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Abstract

Oestrogen receptor (ER)-positive breast cancer (BC) constitutes a heterogeneous group of tumours with regard to outcome and response to therapy. Accurate stratification of ER-positive BC according to risk of relapse and response to therapy will be achieved through an improved understanding of ER and ER-related biological pathways. Recent studies have identified FOXO3a (Forkhead box O3a) transcription factor as an intracellular mediator of ERα expression and as an important downstream target of the Akt/PI3K pathway indicating a biological and potential clinical role for FOXO3a in ER-positive BC.

In this study, we investigated the clinical relevance and biological associations of FOXO3a protein expression, using tissue microarrays and immunohistochemistry, in a large series of patients with invasive breast cancer.

FOXO3a protein expression showed both nuclear and/or cytoplasmic staining patterns. FOXO3a predominant nuclear expression was positively associated with biomarkers of good prognosis including PgR, FOXA1, and p27 expression. There was an inverse association with mitotic counts, MIB1 growth fraction, C-MYC and PIK3CA expression. With respect to patient outcome, FOXO3a nuclear localisation was associated with longer BC specific survival ($p<0.001$) and longer distant metastasis free interval ($p=0.001$), independently of the well-established breast cancer prognostic factors.

In conclusion, our results demonstrate the biological and prognostic role of FOXO3a protein expression and its subcellular localization in ER-positive/luminal-like BC possibly through its involvement in controlling cell proliferation.

Introduction

Oestrogen receptor (ER) positive tumours comprise the majority of breast cancers, accounting for 60-70% of cases and are generally expected to show good response to hormonal treatment and favourable clinical outcome [1]. However, ER-positive tumours are heterogeneous with respect to their clinical behaviour and biology. By studying novel biomarkers with strong association with ER signalling it should be possible to gain a better understanding of the biology of ER-positive disease.

FOXO3a (FKHRL1) gene belongs to the forkhead family of transcription factors [2] and their activity is regulated by several post-translational modifications, including phosphorylation and acetylation [3]. Zou et al 2008 [4] have reported that FOXO3a can suppress ERα-dependent breast cancer cell proliferation and tumourigenesis in the MCF-7 breast cancer cell line, suggesting a crosstalk between FOXO3a and ER signalling pathways in ER dependent breast cancer [4]. Other studies demonstrated FOXO3a has an important intracellular mediator role in ERα expression, suggesting possible therapeutic intervention [5]. Importantly, FOXO3a is a downstream target in the Akt/PI3K pathway and when phosphorylated, is prevented from translocating to the nucleus resulting in its loss of functional activity. In contrast, FOXO3a dephosphorylation leads to nuclear localisation and subsequent target gene activation [6-10]. Therefore, as a target within the Akt/PI3K signalling pathway FOXO3a regulates the expression of proapoptotic genes, cell cycle–regulated genes, and genes that control cellular homeostasis [6, 11]. Alvarez el al [12] suggest that an efficient mitotic programme depends on downregulation of Akt/PI3K and consequent induction of FOXO3a transcriptional activity. However, there is also evidence that an Akt-independent mechanism of FOXO3a regulation exists. In vitro co-transfection of

FOXO3a and IKK resulted in strong inhibition of FOXO3a activity independent of Akt [13].

In breast cancer, FOXO3a activity has been shown to elevate p27 resulting in cell cycle arrest [14]. Furthermore, nuclear FOXO3a can induce cellular apoptosis through upregulation of Fas ligand (Fas-L) [6] and Bim [11, 15] and has been implicated in resistance to oxidative stress and longevity [16]. Other studies have highlighted the importance of FOXO3a for maintenance of the hematopoietic stem cell pool [17, 18]. It has been reported that activation of FOXO3a could induce p53-dependent apoptosis even in cells with a transcriptionally inactive p53 [19].

FOXO3a may have therapeutic implications because some clinical anticancer treatments target FOXO3a through three main oncogenic kinases (Akt, IKK, and ERK) [2, 20, 21]. For instance, nuclear localization of FOXO3a could potentially improve the effectiveness of anti-EGFR agents such as gefitinib by mediating proliferative arrest [22]. Gefitinib treatment causes cell cycle arrest and induces apoptosis due to the effects of FOXO3a dephosphorylation and nuclear translocation at Akt sites. In contrast, resistant cells show phosphorylated FOXO3a are restricted to the cytoplasm [22]. Furthermore, up-regulation of FOXO3a by paclitaxel has been reported to increase Bim mRNA and protein level with subsequent induction of apoptosis in breast cancer cells [23, 24].

The value of FOXO3a as a prognostic biomarker for ER-positive luminal-like breast cancer remains unclear. Therefore, in this study, we have investigated the clinical relevance and biological associations of FOXO3a protein expression in a large series of patients with luminal-like ER-positive invasive breast cancers using high-throughput tissue microarrays (TMAs) and immunohistochemistry.

Materials and Methods

Patient selection and tissue microarray construction

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetics classification of breast cancer".

Formalin fixed paraffin embedded (FFPE) tissue microarrays (TMAs) were prepared from a series cases of primary operable (stage I and II) breast carcinoma cases from patients age <70 presenting consecutively to the Nottingham Breast Unit with tumours of less than 5 cm diameter between 1989 and 1998 as previously reported [25]. This consecutive patient series is well characterized and contains patients' clinical and pathological data including patients' age, histologic tumour type, primary tumour size, lymph node status, mitotic count and histologic grade [26], Nottingham prognostic index (NPI) [27], vascular invasion (VI), therapy and follow-up data. In addition, data on a large panel of biomarkers with strong relevance to breast cancer were available including oestrogen receptor-α (ER), ER-related genes (progesterone receptor (PgR), androgen receptor (AR), FHIT, FOXA1, CARM1, and PELP1), proliferation, apoptosis and cell cycle-related genes (BCL2, p27, p53, C-MYC, and MIB1), and biomarkers related to PI3K pathway (PIK3CA) [25, 28, 29].

Patient management was based on the Nottingham Prognostic Index (NPI) score and ER status as previously described [28, 30], patients within the good prognostic NPI group (≤3.4) did not receive adjuvant systemic therapy. Hormonal therapy (Tamoxifen ± Zoladex if premenopausal) was given to patients with ER-positive tumours and NPI scores of >3.4. Pre-menopausal patients with moderate and poor prognostic NPI groups were given chemotherapy (Cyclophosphamide, Methotrexate, and 5-Flourouracil). ER-positive postmenopausal patients with moderate or poor NPI were offered Hormonal therapy, while ER negative patients received CMF if fit to

receive these cytotoxic agents with no concurrent disease that were considered as potential contraindication to the use of chemotherapy.

Data are maintained on prospective basis for breast cancer specific survival (BCSS), development of distant metastases (DM) and/or locoregional tumour recurrence.

BCSS was defined as the time (in months) from the date of the primary surgical treatment to the time of death from breast cancer. Distant metastasis free interval DMFI was defined as the interval (in months) from the date of the primary surgical treatment to the date of development of the first distant metastasis. FOXO3a expression was assessed in the whole patient series (n=934), and a cohort of ER-positive / luminal-like patients (n=633).

Immunohistochemistry

Rabbit polyclonal antibody to FOXO3a (Antibody 9467, Cell Signalling Technology, Danvers, MA) was optimized at a working dilution of 1:50 using FFPE TMA sections and full-face sections of breast cancer tissue to assess the staining distribution. Immunohistochemical optimization and staining of FOXO3a was performed using a DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) immunostainer with a linked streptavidin biotin (LSAB) technique in accordance with the manufacturer's instructions after microwave antigen retrieval in 0.01M citrated buffer pH6 and as previously described [25]. Negative controls were performed by omitting the primary antibody and substitution with a diluent. Sections were counterstained in haematoxylin and coverslipped using DPX mounting medium.

Western blotting was performed on breast cancer cell lysates of the human breast cancer cell line MCF-7 to confirm the specificity of the FOXO3a antibody used in immunohistochemistry. MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 medium in T75 flasks

supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ ml). The sub-confluent cells were washed with PBS, then 30µl of protease inhibitors (Sigma Aldrich) were added to 470µl of ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS). Western blotting was done on the cell lysates to confirm the specificity of the antibody used in immunohistochemistry. Lysates (20µg) were added to 4X SDS loading buffer with 5% β-mercaptoethanol (Sigma Aldrich, UK) and denatured by heating at 100ºC for 10 minutes prior to loading then added for 5 minutes into ice. Samples were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a 10% resolving polyacrylamide gel and transferred onto a Hybond-P PVDF membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 5% milk powder 0.1% TPBS (Tween20 in PBS solution) for 60 minutes, the membrane was then incubated with 1:1000 dilution of the FoxO3a rabbit polyclonal Antibody (9467) at 4°C overnight.

The membrane was washed with 0.1% PBS/Tween20 3 times for 5 minutes each then incubated for 1 h at room temperature with a horseradish peroxidase-linked secondary antibody (Sigma Aldrich) (1: 4000, anti-rabbit) diluted with 5% milk powder PBS containing 0.1% Tween20. After a further 3 washes, the membrane was visualized enhanced chemiluminescence reagents (Amersham Bioscience, Buckinghamshire, UK). The monoclonal Anti-β-actin antibody (Sigma Aldrich) in a dilution of 1:2000 against the ubiquitous β-actin protein was used.

Assessment of IHC staining results

Staining was initially evaluated on full face sections to assess the expression and the distribution of staining. Firstly, FOXO3a TMAs IHC staining results were categorised into negative and positive expression, regardless of FOXO3a localisation.

Examination of the TMAs has shown that some cases showed nuclear pattern and others were mainly cytoplasmic. Since the expression pattern and localisation of FOXO3a protein expression show variable biological functions, we categorised the positive cases according to whether they showed predominant nuclear (N) or predominant cytoplasmic (C) localisation. Both patterns scored separately using the percentage of the positive cells in each TMA core. Cases were categorised as showing a nuclear or cytoplasmic pattern in 50% or more of the informative TMA core provided that there is more than 20 % variation between both patterns. We have defined the cases with obvious overlap (less than 20 % variation, n=31) and were excluded to ensure a clear separation in their patterns of expression. The cases were scored without the knowledge of patient outcome.

The H-score (histochemical score) was used to assess the intensity of staining and the percentage of stained cells for FOXA1[28], PELP1[31], CARM1, and PIK3CA[32]. The H-score (Histochemical score) was used to assess staining intensity and percentage of stained tumour cells following immunohistochemistry. Staining intensity was scored 0, 1, 2 or 3 (negative to strong) and the percentage of positive cells at each intensity subjectively estimated to produce a final score in the range 0–300. For other biomarkers we used the percentage of the positive cells. Intensity of C-MYC staining was scored as negative, low, moderate or strong. MIB1 staining was done on full face breast cancer sections. Suppliers, dilutions, antigen retrieval methods, cutoffs, and methods of staining assessment used for the biomarkers included in this study is summarised in (Table 1).

Statistical analysis

We have reported our results according to the REMARK criteria for reporting tumour marker prognostic studies [33, 34]. Statistical analysis was performed using SPSS 16

statistical software (SPSS Inc., Chicago, IL, USA). Association between FOXO3a staining patterns and different clinicopathological parameters was evaluated using Chi-square test or Fishers exact test. Survival analysis was estimated by the Kaplan-Meier plots and Log Rank test to assess significance. Multivariate Cox proportional hazard regression models were used to evaluate any independent prognostic effect of the variables with 95% confidence interval. A *p*-value of <0.05 was considered to reflect a significance.

Results

The median age of the patients was 56 years (range 27-70). At the time of the primary diagnosis, Forty seven percent (47%) of patients had tumours less than 2 cm in size and 31.5% had grade 2 tumours. During follow-up, 30.6% of the patients developed metastatic disease.

The specificity of the FOXO3a antibody was confirmed using Western blotting (Fig 1). In normal breast tissue, FOXO3a expression was detected mainly in the nuclei of mammary epithelial cells (Fig 2a). In the malignant tissues, FOXO3a showed both nuclear and cytoplasmic staining patterns but one pattern were obviously dominant.

In whole patient, about 23 % showed predominant nuclear expression pattern and 34 % cytoplasmic pattern while 3 % showed both with less 20% difference in the predominant pattern. In ER-positive patient cohort, 26 % showed nuclear expression pattern (Fig 2b) and 31 showed % cytoplasmic pattern (Fig 2c).

Correlation of FOXO3a protein expression and other clinicopathological variables

The tumour-specific FOXO3a IHC staining characteristics were initially categorised into negative and positive expression (showing either nuclear or cytoplasmic expression), regardless of FOXO3a localisation. FOXO3a expression status did not show significant associations with the other clinicopathological variables including tumour grade, size, stage, NPI and vascular invasion ($p>0.05$).

Subsequently, the cases were categorised according to the pattern of expression into nuclear and non-nuclear, the latter including cytoplasmic predominant localisation and negative expression. Analysis of whole patient series revealed that FOXO3a nuclear localisation is positively associated with low mitotic counts, lower grade tumour, less frequent development of distant metastasis ($p<0.001$) and tumour

recurrence (p=0.001) (Table 2). Its expression was significantly associated with markers of good prognosis (Table 3).

In the luminal-like cohort (ER cutoff 10%), FOXO3a nuclear expression pattern was associated with low mitotic counts (p=0.008), and less frequent development of distant metastasis (p<0.001) and tumour recurrence (p=0.035) (Table 4). The nuclear pattern showed significant positive associations with molecular biomarkers associated with good prognosis including PgR (p=0.004), FHIT (p=0.008), FOXA1 (p<0.001) and p27 (p=0.004) expression. It also showed an inverse correlation with the expression of cell proliferation-related markers, MIB1 expression (p=0.039) and with PIK3CA (p=0.006) (Table 5).

Patients' outcome

In whole patient series, initial univariate analysis of FOXO3a expression status (as positive versus negative) was not associated with BCSS (Log Rank (LR) =0.005, p=0.942) nor DMFI (LR =0.015, p=0.904) but when the localisation of expression was considered FOXO3a nuclear expression was associated with better outcome in terms of longer BCSS (LR=24.079, p<0.001) and longer DMFI (LR =15.996, p<0.001).

In the luminal-like ER-positive cohort of patient (n=633), (median follow up time =126 months), univariate analysis of survival showed no associations between FOXO3a expression status (as positive versus negative) and patient outcome in terms of BCSS (LR =0.234, p=0.628) (Fig 3a) or DMFI (LR=0.198, p=0.656). However, FOXO3a nuclear localisation showed a significant association with both longer BCSS (LR =15.813, p<0.001) (Fig 3b) and longer DMFI (LR=11.836, p=0.001) (Fig 3c).

When we analysed patient survival using categorisation of the cohort into three groups: predominant nuclear, predominant cytoplasmic and negative, our results

showed that subcellular localisation differences of FOXO3a are associated with striking survival differences. Specifically there is a contrast between nuclear and cytoplasmic expression localisation where nuclear pattern showed the most favourable BCSS (LR =18.279, p<0.001) (Fig 3e) and longer DMFI (LR=14.775, p=0.001) (Fig 3f) in ER+ luminal-like cancer.

According to systemic therapy groups

When systemic therapy was considered, similar associations of longer survival were found in the subgroup of ER-positive patients who did not receive adjuvant systemic therapy (n=222) with regards DMFI (LR=10.110, p= 0.001) (Fig 3d) and in the subgroup of patients who received tamoxifen monotherapy (n=221) with regards BCSS (Log Rank (LR) =5.201, p=0.023).

Multivariate analyses

Since many potential prognostic factors may interact with specific therapies and therefore are compounded by the effect of adjuvant hormone therapy and chemotherapy, we have included the systemic therapy groups (given versus not given) in the multivariate analysis together with the other well established prognostic variables such as MIB1, PgR, tumour size, stage, grade to assess the prognostic independence of nuclear FOXO3a expression in the ER+ patient cohort.

FOXO3a nuclear expression was an independent prognostic factor for predicting better outcome in terms of longer BCSS (Hazard ratio (HR)=0.392, p=0.006, 95%CI=0.202-0.760)(Table 6) and longer DMFI (HR=0.530, p=0.020, 95%CI=0.310-0.906) (Table 7) in ER-positive / luminal-like breast cancer.

Discussion

Oestrogen receptor (ER) plays an important role in breast cancer progression and response to therapy and is typically expressed in the most frequent biological class of breast cancers. However ER-positive breast cancer does not appear to be a homogenous group; some tumours respond to therapy and others do not. There is therefore a requirement for improved understanding of the biology of ER-positive breast cancer which could be achieved through studying ER-related pathways and their downstream targets. One of the important pathways that has been implicated in the pathogenesis of the poor prognosis ER-positive (luminal B) subtype is the Akt/PI3K pathway and its related genes [35]. FOXO3a is a key downstream target in this pathway which prompted us to study its expression and associations in breast cancer particularly in ER-positive / luminal-like subtype.

The Akt/PI3K pathway regulates the sub-cellular localization of FOXO3a by phosphorylation which prevents the protein from translocating to the nucleus to regulate transcription [6]. This indicates that absence of nuclear FOXO3a expression in a tumour, with either complete absence or cytoplasmic localisation, should indicate phosphorylation by Akt. This may represent an important biological mechanism responsible in part for poor prognosis in ER-positive breast cancer, thus removing a constraint to cellular proliferation and potentially to tumourigenesis through an active Akt/PI3K pathway. This proposal is supported by our finding that absence of nuclear expression of FOXO3a is associated with poorer outcome and shows a significant association with PIK3CA, a marker strongly related to Akt function. Other breast cancer studies have also shown an association between Akt/PI3K activation and

cytoplasmic FOXO3a expression pattern with decreased patient survival, in agreement with our findings [13].

In this study we did not find a significant association with survival when patients were categorised into either negative or positive FOXO3a expressers *per se*. Instead, we found that subcellular localisation indicates functional relevance as evidenced here by more favourable outcome in patients with predominant nuclear expression. Supporting these findings, previous studies have shown that nuclear FOXO3a induces the expression of genes that inhibit cell cycle progression such as the CDK inhibitors [4, 6]. Subsequently, we found a significant positive association between nuclear FOXO3a and the expression of the cell cycle inhibitor p27 implying a role in the induction of cell cycle arrest.

In this patient series including ER-positive / luminal-like subtype, nuclear localisation of FOXO3a was associated with markers of good prognosis such as FHIT, PgR[36], and FOXA1 expression which is required for the expression of 50% of ER-regulated genes [37]. Furthermore, we have also shown that nuclear FOXO3a expression is significantly associated with longer BCSS and DMFI which implies its role in stratification of ER-positive groups into prognostic subgroups, possibly explained by a tumour suppressor function associated with cell cycle arrest.

Previous studies have shown that loss of FOXO3a function indicated by its absence or by cytoplasmic localisation is positively associated with markers of increased proliferation [14]. We have found that BC, especially luminal-like cases, expressing nuclear FOXO3a are characterised by low proliferation, specifically low mitotic frequency and low MIB1 expression. In addition, we found an inverse association between FOXO3a nuclear expression and C-MYC protein supporting the proposal that the Akt/PI3K/FOXO pathway modulates MYC function by inhibition of MYC-

dependent transcription resulting in decreased cellular proliferation [38]. Taken together, our findings support the interaction of FOXO3a as a downstream target of Akt/PI3K pathway with markers related to proliferation and cell cycle, a role which is independent of the systemic therapy as shown here by our multivariate analysis results.

In conclusion, our results demonstrate the biological and prognostic role of FOXO3a protein expression, as a downstream target of AKT pathway, and its subcellular localization in BC. Loss of nuclear translocation of FOXO3a could tilt the balance in favour of increased proliferation and more aggressive behaviour in luminal-like breast cancers.

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Table 1: Suppliers, dilutions, antigen retrieval methods, cutoffs, and methods of staining assessment used for the biomarkers included in this study

Antibody (clone)	Dilution	Source	Pretreatment	Cut-off
Hormone receptors and ER-related markers				
ER (clone 1D5)	1:80	DakoCytomation	Microwave	10%
PR (clone PgR 636)	1:100	DakoCytomation	Microwave	10%
AR (clone F39.4.1)	1:30	Biogenex	Microwave	10%
FOXA1(clone2F83)	1:2000	Abcam, UK	Microwave	10*
CARM1	1:300	Novus Biological	Microwave	30,150*
PELP1	1:100	Novus Biological	No	5,170*
Tumour suppressor genes				
p53 (clone DO7)	1:50	Novocastra	Microwave	10%
FHIT (cloneZR44)	1:600	Zymed Laboratories		5%
Cell cycle associated, proliferation and Akt/PIK3 pathway associated markers				
p27	1:40	Dako, Denmark	Microwave	50%
Ki67 (MIB1)	1:100	Dako, UK		10%
PIK3CA	1:50	Sigma Aldrich		100*
C-MYC(clone 9E100)	1:100	Abcam, UK		^
BCL2 (clone 124)	1:100	Dako, UK		30%

^=Scored as (0, absent; 1, weak; 2, moderate; 3, strong expression)
*=H score

Table 2: Associations between FOXO3a immunostaining patterns and various clinicopathological parameters in the whole series

Variable	Non-Nuclear localisation	Predominant Nuclear localisation	Total	χ^2	*p*-value
Age					
<40	59(85.5)	10(14.5)	69	5.276	0.153
40-50	215(78.2)	60(21.8)	275		
51-60	235(79.2)	61(20.6)	295		
>60	194(73.8)	69(26.6)	263		
Tumour size					
≤2 cm	335(78.1)	94(21.9)	429	0.032	0.873
>2 cm	367(77.6)	106(22.4)	473		
Lymph node stage					
1(Negative)	413(76.6)	126(23.4)	539	1.169	0.557
2(1-3 LN)	219(79.1)	58(20.9)	277		
3(>3 LN)	69(81)	16(19)	84		
Tumour grade					
1	97(71.3)	39(28.7)	136	9.115	0.010
2	214(74.6)	73(25.4)	287		
3	391(81.6)	88(18.4)	479		
Vascular invasion					
No/Probable	457(77.9)	130(22.10	587	0.003	1.000
Definite	244(77.7)	70(22.3)	314		
NPI					
Good	164(73.5)	59(26.5)	223	4.364	0.113
Moderate	408(78.3)	113(21.7)	521		
Poor	131(82.4)	28(17.6)	159		
Mitotic counts					
1	204(73.6)	73(26.4)	277	20.308	<0.001
2	109(67.7)	52(32.2)	161		
3	365(83.5)	72(16.5)	437		
DM					
No	451(73.5)	163(26.5)	614	21.375	<0.001
Positive	242(87.4)	35(12.6)	277		
Recurrence					
No	374(73.9)	132(26.1)	506	10.000	0.002
Positive	314(82.8)	65(17.2)	379		

Table 3: Associations between FOXO3a immunostaining patterns and other biomarkers in the whole series

Variable	Non-Nuclear localisation N (%)	Predominant Nuclear localisation N (%)	Total	χ^2	*p*-value
ER				6.356	0.013
Negative	202(83.8)	39(16.20	241		
Positive	466(75.9)	148(24.1)	614		
PgR				17.014	<0.001
Negative	319(84.4)	59(15.6)	378		
Positive	344(72.6)	130(27.4)	474		
AR				4.364	0.035
Negative	257(82.5)	55(17.5)	312		
Positive	378(75.9)	120(24.1)	498		
FOXA1				15.145	<0.001
Negative	319(85.1)	56(14.9)	375		
Positive	234(73.1)	86(26.9)	320		
CARM1				1.806	0.405
Negative/low	159(80.7)	38(19.30)	197		
Moderate	281(81.2)	65(18.8)	346		
Strong	97(75.8)	31(24.20	128		
PELP1				2.612	0.271
Negative/low	76(74.5)	26(25.5)	102		
Moderated	412(80.7)	99(19.3)	511		
Strong	69(75.8)	22(24.2)	91		
p53				0.080	0.856
Negative	474(77.8)	135(22.2)	609		
Positive	196(78.8)	53(21.3)	249		
MIB1				5.180	0.028
Low	126(71.2)	51(28.8)	177		
High	407(79.5)	105(20.5)	512		
PIK3CA				11.224	0.001
Negative	175(70.9)	72(29.1)	247		
Positive	444(81.5)	101(18.5)	545		
p27				11.229	0.001
Negative	318(83.9)	61(16.1)	379		
Positive	272(73.9)	96(26.1)	368		
C-MYC				8.113	0.044
Negative	87(88.8)	11(11.2)	98		
Low	203(80.6)	49(19.4)	252		
Moderate	203(75.5)	66(24.5)	269		
Strong	113(80.10	28(19.9)	141		
FHIT				11.092	0.001
Negative	118(88.7)	15(11.3)	133		
Positive	475(75.5)	154(24.5)	629		
BCL2				1.896	0.193
Negative	236(80.8)	56(19.2)	292		
Positive	312(76.5)	96(23.5)	408		

Table 4: Associations between FOXO3a immunostaining patterns and various clinicopathological parameters in the luminal-like cohort

Variable	Non-Nuclear localisation N (%)	Predominant Nuclear localisation N (%)	Total	χ^2	*p*-value
Age					
<40	24(72.7)	9(27.3)	33	1.535	0.674
40-50	135(73.8)	48(26.2)	183		
51-60	159(78.7)	43(21.3)	202		
>60	147(75.4)	48(24.6)	195		
Tumour size					
≤2 cm	231(75.2)	76(24.8)	307	0.126	0.777
>2 cm	234(76.5)	72(23.5)	306		
Lymph node stage					
1(Negative)	269(73.9)	95(26.1)	364	2.135	0.344
2(1-3 LN)	153(77.7)	44(22.3)	197		
3(>3 LN)	41(82)	9(18)	50		
Tumour grade					
1	88(71.5)	35(28.5)	123	4.905	0.086
2	185(73.5)	67(26.5)	253		
3	191(80.6)	46(19.34)	237		
Vascular invasion					
No/Probable	302(76.1)	95(23.9)	397	0.040	0.844
Definite	162(75.3)	53(24.70	215		
NPI					
Good	142(72.4)	54(27.6)	196	5.078	0.079
Moderate	247(75.5)	80(24.5)	327		
Poor	77(84.6)	14(15.40	91		
Mitotic counts					
1	184(73.3)	67(26.7)	251	9.643	0.008
2	90(68.2)	42(31.8)	132		
3	172(82.3)	37(17.7)	209		
DM					
No	306(71.5)	122(28.5)	428	13.825	<0.001
Positive	155(85.6)	26(14.4)	181		
Recurrence					
No	251(72.3)	96(27.7)	347	4.627	0.035
Positive	207(79.9)	52(20.1)	259		

Table 5: Associations between FOXO3a immunostaining patterns and other biomarkers in the luminal-like cohort

Variable	**Non-Nuclear localisation N (%)**	**Predominant Nuclear localisation N (%)**	**Total**	χ^2	***p*-value**
PgR					
Negative	123(84.2)	23(15.8)	146	7.968	0.004
Positive	333(72.7)	125(27.3)	458		
AR					
Negative	110(79.1)	29(20.9)	140	0.860	0.423
Positive	326(75.3)	60(24.7)	433		
FOXA1					
Negative	183(84.7)	33(15.3)	216	14.177	<0.001
Positive	180(70)	77(30)	257		
CARM1					
Negative/low	121(78.1)	34(21.9)	155	2.128	0.345
Moderate	191(79.6)	49(20.4)	240		
Strong	44(71)	18(29)	62		
PELP1					
Negative/low	57(74)	20(26)	77	2.136	0.344
Moderated	278(79)	74(21)	352		
Strong	40(71.4)	16(28.6)	56		
p53					
Negative	374(76.8)	113(23.2)	487	0.0366	0.545
Positive	86(74.1)	30(25.9)	116		
MIB1					
Low	102(68.9)	46(31.1)	148	4.580	0.039
High	253(78.1)	71(21.9)	324		
PIK3CA					
Negative	136(69.4)	60(30.6)	196	8.120	0.006
Positive	276(80.2)	68(19.8)	344		
p27					
Negative	162(83.9)	31(16.1)	193	8.438	0.004
Positive	221(72.7)	83(27.3)	304		
C-MYC					
Negative	56(88.9)	7(11.1)	63	6.756	0.080
Low	137(78.3)	38(21.7)	175		
Moderate	138(73.4)	50(26.60	188		
Strong	69(79.3)	18(20.7)	87		
FHIT					
Negative	64(87.7)	9(12.3)	73	6.573	0.008
Positive	353(73.8)	125(26.2)	478		
BCL2					
Negative	92(78.6)	25(21.40	117	0.351	0.616
Positive	275(76)	87(24)	362		

Table 6: Cox model of predictors of BCSS in the luminal-like breast cancer

Variable	P value	HR	95% CI	
			Lower	Upper
FOXO3a nuclear localisation	0.006	0.392	0.202	0.760
PgR expression	0.049	0.642	0.413	0.997
MIB1 expression	0.011	2.105	1.184	3.742
Tumour size	0.001	2.228	1.411	3.520
LN stage	0.000	1.746	1.290	2.363
Tumour grade	0.006	1.629	1.152	2.302
Endocrine therapy	0.502	0.846	0.519	1.379
Chemotherapy	0.305	0.715	0.377	1.358

Table 7: Cox model of predictors of DM in the luminal-like breast cancer

Variable	P value	HR	95% CI	
			Lower	Upper
FOXO3a nuclear localisation	0.020	0.530	0.310	0.906
PgR expression	0.035	0.641	0.424	0.970
MIB1 expression	0.008	1.989	1.194	3.311
Tumour size	0.001	2.094	1.380	3.177
LN stage	<0.001	1.836	1.380	2.442
Tumour grade	0.048	1.375	1.003	1.887
Endocrine therapy	0.889	0.967	0.604	1.548
Chemotherapy	0.938	0.977	0.536	1.779

Table legends

Table 1: Suppliers, dilutions, antigen retrieval methods, cutoffs, and methods of staining assessment used for the biomarkers included in this study

Table 2: Associations between FOXO3a immunostaining patterns and various clinicopathological parameters in the whole series

Table 3: Associations between FOXO3a immunostaining patterns and other biomarkers in the whole series

Table 4: Associations between FOXO3a immunostaining patterns and various clinicopathological parameters in the luminal-like cohort

Table 5: Associations between FOXO3a immunostaining patterns and other biomarkers in the luminal-like cohort

Table 6: Cox model of predictors of BCSS in the luminal-like patient cohort

Table 7: Cox model of predictors of DM in the luminal-like patient cohort

Figures legends

Figure 1: Western blotting analysis of MCF-7 cell lysates using the FOXO3a rabbit polyclonal antibody. Enhanced chemiluminescence was used to visualize the membrane. The expected band size ranges from 82 -97 KD. Lane (A) is FOXO3a and lane (B) is β-actin.

Figure 2a: FOXO3a expression in normal tissue (mainly nuclear).

Figure 2b: Predominant FOXO3a nuclear expression in low grade ductal cancer.

Figure 2c: Predominant FOXO3a cytoplasmic expression in high grade ductal cancer.

Figure 3a: Kaplan Meier plot of FOXO3a expression status regardless of its subcellular localisation shows no significant difference in BCSS.

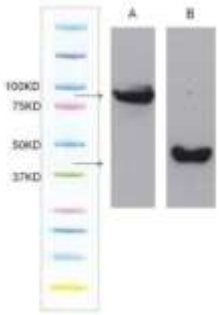
Figure 3b: Kaplan Meier plot of FOXO3a nuclear verse non-nuclear protein expression with respect to BCSS. Nuclear expression was significantly associated with improved survival.

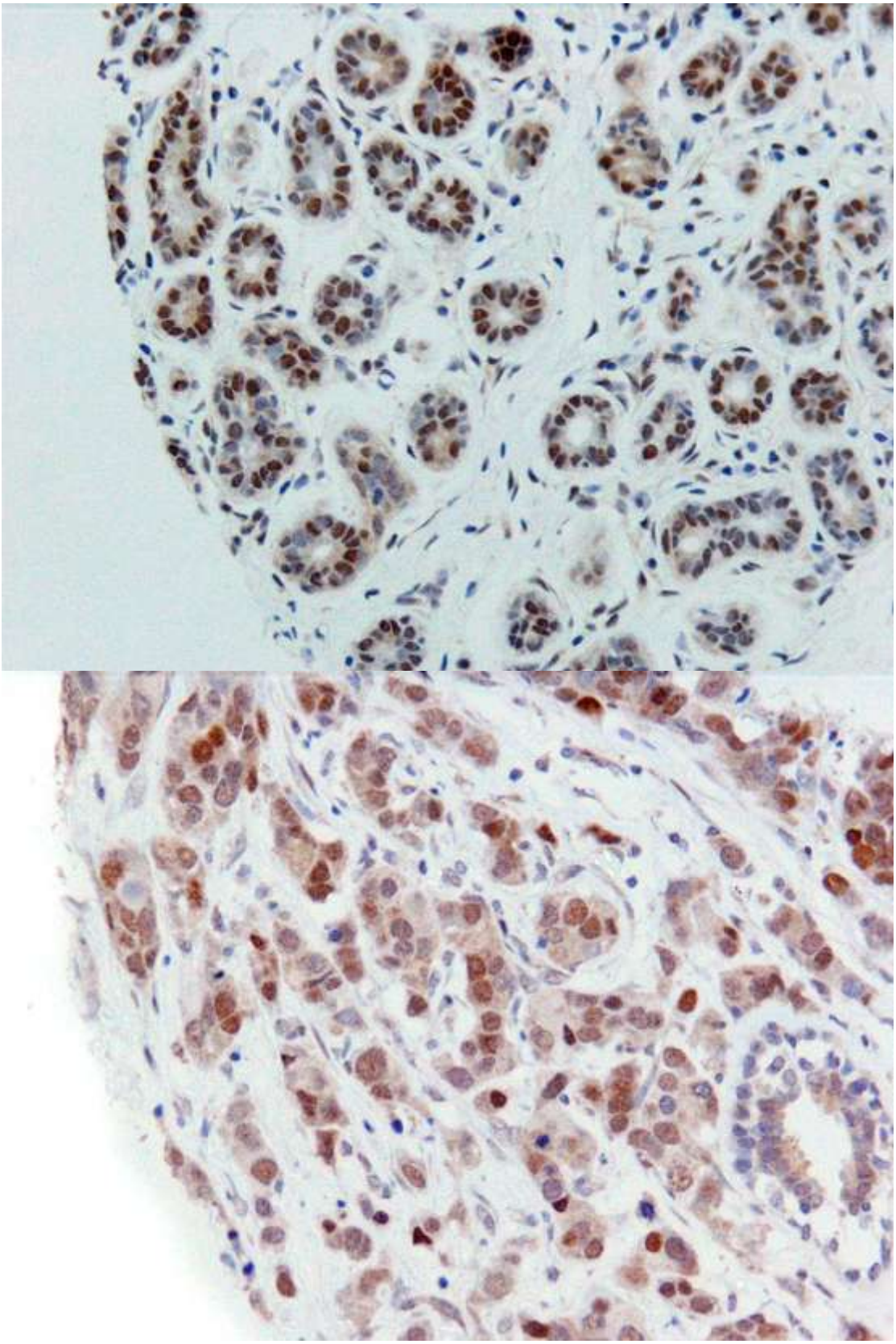
Figure 3c: Kaplan Meier plot of FOXO3a nuclear expression with respect of DMFI shows a significant longer DMFI in patients with nuclear FOXOa3 expression

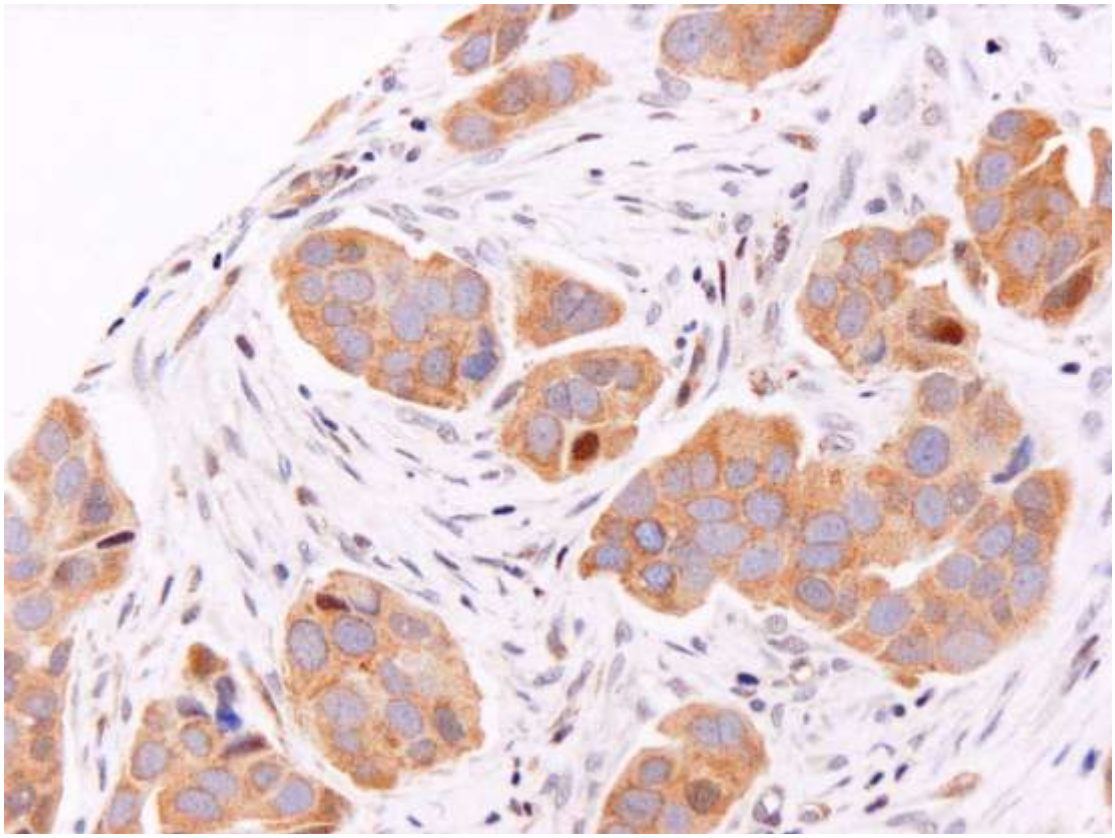
Figure 3d: Kaplan Meier plot of FOXO3a nuclear expression in non-treated cohort with respect of BCSS. Nuclear expression was significantly associated with improved survival in untreated patients.

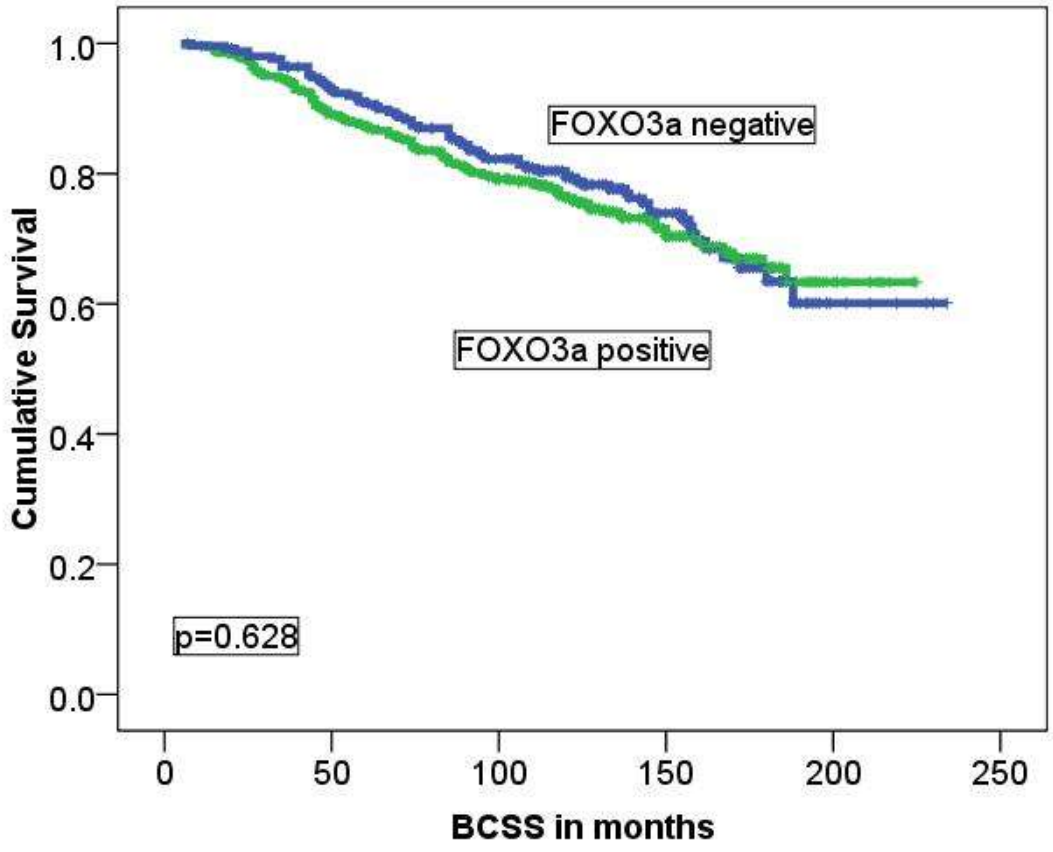
Figure 3e: Kaplan Meier plot of FOXO3a expression patterns with respect of BCSS. Nuclear expression was significantly associated with improved survival in comparison to cytoplasmic expression.

Figure 3f: Kaplan Meier plot of FOXO3a expression patterns with respect of DMFI. Nuclear expression was significantly associated with improved DMFI in comparison to cytoplasmic pattern.





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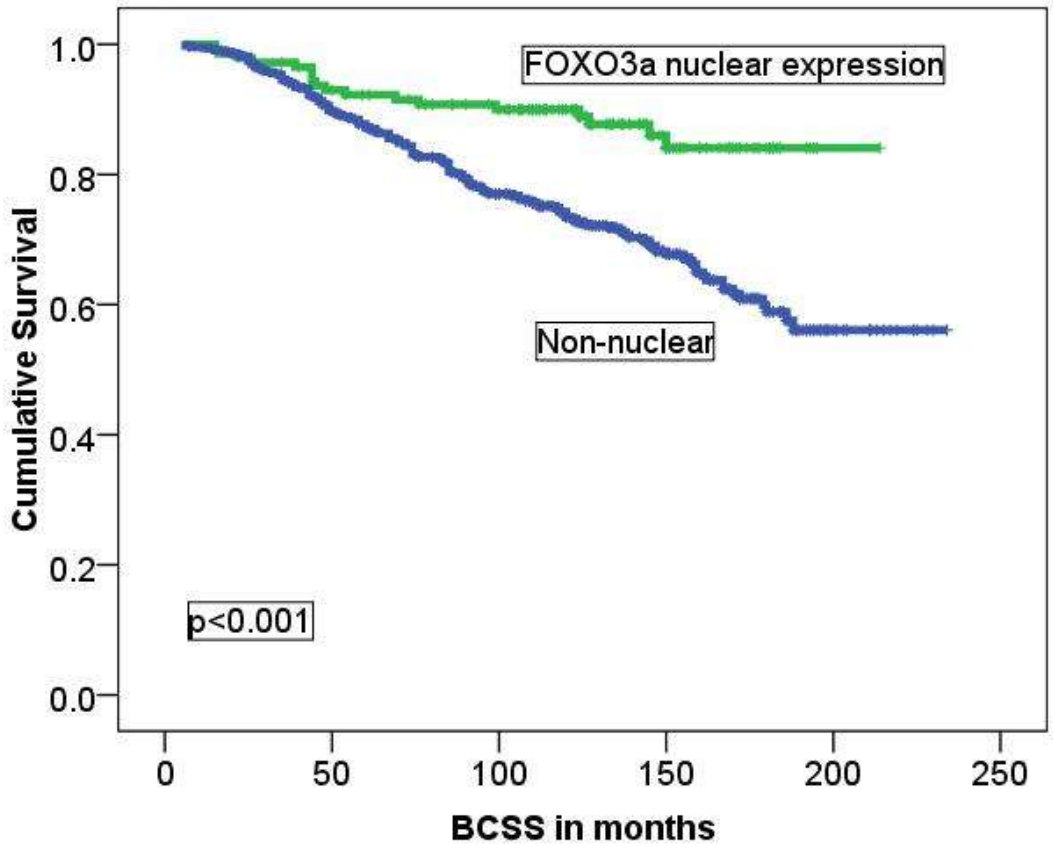
100
150
200
250
BCSS in months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0.4
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FOXO3a nuclear expression
Non-nuclear
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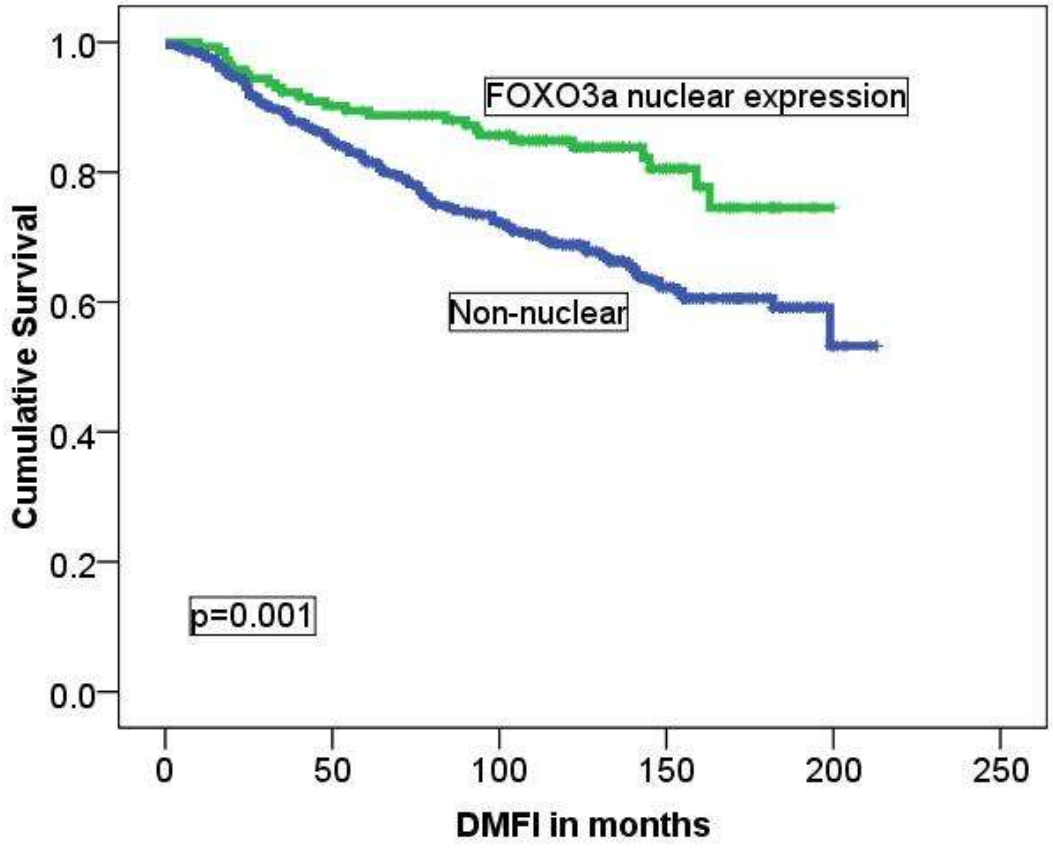
100
150
200
250
BCSS in months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0.6
0.4
0.2
0.0
Cumulative Survival
FOXO3a nuclear expression
Non-nuclear
p=0.001
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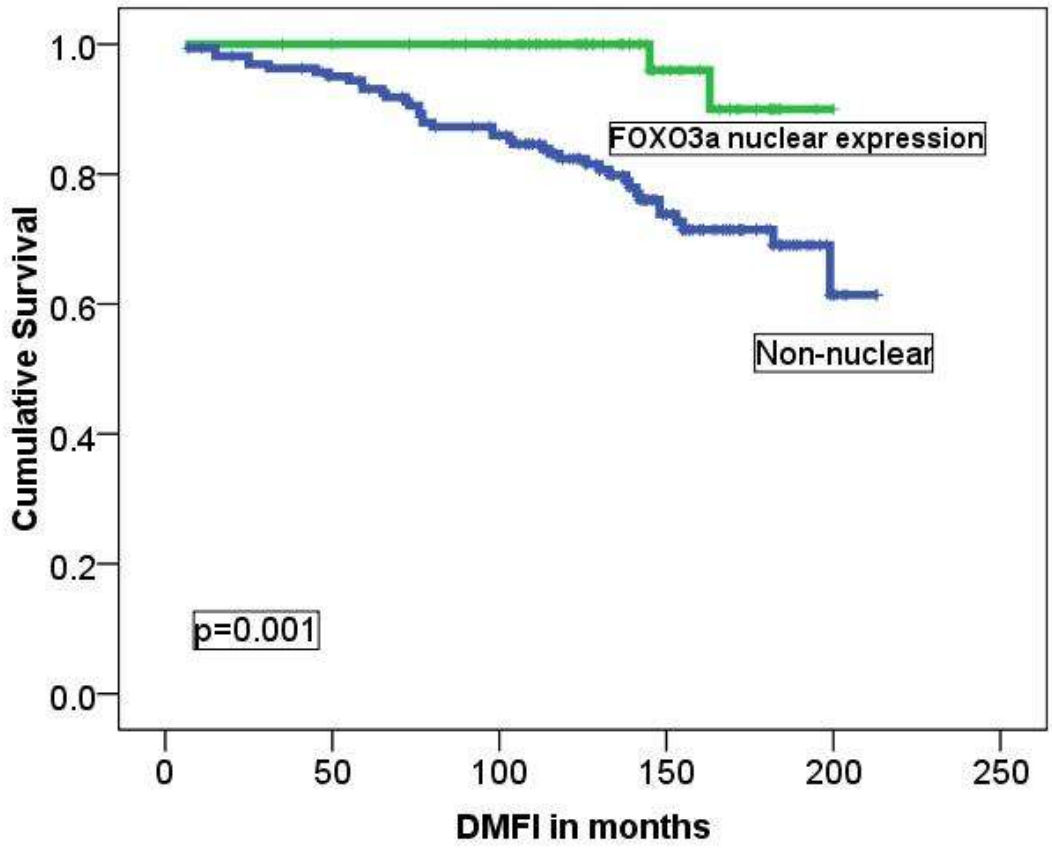
50
100
150
200
250
DMFI in months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FOXO3a nuclear expression
Non-nuclear
p=0.001
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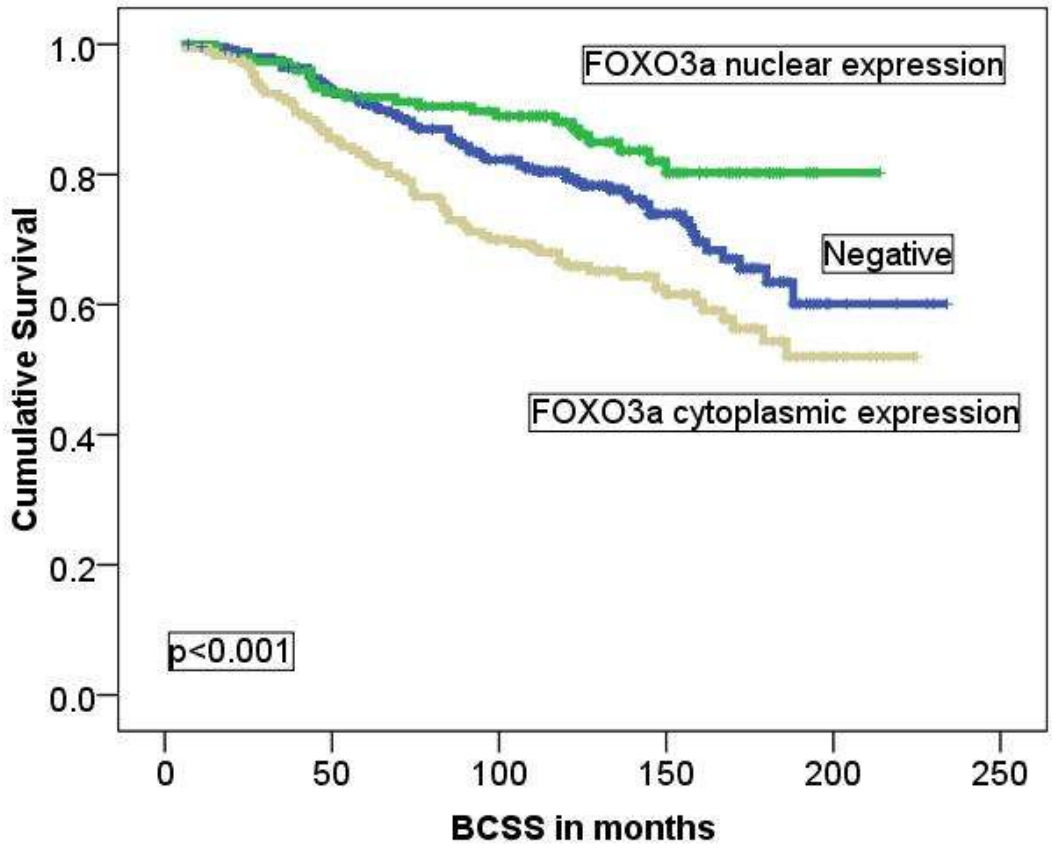
200
250
DMFI in months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0.4
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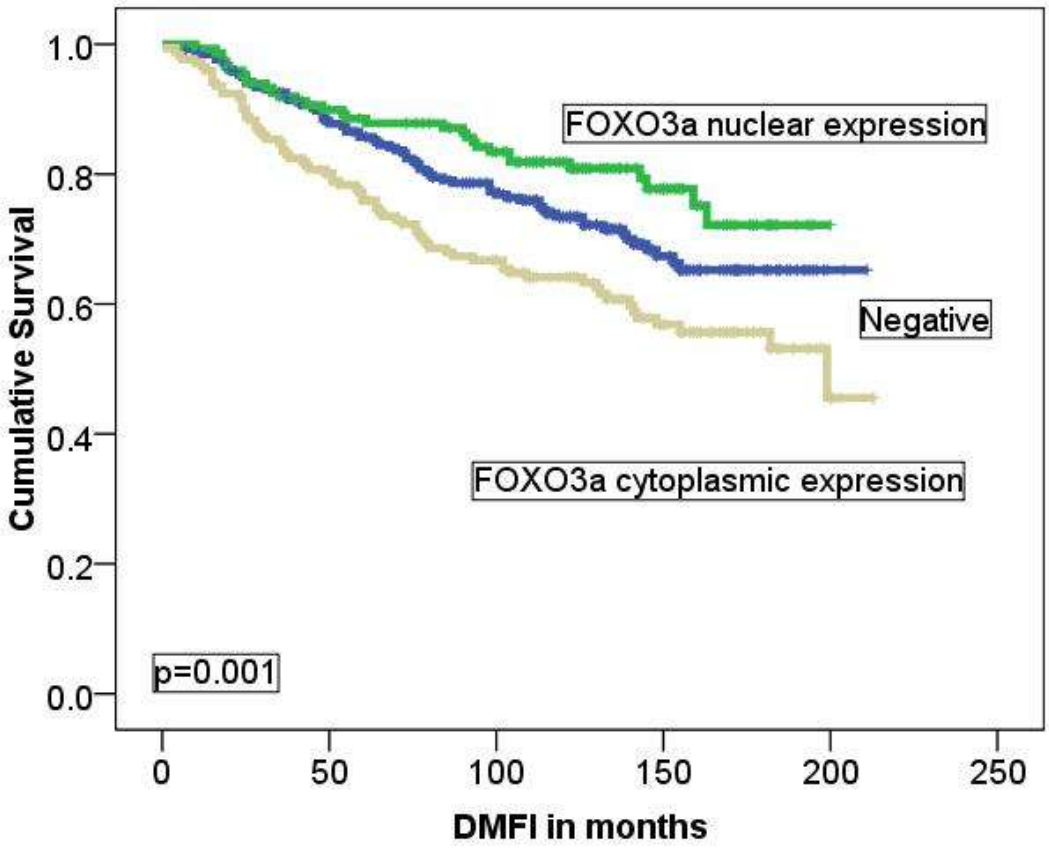
0.0
Cumulative Survival
FOXO3a nuclear expression
Negative
FOXO3a cytoplasmic expression
p<0.001
0
50
100
150
200
250
BCSS in months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0.4
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Cumulative Survival
FOXO3a nuclear expression
Negative
FOXO3a cytoplasmic expression
p=0.001
0
50
100
150
200
250
DMFI in months