Mettl14 inhibits bladder TIC self-renewal and bladder tumorigenesis through $N^6$-methyladenosine of Notch1

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Abstract
Background: $N^6$-methyladenosine (m$^6$A) emerges as one of the most important modification of RNA. Bladder cancer is a common cancer type in developed countries, and hundreds of thousands of bladder cancer patients die every year.

Materials and methods: There are various cells in bladder tumor bulk, and a small population cells defined as tumor initiating cells (TIC) have self-renewal and differentiation capacities. Bladder TICs drive bladder tumorigenesis and metastasis, and their activities are fine regulated. However, the role of $N^6$-methyladenosine in bladder TIC self-renewal is unknown.

Results: Here, we found a decrease of $N^6$-methyladenosine in bladder tumors and bladder TICs. $N^6$-methyladenosine levels are related to clinical severity and outcome. Mettl14 is lowly expressed in bladder cancer and bladder TICs. Mettl14 knockout promotes the proliferation, self-renewal, metastasis and tumor initiating capacity of bladder TICs, and Mettl14 overexpression exerts an opposite role. Mettl14 and m$^6$A modification participate in the RNA stability of Notch1 mRNA. Notch1 m$^6$A modification inhibits its RNA stability. Notch1 plays an essential role in bladder tumorigenesis and bladder TIC self-renewal.

Conclusion: This work reveals a novel role of Mettl14 and $N^6$-methyladenosine in bladder tumorigenesis and bladder TICs, adding new layers for bladder TIC regulation and $N^6$-methyladenosine function.

Keywords: $N^6$-methyladenosine, Bladder tumorigenesis, Bladder TIC, Notch1, Mettl14, Self-renewal

Introduction
Bladder cancer is a serious cancer in the world, especially in advanced countries [1]. There are many kinds of cells in bladder tumor, including bladder cancer stem cells (CSC), or tumor initiating cells (TIC) [2]. Bladder TICs, a small population of cells in bladder tumors, have self-renewal, differentiation and tumor-initiating capacities [2]. Recently, increasing markers of bladder TICs were identified, and CD44 is one of the most widely-accepted markers [3]. Compared with CD44− cells, CD44+ cells show enhanced self-renewal and tumor-initiating capacities. Bladder TICs escape anoikis and initiate oncospheres in FBS-free medium, but bladder non-TICs can't survive [4]. Accordingly, sphere formation assay emerges as one of the most important assays to detect bladder TIC self-renewal. Besides sphere formation, transwell invasion assay can also be used for bladder TICs because of the critical role of bladder TICs in bladder metastasis and invasion [2, 5]. Like TICs in many other tumors, bladder TICs harbor enhanced tumor-initiating capacities, which can be examined by gradient tumor initiating assay [6–8]. Highly expressing ABCG2 and other pump molecules, TICs are resistant to drug treatment [9]. Despite of the critical role of bladder TICs in bladder tumor formation, metastasis, drug resistance and recurrence, the biological characteristics of bladder TICs are largely unknown.

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Like TICs in other tumors, bladder TICs are fine regulated, and the precise regulations of bladder TIC self-renewal are still largely unknown [10, 11]. N6-methyadenosine (m6A) is the most abundant modification of mRNA in human and mice, and is very conserved among species [12]. m6A modification is identified in plants, yeast, insects, virus and so on. Recently, m6A modification was also found on some non-coding RNAs, including tRNA, rRNA, IncRNA and snRNA [13]. m6A modification is reversible, which is added by methyltransferase complex and removed by m6A demethylases. m6A methyltransferase complex is comprised of METTL3, METTL4, METTL14, WTAP, VIRMA and so on [14, 15]. On the contrary, FTO and AlkBH5 induce METTL3, METTL4, METTL14, WTAP, VIRMA and so on [16, 17]. The influence of m6A in RNA stability is dependent on m6A readers, including YTHDF1, YTHDF2, YTHDF3 and YTHDC1 [18]. m6A modification participates in many biological processes, including spermatogenesis and embryonic development [19–21], circadian period [22], DNA damage [23], hematopoietic stem cells [24] and tissue homeostasis [25]. As for tumor biology, m6A modification exerts its role in tumorigenesis, proliferation and metastasis. Mettl14 is lowly expressed in hematopoietic stem cells and liver tumor cells. m6A methyltransferase Mettl14 attenuates the tumorigenesis of AML [26]. Recently the inhibitory role of Mettl14 in liver tumorigenesis and metastasis was also revealed. Demethylase FTO drives tumorigenesis of acute myeloid leukemia [27]. Another demethylase, ALKBH5 also exerts an oncogenic role in glioblastoma tumorigenesis [11]. However, the roles of N6-methyadenosine and related enzymes in bladder tumorigenesis and bladder TIC self-renewal are largely unknown.

Here, we found that m6A modification and Mettl14 were lowly expressed in bladder tumorigenesis and bladder TICs. m6A level and Mettl14 expression were negatively related to the bladder cancer severity and clinical outcome. m6A modification and Mettl14 inhibited bladder tumorigenesis and bladder TIC self-renewal through Notch1 signaling, adding a new layer of bladder TIC regulation and m6A function.

Materials and methods

Reagents and samples

Anti-β-actin (cat. no. A1978) and DAPI (cat. no. 28718–90-3) were obtained from Sigma-Aldrich. Anti-m6A antibody was purchased from Synaptic Systems. Anti-Mettl14 (ab220030) antibody and m6A quantification kit (ab185912) were from Abcam. Fluorescence-conjugated secondary antibodies were obtained from Molecular Probes Life Technologies.

Primary bladder cancer samples were obtained from the Departments of Urology and Henan Institute of Urology, The First Affiliated Hospital of Zhengzhou University with informed consent. The details for bladder tumors used in this work were: #1, early bladder cancer, 63 years old, female, tumor size, 23.1 × 17.3 × 16.2 mm, non-invasive, stage I, non-metastasis. #2, advanced bladder cancer, 72 years old, male, tumor size, 38.3 × 21.8 × 19.9 mm, invasive, stage IV, metastasis. #3, advanced bladder cancer, 69 years old, female, tumor size, 35.5 × 26.8 × 21.9 mm, invasive, stage III, non-metastasis. #4, early bladder cancer, 78 years old, male, tumor size, 29.2 × 18.9 × 17.2 mm, non-invasive, stage II, non-metastasis. #5, advanced bladder cancer, 73 years old, male, tumor size, 31.3 × 28.8 × 28.1 mm, non-invasive, stage II, non-metastasis. #6. advanced bladder cancer, 73 years old, male, tumor size, 31.9 × 29.1 × 19.8 mm, non-invasive, stage II, non-metastasis.

Primary cell isolation and culture

Primary bladder cancer cells were obtained from bladder cancer patients. For primary cell isolation, a portion of excised tumor was incubated in Hanks balanced salt solution (HBSS; Gibco) and transported quickly to the laboratory on ice. Then the samples were cut into small fragments, and digested in HBSS containing 0.1% type I collagenase, 0.05% type IV collagenase, 0.03% pronase, and 0.01% deoxyribonuclease at 37 °C for 30 min. The sample was filtered through 70-μm-nylon filter and centrifuged for 4 min at 50 x g in 4 °C. Bladder cancer primary cells were in precipitation, and cell survival & purification were examined. For bladder TIC enrichment, bladder cancer primary cells were incubated with CD44 antibody for FACS, and TICs were used for sphere formation assay, m6A detection and other assays.

Sphere formation

Sphere formation assay was detected to examine the self-renewal of bladder TICs. 500 CD44+ bladder TICs were cultured in FBS-free DMEM/F12 medium (supplemented with 1 × B27 supplement, 1 × N2 supplement, 20 ng/ml bFGF and 20 ng/ml EGF) and seeded into Corning® Costar® Ultra-Low Attachment Multiple Well Plate (cat. no. 3471, Corning). Two weeks later, sphere images were taken and sphere numbers were counted. For cell line detection, 1000 T24 cells were used for sphere formation.

CRISPR/Cas9 knockout

Mettl14 and Notch1 knockout cells were constructed through CRISPR/Cas9 approach. For knockout, sgRNAs were designed according to online tool (http://crispr.mit.edu/) and cloned into lentICRISPRv2, which were transfected into 293 T cells for lentivirus package, and the lentivirus was concentrated with PEG-it Virus Precipitation Solution (System Biosciences). Bladder cancer
primary cells were infected with lentivirus, and the transfected cells were collected by puromycin selection. Knockout efficiency was confirmed by Western blot, and then used for sphere formation assay, transwell invasion assay or other functional assays.

Immunohistochemistry
For immunohistochemistry, 5-μm bladder cancer sections were treated with xylene (10 min × 2), 100% alcohol (5 min × 2), 95% alcohol (5 min), 75% alcohol (5 min), PBS (5 min), 3% H2O2 (20 min) and then boiled in Tris/EDTA buffer (PH 9.0) for antigen retrieval (20 min). Then the samples were incubated with anti-m6A (1:500 dilution in PBS) or anti-Mettl14 (1:500 dilution in PBS) antibodies for 2 h. After washing three times, HRP-conjugated secondary (1:500 dilution in PBS) and 3,3′-diaminobenzidine were used for visualization.

Dot blot
For m6A dot blot, RNA were extracted from bladder tumor, TICs and spheres using standard Trizol method, and then spotted onto nylon membrane. The samples were crosslink with UV treatment, and followed by m6A antibody incubation (1:2000 dilution in PBS, supplemented with 5% milk) and subsequent HRP-conjugated secondary antibody (1:5000 dilution in PBS, supplemented with 5% milk), finally the samples were detected with 3,3′-diaminobenzidine. For loading control, 0.02% methylene blue was used to stain the same RNA samples.

FACS
For FACS sorting or detection, samples were incubated with Phycoerythrin (PE)-conjugated CD133 (1:300 dilution in FACS buffer) or control antibodies (1:300 dilution in FACS buffer) for 30 min on ice, and then subjected to FACS. For FACS sorting, CD133+ bladder TICs and CD133− non-TICs were enriched. For detection, FlowJo software (FlowJo v10) was used for data analyses.

Statistical methods
For bladder TIC ratio analysis, 10, 1 × 10^2, 1 × 10^3, 1 × 10^4 and 1 × 10^5 cells were injected into BALB/c nude mice for three months’ tumor formation. The ratios of bladder TICs were calculated by ELDA (extreme limiting dilution analysis) with online software (http://bioinf.wehi.edu.au/software/elda/). For most experiments, two tailed unpaired Student’s t-test was used for statistical analysis.

Results
Decreased content of m6A modification in bladder cancer
As the most widely distributed RNA modification in mammalian cells, m6A modification exerts critical roles in many biological processes. However, its role in bladder tumorigenesis and bladder TICs is unknown. In this work, we focused on the role of m6A modification in bladder tumorigenesis and bladder TICs, and we detected the content of m6A modification in bladder tumor first.

m6A modification was detected in non-tumor, early and advanced bladder tumors, and decreased m6A content was observed along with bladder tumorigenesis. Moreover, m6A modification was related to clinical severity (Fig. 1a). The reduction of m6A modification in bladder cancer was also validated by RNA dot blot (Fig. 1b), immunohistochemistry (Fig. 1c) and bladder cancer tissue array (Fig. 1d, e). As expected, lower content of m6A modification was also detected in advanced tumors by bladder cancer tissue array and immunohistochemistry (Fig. 1e). Moreover, m6A modification was also related to the clinical outcome of bladder tumor patients (Fig. 1f). Taken together, m6A modification content was lower in bladder tumors and related to clinical severity.

Decreased m6A modification in bladder TICs
To further examine m6A modification in bladder TICs, we enriched CD44+ bladder TICs and CD44− non-TICs, and detected m6A modification. Compared with non-TICs, lower m6A modification levels were found in bladder TICs (Fig. 2a), which was confirmed by RNA dot blot and immunofluorescence (Fig. 2b, c).

TICs can survive in sphere formation medium and sphere formation assay emerges as a standard method to enrich bladder TICs. Accordingly, sphere formation assay was performed and spheres were collected to detect m6A modification. Compared with non-spheres, lower m6A modification levels were found in oncospheres (Fig. 2d). RNA dot blot and immunofluorescence also confirmed the decreased content of m6A modification in bladder cancer spheres (Fig. 2e, f). Meanwhile, lower m6A modification was also observed in T24 spheres (Fig. 2g). Taken together, m6A modification was decreased in bladder TICs.

Mettl14 was lowly expressed in bladder TICs and accounted for m6A suppression
Considering the importance of methyltransferases, demethylases and m6A readers in m6A modification, we then detected the expression profiles of m6A-related genes. Among these genes, Mettl14 was lowly expressed in bladder cancer, especially in advanced bladder cancer samples (Fig. 3a). What’s more, lower expression of Mettl14 was detected in bladder TICs and spheres (Fig. 3b). To further examine Mettl14 expression pattern, bladder cancer tissue array was performed and confirmed the lowly expression of Mettl14 in bladder cancer (Fig. 3c). Interestingly, Mettl14 was also lowly expressed...
in advanced bladder tumors and related to the prognosis of bladder tumor patients (Fig. 3d, e).

To further confirm the role of Mettl14 in m^6^A modification, we generated Mettl14 knockout cells through CRISPR/Cas9 approach (Fig. 3f). Mettl14 knockout led to decreased content of m^6^A modification, indicating the critical role of Mettl14 in m^6^A modification (Fig. 3g, h). Moreover, a positive correlation of Mettl14 expression and m^6^A content was observed in bladder tumors (Fig. 3i). What’s more, in vitro RNA N^6^-adenosine methylation assay also confirmed the activity of Mettl14 in RNA m^6^A modification (Fig. 3j). Altogether, Mettl14 was lowly expressed in bladder cancer and accounted for the decreased content of m^6^A modification.

**Mettl14 knockout drove bladder TIC self-renewal**

To further explore the role of Mettl14 in bladder tumorigenesis and bladder TICs, we utilized Mettl14 knockout cells to perform sphere formation assay. Mettl14 knockout cells showed increased capacity of sphere formation, indicating the inhibitory role of Mettl14 in bladder TIC self-renewal (Fig. 4a). Moreover, Mettl14 knockout cells contained increased bladder TICs, which confirmed the inhibitory role of Mettl14 in bladder TIC maintenance (Fig. 4b). Cell proliferation was detected by Ki67 staining, and Mettl14 knockout cells showed enhanced proliferation capacity (Fig. 4c). Bladder TICs account for bladder tumor invasion and metastasis, and thus tumor invasion was also detected. Mettl14 knockout cells have enhanced invasion capacity, revealing the inhibition of Mettl14 in bladder tumor invasion (Fig. 4d, e). We then detected tumor propagation of Mettl14 knockout cells through in vivo tumor propagation assay. Increased tumor propagation was found in Mettl14 knockout cells (Fig. 4f).

To further explore the role of Mettl14 in tumor initiation, Mettl14 knockout cells were used for tumor initiation assay in vivo. Gradient Mettl14 knockout cells
were injected into BALB/c nude mice for tumor initiation. Mettl14 deleted cells showed enhanced tumor formation ability (Fig. 4g). Consist with tumor initiation, higher ratios of bladder TICs were also detected in Mettl14 knockout cells (Fig. 4h). Meanwhile, Mettl14 deleted T24 cells showed enhanced oncosphere formation capacity (Fig. 4i), enhanced invasion capacity (Fig. 4j) and tumor propagation ability (Fig. 4k). Altogether, Mettl14 knockout promoted the self-renewal of bladder TICs.

**Mettl14 overexpression inhibited TIC self-renewal**

We then overexpressed Mettl14 in bladder TICs and examined the self-renewal. Mettl14 overexpressing cells were generated through lentivirus and confirmed by Western blot (Fig. 5a). Mettl14 overexpression led to a decrease in sphere formation (Fig. 5b), cell proliferation (Fig. 5c) and TIC ratios (Fig. 5d). Meanwhile, Mettl14 overexpressing cells showed impaired role in tumor invasion (Fig. 5e, f) and propagation (Fig. 5g).

Mettl14 overexpressing cells were also used for tumor-initiating assay, and impaired tumor initiation was observed (Fig. 5h). Decreased ratios of bladder TICs were also calculated through extreme limiting dilution analysis (Fig. 5i). Meanwhile, the inhibitory role of Mettl4 in bladder cancer sphere formation, invasion and tumor propagation was also confirmed in T24 cells (Fig. 5j-l). Altogether, Mettl14 overexpression blocked the self-renewal of bladder TICs.

**Mettl14 targeted Notch1 mRNA stability in bladder TICs**

Finally we explored the functional target genes in bladder TICs. We focused on the target genes of three major pathways, including Wnt/β-catenin, Notch and Hedgehog pathways, and found Notch1 was highly expressed in Mettl14 knockout cells, and lowly expressed in Mettl14 overexpressing cells (Fig. 6a). The inhibitory role of Mettl14 in Notch1 expression was confirmed by Western blot (Fig. 6b, c). Considering the role of m^6^A modification in mRNA stability [18, 19], we examined the stability of Notch1 mRNA in Mettl14 knockout cells. Interestingly, enhanced stability of Notch1 was observed upon Mettl14 knockout (Fig. 6d). Moreover, a negative correlation of Mettl14 expression and Notch1 expression was observed in bladder tumors (Fig. 6e).

Although the role of Notch signaling in TICs is well-known, its role in bladder TICs remains elusive. To explore the role of Notch1 in bladder TICs and Mettl14 function, we generated Notch1 knockout cells through CRISPR/Cas9 approach (Fig. 6f). Compared with control cells, Notch1 knockout cells showed impaired sphere formation and invasion capacities, revealing the essential role of Notch1 in bladder TICs (Fig. 6g-j). More importantly, in Notch1 knockout cell, Mettl14 knockout showed impaired role for sphere formation and metastasis, indicating the critical role of Notch1 in Mettl14 function (Fig. 6g-j). The essential role of Notch1 in Mettl14 function was confirmed in T24 bladder cancer cell line (Fig. 6k). Altogether, Mettl14 targeted Notch1 that was essential for bladder TICs.

**Discussion**

As the most abundant modification in human mRNA, m^6^A modification participates in many physiological and pathological processes [28]. However, its role in bladder tumorigenesis and bladder TICs is unknown. In this work, we discovered the low content of m^6^A modification in bladder tumorigenesis and bladder TICs (shown in Figs. 1 and 2), and identified the role of m^6^A modification and Mettl14 through various functional assays, including sphere formation, transwell invasion assay, gradient tumor initiation assay, tumor propagation assay and Ki67 staining (shown in Figs. 4 and 5). Our work defined Mettl14 as a tumor suppressor gene in bladder and a negative modulator in bladder TICs (shown in Figs. 3, 4 and 5).

The self-renewal of bladder TIC is precisely regulated, and the regulation mechanism is largely unknown. Here, we revealed a novel regulatory axis of bladder TICs. Mettl14 knockout drove the self-renewal of bladder TICs (shown in Fig. 4), and Mettl14 overexpression inhibited bladder TIC self-renewal (shown in Fig. 5). Mettl14 largely attenuated Notch1 expression, and participated in bladder TICs through Notch1 (shown in Fig. 6). Mettl14-m^6^A-Notch1 pathway plays a critical role in bladder tumorigenesis and bladder TICs.
Fig. 3 Down-expression of Mettl14 in bladder TICs drove decreased m^6^A modification. a Realtime PCR was performed to detect the expression of m^6^A-associated genes in 20 peri-tumors, 20 eBC and 20 aBC samples. 10 bladder cancer samples were used for TIC enrichment and sphere formation, followed by realtime PCR analyses for the expression of indicated genes. c Immunohistochemistry for Mettl14 expression in tissue array containing 46 peri-tumors, 20 stage I, 27 stage II and 12 stage III bladder tumors. Typical images were shown in C and quantitative results of m^6^A modification were shown as scatter diagram (d). e Bladder cancer patients were grouped into Mettl14^high^ and Mettl14^low^ groups, followed by Kaplan–Meier survival analysis. f Mettl14 knockout bladder cancer cells were generated through CRISPR/Cas9 strategy, and knockout efficiency was confirmed by Western blot. g The indicated Mettl14 knockout cells were used for m^6^A detection. h m^6^A content in Mettl14 knockout cells was detected by RNA dot blot. i Correlation of m^6^A modification content and Mettl14 expression. The intensity of m^6^A and Mettl14 was used for Pearson correlation coefficient (R) and P-value analyses. j The in vitro RNA N^6^-adenosine methylation activities of Flag-tagged METTL14 were tested using different RNA probes. *P < 0.05, **P < 0.01, ***P < 0.001, by two-tailed t test.
Fig. 4 Mettl14 knockout promoted bladder TIC self-renewal. 

**a** Mettl14 knockout cells were used for oncosphere formation. Two weeks later, typical images of spheres were taken and sphere-formation ratios were quantitated. **b** The ratios of CD44⁺ bladder TICs in Mettl14 knockout cells were analyzed by FACS. **c** Mettl14 knockout and control cells were used for Ki67 staining, and counterstained with DAPI. Typical images were shown in upper panel and Ki67⁺ cell ratios were shown in lower panel. **d** Mettl14 knockout and control cells were used for transwell invasion assay. 36 h later, invasive cells were stained with crystal violet for visualization. Typical images were shown in D and cell numbers were counted in e. **f** 5×10⁵ Mettl14 knockout cells were subcutaneously injected into BALB/c nude mice, and tumor weight was measured one month later. n = 6 for each sample. **g** 10, 1×10⁴, 1×10⁵, and 1×10⁶ Mettl14 knockout and control cells were used for 3 months’ tumor initiation assay. Six BALB/c nude mice were used per sample and the ratios of tumor formation mice were shown. **h** Bladder TIC ratios were analyzed by extreme limiting dilution analysis. **i** Mettl14 knockout T24 cells were used for oncosphere formation, and typical images of spheres were taken two weeks later. **j** Mettl14 knockout T24 cells were used for transwell invasion assay. 36 h later, invasive cells were stained with crystal violet for visualization. **k** Mettl14 knockout T24 cells were used for tumor propagation assay, and the picture of established tumors was taken. *P < 0.05, **P < 0.01, ***P < 0.001, by two-tailed T test. At least three independent experiments were performed and got similar results.
Wnt/β-catenin, Notch and Hedgehog pathways were the most important signaling pathways in TICs of various tumors [29–32]. We detected the related genes of Wnt/β-catenin, Notch and Hedgehog signaling, and identified Notch1 was a functional target gene of m6A modification and Mettl14 (Fig. 6a). Then we generated Notch1 knockout cell and re-evaluated the role of Mettl14. Impaired Mettl14 role was found upon Notch1 knockout, indicating Mettl14 mainly target Notch1 to inhibit bladder TIC (Fig. 6). As a common enzyme, there must be many target genes of Mettl14, but here we defined Notch1 was the functional target gene through loss of function assay.

Fig. 5 Mettl14 overexpression inhibited bladder TIC self-renewal. a Mettl14 overexpressing cells were generated by lentivirus and confirmed by Western blot. oe, overexpression. b Mettl14 overexpressing cells were used for oncosphere formation assay. Spheres were shown in left panel and sphere-initiating ratios were shown in right panel. c Ki67 immunohistochemistry was performed using Mettl14 overexpressing and control cells. Typical images derived from patient #1 were shown. d Mettl14 overexpressing cells were used to detect CD44 bladder TICs by FACS. The ratios of bladder TICs were shown. e Mettl14 overexpressing cells were used for transwell invasion assay. Typical images derived from patient #1 and invasive cell numbers were shown in E and F, respectively. g 5×10⁶ Mettl14 overexpressing cells were utilized for tumor propagation and tumor weight was detected one month later. h, i 1×10², 1×10³, 1×10⁴ and 1×10⁵ Mettl14 overexpressing cells were used for tumor initiation assay. Tumor formation ratios and bladder TIC ratios were shown in H and I, respectively. Six patients were all examined for tumor initiation assay, and only the results of patient #1 were shown. j, k Mettl14 overexpressing T24 cells were established, and used for sphere formation assay (j) and transwell invasion assay (k). l Mettl14 overexpressing T24 cells were used for tumor propagation assay, and the picture of established tumors was taken.
Fig. 6 (See legend on next page.)
We think some other target genes may also involve in other biological processes, but in bladder tumorigenesis and bladder TIC self-renewal, Mettl14 exerted its role mainly through Notch1-dependent manner.

TICs were regulated by various signaling pathways, including Wnt/β-catenin, Notch and Hedgehog pathways. Increasing works revealed the critical role of Wnt/β-catenin and Hedgehog pathway in bladder tumorigenesis and bladder TIC self-renewal. However, the role of Notch signaling in bladder tumorigenesis and TICs remains elusive. Here we generated Notch1 knockout cells and revealed an impaired activity of bladder TICs (shown in Fig. 6). Our work defined the oncogenic role of Notch1 in bladder cancer.

The mRNA modification is a complicated process, with various modifications for various RNA molecules. Besides m6A, N1-methyladenosine, m5C and pseudouridine also emerge as critical modulators in various biological processes [33, 34]. m6A modification has been identified on mRNA and some non-coding RNA, including microRNA, lncRNA and snoRNA [35]. Increasing evidences reveal that lncRNA emerges as critical modulator in the self-renewal of many kinds of TICs [36–38]. The role of m6A and other modifications in non-coding RNAs also need to be further investigated.

Conclusion
Bladder TICs drive bladder tumorigenesis and metastasis, and their regulation remains largely unknown. Here we revealed the role of N6-methyladenosine in bladder TIC self-renewal, adding new layers of bladder TIC regulation and N6-methyladenosine function. As a novel modulator of TICs, Mettl14-N6-methyladenosine-Notch1 pathway may be a potential target for bladder TIC elimination.

Abbreviations
CSC: Cancer stem cells; m6A: N6-methyladenosine; TIC: Tumor initiating cells

Acknowledgments
Not Applicable.
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