Int J Clin Exp Pathol 2016;9(10):9938-9950
www.ijcep.com /ISSN:1936-2625/IJCEP0033922

Original Article

Value of immunohistochemical detection of FOXO3a as a prognostic marker in human breast carcinoma

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Received June 17 2016; Accepted July 12, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Breast cancer is the most common neoplasm in women. Forkhead box-O (FOXO) transcription factors act as transcriptional regulators in the nucleus by modulating cell differentiation, cell cycle arrest, and apoptosis. Based on the their ability to control cell cycle and apoptosis, FOXOs may be a useful prognostic biomarker for breast cancer. Thus, we aimed to investigate the expression patterns of FOXO3a in human breast cancer tissue, immunohistochemically and to evaluate the whether a relationship between FOXO3a and the prognostic factors of breast tumor. FOXO3a expression patterns were examined in thirty tissue specimens from patients with primary operable breast carcinoma who underwent mastectomy. The score of cytoplasmic staining were ranged from 3 to 4 and the score of nuclear staining were ranged from 0 to 3. 100% of specimens showed cytoplasmic staining, 80% of specimens showed both cytoplasmic and nuclear staining and 20% of specimens showed no nuclear staining. FOXO3a nuclear staining scores showed negative correlation with grade, stage, metastatic axillary lymph node, HER2 score, and Ki-67 proliferative index ($P=0.02$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P=0.003$, respectively) and a positive correlation with estrogen receptor ($P=0.012$). There was not found a relationship between between clinicopathological parameters and cytosplasmic staining scores. Consequently, the nuclear FOXO3a accumulation appears to be closely associated with the malignancy of breast tumors. Therefore, consideration of the nuclear FOXO3a staining pattern rather than cytoplasmic staining or total staining pattern will be a more useful prognostic biomarker for breast cancer.

Keywords: Breast neoplasms, FOXO3 protein, human, immunohistochemistry

Introduction

Breast cancer is the most common neoplasm and is the second cause of cancer death in women. It constitutes 21% of all new cancer diagnoses. Survival rates have been increasingly extending over the past 50 years due to improvements in diagnosis and treatment [1]. Clinical-pathological characteristics of tumor such as size, histological grade, hormone receptor status, and number of metastatic axillary lymph nodes estimates the breast cancer prognosis and risk of recurrence. Estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2; Human Genome Organisation, HUGO nomenclature ERBB2) expression levels can be determined using immunohistochemical stainings. The expression levels of ER, PgR, and HER2 are used as predictive markers to determine anti-estrogen- or anti-HER2-directed therapies. They are also used to quantify risk of recurrence. Although any standard test is not available and any test is not uniformly accepted, many clinicians have preferred gene assays as an effective tool to make treatment decisions in early stage of breast cancer cases [2].

Forkhead box-O (FOXO) transcription factors are an important family of proteins that act as transcriptional regulators in the cell nucleus and they bind to their DNA binding sites. They are components of signal-transduction pathways that link growth and stress signals to the control of gene expression. They modulate the expression of genes such as cell differentiation,

resistance to oxidative stress, DNA damage repair, cell cycle arrest, and apoptosis. To date, four isoforms of FOXOs are known in humans: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX1), and FOXO6 [3, 4]. While the FOXO1 and FOXO4 are mostly expressed in adipose tissue and skeletal muscle, FOXO3a is expressed in various tissues including brain, heart, kidney, and spleen and FOXO6 is expressed predominantly in the brain [5]. Subcellular localization of FOXO proteins plays an essential role in the regulation of their activity. FOXO proteins activate or repress transcription of target genes through their DNA-binding FOX domain in the nucleus. Subcellular localization and transcriptional functions of FOXO proteins are regulated by their post-translational modifications, such as phosphorylation, acetylation, and ubiquitination via their specific nuclear export and import signal network. In the absence of growth and survival factors such as insulin and insulin-like growth factor 1, FOXO proteins are localized in the nucleus and are transcriptionally active. On the contrary, phosphorylation of FOXOs by several kinases in response to growth and survival factors leads to the translocation of FOXOs from the nucleus into the cytoplasm. This nuclear exclusion and translocation of FOXO into the cytoplasm inhibits FOXO-dependent transcription. Phosphorylation, monoubiqutination, deacetylation of FOXO proteins in response to increased cellular oxidative stress leads to retention of FOXOs in the nucleus even in the presence of growth and survival factors [4-7].

Akt-dependent phosphorylation is most important mechanism in their regulation and function. Akt is a serine-threonine kinase regulated by activation of phosphoinositide kinase (PI3k). PI3k/Akt pathway has been shown to be activated in numerous tumours [8]. Phosphorylation of FOXOs by Akt impairs their DNA binding activity and promotes their interaction with the chaperone protein 14-3-3, resulting in nuclear exclusion, cytoplasmic accumulation, and ubiquitin-proteasome pathway-dependent degradation, thus promoting cell survival. In the presence of oxidative stress, FOXO proteins are activated and released from 14-3-3 via Jun N-terminal kinase (JNK) signalling [9]. In most cancers, the PI3k pathway is overactivated and this overactivation leads to inactivation of FOXO proteins. On the contrary, reduced activation of PI3k causes activation of FOXOs, induction of apoptosis, decrease of cell viability and G1 cell cycle arrest [10, 11].

Since FOXO proteins can act as tumor suppressors, the loss of FOXO function leads to increased cellular survival and a predisposition to neoplasia. Cancer researchs related to FOXO proteins are increasing each passing day as their function is directly associated with cell-cycle arrest and apoptosis. Therefore, based on the their ability to control cell cycle and promote apoptosis, FOXO proteins appear to be potentially key targets for new drug discovery blocking tumorigenesis [7, 12]. Thus, in this study, we aimed to investigate the expression and subcellular localization of FOXO3a protein in human breast cancer tissue by immunohistochemical staining method. In addition, we also evaluated the relationship between FOXO3a and the prognostic factors of breast tumor such as size, stage, histological grade, hormone receptor status, proliferation index, HER2 expression levels, and the number of metastatic axillary lymph nodes.

Materials and methods

Sample collection

The study cases consisted of thirty formalin-fixed paraffin-embedded tissue specimens from patients with primary operable breast carcinoma who underwent mastectomy at the Department of General Surgery, Dumlupinar University Faculty of Medicine between 2013 and 2015. All patients underwent mastectomy without preoperative chemotherapy or radiotherapy. Formalin-fixed paraffin-embedded tissue specimens of breast carcinoma were randomly collected from the database of Department of Pathology, Dumlupinar University Faculty of Medicine. The study was in accordance with the principles outlined in the Declaration of Helsinki. Ethical approval was received from the local Human Clinical Research Ethics Committee. The informed consent was not requested, since the study was retrospective and the data were analyzed anonymously.

Histopathological examination of breast carcinoma tissues

Formalin-fixed paraffin-embedded breast carcinoma tissues were re-examined and pathologi-

Table 1. Demographic and clinicopathological parameters of the breast carcinoma cases

Parameters	n=30
Age (years)	59.6 ± 14.8
Histological type of the tumor, n (%)	
Invasive ductal carcinoma	28 (93)
Invasive lobular carcinoma	2 (7)
Grade (Nottingham histological grade), n (%)	
1	1 (3)
2	13 (43)
3	16 (54)
Pathological stage (pTNM), n (%)	
1A	2 (7)
2A	7 (23)
2B	7 (23)
3A	5 (17)
3C	9 (30)
Size of the tumor (cm)	3.1 (2.5-4.6)
The number of metastatic axillary lymph node, n (%)	2.5 (0.0-11.0)
Lymphovascular invasion, n (%)	26 (87)
Perineural invasion, n (%)	23 (77)
Microcalcification, n (%)	18 (60)
ER positivity, n (%)	24 (80)
PR positivity, n (%)	18 (60)
HER2 positivity, n (%)	27 (90)
FOXO3a positivity, n (%)	30 (100)
ER score	5.0 (2.0-5.0)
PgR score	3.0 (0.0-5.0)
HER2 score	3.0 (2.0-3.0)
Ki-67 proliferative index (%)	30.0 (20.0-42.5)
FOXO3a nuclear staining score	1.0 (1.0-2.0)
FOXO3a cytoplasmic staining score	4.0 (4.0-4.0)
FOXO3a total staining score	5.0 (4.0-6.0)
FOXO3a staining intensity score	3.0 (2.0-3.0)

Abbrevations: ER: Estrogen receptor, PgR: Progesteron receptor. Data are presented as mean ± standard standart deviation (SD) or median and interquartile ranges (IQRs) or number and %.

cal reports were updated. Microscopic histopathological examinations were performed using routine Haematoxylin and Eosin (H&E) stain. Tissue processing procedures were performed with a tissue processor system (Shandon Excelsior, Thermo Fisher Scientific Inc., Waltham, MA, USA). Tissues were sectioned at 5 μm thickness using a semi-automated rotary microtome (Leica RM2245, Leica Microsystems Inc., Bannockburn, IL, USA). These sections were stained with H&E using an automated side stainer and coverslipper (Tissue-Tek Prisma/Film, Sakura Finetek Inc., CA, USA). Then, the slides were examined under a light microscope (Olympus BX51, Tokyo, Japan). The slides were histologically graded according to the Nottingham (Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system [13]. Pathological staging was performed using the American Joint Committee on Cancer (AJCC's) tumor, node, metastasis (pTNM) staging system [14].

Immunohistochemical examinations

Immunohistochemical examinations were performed through standard routine procedure. Formalin-fixed paraffin-embedded blocks were sectioned at 4 μm thickness using a semi-automated rotary microtome (Leica RM2245) for immunohistochemical analysis. Antigen retrieval and immunohistochemical stainings were performed using an automated immunohistochemistry slide staining system (Roche Ventana BenchMark GX, Ventana Medical Systems, Inc., Tucson, AZ, USA). ER (rabbit monoclonal antibody, clone SP1, Roche, Tuscon, USA), PgR (rabbit monoclonal antibody, clone 1E2, Roche), HER2 (rabbit monoclonal antibody, clone 4B5, Roche), and Ki-67 (rabbit monoclonal primary antibody, clone 30-9, Roche) were analyzed on whole formalin-fixed paraffin-embedded tissue sections during standard pathological examination of the tumor. FOXO3a immunostaining was performed using the goat polyclonal antibody (anti-FKHRL1-N-15, sc-34-897, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) following the manufacturer's instructions. The primary antibodies were omitted For negative controls. The immunohistochemical stainings were evaluated microscopically under a light microscope (Olympus BX51).

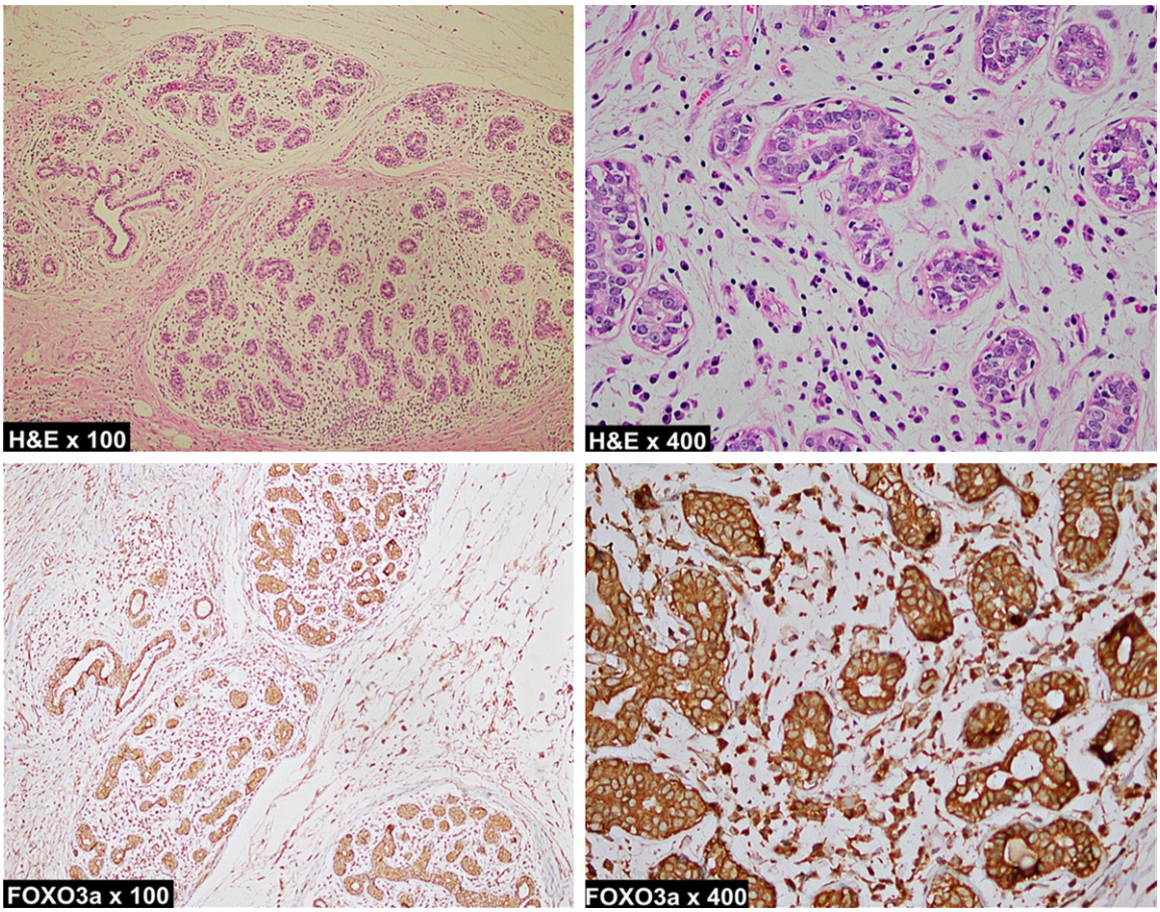

Figure 1. Corresponding adjacent non-tumorous normal breast tissue (H&E × 100, × 400; FOXO3a × 100, × 400). In normal breast tissue, FOXO3a was expressed with strong staining in the both nucleus and cytoplasm of epithelial cells but cytoplasmic staining pattern was more obvious.

Assessment of immunohistochemical staining results

ER and PgR staining was scored according to the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines. The guidelines recommend classifying all cases with at least 1% positive cells as receptor positive [15]. Only nuclear staining is considered positive. Quantification of ER and PgR were performed by using the proportion of positive cells and carcinomas were scored as 0 (< 1% positive), 1 (1% to 25% positive), 2 (> 25% to 75% positive), and 3 (> 75% positive) [16].

HER2 staining was scored according to the ASCO and the CAP guidelines [17]. This system included four scores from 0 to 3: no staining or incomplete, faint/barely perceptible membrane staining in ≤ 10% of invasive tumor cells (score 0), incomplete, faint/barely perceptible membrane staining in > 10% of invasive tumor cells (score 1), incomplete and/or weak to moderate circumferential membrane staining in > 10% of invasive tumor cells or complete, intense, circumferential membrane staining in ≤ 10% of invasive tumor cells (score 2), complete, intense, circumferential membrane staining in > 10% of invasive tumor cells (score 3).

Quantification of Ki-67 were performed by using the percentage of Ki-67 positive tumor cells [18].

The expression and subcellular localization of FOXO3a were evaluated. Corresponding adjacent non-tumorous normal breast tissue was used as positive control. The FOXO3a immunostaining was evaluated as both the positively stained tumor cells and the staining intensity. The staining intensity was scored as follows: 0

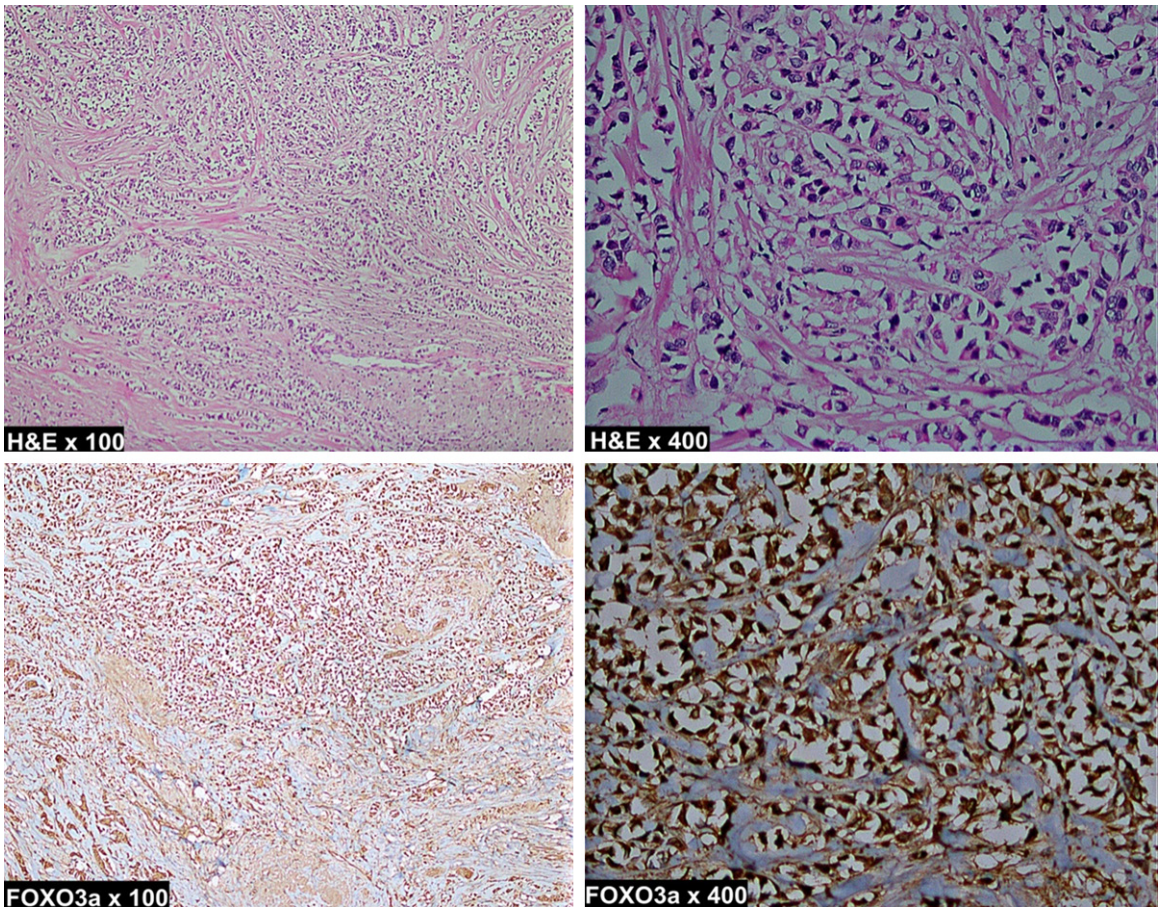

Figure 2. Grade 1 invasive lobular carcinoma (H&E × 100, × 400; FOXO3a × 100, × 400). In tumor tissue, the percentages of nuclear positive cells were higher and FOXO3a was mainly localized in the nucleus of positive stained cells.

(no staining), 1 (weakly stained), 2 (moderately stained), or 3 (strongly stained) [19]. Positively staining cases were categorised according to whether they showed nuclear or cytoplasmic localization. Each of nuclear and cytoplasmic stainings were scored separately according to the percentage of the positive cells as follows: 1 ≤ 25%, 2 ≤ 50%, 3 ≤ 75%, 4 > 75%. For each case, a total score was obtained with sum of the nuclear and cytoplasmic scores [19].

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.05 (GraphPad Software, Inc., CA, USA). All data sets were tested for normality using Shapiro-Wilk test. Data were expressed as mean ± standard deviation (SD) or median and interquartile range (IQRs) according to the distribution of data. The comparisons of categorical variables were analyzed using two tailed Fisher's exact or Chi-square test. The relationships between nuclear, cytoplasmic FOXO3a scores and clinicopathological characteristics of the tumor were tested using the nonparametric Spearman's rank correlation coefficient, since data were not normally distributed. A P value < 0.05 was considered statistically significant.

Results

Demographic and histopathological features of cases are presented in **Table 1**. The patients' ages at diagnosis ranged from 26 to 85 years old (mean age ± SD: 59.6 ± 14.8). Histopathological types of tumors included in the study were invasive ductal carcinoma (93%, n=28) and invasive lobular carcinoma (7%, n=2). According to the Nottingham grading system [13], grades of cases were as follows; grade 1 (3%, n=1), grade 2 (43%, n=13), and grade 3

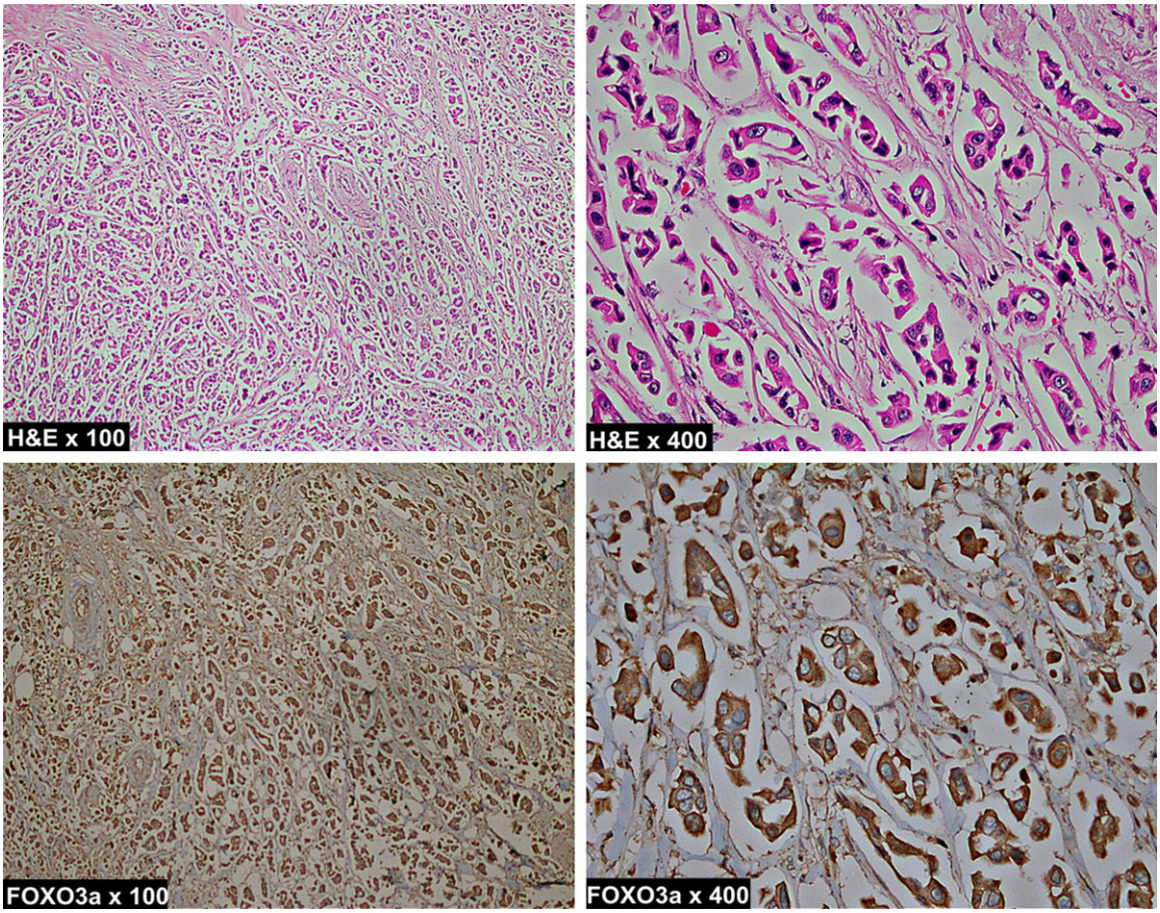

Figure 3. Grade 2 invasive ductal carcinoma (H&E × 100, × 400; FOXO3a × 100, × 400). In tumor tissue, the percentages of nuclear and cytoplasmic positive cells were nearly similar and FOXO3a was mainly localized in the both cytoplasm and nucleus of positive stained cells.

(54%, n=16). According to the AJCC's pTNM staging system [14], stages of cases at the time of histopathological diagnosis were as follows; stage 1A (7%, n=2), stage 2A (23%, n=7), stage 2B (23%, n=7), stage 3A (17%, n=5), and stage 3C (30%, n=9). ER was positive in 24 cases (80%), PR was positive in 18 cases (60%), and HER2 was positive in 27 cases (90%). Thirty percent (30%, n=10) of tumours were less than 2.5 cm in size. Twenty seven percent (27%, n=8) of patients had no lymph node metastasis at the time of surgery.

The epithelial cells from control corresponding adjacent normal breast tissue and the carcinoma cells from malignant breast tumors showed brown cytoplasmic and nuclear staining for Foxo3a (**Figures 1**-**4**). In non-tumorous normal breast tissue, although FOXO3a showed both nuclear and cytoplasmic staining patterns, cytoplasmic staining pattern was dominant in the normal mammary epithelial cells (**Figure 1**). In the all breast tumor tissues, FOXO3a was expressed at various intensity and all cases were positively stained for FOXO3a. FOXO3a staining intensity score was ranged from 1 to 3 (median and IQRs, 3.0; 2.0-3.0). In the malignant tissues, FOXO3a showed both nuclear and cytoplasmic staining patterns but the percentage score of nuclear and cytoplasmic staining showed differences among the specimens. When positively staining cases were categorised according to the subcellular localization, the percentage score of cytoplasmic staining were ranged from 3 to 4 (median and IQRs, 4.0; 4.0-4.0). Whereas, the percentage score of nuclear staining were ranged from 0 to 3 (median and IQRs, 1.0; 1.0-2.0). Although the percentages of cytoplasmic stainings were nearly similar in all cases, the percentages of nuclear stainings showed markedly differences among the cases (**Figures 2**-**4**). While 100% of speci-

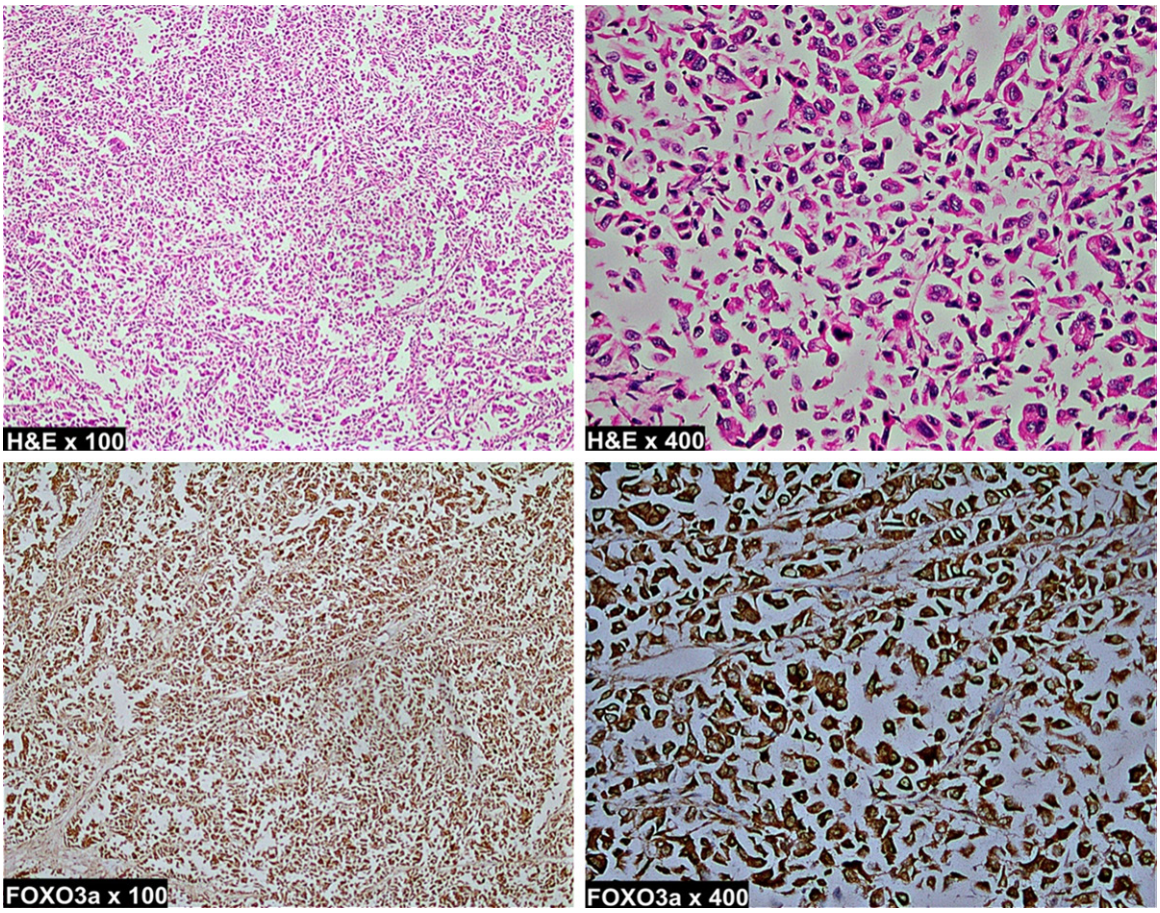

Figure 4. Grade 3 invasive ductal carcinoma (H&E × 100, × 400; FOXO3a × 100, × 400). In tumor tissue, the percentages of nuclear positive cells were lower and FOXO3a was mainly localized in the cytoplasm of positive stained cells.

mens showed cytoplasmic staining patterns, 80% of specimens showed both cytoplasmic and nuclear staining pattern. In 20% of specimens, nuclear staining pattern was negative.

The differences between nuclear staining positive and negative cases for various clinicopathological characteristics of breast tumors are demonstrated in **Table 2** and **Figure 5**. Statistically significant differences were found between nuclear staining positive and negative specimens for age (P=0.002), tumor grade (P < 0.001), tumor size (P=0.009), axillary lymph node involvement (P < 0.001), tumor stage (P < 0.001), HER2 status (P=0.03), and Ki-67 proliferative index (P < 0.001). The percentage of negative nuclear staining pattern was higher in low age, high tumor grade, stage, size, proliferative index, more axillary lymph node involvement, and HER2 positivity.

The relationships between FOXO3a nuclear staining score, FOXO3a cytoplasmic staining score and clinicopathological characteristics of the tumor are presented in **Table 3**. The Spearman's correlation analysis revealed that the nuclear staining score of FOXO3a in tumor tissues was significantly correlated with various clinicopathological parameters. FOXO3a nuclear staining scores showed significant negative correlation with grade, stage, the number of metastatic axillary lymph node, HER2 score, and Ki-67 proliferative index (r=-0.424, P=0.02; r=-0.679, P < 0.001; r=-0.717, P < 0.001; r=-0.694, P < 0.001; r=-0.526, P=0.003, respectively). Significant positive correlation was found between ER and FOXO3a nuclear staining scores (r=0.451, P=0.012). There was not found a relationship between between clinicopathological parameters and FOXO3a cytosplasmic staining scores.

Table 2. The differences between nuclear staining positive and negative cases for various clinicopathological characteristics of breast tumors

	Total n (%)	Nuclear staining negative	Nuclear staining positive	P
Total	30 (100)	6 (20)	24 (80)	
Age (years)				
≤ 50	9 (30)	3 (33)	6 (66)	0.002
> 50	21 (70)	3 (14)	18 (86)	
Histological grade				
Grade 1, 2	14 (47)	1 (7)	13 (93)	< 0.001
Grade 3	16 (53)	5 (31)	11 (69)	
Tumor size (cm)				
≤ 2.5	10 (30)	1 (10)	9 (90)	0.009
> 2.5	20 (60)	5 (25)	15 (75)	
Axillary lymph node				
0 LN	8 (27)	0 (0)	8 (100)	< 0.001
1-3 LN	10 (33)	1 (10)	9 (90)	
> 3 LN	12 (40)	5 (42)	7 (58)	
pTNM stage				
Stage 1A, 2A, 2B	16 (53)	0 (0)	16 (100)	< 0.001
Stage 3A, 3C	14 (47)	6 (43)	8 (57)	
ER status				
Negative	6 (20)	1 (17)	5 (83)	
Positive	24 (80)	5 (21)	19 (79)	0.590
PR status				
Negative	12 (40)	2 (17)	10 (83)	
Positive	18 (60)	4 (22)	14 (78)	0.476
HER2 status				
Negative	3 (10)	1 (33)	2 (67)	
Positive	27 (90)	5 (19)	22 (81)	0.03
Ki-67 index				
≤ 20%	15 (50)	1 (7)	14 (93)	
> 20%	15 (50)	5 (33)	10 (66)	< 0.001

Abbrevations: ER: Estrogen receptor, PgR: Progesteron receptor, LN: Lymph node. Data are presented as number and percentage. The comparisons of categorical variables were analyzed using two tailed Fisher's exact test or Chi-square test. A *P* value < 0.05 was considered statistically significant.

The relationships between FOXO3a staining intensity score, total score and clinicopathological characteristics of the tumor are shown in **Table 3**. The Spearman's correlation analysis revealed that the FOXO3a staining intensity scores in tumor tissues was negatively correlated with HER2 score (r=-0.531, P=0.003), but not with the other clinicopathological characteristics of the tumor. Although there was not a statistically significance, a positive correlation was observed between FOXO3a staining intensity scores and ER, PgR status (r=0.320, P=0.085; r=0.324, P=0.081). In addition, total FOXO3a staining score was negatively correlated with stage of the tumor, metastatic axillary lymph node status, and HER2 score (r=-0.638, P < 0.001; r=-0.723, P < 0.001; r=-0.642, P < 0.001, respectively). Total FOXO3a staining score was positively correlated with ER status (r=0.464, P=0.01). Although there was not a statistically significance, a positive correlation was observed between FOXO3a total staining score and PgR status and a negative correlation was observed between FOXO3a total staining score and Ki-67 proliferative index (r=0.353, P=0.055; r=-0.355, P=0.055, respectively).

Discussion

Although various predictive factors, including age, tumor size, histological type, axillary node involvement, histological grade, hormon receptor status, and HER2 amplification have been used, it is still needful clinically useful, readily available prognostic markers in the management of breast cancer. Thus, we examined immunohistochemical FOXO3a expression in 30 breast cancer specimens and compared its association with clinical significant and prognostic parameters using immunohistochemical staining method. Our results revealed that nuclear staining of FOXO3a in breast tumor tissue was a more important prognostic marker rather than cytoplasmic FOXO3a staining or the intensity of FOXO3a staining. FOXO3a nuclear staining was negatively correlated with grade, stage, the number of metastatic axillary lymph node, HER2 score, Ki-67 proliferative index and positively correlated with ER status. The percentage of negative nuclear staining was higher in low age, high tumor grade, stage, size, proliferative index, more axillary lymph node involvement, and HER2 positivity.

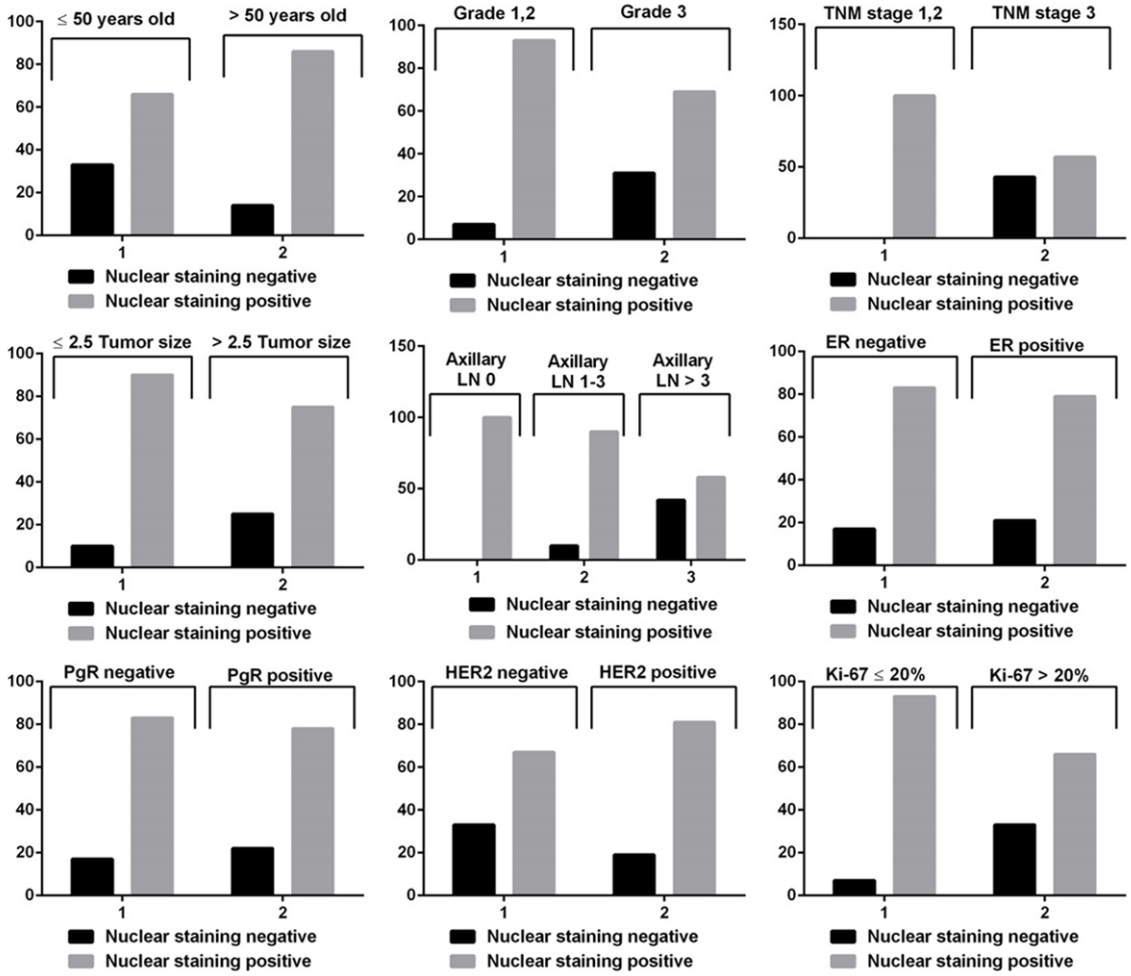

Figure 5. The differences between nuclear staining positive and negative cases for various clinicopathological characteristics of breast tumors. ER: Estrogen receptor, PgR: Progesteron receptor, LN: Lymph node. Data are presented as percentage.

Akt-dependent phosphorylation regulates subcellular localization of FOXO3a by preventing the FOXO3a translocation from cytoplasm to the nucleus. Activation of PI3k by insulin or other growth factors induces the Akt protein kinases. Phosphorylation of FOXO by Akt results in nuclear exclusion and translocation of FOXO into the cytoplasm and consecutive FOXO inactivation. When PI3k and Akt are inactive, FOXOs locate in the nucleus and cause cell cycle arrest and apoptosis. Whereas, when FOXOs are phosphorylated, they accumulate in the cytoplasm and FOXO-mediated transcription is inhibited [20, 21]. While FOXO proteins are mainly regulated through reversible shuttle in subcellular localization, the degradation of FOXOs by ubiquitin-proteosome pathway causes irreversible regulation. Cytoplasmic localization are necessary for FOXO ubiquitination and subsequent degradation. FOXO degradation enables increased cell proliferation that can lead to cell transformation and carcinogenesis involving FOXO as a player in the carcinogenesis [22]. In the absence of growth and survival factors or in the event of phosphorylation of FOXO by JNK in response to increased cellular oxidative stress, FOXOs relocate from the cytoplasm to the nucleus enabling transcription of FOXO target genes. When FOXO proteins are predominantly nuclear, FOXOs are active and FOXO-mediated transcription causes controlled cell cycle arrest and apoptosis, therefore FOXO proteins can behave as a tumor suppressor [23, 24]. The findings of this study also demonstrated an association between nuclear FOXO3a expression with better prognostic factors of breast

Table 3. The relationships between FOXO3a staining patterns and clinicopathological characteristics of the tumor

	FOXO3a nuclear staining score (n=30)		FOXO3a cytoplasmic staining score (n=30)		FOXO3a staining intensity score (n=30)		FOXO3a staining total score (n=30)	
	r	P	r	P	r	P	r	P
Tumor Grade (Nottingham histological grade)	-0.424	0.02	0.200	0.288	0.001	0.996	-0.230	0.221
Tumor Pathological Stage (pTNM)	-0.679	< 0.001	-0.093	0.624	-0.023	0.903	-0.638	< 0.001
The number of metastatic axillary lymph node	-0.717	< 0.001	-0.241	0.200	-0.103	0.589	-0.723	< 0.001
Size of tumor (cm)	-0.217	0.249	-0.018	0.929	-0.208	0.271	-0.213	0.259
ER proportion score	0.451	0.012	0.218	0.248	0.320	0.085	0.464	0.01
PgR proportion score	0.254	0.176	0.315	0.09	0.324	0.081	0.353	0.055
HER2 score	-0.694	< 0.001	0.029	0.878	-0.531	0.003	-0.642	< 0.001
Ki-67 proliferative index (%)	-0.526	0.003	0.310	0.106	-0.209	0.268	-0.355	0.055

Abbrevations: ER: Estrogen receptor, PgR: Progesteron receptor, r: Spearman's correlation coefficient. To analyse relationship between FOXO3a cytoplasmic, nuclear score and stage of the tumor, stage 1A was denoted as 1, stage 2A was denoted as 2, stage 2B was denoted as 3, stage 3A was denoted as 4, stage 3C was denoted as 5. Data were tested using the Spearman's correlation analysis. A *P* value less than 0.05 was considered statistically significant.

tumor including size, stage, grade, hormone receptor status, proliferation index, HER2 expression levels, and the number of axillary lymph node involvement.

A role of FOXO proteins in carcinogenesis was initially suggested by the studies that FOXO gene alterations were found in several human cancers such as rhabdomyosarcomas and acute myeloid leukemias [25, 26]. A role for FOXO3a in cancer prognosis has been reported for colorectal cancer [27], liver cancer [28], over cancer [29], lung cancer [30], and bladder cancer [31]. As mentioned above, several studies executed in various cancer types have demonstrated that subcellular localization of FOXO3a is an important marker for tumor prognosis. Karger et al. [32] reported that while significant cytoplasmatic accumulation of FOXO3a was observed in thyroid cancers, an exclusive nuclear accumulation was observed in normal thyroid tissue. Shukla et al. [33] reported that marked cytoplasmic accumulation of FOXO3a correlated with increased Gleason grade, in contrast to exclusive nuclear accumulation seen in benign prostate cells. Chen et al. [34] found that nuclear accumulation of FOXO3a in tumor cells was correlated with increased radiosensitivity and survival rate in patients with esophageal squamous cell carcinoma. In a study by He et al. [27], the nuclear expression of FOXO3a in colorectal carcinoma tissue was significantly lower than that of normal colorectal tissues. Fei et al. [35] found that FOXO3a immunostaining in ovarian tumor tissues was mainly located in the nucleus, although some weak or variable staining remained in the cytoplasm. In addition, the expression of FOXO3a was decreased from normal ovarian tissues to benign tumor to malignant tumor.

The role of FOXO3a in breast cancer has been examined in several studies. In a previous study by Hu et al. [36], FOXO3a was mostly located in the cytoplasm of breast tumor tissues with a high level of Akt, while FOXO3a was largely located in the nucleus of many tumors with negative Akt. FOXO3a was positive in 113 of 131 examined breast tumor specimens and cytoplasmic localization of FOXO3a was observed in 90% of these 113 tumors with positive Akt. They also found that while cytoplasmic localization of FOXO3a was significantly correlated with poor survival, nuclear localization of FOXO3a were significantly associated with an increased survival in patients with breast cancer. These findings are consistent with those of our study. In agreement with this study, Habashy et al. [37] found that FOXO3a predominant nuclear expression was positively associated with biomarkers of good prognosis including PgR and p27 expression, longer survival, and longer distant metastasis free interval. Furthermore, FOXO3a nuclear localisation was negatively associated with mitotic counts, MIB1 growth fraction, and C-MYC expression. In a recent study by Jiang et al. [38], FOXO3a protein expression correlated with ER positivity, histological grade, axillary lymph node negativity, TNM stage, and long-term survival consistent with this study. Furthermore, Smit et al. [39] reported that patients without FOXO3a expression had a higher recurrence rate, however differential FOXO3a expression between

nucleus or cytoplasm had no influence on recurrence rate. Furthermore, they also found that suppression of FOXO3a increased the number of breast cancer stem cells responsible for metastasis and recurrence of the tumor and consequently in therapy resistance in MCF7 human breast cancer cell lines. In agreement with our study, Sisci et al. [40] found that in non-invading, well-differentiated ER (+) ductal carcinomas in situ, FOXO3a was strongly expressed, showing a very high nuclear localization, however nuclear FOXO3a positivity was gradually lost in invading and less differentiated cells and cytoplasmic localization was not as indicative. In addition, FOXO3a nuclear expression was inversely correlated with tumor grade and the invasive potential, while cytosolic FOXO3a was not significantly correlated with any clinicopathological feature.

This study had several limitations. First, we could not analyse overall survival of patients, because we could not obtain retrospective follow-up data related to survival, such as distant metastasis, tumor recurrence, response to chemotherapy or radiotherapy, and survival status. Second, the number of cases examined in the study was relatively small.

Consequently, given the their ability to control cell cycle and apoptosis, FOXO3a may be a potential target for novel drug discovery supressing tumorigenesis via inducing nuclear translocation of FOXO3a and a useful prognostic marker for the breast cancer management. Further studies elucidating the molecular biology of FOXOs are needed to investigate these possibilities and to develop more effective therapeutic approaches for treatment of breast cancer.

In conclusion, the findings of this study suggested that while the nuclear FOXO3a translocation may be closely associated with the malignancy of breast tumors, the nuclear accumulation of FOXO3a is associated with a better prognosis for breast cancer patients. Loss of nuclear translocation of FOXO3a will cause more aggressive behaviour in breast cancers. To take into account of nuclear FOXO3 staining pattern rather than cytoplasmic staining or the intensity and density of total staining pattern will be a more useful approach for the breast cancer management. Therefore, we suggest that the nuclear FOXO3a may become a useful prognostic biomarker for breast cancer.

Disclosure of conflict of interest

None.

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