

Expression and Role of Methylenetetrahydrofolate Dehydrogenase 1 Like (MTHFD1L) in Bladder Cancer



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Abstract

Cancer cells utilize vitamin folate to fulfill their excessive demand for nucleotides and amino acids. Dihydrofolate reductase (DHFR), an enzyme involved in folate metabolism converts dihydrofolate into tetrahydrofolate, which is required for the *de novo* synthesis of purines, and certain amino acids. DHFR inhibitors are used as a chemotherapeutic agent. Cancer sequencing analysis has identified additional enzymes in folate metabolism that are dysregulated in cancer. Methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L), one such enzyme is overexpressed in bladder cancer. MTHFD1L is a mitochondrial enzyme involved in the folate cycle by catalyzing the reaction of formyl-tetrahydrofolate to formate and tetrahydrofolate (THF). THF is crucial for *de novo* purine and thymidylate synthesis and is also involved in the regeneration of methionine. Cancer cells rely on purines derived from the *de novo* pathway for the nucleotides whereas normal cells favor the salvage pathway. In this study we examined MTHFD1L expression in bladder cancer. By using publicly available cancer transcriptome data analysis web-portal UALCAN, we found overexpression of MTHFD1L in bladder cancer and expression was associated with overall survival. RT-PCR and immunoblot analysis confirmed the overexpression of MTHFD1L in muscle invasive bladder cancer tissues compared to normal urothelium. Furthermore, our investigations suggested a critical role for MTHFD1L in bladder cancer cell proliferation, colony formation and invasion. Thus, in this study, we show the significance of the folate metabolic enzyme MTHFD1L in aggressive bladder cancers and suggest that being an enzyme, MTHFD1L serves as a valuable therapeutic target.

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Introduction

Bladder cancer is the most common malignancy of the urinary tract with estimated 80,470 new cancer cases in 2019 in the USA [1]. Bladder cancer most commonly manifests as superficial disease with high recurrence rates. However about 10–15% of tumors will progress to muscle invasive bladder cancer (MIBC) [2]. For these tumors surgical removal with or without neoadjuvant chemotherapy remains the standard treatment option [3,4]. Once metastatic, urothelial carcinoma of the bladder is generally incurable by current chemotherapy and leads to early mortality [5]. Immune checkpoint inhibitors (e.g. atezolizumab, nivolumab, pembrolizumab) have recently provided durable benefits to a minority (~20%) of post-platinum or selected untreated PD-L1 low cisplatin-ineligible patients and platinum-ineligible patients [6–9]. Erdafitinib was recently approved for selected post-platinum patients with somatic FGFR2/3 alterations [10]. However, these agents are not curative and benefits are modest, suggesting a major role for new tolerable agents and combinations for this elderly population.

Multiple molecular alterations play a role in the progression of this aggressive disease. Recent studies have identified molecular subtypes in MIBC with different sensitivities to frontline therapy suggesting the heterogeneity in these tumors and the importance of molecular characterization of MIBC to provide effective treatment [11–13].

Cancer cells harbor specific metabolic requirements due to their enhanced proliferation rate [14–16]. One of the common dysregulated pathways in this setting is folate metabolism. Inhibitors of this pathway predominantly target *dihydrofolate reductase (DHFR)*, an enzyme that reduces folate to dihydrofolate and converts it to tetrahydrofolate (THF). Methotrexate, a component of one of the standard regimens used to treat metastatic urothelial carcinoma, *i.e.* MVAC (methotrexate, vinblastine, doxorubicin, cisplatin), is a DHFR inhibitor [17–20]. In the present study, we have identified and characterized another folate metabolic enzyme methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L) in bladder cancer. MTHFD1L is a mitochondrial enzyme that catalyzes the reaction of formyl-tetrahydrofolate to formate and THF [21–23]. THF is crucial for *de novo* purine and thymidylate synthesis and is also involved in the regeneration of methionine [24]. Cancer cells rely on purines derived from the *de novo* pathway for nucleotides whereas normal cells favor the salvage pathway [25,26]. Our previous studies have shown that PAICS, an enzyme involved in *de novo* purine biosynthesis is overexpressed in bladder cancer and plays a critical role in bladder cancer growth [27]. Similarly, targeting enzymes involved in the associated folate pathway, like SHMT2, MTHFD2, and MTHFD1L, may harbor a therapeutic potential [28,29]. MTHFD1L, being a potential therapeutic target overexpressed in bladder cancer, warrants further investigation.

In this study we characterized the expression and investigated the role of MTHFD1L in bladder cancer. Our studies show significant overexpression of MTHFD1L in primary bladder cancer. Furthermore, knockdown of MTHFD1L in multiple bladder cancer cells showed a decrease in cancer cell proliferation. In addition, our studies also revealed a reduced colony forming and invasion ability of bladder cancer cells upon MTHFD1L knockdown indicating the therapeutic potential of targeting this gene. Further studies are needed to target MTHFD1L with small molecule inhibitors.

Materials and Methods

Gene Expression from The Cancer Genome Atlas (TCGA)

MTHFD1L gene expression levels in bladder cancer and normal bladder were interrogated utilizing UALCAN [30], a web portal that provides boxplots depicting each gene's expression based on 3 RNA-sequencing data from the TCGA transcriptome sequencing datasets.

Benign and Bladder Cancer Tissue Samples for PCR and Immunoblot Blot Analysis

Fresh frozen MIBC (\geq pT2 stage) tissue samples (T) with adjacent normal tissue (N), were obtained from the Cooperative Human Tissue Network (CHTN) based at the University of Alabama at Birmingham (UAB). CHTN complies with federal human subjects regulations (The "Common Rule;" 45 CFR part 46) to collect and distribute biospecimens. Tumor samples were obtained from patients undergoing radical cystectomy surgery without preceding neoadjuvant systemic therapy, snap frozen and stored in liquid N₂ tanks (23). Specimens underwent central pathological assessment for confirmation of diagnosis. Then, macrodissection of tissue was conducted after histologic demarcation of tumor and normal bladder epithelial tissue. The study was IRB approved (IRB-120917005) at UAB.

Tissue Immunohistochemistry (IHC)

To evaluate MTHFD1L expression in bladder cancer and benign urothelium formalin-fixed, paraffin-embedded tissue sections were stained with rabbit polyclonal antibody against MTHFD1L (#16113–1-AP, PTG Labs, Rosemont, IL). For antigen retrieval tissue sections were boiled for 10 minutes in citrate buffer (#C9999-1000ML, Sigma Aldrich, MO). Immunostaining was performed using Vector Laboratories staining kit following the manufacturer's protocol. Primary antibody was added in a 1:250 dilution for 1 h at room temperature according to dilution protocol optimized in our laboratory. After washing with PBS, secondary antibody (anti-rabbit, #MP-7401 Vector Laboratories, Burlingame, CA) was added for 45 min at room temperature. Antibody signals were detected using ImmPACT DAB (#SK-4105, Vector Laboratories, Burlingame, CA). Hematoxylin QS (# H-3404, Vector Laboratories, Burlingame, CA) was used as a counterstain.

Cell Culture

RT-112 and VMCUB-1 cells were obtained from Leibniz Institut DSMZ, Germany. HT-1376, 5637, HT-1197 and T24 were purchased from ATCC. RT-112, 5637, VMCUB-1 cells were cultivated at 37 °C in a humidified environment with 5% CO₂ in RPMI medium (Gibco™ RPMI 1640 Medium, Life Technologies™), while HT-1376 and HT-1197 were cultivated in MEM medium (Gibco MEM (1x), Life Technologies™) and T24 in McCoy's 5a modified medium (Gibco™ McCoy's 5A (Modified) Medium, Life Technologies™). All media was supplemented with 10% fetal bovine serum and 100 U/ml penicillin G and 100 µg/ml streptomycin.

Immunoblot Analyses

For immunoblot analysis, protein samples were separated on SDS–PAGE (NuPAGE 4–12%, # WG1402BOX, Thermo Fischer Scientific, Waltham, MA). Equal amounts of proteins were loaded and transferred for 2 h at 0.35 A onto an Immobilon1-P PVDF membrane (EMD Millipore, Billerica,

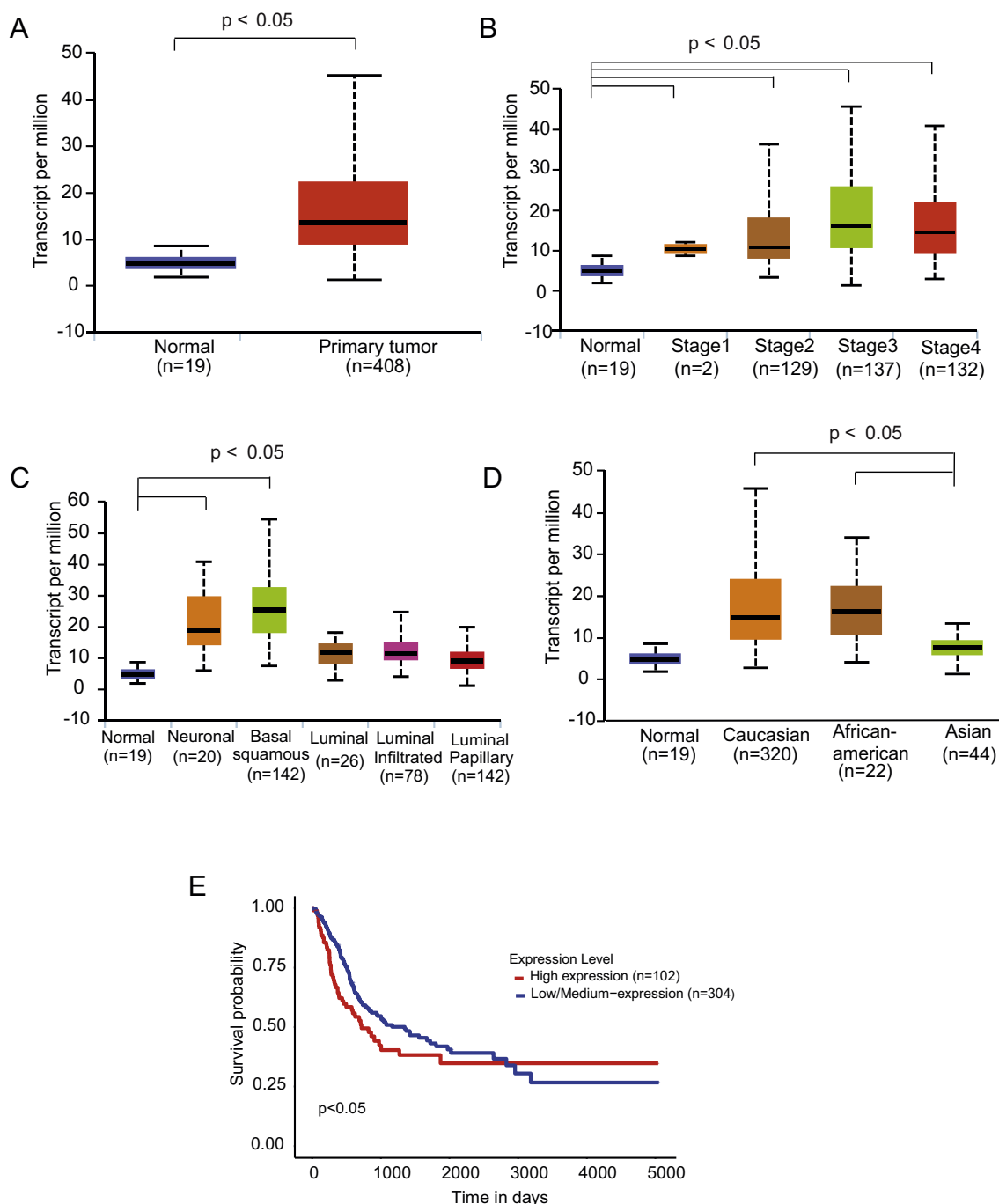


Figure 1. The folate metabolic pathway enzyme MTHFD1L is overexpressed in bladder urothelial carcinoma and predicts poor patient survival. (A) Expression of MTHFD1L in normal bladder and primary tumor samples using TCGA data analyzed by the UALCAN web portal. (B) Boxplots of MTHFD1L expression analyzed by stage. (C) Boxplots represent MTHFD1L expression level across normal bladder and molecular bladder cancer subtypes. (D) Analysis of MTHFD1L expression by race. (E) Overall survival analysis of patients with high vs low/medium MTHFD1L expression.

MA). To block non-specific binding, the membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween 20 [TBS-T], 5% nonfat dry milk) followed by incubation overnight at 4 °C with the primary antibody. After two washes with TBS-T for 5 min, the blot was incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature. The membrane was again washed with TBS-T and TBS twice for 5 min each and signals were visualized by Luminata™ Forte

Western HRP Substrate (#WBLUF0500, EMD Millipore, Billerica, MA) as per manufacturer's protocol. β -actin served as a loading control. Antibodies were implemented as follows: anti-MTHFD1L: #16113-1-AP (1:1000; PTG Labs, Rosemont, IL), anti-HRP- β -actin: # HRP-60008 (1:200000; PTG Labs, Rosemont, IL), and anti-rabbit IgG HRP: # SA00001-2 (1:5000; PTG Labs, Rosemont, IL). All antibodies were employed at dilutions optimized in our laboratory.

Table 1. Clinical and demographic characteristics of TCGA Bladder urothelial carcinoma (BLCA) samples categorized based on MTHFD1L expression level (Clinical and demographic information are not available for few samples)

Clinical/demographic parameters		TCGA BLCA samples with high MTHFD1L expression (n = 102)	TCGA BLCA samples with low/medium MTHFD1L expression (n = 304)
Gender	Male	76 (74.51%)	219 (72.04%)
	Female	26 (25.49%)	79 (25.99%)
Race	Caucasian	89 (87.25%)	230 (75.66%)
	African American	5 (4.9%)	17 (5.59%)
Pathologic N	Asian	4 (3.92%)	39 (12.83%)
	N0	61 (59.8%)	175 (57.57%)
	N1	11 (10.78%)	25 (8.22%)
	N2	14 (13.73%)	32 (10.53%)
	N3	13 (12.75%)	62 (20.39%)
Pathologic T	NX	1 (0.98%)	6 (1.97%)
	T0	0	1 (0.33%)
	T1	1 (0.98%)	2 (0.66%)
	T2	5 (4.9%)	32 (10.53%)
	T2a	3 (2.94%)	22 (7.24%)
	T2b	16 (15.69%)	40 (13.16%)
	T3	14 (13.73%)	29 (9.54%)
	T3a	17 (16.67%)	52 (17.11%)
	T3b	29 (28.43%)	52 (17.11%)
	T4	4 (3.92%)	6 (1.97%)
	T4a	6 (5.88%)	37 (12.17%)
	T4b	0	5 (1.64%)
	TX	0	1 (0.33%)
	Stage	Stage I	0
Stage II		26 (25.49%)	102 (33.55%)
Stage III		45 (44.12%)	92 (30.26%)
Stage IV		31 (30.39%)	100 (32.89%)
Histology	Papillary	21 (20.59%)	109 (35.86%)
	Non-papillary	78 (76.47%)	193 (63.49%)

MTHFD1L Knockdown in Bladder Cancer Cells

MTHFD1L (siGenome SMARTpool, # M-009949-01-0005) and non-targeting small interfering RNA (siRNA) were obtained from Dharmacon (Lafayette, CO) and transfection experiments were performed following the manufacturer's protocol. For transfection Lipofectamine RNAiMAX reagent (#13778150, Thermo Fisher, Waltham, MA) was applied. Cells were seeded 1×10^5 in a 6-well plate and simultaneously transfected with MTHFD1L siRNA and control non-targeting siRNA. Cells were harvested 72 h after transfection for RNA isolations and immunoblot experiments.

RNA Extraction and qRT-PCR

Total RNA from bladder cancer cells was extracted by using the Direct-zol RNA MiniPrep Plus kit (#R2071, Zymo Research, Irvine, CA) according to manufacturer's protocol. For bladder cancer tissue RNA was harvested by employing the Qiagen RNeasy kit. Each sample was transcribed into complementary DNA by using Superscript III Reverse Transcriptase (#18080093, Thermo Fischer Scientific, Waltham, MA). For each qRT-PCR amplification, 2 μ l of the cDNA product (200 ng/ μ l), 5 μ l SYBR green PCR Master Mix (Applied Biosystems, Waltham, MA), 1 μ l primer solution, and 2 μ l of DNase/RNase free water was added for a final volume of 10 μ l. Thermocycling conditions were as suggested by the manufacturer: 95° for 10 min to activate the polymerase followed by 40 cycles of 95° for 15 sec and 60° for 1 min. SYBR green was used to determine the mRNA expression level of a gene of interest. Levels were normalized to beta-Actin and analyzed using the $\Delta\Delta$ CT method. All primers for SYBR green were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are: MTHFD1L: forward primer, TGTGCCAAGGGACTTCATCT and reverse primer,

AGTCCTGGCATGGTGCTC, ACTB: forward primer, GCACA-GAGCCTCGCCTT, and reverse primer, GTTGTGCGACGAC-GAGCG. All PCR reactions were performed in triplicates.

Cell Proliferation Assays

Cell proliferation was measured by cell counting. For this, transient MTHFD1L knockdowns cells were used. Non-targeting siRNA and untreated cells served as control. After 48 hours of transfection using pooled siRNA, the cells were trypsinized and seeded at a density of 1500 cells/well in 12-well plates. Then, the cells were trypsinized and counted at specified time points by Z2 Coulter particle counter (Beckman Coulter, Brea, CA). Each experiment was performed with three replicates.

Matrigel Invasion Assay

For Matrigel Invasion assay cells in medium without fetal bovine serum were seeded onto Corning BioCoat Matrigel matrix (# 354480, Corning, New York, NY) in the upper chamber of a 24-well culture plate. The lower chamber containing adequate medium was supplemented with 10% fetal bovine serum as a chemoattractant. After 48 hours, the non-invading cells and Matrigel matrix were gently removed with a cotton swab. Invasive cells located on the lower side of the chamber were fixed with 10% (v/v) glutaraldehyde (#BP25471, Fisher Scientific, Pittsburg, PA) for 15 minutes and stained with crystal violet (# HT901-8FOZ, Sigma-Aldrich, St. Louis, MO) for 15 minutes and photographed using an inverted microscope (4 \times).

Colony Formation Assay

After 48 hours of transfection, the various test cells were counted and seeded at a density of 1000 cells per 1 well of 6-well plates (triplicates) and incubated at 37 °C, 5% CO₂ for 6 days. Colonies were fixed with 10% (v/v) glutaraldehyde (#BP25471, Fisher scientific, Pittsburg, PA) for 15 minutes and stained with crystal violet (#HT901-8FOZ, Sigma-Aldrich, St. Louis, MO) for 15 minutes. Then, the photographs of the colonies were taken using Amersham Imager 600RGB (GE Healthcare Life Sciences, Pittsburgh, PA).

Statistical Analysis

To determine significant differences between two groups, the Wilcoxon rank sum test was applied for continuous variables using JMP® 13.1.0. For paired samples the pairwise t-test was utilized. P values <.05 were considered significant.

Results

The Mitochondrial Folate Enzyme MTHFD1L is Overexpressed in Bladder Cancer and Its Expression Predicts Poor Patient Survival

Transcriptome sequence analysis of the TCGA bladder cancer dataset revealed an overexpression of MTHFD1L in bladder cancer (Figure 1A). This overexpression was observed in all stages (Figure 1B). Looking at the molecular TCGA subtypes described by Robertson et al. [13], demonstrated higher MTHFD1L levels in the basal/squamous and neuronal subtypes (Figure 1C) and in patients with African American and Caucasian race compared to patients of Asian race (Figure 1D). Furthermore, Kaplan–Meier survival analysis indicated decreased overall survival in patients whose tumors expressed higher MTHFD1L levels (Figure 1E). The clinical and demographic characteristics of the bladder cancer samples from TCGA are given in Table 1.

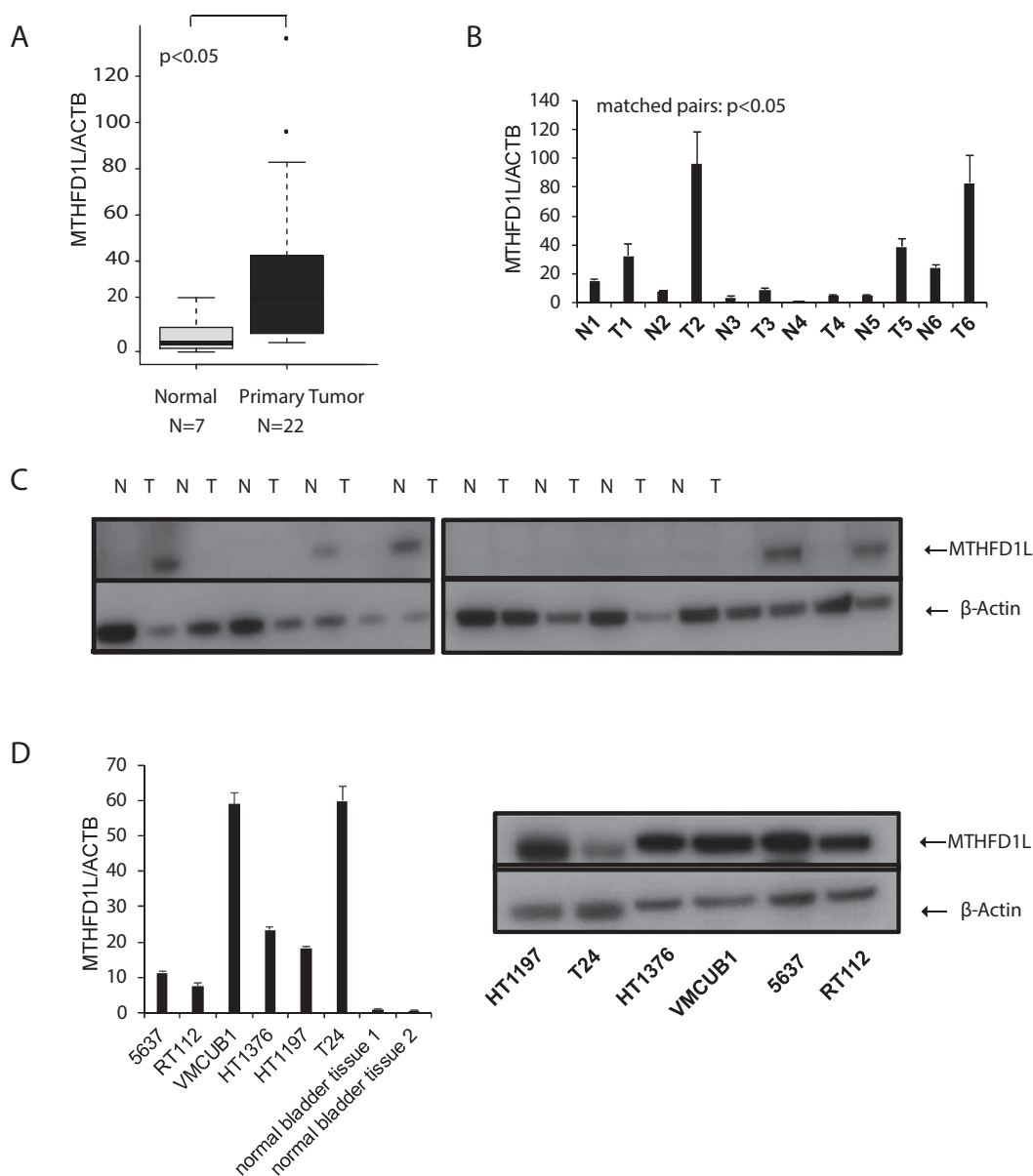


Figure 2. MTHFD1L expression in bladder cancer tissue and cell lines. (A) QRT-PCR analysis in normal bladder and primary bladder urothelial carcinoma. (B) MTHFD1L mRNA levels in cases with available matched normal bladder and tumor tissues. (C) Immunoblot analysis showing MTHFD1L expression in matched normal bladder and tumor tissues. β -actin served as a loading control. (D) MTHFD1L mRNA and protein levels in various bladder cancer cell lines by qRT-PCR and immunoblot analysis.

Next, we validated these *in silico* findings by performing additional analysis. We used bladder cancer tissue and adjacent normal bladder tissue for further analysis. qRT-PCR and immunoblot analysis of bladder tissue using a specific antibody showed overexpression of MTHFD1L in bladder cancer compared to normal bladder tissue (Figure 2, A and C). We found that in all cases with available corresponding uninvolved tissue, MTHFD1L mRNA expression and protein levels to be higher in the malignant tissue compared to normal tissue (Figure 2, B and C). Similarly, elevated levels of MTHFD1L were observed in bladder cancer cell lines compared to normal bladder tissue (Figure 2D). To visualize the localization of MTHFD1L in bladder cancer and paired benign tissue, we performed IHC showing a granular cytoplasmic staining pattern (Figure 3).

Knockdown of MTHFD1L Inhibits Bladder Cancer Cell Proliferation

Since folate metabolism is a critical requirement for the cancer cells, we went on to evaluate the role of MTHFD1L using multiple bladder cancer cell lines. To investigate the role of MTHFD1L in bladder cancer cell proliferation, we performed RNA interference using highly specific pooled siRNA in the bladder cancer cell lines 5637, RT112, HT1376, and VMCUB-1. The knockdown efficiency of MTHFD1L was evaluated by performing immunoblot analysis (Figure 4A). Proliferation experiments were performed over a period of 6 days. Our results indicated a dramatic decrease in cancer cell growth upon knockdown in all four bladder cancer cell lines (Figure 4B). This experiment clearly shows the requirement of MTHFD1L for cancer cell proliferation.

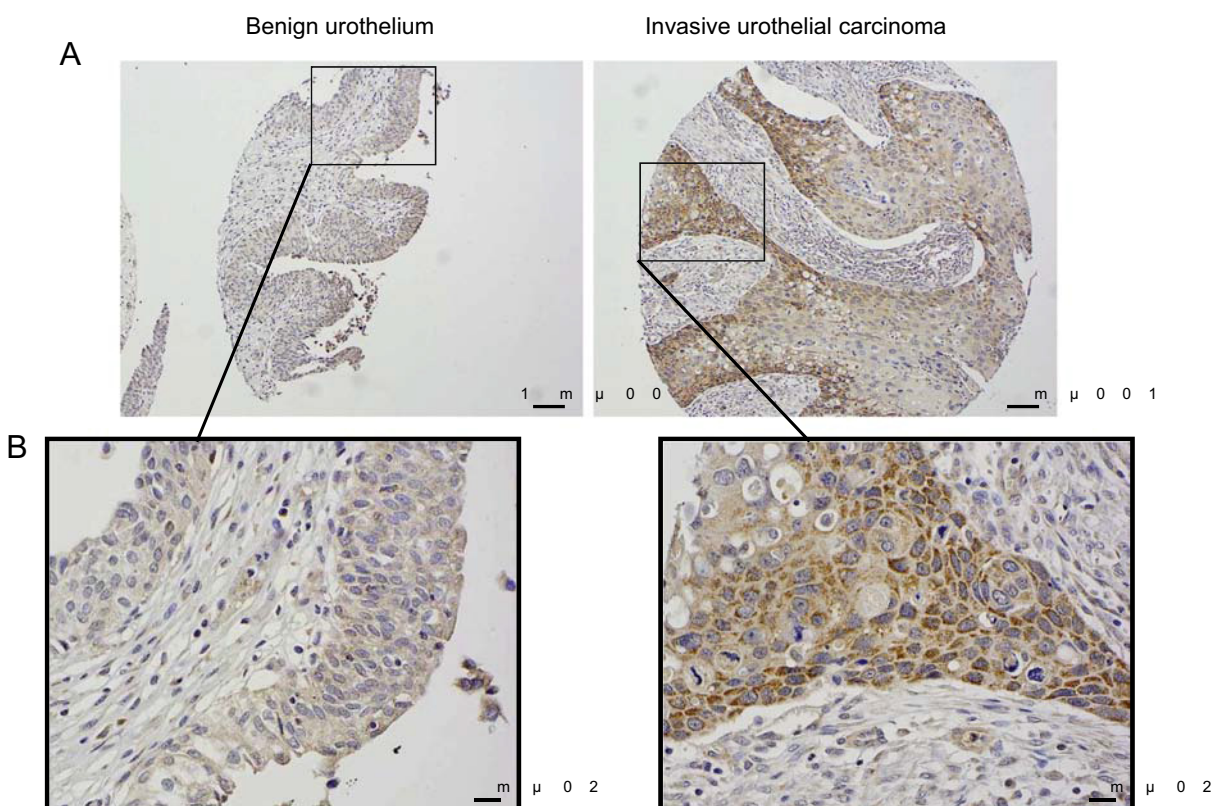


Figure 3. Immunohistochemical analysis of MTHFD1L in urothelial carcinoma of bladder. Overview (A) and detailed photo micrographic (B) depiction of MTHFD1L immunohistochemical staining in invasive urothelial carcinoma and paired benign urothelium.

MTHFD1L Regulates Bladder Cancer Cell Colony Formation and Invasion

Next, in order to further investigate the role of MTHFD1L in other oncogenic phenotypes, we performed cancer cell colony formation and invasion assays. MTHFD1L knockdown resulted in formation of fewer colonies compared to non-targeting and untreated cells (Figure 5A). Boyden Chamber Matrigel invasion assay (Figure 5B) showed reduced cancer cell invasion upon MTHFD1L knockdown suggesting potential role of MTHFD1L in bladder cancer invasion and metastasis.

Discussion

Alterations in cancer cell energetics and metabolism is one of the emerging hallmarks in cancer [14]. It is a field of active investigations in cancer and serves as an avenue to establish new treatment options. Cancer cells have a higher metabolic rate than benign cells [14,15]. Due to increased growth rates and proliferation, tumor cells show an increased demand for nucleotides and amino acids. One of the main pathways that provide these nutrients is the folate cycle (also known C1 metabolism or one carbon metabolism), which helps to supply cells with one-carbon (C1) groups needed for amino acids and nucleotide synthesis [29,31]. In the initial step of folate metabolism folate is converted to dihydrofolate (DHF) and further to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR) [32]. THF can then be used for DNA synthesis. Many chemotherapeutics, such as methotrexate act by compromising the folate metabolism through inhibition of DHFR. However, given that normal cells also require THF, these drugs cause non-specific toxicity in both cancer cells and in non-cancerous cells. As C1 metabolism has

a mitochondrial and cytoplasmic component, studies have hypothesized that targeting only the mitochondrial pathways could potentially cause less side effects, as a parallel pathway in the cytoplasm exists [32]. The mitochondrial enzymes involved in the folate cycle are MTHFD1L, ALDH1L2, SHMT2, AMT and MTHFD2 [24,33].

In our study, by performing *in silico* analysis, we show a significant upregulation of MTHFD1L in bladder cancer. We validated these findings by performing RT-PCR, immunoblot and immunohistochemistry using bladder cancer tissue mRNA and protein lysates. The TCGA cancer RNA sequencing data using the UALCAN web portal indicated decreased overall survival in bladder cancer patients with high MTHFD1L expression [30]. To investigate the biological significance of MTHFD1L we conducted proliferation, colony formation and invasion assays by knocking down MTHFD1L in bladder cancer cell lines. We showed that knockdown of MTHFD1L resulted in decreased proliferation, reduced colony forming and invasion, all of which suggest a critical role of this enzyme in regulating bladder cancer growth and invasion. Earlier studies have shown that MTHFD1L is upregulated in esophageal squamous cell carcinoma [34]. Investigations in hepatocellular carcinoma (HCC) indicated that MTHFD1L is transcriptionally upregulated through nuclear factor (erythroid-derived2)-like 2 (NRF2), which is a key player in the activation of antioxidant genes under oxidative stress. This suggests that metabolic reprogramming in cancer cells produce antioxidants for neutralizing high levels of reactive oxygen species (ROS) [24,35]. Furthermore, the folate cycle was shown to be a major supplier of nicotinamide adenine dinucleotide phosphate (NADPH) which is an important antioxidant [36,37]. Thus inhibition of the folate cycle resulted in reduced ROS anti-oxidant production, which induces intracellular oxidative stress and was shown to inhibit HCC growth [36].

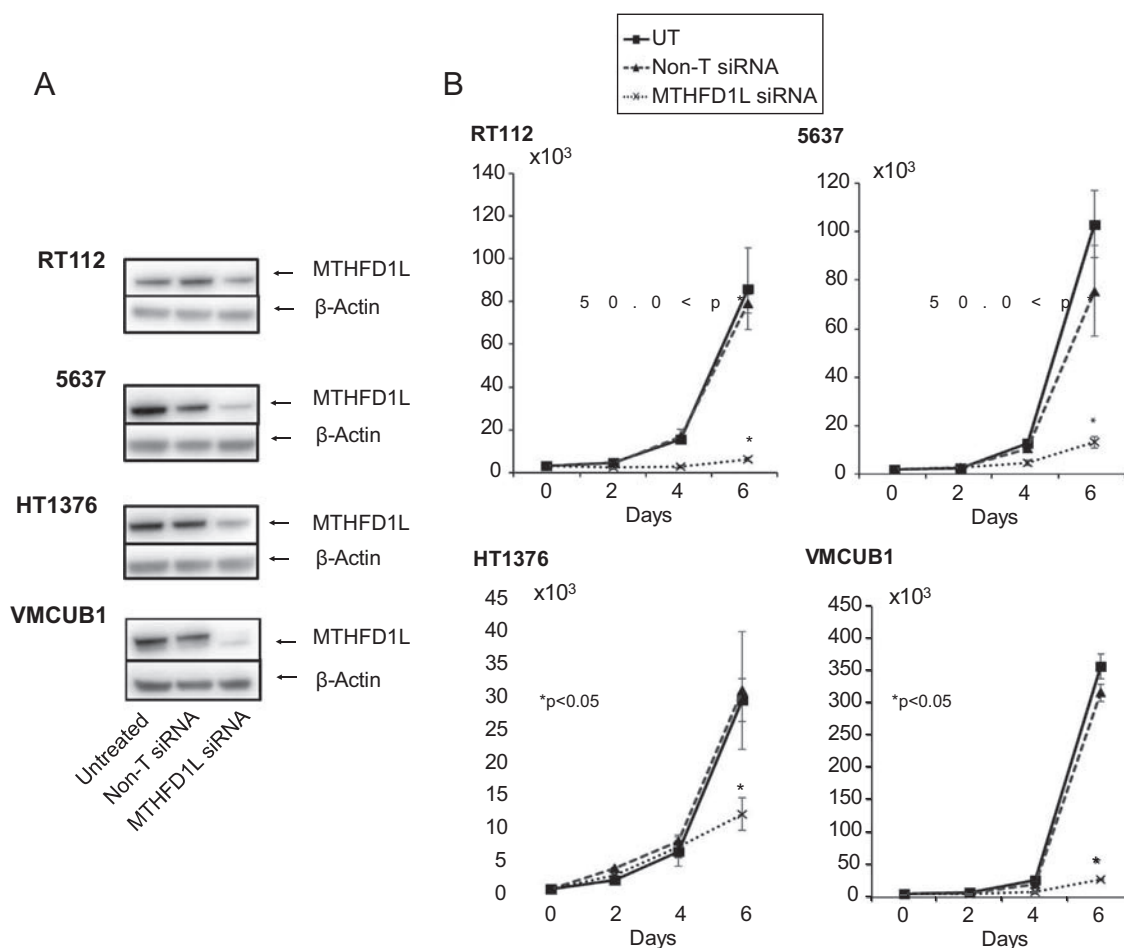


Figure 4. MTHFD1L regulates bladder cancer cell proliferation. (A) Immunoblot analysis showing MTHFD1L protein in RT112, 5637, HT1376, and VMCUB1 cells transfected with a MTHFD1L siRNA or control non-targeting siRNA (non-T siRNA) and untreated cells. (B) Transient knockdown of MTHFD1L reduces cell proliferation of bladder cancer cell lines RT112, 5637, HT1376, and VMCUB1.

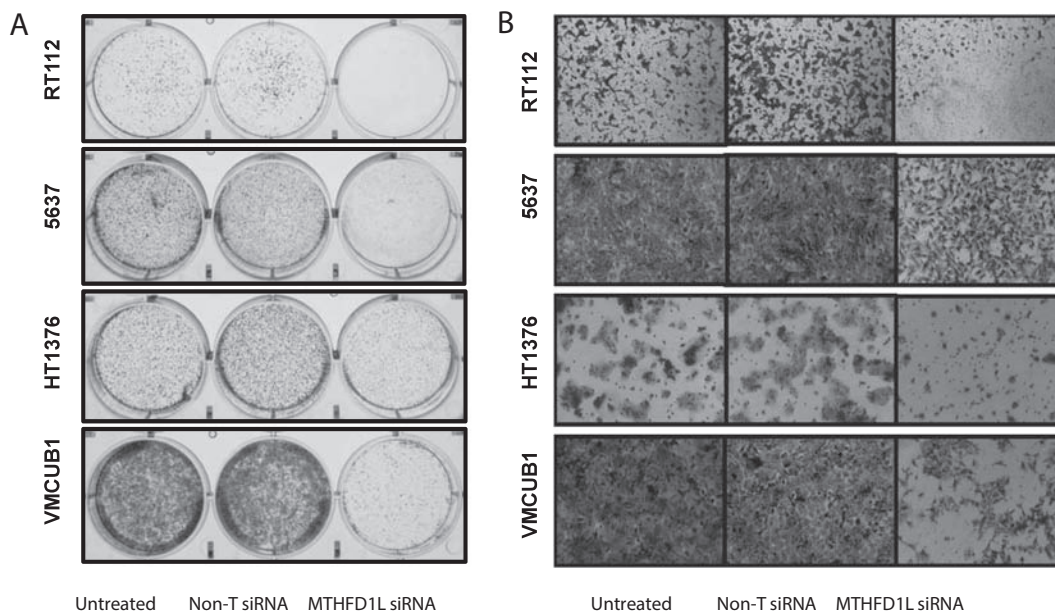


Figure 5. MTHFD1L regulates bladder cancer cell colony formation and invasion. (A) Photomicrographs of colony formation assay using untreated, non-targeting siRNA (non-T siRNA) and MTHFD1L siRNA bladder cancer lines RT112, 5637, HT1376, and VMCUB-1 (B) MTHFD1L siRNA-treated bladder cancer cells showed reduced invasion in Boyden chamber Matrigel invasion assay. Invaded cells were stained with crystal violet.

Through its involvement in the folate cycle MTHFD1L is not only involved in purine and thymidylate synthesis but also helps cells in the methionine cycle [24]. S-Adenosylmethionine (SAM) is an important methyl donor utilized by histone and DNA methyltransferases [38]. In prostate cancer the availability of SAM was shown to lead to aggressive prostate cancer [39]. Thus, reduced THF levels through MTHFD1L inhibition potentially impair cells not only by reducing *de novo* purine and thymidine synthesis, but also through its involvement in the methionine cycle.

In summary we found that overexpression of MTHFD1L in bladder cancer portends to decreased overall survival. Furthermore, our studies show that MTHFD1L plays a critical role in bladder cancer growth and invasion, which suggests its potential as a valuable therapeutic target. Future studies will focus on rationally developing small molecule inhibitor to target MTHFD1L, as single agents and in combination with chemotherapy and checkpoint inhibitors, in biomarker selected patients with high tumor MTHFD1L expression.

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