Jumonji domain-containing protein 6 functions as a marker of head and neck squamous cell carcinoma at advanced stage with no effect on prognosis

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Abstract. A number of studies have reported the upregulation and functional roles of Jumonji domain-containing protein 6 (JMJD6) in various types of cancer. However, little is known regarding the clinical significance of JMJD6 in head and neck squamous cell carcinoma (HNSCC), particularly in terms of large-cohort data. In the present study, bioinformatics analysis was performed, using the University of California Santa Cruz Xena Browser and the Gene Expression Profiling Interactive Analysis 2 server, based on the Cancer Genome Atlas HNSCC cohort. In addition, a validation cohort was constructed based on 98 HNSCC cases. JMJD6 overexpression and knockdown, colony-formation, Transwell and cell viability assays were performed. JMJD6 was highly expressed in HNSCC samples and was associated with advanced pathological stage. However, no significant association was observed between JMJD6 expression levels and overall survival or disease-free survival times of patients with HNSCC. Subsequent *in vitro* assays indicated that overexpression of JMJD6 promoted malignant progression of HNSCC, by regulating epithelial-mesenchymal transition. Nevertheless, JMJD6 overexpression had no significant effects on the viability of HNSCC cells treated with 5-fluorouracil or cisplatin. Thus, it can be concluded that JMJD6 may function as a marker of HNSCC at advanced stage, however with no effect on drug resistance or prognosis. For patients with advanced HNSCC and high JMJD6 expression, rational chemotherapy may be more beneficial than radical surgery, considering their quality of life.

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Introduction

Jumonji domain-containing protein 6 (*JMJD6*) is a member of the Jumonji C domain-containing metalloenzymes (1,2). For the past 17 years, studies have indicated that JMJD6 as a multidimensional protein in biological processes that interacts with multiple protein substrates in distinct molecular signaling pathways [such as U2 small nuclear RNA auxiliary factor 2 and p21 (RAC1) activated kinase 1] (3,4). According to the current understanding, JMJD6 acts as either a histone demethylase, which removes methyl moieties on histone H3 at arginine 2 and H4 at arginine 3, or as a lysyl hydroxylase that targets the RNA splicing factor U2 small nuclear RNA auxiliary factor 2 (2,5,6). In summary, JMJD6 serves diverse roles at the transcriptional, splicing, posttranscriptional and biochemical levels (7).

The upregulation and functional role of JMJD6 in cancer areas have been recently demonstrated (8). In breast cancer, overexpression of JMJD6 has been reported to be associated with poorer outcomes and more aggressive characteristics, via modulating cell proliferation and motility (3,7). In hepatocellular carcinoma, JMJD6 promotes progression of malignancy via regulation of CDK4 by directly targeting its promoter (9). In melanoma, a positive feedback loop (JMJD6- serine/threonine-protein kinase PAK 1-mitogen-activated protein kinase 1-JMJD6) associated with the promotion of melanogenesis, cell proliferation, invasion and angiogenesis has been investigated (10). In oral squamous cell carcinoma (OSCC), JMJD6 has been demonstrated to be a novel regulator of cancer stem cells via upregulation of interleukin 4 expression (11).

However, little is known regarding the clinical significance of JMJD6 in head and neck squamous cell carcinoma (HNSCC), particularly in terms of large-cohort data. The Cancer Genome Atlas (TCGA) project provides an opportunity for the comprehensive understanding of human cancer, based on powerful and detailed information from a large cohort (12-14). In the present study, bioinformatics analysis was conducted using two online tools, the University of

California Santa Cruz (UCSC) Xena Browser and Gene Expression Profiling Interactive Analysis 2 (GEPIA2), based on the TCGA primary HNSCC cohort data. The expression patterns, clinical significance and biological roles of JMJD6 in HNSCC were systematically illustrated based on the TCGA cohort and a validation cohort. Subsequent *in vitro* assays were performed to investigate the biological roles of JMJD6 in HNSCC.

Materials and methods

Bioinformatics analysis and validation. Bioinformatics analysis was performed based on the TCGA HNSCC cohort using the web-based tools GEPIA2 (http://gepia2.cancer-pku.cn) and the UCSC Xena Browser (http://xena.ucsc.edu) (12-14). Only cases of primary HNSCC were included in further analyses of the expression patterns of *JMJD6*, snail family transcriptional repressor 1 (*SNAI1*), vimentin (*VIM*), twist family bHLH transcription factor 1 (*TWIST1*), cadherin 2 (*CDH2*) and cadherin 1 (*CDH1*). Heatmaps of defined gene sets were generated online (https://xenabrowser.net/heatmap), and detailed data were downloaded for further analysis (12-14).

A retrospectively validation HNSCC cohort was constructed, based on 98 primary HNSCC cases admitted to the Department of Oral Maxillofacial-Head and Neck Oncology, Shanghai 9th People's Hospital between January 2010 and December 2017. The clinicopathological characteristics of each case were collected for further analysis. All patients involved in the present study provided written informed consent, and the study was approved by the Medical Ethics Committee of the 9th People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China) (SH9H-2019-0421).

Immunohistochemistry staining and scoring. Tissues were fixed in 10% formalin for 24 h at room temperature, embedded in paraffin and cut into 5-μm sections. Following dewaxing (with xylene twice for 10 min), rehydration (with 100% ethanol twice for 5 min, 95% ethanol twice for 2 min, and 85% ethanol twice for 2 min at room temperature) and antigen retrieval (with citrate buffer at 98˚C for 15 min), the endogenous peroxidase activity (with 3% hydrogen peroxide for 10 min at room temperature) of each section was quenched. Each slide was incubated with the primary antibody (rabbit polyclonal antibody against JMJD6; 1:250 dilution; cat no. ab96795; Abcam) overnight at 4˚C. Subsequently, the samples were incubated with a biotinylated secondary antibody for 50 min at 37˚C, and stained with a 3'-diaminobenzidine kit (GT Vision Ltd.) for 1 min at room temperature. Consecutive sections were counterstained with hematoxylin and eosin (hematoxylin staining for 5 min and 0.5% eosin staining for 1 min, at room temperature).

The immunoreactivity score of JMJD6 staining was recorded by two independent observers under a light microscope (Olympus Corporation) at x100 and x200 magnification, according to staining intensity and percentage of positive cancer cells. The staining intensity was scored as follows: Weak, scored 1; moderate, scored 2; and intense, scored 3. For the percentage of positive cancer cells, the score was defined as follows: ≤25%, scored 1; >25%, ≤50%, scored 2; >50, ≤-75%, scored 3; >75%, scored 4. Finally, a total score (ranging between 1 and 12) was acquired by multiplying the scores for staining intensity and for the percentage of positive cancer cells. A score of 1-6 was considered to indicate low expression, whereas a score of 7-12 was considered to indicate high expression.

Cell culture and plasmid transfection. The Cal27 and HN12 HNSCC cell lines were cultured in DMEM (Shanghai Basal Media Technologies Co., Ltd.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2.

Cal27 cells function as a classical HNSCC cell line, and HN12 cells possess an increased epithelial-mesenchymal transition (EMT) potential. In the present study, JMJD6 was overexpressed in Cal27 cells and knocked down in HN12 cells. The mammalian expression vector used was pcDNA3.1(+)-myc-JMJD6 and pcDNA3.1(+) was used as an empty vector control (Genomeditech). The plasmids (2 μg) were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) diluted in OPTI-MEM® I (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Small interfering RNAs (siRNAs) were constructed (Genomeditech) and validated at the mRNA and protein levels. Validated small interfering RNAs (siRNAs, 50 nM) specific to JMJD6 were transfected into HN12 cells. The sequence of the siRNA targeting JMJD6 was 5'-GAG GGA ACC AGC AAG ACG A-3', and the sequence for the scrambled siRNA was 5'-UUC UCC GAA CGU GUC ACG UdT dT-3' (Genomeditech, Co., Ltd.). For the subsequent analysis, RNA was extracted after 24 h transfection, and protein was extracted 48 h after transfection.

Western blot analysis. Cellular extracts were acquired by using whole-cell lysis buffer (Yeasen Biotechnology, Co., Ltd.) containing a proteinase inhibitor cocktail (1 mM PMSF, Yeasen Biotechnology, Co., Ltd.; cat. no. 20104ES03). The protein concentration was determined using BCA assays (Thermo Fisher Scientific, Inc.). After subjecting the lysates (30 μg protein per lane) to 10% SDS-PAGE, proteins were transferred to a PVDF membrane by electroblotting. Subsequently, the membranes were blocked in 5% skimmed milk for 1 h at room temperature and incubated overnight at 4˚C with specific primary antibodies against JMJD6 (1:1,000; cat. no. ab50720), Snai1 (1:1,000; cat. no. ab53519), Vimentin (1:1,000; cat. no. ab16700), Twist1 (1:1,000; cat. no. ab175430), N-cadherin (1:1,000; cat. no. ab98952), E-cadherin (1:1,000; cat. no. ab40772) (all from Abcam) and GAPDH (1:5000; cat. no. BM3876; Wuhan Boster Biological Technology, Ltd.). Specific secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG; both 1:5,000; cat. nos. 33201ES60 or 34301ES60, respectively; both from Yeasen Biotechnology, Co., Ltd.;) were incubated in room temperature for 1 h. Specific antibody-bound protein bands were detected with ECL Plus reagent (EMD Millipore) using an Amersham Imager 600 (GE Healthcare).

Reverse transcription-quantitative PCR. Total RNA was extracted from treated HNSCC cells using lysis buffer (Takara Bio, Inc.) according to the manufacturer's protocol. Then,

Table I. Primer sequences for each gene.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
JMJD6	TTGGACCCGGCACAACTACTA	TCTGCCCTTTCCACGTTATCC
SNAI1	TCGGAAGCCTAACTACAGCGA	AGATGAGCATTGGCAGCGAG
VIM	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCGGT
TWIST1	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTGCT
CDH1	ATTTTTCCCTCGACACCCGAT	TCCCAGGCGTAGACCAAGA
CDH2	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
GAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAGT

CDH1, cadherin 1; CDH2, cadherin 2; JMJD6, Jumonji domain-containing protein 6; SNAI1, snail family transcriptional repressor 1; TWIST1, twist family bHLH transcription factor 1; VIM, vimentin.

reverse transcription was performed by using the PrimeScript RT reagent kit (Takara Bio, Inc.) with the following RT protocol: 15 min at 37˚C and 5 sec at 85˚C. The cDNA was subjected to PCR detection using a SYBR Green Premix kit (Takara Bio, Inc.) with the following protocols: 95˚C for 30 sec; and followed by 40 cycles, 95˚C for 5 sec and 60˚C for 34 sec. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method by using GAPDH as reference (15). The primers for each gene are listed in Table I.

Transwell assays. For cellular migration, $2x10^4$ cells suspended in 200 μl fresh DMEM were plated in Millicell chambers (8-μm pore size; EMD Millipore), and 600 μl DMEM containing 10% FBS was plated in the lower chamber. Cells that migrated through the membrane were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.1% crystal violet for 15 min at room temperature. For cellular invasion assays, the chamber was coated with 50 μl Matrigel for 1 h at room temperature, which was then hydrated for 2 h at 37˚C (1:8 dilution; BD Biosciences) prior to the assay. Images of the membrane were captured and the number of migratory or invasive cells was observed under a light microscope (Olympus Corporation) and counted from five fields of view (magnification, x100).

Colony formation assay. A cellular suspension was diluted and seeded at $1x10^3$ cells/well on 6-well plates in triplicate. The cells were cultured with culture medium (DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) for 10 days, and then harvested and fixed with 4% paraformaldehyde for 15 min at room temperature. The cell colonies were observed by staining with Coomassie brilliant blue for 30 min at room temperature. The number of large colonies (consisting of ≥50 cells) was counted and analyzed under a light microscope, at x100 magnification (Olympus Corporation).

Cell viability analysis. MTT assays were performed to examine the viability of cells. Briefly, cells were diluted and seeded at $1x10^4$ cells/100 μl in 96-well plates for 12 h for attachment, and then incubated with 5-fluorouracil (5-FU; Selleck Chemicals) or cisplatin (Selleck Chemicals) at various concentrations for 48 h at 37˚C with 5% CO_2. Absorbance measurements following DMSO resolution were performed at a wavelength of 490 nm (Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were conducted using SPSS v16.0 software (SPSS, Inc.). Statistical comparisons of the transcriptional level of JMJD6 among different stages were made using one-way ANOVA and least significant difference post hoc test (LSD). The different Kaplan-Meier curves were compared by using the log-rank test. Bioinformatically, based on the detailed data of each biomarker, all cases involved were divided equally into two groups; the high-expression group and the low-expression group (the median expression levels were used as cut-off values for each biomarker: 9.318 for JMJD6, 6.433 for SNAI1, 13.53 for vimentin, 7.299 for Twist1, 5.103 for CDH2, and 13.23 for CDH1). To illustrate the underlying association between JMJD6 and EMT markers, crosstabs analyses were performed, and the χ^2 test and Pearson's R correlation coefficient test were used to assess the associations between the gene expression levels of JMJD6 and the gene expression levels of EMT markers (16). Significant differences between two groups were determined using unpaired Student's t-test. The IC_{50} values were calculated with GraphPad Prism (version 6; GraphPad Software, Inc.). All experiments were performed in triplicate and representative results are presented. The values presented correspond to the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

JMJD6 is highly expressed in HNSCC tissues. Based on the bioinformatics results from GEPIA2, the mRNA expression levels of *JMJD6* were significantly upregulated in HNSCC tissues compared with normal tissues in the head and neck area (Fig. 1A and B). However, when further prognostic analysis was performed, no obvious significant differences were observed in overall survival (OS) or disease-free survival (DFS) times in HNSCC for different JMJD6 expression levels (Fig. 1C and D). Therefore, the exact roles of highly expressed JMJD6 in HNSCC remain to be determined. In the present study, bioinformatics analysis based on TCGA HNSCC database revealed a significant association between the expression level of JMJD6 and the pathological stage of HNSCC (Fig. 1E; Table II). These

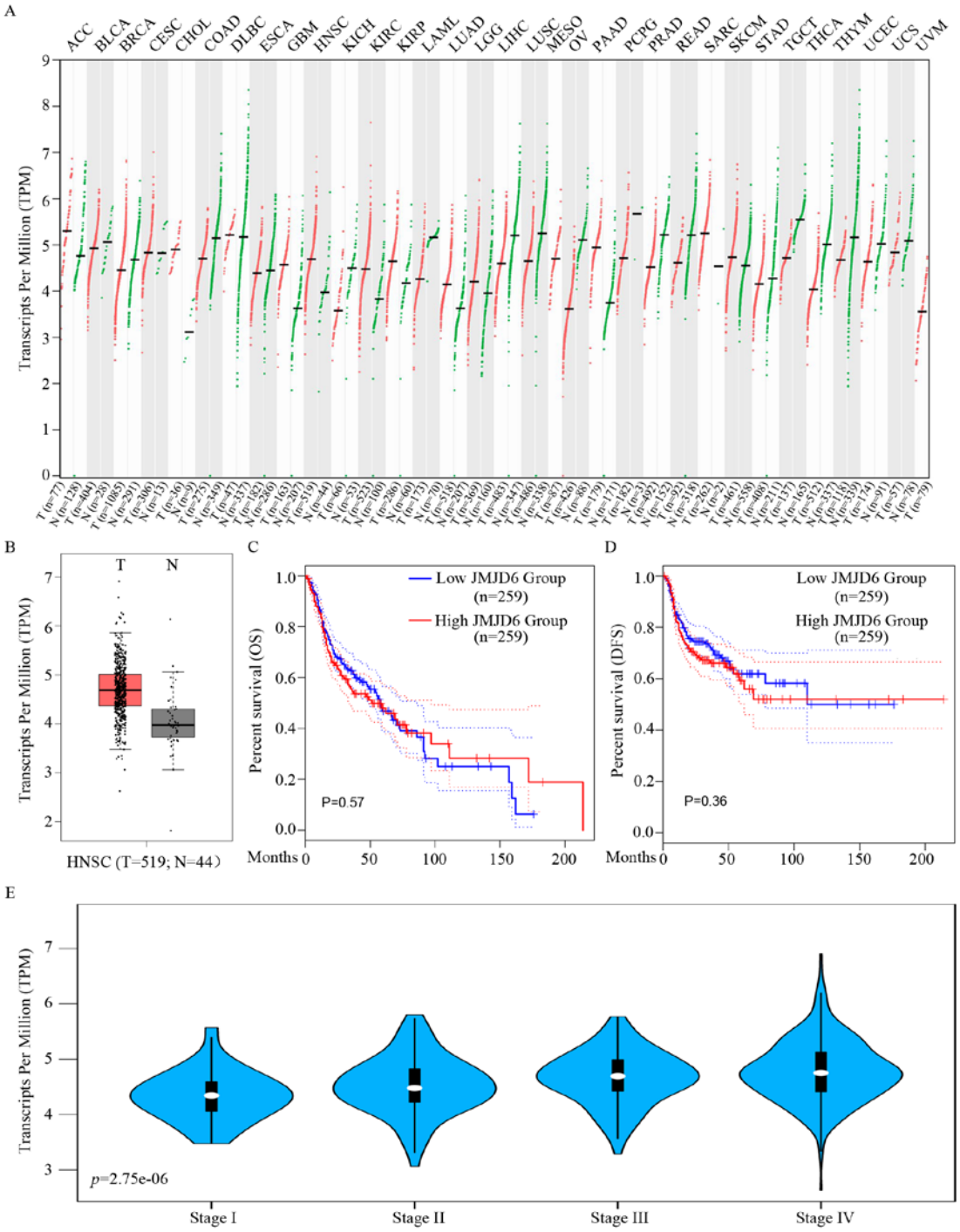

Figure 1. Bioinformatics analysis of the clinical significance of JMJD6 in HNSCC. (A) Expression patterns of JMJD6 in different types of cancer. (B) Expression pattern of JMJD6 in HNSCC. Kaplan-Meier curves for (C) OS time and (D) DFS time analyses, based on JMJD6 expression in HNSCC. (E) Schematic diagram of the association between JMJD6 expression and pathological stage in HNSCC (**P<0.01). JMJD6, Jumonji domain-containing protein 6; HNSCC, head and neck squamous cell carcinoma, T, tumor; N, normal; OS, overall survival; DFS, disease-free survival.

results indicated that JMJD6 could predict HNSCC cases with an advanced stage but had no predictive value for prognosis.

High JMJD6 expression acts as a risk factor for the malignant progression of patients with HNSCC. Based on 566 cases from the TCGA primary HNSCC cohort, increased JMJD6 expression was significantly associated with pathologic tumor size, pathologic lymph node status, pathologic stage and pathological nodal extracapsular spread (the AJCC Cancer Staging manual for head and neck cancer 8th Edition, 2017) (17) (Table II). In the retrospectively validation cohort, based on 98 HNSCC cases, all cases were divided into two group under the expression level of JMJD6 (Fig. 2). Besides, significant associations between increased JMJD6 expression and tumor size and nodal status were observed (Table III). These results suggested that JMJD6 may promote the malignant progression of HNSCC.

Table II. Pathological characteristics of head and neck squamous cell carcinoma cases, based on The Cancer Genome Atlas cohort, according to JMJD6 expression.

		JMJD6 expression		
Variables	Patients, n	≤9.318, n	>9.318, n	P-value
All cases	566	283	283	
Pathologic T				0.005
T1+T2	208	119	89	
T3+T4	295	131	164	
Pathologic N				0.013
N0	193	107	86	
N1+	260	113	147	
Pathologic M				/
M0	190	85	105	
M1	1	1	0	
Pathologic stage				<0.001
I+II	119	77	42	
III+IV	374	167	207	
Neoplasm histologic grade				0.143
G1+G1	398	206	192	
G3+G4	142	63	79	
Lymphovascular invasion present				0.052
Yes	136	54	82	
No	239	121	118	
Pathological nodal extracapsular spread				<0.001
Yes	118	36	82	
No	262	136	126	
Perineural invasion present				0.613
Yes	186	83	103	
No	208	99	109	

Some clinicopathological parameters had missing data and so were excluded. JMJD6, Jumonji domain-containing protein 6; /, not applicable.

JMJD6 regulates the EMT behavior of HNSCC. JMJD6 has been demonstrated to induce EMT and promote malignant migration and invasion in breast cancer (18). In the present study, bioinformatics analysis based on the TCGA primary HNSCC cohort was performed to analyze the association between *JMJD6* expression and EMT status (based on the expression of *SNAI1*, *VIM*, *TWIST1*, *CDH2* and *CDH1*). As shown in Fig. 3A, significant positive correlations between *JMJD6* and *SNAI1*, *TWIST1* and *CDH2* expression and a significant negative correlation between *JMJD6* and *CDH1* expression were observed. Subsequently, a JMJD6 overexpression model in Cal27 cells and a JMJD6 knockdown model in HN12 cells were constructed (Fig. 3B). Overexpression of *JMJD6* in Cal27 cells resulted in increased expression levels of *SNAI1*, *VIM*, *TWIST1* and *CDH2*, and significantly decreased the expression levels of *CDH1* (Fig. 3B and D). Similarly, JMJD6 was knocked down in HN12 cells, which resulted in decreased expression levels of *SNAI1*, *VIM*, *TWIST1* and *CDH2*, while significantly increased expression levels of *CDH1* were observed (Fig. 3C and D). Morphologically, Cal27 cells exhibited an epithelial multiangle shape, and a long, spindle shape was observed in Cal27 cells overexpressing JMJD6 (Fig. 3E). In HN12 cells, knockdown of JMJD6 resulted in a morphological alteration from a long, spindle shape to a multi-angle shape (Fig. 3E). Therefore, increased JMJD6 expression could regulate the EMT status of HNSCC cells.

JMJD6 overexpression promotes the colony formation, and migration and invasion abilities of HNSCC cells. Clinicopathological analysis based on the TCGA primary HNSCC cohort and the validation cohort indicated roles of JMJD6 in the regulation of the metastatic ability of HNSCC. Furthermore, additional roles of JMJD6 in the regulation of EMT were demonstrated in the present study. Colony formation assays indicated markedly increased colony-forming abilities in JMJD6-overexpression Cal27 cells, whereas the knockdown of JMJD6 in HN12 cells significantly decreased their colony-forming abilities (Fig. 4A and B). Transwell assays were performed to investigate the effects of JMJD6 on the migration and invasion ability of HNSCC cells. Overexpression of

Table III. Clinicopathological characteristics of patients divided by JMJD6 expression based on a validation head and neck squamous cell carcinoma cohort.

Variable	Patients, n	JMJD6 expression		P-value
		IRS=1-6, n	IRS=7-12, n	
All cases	98	48	50	
Age, years				0.837
<60	59	28	31	
≥60	39	20	19	
Sex				0.843
Male	52	26	26	
Female	46	22	24	
Smoking				0.999
Yes	51	25	26	
No	47	23	24	
Alcohol				0.999
Yes	48	24	24	
No	50	24	26	
Histological grade				0.110
Well/moderate-differentiation	53	30	23	
Poor-differentiation	45	18	27	
Tumor size				0.017
T1+T2	51	31	20	
T3+T4	47	17	30	
Nodal status				0.003
N0	52	33	19	
N1+	46	15	31	
Metastasis				/
M0	98	48	50	
M1	0	0	0	

IRS, immunoreactivity score; JMJD6, Jumonji domain-containing protein 6; /, not applicable.

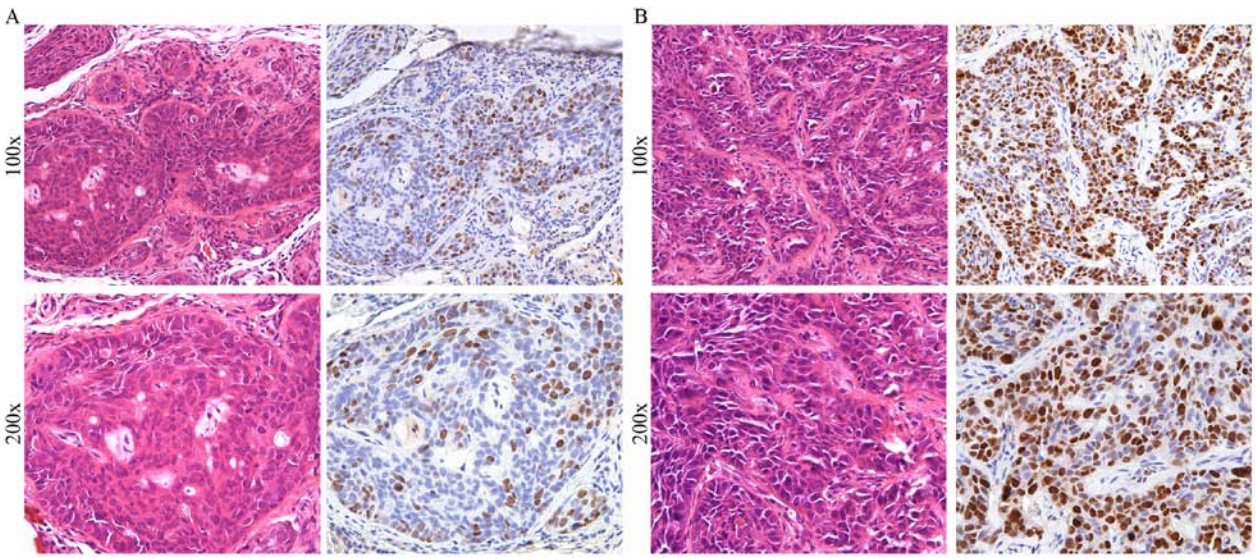

Figure 2. Representative hematoxylin and eosin staining, and JMJD6 immunohistochemical staining of consecutive sections of head and neck squamous cell carcinoma cases. Representative staining for cases with (A) low and (B) high JMJD6 expression. JMJD6, Jumonji domain-containing protein 6.

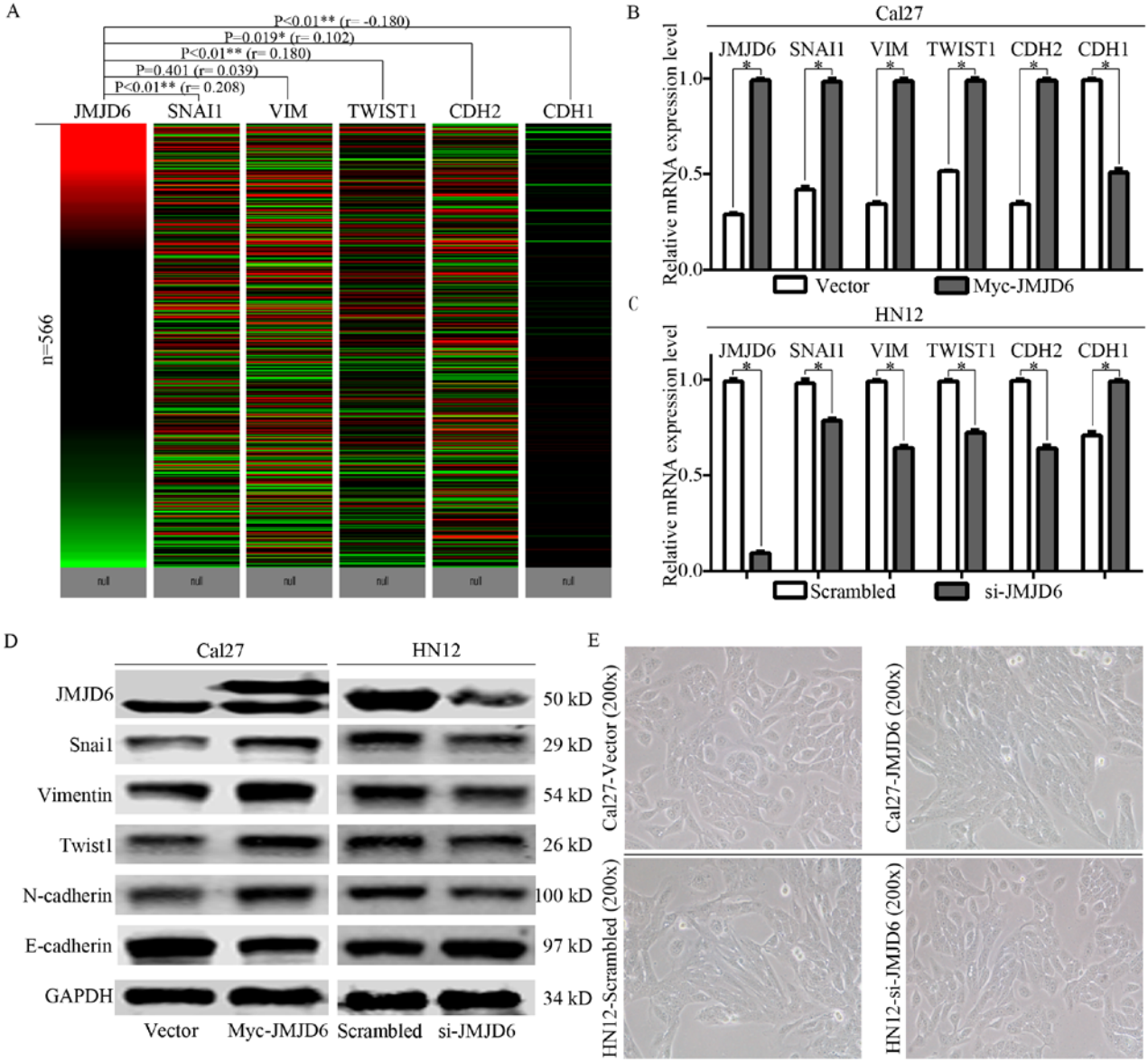

Figure 3. Effects of JMJD6 expression on the epithelial-mesenchymal transition status of HNSCC. (A) Heat map from the UCSC Xena Browser, based on the primary HNSCC cohort from The Cancer Genome Atlas revealing the associations between *JMJD6* and *SNAI1, VIM, TWIST1, CDH2* and *CDH1* gene expression. B A significant correlation with JMJD6 expression was observed for *SNAI1, TWIST1* (Positive correlation) and *CDH1* (negative correlation). (B) mRNA expression levels of *JMJD6, SNAI1, VIM, TWIST1, CDH2* and *CDH1* in Cal27 cells transfected with Myc-JMJD6 for 72 h. (C) mRNA expression levels of *JMJD6, SNAI1, VIM, TWIST1, CDH2* and *CDH1* in HN12 cells transfected with siRNA-JMJD6 for 72 h. (D) Western blot analysis of the expression of JMJD6 in transiently transfected Cal27 and HN12 cells. The upper band of JMJD6 for Cal27 cells indicated the exogenous myc-JMJD6. (E) Representative morphological images of Cal27 cells transfected with Myc-JMJD6 and HN12 cells transfected with si-JMJD6 for 4 days. Magnification, x200. *P<0.05. **P<0.01. CDH1, cadherin 1; CDH2, cadherin 2; JMJD6, Jumonji domain-containing protein 6; HNSCC, head and neck squamous cell carcinoma; siRNA, small interfering RNA; SNAI1, snail family transcriptional repressor 1; TWIST1, twist family bHLH transcription factor 1; VIM, vimentin.

JMJD6 in Cal27 cells resulted in significantly increased migration and invasion (Fig. 4C and D). By contrast, knockdown of JMJD6 in HN12 cells resulted in significantly decreased migration and invasion (Fig. 4C and D).

JMJD6 has no effect on the sensitivity of HNSCC to drugs. The aforementioned data indicated that JMJD6 is significantly associated with malignant behaviors but not with the clinical outcomes of HNSCC. The viability of Cal27 and HN12 cells with different JMJD6 expression levels in response to treatment with 5-FU or cisplatin was investigated, and the IC_{50} values were calculated. No obvious differences were observed in the IC_{50} values of HNSCC cells treated with 5-FU or cisplatin, in association with the different expression levels of JMJD6 (Fig. 5A and B). Therefore, HNSCC with high JMJD6 expression may exhibit a more advanced stage; however, to the best of our knowledge, this is not associated with responsiveness to chemotherapy.

Discussion

The dysregulation of JMJD6 has been reported to contribute to several types of human cancer by controlling a wide range of biological functions (3,4). To the best of our knowledge, the

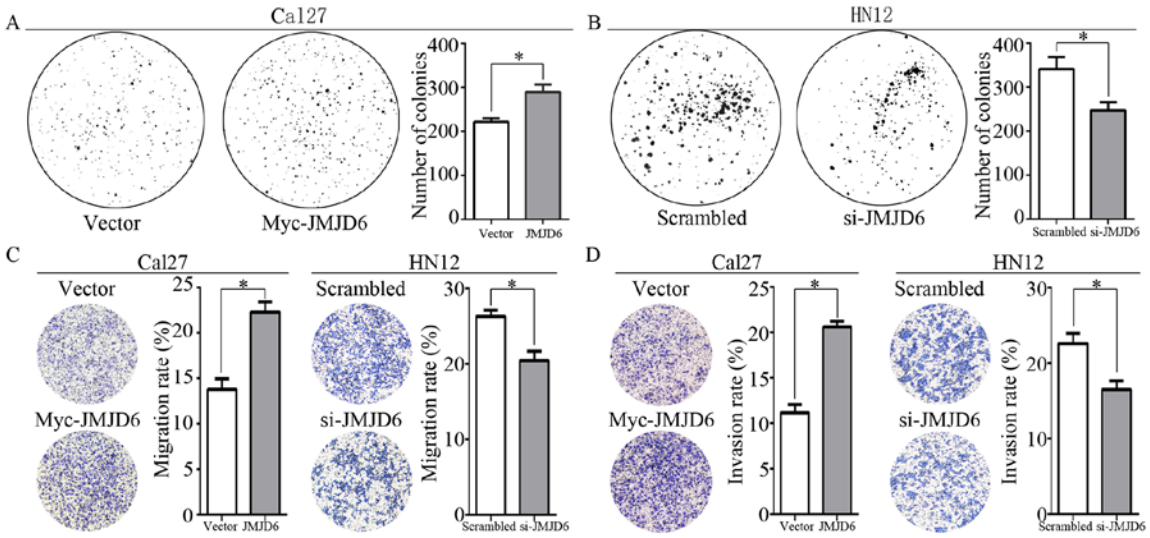

Figure 4. Effects of JMJD6 expression on the behaviors of head and neck squamous carcinoma cells. Colony-formation assay in (A) Cal27 cells transfected with Myc-JMJD6 and (B) HN12 cells transfected with si-JMJD6. (C) Transwell assays for evaluating the effects of JMJD6 overexpression on the migration ability of Cal27 cells and the effects of JMJD6 knockdown on the migration ability of HN12 cells. (D) Transwell assays for evaluating the effects of JMJD6 overexpression on the invasion ability of Cal27 cells and the effects of JMJD6 knockdown on the invasion ability of HN12 cells. Three independent experiments were performed and representative images are shown. *P<0.05. JMJD6, Jumonji domain-containing protein 6; si, small interfering RNA.

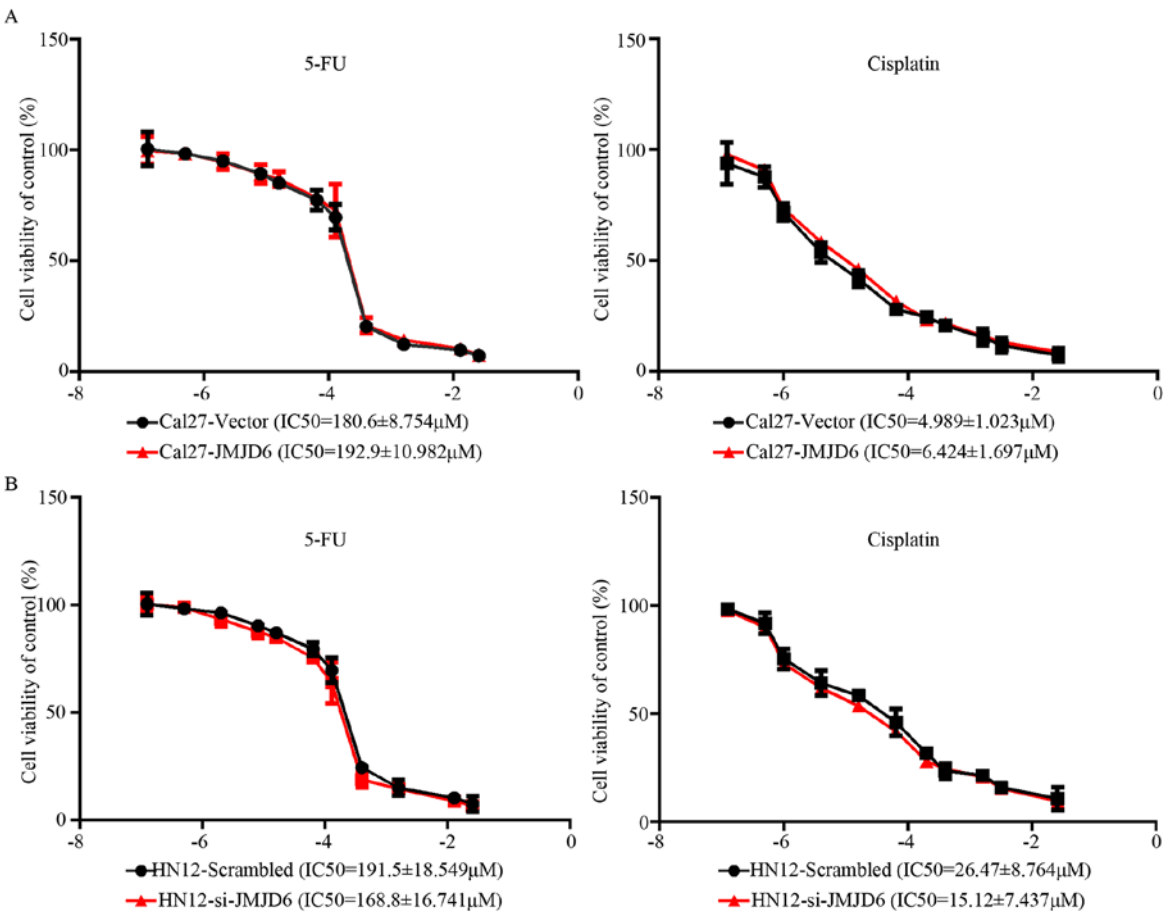

Figure 5. Effects of JMJD6 expression on the drug responses of head and neck squamous cell carcinoma cells. Determination of IC_{50} of (A) Cal27 cells following JMJD6 overexpression and (B) HN12 cells following JMJD6 knockdown in response to 5-FU and cisplatin. 5-FU, 5-fluorouracil; JMJD6, Jumonji domain-containing protein 6; si, small interfering RNA.

biological roles and clinical significance of JMJD6 in HNSCC remain uncertain. In the present study, the clinical significance of JMJD6 based on the TCGA primary HNSCC cohort and a validation HNSCC cohort was investigated. Subsequently,

the roles of JMJD6 in HNSCC were revealed through a series of cellular assays. Consequently, it can be concluded that JMJD6 contributes to the malignant progression of HNSCC by regulating EMT, whereas JMJD6 overexpression exerts no significant effects on the drug responsiveness and prognosis of HNSCC.

The data from the present study demonstrated that JMJD6 was significantly upregulated in HNSCC samples compared with normal samples. Overexpression of JMJD6 did not affect the prognosis (OS and DFS times) of patients with HNSCC; however, it was strongly associated with advanced pathological stage (particularly in terms of tumor size, nodal status and nodal extracapsular spread), based on the TCGA cohort and a validation cohort. Previous studies have demonstrated that the upregulation of JMJD6 indicates aggressive phenotypes and a poor prognosis in multiple types of human cancer (3,11). The present study suggested that patients with HNSCC and high JMJD6 expression are liable to develop advanced-stage disease but exhibited no difference in drug sensitivity. Further studies are required to analyze the expression of JMJD6 across different HNSCC subtypes.

Based on the clinical significance of JMJD6, it was speculated that JMJD6 serves key roles in the regulation of migration/invasion behaviors in HNSCC. Invasion and migration are the main characteristics of the EMT process (19). In the present study, bioinformatics analysis based on the TCGA primary HNSCC cohort was carried out in order to detect the association between JMJD6 expression and EMT status (based on the expression of *SNAI1*, *VIM*, *TWIST1*, *CDH2* and *CDH1*). As expected, JMJD6 was associated with EMT status in HNSCC. Additionally, overexpression of JMJD6 in HNSCC cells resulted in increased migration and invasion rates. Thus, JMJD6 promoted the malignant progression of HNSCC by regulating the EMT process. In lung cancer, JMJD6 has been reported to cooperate with c-Myc to enhance the EMT process (18). In HNSCC, the underlying mechanisms will be further investigated in subsequent studies.

Based on the results of IC50 experiments, the present study revealed that overexpression of JMJD6 had no effect on the drug responses of HNSCC to chemotherapy. These data may illustrate that JMJD6 contributed to a more advanced stage, however not a poorer prognosis. To the best our knowledge, the present study was the first to demonstrate that high JMJD6 expression promoted malignant progression but not the development of drug resistance in HNSCC. Treatment strategies for HNSCC with an advanced stage should be based on combined and sequential therapy. For patients with advanced HNSCC, radical surgeries always lead to poor quality of life without a markedly improved prognosis (20). According to the data obtained in the present study, rational chemotherapy may be beneficial for patients with advanced HNSCC who exhibit high JMJD6 expression.

In conclusion, the present study demonstrated the clinical significance of JMJD6 in HNSCC, and that high JMJD6 expression was significantly associated with an advanced stage, with no effect on drug responsiveness or prognosis. However, additional studies are required to investigate the underlying mechanisms in the future.

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Availability of data and materials

The datasets analysed during the present study are available in the TCGA-HNSC repository (https://xenabrowser.net), or available from the corresponding author upon reasonable request.

Authors' contributions

BG, YS and CM conceived and designed the study. BG and LW performed the experiments. BG and XQ compiled and analyzed the data. BG, LW and XQ contributed to writing and revising the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients involved in the present study provided written informed consent, and the study was approved by the Medical Ethics Committee of the 9th People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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