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.

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
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% CO₂.

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 AC GC-3', and the sequence for the scrambled siRNA was 5'-UUC UCC GAAGC GUU CAG UGdT 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 (1:5,000; 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 from Abcam) and PMSF (Yeasen Biotechnology Co., Ltd.) 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,
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 χ² 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 IC₅₀ 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

Table I. Primer sequences for each gene.

| Gene name | Forward primer (5'-3') | Reverse primer (5'-3') |
|-----------|------------------------|------------------------|
| JMID6     | TGGACCCCGCACAAGTCTACTA | TCTGCCTTTTCCACGGTATCC |
| SNAI1     | TCGGAAAACTTAATTAGGAGGA | AGATGACAATGGCACCGAGAG |
| VIM       | GACGCCATCAACACCGAATT  | CTGTTGCTTGGTTAGCGGT   |
| TWIST1    | TGGCCGAGTCTTTGAGGAG   | GCTTGGGTTCTAGATCTGCT  |
| CDH1      | ATTTTTTTCTCCACACCCCGAT| TCCAGGCGTAGACCAAGA    |
| CDH2      | AGCCAAACTTAACTGAGGAGT | GGCAGTTGTATGAGAGGATG  |
| GAPDH     | AACGTTGCTAGTGGTTGCCCTG | AGTTGGGTCTGCCTGGTATG |

CDH1, cadherin 1; CDH2, cadherin 2; JMID6, Junmoji-domain-containing protein 6; SNAI1, snail family transcriptional repressor 1; TWIST1, twist family bHLH transcription factor 1; VIM, vimentin.
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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.
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 multiangle 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

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

| Variable                      | Patients, n | IRS=1-6, n | IRS=7-12, n | P-value |
|-------------------------------|-------------|------------|-------------|---------|
| 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.
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
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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