Overexpression of BMI1, a Polycomb Group Repressor Protein, in Bladder Tumors
A Preliminary Report

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Introduction: A Polycomb group repressor protein named BMI1 represses the genes that induce cellular senescence and cell death, and it can contribute to cancer when improperly expressed. We aimed to evaluate expression of *BMI1* gene in bladder tumors.

Materials and Methods: Tissue specimens containing bladder tumor were evaluated and compared with intact tissues from tumor margins and normal bladders. There were 40 tumor specimens of patients with transitional cell carcinoma of the bladder, 20 tumor-free tissues taken from the margin of the tumors, and 8 specimens from patients without tumor. Specific primers for *BMI1* and *B2M* (as an internal control) were used for reverse transcript polymerase chain reaction technique. The production and distribution of BMI1 protein was also examined by western blotting and immunohistochemistry techniques.

Results: Polymerase chain reaction generated a 683-bp product, corresponding to the expected size of *BMI1* amplified region. The identity of the amplified fragment was then confirmed by direct DNA sequencing. The mean of expression of *BMI1* detected in tumor tissues was significantly higher than that in intact tissues, and there was also a significant association between the mean of gene expression and the stage of malignancy ($P < .001$). The expression of *BMI1* at protein level was further confirmed by western blotting and immunohistochemistry.

Conclusion: *BMI1* is a potent repressor of retinoblastoma and p53 pathways, and hence, elucidating its role in tumorigenesis is very important. We reported for the first time the expression of *BMI1* and its correlation with incidence and progress of bladder tumors.

INTRODUCTION

Our understanding of the tumor biology has improved measures to prevent progression of superficial tumors to advanced and metastatic disease.[1] In bladder cancer for instance, we know that genetic alterations may occur at early stages and that they are retained in the recurrent tumors. Consequently, information on changes in the cell-cycle regulatory genes at early diagnosis may be useful for predicting tumor progression and recurrence. Today, cancer studies are focused on the identification of molecular markers that would predict which superficial bladder tumors will progress to invasive forms. However, our knowledge of predictive markers has not reached their reliable usage in practice.[1]

The human *BMI1* is located on

chromosome 10p13, a region known to be involved in translocations in various leukemias and rearrangements in malignant T-cell lymphomas.[2-4] It is responsible for expression of BMI1 protein, a member of the polycomb group (PcG) proteins which form multiprotein complexes that function as transcriptional repressors.[5] *Bmi1* was first identified as an oncogene that cooperates with *c-myc* in the generation of mouse pre-B-cell lymphomas.[6,7] The oncogenic activity of *BMI1* may be linked to its another fundamental function; several recent reports showed that *BMI1* is essential for the self-renewal of both hematopoietic and neuronal stem cells, as well as cancer stem cells.[8-11] This function of *BMI1* depends on its ability to repress the *INK4A/ARF* locus. Along with a role of *BMI1* in stem-cell biology, *BMI1* expression in the bone marrow is strong in undifferentiated precursor cells, but it gradually declines in the course of differentiation.[12] The *BMI1* gene is amplified in certain mantle-cell lymphomas and is overexpressed in a subset of non-small-cell lung cancer and colorectal carcinomas.[13-15] Moreover, it plays a role in the development of human breast cancer and increases in metastatic prostate cancer.[16-19]

Despite many studies on the potential involvement of *BMI1* in the oncogenesis of various lymphomas and leukemias, little is known about its role in the pathogenesis of solid tumors, including urothelial carcinomas. To our knowledge, the only available report is that of Glinsky and colleagues who employed a genomics approach to identify an 11-gene, including *BMI1*, signature that consistently displays a stem-cell-resembling expression profile in distant metastatic lesions of different cancers, including prostate and bladder cancers.[20] To investigate the potential involvement of *BMI1* in human bladder cancer, we examined the expression of *BMI1* in the bladder specimens with and without malignant lesions.

MATERIALS AND METHODS

Human Clinical Specimens

Fresh tissue biopsies were obtained from patients who were referred to Shahid Labbafinejad Medical Center. The tissues were immediately snap-frozen in liquid nitrogen and categorized

Characteristics of Patients With Bladder Cancer*

Characteristics	Values
Patients number	40
Mean age, y	65.4 ± 11.1
Age range, y	33 to 84
Sex	
Male	36 (90.0)
Female	4 (10.0)
Stage	
Ta	7 (17.5)
T1	24 (60.0
T2	9 (22.5)
Grade	
Low	25 (62.5)
High	15 (37.5)

*Values in parentheses are percents.

into 3 groups: 40 tumor specimens prepared by transurethral resection from patients with transitional cell carcinoma of the bladder (Table), 20 tumor-free tissues taken from the margin of tumors (cystoscopically normal appearance), and 8 specimens from patients with no symptoms and signs of bladder cancer who had undergone surgical treatment for benign prostatic hyperplasia. Histopathological parameters were evaluated according to the grading and TNM system for stage classification of the World Health Organization. The experimental design was approved by the Ethics Committees of Tarbiat Modares University and the Urology and Nephrology Research Center of Shahid Beheshti University (MC). The patients' written informed consents were obtained prior to participation.

RNA Extraction

Total RNA was isolated from frozen tissues using the RNX plus solution (Cinnagen, Tehran, Iran) according to the manufacturer's instructions. The quality of RNA was evaluated by gel electrophoresis, and the concentration of RNA was measured by optical density at 260 nm.

Semiquantitative Polymerase Chain Reaction

Two micrograms of the total RNA was used for cDNA synthesis using random hexamer primer (Fermentas, Vilnius, Lithuania) and RevertAid MMuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) in a 20 μL reaction according

to the manufacturer's instructions. Reverse transcription polymerase chain reaction (PCR) primers were designed using previously described human *BMI1* and β2-microglobulin (*B2M*) sequences (GenBank accession numbers: NM_005180 and NM_004048, respectively). The appropriate PCR primers were designed using Genrunner software (version 3.02; Hastings Software, New York, USA) as follows:

*BMI1*F: 5'-GAG GGT ACT TCA TTG ATG CCA C-3'

*BMI1*R: 5'-CCA GTT CTC CAG CAT TTG TCA G-3'

*B2M*F: 5'-CTA CTC TCT CTT TCT GGC CTG-3'

*B2M*R: 5'-GAC AAG TCT GAA TGC TCC AC-3'

Polymerase chain reaction amplifications were performed using 2 μL of cDNA with 1 U of Taq polymerase (Cinnagen, Tehran, Iran), 1.5 mM of MgCl2, 200 μM of dNTPs, and 0.4 μM of each primer in a 25-μL PCR reaction. The PCR cycling conditions were as follows: initial denaturation at 94°C for 4 minutes and following 38 (*BMI1*) or 32 (*B2M*) cycles of 94°C for 30 seconds, 59°C (for both *BMI1* and *B2M*) for 40 seconds, 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. The primers amplified 683-bp and 191-bp fragments for *BMI1* and *B2M* cDNA, respectively. The PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under the ultraviolet light. The intensity of bands was determined using Uvitec software (Uvitec, Cambridge, UK). The identity of PCR products was confirmed by direct DNA sequencing (Millegen, Toulouse, France).

Western Blotting

Frozen tissue samples were homogenized and lysed in modified RIPA buffer (Tris-HCl, 50 mM, pH 7.4; NaCl, 150 mM; phenylmethylsulfonyl fluoride, 1 mM; ethylenediamine tetra-acetic acid, 1 mM; Triton X-100, 1%; sodium deoxycholate, 1%; sodium dodecyl sulfate, 0.1%; trypsin inhibitor, 10 μg/mL). The concentration of proteins in cell lysates was quantified by means of Bradford assay, and 20 μg of total protein was loaded in each lane. Samples were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) and blotted for 2 hours onto Hyband-P polyvinylidene difluoride

membrane (Amersham Biosciences Europe GmbH, Freiburg, Germany). Membranes were then blocked for 2 hours in ECL advance blocking solution (Amersham Biosciences, Piscataway, New Jersey, USA), according to the manufacturer's instructions. Blots were incubated with the anti-Bmi1 antibody for 3 hours and anti-β-actin antibody for 1 hour, and then with the secondary antibodies for 1 hour in room temperature, before being visualized by ECL Advance Western Blotting detection kit (Amersham Biosciences, Piscataway, New Jersey, USA). Anti-Bmi1 antibody (mouse monoclonal antibody to *Bmi1* (ab14389) and anti-β-actin antibody (Prosci, Poway, California, USA) were used at dilutions 1:1000, and horse raddish peroxidase-conjugated antimouse IgG A4416 (Dako, Glostrup, Denmark) and horse-radish peroxidase-conjugated antirabbit IgG (Sigma, St Louis, Missouri, USA) were used at 1:42000 dilution. All antibodies were diluted in ECL Advance Blocking solution (Amersham Biosciences, Piscataway, New Jersey, USA), according to the manufacturer's instructions.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections (5 μm) were deparaffinized with xylene, rehydrated in descending concentrations of ethanol, and boiled for 15 minutes in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase activity was suppressed with 3% hydrogen peroxidase for 20 minutes. Slides were serum blocked and incubated with mouse monoclonal antibody to Bmi1 (ab14389, 1:200 dilution) for 2 hours at room temperature followed by staining with secondary horse raddish peroxidase-conjugated antimouse antibody (A4416, 1:200 dilution). In negative controls, all the conditions were kept the same, except that the first antibody was eliminated.

Statistical Analyses

All experiments were replicated 2 or 3 times, and the results were analyzed by performing analysis of variance test to determine the relative intensity of *BMI1* expression among different biopsy groups. Also, the least significant difference test

was used to study the differences between pairs of tumor stages. The SPSS software (Statistical Package for the Social Sciences, version 15.0, SPSS Inc, Chicago, Illinois, USA) was used for statistical analyses.

RESULTS

Expression of *BMI1*

We collected a total number of 68 specimens of tumoral tissue, margin of tumor, and apparently normal bladder tissue. To make sure of using the same amount of RNA for each PCR reaction, *B2M* gene expression was employed as an internal control. For each sample, the reverse transcript PCR reaction was carried out for both *B2M* and *BMI1* genes, in separate tubes and under similar conditions (except for the number of cycles). Furthermore, in each reaction, a tube with no cDNA was used as a negative control.

We determined the relative expression of *BMI1* in 40 tumors, 20 tumor margins from the same patients, and 8 apparently normal tissue samples of the bladder. An expected 191-bp PCR product corresponding to amplified *B2M* segment was visualized in all of the examined samples (Figure 1). The designed primers for *BMI1* amplified an expected 683-bp segment in most of the samples. The accuracy of the amplified products was further confirmed by DNA sequencing. We detected the expression of *BMI1* in 21 of 40 (52.5%) examined tumoral specimens of the bladder. The expression was also detected in 5 of 20 (25.0%) tumor margin specimens, as well as in 2 of 8 (25.0%) apparently normal specimens. However, the intensity of expression was much higher in tumoral tissues compared to

the other ones (Figure 1).

Because we have used a semiquantitative reverse transcript PCR approach, a densitometric evaluation and comparison of the relative expression of *BMI1*/*B2M* between different tissue samples was feasible. The intensity of *BMI1* expression was significantly higher in neoplastic tissues compared to the specimens with no neoplastic changes (P = .004; Figure 2). Then, we examined the potential correlation between *BMI1* expression and the clinicopathological features (eg, tumor grade and stage) of the patients. Despite the fact that the expression level of *BMI1* in high-grade tumors was more than that of the low-grade ones, the difference was not significant (Figure 2). Furthermore, the intensity of *BMI1* expression was significantly lower in stage Ta malignant tumors compared to that in tumors with higher stages of T1 or T2 (P = .03; Figure 2).

Expression of *BMI1* at Protein Level

We employed western blotting and immunohistochemical techniques to confirm the expression of BMI1 protein in bladder tumors and also to determine its tissue distribution and subcellular localization. The western blot data showed a single band of ∼ 45 kDa in tumor specimens, corresponding to the expected size of the protein (Figure 3). We also used the embryonic carcinoma cell line, NTERA2 (NT2), as a negative control. Based on our reverse transcript PCR results, there was no expression of *BMI1* in the cell line (data not shown). As it is evident in Figure 3, there was no signal relevant to BMI1 protein expression in the cells.
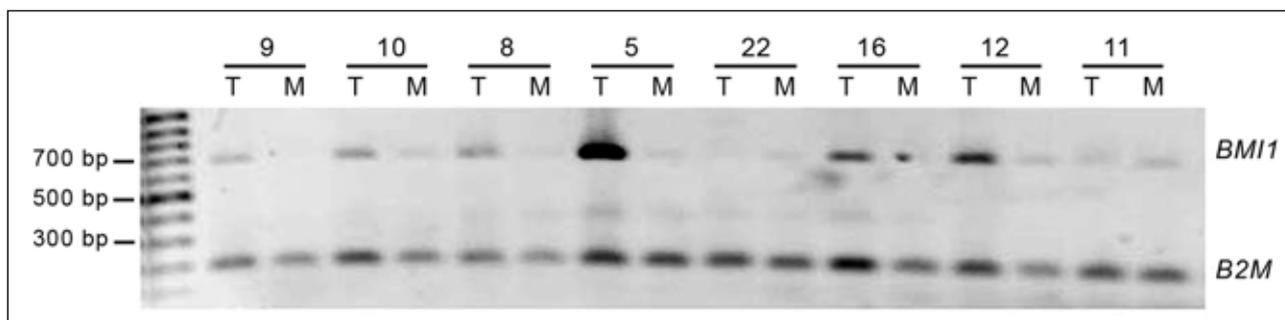
To determine the tissue distribution and

Figure 1. Reverse transcript polymerase chain reaction analysis of the expression of *BMI1* and *B2M* (as an internal control) in the bladder tissues obtained from tumors (T) and the margin of tumors (M) from the same patients (numbers).
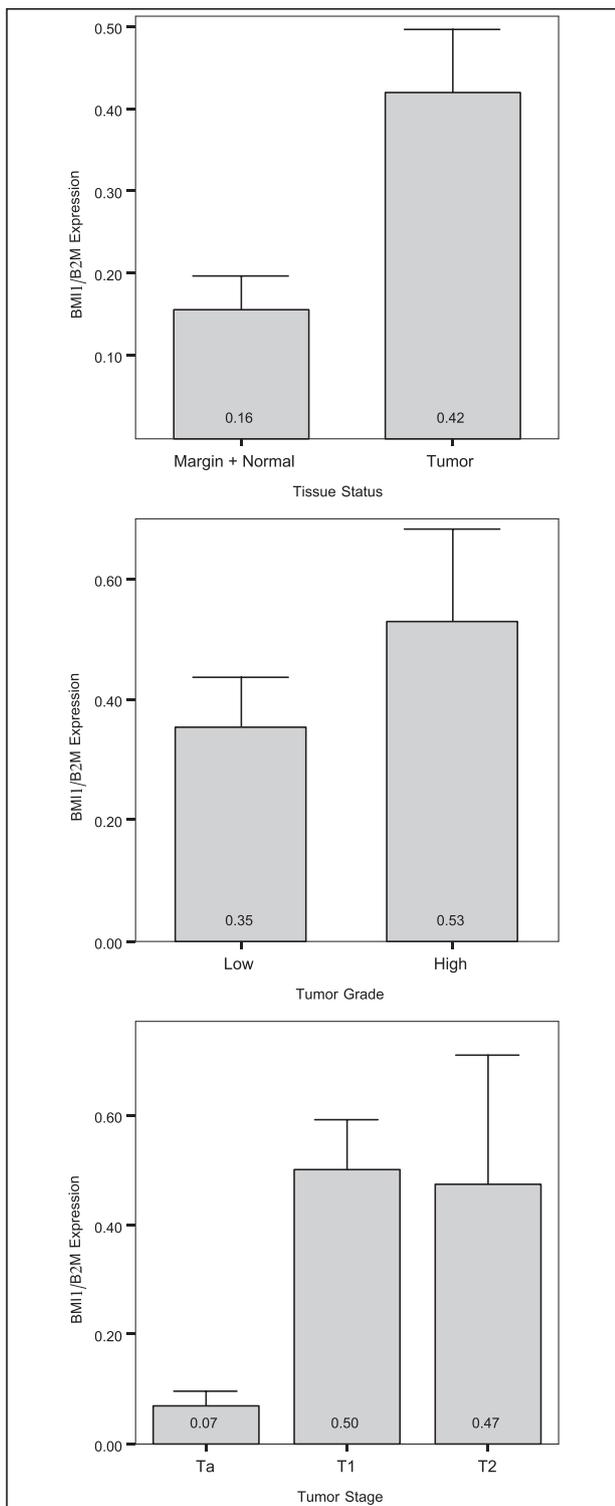
Figure 3. Western blot analysis of BMI1 protein expression in the bladder tissues. Total proteins were isolated from NT2 cell line (that showed no expression in reverse transcript polymerase chain reaction) and 2 representative tumor (T1 and T2) bladder biopsies. The experiments confirmed the expression of an approximately 45 kDa form of BMI1 protein in the bladder tissues. The expression of β-actin was used as a loading control.

subcellular localization of BMI1 in bladder tumors, formalin-fixed paraffin-embedded blocks were collected from archival collections of Shahid Labbafinejad Medical Center and 5-μm tissue sections prepared from the samples. The immunohistochemical data revealed that the BMI1 protein is primarily localized in the nuclei of the tumor cells (Figure 4). To asses the specificity of the antibody, a negative control slide was accompanied each immunohistochemical processing (Figure 4). Similar conditions were used for both positive and negative slides, except for the omission of the first antibody in the negative slides.

DISCUSSION

The cell cycle is a complex process in which many molecules are involved. Among these molecules, inhibitors of cyclin-dependent kinase p16INK4a and p14ARF (coded from *CDKN2a* locus) correlates inversely with the markers of eukaryotic cell proliferation pRB and p53, respectively.[21] The unique genomic structure and compact organization of these genes, which have common reading frames, may be essential for maintaining a balanced Rb and p53 pathway function.[22] In terms of cancer, the *INK4A/ARF* locus which is negatively regulated by *BMI1* is also a frequent target for mutations, deletions, and epigenetic silencing in a wide spectrum of human tumors.[23-25] This raises the possibility that transcriptional regulators of the locus may also be involved in cancer progression. In line with this prediction, it has recently been shown that

Figure 2. Relative (*B2M*-normalized) intensities of *BMI1* expression (mean + standard error). **Top**, Tumoral and intact bladder tissues. **Middle**, Low-grade and high-grade bladder tumors. **Bottom**, Different stages of bladder tumors. Relative band intensities for *BMI1* for each sample were quantitated by densitometry, normalized to *B2M* expression. Statistical analysis revealed that the expression of *BMI1* is significantly higher in tumor specimens compared to that in normal tissues and also in stage T1 and T2 compared to Ta ($P < .001$).
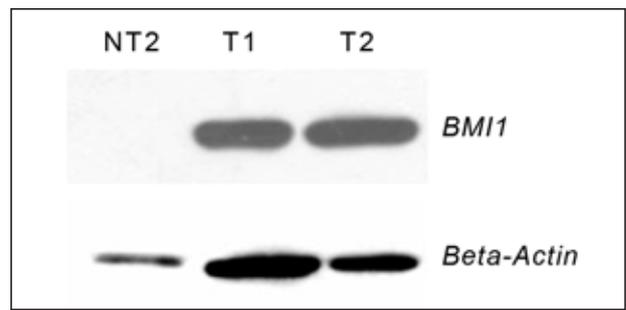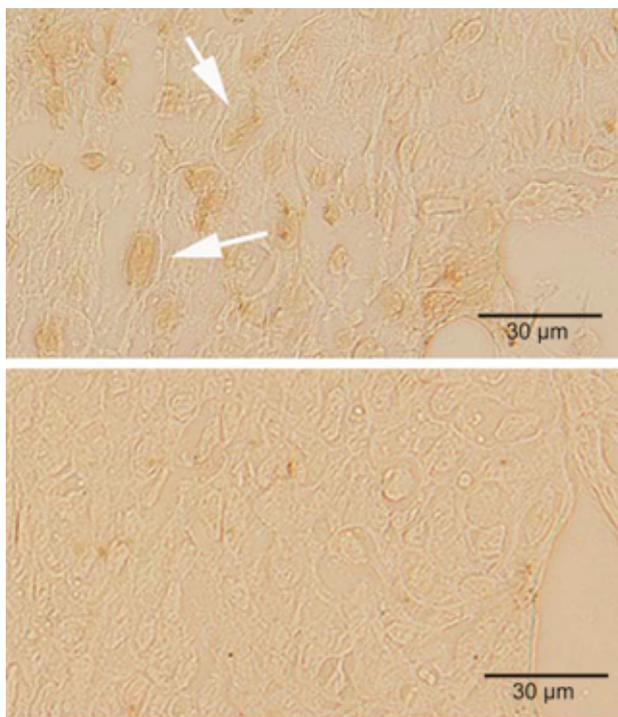
Figure 4. Representative immunohistochemical data of BMI1 protein expression in formalin-fixed, paraffin-embedded sections of tumor tissues. Bladder sections were deparaffinized and subsequently incubated with BMI1 antibody and avidin-horse-radish peroxidase before being visualized with diaminobenzidine. **Top**, *BMI1* expression was primarily localized in the nuclei (in brown color, white arrows) of the cells. **Bottom**, The negative control, sections from the same specimen was identically processed, except for the omission of the first BMI1 antibody.

BMI1 is amplified in some hematologic disorders, such as mantle-cell and non-Hodgkin lymphomas, and is also overexpressed in solid tumors such as non-small-cell lung cancer, medulloblastoma, colorectal cancer, breast cancer, and prostate cancer.[13-19]

In this study, the suitability of the *BMI1* gene expression was evaluated as a potential molecular marker in diagnosis and molecular classification of bladder tumors. *BMI1* is a transcriptional repressor that involves in many cellular mechanisms including tumorigenesis. Despite several reports demonstrating overexpression of *BMI1* in a series of cancers,[13-19] to date, there is no evidence on potential involvement and causative role of *BMI1* in bladder cancer. The main goal of this study was to determine and compare the relative expression of *BMI1* gene in neoplastic tissue versus intact tissue of bladder cancer and its potential involvement in induction and

progression of bladder cancer. Our data revealed a differential expression of *BMI1* in tumoral tissues versus apparently normal tissues and tumor margins of bladder cancer. Furthermore, the data suggests that *BMI1* may play a role in bladder tumor progression rather than initiating the process of tumorigenesis. Based on our data, the expression of the gene is not a good indicator for early detection of bladder tumors (eg, at Ta stage). However, it could be employed to distinguish Ta lesions, which are papillary in nature and limited to the mucosa from T1 lesions, which invade the submucosa or the lamina propria, and hence, are more aggressive.[26]

Despite overexpression of *BMI1* in high-grade tumors compared to the low-grade ones, the difference was not statistically significant in our series. This is probably due to the small size of the patients' population. The data is also in accordance with our previous report on the expression of *OCT4* in bladder cancer,[27] where we found no significant correlation between the expression level of *OCT4* and the grade of the tumors.

CONCLUSION

The present study suggests that alteration of the *BMI1* might play a role in the development and progression of bladder cancer. Furthermore, the current study provides some novel data that further elucidate the complex biology of bladder tumor cells and could potentially be used in diagnosis, prognosis, and treatment of bladder cancer. In summary our data has revealed for the first time that (1) the relative expression of *BMI1* in tumor tissues is much higher than the nontumor samples ($\sim$ 5 times more), and (2) there is a statistically significant correlation between the level of *BMI1* expression and the stage of the bladder tumors. Accordingly, the relative amount of *BMI1* expression in stage Ta is significantly lower than that in stages T1 and T2. In other words, the *BMI1* overexpression is not a primary event in the genetics of bladder cancer, and probably, the gene is involved in the progression of the tumor. This finding is in accordance with previous reports showing a gain and amplification of 10 p (the *BMI1* locus) in stages T1 and T2, but not in stage Ta of bladder cancer.[16]

CONFLICT OF INTEREST

None declared.

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