Forkhead box O-class 1 and Forkhead box G1 as Prognostic Markers for Bladder Cancer

Forkhead box O-class 1 (FOXO1) is a key regulator of glucose homeostasis, cell-cycle progression, and apoptosis. Its functions are modulated by forkhead box G1 (FOXG1), which acts as a transcriptional repressor with oncopgenic potential. Real-time PCR and immunohistochemical staining were performed in 174 primary bladder cancer specimens and 21 normal bladder mucosae to evaluate these genes. FOXO1 and FOXG1 mRNA expression in cancer tissues were higher than in normal mucosa (each P<0.001). FOXO1 mRNA levels were significantly higher in samples of non-progressed patients (P<0.001), but FOXG1 were enhanced in those of progressed patients (P=0.019). On univariate analysis, FOXO1 mRNA expression was significantly associated with grade, stage, recurrence, progression, and survival (each P<0.05). On multivariate analysis, increased FOXO1 mRNA expression was associated with both reduced disease progression (odds ratio [OR], 0.367; 95% confidence interval [CI], 0.163-0.826, P=0.015) and enhanced disease-free survival (OR, 3.262; 95% CI, 1.361-7.820, P=0.008). At a median follow-up of 33 months (range 2 to 156), the patients with a high FOXO1 mRNA expression had a significantly prolonged survival (P=0.001). Immunohistochemical findings of FOXO1 were generally concordant with mRNA expression levels. In conclusion, FOXO1 may be a promising marker for predicting progression in human bladder cancers.

Key Words: FOXO1; FOXG1; Urinary Bladder Neoplasms; Prognostic Factor; Real Time PCR; Immunohistochemistry

INTRODUCTION

Although epidemiologic and experimental evidences favor a strong role of chemical carcinogens in the etiology of bladder cancer, many cases arise without obvious exposure to known carcinogens (1). It is likely that all malignancies involve aberrations of normal mechanisms regulating cell differentiation and proliferation, often with derangements in the genetic composition of malignant cells. Since mechanisms usually exist in all cells to repair mutated or miscopied DNA or to affect the death of cells containing such altered DNA, the failure of these safeguard mechanisms must occur in most, if not all, malignancies. All of these influences undoubtedly play important roles in determining the development of bladder cancer.

Apoptosis is clearly an advantageous response to DNA damage if DNA repair fails, because it allows multicellular organisms to eliminate potentially harmful cells. Eliminated cells can be replaced from the organism’s pool of undamaged cells. Depending on the location, environment, or extent of damage, apoptosis may even be a primary response (2). Therefore, it is not surprising that aberrations in apoptosis can be detrimental and that the failure of dividing cells to initiate apoptosis after sustaining severe DNA damage contributes to cancer (3).

Survival factors suppress the intrinsic cell-death machinery, thereby preventing apoptosis (4). Forkhead box O-class (FOXO) transcription factors, including FOXO1, FOXO3a, and FOXO4, function as tumor-suppressor proteins by inhibiting cell proliferation, promoting apoptosis, and protecting cells from oxidative stress and DNA damage. The potency of these functions is tightly regulated by phosphorylation, acetylation, and ubiquitination. FOXO1 is a key regulator of glucose homeostasis, cell-cycle progression, and apoptosis (5). Emerging evidence indicates that protein levels of FOXO1 are regulated by phosphoinositide-3-kinase-protein kinase B (PI3K-PKB/Akt)-mediated phosphorylation. Akt is highly active in human cancers due to the loss of phosphatase and tensin homolog (PTEN) (6). FOXO1 is modulated by forkhead box G1 (FOXG1), another member of the Fox transcription factor family, which acts as a transcriptional repressor with oncogenic potential (7). FOXG1 recognizes some of the same targets as FOXO, but functions as a repressor rather
than an activator (8).

However, little is known about the properties and roles of FOXO1 and FOXG1 in human bladder cancer. In this study, we explored relationships between these genes and clinicopathologic characteristics in bladder cancer patients using real-time polymerase chain reaction (PCR) and immunohistochemical staining.

**MATERIALS AND METHODS**

**Patients and tissue samples**

One hundred and seventy-four primary bladder cancer samples were taken in the Chungbuk National University Hospital, Cheongju, Korea. Histological diagnoses revealed that all patients had transitional cell carcinoma. Table 1 lists demographic data. Twenty one normal bladder tissues were obtained from patients with benign diseases. These were dissected in order to separate from the mucosa from the underlying smooth muscle, which were histologically confirmed normal mucosae on frozen sections. Informed consent was obtained from each subject and the study was approved by the Institutional Review Board of the Chungbuk National University College of Medicine. Median follow-up was 33 months (range 2 to 156). In this study, we defined the superficial recurrence as the cancer recurrence of primary superficial bladder cancer without progression, and the progression as the cancer progression both of superficial bladder cancer to invasive or metastatic disease and of invasive cancer to metastatic disease after adequate treatment. All specimens were rapidly frozen in liquid nitrogen and stored at -80°C until the RNAs were extracted.

**Real-time PCR**

Total RNA was isolated from the tissues with TRIzol reagent (Life Technologies, NY, U.S.A.) according to the manufacturer’s instructions. cDNA was prepared from 1 μg of total RNA by random priming using a First-Strand cDNA Synthesis Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) according to the manufacturer’s protocol. To quantify the expression levels of FOXO1, real-time PCR amplification was performed with a Rotor Gene 3000 PCR instrument (Corbett Research, Mortlake, Australia). Quantitative values were obtained from the cycle threshold (Ct) number at which increase in the signal associated with exponential growth of PCR products began to be detected. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous RNA reference gene. Each sample was checked on the basis of its GAPDH content. Real-time PCR assays using SYBR Premix EX Taq (TAKARA BIO INC.,

| Variables                      | No. of patients (%) | FOXO1 (×10³ copies/μL) | P* | FOXG1 (×10³ copies/μL) | P* |
|-------------------------------|---------------------|------------------------|----|------------------------|----|
| Sex                           | Male 145 (83.3)     | 14.57 ± 1.11           | <0.001 | 0.92 ± 0.1           | <0.001 |
|                               | Female 29 (16.7)    | 71.17 ± 1.11           |     | 2.58 ± 0.4            |     |
| Age (yr)                      | 64.58 ± 0.89        |                        |     |                        |     |
| Normal versus cancer          |                     |                        |     |                        |     |
| Normal                        | 21                  | 14.57 ± 1.11           | <0.001 | 0.92 ± 0.1           | <0.001 |
| Cancer                        | 174                 | 71.17 ± 1.11           |     | 2.58 ± 0.4            |     |
| Stage                         |                     |                        |     |                        |     |
| Superficial                   | 111 (63.8)          | 93.86 ± 1.14           | 0.001 | 2.57 ± 0.42           | 0.966 |
| Invasive                      | 63 (36.2)           | 43.71 ± 1.18           |     | 2.60 ± 0.61           |     |
| Grade                         |                     |                        |     |                        |     |
| Low                           | 116 (66.7)          | 82.44 ± 1.14           | 0.048 | 2.08 ± 0.81           | 0.134 |
| High                          | 58 (33.3)           | 53.03 ± 1.19           |     | 1.81 ± 0.46           |     |
| Superficial recurrence        |                     |                        |     |                        |     |
| No recurrence                 | 72 (64.9)           | 113.92 ± 1.18          | 0.042 | 2.53 ± 0.55           | 0.173 |
| Recurrence                    | 39 (35.1)           | 65.64 ± 1.23           |     | 3.49 ± 0.85           |     |
| Progression                   |                     |                        |     |                        |     |
| No progression                | 135 (77.6)          | 87.38 ± 1.13           | <0.001 | 2.37 ± 0.38           | 0.019 |
| Progression                   | 39 (22.4)           | 34.98 ± 1.20           |     | 4.84 ± 1.07           |     |
| Survival                      |                     |                        |     |                        |     |
| Alive                         | 137 (78.7)          | 87.66 ± 1.13           | 0.001 | 2.68 ± 0.42           | 0.239 |
| Dead                          | 37 (21.3)           | 34.33 ± 1.19           |     | 3.96 ± 0.99           |     |

* Determined using the t-test.

FOXO1, Forkhead box O-class 1; FOXG1, Forkhead box G1.
staining was mainly cytoplasmic. As cognate anti-FOXG1 antibody was not commercially available, we were unable to study this factor immunohistochemically.

For semiquantitative assessment of the immunohistochemical results, the mean percentage of positive tumor cells was determined in at least 10 random fields at \( \times 400 \) magnification in each section. It was graded as focal (10%), regional (11-50%), or diffuse (>50%). The intensity of immunoreaction was graded weak, moderate, or intense. The mean percentage of positive tumor cells and the staining intensity were then combined to produce immunohistochemical results. The results were graded as negative (0), moderate regional (1), moderate diffuse (2), intense regional (3), and intense diffuse (4). Inter-observer differences were minimal. The consensus opinions were used to assign final scores to the disputed cases before data analysis.

Statistical analysis

All statistical analyses were carried out using the SPSS package, Release 10.0 (SPSS Inc., Chicago, IL, U.S.A.). Because of its highly skewed distribution, the FOXO1 and FOXG1 mRNA data were examined as the natural log function and subsequently back transformed for the interpretation of the model results. The mRNA data were presented as the means and SEMs. Student’s t-tests were applied to assess the association of the mRNA expression levels with the development, progression, recurrence of the cancer and with survival. Pearson’s correlation was used for the relation between the expression level of FOXO1 and FOXG1. Multivariate logistic regression analyses were also performed to identify the factors that had a significant effect on cancer progression and survival. We used receiver-operating-characteristic (ROC) curves to analyze FOXO1 mRNA levels in order to determine the cutoff point (51.48 \( \times 10^3 \) copies/\( \mu \)L) that yielded the highest combined sensitivity and specificity for disease-free survival. Kaplan-Meier curves were generated and compared by the log rank test for survival rates. The immunohistochemical data were analyzed using chi-square method.

RESULTS

Quantification of FOXO1 and FOXG1 mRNA expression levels

Considerably higher FOXO1 and FOXG1 expression were observed in bladder cancer tissues than in normal mucosae (each \( P < 0.001 \)). Table 1 summarizes the expression levels of FOXO1 and FOXG1 according to the stage, grade, superficial recurrence, progression, and disease-free survival of bladder cancer. The FOXO1 expression levels in superficial bladder cancers were significantly higher than in invasive cancers (\( P < 0.001 \)). FOXO1 expression levels were significantly ele-
vated in low-grade compared with high-grade bladder cancers \((P=0.048)\). FOXG1 did not show any relations with the stage and grade of bladder cancer (each \(P>0.05\)).

In the superficial bladder cancers, the expression levels of FOXO1 were enhanced in non-recurred patients compared with recurred patients \((P=0.042)\), whereas FOXG1 did nor. The FOXO1 mRNA levels were significantly higher in bladder cancer tissues from the patients without progression than in progressed cases \((P<0.001)\). In contrast, the expression of FOXG1 mRNA in primary bladder cancer that had progressed was enhanced compared to cases that had not \((P=0.019)\). In addition, a weak negative correlation between mRNA expression levels of FOXO1 and FOXG1 was observed in bladder cancer tissues (Pearson’s sample correlation coefficient \(r=-0.220\), \(P=0.008\)).

The association between the FOXO1 expression levels and the disease-free survival of patients was also analyzed. Our data showed that the patients with elevated FOXO1 mRNA expression in their primary bladder cancers had significant survival benefits compared to those with low expression. Expression levels of FOXO1 in primary cancer tissues of living patients were significantly higher than in those of deceased cases \((P<0.001)\).

Multivariate analysis results showed that the cancer stage and FOXO1 mRNA expression level were strong predictors of cancer progression and disease-free survival (Table 2). In particular, increased FOXO1 mRNA expression levels were associated with both reduced disease progression (odds ratio \([\text{OR}], 0.367; 95\% \text{ confidence interval } [\text{CI}], 0.163-0.826, P=0.015\) and enhanced disease-free survival \([\text{OR}], 3.262; 95\% \text{ CI}, 1.361-7.820, P=0.008\).

Fig. 1 shows the Kaplan-Meier analyses of bladder cancer disease-free survival stratified by FOXO1 mRNA expression level. The patients with elevated FOXO1 mRNA expression level in their primary bladder cancers had significant survival benefit compared to those with low-expression \((P=0.001)\).

Expression of FOXO1 by immunohistochemical staining

Immunohistochemistry revealed cytoplasmic staining of FOXO1 in urinary bladder cancers (Fig. 2). Table 3 shows the relationship between expression levels of FOXO1 and bladder cancer. FOXO1 expression levels were significantly higher in low-grade cancers than in high-grade cancers \((P<0.001)\). FOXO1 was more strongly expressed in superficial bladder

**Table 2.** Multivariate logistic regression analyses of age, sex, grade, stage, and FOXO1 mRNA expression levels in bladder cancers for the prediction of bladder cancer progression and disease-free survival

| Variables                  | Odds ratio | 95% CI     | \(P\)  |
|----------------------------|------------|------------|--------|
| Prediction of progression  |            |            |        |
| Age                        | 1.198      | 0.536-2.678| 0.660  |
| Sex                        | 1.964      | 0.766-5.032| 0.160  |
| Grade                      | 0.904      | 0.336-2.435| 0.842  |
| Stage                      | 4.308      | 1.598-11.615| 0.004  |
| mRNA expression levels     | 0.367      | 0.163-0.826| 0.015  |
| of FOXO1                   |            |            |        |
| Prediction of disease-free survival | |            |        |
| Age                        | 0.326      | 0.271-1.543| 0.326  |
| Sex                        | 0.613      | 0.223-1.685| 0.343  |
| Grade                      | 1.496      | 0.523-4.280| 0.452  |
| Stage                      | 0.111      | 0.037-0.329| <0.001 |
| mRNA expression levels     | 3.262      | 1.361-7.820| 0.008  |
| of FOXO1                   |            |            |        |

FOXO1, Forkhead box O-class 1; 95\% CI, 95\% confidence interval.

**Fig. 2.** The FOXO1 expression shows strong cytoplasmic reactivity on urothelial cancer cells (\(\times 200\)).
cancers than in invasive cancers (P=0.001). Although FOXO1 expression was higher in patients with recurrence than in those without recurrence, the difference was not statistically significant (P=0.143). FOXO1 expression in bladder cancer tissues from non-progressed patients was significantly higher than in those from progressed cases (P=0.005). A significant correlation was also found between FOXO1 expression levels and disease-free survival (P=0.003).

**DISCUSSION**

The FOXO family is regulated by the PI3K-PKB/c-Akt pathway. PKB-induced phosphorylation inhibits transcriptional activity of the FOXO members, which control the cell cycle, cell death, cell metabolism, and response to oxidative stress (9). Studies in mammalian cells have shown that the overproduction of FOXO1 induces either cell-cycle arrest or apoptosis (9). By increasing the production of the cyclin-dependent kinase inhibitor p27kip1, FOXO transcription factors cause cell-cycle arrest in the G1 phase of the cell cycle (10). Recently, FOXO factors have also been implicated in the control of proper progression through the G2-M phase of the cell cycle (11). The production of FOXO family members can also cause cell death by apoptosis (12).

Support for a connection between FOXO proteins and oncogenic transformation is provided by another Fox family protein, FOXG1. This transcription factor was originally identified as brain factor 1 and has also been recovered as the retroviral oncprotein Qin in ASV31 (13, 14). The oncogenic transformation induced by retroviral expression of FOXG1 directly correlates to transcriptional repression (7, 15). In addition, FOXG1 binds the consensus sequence tgtaaaacaaa (15), which is similar to the binding motif for FOXO proteins (gtttaaaacaa) (16). Using cotransfections of FOXO1 and FOXG1 with the reporter pGL3-CMV-3xIRS, Aoki et al. demonstrated that FOXG1 is a strong inhibitor of FOXO1-mediated transcriptional activation. They suggested that FOXG1 and FOXO1 might negatively and positively regulate a shared suite of target genes, respectively (17).

In this study, we investigated the mRNA expression levels of FOXO1 and FOXG1 in human bladder cancer. This is the first study to investigate the relationships between the expression of these genes and clinico-pathological parameters in bladder cancer to our knowledge. Our data shows that FOXO1 and FOXG1 are more highly expressed in bladder cancer tissues than in normal bladder mucosa, which suggests that FOXO1 might be activated during tumorigenesis in the bladder. At present, the exact mechanisms for enhanced expression of FOXO1 and FOXG1 in bladder cancer are unknown. One possible explanation is based on the relationship between FOXO1 and FOXG1. The function of FOXO1 is further modulated by FOXG1. FOXG1 acts as a transcriptional repressor, has oncogenic potential (7), and can repress some of the same targets that are activated by FOXO1 (8). Therefore, FOXO1 expression might increase to compete with FOXG1 suppression. Another possible explanation for enhanced FOXO1 expression may be that, a variety of much stronger oncogenic activity mask the protective effect of FOXO1.

We also analyzed the expression levels of FOXO1 in relation to the clinical findings in bladder cancer patients. FOXO1 immunohistochemical findings were generally in concordance with mRNA expression levels, suggesting that FOXO1 expression might be primarily determined at the transcription-
Our data demonstrate that FOXO1 expression is enhanced in superficial bladder cancer compared to invasive cancer and that FOXO1 expression levels are higher in low-grade cancers (grades 1 and 2) compared to high-grade cancers (grade 3). Bladder cancers with increased FOXO1 expression exhibited pathological features of less aggressive, superficial and low-grade bladder cancers. There is a clear survival advantage for the advanced cancer cell that can protect itself from apoptosis. However, a rapidly growing, infiltrative, advanced tumor that is outgrowing its blood supply and mutating its DNA may have enhanced activation of an apoptosis-related pathway despite protective mechanisms acquired by the tumor cells. However, it remains unclear why FOXO1 expression increases in superficial cancers as compared to invasive tumors.

Although the immunohistochemical findings were not statistically significant, the expression levels of FOXO1 mRNA in primary superficial bladder cancer tissues were significantly higher in non-recurred than in recurred patients in our study. When comparing primary bladder cancer cases that had progressed with those that had not, we observed significantly higher expression of FOXO1 in the latter group and higher levels of FOXG1 in the former. We observed a negative correlation between the levels of FOXO1 and FOXG1 mRNA expression in bladder cancer tissue, a finding supported by Aoki et al., who demonstrated that FOXG1 strongly inhibited FOXO1-mediated transcriptional activation (17). These results indicate that FOXO1 and FOXG1 expression may be useful prognostic markers for bladder cancer progression. Patients with enhanced FOXO1 expression exhibited a higher disease-free survival rate than those with low FOXO1 expression, implying that enhanced FOXO1 expression might suppress disease progression and provide a survival benefit for bladder cancer patients. These findings are further supported by the multivariate analysis, which showed that the cancer stage and FOXO1 mRNA expression level were strong predictors of cancer progression and disease-free survival.

In conclusion, enhanced expression of FOXO1 and FOXG1 are strongly associated with bladder cancer development. Moreover, enhanced expression of FOXO1 is positively associated with low rate of recurrence or progression and with survival, whereas increased expression of FOXG1 correlates to disease progression. Thus these genes may be useful prognostic markers for human bladder cancers.

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