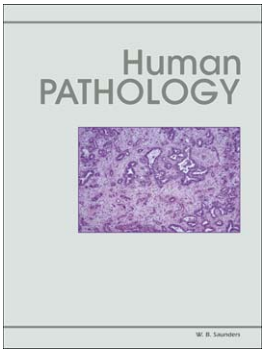

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FOXO1 downregulation is associated with worse outcome in bladder cancer and adds significant prognostic information to p53 overexpression

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Summary

Nuclear FOXOs mediate cell cycle arrest and promote apoptosis. FOXOs and p53 could have similar effects as tumor suppressor genes. In spite of extensive literature, little is known about the role of FOXO1 and its relationship with p53 status in bladder cancer. Expression of FOXO1 and p53 were analyzed by immunohistochemistry in 162 urothelial carcinomas (UC). Decreased FOXO1 expression, p53 overexpression and the combination FOXO1 downregulation/p53 overexpression were strongly associated with high grade (p=0.030; p=0.017; p=0.004, respectively), high stage (p=0.0001; p<0.0001; p<0.0001, respectively) or both (p=0.0004; p<0.0001; p<0.0001, respectively). In the overall series of cases, p53 overexpression was associated with tumor progression (HR=3.18, 95% CI 1.19-8.48 p=0.02), but this association was even stronger if having any alteration in any of the two genes was considered (HR=3.51, 95% CI 1.34-9.21 p=0.01). Having both FOXO1 downregulation and p53 overexpression was associated with disease recurrence (HR=2.75, 95% CI 1.06-7.13 p=0.03). In the analysis of the different subgroups, having any alteration in any of the two genes was associated with progression in low grade (p=0.005) and pTa (p=0.006) tumors. Finally, the combined FOXO1 downregulation/p53 overexpression was associated with disease recurrence specifically in high grade (p=0.04) and in pT1 stage tumors (p=0.007). Adding FOXO1 expression to the immunohistochemical analysis of p53 can provide relevant prognostic information on progression and recurrence of bladder cancer. It may be particularly informative on the risk of progression in the more indolent and on the risk of recurrence in the more aggressive tumors.

1. Introduction

FOXO genes represent a family of four members with high protein homology that are involved in different cellular processes. In mammals, FOXO subfamily consists of four members: FOXO1, FOXO3a, FOXO4 and FOXO6. Nuclear localization of FOXOs stops cell cycle progression, promotes apoptosis, and negatively regulates angiogenesis [1,2].

Several kinases can phosphorylate and regulate FOXO members, promoting FOXO inhibition by nuclear export and degradation by proteosome. Phosphorylation-dependent nuclear/cytoplasmic shuttling of FOXOs is mainly regulated by AKT and PTEN. FOXO dephosphorylation leads to nuclear translocation and target gene activation [3,4].

The control of FOXOs by acetylation and ubiquitylation has been well-characterized [3,5]. On the other hand, several microRNAs can regulate the expression of different members of the FOXO family [4,6]. FOXOs interact with major transcription regulators such as p53, MYC and β-catenin [4]. Different evidence suggests that FOXO and p53 play similar roles as tumor suppressor genes [1,4,7].

Urothelial carcinoma *in situ* and invasive urothelial carcinomas (UC) of the bladder have deregulated p53 and retinoblastoma pathways [8-10]. Numerous studies have shown the association of p53 alterations with high grade and with muscle-invasive tumors, and it is well known that the prevalence of p53 alterations in bladder cancer increases with stage and grade [10-13]. p53 has been extensively studied as a marker to predict recurrence and progression in urothelial cell carcinoma [14-17]. However, the prognostic value of p53 is still not fully clear in advanced disease nor in high grade superficial tumors. In addition, there is no definite evidence that p53 overexpression is an independent prognostic factor [10-12,15,18,19].

The role of FOXOs in tumorigenesis was initially discovered because of their involvement in chromosomal translocations in human cancers [1]. FOXO1 is downregulated in tumors from different organs, such as breast, uterine cervix, kidney and prostate, among others [20–23]. However, at present very few studies have investigated the alterations of FOXO1 in bladder tumors [24-26], and little is known about its association with p53 status in bladder cancer. The purpose of the present study has been to analyze the relationship of FOXO1 with different clinical and pathological varieties of urothelial carcinoma, as well as the effects of the coexistence of alterations in both FOXO1 and p53 in these tumors.

2. Materials and methods

2.1. Tumor Samples and Patients

A total of 162 formalin fixed paraffin embedded tumors (FFPE) were selected retrospectively and obtained from the Parc de Salut MAR-Biobank (MARBiobanc) of Barcelona, Spain. Consecutive cases of primary papillary urothelial carcinoma were included in the study. The exclusion criteria were: lack of muscularis propria in the biopsy, associated in-situ carcinoma, and lack of adequate tissue for TMA construction. With these criteria, 199 cases were excluded. All the cases were first diagnoses of urothelial carcinoma. No patient had received any treatment prior to transurethral resection biopsy (TURB). On the other hand, 72,1% of patients were treated with Bacillus Calmette-Guerin (BCG) after biopsy. Table 1 summarizes the clinical-pathological features of the patients/tumors. Tumor grading and staging was carried out according to the TNM classification and the WHO 2004 (two-tiered classification) criteria. In addition, the same analysis was also performed using the WHO 1973 (three-tiered classification) to test if using a three-tiered scheme yielded any additional

prognostic information. According to stage, the FFPE tumors were 97 pTa, 46 pT1 and 19 pT2; in the two-tiered grade classification, 41 tumors were low grade and 121 high grade. Distribution according to the combination of stage and grade was 41 pTa-low grade (pTa-LG), 56 pTa-high grade (pTa-HG), 46 pT1-high grade (pT1-HG) and 19 pT2-high grade (pT2-HG).

2.2 TMA construction and immunohistochemistry of FOXO1 and P53

Tissue microarrays (TMA) were constructed using a manual tissue arrayer (Chemicon ATA-100, California, US). Tissue cores of 1mm in diameter were obtained from the most representative tumor areas of FFPE tissue donor blocks and were arranged in recipient TMA blocks. The TMAs contained cores from 162 patients. For each case, one core was selected from a hematoxylin-eosin (H&E) stained section of a donor block. From the three resulting TMA blocks, 3 μm sections were transferred to glass slides. H&E stained sections of the TMA blocks were assessed to confirm grade and stage in each core.

Immunohistochemical staining for FOXO1 (clone C29H4, Cell Signaling Technology Inc, Beverly, MA, US) was performed in the 162 FFPE tumor bladder samples. To obtain a final FOXO1 histoscore, the histoscores for nuclear and cytoplasmic FOXO1 staining were added. Results were quantified using the histoscore method: the sum of the product of the staining and the corresponding tumor percentage (histoscore = 1 x (%1+ cells) + 2 x (%2+ cells) + 3 x (%3+ cells)). Histoscore < 10 was considered loss of protein expression [22].

Immunohistochemical staining for p53 (clone 1801, Leica/Novocastra, Buffalo, NY, US) was successfully performed in a total of 155 of the FFPE tumor bladder

samples from the TMA sections. Aberrant p53 protein (nuclear) immunostaining was considered for histoscore values ≥ 20 [11,27].

2.3 Statistical analysis

Categorical variables are presented as frequencies and percentages. Chi-square or Fisher's Exact test were used to compare categorical variables among groups. Data on recurrence and progression were collected from 161 patients. The first event of cancer recurrence was defined as the first date after an initial transurethral resection (TUR) at which another TUR, biopsy or cytology emerged with a diagnosis of urothelial malignancy [28]. These criteria for recurrence were not applied when a positive TUR, biopsy or cytology were found within the first three months after patient's diagnosis, a situation that was considered to be primary residual disease. In addition, tumor progression was considered when patients presented with a recurrence event of higher grade and/or stage than the primary tumor. Cases with no recurrence or no progression were right censored.

The relationship with disease recurrence (first event) and progression was analyzed using Kaplan–Meier analysis (Log-Rank test to study differences among groups) in 161 patients (1 case was lost for follow-up). Cox proportional hazard ratios were estimated to obtain risks of recurrence and progression after adjusting for established prognostic variables as grade, stage, sex, age and tobacco consumption. The assumption of proportional hazards was checked for each variable.

P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using the SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA) and the statistical environment R (v 3.1.1).

3. Results

3.1 Decrease in FOXO1 expression: Relationship with stage and grade in UC

The distribution of FOXO1 expression loss according to grade and stage is shown in Table 1. Immunohistochemistry of FOXO1 was performed in the 162 cases on TMA sections. Total FOXO1 histoscore ranged from 0 to 465. Histoscore values < 10 were considered as low FOXO1 expression: 79 (48.8%) tumors showed values < 10, and 83 (51.2%) tumors had histoscore values from 10 to 465. Thirty-one tumors showed values from 10 to 60, 22 cases from 61 to 150 and 30 from 151 to 465.

According to the two-tiered grading system (WHO 2004) 14 of 41 (34.1%) low grade and 65 of 121 (53.7%) high grade UC showed FOXO1 downregulation (Pearson Chi-Square, p = 0.030) (Table 2). High grade tumors are statistically associated with decreased FOXO1 expression. According to stage, 37 of 97 (38.1%) pTa, 25 of 46 (54.3%) pT1 and 17 of 19 (89.5%) pT2 tumors showed low FOXO1 expression (Pearson Chi-Square, p = 0.0001) (Table 2). Thus, there is a statistical association between higher stage tumors and low FOXO1 expression. Combining stage and grade, 14 of 41 (34.1%) pTa-LG; 23 of 56 (41%) pTa-HG; 25 of 46 (54.3%) pT1-HG and 17 of 19 (89.5%) of pT2-HG tumors showed low FOXO1 expression (Pearson Chi-Square, p = 0.0004). Also, low FOXO1 expression was statistically associated with high stage and grade UC.

3.2 Immunohistochemistry of p53 and relationship with FOXO1 expression

p53 IHC was available in 155 cases. Nuclear histoscore ranged from 0 to 220. As previously reported [11,27], histoscore values ≥ 20 were considered as p53 overexpression; 123 (79.4%) tumors showed values ranging from 0 to < 20, and 32

(20.6%) tumors overexpressed p53, with values ranging from 20 to 220. The relationship between p53 and FOXO1 immunostaining was analyzed in these 155 cases. Fifty-eight tumors (37.4%) showed downregulation of FOXO1 with *wt* p53, 14 tumors (9%) showed p53 overexpression but normal FOXO1 levels, 65 (50%) were *wt* for both genes, and 18 (11.6%) showed alterations in both (Fig. 1A-D). There was no relationship between p53 and FOXO1 expression (Pearson Chi-Square, $p = 0.359$)

3.3 p53 overexpression and relationship with grade and stage in UC

The distribution of the p53 alterations according to grade and stage is shown in Table 2. Only 3 of 40 (7.5%) low grade tumors showed p53 overexpression, with a histoscore equal or higher than 20, whereas 29 of 115 (25.2%) high grade tumors presented p53 alterations (Pearson Chi-Square, $p = 0.017$). According to stage, 6 of 92 (6.5%) pTa, 15 of 44 (34%) pT1 and 11 of 19 (57.9%) pT2 (Pearson Chi-Square, $p < 0.0001$), and according to both stage and grade, 3 of 40 (7.5%) pTa-LG, 3 of 52 (5.8%) pTa-HG, 15 of 44 (34%) pT1-HG and 11 of 19 (57.9%) pT2-HG showed p53 overexpression (Pearson Chi-Square, $p < 0.0001$). As expected, p53 overexpression was associated with high grade, high stage and high grade and stage.

3.4 Combination of FOXO1 and p53 alterations and relationship with the clinical-pathological features

We have analyzed the distribution of the FOXO1 and p53 alterations in the different groups of bladder tumors and the relationship with the clinical-pathological features of the patients. For this purpose, the following comparisons were made: FOXO1*wt*/p53*wt* *vs* alteration in FOXO1 and/or p53 (i.e. *wt* cases compared with all the abnormal cases in one or both genes) and FOXO1*wt*/p53*wt* *vs* 1 alteration and 2

alterations (i.e. *wt* cases compared with tumors having one abnormal gene and with tumors having two abnormal genes).

To harbor one alteration either in FOXO1 or in p53, or alterations in both genes was associated with high grade (Pearson Chi-Square, $p = 0.0022$), high stage (Pearson Chi-Square, $p < 0.0001$), and with high grade and stage tumors (Pearson Chi-Square, $p < 0.0001$) (Table 2).

If we compared *wt* cases *vs* those harboring 1 or 2 alterations (Fig. 2), only 18 cases had alterations in the two genes, p53 overexpression and FOXO1 downregulation. The prevalence of one alteration increased moderately in higher grade tumors, but having two alterations, this is having the combination p53 overexpression/FOXO1 downregulation was strongly associated with high grade, high stage, and high stage/grade bladder tumors.

The p53 overexpression/FOXO1 downregulation phenotype was present in 1 of 40 (2.5%) low grade, but in 17 of 115 (14.8%) high grade bladder tumors (Pearson Chi-Square, $p = 0.0044$) (Table 2). With regard to the stage, 1 of 92 (1.1%) pTa, 7 of 44 (16%) pT1 and 10 of 19 (52.6%) pT2 tumors presented alterations in both genes (Pearson Chi-Square, $p < 0.0001$) (Table 2). Finally, the distribution according to both stage and grade was 1 of 40 (2.5%) pTa-LG, 0 of 52 pTa-HG, 7 of 44 (15.9%) pT1-HG, and 10 of 19 (52.6%) pT2-HG (Pearson Chi-Square, $p < 0.0001$) (Table 2).

3.5 FOXO1 and p53 alterations and relationship with disease recurrence and progression

Kaplan-Meier analysis was performed with respect to bladder cancer disease recurrence and progression for FOXO1 downregulation *vs* FOXO1 *wt*, p53 overexpression *vs* p53 *wt*, FOXO1 and/or p53 alteration *vs wt*, and the group with both

FOXO1 downregulation and p53 overexpression *vs* the other combinations (*wt* for both genes, and FOXO1 downregulation alone and isolated p53 overexpression). p53 overexpression was associated with tumor progression (p = 0.016; Fig. 3A) and decreased FOXO1 expression also showed a trend towards an association with progression (p = 0.062; Fig. 3B). To harbor any alteration, either FOXO1 downregulation or p53 overexpression, or both, was even more strongly associated with bladder tumor progression (p = 0.005; Fig. 3C). With respect to disease recurrence, there was no association with FOXO1 or p53 alterations, in the global series of cases.

The Kaplan-Meier analysis was performed also according to the different clinical-pathological groups. As low grade tumors had a very low frequency of p53 alterations, the log rank test taking into account only p53 overexpression *vs* p53 *wt* was not performed. In pTa stage tumors, p53 overexpression was statistically associated with progression (p = 0.0001). To have one or two altered genes (FOXO1 and/or p53 overexpression) was also statistically associated with progression in low grade (p = 0.005; Fig. 4A) and in pTa stage (p = 0.006; Fig. 4B) and showed a trend to be associated with it in high grade bladder tumors (p = 0.063). Moreover, the combination FOXO1 downregulation plus p53 overexpression showed a trend to be associated with progression in T1 stage tumors (p = 0.058).

With regard to bladder cancer disease recurrence (first event after initial diagnosis) no gene alteration was associated with disease recurrence when we considered all patients grouped together, as indicated above. However, when the cases were stratified according to the different clinical-pathological groups, the combination FOXO1 downregulation plus p53 overexpression was statistically associated with disease recurrence in high grade (p = 0.046; Fig. 5A) and pT1 tumors (all of them also high grade) (p = 0.007; Fig. 5B).

For the multivariate analysis the whole group of tumors was considered (Table 3). On one hand cases with p53 overexpression (HR 3.18, 95% CI 1.19-8.48 p = 0.02) and on the other hand, the group of cases with the combination of FOXO1 and/or p53 alterations (HR 3.51, 95% CI 1.34-9.21 p = 0.01) were both independently associated with a higher probability of progression. In addition, FOXO downregulation plus p53 overexpression were independently associated with a higher probability of disease recurrence (HR 2.75, 95% CI 1.06-7.13 p = 0.037). A multivariate analysis was not possible in the different clinical-pathological groups due to the small number of cases in each of them.

3.6 FOXO1 and p53 alterations and relationship with clinical-pathological features and outcome according to three-tiered classification (WHO 1973)

According to three-tiered grade classification (WHO 1973), 14 of 41 (34.1%) grade 1, 31 of 66 (47%) grade 2 and 34 of 55 (61.2%) grade 3 UC showed decreased FOXO1 expression (Pearson Chi-Square, p = 0.025). In addition, 3 of 40 (7.5%) grade 1, 7 of 62 (11.3%) grade 2 and 22 of 53 (41.5%) grade 3 harbored p53 alterations (Pearson Chi-Square, p < 0.0001). If we considered *wt* cases *vs* those harboring 1 or 2 alterations, the combination of p53 overexpression plus FOXO1 downregulation was dramatically associated with grade 3 tumors (Pearson Chi-Square, p < 0.0001). Downregulation of FOXO1 or p53 overexpression, considered separately, was also associated with grade 3 (Supplementary Table).

To have at least one altered gene was also statistically associated with progression in grade 1 (Log Rank test, p = 0.005) and grade 2 tumors (Log Rank test, p = 0.032). Furthermore, p53 overexpression was statistically associated with progression in grade 2. Thus, the finding of any change in either FOXO1, p53 or both was also associated with progression, independently in grade 1 and grade 2 groups.

4. Discussion

FOXO family members transcriptionally activate or inhibit downstream target genes that are involved in different cellular processes, such as cell cycle progression and apoptosis among others [2,3]. FOXOs mediate cell cycle arrest at the G1/S and G2/M transition, by upregulating the expression of cell cycle inhibitors such as p21, p27, p15, and p19 [29,30]. FOXO transcriptional activity is regulated by a complex array of posttranslational modifications [2]. Different evidence suggests that FOXO and p53 play similar roles in their function as tumor suppressor genes [1,4,7]. The role of FOXOs in tumorigenesis was initially discovered by their involvement in chromosomal translocations in human cancers [1,4]. FOXO1 is downregulated in various types of cancer [20–23]. However, at present very few studies have analyzed the role of FOXO1 in bladder tumors [24-26] and little is known about it.

In order to gain insight on the role of FOXO1 in urothelial carcinoma and its relationship with the status of p53, we have performed FOXO1 and p53 immunohistochemistry in a series of bladder tumors. Our results are in concordance with previous literature regarding FOXO1 in bladder tumors [24-26]. Kim *et al* [25] described for the first time an association between *FOXO1* mRNA expression and grade, stage, recurrence, progression and survival. Guo *et al* [24] showed that miR-96 expression was upregulated in bladder tumors, and consequently *FOXO1* expression was reduced, regulating FOXO1 mediated cell apoptosis. miR-135 is able to downregulate FOXO1, leading to increased cell proliferation in urothelial cell carcinomas [26]. Our results show that there is also FOXO1 downregulation at the protein level in bladder tumors, and that this loss of expression is associated with an aggressive tumor phenotype (high grade and stage). In other tumor types, such as cervical [21] and renal cell carcinoma [22] decreased FOXO1 levels were also associated with aggressive cases.

It is well known that p53 alterations are associated with muscle-invasive and high grade bladder tumors, and that they increase with stage and grade [10–12,17,18]. p53 has been extensively studied as a marker to determine the risk of recurrence and progression in urothelial cell carcinoma [14–17,31]. However, the impact of p53 alterations on recurrence and survival in urothelial cell cancer remains incompletely understood and conflicting results have been reported [11,16,31]. Different studies have analyzed the presence of p53 alterations by IHC and in many of these studies a good correlation between *TP53* gene mutations and protein status has been found [12,18,32–34]. In keeping with previous papers, the results of the present study confirm the association between p53 alterations and high grade and stage.

It is known that FOXOs can interact with major transcription regulators, such as p53, MYC or β-catenin, and that FOXO and p53 play similar roles as tumor suppressor genes [1,4,7]. FOXO may induce apoptosis in a p53-dependent or p53-independent manner [35]. Thus, we also have investigated the relationship between FOXO1 downregulation and p53 alterations in our series of bladder tumors.

There was an association between the presence of any alteration in these genes and high grade, high stage and high grade/stage tumors. Considering the number of gene alterations, the prevalence of one alteration increased moderately in high grade, but the combination of FOXO1 downregulation/p53 overexpression was strongly associated with high grade, high stage and high stage/grade, as only the more aggressive tumors harbored both changes.

In tumors of other locations [22,36,37], FOXO1 expression has been found to convey a favourable prognosis. In bladder cancer, levels of *FOXO1* mRNA were significantly higher in tumor samples of non-progressing patients, and these cases showed significantly prolonged survival [25].

In order to assess the impact of FOXO1 downregulation and p53 overexpression on prognosis in the present series, a Kaplan-Meier analysis was performed. In the univariate analysis, p53 overexpression was associated with tumor progression, while decreased FOXO1 showed only a trend to be associated with it. Having any change in either FOXO1, p53 or both genes was even more strongly associated with progression when we analyzed the whole series of cases. Moreover, the multivariate analysis confirmed the already reported fact that p53 overexpression is independently associated to progression. Our results indicate that adding information on FOXO1 status results in an even stronger association with outcome. Thus, harboring at least one alteration, either FOXO1 downregulation or p53 overexpression, or both, is independently associated with progression, and having alterations in both genes is independently associated with disease recurrence.

Focusing in the different clinical-pathological groups, we found that p53 overexpression was statistically associated with progression in pTa stage tumors, in the univariate analysis. The finding of any change in either FOXO1, p53 or both was also associated with progression, independently in low grade and also in pTa tumors. In the univariate analysis, concurrent FOXO1 downregulation and p53 overexpression were also statistically associated with disease recurrence (first recorded event) in high grade and in pT1 tumors. A multivariate analysis was not possible due to the small number of cases in some clinical-pathological groups.

Thus, in conclusion, the present study indicates that the combination of FOXO1 downregulation and p53 overexpression characterizes a group of high grade, pT1 stage bladder tumors with an increased probability of disease recurrence. This combination of markers could be useful in stratifying different subgroups of patients with bladder cancer. Having any alteration in any of the two genes is strongly associated with

progression in lower stage and grade categories. In addition, having both alterations is associated with disease recurrence in high grade and pT1 UC. Therefore, although p53 is very informative by itself, including FOXO1 in the study confers an added prognostic value.

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Table legends

Table 1. Clinical features of the patients and pathological features of the tumors.

Table 2. Relationships between FOXO1 downregulation and/or p53 overexpression with clinical-pathological features of bladder tumors.

Table 3. Relationships between FOXO1 downregulation and/or p53 overexpression with risk of tumor progression or disease recurrence. Hazard ratios (HR), 95% confidence intervals (95%CI) and *P*-values after adjusting for other prognostic variables.

Figure legends

Fig. 1. Immunostaining of FOXO1 and p53 in UC (x200). Case 1, pT1-HG (grade 3) UC: loss of FOXO1 expression (A) and p53 overexpression (B), and case 2, pTa-LG UC: high FOXO1 expression (*wt*) (C) and lack of p53 overexpression (D).

Fig. 2. Combined p53 overexpression plus FOXO1 decreased expression was statistically associated with high grade ($p = 0.004$) (A), and high stage bladder tumors ($p < 0.0001$) (B).

Fig. 3. Altered FOXO1 and p53 expression predict bladder cancer progression. p53 overexpression was associated with progression (Log Rank, $p = 0.016$) (A). FOXO1 downregulation showed a trend to be associated with progression (Log Rank, $p = 0.062$) (B). To harbor at least one alteration was strongly associated with bladder tumor progression (Log Rank, $p = 0.005$) (C).

Fig. 4. Altered FOXO1 and p53 expression predict progression of low grade and Ta stage bladder tumors. To harbor at least one alteration was statistically associated with progression in low grade (Log Rank, $p = 0.005$) (A), and in Ta stage (Log Rank, $p = 0.006$) (B).

Fig. 5. Altered FOXO1 and p53 expression predict recurrence of high grade and T1 stage bladder tumors. Combined FOXO1 downregulation plus p53 overexpression was statistically associated with disease recurrence in high grade (Log Rank, $p = 0.046$) (A) and in T1 tumors (Log Rank, $p = 0.007$) (B).

Table 1. Clinical-pathological features of the patients/tumors.

	N=162
Age	
Mean (range)	68 (24-92)
Gender	
Male (%)	138 (85%)
Female (%)	24 (15%)
Tumor size	
< 3 cm (%)	104 (64,2%)
> 3 cm (%)	54 (33,3%)
Not available (%)	4 (2,5%)
Localization	
Unifocal (%)	88 (54,3%)
Multifocal (%)	57 (35,2%)
Not available (%)	17 (10,5%)
Treatment	
TURB (%)	27 (16,7%)
TURB + BCG (%)	99 (61,1%)
TURB + Chemotherapy (%)	12 (7,4%)
TURB + BCG + Chemotherapy (%)	11 (6,8%)
Not available (%)	13 (8%)
Smoking history	
Non smoker (%)	26 (16%)
Smoker (%)	84 (52%)
Ex-smoker (%)	44 (27%)
Not available	8 (5%)
Year of biopsy	1998-2003
Follow-up (months) (range)	89 (28-203)
Recurrence (%)	62 (38,3%)
Progression (%)	27 (16,7%)

Table 2

FOXO downregulation and/or p53 overexpression, relationship with the clinical-pathological features of bladder tumors				
	FOXO1 downregulation	p53 overexpression	FOXO1 and/or p53	FOXO1 plus p53
Grade				
Low	34,1%	7,5%	37,5%	2,5%
High	53,7%	25,2%	65,2%	14,8%
P- value [a]	0,030 [b]	0,017 [b]	0,002 [b]	0,004 [b]
Stage				
Ta	38,1%	6,5%	43,5%	1%
T1	54,3%	34,0%	72,7%	16%
T2	89,5%	58%	94,7%	52,6%
P- value [a]	0,0001 [b]	< 0,0001 [b]	< 0,0001 [b]	< 0,0001 [b]
Stage and Grade				
Ta-Low grade	34,1%	7,5%	37,5%	2,5%
Ta-High grade	41,0%	5,8%	48,0%	0%
T1-High grade	54,3%	34,0%	73%	15,9%
T2-High grade	89,5%	58%	94,7%	52,6%
P- value [a]	0,0004 [b]	< 0,0001 [b]	< 0,0001 [b]	< 0,0001 [b]

[a] All statistical results using Pearson chi-square test
[b] Statistically significant

Table 3

PROGRESSION	HR	95%CI	*P* - value
FOXO1 downregulation	1.98	(0.88-4.44)	0.099
p53 overexpression	3.18	(1.19-8.48)	0.020 [b]
FOXO1 and/or p53	3.51	(1.34-9.21)	0.010 [b]
FOXO1 plus p53	2.41	(0.60-9.64)	0.213
RECURRENCE	**HR**	**95%CI**	***P* - value**
FOXO1 downregulation	1.28	(0.75-2.18)	0.371
p53 overexpression	1.19	(0.60-2.39)	0.617
FOXO1 and/or p53	1.03	(0.59-1.81)	0.906
FOXO1 plus p53	2.75	(1.06-7.13)	0.037 [b]

[b] Statistically significant

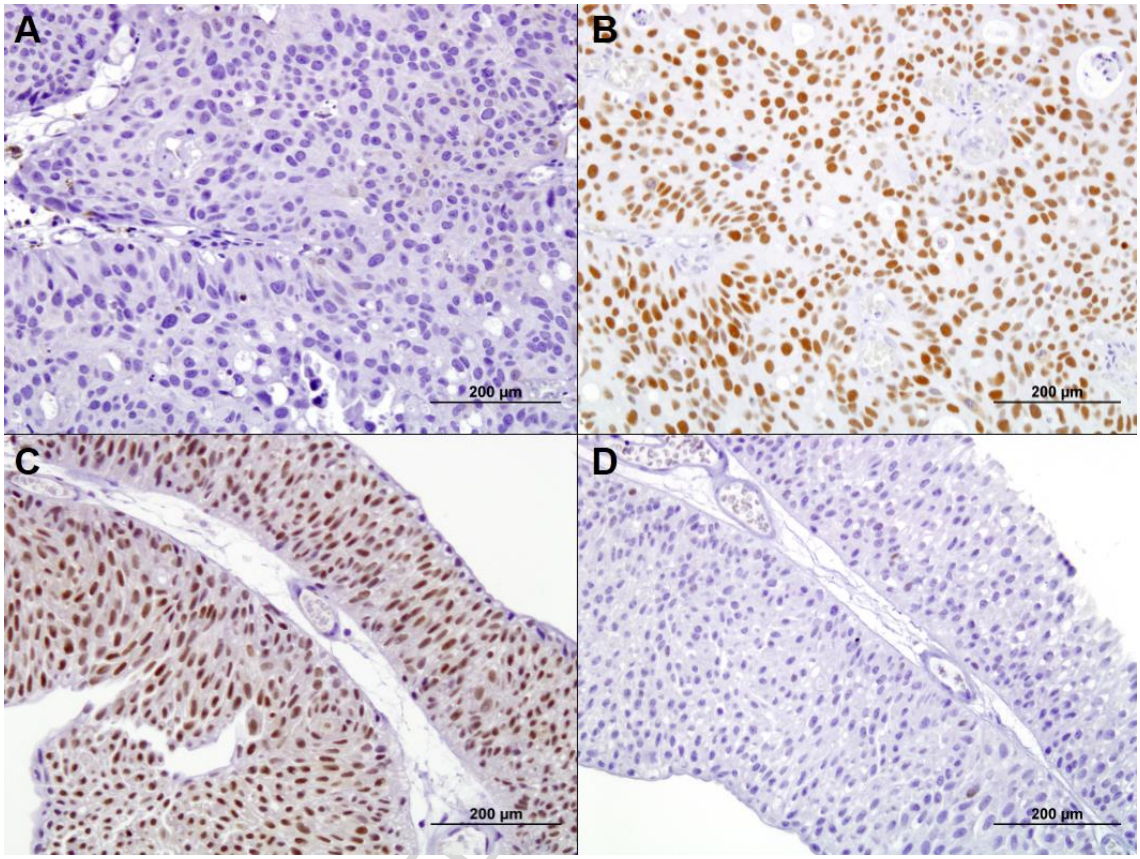

Figure 1

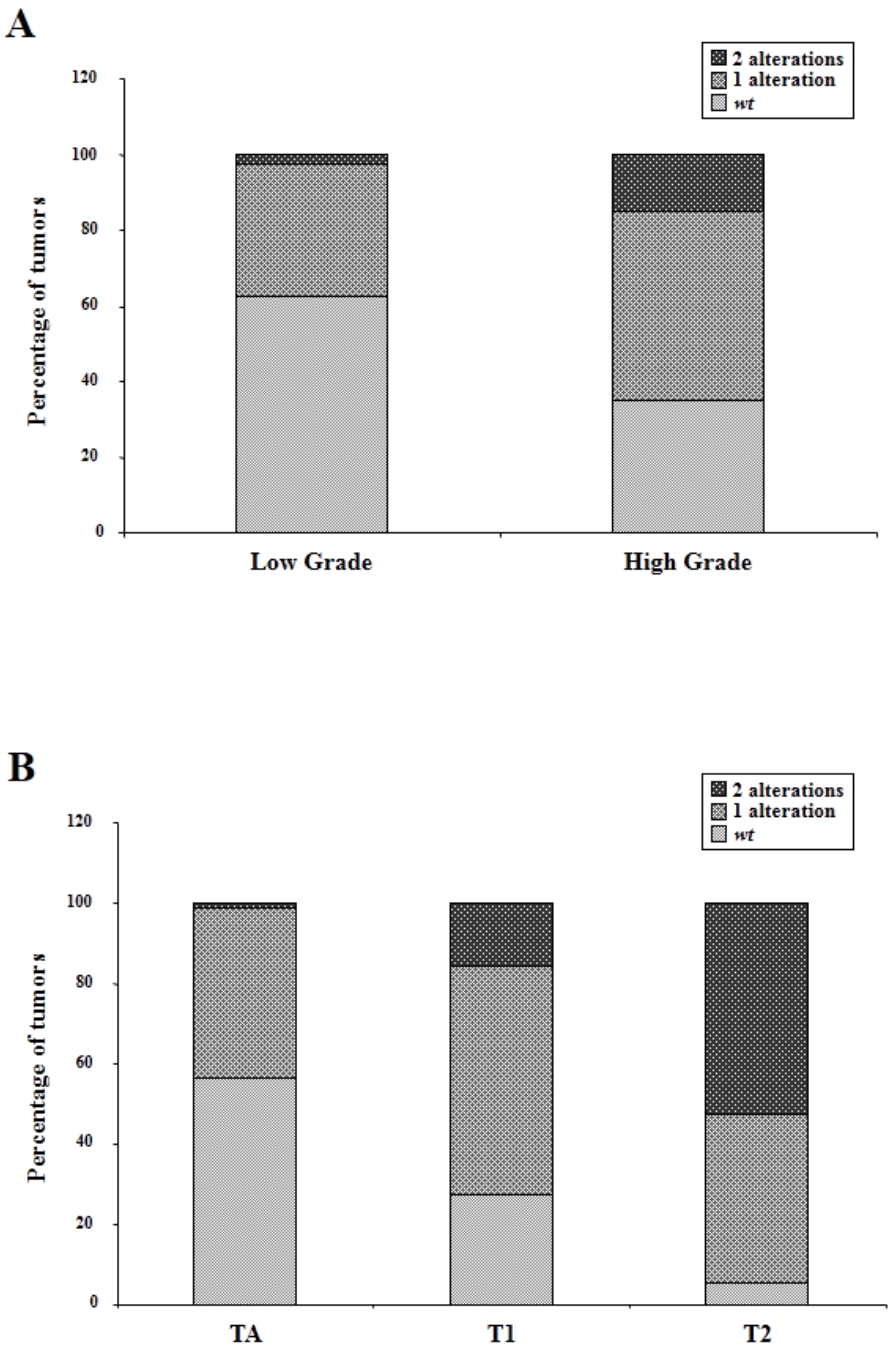

Figure 2

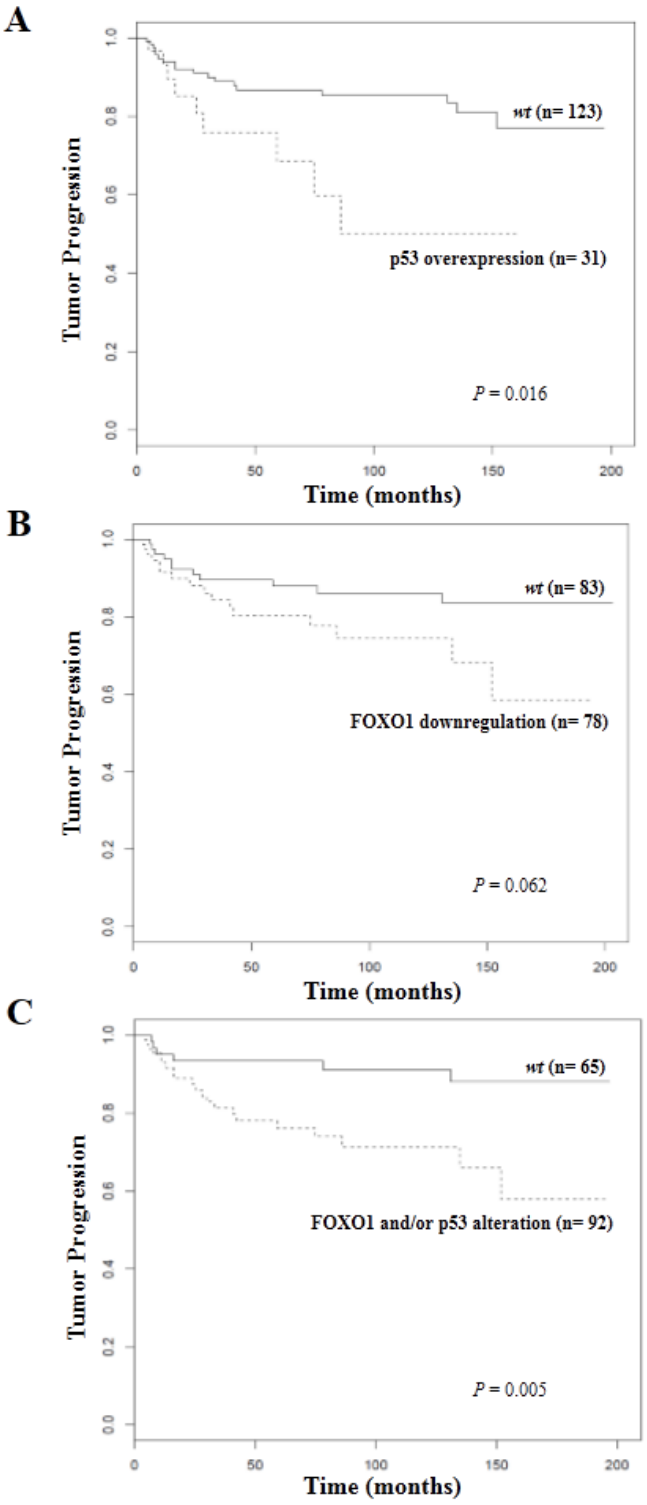

Figure 3

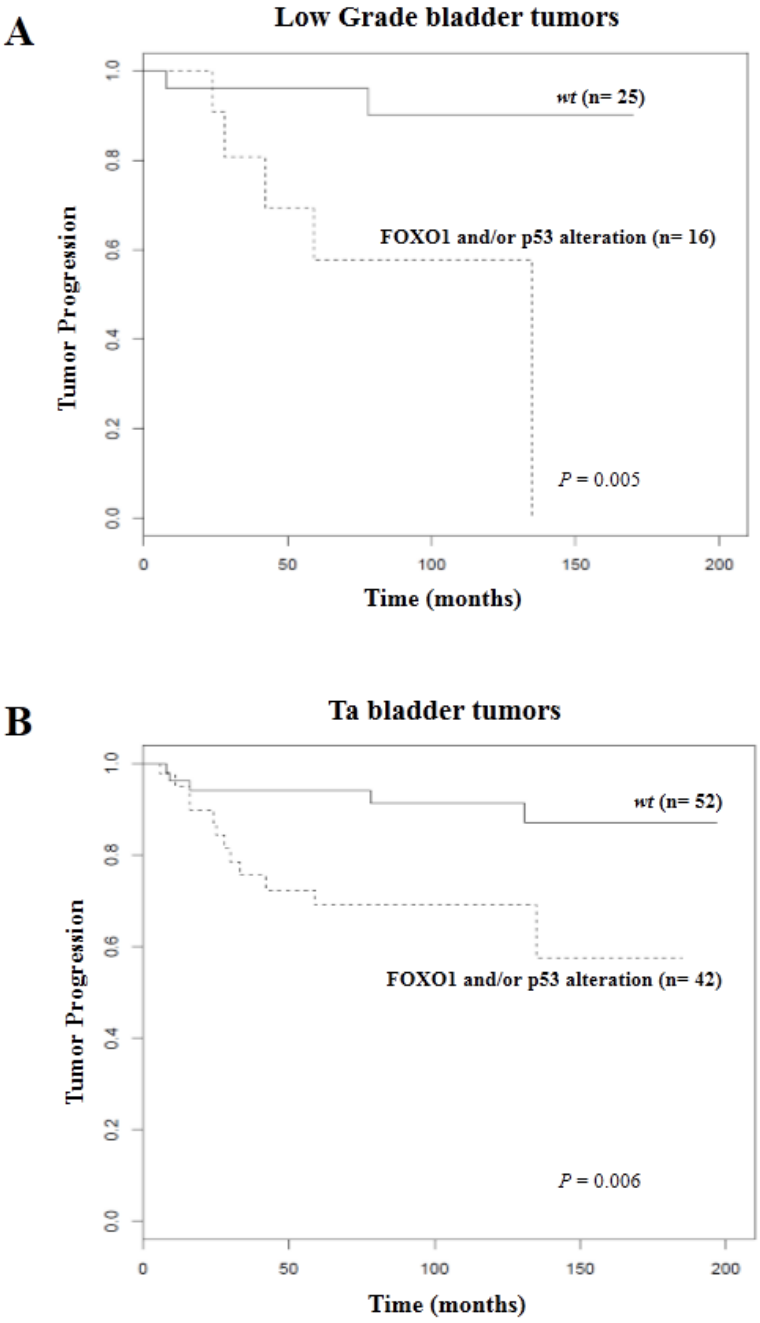

Figure 4

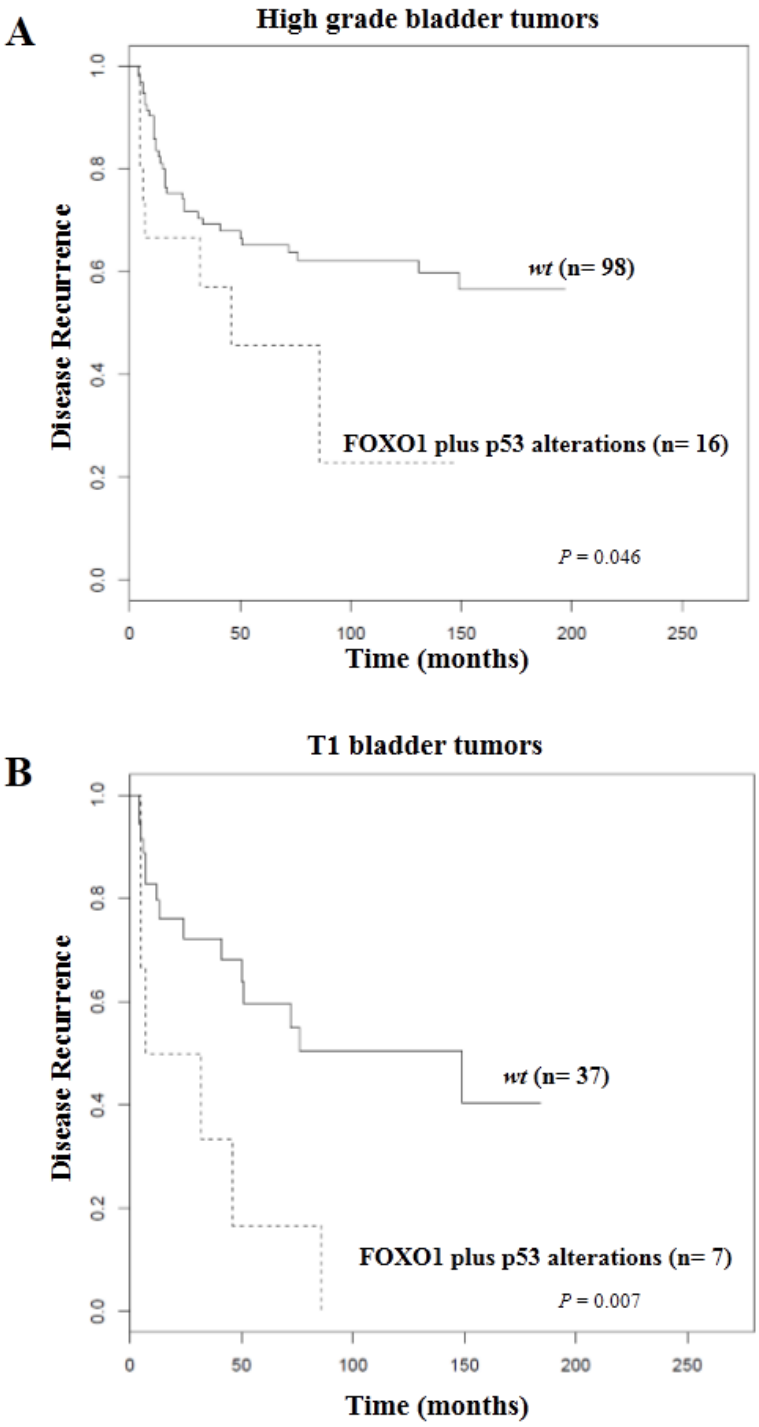

Figure 5

- FOXO1 and p53 may have similar or synergistic effects in bladder cancer.
- The combined study of FOXO1 and p53 expression may be a better prognostic indicator than p53 alone.
- Having abnormal expression of any of the two genes is associated with disease progression in low grade and pTa bladder urothelial carcinomas.
- Having abnormal expression of both genes is associated with disease recurrence in high grade and pT1 bladder urothelial carcinomas.