Downregulation of MTHFD2 Inhibits the Progression of Bladder Cancer Through PI3K/AKT Signaling Pathways

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Research

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Abstract

Background: Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is related to the pathogenesis of many human malignant tumors, but its role in bladder cancer remains poorly understood. We aimed to determine the effect of downregulation of MTHFD2 on the progression of bladder cancer. First, the relationship between MTHFD2 expression and survival time in patients with bladder cancer was analyzed by GEPIA and the UALCAN online database. The expression of MTHFD2 in bladder cancer and adjacent tissues was detected by reverse transcription-quantitative PCR (RT-PCR), Western blot (WB), and tissue microarray. Second, the effects of low expression of MTHFD2 on the proliferation of bladder cancer cell lines were evaluated by CCK-8, Transwell, cell wound scratch, cell cloning, and flow cytometry assays. In vivo, the effect of MTHFD2 silencing on tumorigenicity was determined in nude mice. Furthermore, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway was confirmed by western blotting after RNA sequencing (RNA-seq).

Results: The expression of MTHFD2 in bladder cancer tissues was significantly higher and positively correlated with tumor stage and negatively correlated with overall survival. The expression of MTHFD2 in bladder cancer lines was significantly higher and the proliferation, migration, and clone formation ability of bladder cancer cells with low expression of MTHFD2 were significantly decreased in vitro and in vivo. RNA-seq showed that the differential genes were enriched in the PI3K/Akt signaling pathway. WB revealed that the expression of PI3K/AKT protein was downregulated.

Conclusions: Our findings indicated that downregulation of MTHFD2 can reduce the progression of bladder cancer through inhibited PI3K-AKT signal pathway and may be provided a new approach for the diagnosis and treatment of bladder cancer.

Background

Bladder cancer is the most common malignant tumor in the genitourinary system and is seriously harmful to human health. According to statistical analysis, there were 549393 new cases of bladder cancer in 2018, accounting for 3.0% of all new cancer cases; the number of deaths was as high as 199922, accounting for 2.1% of total global cancer deaths[1]. At present, the therapeutic effect of non-muscular invasive bladder cancer is ideal, but there is still a lack of effective treatment methods and predictive indicators for advanced bladder cancer[2], resulting in a poor prognosis of patients[3, 4]. Therefore, more in-depth studies of new therapeutic targets and biological indicators are needed to judge the prognosis and improve the therapeutic effect of advanced bladder cancer.

Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is one of the key enzymes in folate metabolism. In 1985, it was confirmed by Mejia and MacKenzie that it has the double function of both methylenetetrahydrofolate dehydrogenase and cyclohydrolase, which play an important role in maintaining cell growth and viability[5]. At present, an increasing number of studies have shown that MTHFD2 is overexpressed in many kinds of tumor tissues, and its expression level is negatively

correlated with the prognosis of tumor patients[6, 7]; MTHFD2 can promote the proliferation and invasion of tumor cells[8] and then promote the occurrence and progression of tumors[9, 10]. However, there are few reports concerning the effect of MTHFD2 on the progression and prognosis of bladder cancer[11]. The purpose of this study was to investigate the occurrence and progression of bladder cancer in vivo and in vitro and judge its prognosis to provide new ideas for bladder cancer treatment and identify potential drug targets.

Results

Bioinformatics results

Through GEPIA database analysis, the MTHFD2 gene was differentially expressed in 31 kinds of tumors and adjacent tissues and was highly expressed in most tumors (Figure 1A). A UALCAN database search revealed that the mRNA expression of MTHFD2 in bladder cancer was significantly higher than that in adjacent normal tissues. In different pathological stages, there was a significant difference between stage II/III/IV and normal tissues, but no differences were observed between stage I and normal tissue. Compared with no lymph node metastasis, MTHFD2 was highly expressed in tumor tissues with lymph node metastasis (Figure 1B). It was confirmed in the GEPIA database that there was a significant negative correlation between the survival rate and high expression of MTHFD2 (Figure 1C).

MTHFD2 was highly expressed in cancer tissues

To understand the difference in MTHFD2 expression between bladder cancer tissues and adjacent tissues, mRNA expression in 24 pairs of cancer tissues and adjacent tissues was detected by RT-PCR assays (Figure 2A), and MTHFD2 protein expression in 6 pairs of high-grade bladder cancer and adjacent tissues was detected by WB (Figure 2B). The results showed that the expression of MTHFD2 in cancer tissues was significantly higher than that in adjacent tissues, and the difference was statistically significant ($P < 0.05$). Additionally, the results of the tissue microarray, which included 63 cases of bladder cancer and 16 cases of adjacent tissues, also showed similar differences between tumor and normal tissues (Supplementary Table S1). Forty-two cases (68.8%) of cancer tissues showed high expression, while only 3 cases (23.1%) of adjacent tissues showed high expression, and the difference was statistically significant ($P < 0.0001$), as shown in Figure 2C.

MTHFD2 is highly expressed in bladder cancer cell lines

To assess the roles of MTHFD2 in bladder cancer cells, the mRNA expression of MTHFD2 in SV-HUC-1 and bladder cancer cell lines (UM-UC-3, T24, J82, EJ, BIU, 5637) was detected by RT-PCR assays. As shown in Figure 3A, the expression of MTHFD2 in bladder cancer cells was higher than that of SV-HUC-1. These results suggested that MTHFD2 is highly expressed in bladder cancer cell lines. According to the expression of MTHFD2 in different bladder cancer cell lines, we selected T24 and UM-UC-3 cells for subsequent experiments to verify the effect of low expression of MTHFD2 on the phenotype of bladder cancer cells.

Effect of low expression of MTHFD2 on the proliferation and migration of bladder cancer cells

To explore the effect of MTHFD2 on the proliferation and migration of bladder cancer, we stably knocked out the MTHFD2 gene in bladder cancer cells (T24 and UM-UC-3). After viral transfection, puromycin was used for screening. Through RT-PCR and WB assays, it was indicated that the knockdown effect of T24-shRNA2 and T24-shRNA3 was better than that of T24-shRNA1. Therefore, T24-shRNA2 and T24-shRNA3 were used for further studies. The RT-PCR and WB results showed that the expression of MTHFD2 was significantly decreased in T24 and UM-UC-3 cells after viral transfection (Figure 3B,3C). Then, we performed a CCK-8 assay to detect the effects of MTHFD2 silencing on the proliferation of T24 and UM-UC-3 cells. As shown in Figure 4A, the experimental results showed that the proliferation ability of T24-shRNA2 and UM-UC-3-shRNA2 cells was significantly decreased, and the difference was statistically significant ($P < 0.05$). At the same time, silencing MTHFD2 significantly decreased the migration ability of bladder cancer cells (Figure 4B) and reduced cell colony formation (Figure 4C), but Transwell assays confirmed that there was no significant difference in invasive ability. This is consistent with the effect on tumor proliferation and migration after knocking down MTHFD2 in colon cancer and lung cancer [8, 9]. The above results confirmed that the low expression of MTHFD2 decreased the proliferation, migration, and colony formation of bladder cancer cells but had no significant effect on invasion.

Low expression of MTHFD2 promotes cell apoptosis

To further clarify the underlying mechanism of MTHFD2 in bladder cancer proliferation, the effects on the cell cycle and apoptosis after silencing MTHFD2 were determined by flow cytometry. The expression of 7-ADD and Annexin V-APC in bladder cancer cells was measured, and the results suggested that the apoptosis of cells with low expression of MTHFD2 was higher than that of the control group (Figure 5A). Through PI detection of the cell cycle, it was found that the number of cells in G1 phase increased in the shMTHFD2-R2 group, which made the cells stagnant in G0/G1 phase, but there was no significant difference between the two groups (Figure 5B). This is consistent with the effect of MTHFD2 on the cycle of colon cancer cells[8]. These results suggested that low expression of MTHFD2 promotes the apoptosis of bladder cancer cells.

Knockdown of MTHFD2 inhibited bladder tumor growth in vivo

To further evaluate the effect of MTHFD2 silencing on tumorigenicity *in vivo*, T24 cells transfected with shMTHFD2-RNA2 were injected subcutaneously into nude mice. The volume and weight of xenografts in the MTHFD2 silenced group were less than those in the control group ($p < 0.05$) (Figure 6). This finding is consistent with the inhibitory effect of MTHFD2 on the tumorigenesis of non-small cell lung cancer (NSCLC) in nude mice[9]. These results suggested that low expression of MTHFD2 can inhibit the proliferation of bladder cancer in vivo.

The PI3K/AKT pathway is downregulated in bladder cancer T24 cell lines with low expression of MTHFD2

To further study the mechanism of MTHFD2 promoting the proliferation of BC cells, RNA-seq was used to treat 3 cases of T24-shRNA2 and 3 cases of T24-Control. A total of 367 differentially expressed genes were up-regulated and 120 differentially expressed genes were down-regulated (Figure 7A). GO analysis of differentially expressed genes showed that bladder cancer cells with low expression of MTHFD2 were enriched in apoptotic cell clearance, cell adhesion, MAP kinase activation, intracellular signal transduction, and other functions (Figure 7B). KEGG signal pathway enrichment analysis showed that many signal pathways were enriched in bladder cancer cell lines with low expression of MTHFD2, such as rheumatoid arthritis, TNF signal pathway, NF-KB, PI3K/AKT, and cGMP-PKG pathway (Figure 7C). PI3K/AKT signal pathway is involved in the occurrence and development of many kinds of human tumors. This pathway regulates the proliferation and survival of tumor cells, and its abnormal activity can not only lead to malignant transformation but also be related to tumor cell migration, adhesion, tumor angiogenesis, and extracellular matrix degradation. However, the correlation between MTHFD2 and PI3K/AKT signal pathway in BC has not been reported.

Downregulation of MTHFD2 inhibits the progression of bladder cancer through inactive PI3K/AKT signaling pathways

To further confirm the mechanism of MTHFD2 affecting the proliferation of BC cells, the PI3K, AKT protein levels were detected by WB analysis. We observed that the protein levels of p-PI3K/PI3K and p-AKT/AKT decreased significantly after silencing MTHFD2 in T24 cells (Figure 7D). Interestingly, when T24 cells silencing MTHFD2 were treated with PI3K agonists 740Y-P (90ug/ml), the protein levels of p-PI3K/PI3K and p-AKT/AKT were increased, but there were no significant differences in total PI3K and AKT protein levels (Figure 7E). Therefore, the low expression of MTHFD2 suppresses the PI3K/AKT signaling pathway and reduces the proliferation ability of bladder cancer cells.

Discussion

In recent years, the incidence and mortality of bladder cancer have increased annually, which is extremely harmful to human health. Although the treatment and prognosis of non-muscular invasive bladder cancer are ideal[12], the recurrence rate is high and patients will progress to high-grade muscular invasive bladder cancer. Although radical cystectomy or radiotherapy and chemotherapy can improve the therapeutic effect of advanced bladder cancer, the 5-year survival rate is less than 50%. In the case of distant metastasis, the 5-year survival rate is reduced to approximately 10.2%[13], while the mortality rate has not improved[14]. This may be related to the lack of specific therapeutic targets for bladder cancer[15]. Therefore, important research directions in the field of urinary cancer include exploring the molecular mechanism of the occurrence, progression, and prognosis of bladder cancer, finding new

targets for the treatment of bladder cancer, and searching for personalized and accurate treatment methods for bladder cancer[16]. This study confirmed that low expression of MTHFD2 leads to the delayed progression of bladder cancer, which may be used as an independent risk factor to judge the prognosis of bladder cancer and provide a new therapeutic target for bladder cancer.

Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) plays an important role in maintaining cell growth and activity, including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis and methylation, amino acid metabolism, and vitamin metabolism. Changes in tumor cell metabolism are an important mechanism by which cancer cells maintain continuous growth. As one of the key enzymes involved in folate metabolism, MTHFD2 plays a key role in metabolic reprogramming, growth, proliferation, and progression of many kinds of tumors. Through bioinformatics analysis, we found that there was a significant difference in the expression of the MTHFD2 gene between bladder cancer and adjacent tissues, and the expression of the MTHFD2 was closely related to the clinicopathological stage and prognosis of bladder cancer. This analysis also confirmed that the expression of mRNA and protein in bladder cancer was significantly higher than that in adjacent tissues, which was consistent with the high expression of MTHFD2 in 41.12% of breast cancer tissues found by Liu et al. MTHFD2 may be an independent risk factor to guide the staging and prognosis of patients with bladder cancer. This is consistent with a study showing that MTHFD2 can be used as an independent biomarker to predict the prognosis of esophageal cancer, renal cell carcinoma, and liver cancer[17–19].

There are great differences in the role of MTHFD2 in different tumor tissues; for example, breast cancer cells with MTHFD2 knockout show impaired migration and invasion and a reduction in the number of tumor stem cells but did not show an effect on proliferation and apoptosis[20]. Chan et al.[21] found that MTHFD2 can regulate the redox homeostasis and metabolic rearrangement of lung cancer cells, inhibit the vitality, transformation, and self-renewal ability of lung cancer cells, and it has no effect on apoptosis. Low expression of MTHFD2 in hepatocellular carcinoma cells inhibited migration, invasion, and epithelial-mesenchymal transition (EMT) progression, while it did not inhibit cell proliferation and promote apoptosis[18] and had no effect on the cell cycle distribution of neck squamous cell carcinoma[22]. The expression of the MTHFD2 is related to the proliferation, invasion, and migration of renal cell carcinoma[19]. To reveal the effect of MTHFD2 on bladder cancer cells, we found that low expression of MTHFD2 significantly decreased the proliferation, migration, colony formation, and apoptosis of bladder cancer cells but did not change the invasion and cell cycle of bladder cancer cells. Furthermore, through a xenograft experiment in nude mice, we also confirmed that the growth rate of bladder cancer cells with low expression of MTHFD2 was significantly slower. This finding is consistent with the inhibitory effect of MTHFD2 on the tumorigenesis of non-small cell lung cancer (NSCLC) in nude mice[9]. However, the specific mechanism of the effect of MTHFD2 on bladder cancer has not been reported. Therefore, through high-throughput second-generation sequencing, we looked for the differentially expressed genes with low expression of MTHFD2 bladder cancer cells. Through GO analysis and KEGG signal pathway analysis, we found that PI3K/AKT-related functional proteins were enriched. It is further confirmed that MTHFD2 acts on bladder cancer through PI3K/AKT signal pathway, affecting the proliferation and apoptosis of bladder cancer.

PI3K/AKT signaling pathway is involved in the regulation of proliferation, differentiation, apoptosis, and glucose transport, which is closely related to the occurrence and development of human tumors. This signaling pathway's abnormal activity can not only lead to malignant transformation but also be related to tumor cell migration, adhesion, tumor angiogenesis, and extracellular matrix degradation. PI3K/AKT also plays an important role in bladder cancer. For example, Long non-coding RNA (lncRNA) small nucleolar RNA host gene 1 (SNHG1) affects the proliferation, migration, and invasion of bladder cancer through PI3K/AKT signaling pathway[23]. Plumbagin (PL), a natural plant-derived drug extracted from Chinese herbals, has been shown to affect the proliferation of bladder cancer through the PI3K/AKT/mTOR signal pathway[24, 25]. Heterogeneous nuclear ribonucleoprotein F (hnRNP-F) promotes the proliferation of bladder cancer through PI3K/AKT/FOXO1 signal pathway[25, 26]. We found that phosphorylated PI3K (p-PI3K) and phosphorylated AKT (p-AKT) were significantly decreased in bladder cancer cells with low expression of MTHFD2. However, by exposure to the PI3K agonist 740Y-P, the expression of p-PI3K and p-AKT protein in bladder cancer cells with low expression of MTHFD2 was reversed. This further confirms that MTHFD2 affects the proliferation of bladder cancer cells through PI3K/AKT signal pathway.

However, there are also some shortcomings in this study. This experiment did not further confirm whether PI3K agonist reversed the effect of tumor formation in vivo. Second, it was observed that bladder cancer cells treated PI3K agonist only partially reversed the proliferation ability of bladder cancer, indicating that MTHFD2 may affect the occurrence and progression of bladder cancer cells through other signal pathways. Furthermore, this study did not explore the sensitivity of low expression MTHFD2 to drugs. These need to be further studied in the future.

Conclusion

In conclusion, our study confirmed that MTHFD2 is highly expressed in bladder cancer, which affects the occurrence and progression of bladder cancer through PI3K/AKT signaling pathways and has a negative correlation with the clinical prognosis of patients. MTHFD2 expression can be used as an independent risk factor to judge the prognosis, which provides a new approach for the diagnosis and treatment of bladder cancer.

Methods

Bioinformatics analysis

The differential expression of the MTHFD2 gene in 31 kinds of tumors and adjacent tissues was analyzed by GEPIA online software (<http://gepia.cancer-pku.cn>), and the relationship between survival time and MTHFD2 expression in patients with bladder cancer was analyzed. UALCAN online software (<http://ualcan.path.uab.edu>) was used to analyze the expression of MTHFD2 in bladder cancer and adjacent tissues and the expression of MTHFD2 in tumor and adjacent tissues with different pathological stages and lymph node metastasis.

Patients and tissue samples

From March 2015 to January 2017, bladder cancer tissue and adjacent tissues were collected after transurethral resection of bladder tumor or radical cystectomy in The First Affiliated Hospital of Nanchang University. All specimens were confirmed by pathology and then detected by PCR and WB assays. All the patients in this study signed the informed consent form and were approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University [No. (2021)51]. Tissue microarrays were purchased from Jiangxi Ke Rui Si Biotechnology Co., Ltd. (Jiangxi, China) (Supplementary Table S2). The main clinicopathological parameters and their patient numbers were shown in Supplementary Table S3.

Cell culture

Human bladder cancer cell lines, including UM-UC-3, T24, 5637, EJ, J82, BIU, and human bladder cell biochemistry Pilon (SV-HUC-1), were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). T24 cells were cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; HyClone; GE Health care Life Sciences, Logan, UT, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. UM-UC-3 cells were cultured in MEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were cultured at 37°C with 5% CO₂.

Lentiviral construction

Lentivirus packaging was purchased from Hanheng Biological (Shanghai) Co., Ltd. T24 and UM-UC-3 bladder cancer cell lines were infected with lentiviral knockout MTHFD2 (HBLV-h-MTHFD2shRNA1/2/3-ZsGreen-PURO) or negative control (HBLV-ZsGreen-PURONC) constructs. The stably transfected cell lines were screened with puromycin.

Western blot analysis

Total proteins were extracted from cells or preserved bladder cancer tissues and adjacent tissues and were stored in a refrigerator at -80°C. Western blot analysis was performed as previously described[27]. The membranes were incubated with antibodies targeting β-actin (cat. no. 58169, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), MTHFD2 (cat. no. 98116, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-PI3K (cat. no. 17366, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phospho-AKT (cat. no. 4060, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-PI3K (cat. no. 4257, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-AKT (cat. no. 4691, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), followed by incubation with a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (cat. no. ab6721, 1:3,000; Abcam) for 1.5 h at room temperature. ImageJ 1.45 software (NIH) was used to perform densitometric analysis of each band.

Immunohistochemistry

Tissue specimens were fixed with 4% neutral formaldehyde for less than 24 hours and then rehydrated in a graded alcohol series, transparent, paraffin embedded, and sliced; then, microwave antigen repair was performed for 15 min, hydrogen peroxide blocking and serum sealing. The primary antibody MTHFD2 (cat. no. ab151447, 1:200; Abcam) was incubated overnight at 4°C. The secondary antibody was incubated for 30 min and stained with DAB (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Slides were stained with hematoxylin and sealed with neutral gum.

The cytoplasmic staining results were evaluated based on the percentage of positive cells and the intensity of staining by two independent pathologists. The intensity of staining was scored as 0 (no staining), 1 (mild staining), or 2 (deep staining). The percentage of positive cells was given scores of 0 (no tumor cells stained), 1 (<10%), 2 (10%-50%), and 3 (>50%). The results were calculated as the intensity x proportion. A total score <2 was defined as negative expression, and >2 was defined as positive expression.

RNA extraction and reverse transcription-quantitative PCR (RT-PCR)

Total RNA of cells and tissues was extracted by TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.), the concentration and purity of the extracted RNA were judged by the value of OD260/OD280. cDNA synthesized and the RT-PCR experiment were conducted as previously described[27]. mRNA relative expression levels were evaluated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences were as follows: β -actin-forward: 5'-TCTCCCAAGTCCACACAGG-3'; β -actin-reverse: 5'-GGCACGAAGGCTCATCA-3'. MTHFD2-forward: 5'-CAGCAGATCAAGCAGGAAG-3'; MTHFD2-reverse: 5'-GCAGGATTCTCGCCAAC-3'. 2.

CCK-8 assay

A CCK-8 assay was performed to measure cell viability. Approximately 1×10^3 cells were seeded into 96-well plates and cultured for 24 hours. Then, 10 μ l CCK-8 solution (Nanjing Keygen Biotech Co., Ltd., Nanjing, China) was added to each well at 0 h, 24 h, 48 h, and 72 h. After incubation for 2 hours, the absorbance at 450 nm was measured by a TECAN SPARK 10 M microplate reader (Tecan Group, Ltd., Männedorf, Switzerland).

Cell migration and invasion assays

A wound healing assay was performed to measure cell migration. Approximately 5×10^5 cells were seeded in 6-well plates. A wound was made with a 10 μ l pipette tip. The area of the cell-free scratch was photographed using an Olympus CKX41 inverse light microscope (Olympus Corporation, Tokyo, Japan) at 0 h, 24 h, and 48 h.

Cell invasion abilities were determined using a Transwell assay as previously described[27]. Following incubation for 24 h, the cells were fixed using 4% formaldehyde, stained using crystal violet and photographed using an Olympus CKX41 inverse light microscope at 37°C for 30 min.

Flow cytometry

For apoptosis and cell cycle distribution detection, approximately 1×10^6 cells were collected and washed twice in pre-cooled PBS buffer, and then 7-ADD and Annexin V-APC binding solution were added according to the apoptosis kit (Lian Ke biology, China). PI staining solution (Mei Lun Biology, China) was added to detect the cell cycle by flow cytometry.

Colony formation assay

The appropriate cells (approximately 300 cells/well for T24 cells and approximately 400 cells/well for UM-UC-3 cells) were seeded in 6-well plates, and the cells were cultured in an incubator (37°C, 5% CO₂) with 10% FBS medium for 10–14 days. The culture was terminated when the number of cells was more than 50 per colony under a microscope, or the colony was visible under the naked eye. The colonies were stained with crystal violet and photographed.

Tumor formation experiment in nude mice

Four-week-old female BALB/c nude mice were obtained from Hunan Si Lai Ke Jing Da Laboratory Animal Co., and fourteen mice were divided into two groups with 7 mice in each group and fed according to protocols approved by the Animal Care and Use Committees of Nanchang University [Institutional approval number for lab animal studies: SYXK (Gan) 2015-0001]. T24-control and T24-shRNA2 cells at a density of 1×10^8 /ml were resuspended in 200 μ L Matrigel and injected subcutaneously into the flanks of mice. The size of the tumor was recorded every 3–6 days, and the volume ($V = 1/2A \times B^2$ (A is the long axis, B is the short axis)). After 4 weeks of tumor formation, the nude mice were killed, and tumors were photographed, measured, weighed and immunohistochemically stained.

Construction of RNA sequencing libraries and RNA-sequencing (RNA-Seq)

RNA-Seq was performed by NovelBio Bio-Pharm Technology Co.,Ltd. (Shanghai, China). Total RNA was extracted from 3 pairs of T24-Control and T24-shRNA2 by Trizol reagent (Invitrogen) separately. The RNA quality was checked by Agilent 2200 and kept at -80°C . The cDNA libraries were constructed for each RNA sample using the TruSeq Stranded mRNA Library Prep Kit (Illumina, Inc.) according to the manufacturer's instructions. The libraries were quality controlled with Agilent 2200 and sequenced by HiSeq X (Illumina, San Diego, CA) on a 150 bp paired-end run. The clean reads were then aligned to the human genome (GRCh38, NCBI) using the Hisat2. HTseq was used to get gene counts and the RPKM method was used to determine the gene expression.

We applied the DESeq2 algorithm to filter the differentially expressed genes, after the significant analysis, P-value and FDR analysis were subjected to the following criteria: i) Fold Change > 2 or < 0.5; ii), P-value < 0.05, FDR < 0.05. Gene ontology (GO) analysis was performed to facilitate elucidating the biological implications of the differentially expressed genes in the experiment. Fisher's exact test was applied to identify the significant GO categories (P-value < 0.05). Pathway analysis was used to find out the significant pathway of the differentially expressed genes according to Kyoto Encyclopedia of Genes and

Genomes (KEGG) database. We turn to Fisher's exact test to select the significant pathway, and the threshold of significance was defined by $P\text{-value} < 0.05$.

Statistical analysis

GraphPad Prism 8.00 software (La Jolla, CA, USA) was used for data analysis. The quantitative data are expressed as the mean $\pm$ standard deviation ($\bar{x} \pm S$). Student's t-test or one-way analysis of variance (ANOVA) followed by post hoc Tukey's honest significant difference test were used for comparisons between groups. A $P < 0.05$ was considered to indicate a statistically significant difference.

Abbreviations

MTHFD2

Methylenetetrahydrofolate dehydrogenase 2

RT-PCR

Reverse transcription-quantitative PCR, WB:Western blot

CCK-8

Cell Counting Kit-8

NAD

Nicotinamide adenine dinucleotide

EMT

epithelial-mesenchymal transition

BC

bladder cancer. PI3K:phosphoinositide 3kinase

AKT

protein kinase B

p-PI3K

phosphorylated PI3K

p-AKT

phosphorylated AKT

GO

Gene Ontology

KEGG

Kyoto Encyclopaedia of Genes and Genomes

RNA-Seq

RNA-sequencing.

Declarations

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the First Affiliated Hospital of Nanchang University [No. (2021)51] and the Animal Care and Use Committees of Nanchang University [Institutional approval number for lab animal studies: SYXK (Gan) 2015-0001] and have been performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Informed consent was obtained from all patients.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Conceptualization: Bin Fu, Wensheng Zhang; Methodology: Xinxi Deng, Xiaoqiang Liu, Jianyun Liu; Investigation: Xinxi Deng, Xiaoqiang Liu, Bing Hu; Writing - original draft: Xinxi Deng; Writing - review & editing: Wensheng Zhang, Bin Fu, Xinxi Deng, Xiaoqiang Liu; Funding acquisition: Bin Fu; Resources: Jianyun Liu, Xiaoqiang Liu; Supervision: Bin Fu, Wensheng Zhang.

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Figures

Figure 1

The expression of MTHFD2 in cancer tissues. (A) MTHFD2 gene expression profile across all tumor samples and paired normal tissues in GEPIA database. (B) Differential expression of MTHFD2 in BLCA (n=408) and adjacent tissues (n=19) in UALCAN database; Expression of MTHFD2 in BLCA based individual cancer stages in UALCAN database; Expression of MTHFD2 in BLCA based on nodal metastasis status in UALCAN database. (C) The overall survival between low MTHFD2 and high MTHFD2 in GEPIA database.

Figure 2

The high expression of MTHFD2 in cancer tissues. (A) Relative transcript level of MTHFD2 in bladder cancer tissues (n=24) and adjacent tissues (n=24). (B) Western blot showed the relative expression of MTHFD2 in bladder cancer tissues (n=6) and adjacent tissues (n=6). (C) Tissue microarray showed the relative expression of MTHFD2 in bladder cancer tissues (n=61) and adjacent tissues (n=13). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 3

Relative expression of MTHFD2 in bladder cancer cell lines. (A) Relative transcript level of MTHFD2 in SV-HUC-1 and bladder cancer cell. Silencing MTHFD2 efficacy of T24 and UMUC3 was detected with RT-PCR (B) and Western blot (C), *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 4

Silencing MTHFD2 in BC cells T24 and UM-UC-3 inhibited the proliferation and migration of cells. (A) Cell proliferation was determined by CCK8 assay of T24 and UMUC3 in cells transfected with shMTHFD2 and shCtrl for 4d. (B) Cell migration was determined by wound healing assay of T24 and UMUC3 in cells transfected with shMTHFD2 and shCtrl for 24 hours to 48hours. (C) Colony-forming experiment determined the cell proliferative capacity of T24 and UM-UC-3 after MTHFD2 silenced. The values are expressed as mean $\pm$ SD, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 5

Silencing MTHFD2 in T24 and UMUC3 cells promoted apoptosis, but not blocked the cell cycle. (A) Cell apoptosis was analyzed by flow cytometry in T24 and UMUC3 after transfection with si-MTHFD2. (B) The cell cycle was analyzed by flow cytometry in T24 and UMUC3 after transfection with si-MTHFD2. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 6

Knockdown of MTHFD2 inhibits the tumorigenicity of bladder cancer. (A) Tumor growth curves revealed that xenograft tumor growth in nude mice was significantly slower in shRNA2 treated group than that of Control group. (B) Mean tumor weights 30 d after transplantation was shown. (C) Images of the subcutaneous tumors formed in the nude mice after injection of shMTHFD2 and shCtrl transfected T24 cells. (D) Representative immunohistochemical staining of MTHFD2 in tumor xenograft tissues. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 7

PI3K/AKT pathway is downregulated in bladder cancer T24 cell lines with low expression of MTHFD2. (A) Cluster analysis showed that 367 differentially expressed genes were up-regulated and 120 genes were down-regulated between T24-shRNA2 group and T24-Control group. (B) GO analysis showed that bladder cancer cells with low expression of MTHFD2 were enriched in apoptotic cell clearance, cell adhesion, MAP kinase activation, intracellular signal transduction and other functions. (C) KEGG showed that PI3K/AKT signal pathways were enriched in bladder cancer cell lines with low expression of MTHFD2. (D) WB confirmed that the protein levels of p-PI3K/PI3K and p-AKT/AKT decreased significantly after silencing MTHFD2 in T24 cells and the protein levels of p-PI3K and p-AKT increased significantly after treated with PI3K agonists 740Y-P (10, 30, 90ug/ml). (E) T24 cells silencing MTHFD2 were treated with PI3K agonists 740Y-P (90ug/ml) revealed that the protein levels of p-PI3K/PI3K and p-AKT/AKT were increased, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

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