously inhibited (**Figure 4D**). These experiments indicated that JMJD5 could bind on the promoter of Snail to activate its transcription through its H3K36me2 demethylation activity.

Increased JMJD5 expression was an independent unfavorable biomarker for worse overall survival in breast cancer patients

To explore whether the expression of JMJD5 has the prognostic significance in breast cancer patients, we used Kaplan-Meier analysis to measure the correlation of the JMJD5 expression levels with overall survival in 110 breast cancer patients. We found the JMJD5 overexpression was significantly correlated with breast cancer patients' worse overall survival (**Figure 5A**). Furthermore, we found that the higher JMJD5 expression was significantly associated with clinical stage, Triple-negative breast cancer, histological grade, negative estrogen receptor and lymph node metastasis. However, JMJD5 expression was not associated with age and HER2 positive breast cancer (**Table 1**). Finally, we summarized the increased JMJD5 protein expression was a poor independent prognostic factor in breast cancer.

Discussion

Our study showed that in both mRNA and protein level, the expression of JMJD5 was obviously increased in breast cancer samples compared with their paired adjacent normal mammary tissues. In breast cancer cell lines compared with normal mammary epithelial cells, there were obviously higher expressions of JMJD5. Moreover, overexpression of JMJD5 promoted MCF-7 cells invasion and induce EMT. These results implied the fact that JMJD5 may function as an oncogene in breast cancer. Our results revealed that Snail was a direct target of JMJD5 in breast cancer. Increasing studies revealed that Snail might serve as an oncogene to repress E-cadherin in various tumors, such as prostate cancer [17], ovarian cancer [18], glioblastoma [19], hepatocellular carcinoma [20] as well as breast cancer [21]. So it was of great importance to understand the epigenetic regulation of Snail. Those enzymes demethylate specific lysine residues on histone tails such as JMJD2A, LSD1, the dysfunction of which often result in epigenomic aberrations, often play fundamental roles in human diseases such as cancer [22, 23]. In the study, we found that the higher JMJD5 expression was significantly associated with clinical stage, histological grade negative estrogen receptor and lymph node metastasis, all of which were poor prognostic factors in breast cancer. Patients with lower JMJD5 protein expression had better overall survival than those with higher expression.

In conclusion, the present study demonstrated that JMJD5 was increased in breast cancer tissues and cell lines. Overexpression of JMJD5 promoted the cell invasion and EMT of breast cancer. Through catalyze H3K36me2 demethylation on the promoter of Snail, JMJD5 could activate the expression of Snail, by which, is one of the mechanism in JMJD5 influencing breast cancer metastasis. To the best of our knowledge, this is the first study to demonstrate that the JMJD5/Snail regulates the invasion and EMT of breast cancer cells. In the future, more studies are needed to verify the mechanism of JMJD5 as an oncogene and an unfavorable clinical biomarker for breast cancer.

Disclosure of conflict of interest

None.

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