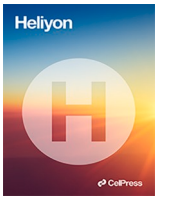
Heliyon 9 (2023) e16004

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Epigenetic regulation of human WIF1 and DNA methylation situation of WIF1 and GSTM5 in urothelial carcinoma

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ARTICLE INFO

Keywords:
WNT inhibitory factor 1 (*WIF1*)
Epigenetic regulation
Bladder cancer
DNA promoter hypermethylation
Tumor biomarker

ABSTRACT

WNT inhibitory factor 1 (*WIF1*) is known to function as a tumor suppressor gene; it inhibits oncogene activation by preventing WNT signaling. This study investigated the epigenetic regulation of WIF1 gene in bladder cancer. We observed a positive relationship between WIF1 mRNA expression and survival probability of bladder cancer patients. The WIF1 gene expression could be enhanced by DNA demethylation drug 5-aza-2′-deoxycytidine (5-aza-dC) and histone deacetylase inhibitor trichostatin A (TSA), suggesting that epigenetic modifications could regulate WIF1 gene expression. Overexpression of WIF1 inhibited cell proliferation and migration in 5637 cells, confirming the tumor suppressor role of WIF1. 5-Aza-dC dose dependently increased WIF1 gene expression while reducing DNA methylation level, suggesting that reversing WIF1 DNA methylation could activate its gene expression. We collected the cancer tissues and urine pellets of bladder cancer patients and only urine pellets from non-bladder cancer volunteers for DNA methylation analysis, but the methylation level of *WIF1* gene −184 to +29 did not differ between patients and controls. We also analyzed glutathione *S*-transferase Mu 5 (GSTM5) gene methylation level because GSTM5 DNA hypermethylation was suggested to be a tumor biomarker in our previous study. It confirmed a higher GSTM5 DNA methylation in bladder cancer patients than in controls. In summary, this study suggests that the 5-aza-dC activated WIF1 gene which showed an anti-cancer effect, while *WIF1* promoter −184 to +29 did not provide a suitable methylation assay region in clinical samples. In contrast, *GSTM5* promoter −258 to −89 is a useful region for DNA methylation assay because it shows a higher methylation level in bladder cancer patients.

1. Introduction

Bladder cancer is a common urogenital cancer and one of the top ten cancers worldwide [1,2]. According to GLOBOCAN data, an estimated 573,278 people were diagnosed with bladder cancer in 2020, accounting for about 3% of all new cancer diagnoses [1,3],

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https://doi.org/10.1016/j.heliyon.2023.e16004
Received 21 September 2022; Received in revised form 26 April 2023; Accepted 28 April 2023
Available online 1 May 2023

Abbreviations

5-aza-dC	5-Aza-2′-deoxycytidine
BSP	Bisulfite-sequencing polymerase chain reaction
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione *S*-transferase
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
IRB	Institutional Review Board
TSA	Trichostatin A
TSS	Transcription start site
WIF1	WNT inhibitory factor 1

while 212,536 people died of bladder cancer, accounting for about 2.1% of all cancer mortality [3]. The incidence of bladder cancer in men is approximately three times higher than in women [3]. Today, study on bladder cancer diagnosis and treatment is still important.

The WNT/β-catenin signaling pathway is a family of proteins that regulate cell proliferation and apoptosis, thereby mediating the occurrence and development of cancer by abnormal regulation of the transcription factor β-catenin [4]. Therefore, new drug development targeting WNT/β-catenin pathway is ongoing [5]. In contrast to WNT, Wnt inhibitory factor 1 (WIF1) is a secreted protein that inhibits the WNT/β-catenin signaling pathway by binding to WNT proteins [6]. Downregulation expression of WIF1 mRNA was found in various cancers [7]. It is known that the balance of WNT and WIF1 is disturbed by WIF1 downregulation, which may be due to epigenetic modification such as DNA methylation [8], non-coding RNA interference [9], histone modification [10] and mRNA methylation [11]. Epigenetics is defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence [12]. Most epigenetic changes mainly include DNA methylation and histone modifications. To date, the WIF1 gene has been demonstrated to be down-regulated in certain tumors, such as lung [13], gastrointestinal [14], breast [15], endometrial adenocarcinoma [16] and bladder cancers [17], through the *WIF1* promoter CpG methylation, and silences the WIF1 expression.

WIF1 is considered to be a tumor suppressor gene that inhibits the transcription of downstream oncogenes by blocking the WNT/β-catenin signaling pathway. In our previous study, it was found that after treatment with inorganic arsenic, the normal human urothelial cell line SV-HUC1 showed increased migration, and with the increase of treatment time, the expression of WIF1 mRNA decreased, and the DNA methylation level of WIF1 gene increased significantly [18]. Glutathione *S*-transferase Mu 5 (GSTM5) is also a tumor suppressor gene in bladder cancer and its DNA methylation ratio could be a tumor biomarker in our previous study [19]. The GSTM5 DNA methylation ratio of bladder cancer tissues is significantly higher than that of normal subject urine pellets [19]. Therefore, the purpose of this study is to explore the relationship between the DNA methylation and histone acetylation of WIF1 gene and bladder cancer cells. Various human bladder cancer cell lines were treated with the DNA demethylation drug 5-aza-2′-deoxycitidine (5-aza-dC) and the histone deacetylase inhibitor trichostatin A (TSA), the expression of WIF1 gene and protein was observed, and the methylation of WIF1 gene promoter was analyzed. The DNA methylation levels of WIF1 were compared among bladder cancer tissues, bladder cancer urine pellets and non-bladder cancer urine pellets; in addition, the GSTM5 was rechecked in these clinical samples.

2. Materials and methods

2.1. Cell culture condition

Human urinary tract epithelial cell line SV-HUC-1 and human bladder carcinoma cell lines RT4, 5637, and T24 were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). SV-HUC-1 was cultured in Ham's F12 medium with 7% fetal bovine serum (FBS); RT4 and T24 were cultured in McCoy's 5a medium with 10% FBS; 5637 were cultured in RPMI-1640 medium with 10% FBS. All culture mediums contained 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated in a CO_2 incubator at 37 °C, with 5% CO_2 and 95% filtered air.

2.2. RNA extraction

After washing the cells by PBS and lysing cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), the RNA was collected according to the manufacturer's instructions. The concentration and purity of the RNA was measured by a NanoDrop 1000 spectrophotometer. Purity was verified using the ratio of the OD260/OD280, OD260/OD230, and gel electrophoresis analysis.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR analysis

WIF1 gene was selectively targeted and GAPDH gene was the internal control. Before PCR, cDNA was prepared by reverse transcription on 2 μg of total RNA by 1.5 μM random hexamer and RevertAid™ reverse transcriptase (Fermentas, Waltham, MA, USA); total reaction volume was 20 μl. In PCR assay, 1 μl of the cDNA reaction mixture was used with human *WIF1*-specific primers (5′CACTCGCAGATGCGTCTTTCT3′, 5′CCAACCGTCAATGTCCCTCT3′, product size 251 bp) or *GAPDH*-specific primers (5′CAAGGTCATCCATGACAACTTTG3′, 5′GTCCACCACCCTGTTGCTGTAG3′, product size 496 bp). The PCR products were analyzed by 1–2% agarose gel electrophoresis. In quantitative real-time PCR assay, each reaction included 1 μl of cDNA mix, 250 nM forward and reverse primers, and 2X Fast SYBR-Green PCR Master mix (Applied Biosystems, Thermo Fisher Scientific, MA, USA). A total of 10 μl reaction volumes were used with the specific primers listed as follows: human *WIF1* forward, 5′GTTCAAGTTGGTTTCCCATGTC3′ and reverse, 5′CGCAGATGCGTCTTTCATTAC3' (196 bp), and *GAPDH* forward, 5′GTCTCCTCTGACTTCAACAGCG3′ and reverse, 5′ACCACCCTGTTGCTGTAGCCAA3′ (130 bp). The StepOne™ System real-time PCR instrument and the StepOne™ System version 2.3 software (both from Thermo Fisher Scientific, Waltham, MA, USA) were used for the experimental setup and data analysis. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method using GAPDH RNA as a reference.

2.4. Western blot

Cell total protein was extracted by PRO-PREP protein extraction solution (iNtRON Biotechnology, Burlington, MA, USA). Fifteen μg of protein were loaded for separation by 8% SDS-polyacrylamide gels electrophoresis and then transferred to polyvinylidene difluoride membranes. Immunodetection was performed using first antibodies against human WIF1 (ab155101, Abcam, Cambridge, UK, 1:20000 dilution), and α-tubulin (GTX112141, GeneTex, Irvine, CA, USA, 1:20000 dilution) as an internal control. Rabbit IgG antibodies coupled to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) were used as the secondary antibodies (1:2500 dilution). Protein expression was visualized by Luminol/Enhancer solution (Thermo Scientific, Waltham, MA, USA) and a luminescence detector. The quantity of each band was determined by the Multi Gauge software version 3.0.

2.5. ELISA of WIF1

The WIF1 ELISA kit ARG81598 was operated according to the manufacturer's instructions (Arigo Biolaboratories, Hsinchu, Taiwan), and the detection range of WIF1 was 23.4–1500 pg/ml. The ELISA analysis tool was calculated using the GainData 4 PL (4 Parameter Logistics) curve (https://www.arigobio.com/elisa-analysis).

2.6. WIF1 overexpression by lentivirus vector

The plasmid DNA of MGC premier cDNA clone for *WIF1* (BC018037) was purchased from Transomic technologies (Huntsville, AL, USA) and was subcloned to pLAS2w.FLuc.Ppuro vector for lentivirus production. The lentiviral vectors for WIF1 overexpression, pLAS2w.*WIF1*–P2A-FLuc.Ppuro and pLAS2w.FLuc.Ppuro (control plasmid) were from the National RNAi Core Facility (Academia Sinica, Taiwan). Lentivirus was prepared following the protocol of the National RNAi Core Facility. Lentivirus infected cells including (i) 5637-Fluc and (ii) 5637-*WIF1*-Fluc were grown in RPMI-1640 supplemented with 1 μg/ml puromycin.

2.7. Extraction of genomic DNA

The clinical sample collection was conducted in accordance with the Declaration of Helsinki 2013, and study protocols were reviewed and approved by the Institutional Review Board (IRB) of Ditmanson Medical Foundation Chiayi Christian Hospital (Chiayi, Taiwan). The approval number is IRB2018092. Bladder tumor tissues and the paired urine pellets from patients (n = 30) with bladder cancer and urine pellets from non-bladder cancer subjects (n = 30) were gathered. All subjects gave their informed consents for inclusion before sample collection. DNA extraction of bladder tumor samples, urine pellets, and culture cells followed the instructions of the DNA extraction kit (Geno Plus Genomic DNA extraction miniprep system, Viogene, New Taipei City, Taiwan).

2.8. Bisulfite conversion of genomic DNA and analysis of DNA methylation level

Five hundred nanograms of genomic DNA were subjected to sodium bisulfite modification using the EZ DNA methylation-Gold™ kit (Zymo Research Corp., Irvine, CA). Human DNA methylation levels were analyzed by bisulfite-sequencing PCR (BSP) method using bisulfite specific primers. The BSP primers for the human *WIF1* gene were forward 5′ GTTGGTTTYGTTAGTTTTGTTAGTTT 3′ and reverse 5′ AACTCCCTCAACCAAAACTATTCC 3′, which amplified −184 to +29 bp of the human *WIF1* gene (213 bp); for human *GSTM5* gene they were forward 5′ ATAGTTAAGTYGTTGTGGATTTAGTAAG 3′ and reverse 5′ RAAACTAAAATCAATAAACCCCCTCC 3′, which amplified −258 to −89 bp of human *GSTM5* gene (170 bp). Next, the PCR products were subcloned into the T&A cloning vector (Yearstern Biotech, Taiwan). To determine the CpG methylation level of the 5′CpG island of each gene, 10 clones of each gene were randomly selected for sequencing.

2.9. Migration assays

It was performed using 24-well Transwell chambers with 8 μm pore membrane (8 μm pore PET filters; EMD Millipore, Burlington, MA). Bladder cancer cells were seeded in the upper chamber at 1×10^4 cells/well (5637-*WIF1*-Fluc or 5637-Fluc) in 0.1 ml of RPMI 1640 serum-free medium. Medium with 10% FBS was placed in the bottom well (0.6 ml/well). After incubation for 24 h at 37 °C in an atmosphere containing 5% CO_2, the medium was discarded. Then cells were fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) at room temperature for 2 min, and then in methanol for 15 min. After PBS wash, cells were stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO) for 5 min. After PBS wash, cells on the inside surface of the Transwell were removed by a cotton swab. The chambers were photographed using a microscope ZEISS Axio Observer A1, and stained cells were dissolved with DMSO for 10 min and then detected for the OD at 580 nm using a microplate reader Molecular Devices SpectraMax 340PC384.

2.10. Wound healing assay

Cells were seeded in 6-well plates. After the cells reached confluence, a wound was made with a 200-μl plastic tip in each well. The wells were then washed twice with PBS to remove cell debris and then incubated with a culture medium. The wound areas were recorded at 0 h and 7 h by taking a photograph in a microscope. The wound areas were analyzed by ImageJ software [20], and the recovery rate of wound area was calculated as 100 - [(wound area of 0 h) - (wound area of 7 h)/(wound area of 0 h)] x 100%.

2.11. Statistical analysis

The values shown are mean ± SE. Data were statistically evaluated by GraphPad Prism 9 student's *t*-test for comparison of the two groups, and for more than two groups we conducted by ANOVA analysis with Dunnett's multiple comparisons test; significant differences are shown as $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. For comparing the sex difference between bladder cancer patients and non-bladder cancer patients, SPSS statistics 25 Pearson's chi-square test software was used.

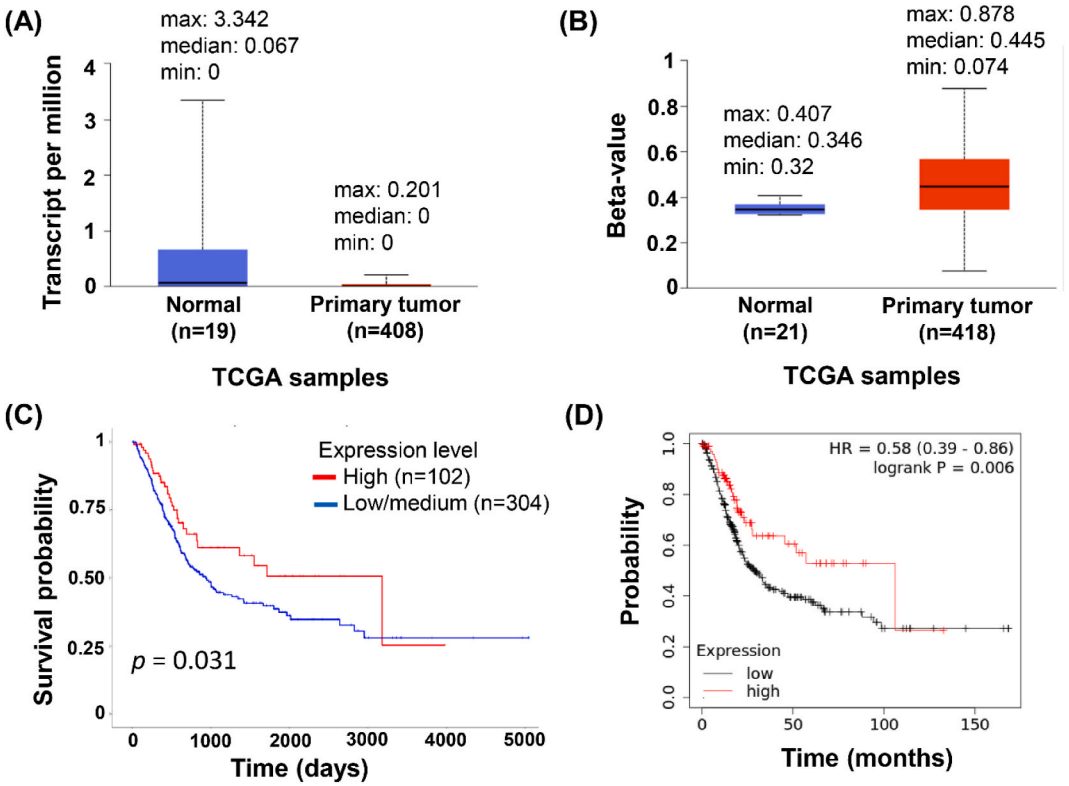

Fig. 1. The database analysis results of WIF1 in bladder cancer patients and normal subjects. (A) The WIF1 mRNA expression values, $p = 0.928$. (B) The WIF1 gene DNA methylation values. The beta value indicates the level of DNA methylation ranging from 0 (unmethylated) to 1 (fully methylated), $p = 5.795 \times 10^{-9}$. (C) Effect of WIF1 expression level on bladder cancer patient survival from Ualcan. n means the subject number in each group. (D) Survival analysis result of Kaplan-Meier analysis from Kaplan-Meier Plotter. The median survival period of WIF1 high expression patients (n = 104) was 106.1 months, and was 28.8 months in low expression patients (n = 300).

3. Results

3.1. WIF1 gene expression and DNA methylation level of bladder cancer patients

The mRNA expression and DNA methylation level were extracted from the publicly available database Ualcan (http://ualcan.path.uab.edu/index.html, accessed date: Aug 7, 2020). The WIF1 mRNA expression was lower in bladder primary cancers than in normal subjects but without statistical significance ($p = 0.928$) (Fig. 1A). In DNA methylation level, the WIF1 DNA methylation level of bladder primary cancers was significant higher than that of normal subjects ($p = 5.795 \times 10^{-9}$) (Fig. 1B). In addition, the Kaplan-Meier survival analysis was extracted from database Ualcan (Fig. 1C) and Kaplan-Meier Plotter (https://kmplot.com/analysis/index.php?p=background, accessed date: Aug 7, 2020) (Fig. 1D). Both results showed that patients with high WIF1 expression had higher survival rates than patients with low/medium WIF1 expression in bladder cancer. All of the above data suggest that *WIF1* was a positive gene for bladder cancer survival, and its mRNA expression was lower, while the DNA methylation level was higher in bladder cancer patients.

3.2. Effect of DNA demethylation and histone acetylation on WIF1 gene expression in bladder cancer cells and normal bladder epithelial cells

In order to understand the epigenetic effect of DNA methylation and histone acetylation on WIF1 gene expression, we used a DNA methylation inhibitor 5-aza-dC and a histone deacetylase inhibitor TSA to assay their effect on WIF1 mRNA expression in various bladder cell lines. First, a grade I bladder cancer cell RT4 was used for this assay. WIF1 mRNA was significantly increased by 1 μM 5-aza-dC and slightly activated by 0.5 μM TSA treatment, and synergistically increased by co-treatment (Fig. 2A). The bladder cancer cell lines of different grades were also analyzed including 5637 (grade II) and T24 (grade III), and a normal urothelial cell line SV-HUC1. Fig. 2B shows that WIF1 mRNA is also increased by 5-aza-dC but not TSA treatment in 5637 cells. In T24 cells, WIF1 mRNA was also increased by 1 μM 5-aza-dC but not 0.5 μM TSA treatment (Fig. 2C). It means that *WIF1* gene is majorly silenced by DNA methylation in RT4, 5637 and T24 cells. Like RT4 cells, when 5-aza-dC was combined with TSA, *WIF1* gene was synergistically activated in 5637 and T24 cells. It suggested that WIF1 DNA methylation might be a major inhibitory factor for gene expression, and histone acetylation

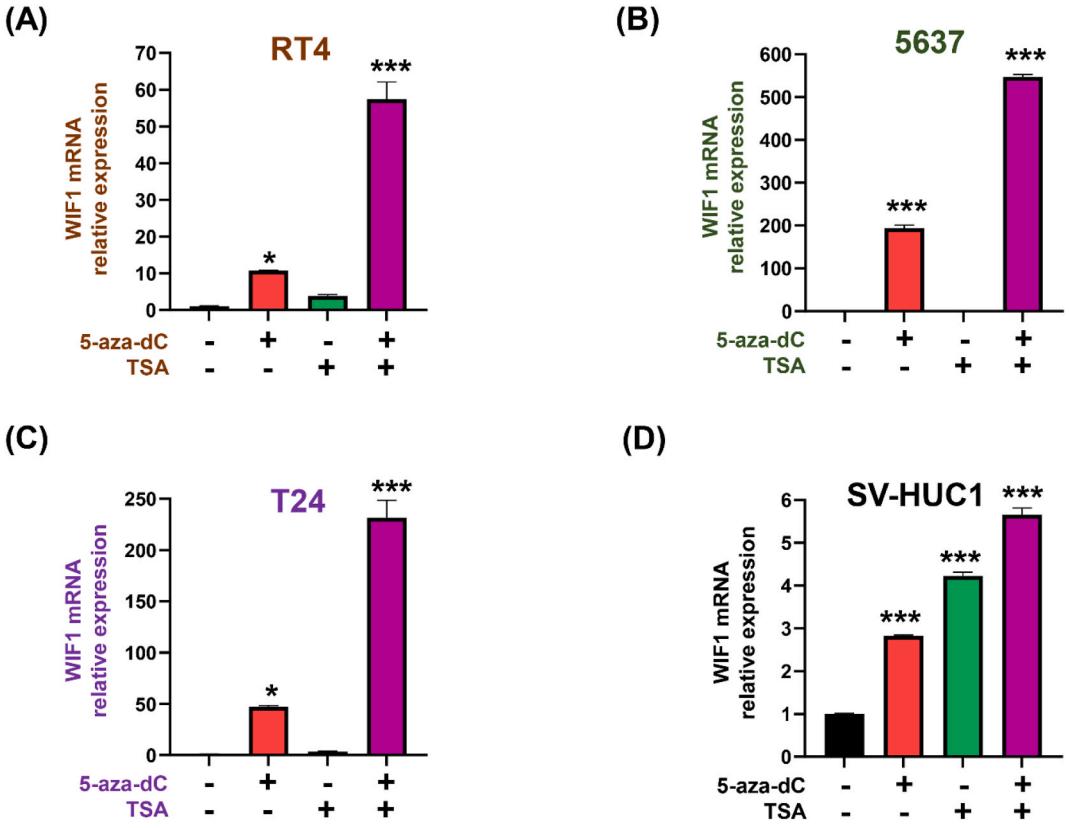

Fig. 2. The WIF1 mRNA expression in human bladder cancer cells and human normal bladder epithelial cells. Cells were treated with 1 μM 5-aza-dC for 72 h and 0.5 μM TSA for 12 h. The quantitative change of WIF1 mRNA expression level was analyzed by real-time PCR using GAPDH as an internal control, and was related to the control group. (A) RT4 cells, (B) 5637 cells, (C) T24 cells, and (D) SV-HUC1 cells. *$p < 0.05$, ***$p < 0.001$.

enhanced WIF1 gene expression after reducing DNA methylation. In the normal urothelial cell line SV-HUC1, although WIF1 mRNA was also increased by both DNA demethylation and histone acetylation (Fig. 2D), the increased fold was much lower than bladder cancer cell lines RT4, 5637, and T24. According to Fig. 2 result, it is suggested that in bladder cancer cells, WIF1 gene could be inhibited by DNA methylation and histone deacetylation, and the inhibitory level is different in different cell lines. It was difficult for the histone deacetylation to activate genes under DNA methylated in RT4, 5637, and T24 cells. But when DNA methylation was decreased by 5-aza-dC, the TSA-activated WIF1 expression is much higher than the 5-aza-dC treatment alone. Even in a higher dose of 5-aza-dC (30 μM), the activation effect of TSA also worked in 5637 and T24 cells (Supplementary Fig. 1), and the lysine 9 acetylation of histone 3 was significantly increased after TSA and 5-aza-dC co-treatment in T24 cells (Supplementary Fig. 2). Conversely, normal urothelium SV-HUC1 cells were simultaneously regulated by DNA methylation and histone deacetylation to inhibit the expression of WIF1, but the inhibition effect was not as strong as that in cancer cells. In summary, the obvious inhibition of *WIF1* gene by DNA methylation might be a biomarker and therapeutic target in bladder cancer.

3.3. Induction of WIF1 protein expression by 5-aza-dC and TSA in bladder cancer cells

In addition to mRNA expression, the WIF1 protein expression was also analyzed. We detected WIF1 protein by Western blot. After 5637 and T24 cells were treated with 1 μM 5-aza-dC for 72 h and 0.5 μM TSA for the last 12 h, the cell lysates were collected for Western blot analysis. It shows that WIF1 protein was slightly increased by combined treatment in 5637 and T24 cells (Fig. 3).

3.4. 5-Aza-dC dose-dependently increases WIF1 gene expression and decreases WIF1 gene methylation levels in T24 cells

In order to confirm the correlation of DNA methylation and gene expression levels of WIF1 gene, the WIF1 mRNA and protein expression and methylation level of the WIF1 gene promoter in the 5-aza-dC-treated T24 cells were analyzed. Because the concentration of 5-aza-dC used in the human endometrial cancer cell literature was 30 μM [16], we observed the changes in WIF1 expression after different 5-aza-dC drug concentrations from 10 μM to 30 μM. After T24 cells were treated with 5-aza-dC for 3 days, the WIF1 mRNA was dose-dependently induced (Fig. 4A and B). In Western blot analysis, WIF1 protein was also slightly induced by 5-aza-dC treatment (Fig. 4C). In DNA methylation assay, the CG content of *WIF1* gene promoter was analyzed by CpG island Finder (http://dbcat.cgm.ntu.edu.tw/, accessed date: Dec 12, 2020). We analyzed 2 regions of *WIF1* promoter, the result showed that there was 50% CG content in −411 to +9 region and 72% in −201 to +35 region (Fig. 4D). Therefore, we designed the BSP primers in the region of −201 to +35. Here, *WIF1* BSP PCR product region contained −184 ~ +29 (213 bp) with 20 CG sites, and the transcription start site (TSS) was set as +1. The methylation level was dose-dependently decreased by 5-aza-dC treatment (Fig. 4E) and the individual methylation patterns were in Fig. 4F. In addition to T24 cells, 5-aza-dC also dose-dependently decreased WIF1 DNA methylation level

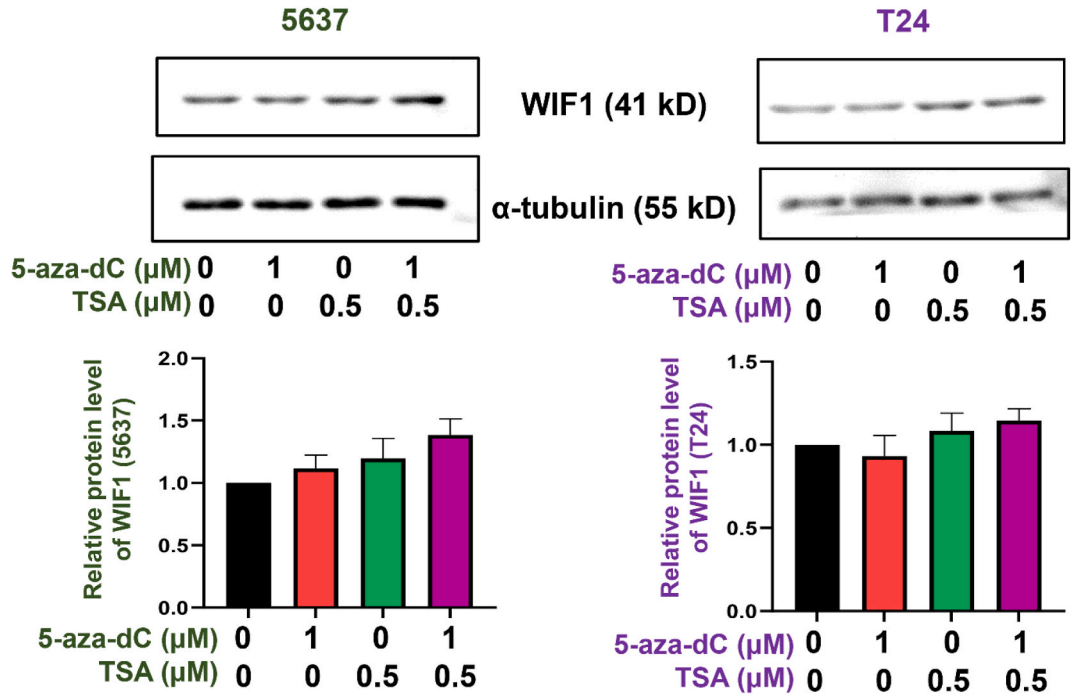

Fig. 3. Analysis of WIF1 protein expression in 5637 and T24 cells. After 5-aza-dC and TSA treatment, total cell lysates were analyzed by Western blot with *anti*-WIF1 and *anti*-α-tubulin antibodies. The quantitative values of WIF1 were normalized by α-tubulin values from 3 experiments.

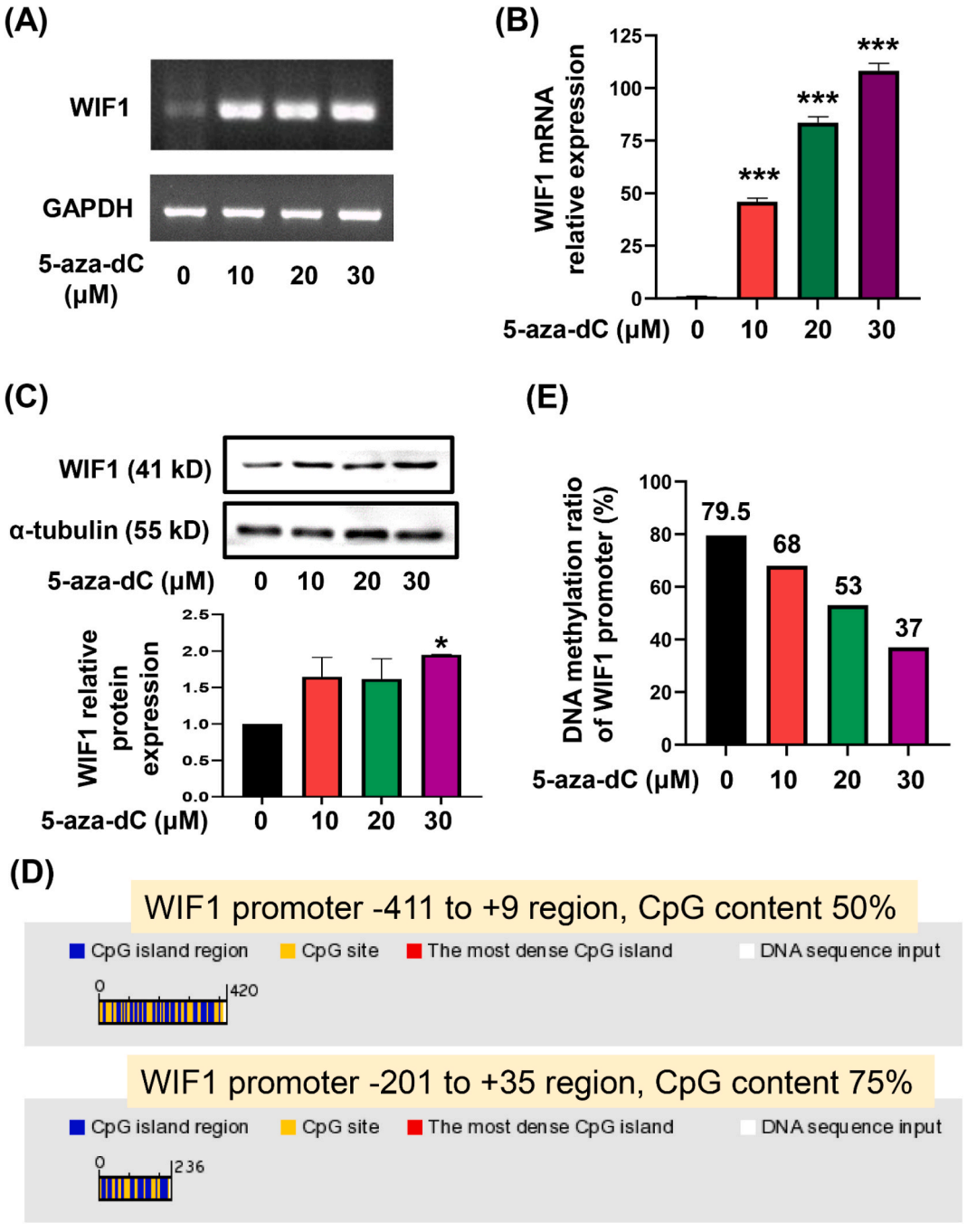

Fig. 4. The WIF1 gene expression and DNA methylation changes after different dose 5-aza-dC treatment in bladder cancer cells. (A) WIF1 mRNA expression by RT-PCR analysis and gel electrophoresis in T24 cells. (B) WIF1 mRNA expression by quantitative real-time PCR analysis in T24 cells. (C) WIF1 protein expression by Western blot analysis in T24 cells. The quantitative WIF1 protein expression was normalized by α-tubulin from 3 experiments. (D) The CpG content and CpG island analysis in two *WIF1* promoter regions. (E) The DNA methylation level of *WIF1* promoter −184 ~ +29 region in T24 cells. The number up each column indicates its DNA methylation level. (F and G) The pattern of methylation was analyzed by bisulfite specific PCR and TA cloning/sequencing in T24 cells (F) and in 5637 cells (G). The number 1–10 in the left means the different clones. A circle represents a CpG site, white circles represent unmethylated, and black circles represent methylated. The black line represents the region of *WIF1* gene −184 ~ +29 with +1 for TSS. *$p < 0.05$, ***$p < 0.001$.

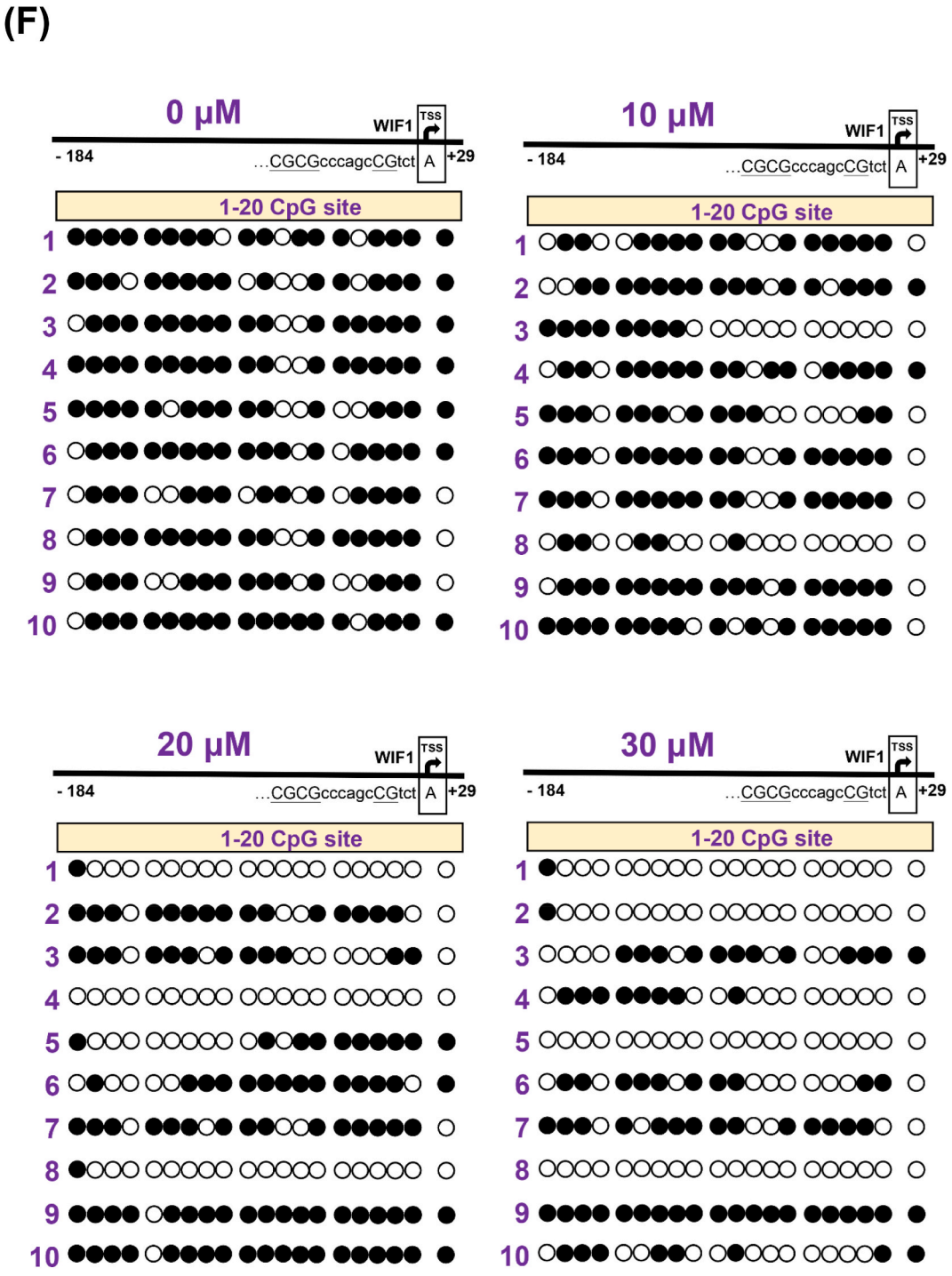

Fig. 4. (*continued*).

from 92.5% (control) to 87% (10 μM), 77% (20 μM) and 69% (30 μM) in 5637 cells (Fig. 4G). It indicates that the WIF1 DNA methylation level in the region of −184 ~ +29 might be related to its gene expression potency.

3.5. Overexpression of WIF1 inhibits cell proliferation and migration of bladder cancer 5637 cells

For the confirmation anti-cancer function of WIF1, we transfected a plasmid with (WIF overexpression) or without (vector control) *WIF1* gene and selected individual stable clones in 5637 cells. The expression of WIF1 mRNA in overexpression 5637 cells (5637-*WIF1*-

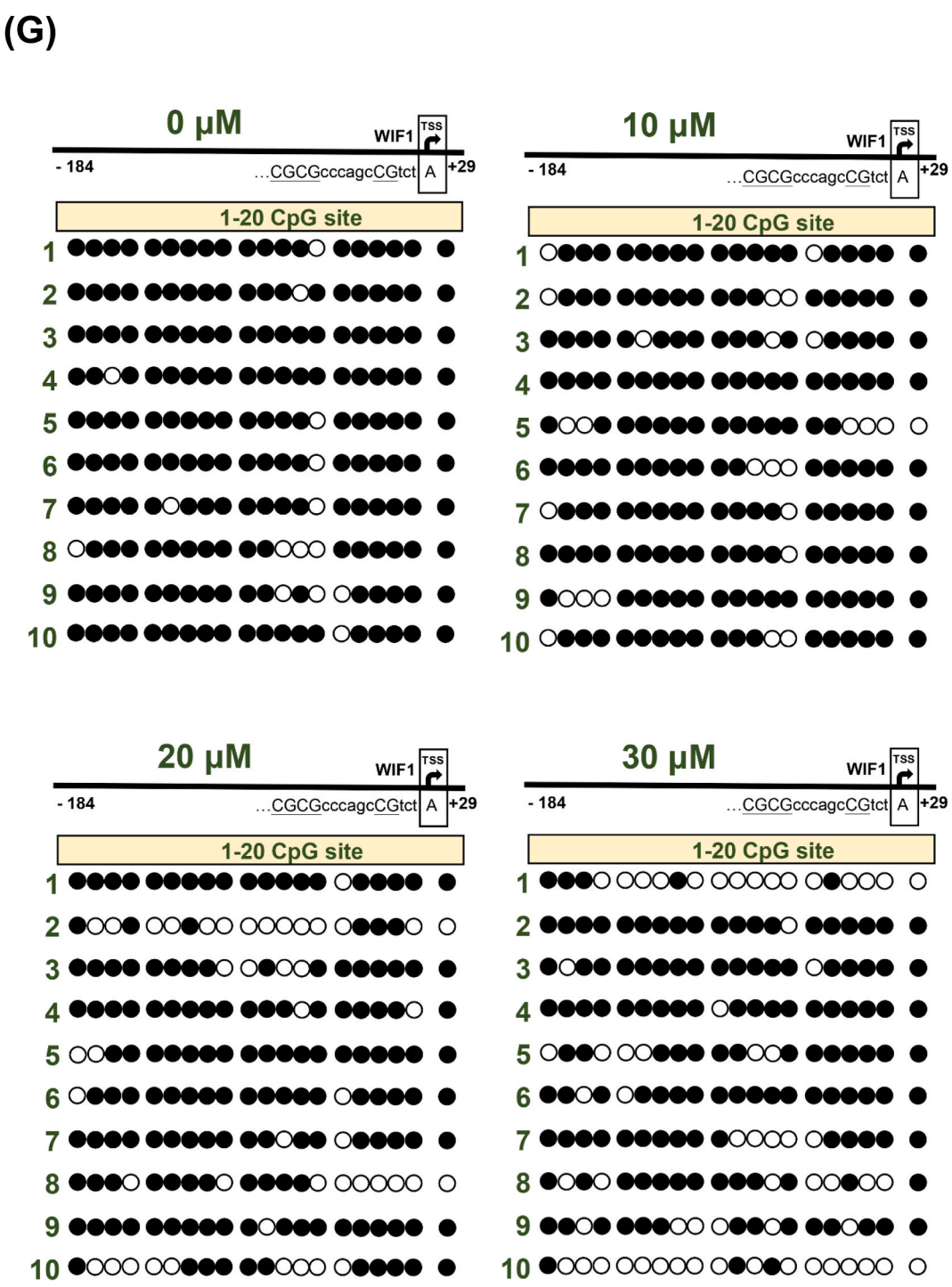

Fig. 4. (*continued*).

Fluc) was detected by RT-PCR (Fig. 5A), and the fold change of WIF1 mRNA expression was quantified by real-time PCR (Fig. 5B). WIF1 overexpression 5637 cells were about 6500 times higher than the vector control 5637 cells (5637-Fluc) at mRNA level. The WIF1 protein was also slightly increased by Western blot analysis (Fig. 5C). Because the WIF1 protein expression was not obvious in Western blot analysis, the cell culture medium was also assayed by ELISA, which showed that the WIF1 protein was 4423 pg/ml in 5637-*WIF1*-Fluc cells and lower than minimum (23.4 pg/ml) in 5637-Fluc cells. Because WIF1 is a secretory protein, it may explain that the fold change in culture medium by ELISA is more obvious than in cell lysates by Western blot assay. In the functional assay, cell growth was

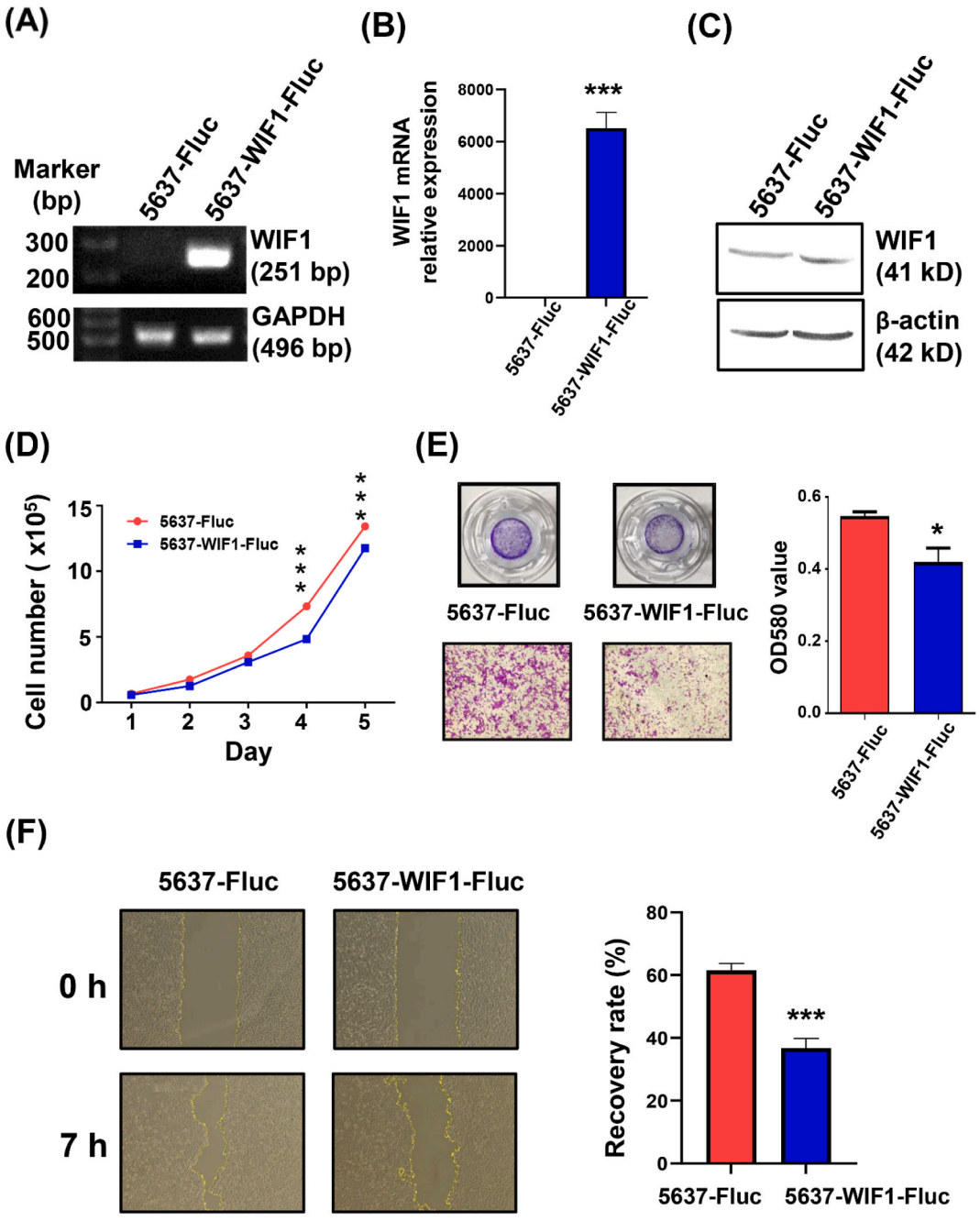

Fig. 5. The WIF1 expression and cell functional assessment in 5637 transfection stable cell lines 5637-Fluc and 5637-*WIF1*-Fluc. (A) The expression level of WIF1 RNA in cells was detected by RT-PCR analysis, and (B) quantitative change of WIF1 mRNA expression by real-time PCR assay. (C) WIF1 protein expression of Western blot analysis. (D) Cell growth was analyzed for five consecutive days by counting cells. (E) Analysis of cell crawling ability by Transwell migration assay. The quantitative OD580 values are on the right. (F) Analysis of the crawling ability of cells in the plane by wound healing assay. The values were the mean of 12 wound areas in each condition and the experiment was repeated 3 times. The quantitative recovery rates are on the right. $*p < 0.05$, $***p < 0.001$.

analyzed by counting cells for five consecutive days (Fig. 5D). The number of cells in the 5637-*WIF1*-Fluc on the fourth and fifth days was lower than that in the control group, indicating that the cell growth was slowed down. Using transwell (8 μm pore PET filters, Millipore) to analyze the migration ability (Fig. 5E), the results showed that the optical density OD580 of 5637-*WIF1*-Fluc was lower than that of the control group (5637-Fluc), indicating that the migration ability of the cells was also inhibited. The migration ability of the cells in the plane was also analyzed by wound healing method (Fig. 5F), and found that the 5637-*WIF1*-Fluc had a weaker migration ability than 5637-Fluc cells.

3.6. Comparison of DNA methylation level of WIF1 and GSTM5 genes in bladder cancer patients and non-bladder cancer patients

Here, *WIF1* gene was proved to be silenced by DNA methylation and has anti-proliferation and anti-migration effects in bladder cancer cells. Next we analyzed the DNA methylation level of *WIF1* gene promoter -184 to +29 region in clinical samples. We simultaneously collected tumors and urine pellets from urothelial carcinoma patients, and collected urine pellets from urolithiasis patients without bladder cancer to compare the DNA methylation level of WIF1 gene promoter. Twenty-one bladder cancer patients and 30 non-bladder cancer patients were included, and their age, sex, and DNA methylation level are listed in Table 1. Comparing the patients and non-bladder cancer patients, we found no significant difference in the DNA methylation level of WIF1 promoter (Table 1 and Fig. 6A).

In addition to analyzing the *WIF1* gene, *GSTM5* gene was also analyzed because in our previous studies, GSTM5 DNA methylation level of bladder carcinoma tissues is higher than that of normal human urine pellets, suggesting that GSTM5 DNA methylation level could be a potential bladder cancer biomarker [19]. Here another batch of human samples, including bladder cancer tissues, urine pellets, and non-bladder cancer urine pellets, was analyzed to recheck the methylation level of GSTM5 promoter. Thirty bladder cancer patients and 30 non-bladder cancer patients were included. The age, sex, and GSTM5 DNA methylation level of patients and non-bladder cancer patients are shown in Table 2. In DNA methylation analysis, the level of GSTM5 in patients, either tumor tissues or urine pellets, were significantly higher than the level of non-bladder cancer urine pellets (Table 2 and Fig. 6B). In summary, *WIF1* gene and *GSTM5* gene [19] could be activated by the DNA methylation inhibitor 5-aza-dC in bladder cancer cells, but the difference in DNA methylation level between bladder cancer patients and non-bladder cancer patients was only found in *GSTM5* promoter −258 to −89 region, not in *WIF1* promoter −184 to +29 region. For clinical data analysis, GSTM5 mRNA expression and DNA methylation level were extracted from Ualcan (http://ualcan.path.uab.edu/index.html, accessed date: Nov 9, 2022). It shows that either in GSTM5 mRNA expression or in DNA methylation level, there was significant difference between normal and bladder primary tumor (Fig. 6C and 6D). Though the survival probability did not reach a significant difference from Ualcan database ($p = 0.51$, data not shown), the relapse-free survival probability was significantly higher ($p = 0.03$) in GSTM5 high expression then in GSTM5 low expression of 187 bladder cancer patients from Kaplan-Meier Plotter database in our previous study [19].

Because the GSTM5 DNA methylation level was significantly different between bladder cancer patients and non-bladder cancer patients (Fig. 6B), and the difference did not present in WIF1 gene (Fig. 6A). Therefore, we analyzed the correlation of DNA methylation level and mRNA expression level of WIF1 and GSTM5 in bladder cancer patients. After analysis by cBioPortal for Cancer Genomics (https://www.cbioportal.org/, accessed date: Jan 5, 2023), it shows that a low correlation in WIF1 (Pearson −0.13) (Fig. 7A) and a middle correlation in GSTM5 (Pearson −0.54) (Fig. 7B). Because the correlation of DNA methylation level and mRNA expression level of GSTM5 is better than the correlation of WIF1, it suggests that other factor in addition to DNA methylation level might also inhibit WIF1 mRNA expression in bladder cancer.

3.7. The different DNA methylation levels of WIF1 gene promoter −184 to +29 in RT4, 5637, and T24 cells

Due to there being no significant difference of WIF1 DNA methylation level in bladder cancer patients and non-bladder cancer subjects (Table 1 and Fig. 6A), and low correlation of WIF1 DNA methylation level and its mRNA expression (Fig. 7A), we next analyzed the DNA methylation level of *WIF1* gene promoter -184 to +29 region in 3 bladder cancer cell lines, RT4, 5637, and T24. According to the results of Fig. 2, WIF1 gene is inhibited by DNA methylation in RT4, 5637, and T24 cells because 5-aza-dC could activate its expression in these three cell lines. In addition, in our previous report [18], the WIF1 mRNA expression in RT4, 5637 and T24 cells is obviously lower than that in SV-HUC1 cells. Also, from the real-time PCR data of Fig. 2, the Ct values of control group in RT4, 5637, and T24 cells were about 31–32, and about 28–29 in SV-HUC1 cells, which matched the previous RT-PCR result [18]. As shown in Fig. 8, the methylation ratio was 7.5% in RT4 cells, 91.5% in 5637 cells, and 75% in T24 cells. Comparing all three cell lines, the methylation level was highest in 5637 cells (91.5%) and lowest in RT4 cells (7.5%); it was much lower in grade I RT4 cells than in grade II 5637 and grade III T24 cells. Although the DNA methylation levels were very different, the mRNA expression levels were all low in these 3 cell lines. Like the result of Fig. 7A, there might be other factor in addition to DNA methylation to inhibit WIF1 mRNA expression in RT4 cells. Furthermore, because WIF1 gene could be activated by 5-aza-dC in RT4 cells, it is possible that there was another DNA methylation region to inhibit WIF1 gene expression, not only the DNA methylation situation of −184 ~ +29 region. This

Table 1
The age, sex and *WIF1* DNA methylation level of 30 non-bladder cancer patients and 21 bladder cancer patients.

		Non-bladder cancer	Bladder cancer		*p*-value
			Tumor tissue	Urine pellet	
Age	min/max	60/80	59/84		–
	mean ± SD	69.1 ± 7.14	73.1 ± 7.04		0.0544
Sex	male/female	16/14	15/6		0.193
WIF1 DNA methylation level (%) (mean ± SD)		60.8 ± 22.17	55.5 ± 19.62	59.4 ± 15.16	Tumor vs. non-cancer 0.3829 Urine vs. non-cancer 0.8015

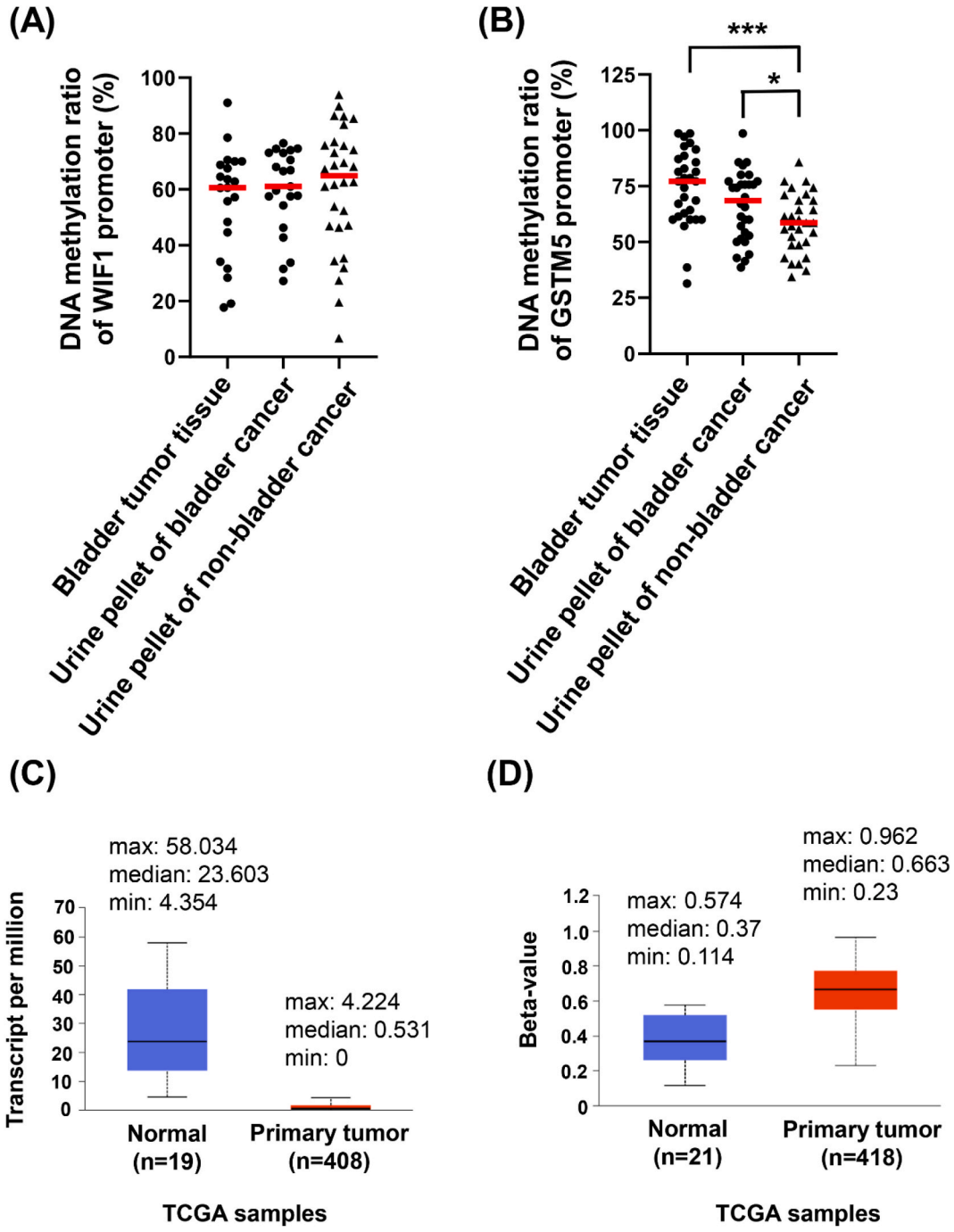

Fig. 6. DNA CpG methylation ratio of human WIF1 and GSTM5 promoter in human samples. The degree of methylation was analyzed by bisulfite specific PCR and TA cloning/sequencing. (A) The WIF1 DNA methylation ratio in 21 bladder cancer tissues and urine pellets, and in 30 urine pellets of patients without bladder cancer. The red lines mean median values. (B) The GSTM5 DNA methylation ratio in 30 bladder cancer tissues and urine pellets, and in 30 urine pellets of patients without bladder cancer. Unpaired *t*-test was used for statistical analysis between different groups. $*p < 0.05$, $***p < 0.001$. (C and D) The Ualcan analysis results of GSTM5 in bladder cancer patients and normal subjects. The GSTM5 mRNA expression values, $p = 3.558 \times 10^{-6}$ (C). The GSTM5 gene DNA methylation values. The beta value indicates the level of DNA methylation ranging from 0 (unmethylated) to 1 (fully methylated), $p = 1.625 \times 10^{-12}$ (D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
The age, sex and *GSTM5* DNA methylation level of 30 non-bladder cancer patients and 30 bladder cancer patients.

		Non-bladder cancer	Bladder cancer		*p*-value
			Tumor tissue	Urine pellet	
Age	min/max	60/80	58/85		–
	mean ±	69.1 ±	73.3 ±		0.0265[a]
	SD	7.14	7.38		
Sex	male/female	16/14	20/10		0.292
GSTM5 DNA methylation level (%) (mean ± SD)		58.5 ± 13.26	74.2 ± 16.81	66.2 ± 15.43	Tumor vs. non-cancer 0.0002[a] Urine vs. non-cancer 0.0428[a]

[a] means statistically significant difference between normal subject and bladder cancer.

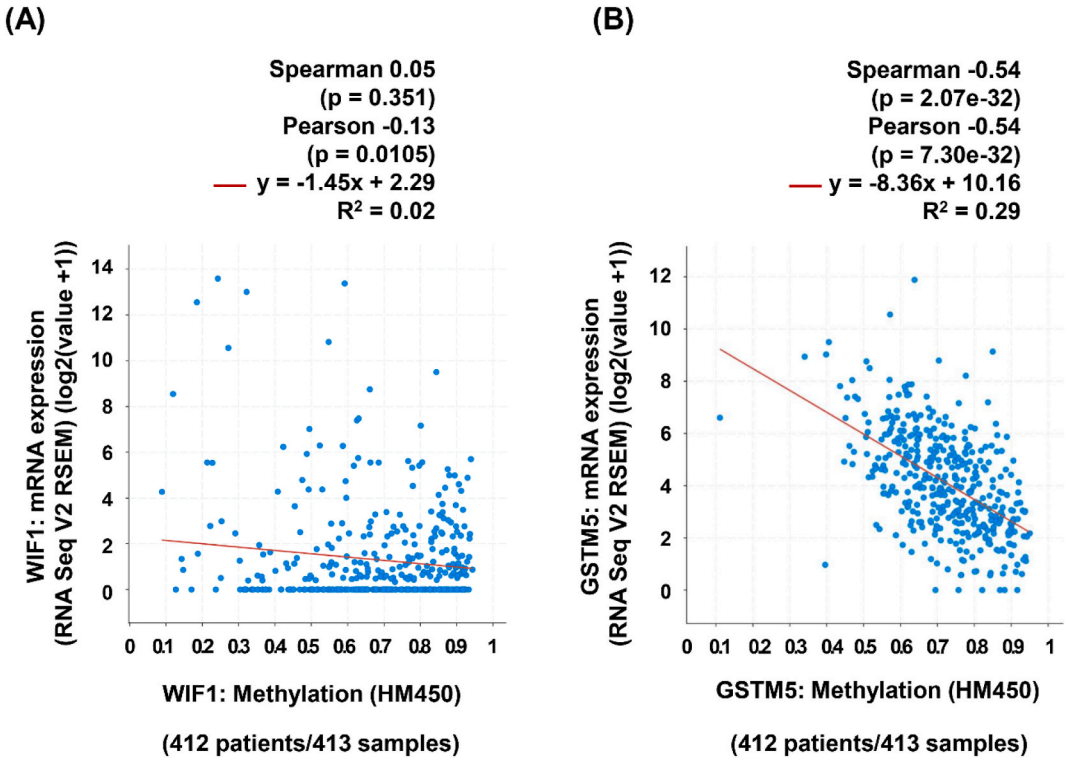

Fig. 7. The correlation of DNA methylation and mRNA expression in bladder cancer patients. The correlation was analyzed from cBioPortal for Cancer Genomics (A) *WIF*1 gene. (B) *GSTM5* gene.

fact might explain why there was no difference in *WIF1* DNA methylation levels of the −184 ~ +29 region between bladder cancer patients and non-bladder cancer patients.

4. Conclusion and discussion

According to previous studies, in some cancers such as lung cancer [13], gastrointestinal cancer [14], etc., it was found that the *WIF1* DNA promoter is hypermethylation, causing that the WIF1 gene is not expressed and loses its cancer suppressor function. This study also confirmed WIF1 gene inactivation under DNA hypermethylation in bladder cancer cell lines. The bladder cancer suppressor role was also found in other genes like GSTM5 [19] and GSTM2 [21] which were downregulated by DNA hypermethylation, too. In addition, we also found that histone acetylation greatly enhanced WIF1 gene expression after DNA demethylation. Because the DNA demethylation drug 5-aza-dC used in this study is a FDA approved drug and there are some marketed histone deacetylase inhibitors, combining these 2 kinds of drug might be beneficial for WIF1 gene re-expression and tumor suppression in bladder cancer. Furthermore, 5-aza-dC activates the expression of GSTM5 [19] and GSTM2 [21] which are also anti-tumor genes in bladder cancer cells.

In a previous study of our research, C57BL/6 mice drank drinking water containing 50 ppm arsenic for two weeks, and 83.3% of the

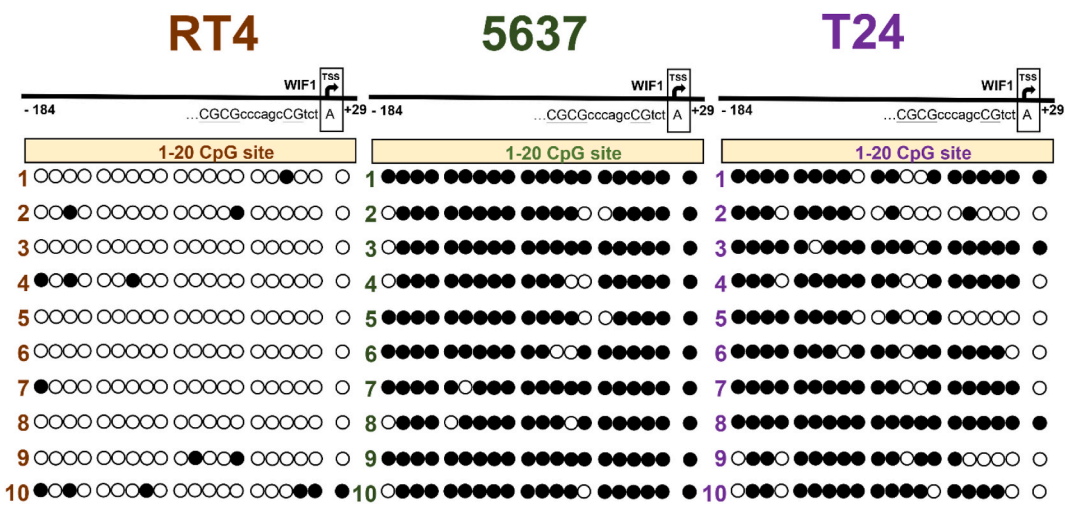

Fig. 8. WIF1 DNA CpG methylation pattern in RT4, 5637, and T24 cells. The degree of methylation was analyzed by bisulfite specific PCR and TA cloning-sequencing. Human WIF1 BSP PCR product is 213 bp in length and contains 20 CpG sites. The sequencing region is −184 ~ +29, and the TSS is +1. The number 1–10 in the left means the different clones. A circle represents a CpG site, white circles represent unmethylated, and black circles represent methylated.

mouse bladders had a tendency to proliferate urothelial cells, a precancerous change in the bladder. The RNA of the bladder tissues were analyzed by DNA microarray chip, and found 4 genes, namely cystathionine β-synthase (*CBS*), adenosine A1 receptor (*ADORA1*), metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), and *WIF1*, whose changes were statistically significant. These 4 genes were then verified in normal human bladder epithelial cells SV-HUC1 where the expression of WIF1 was most significantly down-regulated by sodium arsenite treatment. When SV-HUC1 was treated with sodium arsenite for 14 days and 10 weeks, the expression of WIF1 mRNA was down-regulated with the increase of sodium arsenite treatment time. The degree of WIF1 gene DNA methylation ratio increased with the time of sodium arsenite treatment; in terms of cell viability, cell proliferation and migration capacity also increased with time [18]. In addition, 5 bladder cancer cell lines (5637, J82, RT4, T24, BFTC 905) treated with DNA methyltransferase inhibitor 5-aza-dC increased the expression of WIF1 mRNA, and the WIF1 mRNA was increased most significantly in RT4, 5637, T24 cell line [18]. Therefore, in this study, three bladder cancer cell lines, RT4, 5637, and T24 were selected, and the human normal bladder epithelial cell line SV-HUC1 was used as a normal cell control. The results showed that DNA methylation is a major inhibitory mechanism of WIF1 expression in bladder cancer cells RT4, 5637, and T24, but it does not have an obvious effect in SV-HUC1 cells.

The Ualcan databases showed that the level of WIF1 gene methylation in bladder primary tumors was higher than that in normal bladder tissues. A previous study also suggested that WIF1 DNA hypermethylation presents in bladder tumors of patients, which correlates to the high mRNA expression of c-Myc proto-oncogene (MYC) and cyclin D1 (CCND1) [17]. However, in this study, the WIF1 DNA methylation showed no difference between bladder cancer patients and non-bladder cancer subjects. To compare the *WIF1* DNA region for DNA methylation assay, we analyzed the DNA region (−184 to +29 bp) which was the downstream of MSP/USP assay regions (−371 to −352 bp and −247 to −227 bp) used in Urakami's study [17]. The different assay regions may account for the different results of this study and Urakami's study. Another study of primary normal and cancer cervical tissues, analyzed −555 to −140 bp of *WIF1* gene which has a partial overlapping region with our study region (−184 to +29 bp) and showed higher methylation levels in primary cancer cervical tissues than in primary normal cervical tissues [22]. *WIF1* gene promoter also shows a higher DNA methylation level in 3 nasopharyngeal carcinoma cell lines than in one normal nasopharyngeal cell line [23]. The above study analyzed −402 to +61 bp of *WIF1* gene which included our study region (−184 to +29 bp). In this study, the limitation is that *WIF1* DNA methylation levels didn't show the difference between patient and control, the regulatory CpGs of the *WIF1* gene should be further investigated. Because the DNA methylation levels of GSTM5 gene is higher in patient than in control, it provides a positive control for this clinical samples. Three bladder cancer cell lines were analyzed in this study, and only 5637 and T24 had high WIF1 DNA methylation levels (91.5% and 75%); the level was low in RT4 cells (7.5%). Because the mRNA expression is low in all three of these cell lines, it suggests that there are other mechanisms to inhibit WIF1 gene expression in RT4 cells. For WIF1 gene methylation assay, the best region for DNA methylation assay in bladder cancer needs to be further investigated.

Plasma cell-free DNA of advanced gastric cancer patients presents a correlation between WIF1 promoter methylation and lower progression free survival and overall survival [24]. In a report of meta-analysis and literature review, the frequency of WIF1 hypermethylation is significantly increased in non-small cell lung cancer compared with normal lung tissue with the pooled odds ratio (OR) of 8.67 [25]. Another meta-analysis of colorectal cancer shows WIF1 hypermethylation as a biomarker with OR 30.1 [26]. They suggest WIF1 hypermethylation as a useful biomarker in advanced gastric cancer, lung cancer and in colorectal cancer. WIF1 promoter methylation also plays a biomarker role in bladder cancer by using the MSP/UPS analysis [17], GSTM5 promoter hypermethylation is also another reference biomarker for bladder cancer (Fig. 6B) [19].

Previous literature confirmed that *WIF1* is a tumor suppressor gene, inhibiting the WNT/β-catenin pathway through WIF1 protein, down-regulating S-phase kinase associated protein (*SKP2*) and *MYC*, stopping the cell cycle in the G1 phase, and inhibiting the growth of bladder tumors in nude mice [27]. In this study, we confirmed the anti-cancer effect of WIF1 in bladder cancer cells. In addition, our previous study also suggests the anti-bladder cancer role of GSTM5 [19] and GSTM2 [20]. In conclusion, we suggest that WIF1 and GSTM5 could not only be the biomarkers of bladder cancer, as well as WIF1 and GSTM5 could also be activated by 5-aza-dC to assist bladder cancer therapy.

Author contribution statement

Cheng-Huang Shen: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pei-Yu Li: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shou-Chieh Wang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sin-Rong Wu: Yuan-Chang Dai: Analyzed and interpreted the data.

Chih-Yu Hsieh: Performed the experiments.

Yi-Wen Liu: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research was supported in part by the Ministry of Science and Technology, Taiwan, R.O.C. (grant number MOST108–2320-B-415-006-MY3), Ditmanson Medical Foundation Chiayi Christian Hospital, Chiayi, Taiwan (grant number R109-10) and SKBIO Technology Corp. (grant number Shen-1). The sponsor or funding organization had no role in the design or conduct of this research. The authors thank the Biobank of Ditmanson Medical Foundation Chiayi Christian Hospital for providing tissue samples.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16004.

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