Abstract. The development and acquisition of multiple drug resistance in cancer cells remain a major obstacle in the treatment of bladder cancer. Nuclear translocation of Y box binding-1 (YB-1), which is a member of a family of DNA-binding proteins that contain a cold shock domain, plays a significant role in the acquisition of drug resistance by upregulating expression of the multidrug resistance-1 (MDR-1) gene product, P-glycoprotein. The tumor suppressor protein p53 is thought to be essential for nuclear translocation of YB-1. We hypothesized that nuclear translocation of YB-1 might be associated with drug resistance of bladder cancer with an abnormality of the TP53 gene that results in a mutated p53 protein. To test this hypothesis, we analyzed the association of YB-1 with drug resistance of TP53-mutated bladder cancer, including immunohistochemical analysis of YB-1, P-glycoprotein and p53 in vivo as well as the function of YB-1 nuclear translocation and regulation of its translocation by p53 in vitro. Additionally, we examined the association between the nuclear translocation of YB-1 and gemcitabine, a major anticancer-drug for bladder cancer, in cancer cell lines. Nuclear expression of YB-1 was correlated with the expression of P-glycoprotein and p53 in bladder cancer cases (P<0.05). In vitro, both introduction of TP53 and gemcitabine induced nuclear translocation of YB-1. These data indicate that YB-1 translocates to the nucleus coordinately with p53 expression and is involved in gemcitabine resistance in bladder cancer. Nuclear expression of YB-1 is important for resistance to chemotherapy including gemcitabine in TP53-mutated bladder cancer.

Introduction

Bladder cancer is thought to develop along two major, independent pathways and these progression pathways have different genetic abnormalities (1). One is the ‘papillary pathway’ that is characterized as low-grade papillary cancer via epithelial hyperplasia. Approximately 80% of bladder cancers arise from the papillary pathway and these cancers can be treated by transurethral resections and have a relatively good prognosis compared to other types. This pathway is associated with genetic abnormalities such as activating mutations of the oncogene, HRAS and the receptor tyrosine kinase gene, FGFR3. The other pathway is the ‘carcinoma in situ (CIS) pathway’. This pathway is associated with genetic abnormalities of the tumor suppressor genes, TP53 and Rb. Approximately 80% of muscle-invasive bladder cancer is thought to arise from this pathway. CIS is a distinct entity with a high-grade malignancy and a high tendency to progress to muscle-invasive bladder cancer (2). CIS is commonly treated by intravesical infusion therapy. Although therapy has been developed for CIS, there remain therapy-resistant cases in ~30% of patients treated with Bacillus Calmette-Guérin (BCG) therapy and in 50% of cases treated with intravesical chemotherapy (3). Moreover, in more than 50% of cases, residual CIS lesions are found in radical cystectomy specimens after preoperative chemotherapy (4). Thus, CIS is considered to have the potential to develop anticancer drug resistance.

Recently, gemcitabine combined with cisplatin(GC) therapy has been accepted as a new standard treatment for advanced bladder cancer because of fewer adverse events than those with methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) therapy (5). However, despite a reasonable response rate after initial chemotherapy, 60-70% of patients relapse within the first year probably because of drug resistance to gemcitabine.
The mechanism of drug resistance to gemcitabine has not been elucidated. Recently, gemcitabine had been used not only as a systemic chemotherapy for metastatic bladder cancer, but also as intravesical therapy for CIS (6). It is therefore necessary to clarify the mechanism of CIS drug-resistance to gemcitabine, which could provide a basis for the development of novel strategies for bladder cancer.

Y box binding-1 (YB-1) is a member of a family of DNA-binding proteins that contain a cold shock domain and it is directly involved in the cellular response to genotoxic stress, such as DNA-damaging agents and UV irradiation (7-9). YB-1 is predominantly localized to the cytoplasm in many cancer cells but its translocation to the nucleus is directly induced by phosphorylated Akt, in response to these environmental stresses (10-14). Nuclear translocated YB-1 upregulates transcription of the multidrug resistance-1 (MDR-1) gene. YB-1 associates with p53 and p53 is thought to be essential for nuclear translocation of YB-1 (15-17).

In the present study, we aimed to analyze YB-1 association with the drug resistance of TP53-mutated bladder cancer, including immunohistochemical analysis of YB-1, P-glycoprotein and p53 in vivo and analysis of the role of p53 in the nuclear translocation and nuclear function of YB-1 in vitro. Additionally, we examined the association between gemcitabine and the nuclear translocation of YB-1.

Materials and methods

Patients. Transurethral resected specimens, and clinical data were obtained retrospectively from 81 patients newly diagnosed with CIS from 2000 to 2013 at the Department of Pathology of Saitama Medical Center, Saitama Medical University. The examined specimens were from patients previously untreated for CIS. The staging was performed according to the TNM classification (7th edition). All specimen collections were approved by the ethics committee of Saitama Medical Center, Saitama Medical University (no. 992).

Immunohistochemical analysis of clinical samples. The following antibodies were used for immunohistochemical detection; anti-YB-1 mouse monoclonal IgG antibody (clone: 21A3, 1:100; Immuno-Biological Laboratories, Gunma, Japan), anti-p53 mouse monoclonal IgG antibody (clone: DO-7, 1:40; Dako, Tokyo, Japan) and anti-P-glycoprotein mouse monoclonal IgG antibody (clone: C494, 1:100; Thermo Fisher Scientific, Waltham, MA, USA) antibodies. Immunohistochemical staining for p53 was performed using the Ventana iVIEW DAB kit reagents (Ventana Medical Systems, Inc., Tucson, AZ, USA) and an auto-immunostainer (Ventana ULTRA). For YB-1 and P-glycoprotein detection, the BOND polymer system refine kit and an auto-immunostainer (BOND III; Leica Microsystems GmbH, Wetzlar, Germany) were used according to the manufacturer's instructions. Protein expression was blindly assessed by two pathologists (T.Y. and J.T.). Expression of YB-1 and p53 was categorized into two groups according to the proportion of expressing cells in 5 hot spots of the specimens: positive, ≥30% of cancer cells stained; negative, <30% of cancer cells stained. Nuclear expression of YB-1 was evaluated according to the presence or absence of staining in the nucleus. For P-glycoprotein expression, positive staining of ≥50% of the cells was defined as positive.

Cell culture, reagents and materials. The human cervical cancer cell line HeLa, the human breast cancer cell line MCF-7 and the human skin cancer cell line A431 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The human bladder cancer cell lines, 5637 and T24 were obtained from the RIKEN BioResource Center (Saitama, Japan). HeLa, MCF-7, T24 and A431 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) and 5637 cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific). Media were supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Inc., Tokyo, Japan). L-glutamine and antibiotics (penicillin and streptomycin). Gemcitabine was purchased from Taiho Pharmaceutical, Co., Ltd. (Tokyo, Japan) and cisplatin was purchased from Nichi-Iko Pharmaceutical, Co., Ltd. (Toyama, Japan).

Plasmids. Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific) and reverse transcribed using the SuperScript™ III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) for polymerase chain reaction (PCR), according to the manufacturer's protocol. YB-1 and p53 cDNA sequence was obtained from the National Center for Biotechnology Information (NCBI) GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/). The cDNAs encoding YB-1 and wild-type p53 were cloned using PCR of normal lymphocyte mRNA from a healthy donor, and that encoding mutant p53 (R273H) was cloned by PCR of the mRNA from A431 cells. The primers for PCR were: YB-1 sense, 5'-GGACTCAGATCTCGAGGAGAGGG-3' and antisense, 5'-GTCGACTGCAGAATTTTTACCTAGCCCCGCTTGC-3'; p53 sense, 5'-GGGTCAGATCAGGACAACTGATGTG-3' and antisense, 5'-GTCGACTGCAGAGGCCGGCAGC-3'. PCR was performed with an initial denaturation step at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 15 sec and extension at 68°C for 90 sec. The PCR fragments from p53 and YB-1 cDNAs were cloned in frame with the enhanced green fluorescence protein (EGFP) or mCherry (Clontech Laboratories, Inc., Mountain View, CA, USA) as a C-terminal fusion using In-Fusion HD cloning kit (Clontech Laboratories). The insert sites were released from EGFP and mCherry by digestion with Xhol and EcoRI. Transfections were performed with Lipofectamine 3000 (Thermo Fisher Scientific) as directed by the manufacturer.

Small interfering RNAs (siRNA). Two siRNA species for knockdown of YB-1, YB-1 siRNA I (cat. no. #6206) and II (cat. no. #6207), as well as control siRNA (cat. no. #6568), were purchased from Cell Signaling Technology (Danvers, MA, USA). Transfections were performed with Lipofectamine 2000 (Thermo Fisher Scientific) as directed by the manufacturer.

Gemcitabine cytotoxicity assay. The 5637 cells transfected with YB-1 siRNA or control siRNA were incubated for
Cell imaging. At 20 to 48 h after transfection the cells were imaged using an inverted fluorescent microscope (TE2000-S Eclipse; Nikon, Corp., Tokyo, Japan) equipped with a cooled CCD camera (CoolSNAP HQ™; Roper Scientific GmbH, Ottobrunn, Germany), with a precentered fiber illuminator as a light source (Intensilight C-HGFI; Nikon) and controlled by Image-Pro Plus® software (Media Cybernetics, Inc., Rockville, MD, USA). Oil immersion objective lenses of x40 or x100 were used for all imaging. For immunofluorescence analysis of YB-1 in cells, the cells were incubated with anti-YB-1 antibody for 1 h after fixation in 4% paraformaldehyde for 10 min and permeabilization in 0.2% Triton X-100 for 10 min. Conjugated antibody was visualized with Fluor Alexa 594-conjugated anti-mouse secondary antibody (Cell Signaling Technology).

Image analysis. Obtained images were analyzed with Fiji software (https://fiji.sc/). The nuclear/cytosolic (N/C) ratio of fluorescence intensity in the region of interest (ROI) of cells was calculated from ~100 cells.

Nuclear/cytosolic protein extraction and western blotting. For preparation of nuclear and cytosolic extracts, cells were suspended in 350 µl of cytosol extraction (CE) buffer (10 mM HEPES pH 7.6, 60 mM KCl, 1 mM EDTA, 0.075% NP-40, 1 mM dithiothreitol, protease inhibitor cocktail), incubated on ice for 3 min and centrifuged at 1,500 g for 4 min (18). After removal of the supernatant (cytosolic extract), the pellet was obtained as a nuclear extract after two washes in 1 ml of CE buffer. Nuclear and cytosolic extracts were dissolved in 120 µl of 1x Laemmli buffer. For whole protein extracts, cells were suspended in 1x Laemmli buffer, followed by SDS-PAGE and western blotting. Protein concentrations were determined using Pierce BCA Protein assay kit (Thermo Fisher Scientific) according to the standard protocol of the manufacturer. Equal amounts of protein (20-40 µg protein/lane) were separated on a 6-12% sodium dodecyl sulfate (SDS) gel via polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The primary antibodies used for western blotting were as follows; anti-YB-1 mouse monoclonal IgG antibody (clone: 21A3, 1:100), anti-phospho-YB-1 rabbit monoclonal IgG antibody (clone: 34A2, 1:100; Cell Signaling Technology), anti-p53 and P-glycoprotein mouse monoclonal IgG antibody (sc-55510; Santa Cruz Biotechnology, Dallas, TX, USA), anti-p53 mouse monoclonal IgG antibody (clone: DO-7, 1:200; Dako), anti-Akt goat polyclonal IgG antibody (clone: C-20, 1:2,000; Santa Cruz Biotechnology), anti-phospho-Akt rabbit monoclonal IgG antibody (clone: D9E, 1:1,000; Cell Signaling Technology), anti-Fibrillarin rabbit monoclonal IgG antibody (clone: 13C3, 1:1,000; Cell Signaling Technology), anti-α/β tubulin rabbit polyclonal IgG antibody (1:1,000; Cell Signaling Technology), and anti-actin goat polyclonal IgG antibody (clone: I-19, 1:1,000; Santa Cruz Biotechnology). Conjugated antibodies were detected by the appropriate secondary antibody: mouse IgG HRP-linked (1:5,000; Cell Signaling Technology) or rabbit IgG HRP-linked (1:5,000; Cell Signaling Technology) antibody or Peroxidase AffiniPure F(ab')2 fragment rabbit anti-goat IgG (1:5,000; IRJ, West Grove, PA, USA). Bands were visualized using the Lumi cube (Liponics, Ltd., Tokyo, Japan).

Statistical analysis. Comparison between the two groups of the immunohistochemical analysis was made using Fisher's exact test. The N/C ratio and viability of cells were analyzed using a Wilcoxon signed-rank test or a Steel's test. In all cases, results were considered significant at p<0.05.

Results

Localization of YB-1 and expression of p53 and P-glycoprotein in CIS. We first investigated the expression of YB-1 and P-glycoprotein in CIS (pTis) lesions in newly diagnosed bladder cancer samples. Table 1 summarizes the characteristics of the patients. Seventy-one (88%) out of the 81 patients were male and ten patients (12%) were female. Lesions of non-invasive papillary carcinoma (pTa) were also observed in 3 patients (4%) whereas 78 patients (96%) had a pTis lesion only. YB-1...
was expressed in 52 cases (64%). Nuclear expression of YB-1 was observed in 19 cases (23%), cytoplasmic expression in 37 cases (46%) and both nuclear and cytoplasmic expression was observed in 4 cases (5%) (Fig. 1A and B). P-glycoprotein expression was observed in 31 cases (38%). Expression of p-glycoprotein was significantly correlated with nuclear expression of YB-1 (P<0.05; Table I and Fig. 1C and D). Next, to clarify the involvement of p53, we investigated the expression of p53 in pTis cancers. p53 was expressed in 41 cases (51%) of pTis lesions (Fig. 1E) and its expression correlated with the nuclear translocation of YB-1 (P<0.05; Table II) and with the expression of P-glycoprotein (P<0.05; Table II). These data suggested that YB-1 coordinately
translocates to the nucleus with expression of p53 in CIS and thereby increases p-glycoprotein expression.

Effect of p53 on YB-1 nuclear translocation. To investigate whether p53 is involved in the nuclear translocation of YB-1, we introduced exogenous P53 into a human cancer cell line and then observed the cellular localization of YB-1. First, we assessed the expression levels of p53 in the HeLa, T24 and 5637 cells by western blotting. It is known that the expression level of the endogenous p53 protein is suppressed to a low level in HeLa cells (Fig. 2A) because of its ubiquitination and degradation by the E6 protein expressed in human papilloma virus transformed HeLa cells (19). T24 cells contain a P53 mutant that has an in-frame deletion of tyrosine 126 and this mutant p53 protein is expressed at a low level compared to cancer cell lines with wild-type P53. The 5637 cells contain a P53 point mutant (R280T) and overexpression of the p53 protein may correlate with P53 mutation (20,21). As expected, in western blotting the p53 protein was undetectable in HeLa and T24 cells, whereas high expression of p53 was seen in the 5637 cells (Fig. 2A). Next, we introduced P53 into HeLa cells. Thirty-six hours after transfection of pEGFP-wild-type p53, some of the YB-1 population had translocated to the nucleus (Fig. 2B) and the nuclear/cytoplasmic (N/C) ratio was increased compared to that of control cells (Fig. 2C). Moreover, introduction of a mutant p53 (R273H), which is known to lead to resistance to a variety of anticancer drugs, increased the N/C ratio compared to that of control or cells in which wild-type p53 had been introduced (22). These data suggested that p53 coordinates the nuclear translocation of YB-1 and that a high amount of p53 protein is essential for this translocation.

Drug-induced YB-1 nuclear translocation and p-glycoprotein expression. Next, we examined whether gemcitabine induces the nuclear translocation of YB-1. First, HeLa cells were transfected with mCherry-fused YB-1 (mCherry-YB-1) and treated with gemcitabine. Treatment with gemcitabine (35 µM) or with cisplatin (13 µM) induced translocation of mCherry-YB-1 to the nucleus (Fig. 3A) (23). Nuclear translocation of mCherry-YB-1 with gemcitabine treatment was also observed in the breast cancer MCF-7 cells and in the urinary bladder cancer 5637 and T24 cells, indicating that this phenomenon was not cell-specific. The nuclear translocation of YB-1 was maintained for up to 4 h after gemcitabine treatment. An immunohistochemical analysis in the 5637 cells showed that endogenous YB-1 also accumulated in the nucleus with

![Figure 3. Gemcitabine treatment induces nuclear translocation of YB-1.](image)
Yamashita et al.: YB-1 is important for resistance to chemotherapy in bladder cancer

Gemcitabine treatment (Fig. 3B), while YB-1 was not accumulated in T24 cells (data not shown). Since Akt-mediated phosphorylation of YB-1 at Ser102 is required for nuclear translocation of YB-1 (24), we checked the phosphorylation status of YB-1 in the nuclear fraction of gemcitabine-treated cells. The phosphorylation of nuclear YB-1 was increased 2 h after gemcitabine treatment (Fig. 4A-a). Furthermore, the increase of the phosphorylated YB-1 corresponded to an increase in P-glycoprotein expression (Fig. 4A-b). In addition, Akt-induced YB-1 mediated P-glycoprotein expression was more increased in the gemcitabine treated 5637 cells vs. control cells (Fig. 4B).

YB-1 knockdown decreased P-glycoprotein expression and sensitized 5637 cells to gemcitabine. To examine the effect of YB-1 knockdown on P-glycoprotein expression, the 5637 cells were transfected with YB-1 siRNA showed decreased resistance to gemcitabine compared with control. After 72 h, the cell survival rates were analyzed by cytotoxicity analysis. Brackets with asterisks indicate statistically significant differences between the data sets based on a Wilcoxon signed-rank test. *p<0.05.

Discussion

We present data indicating that YB-1 translocation to the nucleus correlates significantly with p53 expression in CIS. Nuclear translocation of YB-1 is thought to be involved in drug resistance by increasing MDR-1 transcription and its product, P-glycoprotein expression (9). The present study showed that YB-1 siRNA transfected into a bladder cancer cell line 5637 was analyzed by western blotting at the indicated times after gemcitabine treatment. The 5637 cells were treated with 10 µM gemcitabine for the indicated times following which cell extracts were made and 20 µg of the total extracted proteins were applied to each well of a gel for SDS-PAGE. Electrobotted membranes were stained with anti-P-glycoprotein (p-gp), anti-pYB-1, anti-pAkt, anti-Akt and anti-actin (loading control) antibodies.

Further we showed that the R273H mutation of p53 induced more YB-1 nuclear translocation compared to wild-type p53 in vitro. The R273H mutation of p53 is thought to be a ‘gain-of-function’ mutation that drives oncogenesis (25).
Zhang *et al* (15) reported that some tumor-derived mutants of p53 including R273H also induce nuclear translocation of YB-1 and that nuclear expression of YB-1 is not seen in p53 null cell lines. These data are consistent with recent reports that p53 upregulates YB-1 nuclear import in different cell types (11.15-17). Nuclear accumulation of p53 in bladder cancer is thought to be a result of mutation of p53, including the R273H mutation (26,27). Combined with these previous reports, our findings indicate that urinary CIS may acquire the property of drug resistance during its oncogenesis through overexpression of p53 or mutation of p53.

Third, we showed that gemcitabine induced both nuclear translocation of YB-1 and increased the expression of P-glycoprotein and, in addition, YB-1 knockdown increased the effect of gemcitabine in bladder cancer. To the best of our knowledge, this is the first report that YB-1 is involved in the effect of gemcitabine in bladder cancer. To the best of our knowledge, this is the first report that YB-1 is involved in the effect of gemcitabine in bladder cancer. We showed that gemcitabine treatment increased the nuclear translocation of YB-1 in 5637 cells that have a high expression level of p53, but not in T24 cells that have a low expression level of p53. These results support the notion that a high expression level of mutant p53 protein is necessary for nuclear translocation of YB-1 in bladder cancer. Nowadays, GC therapy is a standard therapy for aggressive bladder cancer. However, gemcitabine-induced drug resistance in bladder cancer compromises the therapeutic efficacy. Although it has been proposed that genes required for gemcitabine transport and metabolism such as human equilibrative nucleoside transporter-1 and deoxycytidine kinase are involved in the mechanism of cellular resistance to gemcitabine, the mechanisms by which gemcitabine resistance occurs are not fully understood (28,29).

Finally, gemcitabine has recently been used for intravesical chemotherapy and it is expected that gemcitabine will be more frequently used for bladder cancer treatment in the future. Our *in vitro* findings indicate that further investigation of the association of p53 expression and P53 gene status and YB-1 expression in bladder cancer with drug-resistance, including gemcitabine resistance, is warranted.

Acknowledgements

The present study was supported by a Grant-in-Aid for Young doctor of SMC (grant no. 26-F-1-05). We thank Kazuko Matsuno, Yuko Ohno, Kumiko Ohsawa and Tomoaki Aoki for their technical assistance, and Akiko Murata of Leica Microsystems for technical advice regarding immunohistochemistry.

References


