FOXO1 is a tumor suppressor in cervical cancer

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ABSTRACT. Forkhead box protein O1 (FOXO1) is an important transcriptional regulator of cell proliferation, and is considered essential for tumor growth and progression. However, the function of FOXO1 in human cervical cancer remains unclear. In this study, we investigated the role of FOXO1 in cervical cancer. Our results showed that FOXO1 expression was lower in cervical cancer than in cervical intraepithelial neoplasia and normal cervix by immunohistochemical analysis ($P < 0.05$). The level of FOXO1 in high-grade lesions was significantly lower than in low-grade lesion ($P < 0.05$), indicating that deficient expression of FOXO1 is involved in tumor progression and significantly associated with late-stage tumors ($P < 0.05$), which was further supported by clinicopathological, real-time polymerase chain reaction, and Western blotting analysis. Moreover, we confirmed that the overexpression of FOXO1 remarkably repressed cell growth and blocked cell proliferation, accompanied by cell-cycle arrest in the G_2/M phase and upregulation of caspases-3 and -9 gene expression. Collectively, our data suggest that FOXO1 plays a vital role in inhibiting cervical cancer development by inducing cell-cycle arrest

and apoptosis. FOXO1 expression is a favorable prognostic factor for human cervical cancer.

Key words: Cell apoptosis; Cell proliferation; Cervical cancer; Cell cycle; Forkhead box protein 01; Immunohistochemistry

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide and infection with oncogenic human papillomavirus (HPV) types, which is an important risk factor of its etiology (Martin, 2007). However, a previous study suggested that HPV infection alone is insufficient for inducing cervical cancer (Shai et al., 2007) and malignant changes (Kim and Zhao, 2005). There is an urgent need to understand the molecular mechanisms governing cervical tumorigenesis. Current knowledge regarding the cause and pathogenesis in cervical cancer is expanding rapidly (Schiffman et al., 2007). The transformation from low-grade lesion to invasive cancer must involve other cellular genetic changes affecting oncogenes, tumor suppressor genes, or the signal transduction pathway.

The FoxO subfamily of Forkhead transcription factors is conserved from *Caenorhabditis elegans* to mammals (Furuyama et al., 2004). Invertebrates possess one FoxO gene, whereas mammals contain 4 FoxO family members: $FoxO_1$ (FKHR), $FoxO_3$ (FKHRL1), $FoxO_4$ (AFX), and $FoxO_6$. FoxO factor has been shown to have a key role in a variety of biological processes, including gluconeogenesis, cell cycle arrest, apoptosis, atrophy, oxidative, and stress response (Calnan and Brunet, 2008). In mammals, FOXO1 has a wide range of organismal functions; it can promote tumor suppression and may also extend the mammalian lifespan (Furuyama et al., 2004), as all FoxO proteins have the ability to induce cell cycle arrest, DNA repair, and apoptosis (Burgering and Kops, 2002; van der Horst and Burgering, 2007; Salih and Brunet, 2008).

Deregulation of FOXO1 has been shown to promote cell proliferation and tumorgenesis in prostate, breast, and endometrial cancer cells (Jackson et al., 2000; Huang et al., 2004; Goto et al., 2008a). FOXO1 has become a major target in preventing tumorigenesis (Arden, 2006; Yang and Hung, 2009). However, the relationship between the clinical significance and FOXO1 expression as well as the underlying mechanisms of FOXO1expression in human cervical tumorigenesis have not been established.

We investigated the expression level of FOXO1 and explored the relationship between its expression and clinical characteristics of patients with cervical cancer. Furthermore, we examined the role of FOXO1 in cell proliferation, cell cycle, and the proapoptotic-mediators caspases in a cervical cancer cell line.

MATERIAL AND METHODS

Patients and clinical samples

The research group included 49 cases of cervical cancer and 20 cases of cervical intraepithelial neoplasia (CIN). The control group included 20 cases with normal cervix. Tissue samples were obtained from patients undergoing hysterectomy without preoperative chemotherapy or radiation and histologically validated for type and grade before tissue collection from Anhui Medical University from 2010-2012. Normal samples were obtained from pre-

menopausal women awaiting *in vitro* fertilization treatment. Written consent from each patient and approval from the Institutional Research Ethics Committee were obtained. Tumors were diagnosed by 2 expert pathologists according to the grading system defined by the 2009 International Federation of Gynecology and Obstetrics (FIGO) for tumors of the cervical cancer (Quinn et al., 2006; Pecorelli, 2009). All samples were acquired at the time of surgery from unfixed tissue, and the samples were immediately frozen and stored at -196°C in liquid nitrogen until further analysis.

Immunohistochemistry

Tumor tissues were fixed in 10% buffered formalin and then embedded in paraffin for routine examination and immunohistochemistry. Next, 4-μm sectioned tissues were immunostained analyzed using histostain TM-plus kits (ZSGB-BIO, Beijing, China) with primary antibodies against FOXO1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 dilution and rabbit anti-beta-actin (Bioss, Woburn, MA, USA) at 1:200 dilution at 4°C overnight. As a negative control, the primary antibody was replaced with Tris-buffered saline. Goat anti-mouse IgG (H+L), horseradish peroxidase (HRP)-conjugated secondary antibodies (bs-0296G-HRP), goat anti-rabbit IgG (H+L), and HRP antibodies (bs-0295G-HRP) were purchased from Bio-Synthesis, Inc. (Lewisville, TX, USA). Diaminobenzidine tetrahydrochloride was used as the substrate to detect antigen-antibody binding, and the sections were counter-stained with Mayer's hematoxylin. Subsequent steps were performed according to manufacturer instructions. Histological and immunohistochemical assessments were performed by 2 independent pathologists.

Cell lines and cell culture

Human cervical cancer cell lines were acquired from Shanghai Institutes for Biological Sciences (Shanghai, China). Cell lines used in the present study were maintained in Dulbecco's modified Eagle medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a 37°C incubator and 5% carbon dioxide humidified atmosphere.

Plasmids and transfection

To study the effects of FOXO1 overexpression, the expressing plasmid, pCMV6-Entry-FOXO1, was used. Experiments were carried out in SiHa cells seeded on 6-well plates that were 70% confluent and then transfected with 5 μg plasmid DNA using 4 μL Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The cells were incubated for 6 h in the transfection mixture, after which the media was replaced with normal fresh culture media.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 1×10^6 cells using Trizol reagent (Invitrogen) according to manufacturer instructions and used for qRT-PCR analysis. Next, 1 g total RNA was reverse-transcribed into complementary DNA using the Prime Script RT reagent kit (Ta-

kara, Shiga, Japan). qRT-PCR reactions were performed in an IQ5 (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex TaqTM (TaKaRa). Relative gene expression was determined using the CT method. For FOXO1 mRNA quantification, the following gene-specific primer pairs were used: β-actin-sense (5'-gacgtggacatccgcaaag-3') and β-actin-antisense (5'-ctggaaggtggacagcgagg-3'); FOXO1-sense (5'-tacgagtggatggtcaagagc-3') and FOXO1-antisense (5'-tgaacttgctgtgtagggaca-3'). β-actin, a non-regulated housekeeping gene, was used as an internal control to normalize input cDNA. Each experiment was performed in triplicate and repeated at least 3 times.

Western blotting

Total protein was quantified using the BCA Reagent Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Equal amounts of proteins (20 μg) from each sample were size-fractionated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene fluoride membrane using a Bio-Rad wet-blot transfer apparatus. The membrane was blocked with 5% skim milk in phosphate-buffered saline containing 0.01% Tween 20 at room temperature for 1 h. The membranes were probed with primary antibody specific for FOXO1 (sc-502) at 1:1000 dilution at 4°C overnight. They were then incubated with goat anti-mouse (bs-0296G-HRP, Bio-Synthesis) or anti-rabbit secondary antibodies (bs-0295G-HRP, Bio-Synthesis) at 1:1000 dilution for 1 h and visualized by enhanced chemiluminescence. Western blot quantification was performed using the Photoshop software. We evaluated the expression of FOXO1 as an optical densitometry (OD) ratio and was scored as the densitometry of FOXO1 relative to the densitometry of β-actin.

Cell proliferation and cell-cycle

Cell viability was assessed using the MTT assay for 5 days as described previously (Yuan et al., 2009). The experiment was performed in triplicate for each time point.

For cell cycle analysis, cells were examined by fluorescence-activated cell analysis with propidium iodide-stained cells on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The cells harvested (1 x 10^6 per well) were washed with phosphate-buffered saline and fixed in cold 70% ethanol overnight. For flow cytometric analysis, the cells were incubated with 40 μg/mL propidium iodide and 100 μg/mL DNase-free RNase A in phosphate-buffered saline at 37°C for 30 min. The cell cycle distribution was analyzed using the Mod Fit LT version 3.0 software (Verity Software House Inc., Topsham, ME, USA). Representative results of 3 experiments with consistent results are shown.

Statistical analyses

All statistical analyses were carried out using the SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are reported as means ± standard deviation for experiments performed at least 3 times. Statistical analyses comparing the 2 groups were performed using Student's t test, the χ^2 test, and Fisher's exact test, as appropriate. The remaining data were analyzed using 1-way analysis of variance. The level for statistical significance was set at P < 0.05.

RESULTS

FOXO1 expression was low in human cervical cancer tissues

To investigate the expression levels of FOXO1 in human cervical tumors, we examined 20 normal cervix, 20 CIN, and 59 cervical cancer tissues using immunohistochemical analysis. Higher expression of FOXO1 was observed in most normal cervix and CIN, whereas weak or undetectable FOXO1 expression was dominant in cervical cancer cases (Figure 1). Moreover, the intensity of the FOXO1 staining in cervical cancer was on average significantly lower than that in normal cervix and CIN ($P < 0.05$). There was no significant difference between normal cervix and CIN ($P > 0.05$) (Table 1). The immunoreactivity of FOXO1 was observed in the nuclei of all cervical tissues.
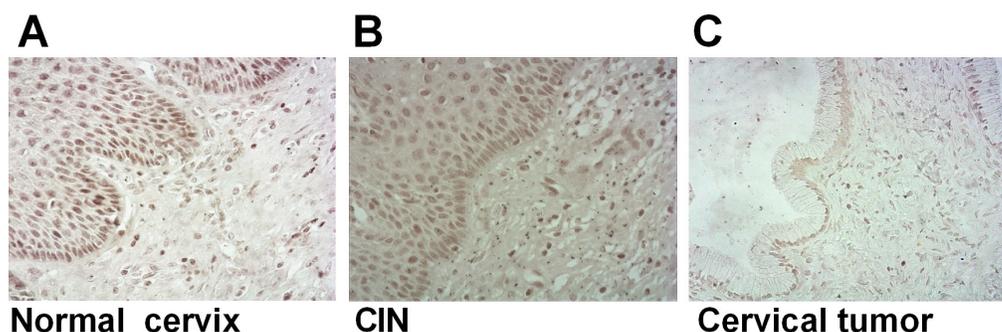

Figure 1. FOXO1 staining in normal cervix, CIN, and cervical cancers. Immunohistochemical analysis of FOXO1 in paraffin-embedded, formalin-fixed endometrial specimens: 20 normal cervix, 20 CIN, and 59 cervical cancer were stained for FOXO1. FOXO1 immunostaining was strong in the normal cervix (**A**) and CIN (**B**), but was undetectable or markedly reduced in cervical cancers (**C**). Based on the proportion of stained cells in each sample, the staining scores were as follows: negative (if 65% of cells were stained by the antibody); weakly positive (>5-20%, including 20%, of cells were stained); positive (>20-50%, including 50%, of cells were stained); strongly positive (>50% of cells were stained). Table 1 summarizes the outcome of statistical analysis of the staining results. Original magnification (200X).

Table 1. Immunohistochemical analyses of FOXO1 expression.

Group	N	FOXO1 expression			χ^2	P value
		Negative	Positive	Positive rate (%)		
Normal cervix	20	3	17	85		
CIN	20	5	15	75	0.229	>0.05
Cervical cancer	59	52	7	11.86	37.74	<0.05

$P < 0.05$ *vs* normal cervix.

Immunohistochemical analysis of FOXO1 in paraffin-embedded, formalin-fixed endometrial specimens: 20 normal cervix, 20 CIN, and 59 cervical cancer were stained for FOXO1. FOXO1 immunostaining was strong in the normal cervix (A) and CIN (B), but was undetectable or markedly reduced in cervical cancers (C). Based on the proportion of stained cells in each sample, the staining scores were as follows: negative (if 65% of cells were stained by

the antibody); weakly positive (>5-20%, including 20%, of cells were stained); positive (>20-50%, including 50%, of cells were stained); strongly positive (>50% of cells were stained). Table 1 summarizes the outcome of statistical analysis of the staining results.

We also examined the relationship between FOXO1 staining and clinicopathological factors. Our results showed that there was a significant positive correlation between FOXO1 expression and differentiation, FIGO grade, or lymphatic invasion ($P < 0.05$) (Table 2). In contrast, no significant correlation was observed between FOXO1-positive staining and patient's age or pathological type in cervical cancer tissue ($P > 0.05$). Interestingly, we observed that FOXO1 expression in high-grade (III-IV) FIGO were significantly lower than that in low-grade (I-II) FIGO and gradually decreased and progressed to FIGO III ($P < 0.05$).

Table 2. Clinicopathological features of patients and FOXO1 expression in cervical cancers.

Group	N	FOXO1 expression			χ^2	P value
		Negative	Positive	Positive rate (%)		
Age (years)						
<45	30	25	5	16.67		
≥45	29	23	6	20.69	0.690	>0.05
Pathological type						
Squamous	8	7	1	12.5		
Adenocarcinomas	51	41	10	19.61	1	>0.05
Differentiation						
Well or moderate	18	11	7	9.76		
Poor	41	37	4	38.89	0.022	<0.01
FIGO grade						
I, II	26	18	3	30.77		
III, IV	33	30	8	9.09	4.506	<0.05
Lymphatic invasion (ly)						
+	22	14	8	36.4		
-	37	34	3	8.11	0.019	<0.01

LN metastasis = Lymph node (LN) invasion (ly).

To confirm these findings, the same samples were analyzed for FOXO1 expression at the mRNA level (Figure 2A) and protein level (Figure 2B). Consistent with our immunohistochemistry data, qRT-PCR and Western blot results showed that FOXO1 was expressed at relatively high levels in the normal cervix (control) and cervicitis, whereas in cervical cancer samples, the levels were much lower or beyond detection ($P < 0.05$). There was no difference between control and cervicitis, I and II grade FIGO, III and IV grade FIGO, squamous cancer and adenocarcinomas cancer ($P > 0.05$).

FOXO1 represses cell proliferation and cell cycle in cervical cancer cells

Given the consistent downregulation of FOXO1 in cervical cancer tissue, we examined whether FOXO1 exerted tumor suppressor functions or inhibited proliferation and/or induce cell death in cervical cancer cell lines.

We investigated FOXO1 expression in the cervical cell lines HeLa, Caski, SiHa, and C-33A. Remarkably, of the 4 cell lines, C-33A showed the highest levels, while SiHa showed the lowest levels of FOXO1 expression at both the mRNA level ($P < 0.05$) (Figure 3A) and protein level ($P < 0.05$) (Figure 3B).
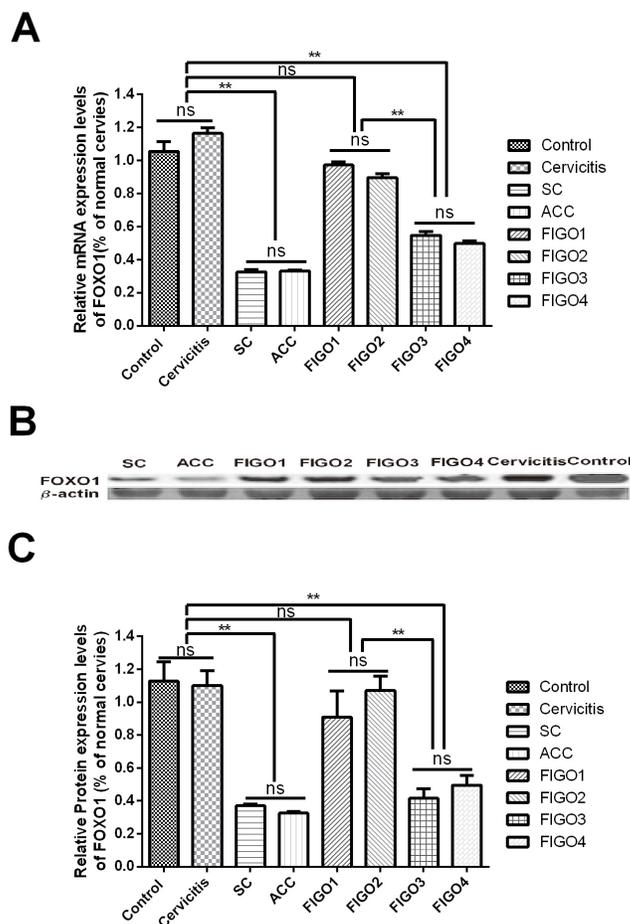
Figure 2. A. Downregulation of FOXO1 expression in human cervical cancer tissues. **B.** Real-time PCR analysis of FOXO1 expression in 20 normal cervix and 59 cervical cancer samples. **C.** Western blot analysis of FOXO1 transcripts in the same samples. β-actin staining was included as a loading control. Statistical analysis of FOXO1 expression after western blot quantification using the Image J software, the average optical densitometry ratio demonstrating significantly lower FOXO1 protein levels in cervical cancer compared with normal cervix and cervicitis. Low-grade FIGO were higher than that in high-grade FIGO cervical tumors. There was no difference between normal cervix and cervicitis, SC, and ACC. Results are presented as arithmetic mean of 3 separate experiments ± standard deviation. Double asterisks (**) indicate a significant difference (**P < 0.05). The experiment was performed 3 times with similar results. SC = squamous cancer; ACC = adenocarcinomas cancer; ns = not significant.

To further characterize the effect of FOXO1 on cell viability and the cell cycle, we expressed FOXO1 in SiHa cells, the lowest FOXO1-expressing cervical cancer cell line (Figure 3). Combined with MTT analysis, we observed that overexpression of FOXO1 significantly repressed cell proliferation of SiHa cells (P < 0.05) (Figure 4A). Using flow cytometric analysis, we found that the gain function of FOXO1 also blocked cell proliferation by regulating the G_1-S and G_2-M transitions, reduced levels of cells in the S-phase accompanied by cell-cycle arrest in the G_0/G_1 phase, and decreased tumorigenic activity (P < 0.05) (Figure 4B-D).
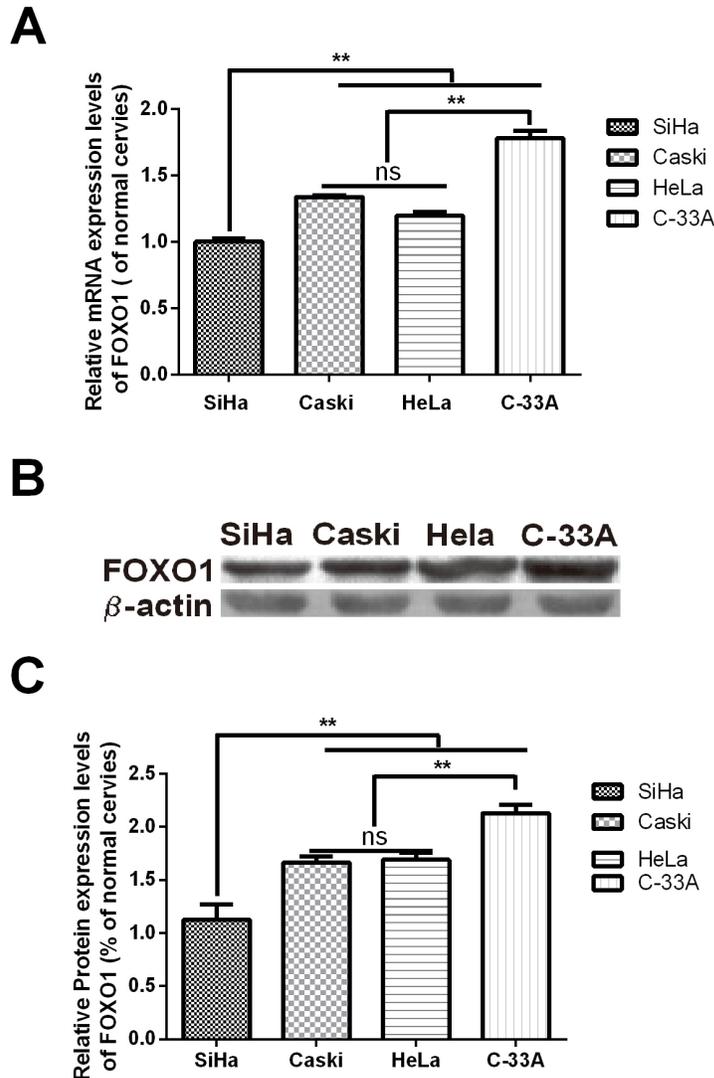
A

B

C

Figure 3. A. Expression of FOXO1 in cervical cancer cell lines. **B.** Real-time PCR analysis of FOXO1 expression in the 4 cell lines. **C.** Western blot analysis FOXO1 expression in the same 4 cell lines, showing that the expression of FOXO1 in SiHa was the lowest compared to in other cell lines. Results are presented as arithmetic mean of 3 separate experiments ± standard deviation. Double asterisks (**) indicate a significant difference (**$P < 0.05$). The experiment was performed 3 times with similar results.

FOXO1 activates caspase activity in cervical cancer cells

To further investigate the impact of FOXO1 on apoptosis, we examined caspase activity in SiHa cells overexpressing FOXO1. As expected, FOXO1 strongly induced an increase in caspase-3 and capase-9 mRNA activity compared with the control group ($P < 0.05$) (Figure 5).
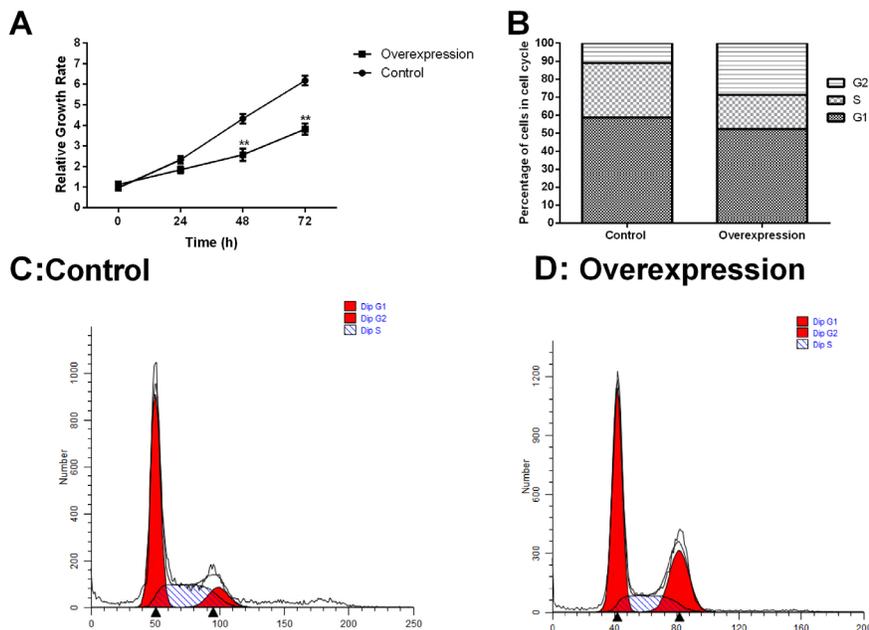
Figure 4. FOXO1 repressed cell proliferation and cell cycle in SiHa cells. **A. B.** Analysis of cell viability. MTT assay showed that overexpression of FOXO1 significantly decreased cell viability. **P < 0.05 for all time points from 24-72 h. **C. D.** Cell-cycle analysis. Cells were seeded at the density 10^6 of the complete medium and cell-cycle distribution was measured by propidium iodide staining. A representative flow cytometric profile and a bar graph showing the corresponding cell cycle distribution in percentage of FOXO1 expression. Percentage of cells in each phase of the cell cycle (sub-G_1, G_1, S, and G_2-M) is indicated. Representative data from 3 independent experiments are shown. The results confirmed that the overexpression of FOXO1 in SiHa cells remarkably repressed cell growth ability and blocked cell proliferation, accompanied by cell-cycle arrest in the G_2/M phase.
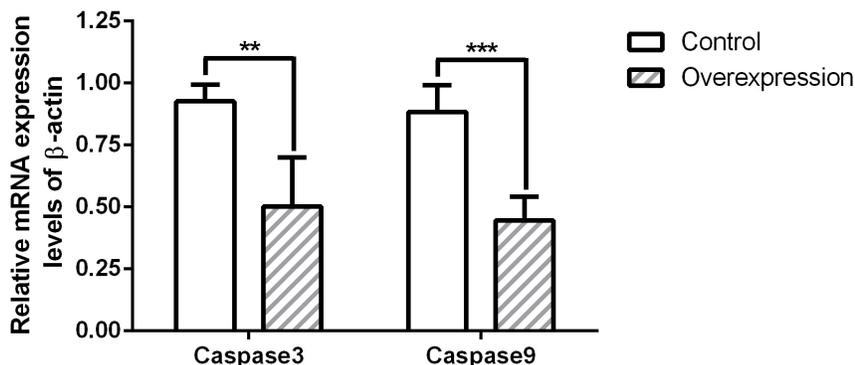

Figure 5. Caspase mRNA levels after overexpression of FOXO1 in SiHa cells. The mRNA levels of selected proapoptotic genes caspase-3 and caspase-9 were tested by real-time PCR, which showed significant difference between the control group and overexpression of FOXO1 group in SiHa cells. Data are reported as means of 3 independent experiments. Asterisks indicate a significant difference (**P < 0.05; ***P < 0.01).

DISCUSSION

Recent studies have reported that in a variety of cancers, FOXO1 was associated with aggressive cell proliferation behavior. The FOXO1 transcription factor orchestrates the regulation of genes involved in the apoptotic response, cell cycle checkpoints, and cellular metabolism. FOXO1 is a putative tumor suppressor and the expression of this gene is dysregulated in some cancers, including prostate and endometrial cancers. However, relatively little is known regarding the role of FOXO1 in cervical cancer.

In this study, we first examined the expression of FOXO1 in cervical tissues using immunohistochemistry analysis and characterized whether the protein was deficient or weakly expressed in human cervical cancer samples compared with normal cervix and CIN, which was consistent with observation in other types of human tumors (Jackson et al., 2000; Huang et al., 2004; Goto et al., 2008b). Interestingly, we noted that FOXO1 expression was restricted in proliferative cells and gradually decreased with the progression of tumor stage. These findings suggest that downregulated expression of FOXO1 contributes to the repression of cervical cancer development.

To further investigate the role of transcriptional factor FOXO1 in modulating the cell cycle and cell apoptosis in cervical cancer cells, we first confirmed expression in 4 cervical cancer cell lines (HeLa, Caski, SiHa, and C-33A). We selected SiHa for further analysis because the level of FOXO1 was the lowest among the 4 cell lines. Functionally, enforced expression of FOXO1 in SiHa cells not only remarkably blocked cell proliferation, but also decreased tumorigenic activity, which was similar to previous findings in other cancers (Goto et al., 2008a; Guttilla and White, 2009). Our findings suggest that FOXO1 has crucial roles in the pathogenesis of human cervical cancer.

Although the pathway(s) by which FoxO family members inhibit tumor development remain unclear, previous studies indicate that FoxO members are involved in the induction of apoptosis. In numerous cell types, activation of the FoxO family leads to apoptosis, particularly when its expression or activation is prolonged (Burgering and Medema, 2003; Gilley et al., 2003). Because of the increased expression of proapoptotic factors, caspase activity is also enhanced, triggering cell apoptosis (Alikhani et al., 2005; Huang and Tindall, 2007).

Cell apoptosis can be initiated by a mitochondria-dependent or -independent apoptosis pathway (Dlamini et al., 2004). Recent reports have demonstrated apoptosis through the activation of caspase-3 by both caspase-8- and caspase-9-dependent pathways (Alikhani et al., 2003, 2004). Both pathways trigger apoptosis by activating different caspase cascades and converging on caspase-3, the executor of apoptosis.

We therefore investigated whether and how FOXO1 modulates cell apoptosis and explored the relationship between FOXO1 and anticancer activity. Our results showed that the mRNA levels of caspase-3 and caspase-9 were upregulated with FOXO1 overexpression in SiHa cells. Although it was unknown whether FOXO1 directly transactivated caspase-9, and our results provide the first evidence that activation of the transcription factor FOXO1 is an important step in apoptosis. Additionally, our results indicate that FOXO1 activates apoptosis through the mitochondria-dependent apoptosis pathway in cervical cancer.

Remarkably, our limited data demonstrated that the HPV-negative cell line, C-33A showed the highest expression of FOXO1, while the HPV-positive cervical cancer cell lines, SiHa, HeLa, and Caski cells, showed lower levels, particularly SiHa. Further studies to investigate the relationship between FOXO1 and E_6/E_7 in tumorigenesis of cervical cancer remains needed.

The traditional trinity of cancer therapies is comprised of chemotherapy, surgical intervention, and radiation. To date, none of these treatments are curative. Thus, continued studies examining innovative therapeutic strategies are necessary. With the development of molecular biological techniques, additional markers for predicting treatment and potential target therapies are becoming increasingly important. Our findings regarding the intrinsic high correlation between FOXO1 and cervical cancer suggest that FOXO1 can be used as gene target therapy for cervical cancer.

ACKNOWLEDGMENTS

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