Original Article
Effect of RNA interference-mediated down-regulation of JMJD2A on cervical cancer

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Received July 16, 2016; Accepted September 1, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: Cervical cancer is an important cause of cancer-related mortality and often has a poor prognosis because of its late diagnosis, aggressive local invasion, early metastasis, and poor response to chemotherapy and radiotherapy. It is expected that development of new strategies to block the tumorigenesis process will improve the disease prognosis. In this study, we tested our hypothesis that JMJD2A, a histone demethylase, may regulate cervical cancer cell motility, and silencing of it adversely affects cell biological characteristics. We used JMJD2A small interfering RNA (siRNA) to silence its expression in a cervical cancer cell line to determine whether JMJD2A regulates cell proliferation, migration and invasion in vitro. JMJD2A was overexpressed in cervical cancer tissue and cells, and its expression level was related to histological differentiation: the poorer the tissue differentiation, the higher the expression of JMJD2A. Furthermore, JMJD2A knockdown led to reduced SiHa cervical cancer cell proliferation, migration and invasion. Our results suggest that JMJD2A could be a novel therapeutic target in cervical cancer.

Keywords: JMJD2A, cervical cancer, siRNA, transfection

Introduction

Worldwide, cervical cancer is the third most common cancer in women, with an estimated 530,000 new cases and 275,000 deaths each year [1]. Considering its 51% mortality rate, cervical cancer has become a public health concern. Although advanced cervical cancer can be treated by radical surgery, radiotherapy and/or chemotherapy, have limited effects, and some patients with high-risk factors still have an unfavorable prognosis [2]. Therefore, new treatment strategies, diagnostic molecular markers and cancer therapeutic targets may prove useful in improving the prognosis of cervical cancer patients. More recently, a number of Jumonji-domain-containing histone lysine demethylases (KDMs) have been identified that can demethylate trimethylated lysine residues of histone proteins [3-5]. The Jumonji domain-2 (JMJD2) family of KDMs consists of four members, Jmjd2A-D. JMJD2A, also named JHDM3A or KDM4A, demethylates histone 1.4 on lysine 26 as well as histone 3 on lysines 9 and 36 [6]. Histone modifications are important due to their role in chromatin structure and organization. Alterations in these modifications can activate oncogenes and inactivate tumor suppressor genes that ultimately result in uncontrollable proliferation [7]. Recently, a growing number of studies have shown that JMJD2A is closely related to the development of different tumors, including breast [8, 9], bladder [10], prostate [11] and colon [12] cancer. These results suggest that over-regulation of JMJD2A plays a role in the development of cancer in diverse tissues. However, the relationships between JMJD2A and cervical cancer have not been reported.

Like other epigenetic changes histone modifications are promising drug targets because they can be reversed relatively easily through chemotherapeutic intervention compared to genetic changes [13]. RNAi is a post-transcriptional regulation and provides a rapid means of depleting mRNAs by introducing double-stranded RNA homologous to a particular message leading to its sequence-specific degradation [14].

In this study, to investigate how JMJD2A regulates cervical cancer progression and/or metastasis, we examined various effects of transient JMJD2A silencing by RNAi on a human cervical
Effect of JMJD2A on cervical cancer

Table 1. Relationship between JMJD2A expression in cervical cancer tissues and clinicopathological features

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>JMJD2A Expression</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 40 years</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>≥ 40 years</td>
<td>53</td>
<td>32</td>
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<tr>
<td>Tumor SIZE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 4 cm</td>
<td>52</td>
<td>32</td>
</tr>
<tr>
<td>≥ 4 cm</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Histological differentiation</td>
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<td></td>
</tr>
<tr>
<td>Well</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>Moderate</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>Poor</td>
<td>22</td>
<td>11</td>
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<tr>
<td>FIGO stage</td>
<td></td>
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</tr>
<tr>
<td>I</td>
<td>42</td>
<td>25</td>
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<tr>
<td>II</td>
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<td>20</td>
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<td>III</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

tissue positivity, that is, the percentage of tissue staining positive (0-100%), and (2) the intensity of positively stained cells (0, none; 1, weak; 2, moderate; and 3, strong). Final score was calculated from a combination of intensity and percentage score. The total score ranged from 0 to 9.

Cell culture and transfection

Human cervical cancer cell line SiHa (Shanghai Cell Bank, China) was routinely cultivated in a complete growth medium comprising Dulbecco’s Minimal Eagle’s Medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Gibco, Invitrogen). JMJD2A siRNA and negative control siRNA were purchased from Qiagen (Hilden, Germany) and were transiently transfected into SiHa cells using the Lipofectamine 2000 reagents (Invitrogen), following the manufacturer’s instructions. Transfection compounds were assigned into three groups as follows: blank control group (without any treatment); siRNA control group (transfected with the JMJD2A siRNA); and negative control group (transfected with the negative control siRNA). Cells were then incubated for 48 h, followed by RNA and protein extraction.

Quantitative real-time PCR

Total RNA was extracted from cultured cells by Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). A reverse transcription kit, the one-step Prime Script RT reagent Kit (TaKaRa, Dalian, China), was used for the synthesis of cDNA. Quantitative RT-PCR (qRT-PCR) for miRNAs was performed using the AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, Korea) in an Bio-Rad CFX96 Real-Time System. The sequences of forward and reverse oligonucleotide primers, specific to JMJD2A and housekeeping genes, were designed using Primer5 software. The primers were as follows:

JMJD2A: F: GTGGTCTTATTGACCTTGCTTTGCG; R: GGACCAACTGAGACGATCTTG; GAPDH: F: AACCCCAAGGCAACCAGCCGAGAAGATGACC; R: GTGTAGACCTGGCCCGTCAGAGCTCGTA.

Material and methods

Tissue specimens

Tissue specimens consisting of 82 cases of cervical cancer and 20 normal cervical tissues were obtained from the Department of Pathology, Second Affiliated Hospital of Harbin Medical University after obtaining approval from the local ethics committees and informed consent from the patients.

Specimens were acquired from December 2007 to December 2012, and the stages and histological grades of the tumors were established according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). All cervical cancer samples were classified as squamous cervical cancer. No patient had received radiotherapy, chemotherapy, or other treatment prior to surgery. Patient characteristics are summarized in Table 1.

Immunohistochemistry

Immunohistochemistry was performed on sections of human cervical cancer tissue using previously reported procedures [15]. Tissue blocks were cut into 4-mm sections, which were stained with JMJD2A antibodies. All stained tissue sections were then graded by two independent pathologists, and separate scores were given to each sample based on: (1)
Effect of JMJD2A on cervical cancer

Primers were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The PCR conditions were: denaturation at 95°C for 10 min, 40 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. Threshold cycle (Ct), which correlates inversely with the target mRNA levels, was calculated using the second derivative maximum algorithm.

Western blotting

After transfection, total protein was extracted from cells with lysis buffer (Beyotime, Shanghai, China) containing a 1% dilution of the protease inhibitor PMSF (Beyotime). We determined the protein concentration with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (40 µg) were loaded onto 10% SDS-polyacrylamide gels, and separated proteins were transferred to PVDF membranes. After blocking the membrane with 5% milk proteins for 2 h at room temperature, the membrane was incubated with monoclonal rabbit anti-human JMJD2A antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:2000; Cell Signaling Technology) at 4°C overnight. Immunolabeling was detected using an enhanced chemiluminescence reagent (ECL kit, Amersham). Western blot data were quantified using ImageJ pixel analysis (NIH Image software). β-Actin was used as an internal control to confirm equal protein loading.

Figure 1. Expression of JMJD2A by immunohistochemistry in benign and malignant cervical tissue with well, moderately, and poorly differentiated cervical cancer. A. JMJD2A protein expression in benign cervical tissue. B. JMJD2A protein expression in well-differentiated cervical cancer tissue. C. JMJD2A protein expression in moderately differentiated cervical cancer. D. JMJD2A protein expression in poorly differentiated cervical cancer (all images taken at 200× total magnification; bar indicates 50 µm). The scores for JMJD2A in tissue from well, moderately, and poorly differentiated cervical cancer were 1, 4, and 9, respectively. There was no expression in benign cervical tissue.
Effect of JMJD2A on cervical cancer

**Cell proliferation assay**

Cell proliferation was measured using Cell Counting Kit-8 assays (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). Human cervical cancer cells (2×10^3 cells/well) transfected with either negative control or JMJD2A siRNA were incubated in 96-well plates in a total volume of 100 μl/well. One day after seeding, the cell number was counted for the first time (defined as day 0) and 2 days later, the cell number was counted again. After 48 h incubation, cell growth inhibition was determined by a reduction assay as follows: 10 μl CCK-8 was added per well, and the cells were incubated for an additional 4 h and the absorbance at 450 nm was recorded using a 96-well plate reader (Infinite M200 PRO, Tecan, Morrisville, NC, USA). All experiments were performed in triplicate.

**In vitro migration and invasion assays**

Cell migration and invasion assays were performed using Transwell chambers (24-well, 8-μm pore size, 6.5-mm diameter; Corning Costar, Cambridge, MA, USA). After transfection, cells (2×10^5 cells/ml) in 0.2 ml serum-free medium were placed in each upper chamber, either coated with (invasion) or without (migration) Matrigel (BD Biosciences, San Jose, CA, USA). Medium with 10% FBS (500 μl) was added to each lower chamber. After 24 h (migration) or 48 h (invasion) incubation, the cells on the top surface of the insert were removed by wiping with a cotton swab. The cells were fixed with 10% carbinol and stained with crystal violet. The cells were counted using light microscopy (200× magnification). Three independent experiments were performed in triplicate.

**Statistical analysis**

Data were analyzed and expressed as mean ± SD for continuous variables. Associations among categorical variables were assessed using Fisher’s exact probability test or the x^2 test. Data for JMJD2A expression levels in the three groups were analyzed by ANOVA. Data for cell proliferation, migration and invasion assays in vitro were also analyzed by ANOVA. When the results of the ANOVA were significant, Dunnett’s test was used to assess the differences in these expression levels among each group. P < 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 17.0 (Chicago, IL, USA).

**Results**

**Pattern of JMJD2A expression in cervical tumor**

To elucidate the expression of JMJD2A in cervical cancer tissue, we compared the degree of JMJD2A protein expression between benign and malignant cervical tissue. JMJD2A protein
expression was detected in 62.2% (51/82) cervical cancer, and in 25% (5/20) normal cervical tissue. Expression of JMJD2A was found in the nucleus as well as the cytoplasm. The difference in JMJD2A expression between cervical cancer and normal cervical tissue was significant ($\chi^2=8.984, P < 0.05$). Expression of JMJD2A was not related to the age, tumor size, histological differentiation and stage ($P > 0.05$). The poorer the tissue differentiation, the higher

the expression of JMJD2A (Figure 1). Quantitative real-time PCR (data not shown) and western blotting (Figure 2) showed that the SiHa cell line expressed high levels of JMJD2A.

Transfection with JMJD2A siRNA inhibited JMJD2A mRNA and protein expression in SiHa cells

According to the results of real-time qRT-PCR (data not shown), JMJD2A mRNA expression levels in the siRNA control group were significantly lower than in the blank control and negative control groups, respectively. However, there was no significant difference between the blank control and negative control groups. Western blot analysis (Figure 2) showed that there was no significant different ($P > 0.05$) in the expression of JMJD2A protein between the blank control (0.777 ± 0.090%) and negative control (0.709 ± 0.085%) groups. JMJD2A protein expression in the siRNA control group (0.398 ± 0.092%) was significantly lower than in the blank control ($P < 0.05$) and negative control ($P < 0.05$) groups. These data indicated that the expression levels of JMJD2A mRNA and protein in SiHa cells were significantly reduced after transfection with JMJD2A siRNA.

Silencing JMJD2A suppresses proliferation of SiHa cells

To investigate the role of JMJD2A in the proliferation of cervical cancer cells, we silenced JMJD2A in SiHa cells by siRNA transfection, and examined whether JMJD2A was associated with cell proliferation. Figure 3 shows a significant decrease in the proliferation rate of cells transfected with JMJD2A siRNA (0.483 ±
Effect of JMJD2A on cervical cancer

0.051%) compared with the blank control (P < 0.05) and negative control (P < 0.05) groups. There was no significant difference (P > 0.05) between the blank control (0.749 ± 0.089%) and negative control (0.739 ± 0.118%) groups. These results demonstrated that transfection with JMJD2A siRNA could reduce the proliferation of SiHa cells.

JMJD2A silencing reduces migration and invasion of SiHa cells

To elucidate the role of JMJD2A in the processes of cervical cancer cells, we inhibited JMJD2A expression in SiHa cells by RNAi, and detected cell migration and invasion. Figure 4A shows that, compared with the blank control and negative control groups, cell migration in the JMJD2A siRNA control group was significantly inhibited (P < 0.05). However, no significant difference (P > 0.05) was observed between the blank control and negative control groups for cell migration. Additionally, we detected that JMJD2A silencing affected cell invasion similarly to migration of the cervical cancer cells (Figure 4B). These results demonstrated that after transfection with JMJD2A siRNA, migration and invasion of SiHa cells were significantly reduced.

Discussion

We measured the expression of histone demethylase JMJD2A in cervical cancer tissues and cells, and aimed to clarify the relationship between JMJD2A and cervical cancer progression, as well as to establish whether JMJD2A might be a potential molecular therapeutic target in cervical cancer. First, we investigated JMJD2A overexpression in cervical cancer tissues and SiHa cells by immunohistochemistry, real-time qRT-PCR and western blotting. Expression of JMJD2A was not related to the age, tumor size, histological differentiation and stage, but the poorer the tissue differentiation, the higher the expression of JMJD2A. We also showed that transient silencing of JMJD2A reduced cell proliferation, migration and invasion of SiHa human cervical cancer cells. Collectively, our results strongly suggest that JMJD2A overexpression plays an important role in cell proliferation, migration and invasion of cervical cancer, and that JMJD2A exhibits features of an oncoprotein and is closely related to tumorigenesis.

We demonstrated that expression of JMJD2A in cervical cancer and downregulation of JMJD2A affected the biological characteristics of cervical cancer cells, such as proliferation, migration and invasion. However, how JMJD2A affected tumorigenesis and tumor biological characteristics in cervical cancer was not clear. Based on recent data, we discuss the probable function of JMJD2A in cervical cancer.

JMJD2 proteins are especially capable of efficiently demethylating trimethylated H3K9 and act either as coactivators or corepressors [11, 16]. Recent reports also relate histone methylation downregulation with tumorigenesis [17]. In recent studies, JMJD2A catalyzed the demethylation of trimethylated H3K9, and also of dimethylated H3K9 and trimethylated H3K36 peptides [3, 6]. Appropriate JMJD2A expression can maintain the balance of histone lysine methylation and demethylation, and have important biological significance in building and maintaining the regulation of heterochromatin in normal cells. JMJD2A overexpression can cause imbalance in histone lysine methylation and demethylation, and affect the stability of the structure of heterochromatin [9]. In accordance with these studies, histone demethylase JMJD2A protein may participate in tumorigenesis.

A recent study reported that JMJD2A formed complexes with p53 in vivo and binds to it in vitro [12]. During tumor development, a large percentage of cells are lost through apoptosis [18]. Such cell death is triggered by a variety of extracellular signals, including growth/survival factor depletion, hypoxia and a loss of cell-matrix interactions, as well as intracellular signals such as DNA damage [19]. It is well known that p53 is a tumor suppressor gene, as well as a DNA-binding transcriptional regulator [20, 21]. It plays a critical role at the G1/S phase of cell cycle transition, where it can either block entry into S phase or activate apoptosis in response to DNA damage [22]. In more than 50% of human cancers, somatic mutations in p53 have been found [23]. Expression of the p53 protein was associated with both poor prognosis and metastasis in cervical cancer [24]. There is a significant association between p53 gene expression and cervical cancer [25]. Apoptosis is controlled by a balance of pro-apoptotic and antiapoptotic genes. Two promi-
Effect of JMJD2A on cervical cancer

Target genes of p53 include the cell cycle inhibitor p21 and the proapoptotic protein Puma [26, 27]. Previous studies have shown that p53 can directly suppress transcription of Bcl-2 protein, which is a prosurvival protein [28, 29]. The data show that JMJD2A depletion can increase p53 protein levels and regulate p53 transcriptional activity and further regulate p53 target genes. Knockdown of JMJD2A can increase expression of p21 and Puma protein. JMJD2A depletion reduces Bcl-2 levels, which suggests that JMJD2A can decrease the ability of p53 to inhibit Bcl-2 transcription [12]. These observations provide evidence that JMJD2A promotes tumorigenesis by the p53 pathway represses p21 and Puma expression and stimulates Bcl-2 expression. There is growing evidence that JMJD2A may play an important role in cervical cancer progression by activation of the p53 signaling pathway.

In conclusion, we showed for the first time that histone demethylase JMJD2A expression in cervical cancer and a possibly clinically significant role of JMJD2A in cervical cancer cell proliferation, migration and invasion. These data strongly indicate that JMJD2A as an oncoprotein may play an important role during tumorigenesis and suggest that JMJD2A could be a novel drug target in cervical cancer therapy. However, our study was based on tissues and a single cell line, therefore, the mechanism of JMJD2A in cervical cancer needs further research.

Acknowledgements

We gratefully acknowledge the assistance of technicians in the Key Laboratory of Myocardial Ischemia, Harbin Medical University who support the equipment and tools for the test and help to conduct the experiment.

 Disclosure of conflict of interest

None.

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References

Effect of JMJD2A on cervical cancer