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KMT2D inhibits the growth and metastasis of bladder Cancer cells by maintaining the tumor suppressor genes

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ABSTRACT

KMT2D, a kind of histone H3 lysine 4 (H3K4) methyltransferase, its abnormal expression confirmed to be associated with diverse tumors, but is lack of defined role in bladder cancer (BC). KMT2D mutation was analyzed using several databases. Immunohistochemistry and clinicopathological analysis of KMT2D in 51 paired of BC tissues and corresponding normal tissues were used to evaluate the relationship between KMT2D and BC. The effects of silencing or over-expressing KMT2D on HTB-9 and T24 cell viability, migration and invasion were performed using MTT, wound scratch and Transwell, respectively. Also, bladder cancer mouse model was established by hypodermic injection of the BC cells. Associated expressions of methylation genes, oncogenes and tumor suppressors were assessed by western blot and quantitative real-time PCR. KMT2D was frequent mutation in various tumors, including BC. It was negative expression in BC tissues and cells, also implicated with tumor stages and lymph node metastasis. In silencing KMT2D HTB-9 and T24 cells, cell viability, migration and invasion were notably promoted. Meanwhile, knockdown of KMT2D benefited to solid tumor formation *in vivo*. However, over-expressing KMT2D represented contrary results. Especially, KMT2D over-expression induced the activity of H3K4 monomethylation (me1), and effectively enhanced PTEN and p53 expressions as well as repressed STAG2 expression. Meanwhile, KMT2D had no obvious effect on Survivin. This work suggested an antitumor role for KMT2D *in vitro* and *in vivo*, as well as provided a possible tumor inhibition mechanism in which KMT2D enhanced H3K4me1 activity to support the expressions of tumor suppressors.

1. Introduction

Bladder cancer (BC) is a common malignant tumor in human urinary system, most of which are urothelial carcinoma. Every year, there are about 430,000 new cases of bladder cancer and 170,000 deaths in the world, as well as the morbidity and mortality are increasing year by year [1,2]. It can be found that bladder cancer always has the highest incidence of malignant tumors in the urinary system in China [3]. In recent decades, the survival rate of patients with renal and prostate cancer has been greatly improved, but the prognosis of patients with bladder cancer has not been essentially enhanced. About 1/3 of the primary bladder carcinomas develop local progression and distant metastasis, with a 5-year survival rate of less than 62% [4]. And high-grade muscle invasiveness is considered as the main cause of death in bladder cancer patients [5–7]. Numerous factors, such as environmental and epigenetic factors, are involved in the progression of BC [8,9]. It is of great significance to further clarify the biological mechanism of the occurrence and development of bladder cancer and to improve the prognosis of bladder cancer patients.

Gene mutation and abnormal regulation of gene expression play an important role in tumorigenesis and progression [10]. In bladder cancer, the increase in activity of oncogene PIK3CA, FGFR3, CCND1, which are presented as point mutation, amplification or up-regulation expression, promotes the progression of bladder cancer [11–14]. At the same time, the deletion or inactivation of tumor suppressor genes such as RUNX3, CDKN2A, TSC1, PTEN, TP53 are also one of the important factors leading to the development of bladder cancer [15–19]. The expressions of these gene mutations are affected by epigenetic regulation, such as acetylation, deacetylation, ubiquitin and methylation of histone [20,21]. Histone methyltransferases contain a class of SET domains that are important components for completing histone methylation. KMT2D/MLL2 is a catalytic lysine methyltransferase containing SET domain [22]. Abnormally expressed KMT2D has been shown to be associated with tumors because its SET region has the activity to

methylate H3K4 and is an important component of the polyprotein complex that plays a role in gene regulation and embryonic development [23–25]. Natarajan detected a positive correlation between the increase in KMT2D expression level and the malignancy of breast cancer and colon cancer in protein level studies [26]. Through microarray analysis, some studies had found that the expression of MLL2 gene was often abnormal in prostate cancer and gastric cancer relative to normal tissues [27,28]. Several studies reported that KMT2D acted as a tumor suppressor gene in non-Hodgkin lympoma [29–31].

Recently, Fantini established a mouse model of muscle-invasive bladder cancer and exome sequencing analysis KMT2D had a high mutation [32]. To the best of our knowledge, there are limited reports on the roles of KMT2D in bladder cancer cells. Therefore, in this work, we aim to investigate the effects of KMT2D on bladder cancer cell proliferation and metastasis, as well as its possible mechanism.

2. Materials and methods

2.1. Patients and tissue samples

51 cases of bladder cancer tissues and adjacent normal tissues were obtained from specimens after surgically removed at Shandong Provincial Hospital Affiliated to Shandong University from May 2015 to June 2017. Samples were kept in 4% formaldehyde solution for pathological diagnosis routinely. Written informed consents had been provided by all patients and the ethics committees of hospital approved the study.

2.2. Immunohistochemistry (IHC)

The bladder cancer and adjacent tissue sections were deparaffinized, dehydrated and blocked in turn. Hot sodium chloride citrate buffer was performed to renovate antigen for 20 min. And then sections were incubated with KMT2D antibody (ab224156, 1:200, Abcam) at 4 °C overnight. After washing with PBS, secondary antibody HRP-conjugated goat anti-Rabbit Ig G (Protein tech, USA) were incubated at room temperature for 30 min. Subsequently, chromogen staining was used by diaminobenzidine. Also, sections were redyed with hematoxylin. Three representative slices were evaluated by two independent experienced pathologists according to staining pattern, which was scored based on the tissue positive ratio and intensity. The pair tissue positive ratio was identified as 0 for negative, 1 for < 1/3, 2 for 1/3-2/3 and 3 for > 2/3. Meanwhile, staining intensity categorised as negative (0), faint yellow (1), brown yellow (2) and dark brown (3). The final KMT2D staining score was obtained by addition tissue positive ratio and intensity rank number, and was defined as follows staining score of 0 was negative, 2–3 was weakly positive (+), 4 was medium positive (+ +), 5–6 was strong positive (+ + +).

Based on the immunohistochemical comprehensive score of the 51 pair cancer and adjacent tissues, we ranked the KMT2D which is greater than the median as high expression, n = 26, and less than the median as low expression, n = 25. The Comparison of KMT2D expression in clinicopathological parameters are shown in Table 1.

2.3. Cell culture and animal

Human uroepithelial cells (SV-HUC-1 cells) and four bladder cancer cell lines (HTB-9, T24, UM-UC-3 and J82 cells) were all purchased from Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. HTB-9 and T24 cells were cultured in RPIM 1640 medium (Gibco, USA), UM-UC-3, J82 and SV-HUC-1 cells were cultured in DMEM medium (Gibco, USA). All of mediums were added into 10% fetal bovine serum, 100 u/ml penicillin and 100 u/ml streptomycin at 37 °C and 5% CO_2 incubator.

The mice experiment was performed use of 24 SPF of BALB/c-nu mice which was purchased from Shanghai Laboratory Animal

Table 1
The Comparison of KMT2D expression in clinicopathological parameters.

Characteristics	N	KMT2D high expression (≥ median)	KMT2D low expression (< median)	P value
Age(year)	31	14	17	0.301
< 60	20	12	8	
≥ 60				
Gender	27	13	14	0.668
Male	24	13	11	
Female				
Tumor size (cm)	25	12	13	0.676
< 3	26	14	12	
≥ 3				
Histology	35	19	16	0.485
Adenocarcinoma	16	7	9	
Squamous carcinoma				
Tumor stage	13	9	4	0.024
I	19	12	7	
II	19	5	14	
IIIa				
Lymph node metastasis	32	21	11	0.007
No	19	5	14	
Yes				

Resources, Chinese Academy of Sciences (Shanghai, China). Animal experiments were performed according to the Shandong Provincial Hospital Affiliated to Shandong University Animal Ethics Committee and Guidelines for the Care and Use of Laboratory Animals. Mice kept in cages at room temperature (22 °C ± 3 °C) with a constant humidity (50% ± 10%) with free access to food/water in a light/dark cycle (12 h/12 h).

2.4. Cell transfection

Lipofectamine 2000 (Invitrogen, USA) was used according to manufacturer's protocol to perform the transient transfection assay in selected HTB-9 and T24 cell lines. Opti-MEM mediums were respectively added with Lipofectamine 2000, empty vector plasmid, silencing and over-expressing KMT2D plasmid and incubated at 25 °C for 5 min. Subsequently, Lipofectamine 2000 was mixed into each well for 20 min and mixtures were cultured in Opti-MEM medium. After 6 h culturing, the fluid was changed back to RPMI 1640 medium containing 10% FBS. In the following experiments, cells were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid).

2.5. Cell viability

MTT reagent (Solarbio Life Sciences, Beijing, China) was used to detect cell viability. Cells (1×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h after transfection. 20 μL MTT was added to each well after washing with PBS buffer and then cultured for another 4 h. Lastly, cells were centrifugation and mixed with 150 μL DMSO. The optical density was measured at 490 nm using a microplate reader (Thermo Fisher, USA).

2.6. Wound scratch and transwell assay

After transfection treatment, 2×10^5 cells were seeded in 6-well plates for 24 h. At the bottom of the plate, sterile 20 μl pipette tip was used to marking off. Afterwards, cells were washed with PBS 3 times and added with serum-free medium. Cell migration was observed by inverted microscope and measured using Image J software.

Also, cells were resuspended in serum-free medium and 2×10^4 cells were added into upper chamber coated with matrigel. In the lower

Table 2
Primers used in qRT-PCR.

Gene	Primer	Sequence
KMT2D	Forward	5'-CGCGGATCCATGCTGCGCCGCGCTCTGCT-3'
	Reverse	5'-CCGGAATTCTTACAGTTCATCTTTCACAGCTTTCTG-3'
PTEN	Forward	5'-CAGCCAAGTCTGTGACTTGCCGTAC -3'
	Reverse	5'-CCGCTCGAGCAGTCGCTGCAACCATCCA -3'
P53	Forward	5'-CTGAGGTTGGCTCTGACTGTACCACCATCC -3'
	Reverse	5'-CTCATTCAGCTCTCGGAACATCTCGAAGCG -3'
Survivin	Forward	5'-TGGCGTAAGATGATGGA -3'
	Reverse	5'-TAGGGACGACGATGAAA -3'
STAG2	Forward	5'-ACGGAAAGTGGTTGAGGG-3'
	Reverse	5'- GTGGAGGTGAGTTGTGGTGT-3'
GAPDH	Forward	5'-AGCCACATCGCTCAGACAC -3'
	Reverse	5'-GCCCAATACGACCAAATCC -3'

24-well chamber, RPIM1640 medium containing 10% fetal bovine serum was added and cells were incubated for 24 h for 37℃. Cells were fixed with 1% formaldehyde for 10 min at 25 ℃ and stained with 0.5% crystal violet for another 5 min.

2.7. Quantitative real-time qRT-PCR

KMT2D, PTEN, p53, Survivin and STAG2 would be detected the mRNA expression in HTB-9 and T24 cells. Total RNA was isolated by using Trizol reagent (Invitrogen, USA) in accordance with manufacturer's protocol. OrimeScript™ RT reagent kit (TaKaRa, Otsu, Japan) was applied as reverse transcription according to the manufacturer's instructions. qRT-PCR was performed by SYBR Fast qPCR Mix (Invitrogen, USA) and primer sequences are listed in Table 2. Samples ran using the following cycling parameters, KMT2D at 94 ℃ for 5 min, 98 ℃ for 30 s, 62 ℃ for 30 s followed by 30 cycles of 68 ℃ for 50 s and 68 ℃ for 5 min; PTEN and p53 at 94 ℃ for 5 min, 94 ℃ for 30 s, 60 ℃ for

30 s followed by 30 cycles of 72 ℃ for 1 min and 72 ℃ for 5 min; Survivin at 94 ℃ for 5 min, 94 ℃ for 30 s, 54 ℃ for 30 s followed by 35 cycles of 72 ℃ for 30 s and 72 ℃ for 5 min; STAG2 at 95 ℃ for 30 s, 95 ℃ for 5 s, 60 ℃ for 34 s followed by 37 cycles of 72 ℃ for 30 s and 72 ℃ for 5 min. GAPDH was used as an internal control. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$.

2.8. Western blotting analysis

Proteins were extracted from HTB-9 and T24 cells using RIPA lysis buffer (Thermo Scientific, USA). BCA protein kit (Beyotime, Shanghai, China) was performed to analysis proteins concentration. Subsequently, samples were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membranes were blocked and incubated with anti-KMT2D antibody (ab224156, 1:1000, Abcam, USA), anti-H3K4me1 antibody (ab8895, 1:500, Abcam, USA), anti-H3K4me2 antibody (ab7768, 1:1000, Abcam, USA), anti-H3K4me3 antibody (ab8580, 1:1000, Abcam, USA), anti-PTEN antibody (ab32199, 1:10000, Abcam, USA), anti-p53 antibody (ab131442, 1:1000, Abcam, USA), anti-Survivin antibody (ab469, 1:5000, Abcam, USA), anti-STAG2 antibody (ab209477, 1:5000, Abcam, USA), anti-GAPDH antibody (ab9485, 1:2500, Abcam, USA) overnight at 4 ℃. After Tween-20 washing for 3 times, HRP-conjugated secondary antibody (Protein tech, USA) was subsequently incubated with membranes. Protein bands were detected with ECL (Thermo Fisher, USA) and visualized using Quantity one (Bio-Rad, USA).

2.9. Xenograft experiment

Both of HTB-9 and T24 cells were transfected with silencing KMT2D, over-expressing KMT2D or empty control plasmid. Bladder cancer model (6 per group) was induced by hypodermic injection of 2×10^6 cells and mice were kept in same raising condition. Tumor
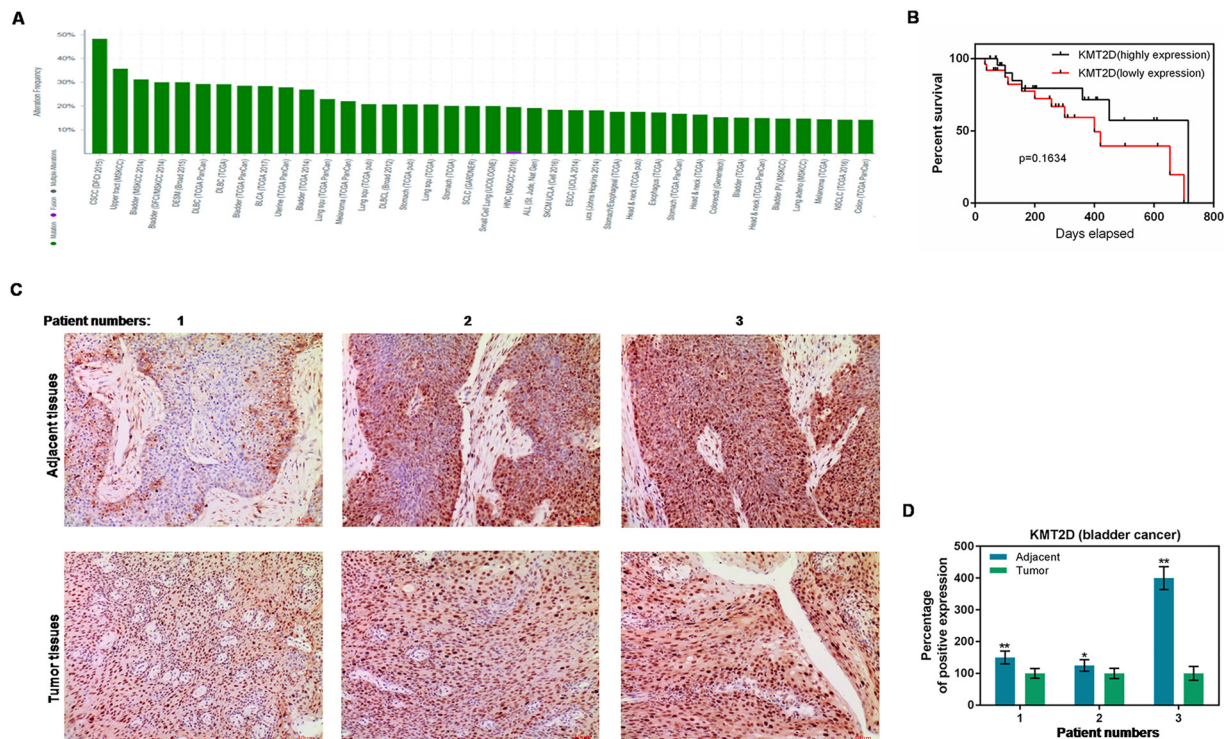

Fig. 1. Mutation frequency and immunohistochemical staining of KMT2D in bladder cancer. A Using several databases and previous data analysis, the alteration frequency of KMT2D in different tumors were showed as bar diagrams. B 51 pair of bladder cancer and adjacent normal tissue was collected to analyze 2-year survival rate by Kaplan-Meier. P = 0.1634. C Randomly selected 3 pair tissues were used to detect the immunohistochemical staining of KMT2D. D The positive expression of KMT2D in selected 3 pair bladder and adjacent tissues. Data were shown as mean ± SD from three independent experiments ([*]compared to adjacent, [*]P < 0.05, [**]P < 0.01).
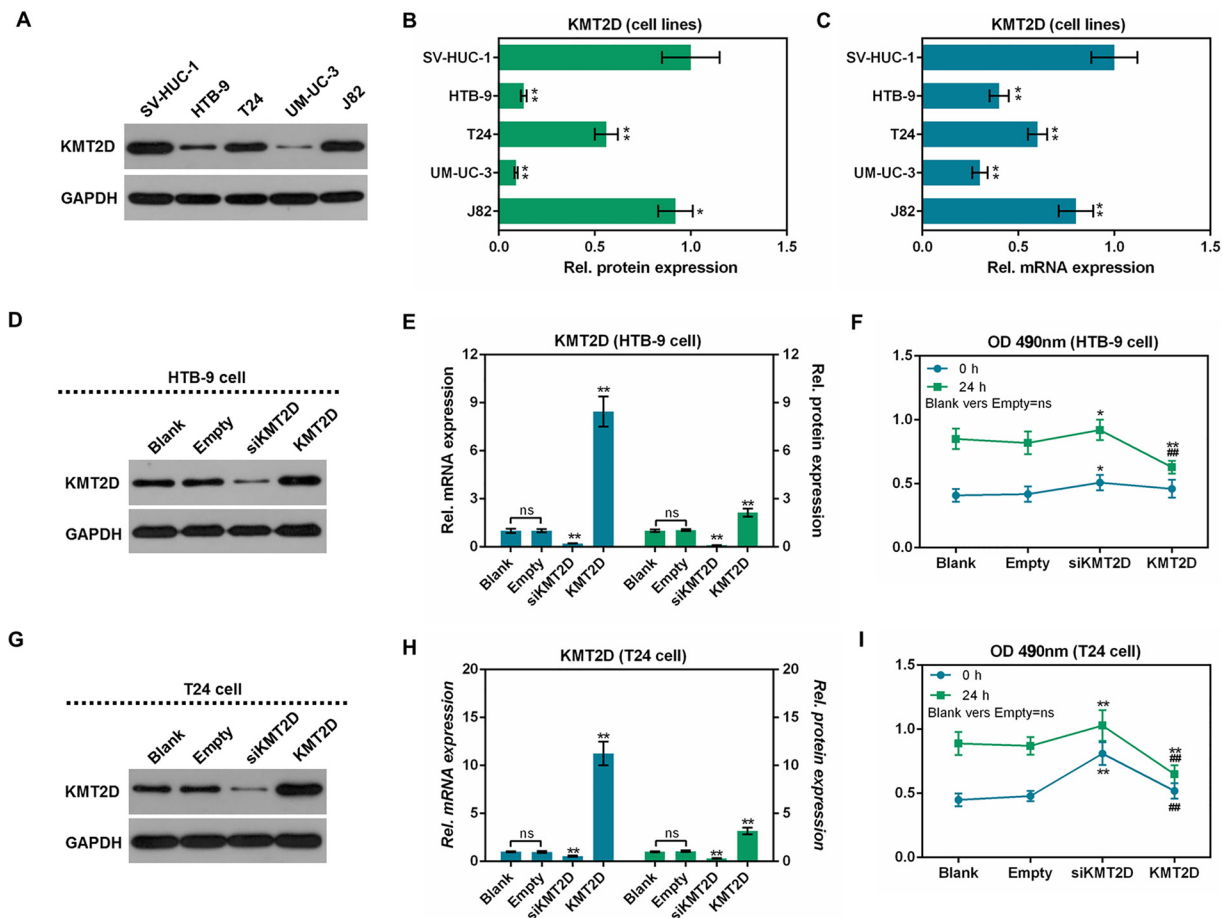
Fig. 2. The low expression of KMT2D in bladder cancer cell lines and the effects of KMT2D on cell viability in HTB-9 and T24 cells. A, B Western blot analysis of KMT2D protein expression in normal uroepithelial cells (SV-HUC-1 cells) and four bladder cancer cell lines (HTB-9, T24, UM-UC-3 and J82 cells). C Quantitative real-time PCR analysis of KMT2D mRNA expression in normal uroepithelial cells (SV-HUC-1 cells) and four bladder cancer cell lines (HTB-9, T24, UM-UC-3 and J82 cells). (*compared to SV-HUC-1 cells, *P < 0.05, **P < 0.01). D Cells were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid). Western blot was performed to detect the effect of silencing or over-expressing KMT2D on KMT2D protein expression in HTB-9 cells. E Related mRNA and protein expression were shown as bar diagrams in HTB-9 cells. F MTT assay was used to assess the effect of silencing or over-expressing KMT2D on cell viability in HTB-9 cells. G Western blot was performed to detect the effect of silencing or over-expressing KMT2D on KMT2D protein expression in T24 cells. H Related mRNA and protein expression were shown as bar diagrams in T24 cells. I MTT assay was used to assess the effect of silencing or over-expressing KMT2D on cell viability in T24 cells. GAPDH served as an internal control. Data were shown as mean ± SD from three independent experiments (Blank vers Empty = ns, *compared to ns, #compared to silencing KMT2D, */#P < 0.05, **/##P < 0.01).

volume and weight were measured every three days and the mice were sacrificed 30 days after the cells transplantation.

2.10. Statistical analysis

Statistical analysis was detected by Prism Graphpad version 6.0 software. All data are presented as mean ± standard deviation (SD). Differences were performed using one-way analysis of variance (ANOVA) following Turkey's multiple comparison. A *p* < 0.05 was considered significant.

3. Results

3.1. KMT2D has high mutation frequency in bladder cancer

Several databases such as TCGA, MSKCC, and previous data analysis showed that KMT2D had frequent mutations in numerous tumors. In bladder cancer, KMT2D presented a higher mutation with alteration frequency of 30% in MSKCC analysis, although less than CSCC and Upper tract (Fig. 1A).

3.2. The protein expression of KMT2D in bladder cancer and adjacent tissues and associated with metastasis

51 pair of bladder cancer and adjacent normal tissues were collected to conduct the IHC staining, as well as analyze the survival rate. We could find KMT2D high expression was somewhat higher 2-year survival rate than that low expression (P = 0.1634, Fig. 1B). Randomly selected 3 pair tissues with IHC staining showed that KMT2D significantly positive expression in adjacent normal tissues (P < 0.05 or P < 0.01, Fig. 1C–D). Combined with the staining score of KMT2D, the relative clinicopathological parameters were also analyzed. As Table 1 revealed, age, gender, tumor size and histology had no obviously effects on the expression of KMT2D (P > 0.05). It could be found KMT2D was particular associated with different tumor stages (P = 0.024) and lymph node metastasis (P = 0.007).

3.3. Down-regulation expression of KMT2D in bladder cancer cell lines

Both of western blot (Fig. 2A–B) and qRT-PCR (Fig. 2C) were performed to assess the expression of KMT2D in normal uroepithelial cells (SV-HUC-1 cells) and four bladder cancer cell lines (HTB-9, T24, UM-UC-3 and J82 cells). The results showed that four bladder cancer cells
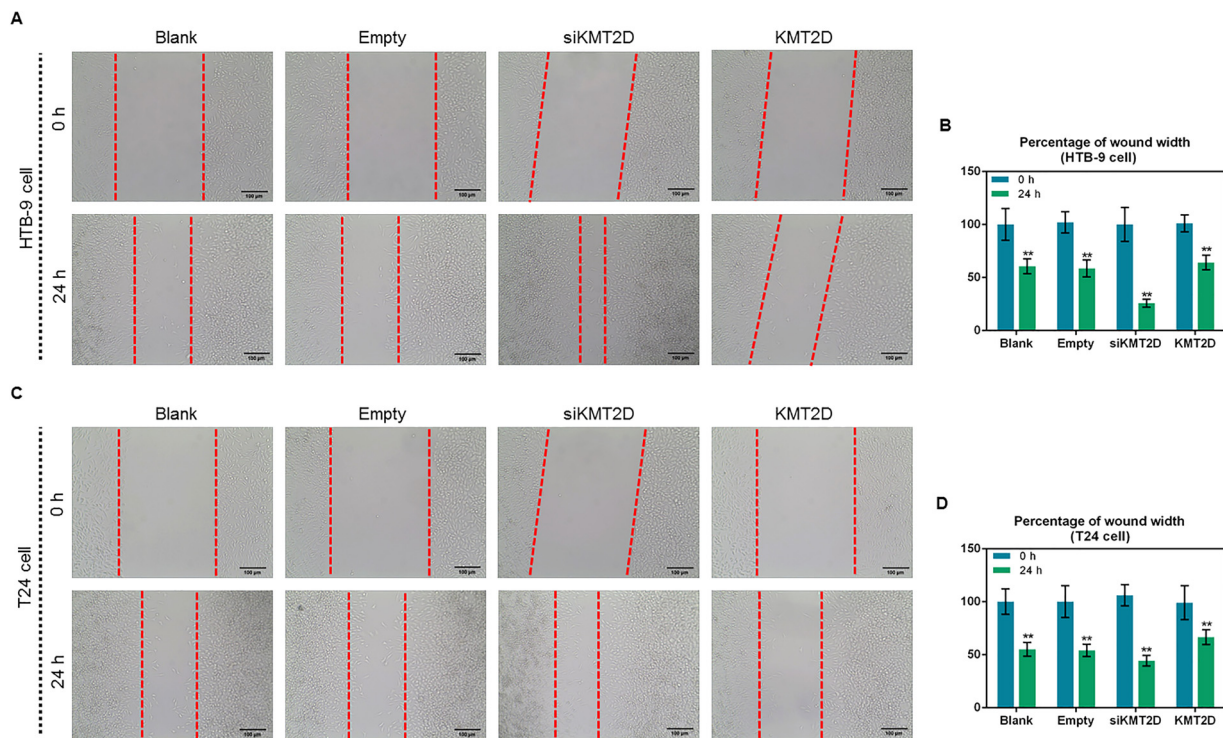
Fig. 3. The effects of KMT2D on cell migration in HTB-9 and T24 cells. Cells were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid). A, C Cell migration was performed by wound scratch assay and observed by inverted microscope at 0 and 24 h. B, D Wound width at 0 and 24 h was quantified as bar diagrams in HTB-9 and T24 cells. Data were shown as mean $\pm$ SD from three independent experiments (*compared to 0 h, *P $<$ 0.05, **P $<$ 0.01).

had a reduced expression of KMT2D in varying degree compared to SV-HUC-1 cells (J82 in protein, P $<$ 0.05, others, P $<$ 0.01). T24 and HTB-9 cells were selected to use in the following experiments for T24 cell line is the most commonly studied cell line in bladder cancer [33,34] and HTB-9 cell line is the least studied cell line in bladder cancer [35].

3.4. KMT2D regulates cell growth and metastasis in HTB-9 and T24 cells

Silencing KMT2D and over-expressing KMT2D were successfully transfected in HTB-9 (Fig. 2D–E) and T24 (Fig. 2G–H) cells. The results could be clearly displayed that silencing KMT2D significantly decreased KMT2D expression and over-expressing KMT2D remarkably increased its expression both in mRNA and protein levels (P $<$ 0.01). Cell viability was detected using MTT assay. In HTB-9 cells, silencing KMT2D could promote cell viability increase starting from initial time (P $<$ 0.05, Fig. 2F). However, over-expressing KMT2D dramatic decreased cell viability just at 24 h in comparison with blank or siKMT2D (P $<$ 0.01). In T24 cells and both in 0 or 24 h, cell viability had robust increases in silencing KMT2D treatment cells (P $<$ 0.01, Fig. 2I). On the other hand, over-expressing KMT2D could significantly reduce cell viability compared to siKMT2D (P $<$ 0.01). As for cell migration which was performed by wound scratch (Fig. 3A and C), the measured wound width showed sharp reductions after cultured for 24 h both in HTB-9 (P $<$ 0.01, Fig. 3B) and T24 cells (P $<$ 0.01, Fig. 3D). Moreover, we found the reduction effect of silencing KMT2D was greater than blank. In addition, cell invasion was also assessed by Transwell assay showed in Fig. 4A and C. It is as expected silencing KMT2D promoted HTB-9 (P $<$ 0.01, Fig. 4B) and T24 (P $<$ 0.01, Fig. 4D) cell invasion. Also over-expressing KMT2D had an opposite results with siKMT2D (P $<$ 0.01).

3.5. KMT2D affects the expression of H3K4me1 in HTB-9 and T24 cells

KMT2D could regulate the activity of H3K4 methylation as described above. As we all known, there are three different forms of methylation of lysine, including monomethylation (me1), dimethylation (me2) and trimethylation (me3). Therefore, the effects of KMT2D on the expression of these three types were detected. In HTB-9 cells, the results showed that silencing KMT2D noticeably decreased the protein expressions of H3K4me1, H3K4me2 and H3K4me3 compared with blank or empty (P $<$ 0.01, Fig. 5A). At the same time, over-expressing KMT2D could significantly up-regulated the H3K4me1 protein level (P $<$ 0.01) but had no obvious changes with H3K4me2 and H3K4me3 in comparison to blank. In T24 cells, it is surprise to see both silencing and over-expressing KMT2D had no complete differences with regulation the protein expressions of H3K4me2 and H3K4me3 compared to blank. Whereas, the effect of KMT2D on the protein expression of H3K4me1 displayed a similar result with that in HTB-9 cells (P $<$ 0.01, Fig. 5G). Taken together, KMT2D could regulate the H3K4 monomethylation in bladder cancer HTB-9 and T24 cells.

3.6. The effects of KMT2D on the regulation the expressions of oncogenes and anti-oncogenes in HTB-9 and T24 cells

Furthermore, western blot and qRT-PCR were performed to assess related oncogenes and anti-oncogenes expressions. We could find PTEN (Fig. 5B–C, H–I) and p53 (Fig. 5B, D, H and J) protein and mRNA expressions had almost the same results that silencing KMT2D down-regulated their expressions and over-expressing up-regulated their expressions both in the HTB-9 and T24 cells (PTEN mRNA in siKMTD2, P $<$ 0.05, others, P $<$ 0.01). As for Survivin gene, the result showed that both in two bladder cancer cells, it is nearly unchanged expression no matter treatment with silencing or over-expressing KMT2D (P $>$ 0.05, Fig. 5B, H, E and K). In regard of STAG2, it had somewhat difference expression in HTB-9 and T24 cells. The expression of STAG2
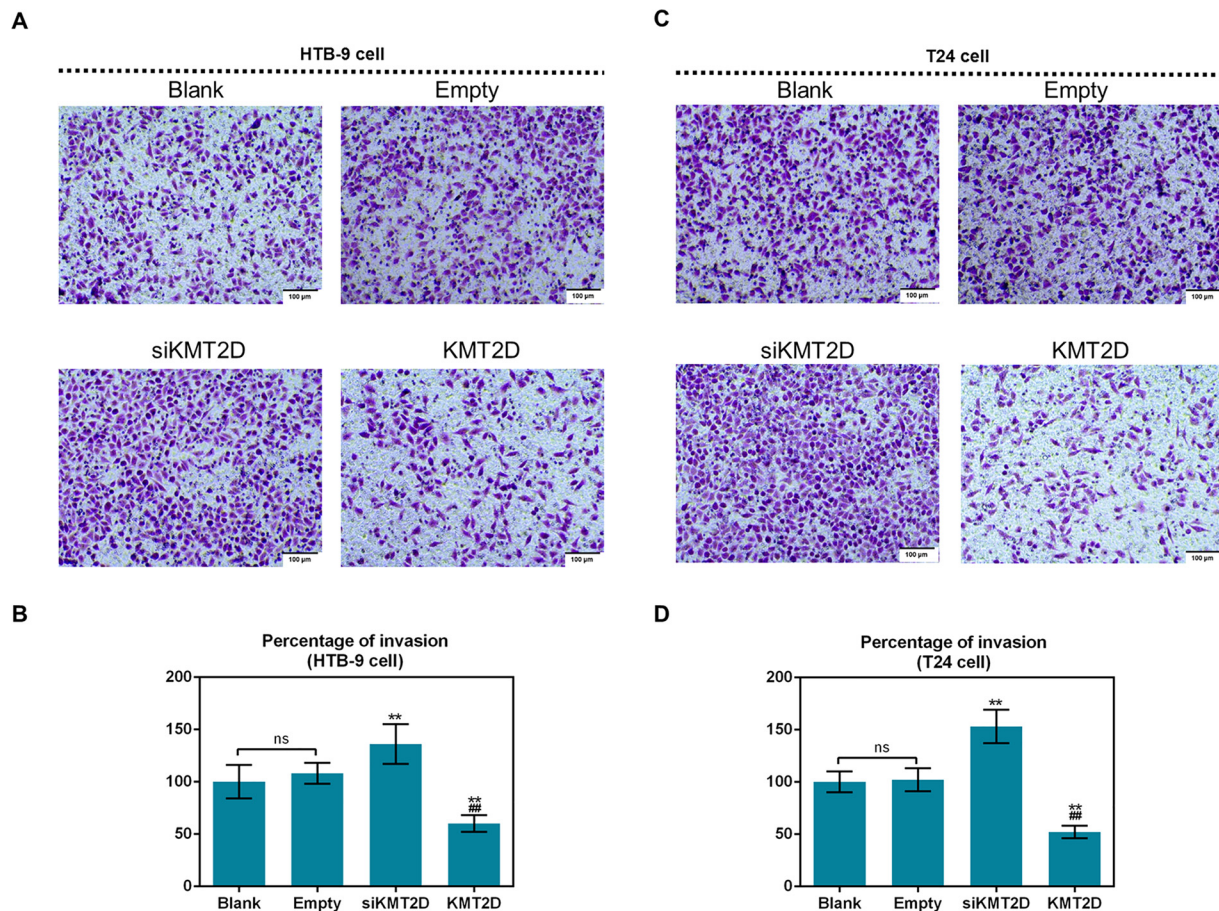
Fig. 4. The effects of KMT2D on cell invasion in HTB-9 and T24 cells. Cells were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid). A, C Cell invasion was performed by Transwell assay and observed by inverted microscope. B, D Cell invasion was quantified as bar diagrams in HTB-9 and T24 cells. Data were shown as mean ± SD from three independent experiments (Blank vers Empty = ns, *compared to ns, #compared to silencing KMT2D, */#P < 0.05, **/##P < 0.01).

was remarkably increased by silencing KMT2D and was decreased by over-expressing KMT2D (P < 0.01, Fig. 5B and F) in HTB-9 cells. In T24 cells, only over-expressing KMT2D could clearly down-regulate the STAG2 protein and mRNA levels (P < 0.01, Fig. 5H and L).

3.7. KMT2D affects the growth of solid bladder cancer in vivo

After HTB-9 bladder cancer cells transplanted into mice, we could find cells transfected with silencing KMT2D extraordinarily induced solid tumor formations (Fig. 6A), of which tumor weight (P < 0.01, Fig. 6B) and volume (P < 0.01, Fig. 6C) increased. However, over-expressing KMT2D significantly reduced tumor weight (P < 0.05), and the tumor volume was also decreased although to a lesser extent. Similarly, KMT2D also could affect the formation of solid tumor when T24 bladder cancer cells were transplanted. Especially, silencing KMT2D promoted tumor weight and volume enhancement (P < 0.01, Fig. 6D–F). On the other hand, over-expressing KMT2D attenuated tumor weight (P < 0.01, Fig. 6E) and volume (P < 0.05, Fig. 6F) to limit tumor growth.

4. Discussion

Tumor formation is mainly owing to the accumulation of DNA mutations in somatic cells, which is an important reason, possibly due to point mutations or large fragments of DNA gain or loss, resulting in corresponding changes in protein expression [36–38]. A large amount studies reported that KMT2D mutations could cause Kabuki syndrome [39,40]. Also, KMT2D mutations were commonly generated in lymph

nodes, blood, brain and so on, which were associated with the progression of malignant tumors [41,42]. In this work, we used multiple databases as well as data analysis, and found KMT2D were frequent mutations in several tumors, mainly in cutaneous squamous cell carcinoma and bladder cancer cells. It is in agreement with da reporting that KMT2D was one of pivotal genes implicated with cutaneous T cell lymphoma [43]. At the meantime, Juhlin suggested that KMT2D acted as a frequently mutated gene, potential involving in pheochromocytomas development [44]. Moreover, various literatures demonstrated that in oesophageal squamous cell, pancreatic cancer, lung and bladder cancer KMT2D were recurrently altered [45–48].

Furthermore, to understand the relationship between KMT2D and bladder cancer, 51 bladder cancer patients were recruited. Combined with immunohistochemical staining and clinicopathological analysis, we found KMT2D was down-regulation expression in bladder cancer tissues and related with tumor stages and lymph node metastasis. It is somewhat different with previous studies that high expression of KMT2D/MLL2 contributed to esophageal squamous cell carcinoma, prostate, pancreatic and gastric cancer progression [23,49–51]. Our experiments concluded that silencing KMT2D could increase cell viability, enhance cell migration and invasion *in vitro*, as well as could promote solid tumor formation *in vivo*. On the other hand, over-expressing KMT2D represented an opposite phenomena. These results are consistent with previous KMT2D gene detection in bladder-associated tissues and cells, as well as are confirmed by several researches, which decimated KMT2D in B cells benefited lymphoma development [52–54].

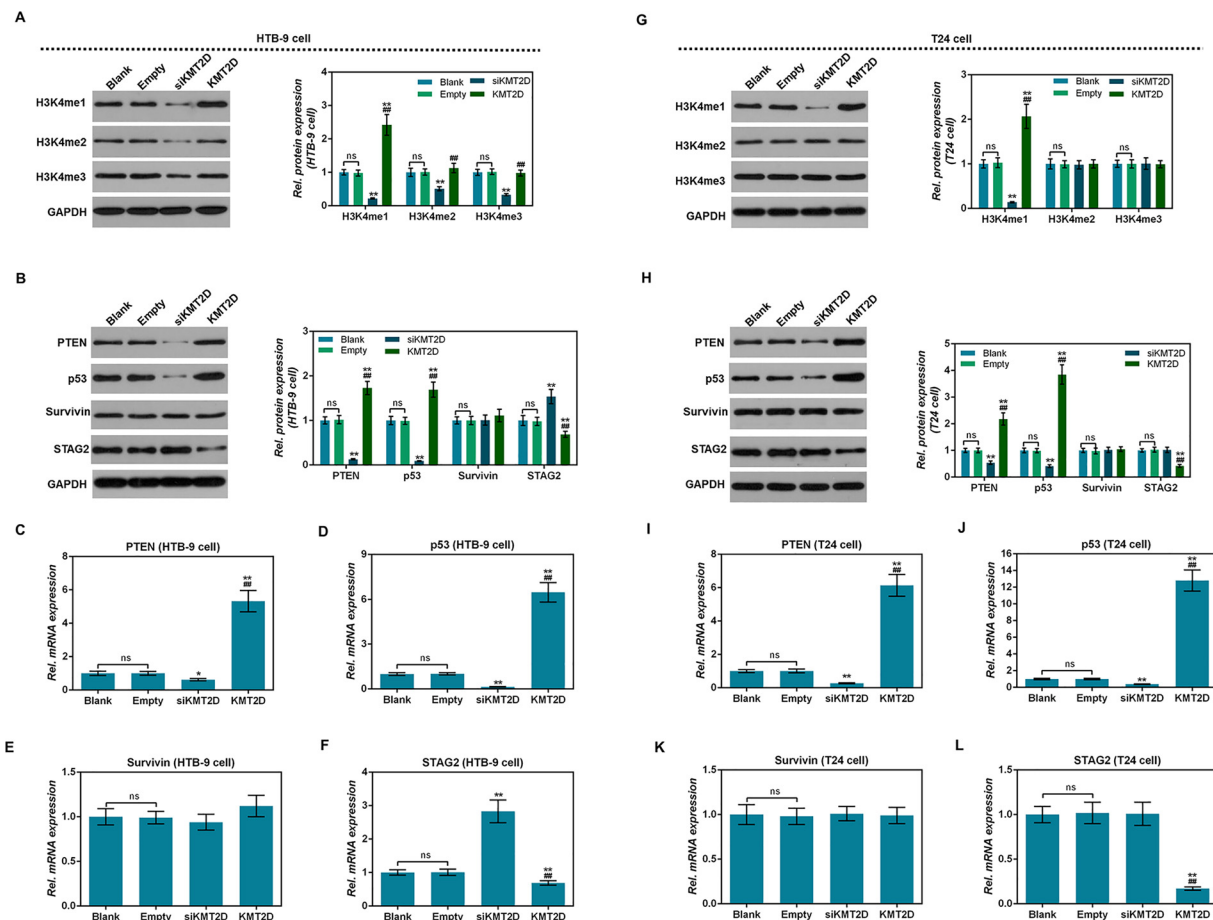It is currently believed that histone modification is no exception to

Fig. 5. The effects of KMT2D on the expressions of H3K4 methylation, oncogenes and anti-oncogenes in HTB-9 and T24 cells. Cells were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid). A Western blot was used to detect the effects of KMT2D on the protein expressions of H3K4me1, H3K4me2 and H3K4me3 in HTB-9 cells. B Western blot was used to detect the effects of KMT2D on the protein expressions of PTEN, p53, Survivin and STAG2 in HTB-9 cells. Quantitative real-time PCR was performed to assess the mRNA expressions of PTEN (C), p53 (D), Survivin (E) and STAG2 (F) in HTB-9 cells. G Western blot was used to detect the effects of KMT2D on the protein expressions of H3K4me1, H3K4me2 and H3K4me3 in T24 cells. H Western blot was used to detect the effects of KMT2D on the protein expressions of PTEN, p53, Survivin and STAG2 in T24 cells. Quantitative real-time PCR was performed to assess the mRNA expressions of PTEN (I), p53 (J), Survivin (K) and STAG2 (L) in T24 cells. GAPDH served as an internal control. Data were shown as mean $\pm$ SD from three independent experiments (Blank vers Empty = ns, [*]compared to ns, [#]compared to silencing KMT2D, [*/#]$P < 0.05$, [**/##]$P < 0.01$).

participate in almost every aspect of cancer development [55,56]. KMT2D also could catalyze lysine methylation. To our knowledge each methylation site can have a different degree of methylation. Similar in lysine which it can undergo monomethylation (me1), dimethylation (me2) and trimethylation (me3) [57]. Our current study provided a result that KMT2D principally regulated the expression of H3K4 monomethylation in bladder cancer cells. Specifically, silencing KMT2D inhibited the activity of H3K4me1 and over-expressing KMT2D boosted H3K4me1 expression. In line with some reports demonstrated that KMT2D played a vital role in sustaining the activity of global H3K4me1 in colon cancer cells and mouse embryonic stem cells [58–60]. Histone methylation is often thought to be implicated in transcriptional activation and inhibition of genes. Usually, H3K4me3 is located in the promoter region of the gene and is closely related to the active transcription of the gene [61]. H3K36me3 is considered as participating in transcription elongation for its SET2 keeping in RNA polymerase II elongation [62]. Naturally, several oncogenes and anti-oncogenes were detected to investigate the possible mechanism of KMT2D on regulating inhibition effects in bladder cancer cells. Our findings suggested that silencing KMT2D attenuated the anti-oncogenes PTEN and p53 proteins and mRNAs expressions. But on the other side, over-expressing KMT2D could maintain the levels of the two tumor suppressors. Survivin and STAG2, the tumor promoters, almost had no clear changes in

transfected with KMT2D bladder cancer cells. The possible reason for the slight differences in these two cell lines is the heterogeneity of bladder cancer cells [63]. Dhar reported that KMT2D knockdown was responsible for the development of medulloblastoma and provided a tumor-suppressive mechanism for establishment of H3K4me3 [64]. Relevant to our studies, KMT2D was necessary to keep the activity of H3K4me1, in further to maintain the effect of tumor suppressors, and ultimately inhibiting the growth and metastasis of bladder cancer cells.

In the future, an in-depth, comprehensive and advanced study is worth performing to make up for the limitations of the present experiment, for example, poor quality of figures in wound scratch assay.

5. Conclusion

Collectively, this work suggested an anti-tumor role for KMT2D *in vitro* and *in vivo*, as well as provided a possible tumor inhibition mechanism in which KMT2D enhanced H3K4me1 activity to support the expressions of tumor suppressors.
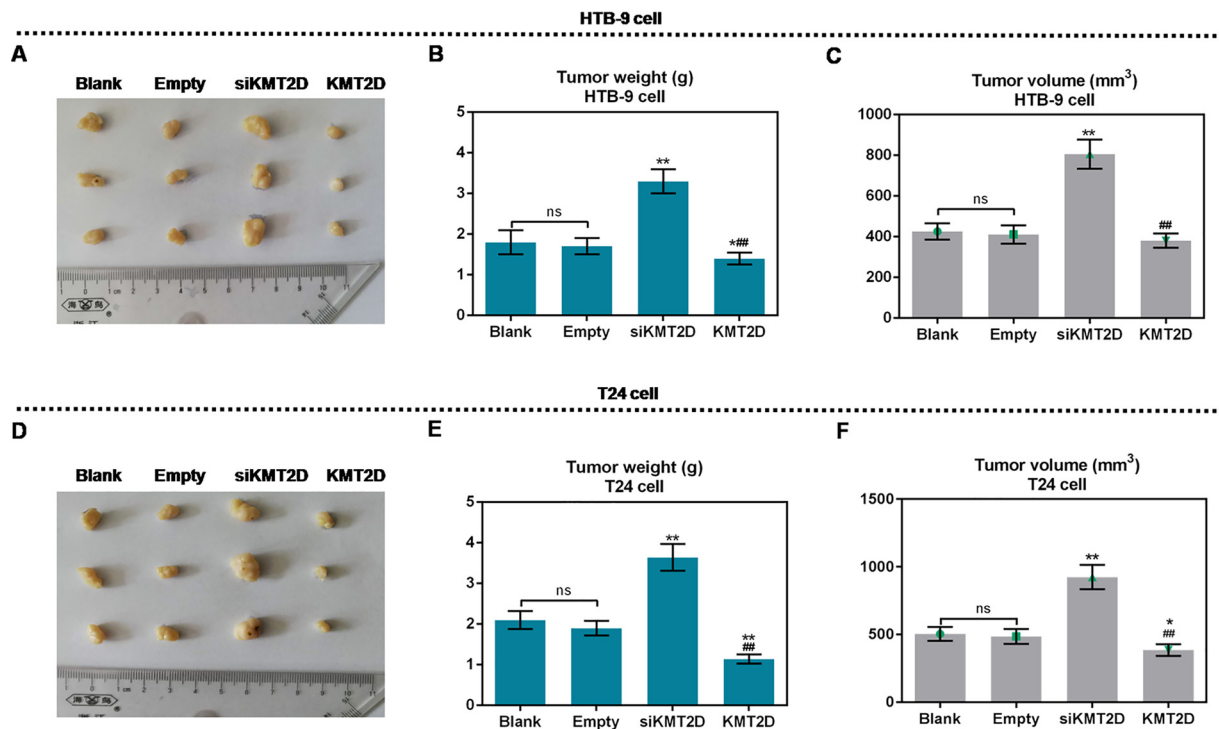
Funding

Fig. 6. The effects of KMT2D on the affecting the growth of solid bladder cancer *in vivo*. 24 SPF of BALB/c-nu mice (6 per group) was used in this research. The experiments were divided into four groups, including control, empty (transfected with empty plasmid), siKMT2D (transfected with silencing KMT2D plasmid) and KMT2D (transfected with over-expressing KMT2D plasmid). A Solid tumor formation after HTB-9 bladder cancer cells were transplanted in BALB/c-nu mice. Tumor weight (B) and volume (C) were determined 30 days after HTB-9 cell injection. D Solid tumor formation after T24 bladder cancer cells were transplanted in BALB/c-nu mice. Tumor weight (E) and volume (F) were determined 30 days after T24 cell injection. Data were shown as mean ± SD from three independent experiments (Blank vers Empty = ns, *compared to ns, #compared to silencing KMT2D, */#P < 0.05, **/##P < 0.01).

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Disclosure of interest

None.

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