# A Plausible Anti-Apoptotic Role of Up-Regulated *OCT4B1* in Bladder Tumors

Jamshid Asadzadeh,[1] Malek Hossein Asadi,[2] Nasser Shakhssalim,[3] Mahmoud-Reza Rafiee,[4] Hamid Reza Kalhor,[5] Mahmoud Tavallaei,[6] Seyed Javad Mowla[1]

[1] Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[2] Department of Biotechnology, Research Institute of Environmental Sciences, International Center for Science, High Technology & Environmental Sciences, Kerman, Iran
[3] Urology and Nephrology Research Center , Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical sciences, Tehran, Iran
[4] Nanomedicine and Tissue Engineering Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5] Omics Research Center, Golestan University of Medical Sciences, Gorgan, Iran
[6] Human Genetics Research Center, Baqiyatallah Medical Sciences University, Tehran, Iran

*Corresponding Author:*

*Seyed Javad Mowla, PhD*
*Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

*Tel: +98 21 8288 3464*
*Fax: +98 21 8288 4717*
*E-mail: sjmowla@modares.ac.ir*

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**Purpose:** To investigate and compare the expression of *OCT4B1* between tumor and non-tumor bladder tissues.

**Materials and Methods:** We investigated the expression of *OCT4B1* in 30 tumor and non-tumor surgical specimens of the bladder, using the TaqMan real-time polymerase chain reaction approach and by carefully designing primers and probes specific for the amplification of the variant.

**Results:** Most tumor and non-tumor samples of the bladder showed *OCT4B1* expression, but its expression level was significantly higher in the tumors ($P < .002$). Moreover, the up-regulation of *OCT4B1* was more significant in high-grade tumors compared to the low-grade ones ($P < .05$). We have also employed the RNA interference strategy to evaluate the functional role of *OCT4B1* in a bladder cancer cell line, 5637. Suppression of *OCT4B1* caused some changes in cell cycle distribution, and significantly elevated the rate of apoptosis in the cells.

**Conclusion:** Our findings suggest that *OCT4B1* plays a potential role in tumor initiation and/or progression of the bladder cancer. Additionally, *OCT4B1* can be regarded as a new tumor marker for detection, classification, and treatment of the bladder cancer. However, more experimental studies are needed to replicate our findings.

**Keywords:** cancer stem cells, urinary bladder neoplasms, apoptosis, neoplasm invasiveness, prognosis

## INTRODUCTION

Bladder cancer is the 9th most common malignancy and the 2nd most common tumor of the genitourinary tract worldwide. It is also the 13th most numerous cause of cancer death.[1,2]

According to the cancer stem cell (CSC) hypothesis, there is a subpopulation of CSCs in each tumor; rendering a number of characteristics to the tumor mass, including self-renewal potential, tumorigenesis, resistance to therapy, etc. Similar to stem cells, CSCs are capable of self-renewing to generate new CSCs as well as partially differentiated tumor cells. Based on this hypothesis, this highly tumorigenic subset of cells could arise as a result of dysregulation of self-renewal process in normal stem/early progenitor cells.[3-7]

Octamer binding protein 4 (*OCT4*), a POU homeodomain transcription factor (also known as *OCT3*, *POU5F1*, and *OTF3*), has been widely studied in embryonic stem cells (ESCs). This transcription factor has been long known to be associated with pluripotency and the self-renewal properties that have been observed in ESCs and germ cells.[8] *OCT4* expression was primarily shown to be restricted to inner cell mass, primitive ectoderm, and primordial germ cells as well as embryonic stem/germ/carcinoma cells (ES, EG, and EC, respectively).[9]

Recent reports from several laboratories have shown that *OCT4* is expressed not only in ESCs, but also in adult and cancer stem cells.[10,11] Subsequently, other research groups have reported *OCT4* overexpression in the bladder, gastric, prostate, and colorectal cancers.[12-16] Based on its main role in regulating self-renewal and pluripotency in ESCs, *OCT4* expression in cancer has been regarded as a possible route for tumor promotion toward malignancy.[17] Furthermore, the ectopic expression of *OCT4* has an anti-differentiation effect on the differentiated host cells.[18] Moreover, it has been recently shown that ectopic expression of a handful of genes, tightly associated with stemness state, in differentiated fibroblasts resulted in complete reprogramming of the host cells to a stem cell-like phenotype; indeed, *OCT4* turned out to be one of the essential players.[19-22]

In addition to the main variant (*OCT4A*), human *OCT4* gene can potentially encode two additional alternatively spliced variants, designated as *OCT4B*[10] and *OCT4B1*.[23] *OCT4B* is mainly localized in the cytoplasm, and lacks DNA binding activities. Furthermore, *OCT4B* cannot maintain the self-renewal and pluripotency of ESCs.[9,10] Our previous reports revealed that *OCT4B1* is expressed in pleuripotent and non-pleuripotent cells,[23] localized within the cytoplasm,[24] and plays a potential role as an anti-apoptotic factor in gastric adenocarcinoma.[15] Although *OCT4B1* expression has been studied in gastric and colorectal cancers.[15,16] little is known about its expression and function in other tumors, including bladder cancer.

In the present study, we have investigated the potential expression of *OCT4B1* in a series of bladder cancer samples and further examined its function in a bladder cancer cell line using RNA interference (RNAi) technology.

## MATERIALS AND METHODS

### Clinical Specimens

Surgical specimens were collected from patients refereed to our hospital from May to September 2008. The samples were quickly snap-frozen in liquid nitrogen before being categorized in two groups: 30 tumor samples and 18 apparently normal tissues as control.

The control samples were taken from the non-tumor parts of the bladder, and all had a tumoric counterpart in the tumor group. The patients' clinicopathological parameters (Table) were evaluated according to ISUP criteria for grading and TNM system for staging. The experimental design was approved by the Ethics Committees of Tarbiat Modares University and Urology and Nephrology Research Center affiliated to Shahid Beheshti University of Medical Sciences, and the written informed consents were obtained from the patients prior to participation.

### RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Frozen tissues were homogenized using glass-made hammer-handle homogenizers. Total RNA was extracted from homogenized tissues using RNX TM-Plus solution (Cinnagen, Iran), according to the manufacturer's instructions, and dissolved in RNase-free water. The quality and quantity of the isolated RNAs were evaluated by gel electrophoresis and ultraviolet-visible (UV) spectrophotometry at

**Clinicopathological characteristics of the patients with bladder cancer.**

| Characteristic | n | % |
|---|---|---|
| Histological type | | |
| TCC | 30 | 100 |
| Tumor grade | | |
| High | 13 | 43.34 |
| Low | 17 | 56.66 |
| Tumor stage | | |
| Ta | 4 | 13.34 |
| pT1 | 19 | 63.34 |
| pT2/pT2a | 6 | 20 |
| pT4 | 1 | 3.34 |
| Gender | | |
| Male | 28 | 6.67 |
| Female | 2 | 93.34 |

260 nm, respectively. Equal amounts of RNA were treated with RNase-free DNase (Fermentas, Lithuania), primed by random hexamers (Fermentas, Lithuania), and reverse transcribed using RevertAidTM MMuLV Reverse Transcriptase (RT) (Fermentas, Lithuania) in a 20 µL reaction, according to the manufacturer's instruction. To check a potential DNA contamination and hence nonspecific amplification, a no-RT reaction was also included in each run of experiments, as a negative control.

Specific primers and probes were designed for *OCT4B1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession numbers: EU518650 and NM-002046, respectively), using AlleleID 4.0 and Gene Runner softwares. The sequence of primers and probes were as follow:

*OCT4 B1* variant:
Forward primer: 5′GGGTTCTATTTGGTGGGTTCC 3′
Reverse primer: 5′TCCCTCTCCCTACTCCTCTTCA 3′
Probe: ATTCTGACCGCATCTCCCCTCTAAG
*GAPDH*:
Forward primer: 5′GTGAACCATGAGAAGTATGACAAC 3′
Reverse primer: 5′ CATGAGTCCTTCCACGATACC 3′
Probe: CCTCAAGATCATCAGCAATGCCTCCTG

TaKaRa Ex Taq Master Mix (2X), supplemented with ROX reference Dye II, was used for all real-time PCR reactions. For compensating variations in the amount of input RNA and the efficacy of reverse transcriptase, *GAPDH* mRNA was also quantified, and the *OCT4B1* expression data were normalized to *GAPDH* expression value in each sample. All real-time PCR reactions were carried out on the ABI 7500 real-time PCR systems (Applied Biosystems, Inc., Foster City, CA) using the following cycling conditions: initiation at 94 ˚C for 30 s, amplification for 45 cycles with denaturation at 94 ˚C for 5 s, and annealing and extending at 60 ˚C for 34 s. To determine the reaction efficiencies for each primer pair and the corresponding probe, standard curves were plotted using serial dilutions of an embryonic carcinoma cell line, NT2, and cDNA. All reaction efficiencies were measured to be close to 100%. All reactions were performed in duplicate or triplicate.

Group-wise comparison and statistical analysis of the relative expression results of real-time PCR were carried out by REST 2008 (Relative Expression Software Tool, V2.0.7, Corbett Research Pty. Ltd. USA). SPSS software (the Statistical Package for the Social Sciences, Version 16.0, SPSS Inc, Chicago, Illinois, USA) was used to plot the charts and Mann-Whitney non-parametric test was used to analyze the correlation between the expression of the variant and the state and grade of the tumors.

**Cell Culture**

The bladder cancer cell line 5637 was obtained from the national cell bank of Iran (Pasteur Institute of Iran, Tehran) and was cultured in RPMI-1640 (Gibco), supplemented with penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) and 10% fetal bovine serum, at 37 ºC in a humidified atmosphere of 5% $CO_2$. The human EC cells, NTERA2 (NT2; kindly provided by Dr Peter Andrews at Sheffield University), were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high concentration of glucose (4500 mg/L), supplemented with 10% fetal bovine serum at 37 ˚C (humidified) and 5% $CO_2$.

**RNAi**

Using the siRNA Selection Program (Whitehead Institute for Biomedical Research; http://jura.wi.mit.edu/), we designed two specific set of siRNAs; two siRNAs to suppress *OCT4B1*

and an irrelevant siRNA (IR-siRNA), with no complementary sequence (more than 16 out of its 21 mer length) within human genome. The siRNAs with the following sequences were synthesized by MWG Company (Germany).

*Oct4B1*-siRNA1 Target sequence: AAG GAG TAT CCC TGA ACC TAG
Sense: (GGA GUA UCC CUG AAC CUA G) dT dT
Antisense: (CUA GGU UCA GGG AUA CUC C) dT dT
*Oct4B1*-siRNA2 Target sequence: AAG AGG TGG TAA GCT TGG ATC
Sense: (CAG GUG GUA AGC UUG GAU C) dT dT
Antisense: (GAU CCA AGC UUA CCA CCU C) dT dT
The OCT4B1 siRNAs were designed on the Exon2b sequence of *OCT4B1*, to discriminate it from other variants of *OCT4*.
IR (Irrelevant, control):
Sense: GCGGAGAGGCUUAGGUGUAdTdT
Antisense: UACACCUAAGCCUCUCCGCdTdT

Twenty-four hours before siRNAs transfection, $2 \times 10^4$ cells per well (30% to 50% of confluency at the time of transfection) were cultured in six-well plates in growth medium without antibiotic. siRNAs were introduced into the cells, using the LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen). Briefly, 5 µL of siRNA (25µM) solution and 4.5 µL RNAi-MAX reagent were diluted in 250 µL Opti-MEM (Invitrogen) and incubated for 10 min in room temperature. The mixture was then added to the cells in a final volume of 2.5 mL. The cells were further incubated for 3 days at 37 ºC in a 5% $CO_2$ incubator.

### Cell Cycle Analysis

For cell cycle analysis, cells were washed with phosphate buffered saline and trypsinized with 0.025% trypsin-ethylenediaminetetraacetic acid to yield single cell suspensions. The cells were stained with 50 µg/mL propidium iodide solution containing 0.1% Triton X-100 and sodium citrate as described elsewhere. The single cell suspensions were then used for flow cytometric analysis (Partec, Germany). Cell cycle profiles were analyzed using Partec FloMax software.

### Optimization of RT-PCR Reactions

Before quantitative analysis, optimization procedures were performed for real-time RT-PCR reactions on the pluripotent embryonic carcinoma cell line NT2. Specific forward and reverse primers and the probe for *OCT4B1* variant were designed carefully to avoid any nonspecific amplification of other *OCT4* variants and pseudogenes. To determine the reaction efficiencies for each primer pair and the corresponding probe, standard curves were plotted using serial dilutions of NT2 cDNA. All reaction efficiencies were measured to be close to 100%. The identity of amplified products was further confirmed by direct DNA sequencing (data not shown). The relative expression of *OCT4B1* spliced variant was compared between bladder tumor and non-tumor tissues. *GAPDH* transcript was used as an internal control for normalization of probable sampling errors.

## RESULTS

### Elevated Expression of *OCT4B1* in Bladder Tumors

We have detected the expression of *OCT4B1* in 90% (27/30) of tumors as well as 83% (15/18) of non-tumor/apparently normal samples. However, the expression level was much stronger in the tumor samples, compared to their non-tumor counterparts, demonstrating a significant overexpression of *OCT4B1* in bladder tumor samples ($P < .05$; Figure 1A). Moreover, a significant correlation was found between the expression of the variant and the tumor/non-tumor state of the samples (0.39; $P < .01$).
Interestingly, the comparison of the expression level of *OCT4B1* in high- and low-grade tumors revealed that *OCT4B1* was expressed in a significantly higher level in high-grade tumors ($P < .05$; Figure 1B). Additionally, a significant correlation was found between *OCT4B1* expression level and the grade of the tumors (0.31; $P < .05$).

### Cell Cycle Alterations Following *OCT4b1* Knock-Down in 5637 Cells

Cell cycle alterations in 5637 cells, transfected with siRNAs against *OCTB1* or IR, were investigated by flow cytometry, three days after the transfection. The level of *OCT4B1* expression sharply reduced in the cells transcfected by specific siRNAs against *OCT4B1*, with no or very little effects on

cells transfected with IR:siRNA (Figure 2A). The cells transfected with *OCT4B1* siRNA showed around 6 times elevation ($P < .05$) in the percentage of the cells in the sub-G1 phase (a characteristic feature of apoptotic cells, Figure 2B), in comparison to IR-siRNA treated cells (± 0.1% versus 6 ± 0.2%, respectively, Figure 2C). Accordingly, the proportion of cells in G1 phase of *OCT4B1* suppressed group declined (36 ± 0.1% versus 55 ± 0.01%, of IR-siRNA treated cells).

## DISCUSSION

Understanding the molecular mechanisms underlying cancer initiation and progression is one of the most important aims of cancer research. Given the lines of evidence confirming the similarities between stem cells and cancer cells,[3-7] several groups have investigated the expression of key stemness markers in cancer cells.[12-16,25-27] *OCT4*, is the most notable example of such markers. *OCT4* was previously thought to be a master regulator of pluripotency and self-renewal exclusively expressed in ESCs. Recently, a number of investigators have shown the *OCT4* expression in some cancers, cancer cell lines, and adult stem cells.[11-16] The latter findings appeared to be controversial, and there is a need to discrimi-

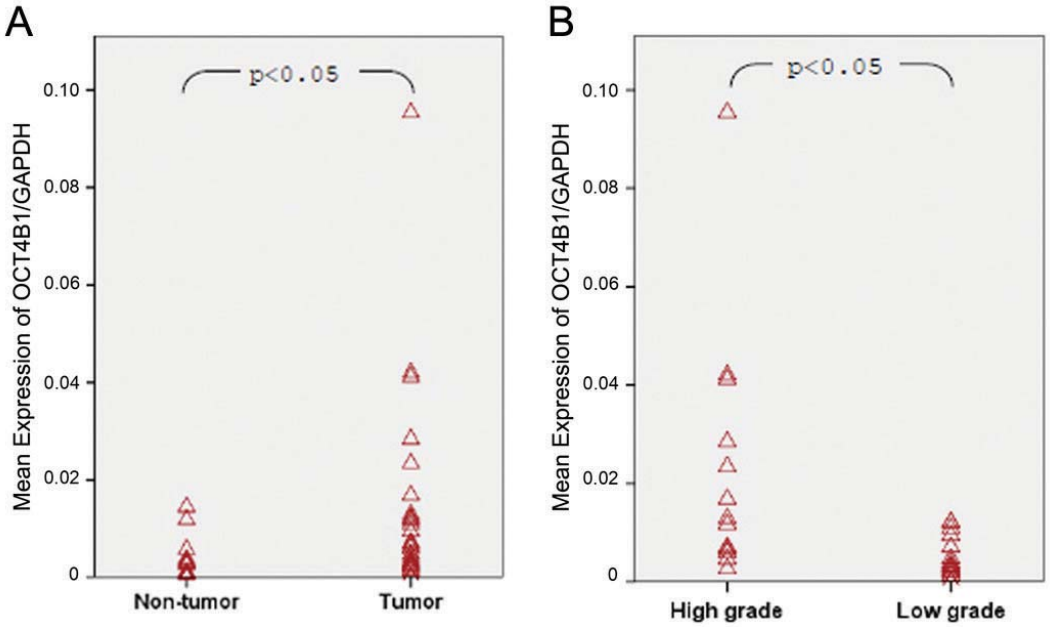


**Figure 1.** (A) Comparison of the relative expression level of the *OCT4B1* between tumor and non-tumor samples. Each triangle represents the mean expression level of a single specimen, obtained by averaging values from two to three independent experimental replicates. (B) Comparison of relative expression of *OCT4B1* between 13 high-grade and 17 low-grade tumor samples, as determined by real-time PCR.

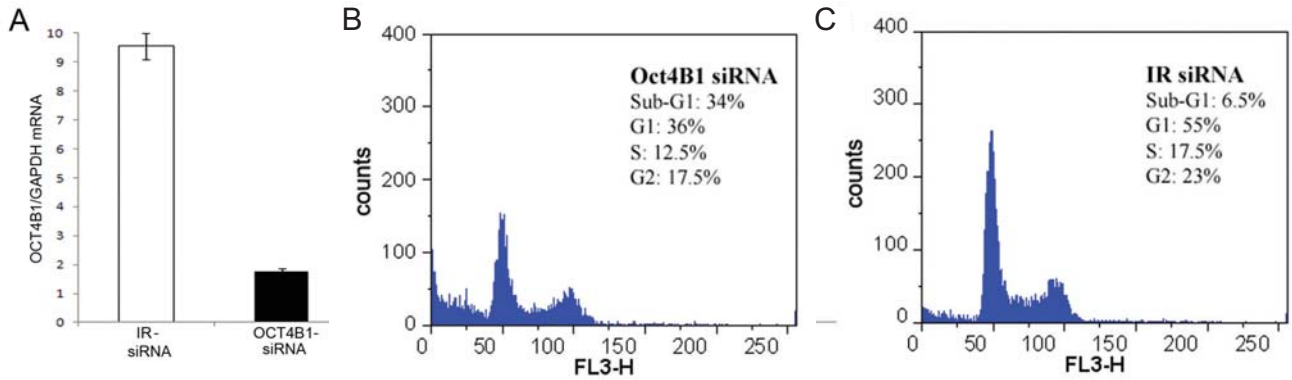


**Figure 2.** (A) Cells treated with *OCT4B1*-siRNA, compared to the ones treated with IR-siRNA, demonstrated a dramatic suppression in *OCT4B1* expression as determined by real-time PCR. GAPDH was used as an internal control. (B and C) Effect of *OCT4B1* knock-down on cell cycle distribution in 5637 cell line.

nate the expression of *OCT4* variants in different types of cancers.

In addition to the previously known *OCT4* variants, *OCT4A* and *OCT4B*, we have recently discovered a new variant of the *OCT4* gene coined *OCT4B1*.[23] This variant is primarily expressed in undifferentiated cells, and might contribute to tumorigenesis with blocking apoptosis and/or stress pathways.[15,23,24] However, there is still little data about its expression and function in different cancers. To gain more insight on the role of the *OCT4* variant in cancer, we undertook this study to investigate and compare the expression of *OCT4B1* between tumor and non-tumor bladder tissues. TaqMan real-time PCR technique was employed in this study using carefully designed specific primer-probe sets. Moreover, by direct sequencing of the PCR products, the accuracy of the amplified products was examined to be exactly as the desired mRNA transcript for *OCT4B1*.

Our results revealed that *OCT4B1* expression was significantly elevated in tumor samples compared to that of non-tumor samples. *OCT4B1* expression also showed correlation with the tumor grades of malignancy; that is, the variant was expressed at higher levels in the high-grade tumors compared to the low-grade ones.

It is noteworthy to mention that *OCT4B1* expression also existed in most of the non-tumor samples, ie, the expression was not zero or undetectable. Two potential possibilities could be considered; first, in agreement with the previous research showing the expression of ESC markers, such as *OCT4* in adult stem cells,[11,15,27] *OCT4B1* is expressed at a basic level in normal tissue of the bladder as a result of existing adult stem cells. Secondly, several lines of evidence exist in supporting a clonal expansion of multifocal carcinomas, suggesting derivation of these tumors from a primary transformed progenitor cell.[28-30] Therefore, it can be deduced that seeding or infiltration of cancer stem cells at other luminal surfaces of the urinary tract could be regarded as a major reason why *OCT4B1* was also detectable in superficially normal tissues locating in the non-tumor parts of the bladder.

Elevated expression of *OCT4B1* in high-grade tumors is quite interesting since Atlasi and colleagues, who examined this variant for the first time, have reported its high expression in stem cells and its sharp down-regulation upon the induction of differentiation.[23] Additionally, in agreement with our pervious data on gastric cancer,[15] this novel variant may play an anti-differentiation role as the tumor grade is related to the degree of differentiation. According to the grading system, the degree of differentiation of a given tumor has an inverse relationship to its grade, ie, the higher the grade of tumor, the lower the degree of tumor differentiation.[31] Furthermore, in line with our pervious data on AGS cells,[15] suppression of *OCT4B1* in 5637 cells significantly increased the percentage of cells in the sub-G1 fraction, suggesting that *OCT4B1* plays a role in blocking apoptosis in bladder cancer cell line, 5637.

## CONCLUSION

Altogether, our data revealed that *OCT4B1* was up-regulated in the bladder cancer and the variant seemed to have a role in tumorigenesis process, probably as an anti-apoptotic factor. Therefore, *OCT4B1* can be considered as a novel tumor marker in bladder, with potential diagnostic and therapeutic values. However, to clarify whether there is a stable and functional *OCT4B1* protein, specific antibody against the variant is needed. Furthermore, determining subcellular localization of *OCT4B1* as well as identifying its intracellular molecular partners would shed more lights on its molecular mechanism and its function.

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## CONFLICT OF INTEREST

None declared.

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