Foxo3a suppression of urothelial cancer invasiveness through twist1, Y-box-binding protein 1, and E-cadherin regulation

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Foxo3a suppression of urothelial cancer invasiveness through Twist1, Y-box-binding protein 1 and E-cadherin regulation

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Running title: Foxo3a regulates cancer cell invasion

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Translational Relevance

The role of Foxo3a in urothelial cancer development has not been investigated and its prognostic relevance is still poorly understood. This study revealed that Foxo3a expression was inversely correlated with tumor stage, and that low Foxo3a expression level was an independent prognostic factor for poor disease-free survival, cancer-cause specific survival, and overall survival. Moreover, Foxo3a expression was also found to regulate the motility of urothelial cancer cells through the regulation of several epithelial-mesenchymal transition-associated proteins. Thus, although Foxo3a is already known to be implicated in cancer cell growth, this study showed that it also regulates cancer cell motility. Taken together, these results suggest that Foxo3a could act as a novel prognostic factor and therapeutic target in urothelial cancer.

Abstract

Purpose: Invasion and metastasis are key steps in the progression of urothelial cancer (UC) into a critical disease. Foxo3a is a member of the Foxo transcription factor family, which modulates the expression of various genes. We aimed to elucidate the role of Foxo3a in UC invasion.

Experimental Design: Foxo3a mRNA and protein expressions in UC samples were investigated by gene expression assays and immunohistochemistry, respectively. Foxo3a expression was compared with clinicopathologic characteristics and patient prognoses based on UC samples. Quantitative real-time polymerase chain reaction, western blotting, and migration assays were also conducted in UC cells.

Results: Foxo3a expression decreased in invasive UC; patients with low Foxo3a expression had poor disease-free survival, cancer-specific survival, and overall survival; Foxo3a knockdown in UC cells increased cellular motility. Foxo3a negatively regulated Twist1 and Y-box-binding protein 1 (YB-1), and positively regulated E-cadherin in KK47 and TCCsup cells that expressed Twist1, but not in T24 cells that did not express Twist1. Foxo3a-associated acetyltransferase p300 and Foxo3a acetylation status also affected UC motility.

Conclusion: The results of this study indicate that Foxo3a regulates motility of UC

through negative regulation of Twist1 and YB-1, and through positive regulation of E-

cadherin. This suggests that Foxo3a could act as an independent prognostic factor in

UC and could represent a promising molecular target for cancer therapeutics.

Introduction

Bladder cancer is the second most common malignancy of the genitourinary tract and the fourth major cause of cancer death among men (1). Urothelial carcinoma of the bladder accounts for more than 90% of bladder cancers (2). Most urothelial carcinomas (about 80%) present as superficially invasive tumors, which include Ta (non-invasive) or T1 (lamina propria-invasive) tumors. Muscle-invasive cancer (T2–4) has a much less favorable prognosis than superficial cancer, despite aggressive multimodal therapy (3). Due to the unfavorable prognosis of muscle-invasive cancer, there is a need to develop markers that can identify superficial cancers with a high risk of progression. The characterization of such markers will help to detect more invasive urothelial cancer (UC), and thus improve the prognosis of this disease.

Foxo3a is a member of the Foxo transcription factor family. Foxo transcription factors, which belong to the 'other' class of the Fox superfamily, are involved in multiple signaling pathways and play critical roles in a number of physiologic and pathologic processes (4). It has been recently reported that the Forkhead transcription factor, Foxo3a, regulates DNA damage response and stimulates DNA repair pathways (5,6). Foxo transcription factors also regulate oxidative detoxification and cell cycle-related genes (7), apoptosis-related genes, such as Bim (8), tumor necrosis factor

receptor ligand (TRAIL) (9) and Fas ligand (10). Foxo transcription factors are known

to be reversibly acetylated and deacetylated by calcium-responsive element binding-

binding protein (CBP)/p300 and Sirt1, respectively (11–16). These modifications

modulate the transcriptional abilities and gene target selections of Foxo transcription

factors, thus affecting biological behavior.

Epithelial–mesenchymal transition (EMT) is a characteristic of cancer cell

intravasation and metastasis. E-cadherin is a cell-to-cell adhesion molecule, and loss of

its expression is a hallmark of EMT. Reduction of E-cadherin increases cell motility

and promotes cancer cell invasion (17,18). Foxe3 (also called HNF3 and forkhead box

E3) and p300 have been shown to play important roles in promoting E-cadherin

expression (19). E-cadherin expression, in turn, is regulated by other factors, including

Twist1 (20). An inverse correlation between Twist1 and E-cadherin expression was

observed in invasive lobular breast carcinoma, suggesting that Twist1 affects cancer

cell intravasation and entry into the circulation to seed metastases (21). Y-box-binding

protein 1 (YB-1) has recently been shown to regulate cancer cell invasion through

translational control of Twist1 (22,23); Twist1 transcriptionally regulates YB-1

expression, while p53 and programmed cell death protein 4 (PDCD4) affect YB-1

expression through interactions with Twist1 (24–26).

In this study, we investigated the correlation between Foxo3a expression and clinicopathologic parameters in patients with UC. We also examined the molecular mechanism of cancer cell motility control by Foxo3a and its associated acetyltransferase, p300.

Materials and methods

Cell culture. Human bladder cancer TCCsup (RPMI1640) cells (kindly provided by Dr. Osamu Ogawa, Kyoto University, Kyoto, Japan), KK47 (MEM) and T24 (MEM) cells were cultured in the media indicated (Invitrogen, San Diego, CA), containing 10% fetal bovine serum. The stable transfectants KK47-Ac green fluorescent protein (GFP) (#1 and #2), KK47-Foxo3a-GFP (#1 and #2) and KK47-Foxo3a-GFP KR (#1 and #2) cells were derived from KK47 cells and stably expressed the corresponding proteins. These transfectants were established as previously described (27). Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies and plasmids. Anti-Foxo3a (EP1949Y) and anti-YB-1 (EP2708Y) antibodies were purchased from Epitomics (Burlingame, CA). Antibodies against p300 (sc-585) and Twist1 (sc-81417) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-E-cadherin and Anti-ß-actin (AC-15) antibodies were purchased from BD Bioscinces (San Jose, CA) and Sigma (St. Louis, MO), respectively. Foxo3a-GFP

plasmid expressing C-terminal-GFP-tagged Foxo3a protein and mutated Foxo3a-GFP

expression plasmid (Foxo3a-GFP KR) were created as described previously (27).

Gene expression assay. Tissues for gene expression assays were collected from 71

patients who underwent transurethral resection or radical cystectomy, without

preoperative chemotherapy, at the Kyushu University Hospital, Japan, between 1992

and 2004, and who provided enough suitable UC tissue for evaluation. Tissues that

were suspected to be necrotic by endoscopic and macroscopic examination were avoided.

The samples were collected under careful endoscopic and macroscopic observation, to

avoid contamination by normal urothelial tissues. If multiple tumors were present,

then samples were collected from the major tumors, in order to select the predominant

representative tumor. Written informed consent was obtained from all subjects. RNA

isolation, reverse transcription and quantitative real-time polymerase chain reaction

(PCR) were performed as described below. The median transcript level corresponded to

1.

Patients and samples for immunohistochemistry. Tissues for

immunohistochemistry were collected from 53 patients who underwent radical

cystectomies, without preoperative chemotherapy, at the Kyushu University Hospital,

Japan, between April 1993 and March 2008, and who provided sufficient UC tissue for

immunohistochemical evaluation. Bladder blocks containing the largest and most

representative area of the tumor were prepared. All surgical specimens were

completely reviewed to establish histologic grading and disease staging of the

respective UCs, based on the criteria of the 2004 WHO classification and 2002 TNM

system. Clinical follow-up data were available for all patients. There were 21 deaths,

24 recurrences and 15 disease-specific deaths during the follow-up period. The median

follow-up period was 25.0 months (mean: 46.5 months).

Immunohistochemistry. Immunohistochemistry was performed as previously

described (28) using anti-Foxo3a antibody (dilution 1:50).

Immunohistochemical analysis. Foxo3a expression was assessed by evaluating the

proportion and intensity of positively stained carcinoma cells. A score was assigned to

represent the estimated percentage of positively stained carcinoma cells as follows: 0 =

none; 1 = $\leq$ 1%; 2 = 1–10%; 3 = 10–33%; 4 = 33–67%: 5 = $\geq$ 67%. An intensity score was

assigned to represent the average estimated intensity of staining in positive carcinoma

cells as follows: 0 = none, 1 = weak, 2 = intermediate, 3 = strong. The proportion score

and intensity score were added to obtain a total score ranging from 0–8 (29). The

immunohistochemistry results were classified based on the total scores, with 0–4

classified as low expression, and 5–8 as high expression. The immunohistochemistry

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results were independently judged by two trained uropathologists (YS and KKi).

Differences in evaluations were discussed using a double-headed microscope until

consensus was reached.

Knockdown analysis using small interfering (si)RNAs. Knockdown analysis using

siRNAs was performed as previously described (27,30). Briefly, the double-stranded

RNA 25-base-pair oligonucleotides presented in Supplementary Table 1 were

commercially generated (Invitrogen). KK47, TCCsup and T24 cells were transfected

with siRNA using Lipofectamine 2000 (Invitrogen), according to the manufacturer's

instructions.

Scratch-wound assay. Scratch-wound assays were performed, as previously

described (30). Briefly, KK47, TCCsup and T24 cells (1×10^5) transfected with 40 nmol/L

of the indicated siRNAs, or KK47 stable transfectants (1×10^5), were seeded into 12-well

plates and incubated for 72 h until they reached confluency. The invasive abilities of

the KK47, TCCsup and T24 cells were represented by the ratios of the decreased

scratch width after 48, 24, and 12 h, respectively, relative to the initial scratch width in

the same field.

Boyden-chamber assay. Boyden-chamber assays were performed, as previously

described (30). Briefly, KK47, TCCsup and T24 cells (5×10^4) transfected with 40 nmol/L

of the indicated siRNAs for 72 h, or KK47 stable transfectants (5×10^4), were placed in

the top compartment of a standard 3-µm pore Boyden chamber (BD Biosciences, San

Jose, CA). The numbers of migrated cells were determined from three random fields.

RNA isolation, reverse transcription and quantitative real-time PCR. These

procedures were performed as previously described (27,30). Quantitative real-time PCR

was performed using the following TaqMan Gene Expression Assays for Foxo3a

(Hs00921424_m1), Twist1 (Hs00361186_m1), YB-1 (Hs00698625_g1) and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs02758991_g1) (Applied

Biosystems, Foster City, CA) and TaqMan Gene Expression Master Mix (Applied

Biosystems), or the following primer pairs: 5′-ATGCTTGCCAGTGTCGGAAT-3′ (Fw)

and 5′-CAACCCGCTTCATCTTCTGG-3′ (Rv) for p300, 5′-

GCTCACTGCAGCCTTGTCCT-3′ (Fw) and 5′-TGCCTGTGGTCCCAGCTACT-3′ (Rv) for

E-cadherin and 5′-GGAACGGTGAAGGTGACAGC-3′ (Fw) and 5′-

AATCAAAGTCCTCGGCCACA-3′ (Rv) for ß-actin and SYBR Premix Ex Taq II (Takara

Bio, Shiga, Japan), on a 7900HT PCR system (Applied Biosystems). The transcript

levels of Foxo3a, Twist1, YB-1 and E-cadherin were corrected according to the

corresponding GAPDH and ß-actin transcript levels. All values represent the results of

at least three independent experiments.

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Western blot analysis. The preparation of whole-cell lysates and western blotting

were performed as previously described (27,30).

Statistical analysis. The Mann-Whitney U-test was used to analyze correlations

between gene expression and clinicopathologic parameters and the *in vitro* experiments.

Fisher's exact test and the χ2 test were used to analyze correlations between Foxo3a

expressions and clinicopathologic parameters. The Kaplan-Meier method was used to

show that patients could be stratified into significantly different risk groups for disease

recurrence, bladder cancer-specific survival, and overall survival. The Cox proportional

hazards model was used for multivariate survival analysis. Values of $P < 0.05$ were

considered to be statistically significant.

Results

Foxo3a mRNA levels decrease in invasive UC. The Foxo3a transcription factor is

implicated in cancer cell growth and clinicopathologic parameters in various cancers

(31–35). We therefore investigated Foxo3a mRNA expression levels in 71 UC samples

(Supplementary Table 2). Foxo3a was expressed, at various levels, in all 71 samples.

The mean level of Foxo3a mRNA expression was lower in high-grade than in low-grade

tumors, although the difference was not significant (Fig. 1A). This was in accordance

with previous results indicating that Foxo3a regulates cell proliferation (27). Foxo3a

expression levels were compared in non-invasive (Ta, T1, Tis) and invasive cancers (T2, T3, T4); surprisingly, Foxo3a mRNA expression levels were clearly lower in invasive than in non-invasive cancers (Fig. 1B; P = 0.049). Moreover, five metastatic urothelial tumors had decreased Foxo3a mRNA expression levels compared with non-metastatic cancers, although the difference was not significant (Fig. 1C).

Foxo3a immunohistochemical expression is downregulated in UC cases with high tumor stage and poor prognosis. To strengthen the finding of gene expression assay, we conducted immunohistochemistry against Foxo3a. Foxo3a staining was detected in the nucleus and cytoplasm of normal urothelial epithelium and carcinoma cells. Stromal cell nuclei from normal bladder parenchyma displayed discontinuous equivocal-to-moderate expression. Foxo3a expression was high in non-invasive UC and low in invasive UC (Fig. 2A). The relationships between Foxo3a expression and clinicopathologic parameters are summarized in Table 1. Foxo3a expression was significantly lower in high pT stage cancer than in low pT stage cancer (P = 0.009), supporting the finding of gene expression assay. Patients with low Foxo3a expression had lower cancer-specific survival rates (Fig. 2B), disease-free survival rates (Fig. 2C), and overall survival rates (Fig. 2D), than the high expression group (log-rank test: P =

0.002; P = 0.020; P = 0.002, respectively). The effects of Foxo3a expression on patient

prognosis was recognized in multivariate survival analysis, even when stage and

lymph node status were included, demonstrating the independent prognostic

significance of Foxo3a expression (Table 2; P = 0.037; hazard ratio = 2.974).

Foxo3a regulates cancer cell motility in KK47 and TCCsup cells, but not in T24

cells. Because Foxo3a expression was inversely correlated with tumor stage, the effect

of Foxo3a on UC motility was investigated using the scratch-wound assay. Foxo3a-

specific siRNA was used to suppress Foxo3a expression, as previously described (27).

Silencing Foxo3a led to increased motility of KK47 and TCCsup cells, but had no

significant effect on the motility of T24 cells (Fig. 3A). Cell motility in the Foxo3a-

knockdown cells was also assayed using a Boyden chamber. As in the scratch-wound

assay, Foxo3a silencing increased the motility of KK47 and TCCsup cells, but not of

T24 cells (Fig. 3B).

Foxo3a regulates YB-1 and E-cadherin expression through negative Twist1

regulation. Twist1 and E-cadherin are known to be master regulators, regulating cell

migration and metastasis (21); YB-1 is also involved in cancer-cell migration (22,23). As

Foxo3a expression levels were inversely correlated with invasive UC phenotypes, we

speculated that Foxo3a might be inversely correlated with Twist1 and YB-1, and

positively correlated with E-cadherin expression. Twist1, YB-1, and E-cadherin

expression levels were examined in KK47, TCCsup, and T24 cells transfected with

Foxo3a-specific siRNAs. Twist1 and YB-1 expression were elevated in Foxo3a-

knockdown KK47 and TCCsup cells, while E-cadherin expression was decreased.

However, Twist1 expression was barely detectable or affected by Foxo3a suppression in

knockdown T24 cells, and consequently had no effect on YB-1 and E-cadherin

expression levels. Twist1 and YB-1 protein levels analyzed by western blotting showed

similar changes to those detected at the mRNA level, although E-cadherin expression

in KK47 and TCCsup cells was not detected as previously reported (31) (Fig. 3C). The

motilities of Foxo3a- and/or Twist1-knockdown KK47 cells were examined to determine

the effect Foxo3a on cell motility mediated via Twist1. As shown in Fig. 3D, Twist1

knockdown abolished KK47 motility which was increased by Foxo3a knockdown,

suggesting that Foxo3a regulated KK47 motility through Twist1.

Foxo3a acetylation level is implicated in KK47 cell motility. Foxo3a is known to

interact with, and be acetylated by p300, which modulates transcriptional ability.

Modulation of Foxo3a, Twist1, YB-1 and E-cadherin expression was therefore

investigated in p300-knockdown cells, using p300-specific siRNA, as previously

described (27). As in Foxo3a-knockdown cells, p300 downregulation increased Twist1

expression and decreased E-cadherin expression in KK47 cells. However, YB-1

expression was not affected; this may be due to the effects of p300 on YB-1

transcription by Twist1. Also as in Foxo3a-knockdown cells, Twist1, YB-1 and E-

cadherin expression were unaffected by p300 knockdown in T24 cells. These findings

were confirmed by western blotting (Fig. 4A). As the transcription co-activator p300

regulated Twist1, YB-1 and E-cadherin expression in KK47 cells, the effects of p300

silencing on KK47 cell motility were then investigated. Knockdown of p300 expression

in KK47 cells led to increased cell motility (Fig. 4B).

Because the expression and acetylation levels of Foxo3a are implicated in cellular

motility, the effects of wild-type and non-acetylation-mimicking Foxo3a overexpression

on cell motility were investigated, using stable transfectants of KK47 cells expressing

GFP, Foxo3a-GFP or Foxo3a-GFP KR protein. These stable transfectants expressed

almost equal amounts of exogenous GFP-tagged proteins and the mutated Foxo3a

protein (Foxo3a-GFP KR) was not acetylated, as shown previously (27). The results of

quantitative real-time PCR showed that Foxo3a-GFP overexpression in KK47 cells

reduced Twist1 and YB-1 expression, whereas E-cadherin expression was increased by

Foxo3a overexpression. However, Foxo3a-GFP KR overexpression in KK47 cells

affected the expression of these genes to a lesser extent, compared with wild-type

Foxo3a overexpression. These findings were confirmed at the protein level by western

blotting (Fig. 4C). The motility of KK47-Foxo3a-GFP (#1 and #2) cells determined by

scratch-wound assay was lower than that of KK47-AcGFP (#1 and #2) cells. However,

KK47-Foxo3a-GFP KR (#1 and #2) cells were more motile than KK47-Foxo3a-GFP (#1

and #2) cells, but less motile than KK47-AcGFP (#1 and #2) cells. Similarly, using

Boyden-chamber assays, the motility of KK47-Foxo3a-GFP (#1 and #2) cells was clearly

lower than that of KK47-AcGFP (#1 and #2) cells. KK47-Foxo3a-GFP KR (#1 and #2)

cells were more motile than KK47-Foxo3a-GFP (#1 and #2) cells, but less motile than

KK47-AcGFP (#1 and #2) cells (Fig. 4D).

Discussion

The results of this study demonstrated that Foxo3a expression levels were

inversely correlated with UC invasiveness, as represented by tumor stage. Although

the samples used for gene expression assay contained some amount of stroma, the bias

by stroma contamination doesn't seem so variable among these samples. In addition,

immunohistochemistry clearly revealed the association between Foxo3a expression and

UC invasiveness although hereafter the method such as micro-dissection might be

more suitable to avoid a potential bias. Human prostate cancer cells, xenografts, and

clinical samples have been shown to harbor heterozygosity losses on chromosome

13q14, which is the known gene locus for Foxo1 (32). Loss of Foxo1 gene expression is

therefore directly related to increased cell proliferation in cancer cells, suggesting that

Foxo1 protects against prostate carcinogenesis. Furthermore, chromosomal

breakpoints in human acute myeloid leukemia were found to involve Foxo3a and Foxo4.

For example, the mixed-lineage leukemia gene can fuse with Foxo3a and Foxo4,

thereby potentially disturbing their tumor suppressive functions (33,34).

Immunohistochemical analyses have also shown a direct relationship between low

levels of Foxo3a and rapid proliferation and a positive correlation between Foxo3a and

the cell cycle inhibitor p27[kip1] in non-Hodgkin's lymphoma cells (35). Expression of

phosphorylated Foxo1, an inactivated form, was higher in early pTNM stages of gastric

cancer, and was inversely correlated with lymphatic invasion and lymph node

metastasis, and positively correlated with longer patient survival (36). On the other

hand, an association between expression of phosphorylated Foxo1 and unfavorable

outcomes in acute myeloid leukemia or advanced-stage colon cancer was reported

(37,38). The biological significance of Foxo expression thus appears to differ among

cancer types.

Twist1 is known to regulate the invasive and metastatic characteristics of cancer cells (21). YB-1 has also recently been shown to regulate cancer cell invasiveness (22,23). In the current study, Foxo3a expression levels in UC were shown to be inversely correlated with tumor stage, and cancer cell motility was augmented in Foxo3a-knockdown cells, an effect that was abolished by Twist1 knockdown. These findings may reflect Foxo3a's negative regulation of Twist1 and YB-1, which are known promoters of tumor invasion. No direct link between Foxo transcription factors and Twist1/YB-1 signaling has been previously reported, but this negative regulation of Twist1 and YB-1 by Foxo3a might be a crucial factor affecting various aspects of cancer biology.

Several researchers have reported that the acetylation status of Foxo transcription factors influences their transcriptional activity (11). Foxo transcription factors were found to interact with Sirt1 and p300, which deacetylate and acetylate, respectively (11–15). Sirt1 represses Foxo3a's ability to transcribe the pro-apoptotic factor Bim (39). The transcription of Fas ligand is also reportedly decreased by Sirt1 (16), while the DNA-repair-related gene GADD45α, and the antioxidant protein MnSOD are induced by Sirt1 overexpression (16,40).

The results of this study showed that Foxo3a regulates Twist1, YB-1 and E-cadherin expression. Twist1 and YB-1 are well-known proto-oncogenes, involved in cancer cell aggressiveness and invasiveness. In addition, Foxo3a silencing increases cell motility, and aggressive UCs express less Foxo3a. Foxo3a may therefore repress cancer cell aggressiveness through negative regulation of Twist/YB-1 signaling, in addition to its positive effects on E-cadherin.

Acknowledgments

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Figure legends

Fig. 1 Foxo3a mRNA level is decreased in invasive urothelial cancer. (A)–(C) Twist1 and YB-1 transcript levels are indicated according to tumor grade (A), tumor stage (B) and metastasis status (C). Middle lines, median; upper lines, upper quartile; lower lines, lower quartile; 75%; bars: ± s.d.

Fig. 2 Foxo3a immunohistochemical expression is downregulated in urothelial cancers (UCs) with high tumor stage and poor prognosis. (A) Foxo3a expression was high in non-invasive UC, and low in invasive UC. Hematoxylin-eosin (H&E) staining of the same samples is also shown. (B)–(D) Disease-free survival curves (B), cancer-specific survival curves (C), and overall survival curves (D) of patients in Foxo3a low-expression and high-expression groups.

Fig. 3 Foxo3a regulates YB-1 and E-cadherin expression through negative Twist1 regulation, and regulates cancer-cell motility in KK47 and TCCsup cells, but not in T24 cells. (A) KK47, TCCsup and T24 cells were transfected with 40 nmol/L of the indicated siRNA. The cells were scratched 72 h after transfection. The scratch widths were measured after 48 h (KK47 cells), 24 h (TCCsup cells) or 12 h (T24 cells) of incubation.

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The motility of cells transfected with control siRNA corresponded to 1. Boxes, mean;

bars, ±s.d. *P < 0.05 (compared with cells transfected with control siRNA). (B) KK47,

TCCsup and T24 cells were transfected with 40 nmol/L of the indicated siRNA. The

cells were applied to a Boyden chamber 72 h after transfection. The mobilized cell

count when transfected with control siRNA corresponded to 1. Boxes, mean; bars, ± s.d.

*P < 0.05 (compared with cells transfected with control siRNA). (C) KK47, TCCsup and

T24 cells were transfected with 40 nmol/L of the indicated siRNA. Quantitative real-

time PCR analysis of Foxo3a, Twist1, YB-1, E-cadherin, glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) and β-actin was performed. The levels of Foxo3a, Twist1, YB-

1 and E-cadherin transcripts in cells transfected with control siRNA corresponded to 1.

Boxes: mean; bars: ± s.d. *P < 0.05 (compared with cells transfected with control

siRNA). Whole-cell extracts were subjected to SDS-PAGE, and western blotting was

performed using the indicated antibodies. (D) KK47 cells were transfected with 20

nmol/L of control siRNA or Foxo3a siRNA #1, and 20 nmol/L of control siRNA or Twist1

siRNA. (Left panel) Whole-cell extracts were subjected to SDS-PAGE, and western

blotting was performed using the indicated antibodies. (Right panel) The cells were

scratched 72 h after transfection and the scratch widths were measured after 48 h of

incubation. The motility when transfected with control siRNA corresponded to 1. Lane
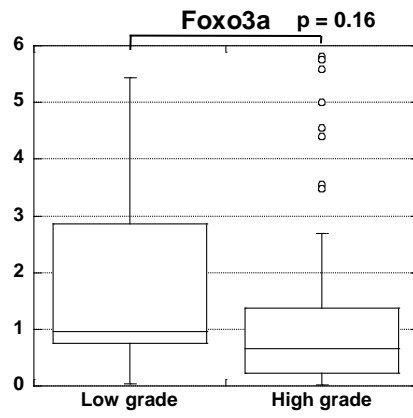
1, control siRNA; lane 2, Foxo3a siRNA #1; lane 3, Twist1 siRNA; lane 4, Foxo3a

siRNA #1 and Twist1 siRNA. Boxes, mean; bars, ±s.d. *P < 0.05 (compared with lane 1).

**P < 0.05 (compared with lane 2).

Fig. 4 Foxo3a acetylation level is implicated in KK47 cell motility. (A) KK47 and T24

cells were transfected with 40 nmol/L of the indicated siRNA. Quantitative real-time

PCR analysis of p300, Foxo3a, Twist1, YB-1, E-cadherin, glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) and β-actin was performed. The levels of p300, Foxo3a,

Twist1, YB-1 and E-cadherin transcripts from cells transfected with control siRNA

corresponded to 1. Boxes: mean; bars: ± s.d. *P < 0.05 (compared with cells transfected

with control siRNA). Whole-cell extracts were subjected to SDS-PAGE, and western

blotting was performed using the indicated antibodies. (B) KK47 cells were transfected

with 40 nmol/L of the indicated siRNA. (Upper panel) The cells were scratched 72 h

after transfection and the scratch widths were measured after 48 h of incubation. The

motility when transfected with control siRNA corresponded to 1. Boxes: mean; bars: ±

s.d. *P < 0.05 (compared with cells transfected with control siRNA). (Lower panel) The

cells were applied to a Boyden chamber 72 h after transfection. Mobilized cells were

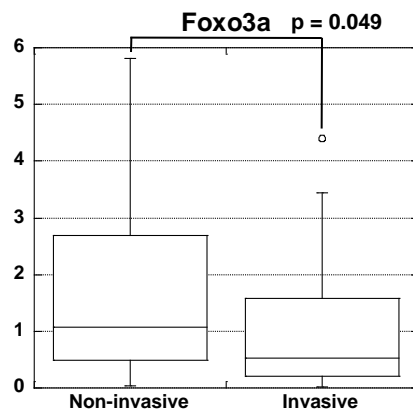then counted. The cell count when transfected with control siRNA corresponded to 1.

Boxes: mean; bars: ± s.d. *P < 0.05 (compared with cells transfected with control

siRNA). (C) Quantitative real-time PCR analysis of Foxo3a, Twist1, YB-1, E-cadherin,

GAPDH and β-actin was performed after extraction of total RNA from the indicated

KK47 stable transfectants and synthesis of cDNA. The levels of Foxo3a, Twist1, YB-1

and E-cadherin transcripts from KK47-AcGFP #1 cells corresponded to 1. Boxes: mean;

bars: ± s.d. *P < 0.05 (compared with KK47-AcGFP #1 cells). **P < 0.05 (compared with

KK47-Foxo3a-GFP #1 cells). Whole-cell extracts were subjected to SDS-PAGE, and

western blotting was performed using the indicated antibodies. (D) The indicated KK47

stable transfectants were seeded. Scratch-wound and Boyden chamber assays were

performed the following day, as described in (B). *P < 0.05 (compared with KK47-

AcGFP #1 cells). **P < 0.05 (compared with KK47-Foxo3a-GFP #1 cells).
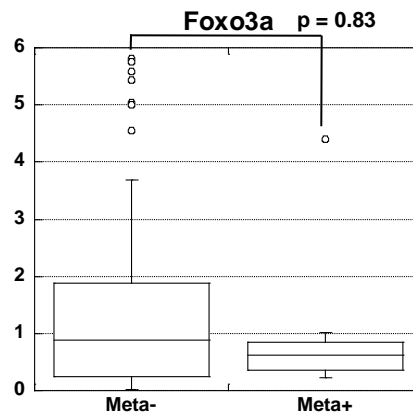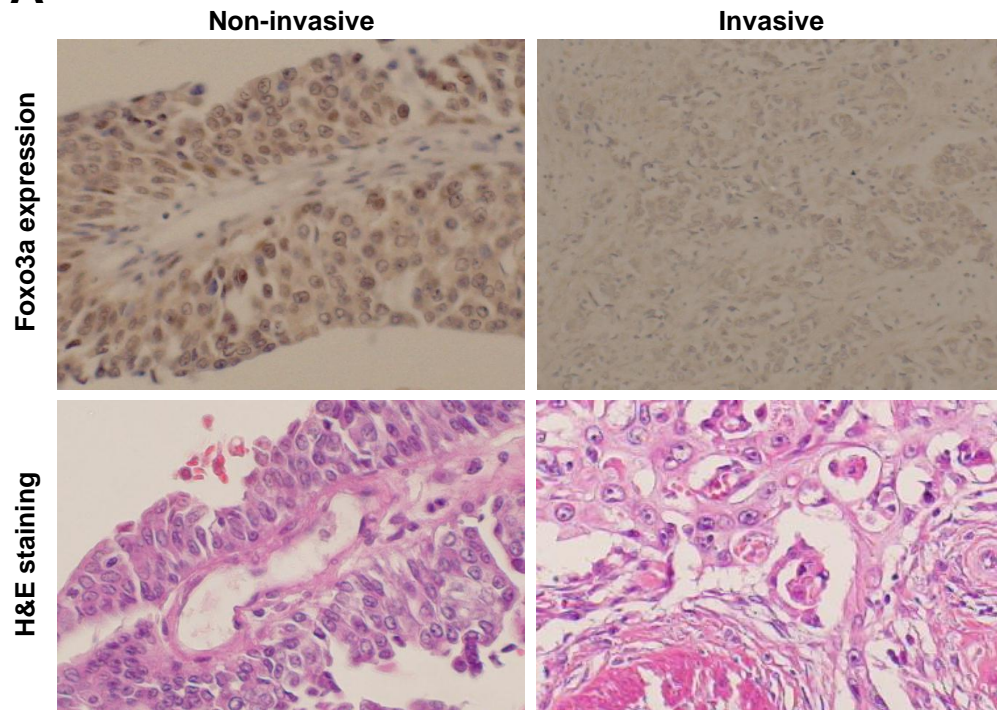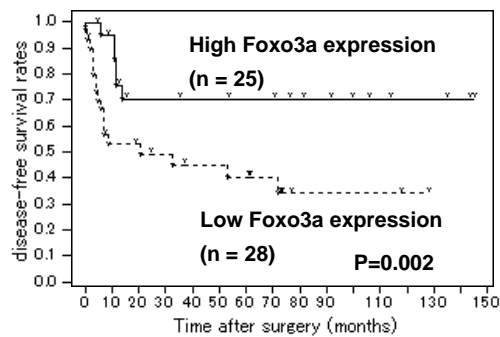
Shiota et al. Figure. 1

A

Foxo3a   p = 0.16

Low grade   High grade

B

Foxo3a   p = 0.049

Non-invasive   Invasive

C

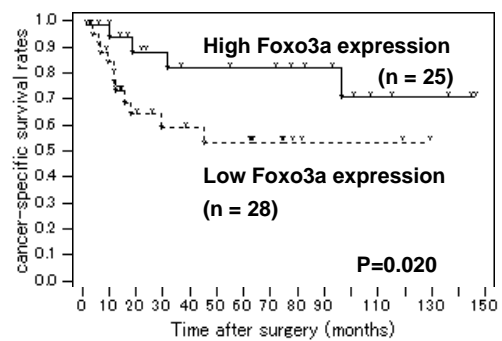Foxo3a   p = 0.83
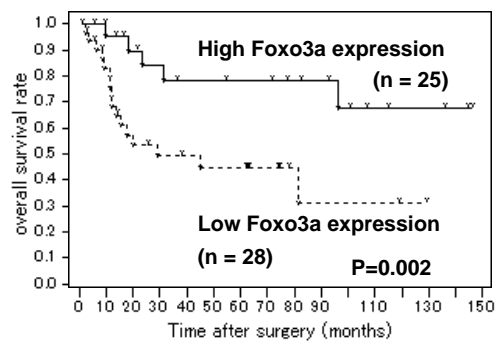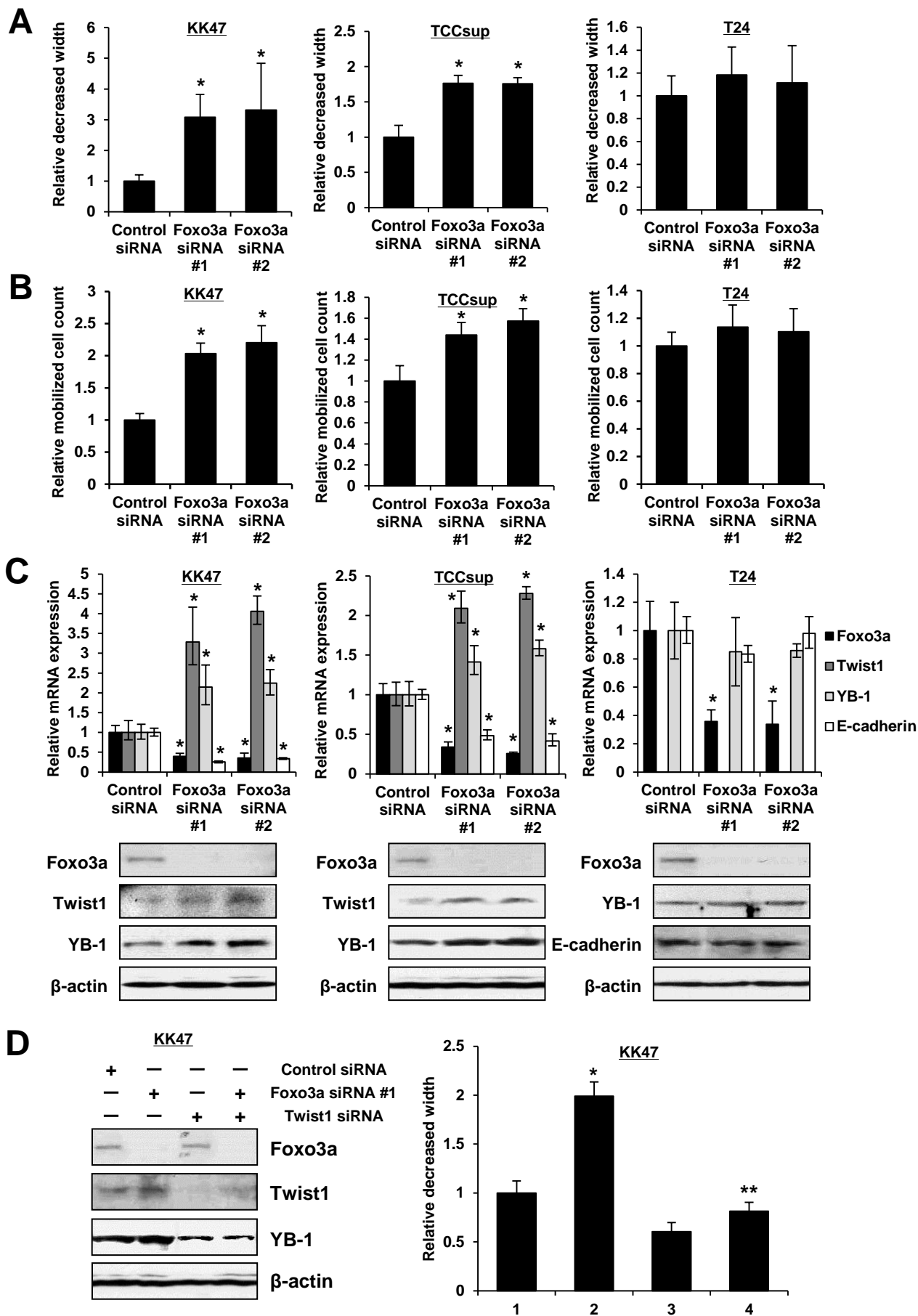
Meta-   Meta+

Shiota et al. Figure. 2
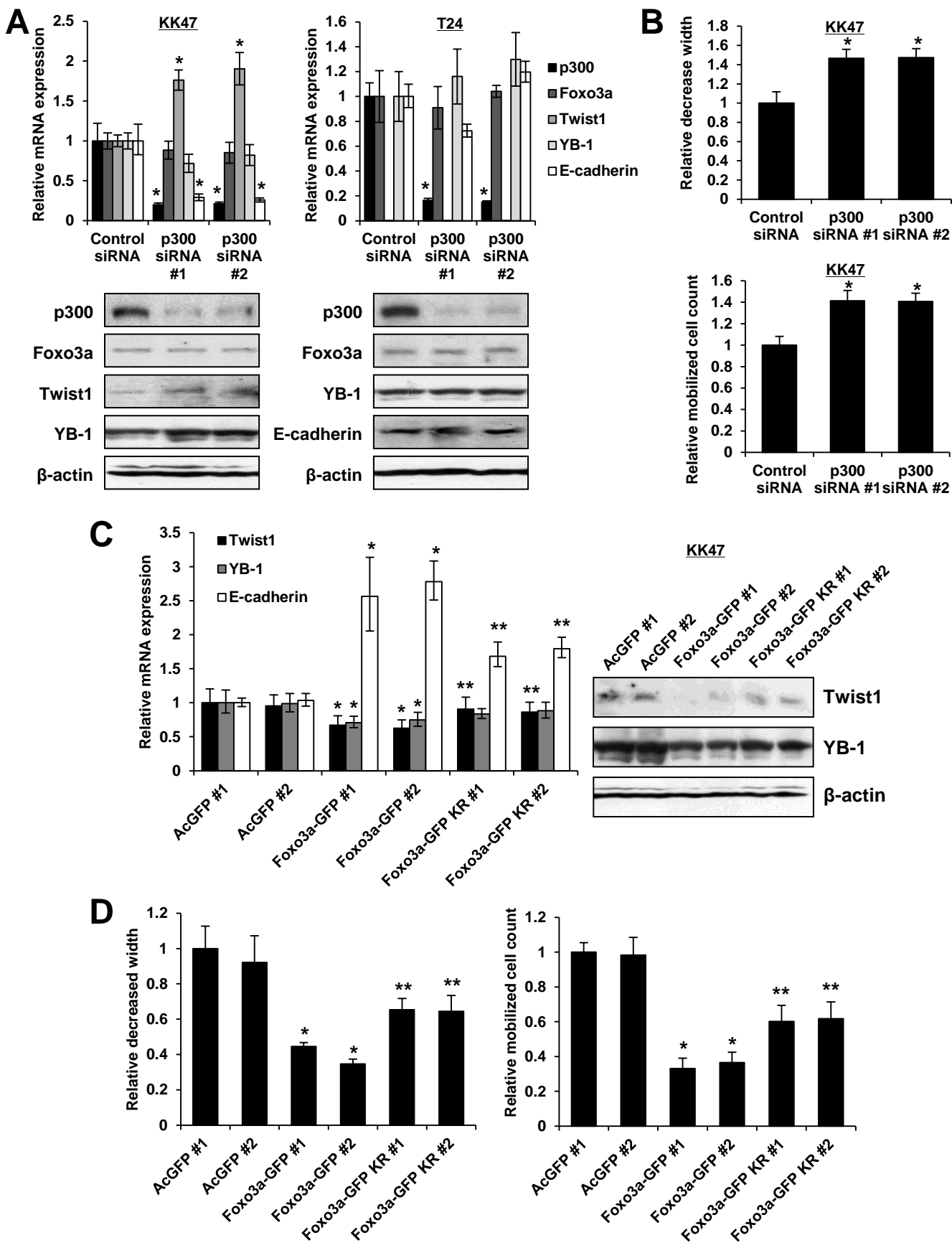
Shiota et al. Figure 3

Shiota et al. Figure 4

Table 1 Relationships between Foxo3a expression and clinicopathologic parameters

Variable	n = 53	Foxo3a expression Low	High	P value
Age, years				
<70	31	13	18	0.057
>=70	22	15	7	
Sex				
Male	42	21	21	0.509
Female	11	7	4	
Grade				
Low	6	2	4	0.404
High	47	26	21	
Stage				
pTa, pT1, pTis	22	7	15	0.009*
pT2, pT3, pT4	31	21	10	
Lymph node status				
N0	40	18	22	0.101
N1, N2	12	9	3	

* Statistically significant ($\chi 2$ test)

Table 2 Overall survial in Cox regression analysis

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Foxo3a						
High expression	1			1		
Low expression	4.421	1.709–13.653	0.002*	2.974	1.063—9.964	0.037*
Sex						
Male	1					
Female	1.021	0.292–2.767	0.971	-	-	-
Grade						
Low	1					
High	3.684	0.765–66.022	0.118	-	-	-
Stage						
pTa, pT1, pTis	1			1		
pT2, pT3, pT4	8.819	2.932–38.015	< 0.001*	4.623	1.244–21.994	0.022*
Lymph node status						
N0	1			1		
N1, N2	10.069	3.618–30.599	< 0.001*	4.407	1.536—14.041	0.006*

HR = hazard ratio.; CI = confidence interval.*Statistically significant (Cox proportional hazards model)

Supplementary Table 1 Sequence of siRNAs used

siRNA	Sense sequence	Antisense sequence
Foxo3a siRNA #1	5′ -UAGAAUUGGUGCGUGAACGGAAGUC-3′	5′ -GACUUCCGUUCACGCACCAAUUCUA-3′
Foxo3a siRNA #2	5′ -UAUACGGGAAGCUAGAGCUCCGCUG-3′	5′ -CAGCGGAGCUCUAGCUUCCCGUAUA-3′
Twist1 siRNA	5′ -CUUCCUCGCUGUUGCUCAGGCUGUC-3′	5′ -GACAGCCUGAGCAACAGCGAGGAAG-3′
p300 siRNA #1	5′ -AUUAUAGGCUGACGUGGCAUUCCUA-3′	5′ -CCUGCCCGGUGAACUCUCCUAUAAU-3′
p300 siRNA #2	5′ -UUAAACAGCCAUCACAGACGAAUCC-3′	5′ -GGAUUCGUCUGUGAUGGCUGUUUAA-3′

Supplementary Table 2 Clinicopathologic characteristics of patients subjected to gene expression assay

Variable	n = 71	Percent (%)
Age at operation, years	44 — 86 (median 68)	
Sex		
Male	57	80
Female	14	20
Grade		
Low	18	25
High	53	75
Stage		
Non-invasive	48	68
Invasive	22	31
Unknown	1	1.4
Metastasis		
Absent	66	93
Present	5	7