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Original Article

Expression and function of SIRT6 in muscle invasive urothelial carcinoma of the bladder

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Abstract: SIRT6, a member of the class III histone deacetylase, has been shown to inhibit glycolysis and promote DNA double strand break repairs. Despite of its proposed tumor suppressor role, no significant differences in SIRT6 mRNA levels among normal bladder urothelium, non-muscle invasive, and muscle invasive urothelial carcinoma were noted in the two largest bladder cancer gene expression datasets available in Oncomine™. We therefore studied the expression and function of SIRT6 in muscle invasive urothelial carcinoma of the bladder. Immunohistochemistry studies of SIRT6 on radical cystectomy samples showed a dramatic decline of SIRT6 expression when bladder cancer progressed from T2 to T4. Functional study with bladder cancer cell lines confirmed its role in inhibiting glycolysis and cell proliferation. Reducing SIRT6 with siRNA, however, did not sensitize bladder cancer cells to drug induced DNA damage. The differential expression patterns of SIRT6 amongst different T stages of muscle invasive bladder cancers indicate less reliance on glycolysis when urothelial carcinoma invades deeper through the bladder and into the adjacent tissues.

Keywords: SIRT6, bladder cancer, glycolysis, metabolism

Introduction

Bladder cancer is the fifth most common cancer in the United States. Approximately 74,690 new cases (56,390 men and 18,300 women) and 15,580 deaths (11,170 men and 4,410 women) from bladder cancer would occur in the United States in 2014 [1]. Most of these deaths are due to metastatic diseases from muscle invasive bladder cancer. Unlike most other solid tumors, there is limited success of targeted agents in treating metastatic bladder cancer [2-5]. After first-line chemotherapy with GC (gemcitabine and cisplatin) or MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin), no standard second-line regimens have been established and the median overall survival for metastatic bladder cancer is merely 13-15 months [6, 7]. There is an urgent need to develop novel therapies to improve the outcome of patients with metastatic urothelial carcinoma of the bladder.

Reprogramming of energy metabolism has emerged as a new hallmark of cancer. Understanding the metabolic pathways these cancer cells are dependent on could provide new treatment opportunities. Recent insights into SIRT6, a member of the class III histone deacetylases, have put it in the center stage of regulating aerobic glycolysis or the Warburg effect, a hallmark of cancer metabolism [8, 9]. Through studies of SIRT6 knockout mice and SIRT6-/-mouse Embryonic Stem (ES) cells, Zhong et al. showed that SIRT6 corepressed HIF-1a by deacetylating histone 3 lysine 9 (H3K9) at the promoter of key glycolytic genes (glucose transporter type1/Glut-1, phosphofructokinase-1/PFK-1, aldolase C/ALDOC, pyruvate dehydrogenase kinase, isozyme 1/PDK1, and lactate dehydrogenase/LDH) [9]. In addition to inhibiting glycolysis, SIRT6 was shown to facilitate DNA repair [10-12], maintain genomic stability [13, 14], and attenuate inflammation through damping NF-κB-dependent gene expression [15]. Overexpression of SIRT6 in human fibroblast stimulated DNA double strand break repair through both the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways [11]. Overexpression of SIRT6 in fibrosarcoma, cervi-

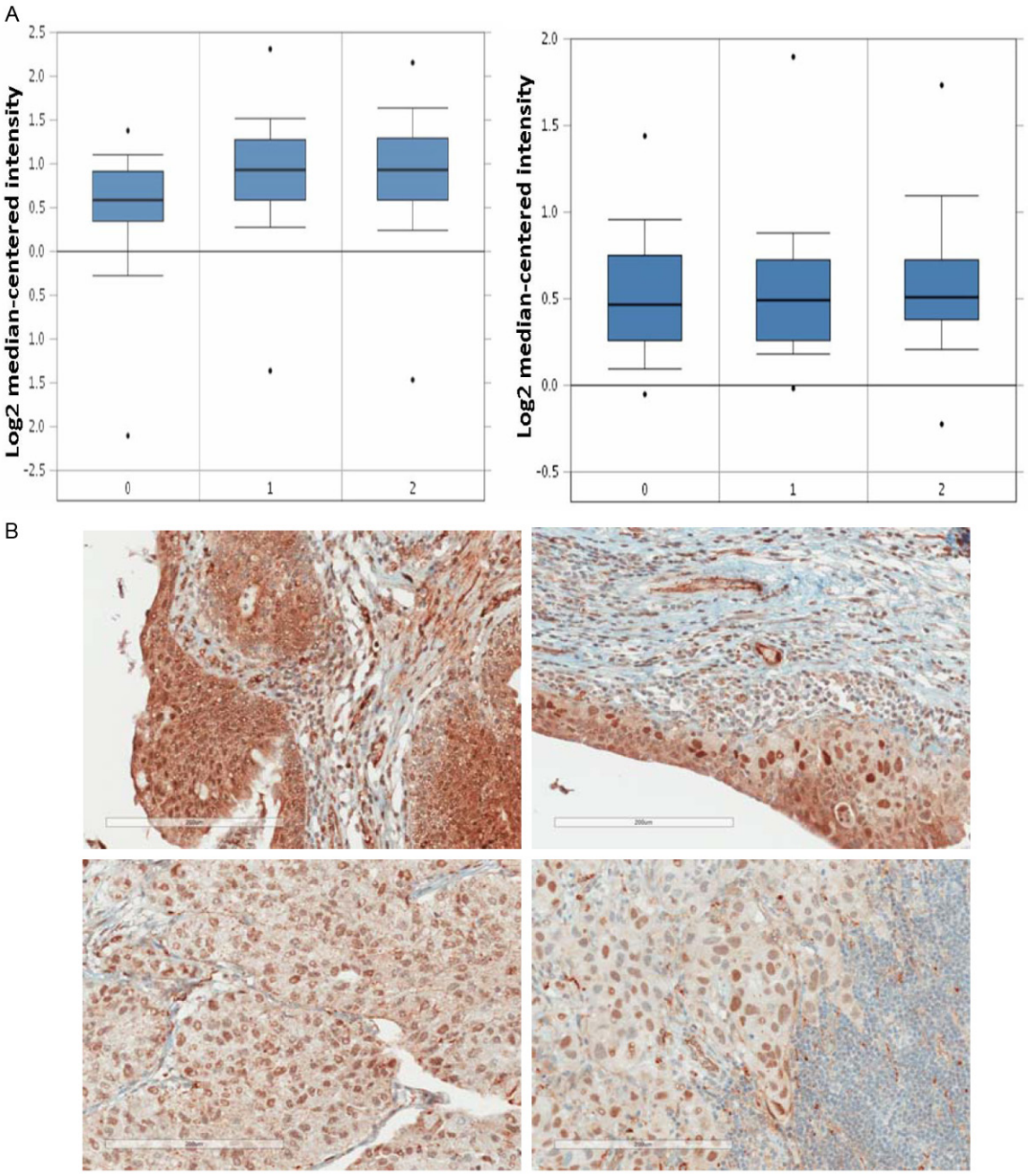


Figure 1. Expression of SIRT6 in urothelial carcinoma of the bladder. A. Relative abundance of SIRT6 mRNA in Oncomine™ *Sanchez-Carbayo Bladder 2* dataset with human genome U133A array on 157 samples (left) and *Lee Bladder* dataset with illumina Human-6 v2 expression beadchip on 256 samples (right). The log2 median-centered intensities are shown for 3 groups: normal urothelium (0), muscle invasive (1) and superficial/non muscle invasive urothelial carcinoma of the bladder (2). For each group, the bold horizontal line represents the median, whereas the error bars represent the 90th and 10th percentile of log2 median-centered intensities. B. Representative picture of SIRT6 IHC nuclear staining (brown) of normal urothelium (upper left panel), carcinoma in situ (upper right panel), T2 urothelial carcinoma (lower left panel), and lymph node metastasis from this T2 urothelial carcinoma (lower right panel). Sections were counterstained with hematoxylin (blue).

cal cancer and breast cancer cell lines led to either p53 or p73 mediated apoptosis [16]. This observation is consistent with the proposed tumor suppressor role of SIRT6 [8].

Here we report a distinct expression pattern of SIRT6 between T2 and T3 or above muscle invasive urothelial carcinoma of the bladder. *In vitro* functional studies of SIRT6 in bladder can-

Table 1. SIRT6 expression in different stages of bladder cancers and their lymph node (LN) metastasis

	Adjacent Normal	T2	T3	T4
SIRT6 positivity	61/61 (100%)	33/52 (63%)	2/23 (9%)	0/6 (0%)
LN Metastasis	Not Applicable	1/3 (33%)	1/13 (8%)	0/3 (0%)

cer cell lines confirmed its role in inhibiting glycolysis. Knocking down SIRT6 expression in these cell lines, however, does not seem to sensitize them to DNA damaging chemotherapy agents.

Materials and methods

Cell culture

5637 (HTB-9), RT4 (HTB-2) and UM-UC-3[UMUC3] (CRL-1749) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in culture mediums as instructed by ATCC, supplemented with 10% FBS and 1% penicillin.

Western blot

Protein lysate preparation and immunoblotting were performed as described previously [17]. Rabbit anti-SIRT6 polyclonal antibody (Cell Signaling, Boston, MA), rabbit anti-PDK1 polyclonal antibody (Cell Signaling, Boston, MA), mouse anti-Glut1 monoclonal antibody (Abcam, Cambridge, MA), and mouse anti-γH2AX monoclonal antibody (Millipore, Billerica, MA) were used as the primary antibodies. Tubulin was used as the loading control (Sigma, St. Louis, MO). Immunoreactive protein was detected using ECL reagents (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Transfection of siRNA and plasmids

SIRT6 siRNA pools (4 target specific siRNAs) were purchased from Santa Cruz (Santa Cruz, CA). Plasmids encoding SIRT6, pcDNA3-SIRT6 was kindly provided by Dr. Edward Seto. Cells were seeded in 6-well plates and transfected with 5 μM siRNAs or 2 μg of plasmids. 6 hours post transfection, the medium was removed and cells were incubated with fresh medium 1-2 days before further treatment. Scrambled siRNA (Dharmacon, Pittsburgh, PA) or an empty pcDNA3 vector (Invitrogen, Carlsbad, CA) was used as control.

Cell proliferation assay

2×10^5 of 5637 or UMUC3 cells were seeded in 6-well plate, and then transfected with siRNA or plasmids. Cell count was initiated 1 day after the transfection. Cell viability is determined by trypan blue and the numbers of viable cells were counted every day for 4 days with Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA). The test was performed in triplicates. The mean and SEM were plotted on the grow curve.

In vitro oxygen consumption study

Extracellular Flux System 24 Instrument (Seahorse XF24, Seahorse Bioscience, North Billerica, MA) was used to assay the oxygen consumption. The rate of oxygen consumption, or OCR, is proportional to mitochondrial respiration. Trypsinized 5637 and UMUC3 cells were re-suspended in grow media. 25,000 cells were plated in each well of a Seahorse XF24 cell culture plate. The cells were grown for 24 h at 37°C. The media was exchanged with unbuffered DMEM XF assay media (Seahorse Bioscience, North Billerica, MA) supplemented with 2 mM glutaMAX, 1 mM sodium pyruvate and 5 mM glucose (pH 7.4 at 37°C), and equilibrated for 30 min at 37°C and ~0.04 % CO_2 before the experiment. Basal Cellular oxygen consumption was monitored before addition of the drugs. Oligomycin (1.25 μM), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (0.4 μM), and Rotenone (1.8 μM) were injected into the XF24 sequentially. Oligomycin blocks ATP synthesis at mitochondrial Complex V and increases cellular glycolysis. FCCP acts as an uncoupling agent and increases mitochondria respiration since proton leakage by FCCP is overcome by cells to consume more O_2 to pump the excess protons across the mitochondrial membrane. Rotenone inhibits mitochondrial Complex I and thereafter the consumption of O_2. Each data point represents mean ± SEM of 4 independent experiments. Data is normalized to total protein.

Immunohistochemistry (IHC) for SIRT6

Tissue sections of neutral-buffered 10% formalin fixed paraffin embedded (FFPE) bladder cancer blocks were obtained from Moffitt cancer center total cancer care tumor bank (protocol

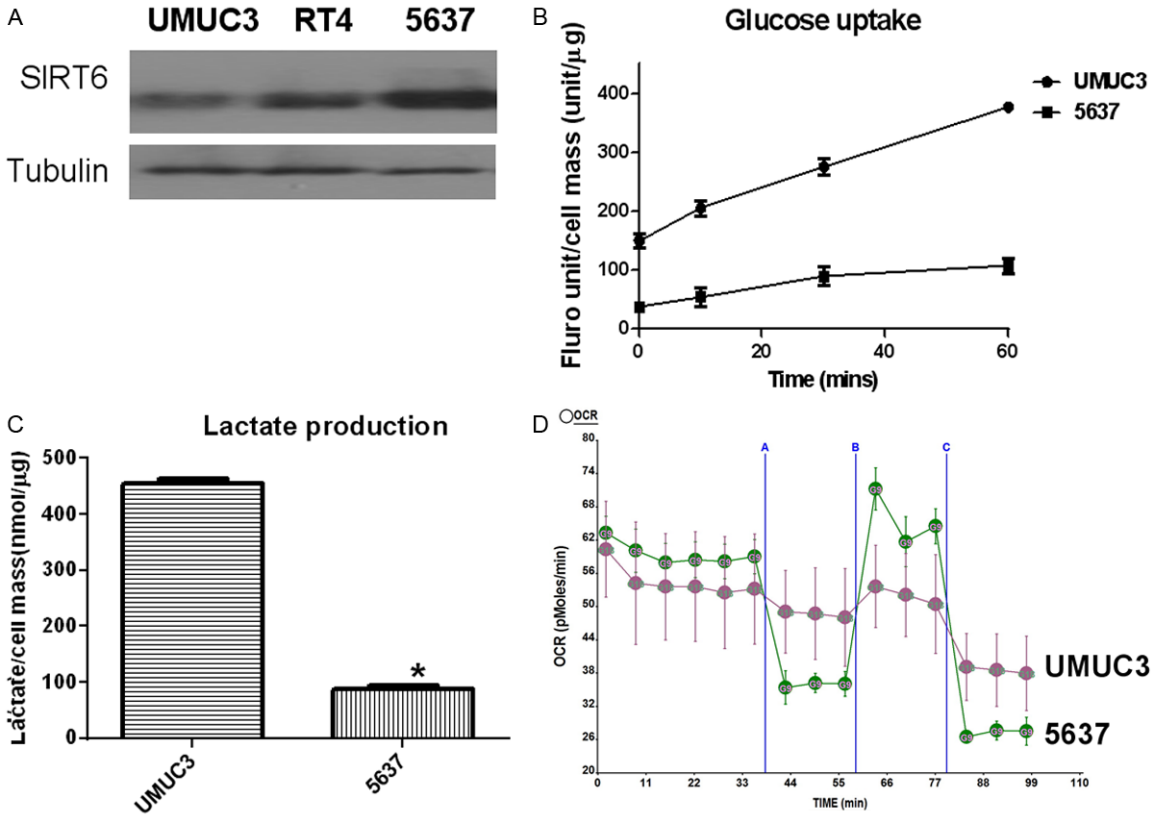


Figure 2. Cell lines with higher SIRT6 have lower aerobic glycolysis and higher mitochondria oxygen consumption. (A) Western blots comparing SIRT6 expression in bladder cancer lines, UMUC3, RT4 and 5637. (B) Negative correlation between SIRT6 levels and glucose uptakes. (C) Negative correlation between SIRT6 levels and lactate productions. (D) Positive correlation between SIRT6 level and mitochondria oxygen consumption was detected with XF24 SeaHorse Analyzer. Each column represents the mean and SEM of 3 independent experiments. (A) treated with oligomycin, (B) treated with FCCP, and (C) treated with rotenone $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

13.06.0037 Liberty IRB). Routine hematoxylin and eosin stained sections and deparaffinized four-micron thick sections were obtained. SIRT6 IHC with rabbit polyclonal anti-SIRT6 (Abcam, Cambridge, MA) was performed at Moffitt Cancer Center's Tissue Core with the standard antigen retrieval method using the avidin-biotin method. The SIRT6 IHC positivity and intensities were reviewed by a genitourinary pathologist. Positive and negative controls were evaluated appropriately for each procedure. Immunostaining for SIRT6 was considered positive if the calculated H score is 10 or above.

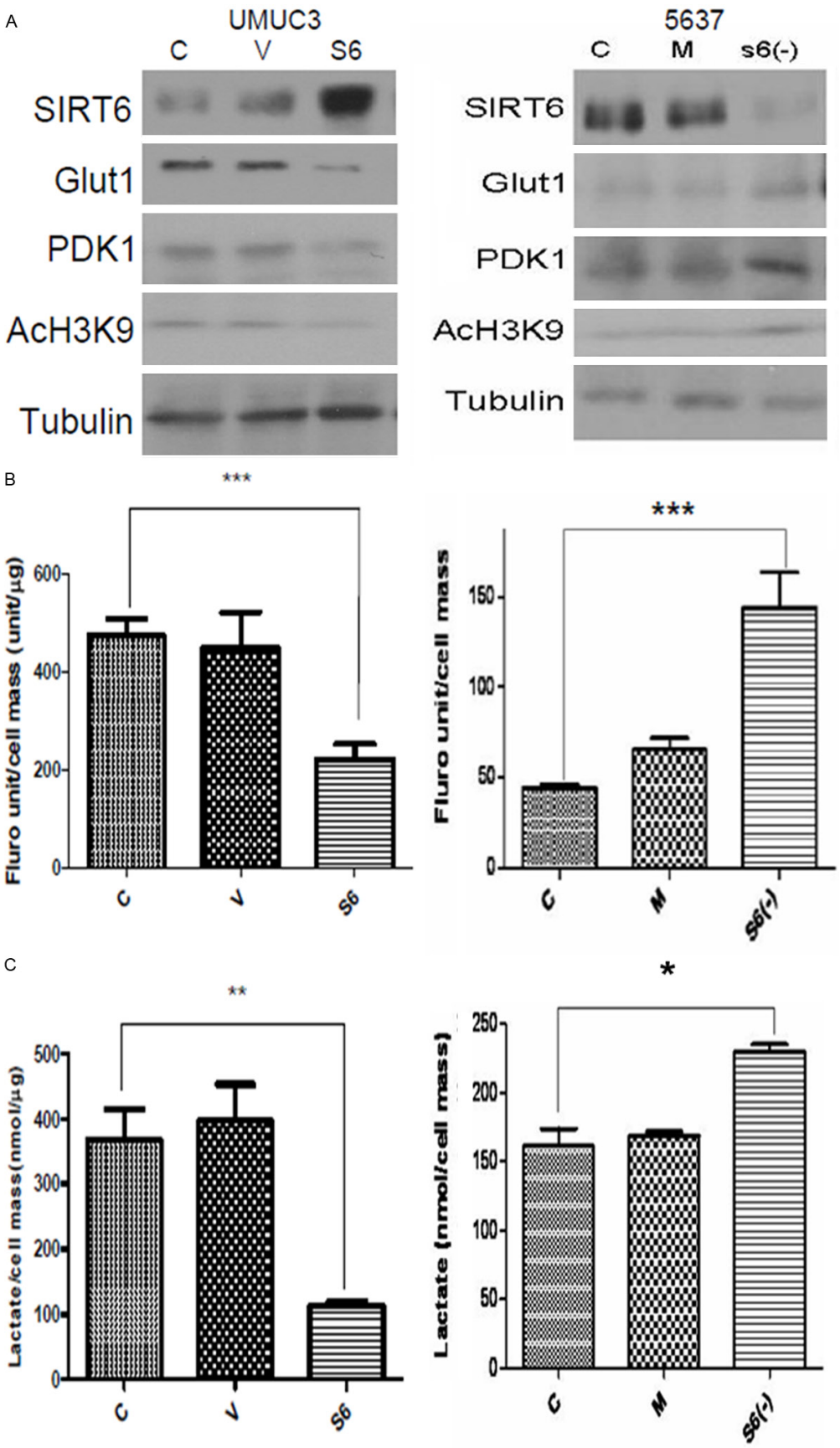
Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.5 software. For one-way ANOVA, Tukey's multiple comparison test was used. T-test was used for two-group comparisons. Data derived from at least 3 independent experiments were shown as means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

SIRT6 expression decreases with progression of bladder cancer from T2 to T4

Inconsistent with its proposed tumor suppressor role, the 2 largest bladder cancer gene expression datasets available in Oncomine did not reveal significant differences in SIRT6 mRNA levels among normal bladder urothelium, superficial/non muscle invasive, and infiltrating/muscle invasive urothelial carcinoma of the bladder (**Figure 1A**) [18, 19]. We therefore compared SIRT6 expression at the protein levels with IHC on FFPE slides of 81 cases of radical cystectomy with lymph node dissection. Immunoreactivity for SIRT6 was found primarily


A
UMUC3
C
V
S6
SIRT6
Glut1
PDK1
AcH3K9
Tubulin
5637
C
M
s6(-)
SIRT6
Glut1
PDK1
AcH3K9
Tubulin
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***
Fluro unit/cell mass (unit/μg)
600
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Figure 3. Comparisons of glucose uptake and lactate production after over expressing SIRT6 in UMUC3 or reducing Sirt6 in 5637. A. Western blots comparing levels of SIRT6, Glut1, PDK1 and acetylation of histone 3 lysine 9 (AcH3K9) in UMUC3 (left) and 5637 (right) cells. B. Glucose uptake assay. C. Lactate production assay. (C) untransfected control, (V) pcDNA3 vector transfected, (S6) pcDNA3-SIRT6 transfected, (M) scrambled/mock siRNA transfected, (S6(-)) SIRT6 siRNA transfected. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

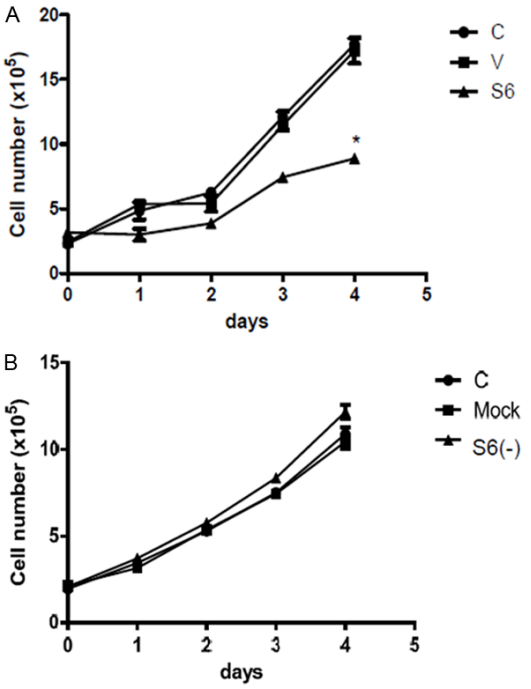

Figure 4. Impacts of SIRT6 expression on cell proliferation. A. Overexpression of SIRT6 decreased the proliferation of UMUC3 cells. B. Reducing SIRT6 in 5637 slightly increased its proliferation. (C) untransfected control, (V) pcDNA3 vector transfected, (S6) pcDNA3-SIRT6 transfected, (M) scrambled/mock siRNA transfected, (S6(-)) SIRT6 siRNA transfected. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

in the nuclei. SIRT6 was expressed in all the adjacent normal bladder urothelium that were present in the tumor blocks. As shown in **Table 1**, positive expression of SIRT6 was seen in 63% of T2 (*invades muscularis propia*), 9% in T3 (*invades perivesical tissue*), and 0% in T4 (*invades prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall or abdominal wall*) urothelial carcinoma of the bladder. The distributions and positivity of the 19 available lymph node metastasis cases were also shown. Among the 2 SIRT6 lymph node positive cases, both primary tumors (one T2 and one T3) were positive for SIRT6 in more than 60% of the tumor cells (**Figure 1B**). Of note, SIRT6 is widely expressed in the matched normal eipithelium and carcinoma in situ (**Figure 1B**). The loss of SIRT6 expression in T3 and T4 urothelial carcinoma indicate that the metabolic pathways are significantly different between early (T2) and late stage (T3, T4) muscle invasive urothelial carcinoma of the bladder.

SIRT6 suppresses glycolysis in bladder cancer cells

To role of SIRT6 in regulating aerobic glycolysis was studied in bladder cancer cell lines. Among UMUC3, RT4 and 5637 cell lines, UMUC3 has the lowest SIRT6 expression whereas 5637 has the highest expression (**Figure 2A**). Consistent with its role in down regulating glycolysis, significantly higher levels of glucose uptake and lactate production were observed in UMUC3 compared to 5637 (**Figure 2B** & **2C**). Real-time oxygen consumption in 5637 and UMUC3 cells was then measured with SeaHorse XF24 analyzer. As expected, oxygen consumption was inhibited after treatments with oligomycin or rotenone; and increased after treatments with FCCP (**Figure 2D**). Compared to UMUC3, such changes in oxygen consumption are more noticeable in 5637, which is consistent with the less glycolysis, higher SIRT6 expression and function observed in 5637.

To test further the role of Srit6 in regulating bladder cancer cells' glycolysis, SIRT6 was overexpressed in UMUC3 with transfection of pcDNA3-SIRT6 and reduced with transfection of SIRT6 siRNA in 5637 cells. Compared to untransfected and empty vector transfected UMUC3 cells, overexpression of SIRT6 reduced H3K9 acetylation and decreased expression of key proteins in glycolysis, Glut1 and PDK1 (**Figure 3A**). Glut1 facilitate the uptake of glucose, whereas PDK1 inactivates/phosphorylates pyruvate dehydrogenase and diverts pyruvate from the Krebs cycle to lactate formation. As expected, the decrease in Glut 1 and PDK1 after overexpressing SIRT6 led to decreased glucose uptake and lactate production (**Figure 3B** & **3C**). Inhibiting SIRT6 expression with siRNA in 5637 cells increased H3K9 acetylation, Glut1 and PDK1 levels, and enhanced glucose uptake and lactate production (**Figure 3**). Taken together, these data show that SIRT6

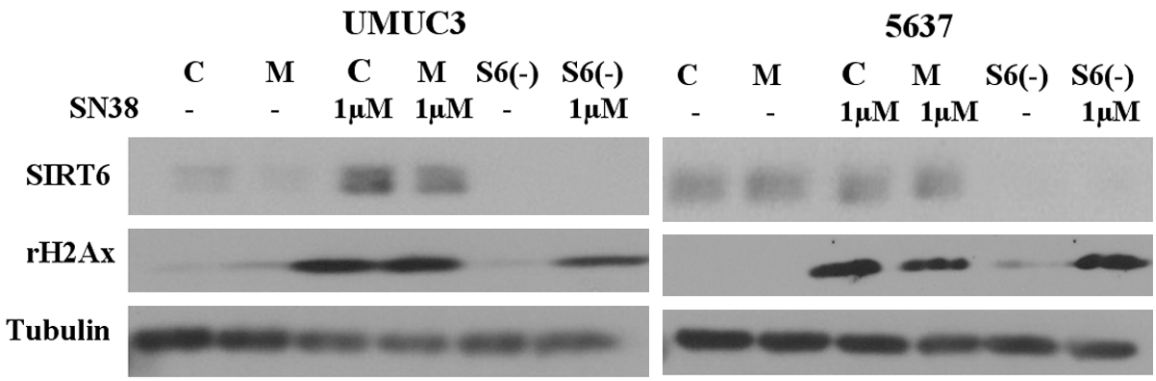

Figure 5. Reducing SIRT6 levels with siRNA does not sensitize bladder cancer cells to SN38 induced DNA damage in untransfected control (C), scrambled/mock siRNA transfected (M) and SIRT6 siRNA transfected [S6(-)] cells.

suppresses glycolysis in urothelial carcinoma of the bladder.

Over expression of SIRT6 inhibits the proliferation of bladder cancer cells

The downstream effects of altering SIRT6 expression on cell proliferation were assessed with trypan blue cell viability and proliferation assay. Consistent with its proposed role as a tumor suppressor, over expression of SIRT6 in UMUC3 cells inhibited its proliferation compared to untransfected and vector transfected cells (**Figure 4A**). No significant apoptosis was observed after SIRT6 overexpression for at least 4 days. This can be attributed to p53 mutation in UMUC3 as suggested previously [16]. Knocking down SIRT6 in 5637 with siRNA only marginally increased the proliferation of 5637 cells (**Figure 4B**). Given these are transient transfections, we checked the expression levels of SIRT6 at day 4 and confirmed that SIRT6 was still over expressed in UMUC3 or knocked down in 5637 (data not shown). Of note, the change in cell proliferation after knocking down SIRT6 in 5637 is not as significant as over expression SIRT6 in UMUC3. Like UMUC3, 5637 has mutant p53. The high basal level of SIRT6 in 5637 and its fast proliferation rate (doubling time around 48 hours) indicate that some bladder cancer cells utilize metabolic pathways other than glycolysis to sustain its growth.

Knock-down of SIRT6 did not sensitize bladder cancer cell lines to DNA damaging drugs

SIRT6 has been reported to facilitate double strand DNA damage repair during oxidative stress and replicative senescence. We therefore studied whether knocking down SIRT6 can sensitize bladder cancer cells to drug induced DNA double strand breaks. SN38 is the active metabolite of irrinotecan, a potent topoisomerase I inhibitor that has good *in vitro* activity but modest clinical activity in bladder cancer. The level of DNA damage is reflected by γH2AX, a well-established marker for DNA double strand breaks. As shown in **Figure 5**, treatment with 1 μM SN38 led to DNA damage in both UMUC3 and 5637 cells. Compared to untransfected control and mock siRNA transfected cells treated with SN38, knocking down SIRT6 did not increase further DNA double strand breaks. Similar results were seen with cisplatin treatment. Compared to cisplatin, treatment with SN38 led to more significant cell death and DNA double strand breaks with *in vitro* cytotoxicity assays (data not shown).

Discussion

Previous studies have shown divergent pathways underlying the tumorgenesis of superficial and muscle invasive urothelial carcinoma of the bladder with mutations in H-RAS and FGFR3 in superficial papillary neoplasm; whereas loss of PTEN and cell cycle G1 phase check points like RB1 and p53 in muscle invasive urothelial carcinoma of the bladder [20-22]. Here we showed a loss of expression of SIRT6 when muscle invasive urothelial carcinoma of the bladder progresses from T2 to T4 stage. Of note, muscle invasive bladder cancer has always been studied and treated as a single entity. To the best of our knowledge, our data are the first to report differential expression pattern of SIRT6 among different T stages of

cancer, indicating more reliance on glycolysis when bladder cancer invades deeper through the bladder and into the adjacent tissues.

Recent insights into SIRT6 function have put it in the center stage of regulating aerobic glycolysis, a hallmark of cancer metabolism [8, 9]. Knocking down SIRT6 in immortalized mouse embryonic fibroblast led to oncogene-independent, glycolysis-dependent tumorgenesis [8]. Unlike colon cancer and pancreatic cancer, muscle invasive bladder cancer, particularly at T2 stage tolerates SIRT6 expression. This can be attributed to its high frequency of p53 mutations [16, 20, 22]. More importantly it indicates less reliance of T2 bladder cancer on aerobic glycolysis to sustain its rapid growth. Chromatin regulatory genes were found to be more frequently mutated in urothelial carcinoma than in any other common cancer studied [20]. Although SIRT6 likely functions as a tumor suppressor, no mutations in SIRT6 have been reported. Based on the TCGA database, copy number variations in SIRT6 are also rare. The function of SIRT6 is likely regulated at the expression level, which can be transcriptional and or post transcriptional [23].

Although studies on normal human fibroblast and non-bladder cancer lines support a role of SIRT6 in facilitating DNA double strand break repair, inhibiting SIRT6 expression in bladder cancer lines do not sensitize them to drug induced DNA damage. Compared to TNM matched chemotherapy naïve cases, we also did not observe notable increase in SIRT6 levels in 11 cases that are refractory (without tumor down staging) to platinum based neoadjuvant chemotherapy (data not shown). Although the number of cases is too small to be statistically meaningful, this observation is consistent with our *in vitro* data that altering SIRT6 expression levels is unlikely to affect bladder cancer cells' sensitivity to DNA damaging agents.

In comparison to genetic rewiring of the metabolic pathway to suit for the uncontrolled growth of cancer, epigenetic regulations provides a more efficient way for cancer cells to adapt to the changing environmental factors like hypoxia, nutrient deprivation and DNA damage. Previous studies of cancer metabolism have also led to the development of a wide array of therapeutic agents that target key enzymes in glycolysis, lipid synthesis, nucleic acid synthesis or amino acid metabolism. Some of these agents like 2-deoxyglucose and dichloroacetate have been tested in early phase clinical trials [24-26]. Further studies on the function and regulation of SIRT6 expression as well as the glycolytic and non-glycolytic metabolic pathways in bladder cancer would lead to the development of novel biomarkers and therapeutic targets for this lethal disease.

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Disclosure of conflict of interest

None.

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