

Correlation of securin and Ki67 in invasive breast carcinoma

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Summary. Aims. To identify the role of securin (PTTG) as a prognostic marker in invasive breast carcinoma and its possible relation to ki67 and to evaluate the use of ImmunoRatio® as a tool for calculating ki67 and securin labelling indices. Methods. Securin and ki67 immunohistochemical staining were performed on tissue microarray sections representative of 118 patients diagnosed with invasive breast carcinoma from 2005 to 2011. Assessment of immunohistochemical staining was carried out using both visual counting and ImmunoRatio®. The 118 cases were categorized into 2 groups according to their clinical outcome; the first group (G1) (n=77) comprised patients who were disease-free while the second group (G2) (n=41) included patients who developed either recurrence and/or metastasis at the end of 24 months follow-up duration. Results. Both securin and ki67 labelling indices (LIs) obtained by visual counting were significantly higher in G2, while only securin LIs acquired by ImmunoRatio® were significantly higher in G2. Securin assessment by visual counting was the most accurate (AUC=0.775) in identifying patients who will likely suffer from recurrence and/or distant metastasis. Pearson correlation showed $r=0.638$, $p<0.001$ for Ki67 and $r=0.671$, $p<0.001$ for securin. Linear regression analysis showed a significant correlation between ki67 and securin, $B=1.75$, $p<0.001$. Conclusion. The present results suggest that securin may add to the prognostic value of

ki67 in highlighting intra-tumoural heterogeneity in invasive breast carcinoma patients with poor clinical outcome. In addition, the study showed that since securin has a visual counting cutoff with more than 1%, making it easier to use as a breast cancer biomarker in conjunction with ki67 to predict the outcome of the cases more accurately than using only ki67. However, a multivariate analysis on a larger cohort of patients is mandatory to test its potential prognostic value.

Key words: Breast carcinoma, Ki67, Image analysis, Immunohistochemistry, Securin

Introduction

Apart from skin malignancies, breast cancer ranks as the second most common malignancy in females worldwide (Ferlay et al., 2013). In Egypt, a registry-based cancer study starting from 2008 ending on 2011 has revealed that breast cancer is the most common type of cancer in females accounting for 33.6% of cases (Ibrahim et al., 2014; Ferlay et al., 2015). The increasing trends of breast cancer have led to a plethora of studies in an attempt to identify the biological markers that may identify patients who may develop local recurrence or metastasis. Among those are proliferation markers which encompass ki67 and Pituitary Derived Tumour-Transforming Growth Protein (PTTG) (Talvinen et al., 2009).

Pituitary Derived Tumour-Transforming Growth Protein (PTTG) family is composed of three members; PTTG-1, PTTG-2 and PTTG-3 (Prezant et al., 1999).

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PTTG-1 is the most widely studied type and referred to as PTTG. It is a proto-oncogene which was first discovered in rat pituitary tumour cells (Pei and Melmed, 1997). Later, its human homologue was detected in human lymphocytes (Dominguez et al., 1998). PTTG has nuclear and cytoplasmic forms. The nuclear form, known as securin, has been shown to regulate chromatid separation during the metaphase-anaphase interface of the cell cycle via inhibition of separase, hence maintaining chromosomal stability (Huang et al., 2005).

Abnormality of securin activity leads to aneuploidy and chromosomal instability (Jallepalli et al., 2001). Securin is implicated in tumorigenesis through a wide range of mechanisms. It mediates epithelial-mesenchymal transition (Shah et al., 2009; Yoon et al., 2012), angiogenesis (Malik and Kakar, 2006) and migration of tumour cells likely via increasing matrix metalloproteinase-2 and inducing alterations in cytoskeleton (Malik and Kakar, 2006; Liao et al., 2012). Thus, elevated expression was detected in both endocrine tumours and non-endocrine tumours (Salehi et al., 2008).

Securin was first detected with a suggested prognostic role in pituitary adenomas (McCabe and Gittoes, 1999; Heaney et al., 2002; McCabe et al., 2002; Filippella et al., 2006; Minematsu et al., 2006). Its overexpression was associated with aggressive tumour behaviour and invasiveness and a higher incidence of recurrence (Filippella et al., 2006). In thyroid cancer, it regulated multiple downstream genes including vascular endothelial growth factor which promote tumour progression and metastasis (Kim et al., 2006). In non-endocrine tumours, astrocytomas showed higher levels of securin compared to normal brain tissue with the highest levels of expression in glioblastoma (Tfelt-Hansen et al., 2004; Genkai et al., 2006). In colorectal cancer, it was suggested as an independent prognostic marker (Heaney et al., 2000; Zhou et al., 2014). Securin overexpression has been reported in other tumours including oesophageal, gastric, ovarian and prostatic carcinoma (Zhou et al., 2005; El-Naggar et al., 2007; Xu et al., 2016). Research on breast carcinoma yielded similar results (Ogbagabriel et al., 2005; Grizzi et al., 2013). Several studies investigated its potential prognostic value in breast cancer (Talvinen et al., 2008; Talvinen, 2010; Gurvits et al., 2016). In this study, securin expression was evaluated using immunohistochemistry (IHC) in order to identify patients who will suffer from poor outcome and compare it to ki67 expression.

Material and methods

All mastectomy specimens submitted to the Pathology Department in Alexandria University Main Hospital were routinely sectioned and fixed in 10% neutral buffered formalin for 36-48 hours prior to tissue processing (Werner et al., 2000). The material of the

present work encompasses 118 formalin fixed paraffin embedded (FFPE) primary tumour blocks of invasive breast carcinoma. These cases were selected out of 753 cases diagnosed as invasive breast carcinoma surgically managed by modified radical mastectomy in Alexandria University Main Hospital starting from 2005 to the end of 2011. Six hundred and thirty-five cases were excluded either due to lack of sufficient clinical data or inadequate tissue material. All the cases received adjuvant therapy in the form of chemotherapy or chemo-endocrine therapy according to the hormonal receptor status. Follow-up data for the cases were retrieved from the patients' files in the Clinical Oncology and Nuclear Medicine Department.

Histopathological examination

The FFPE breast primary tumours of the 118 cases were cut into 5-µm thick sections and stained by Haematoxylin and Eosin (H&E), followed by microscopic examination to assess the histologic type according to the 2012 WHO classification of breast tumours (Lakhani et al., 2012), the histologic grade according to Nottingham grading system (Elston and Ellis, 1991), the lymphovascular invasion (Rosen, 1983) and the in situ component. Nodal status and extranodal extension were evaluated as well.

Tissue microarray

Two representative areas, one from the tumour center and the other from the periphery (hot spot), were selected during histopathologic examination of the tissue sections of each tumour. Two tissue cores (0.6 mm in diameter each) were punched out. These cores were transferred to six recipient tissue microarray (TMA) paraffin blocks as designated by Kononen (Kononen et al., 1998). Positive control for securin in the form of tonsillar tissue was used as shown in the Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000164611-PTTG1/tissue/tonsil>). The positive control was included in two blocks. All the slides were stained in a single run.

Immunohistochemical methods

HER-2 immunostaining was initially performed on whole tissue sections of the 118 cases according to the manufacturer datasheet and the antibody was applied using the following concentration; 1: 300 (HER-2, e2-4001+3B5. Thermo, Fermont, USA). Manual immunostaining for HER-2, ki67 and securin was performed on 5-µm thick sections of the TMA block according to the manufacturer datasheet for each antibody. Antigen retrieval in microwave oven for HER-2 and ki67 was carried out using sodium citrate buffer for 15 minutes and for securin using EDTA for 10 minutes. Antibodies were applied at the following concentrations; 1:300 (HER-2, e2-4001+3B5. Thermo, Fermont, USA), 1:300 (Ki67, Rabbit monoclonal RM-

9106-S, Thermo, Ferment, USA) and 1:50 (Securin, clone DCS-280, ab3305, Abcam, Cambridge, UK). Detection was performed using UltraVision Detection System (Antipolyvalent. HRP, ready-To-Use) with diaminobenzidine (DAB) as chromogen.

Automated immunohistochemical staining for CK 5/6 (mouse monoclonal, clone D5/16, ready-to-use) was performed on the TMA sections using Dako autostainer link 48 (Denmark). Visualization system used was EnVision, FLEX, high. The staining steps and incubation times are pre-programmed into the Autostainer Link software. The application volume was 2x200 μ L per slide.

Immunohistochemical staining assessment

Immunostaining assessment was performed by a consultant pathologist who had no prior knowledge of the clinical outcome of the cases to avoid assessment bias. Tissue microarray sections were evaluated using x100 magnification to check core orientation. Malignant cells per core were counted and cores with malignant cells less than 100 cells were considered non-assessable. Assessment of ki67 and securin immunohistochemical staining was performed by both visual counting and image analysis using ImmunoRatio®.

Ki67 immunohistochemical staining was evaluated visually as follows; in each tissue core, three fields each containing 100 malignant cells per field were selected and evaluated using x400 magnification. One of the fields was a hot spot with the other two fields showing less cells expressing ki67 positivity. The number of positively stained nuclei in the form of brown staining regardless of the intensity was designated as a fraction out of the total number of the malignant nuclei (Dowsett et al., 2011). The average of the three fields percentage was calculated to yield ki67 labelling index (LI) per core. The average of ki67 labelling indices (LIs) of the two cores representative of the case was calculated. Ki67 LI was calculated for one core only if the other core was lost or non-interpretable.

To assess securin expression, the exact percentage of the positively stained nuclei was calculated. Cutoff value was set at 1.5% (i.e perceptible immunopositivity in the form of either nuclear, nuclear membrane or nucleolar staining of tumour cells were considered as cases with high securin expression) (Talvinen et al., 2009) and as illustrated in the Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000164611-PTTG1/tissue/breast>) and registered as fraction out of total number of malignant cells.

Intraclass correlation coefficients for intra- and inter-observer reproducibility were used for assessing ki67 and securin (Talvinen et al., 2009).

Image analysis for ki67 and securin was carried out using ImmunoRatio® plugin for offline analysis which is a part of the ImageJ software. Two images per core were captured using Olympus microscope equipped with a

colour camera and submitted for analysis. Once the analysis was complete, a montage image consisting of every analyzed image and their result pseudo-coloured image appeared. Details about how the plugin executes its function are explained on the following website: <http://jvsmicroscope.uta.fi/sites/default/files/software/immunoratio-plugin/index.html> (Tuominen et al., 2010).

Assessment of HER-2 immunostaining was performed according to the ASCO/CAP recommendations for HER-2 testing (Wolff et al., 2013). Fluorescence in situ hybridization (FISH) was performed for the five equivocal cases after two runs of immunohistochemical staining for HER-2. Three cases showed gene amplification, while two cases were negative. CK5/6 was regarded positive in case of a complete membranous staining.

Estrogen and progesterone receptor status using Allred method were obtained from reports archived in the Pathology Department and used together with the HER-2 status, CK5/6 and ki67 LI to identify IHC surrogate for the molecular class of each case. Molecular classes included luminal-A, luminal-B, HER-2 enriched and triple negative (Goldhirsch et al., 2011; Guin et al., 2012).

Statistical analysis

Data were collected and entered either numerical or categorical, as appropriate to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis (version 21) (Corp, 2012). Descriptive statistics were described using a minimum, maximum, median and inter-quartile range for non-parametrically distributed data (Field, 2009).

Chi-square (χ^2) was used to test association between the categorical variables. The correlation between paired-variables in the same group was performed using Pearson correlation and linear regression.

Monte Carlo (MC) correction was carried out when indicated. Reliability statistics were applied to test the reproducibility of ki67 and securin LIs acquired by visual counting and ImmunoRatio® and expressed by Cronbach's alpha value. Receiver Operator Characteristics (ROC) curves were used to test the possible cut off values of ki67 and securin LIs by each method. Area under the ROC curves (AUC) and comparison between the AUC of both groups was carried out using MedCalc Software version 14 (DeLong et al., 1988).

Results

In the present study, 236 cores representative of 118 cases were included in six TMA blocks. The 118 cases were divided into two groups; first group (G1) (n=77) who were disease free and the second group (G2) (n=41) included cases who developed recurrence or distant

metastasis at the end of the 24 months follow-up. The clinicopathological characteristics of both study groups are summarized in Table 1. Risk association between the clinicopathological features of the patients and the poor clinical outcome was performed. Only axillary nodal

status, extranodal extension, lymphovascular invasion, ER status, HER-2 positivity and molecular classes showed statistically significant risk association (p values are mentioned in Table 1) with recurrence and/or metastasis.

Table 1. Summary of the clinicopathological characteristics of the patient material (n=118).

	G1 (n=77)	G2 (n=41)	
Age at diagnosis			
Median (min-max)	47 (31-90 years)	48 (27-77)	Z=0.255 p=0.799
Primary tumour size			
T1	4 (5.2%)	1 (2.4%)	$\chi^2=4.790$ p _(MC) =0.314
T2	39 (50.6%)	18 (43.9%)	
T3	23 (29.9%)	12 (29.3%)	
T4	3 (3.9%)	6 (14.6%)	
Couldn't be assessed	8 (10.4%)	4 (9.8%)	
Nodal status			
N0	17 (22.1%)	3 (7.3%)	$\chi^2=16.908$ p _(MC) =0.001*
N1	20 (26.0%)	14 (34.1%)	
N2	25 (32.5%)	5 (12.2%)	
N3	15 (19.5%)	17 (41.5%)	
Cannot be assessed	0 (0.0%)	2 (4.9%)	
Extranodal extension			
Positive	26 (33.8%)	26 (63.4%)	$\chi^2=11.092$ p=0.004*
Negative	45 (58.4%)	11 (26.8%)	
Cannot be assessed	6 (7.8%)	4 (9.8%)	
Histologic type			
Invasive carcinoma, NST	69 (89.6%)	38 (92.7%)	$\chi^2=2.865$ p _(MC) =0.451
Invasive lobular carcinoma	4 (5.2%)	1 (2.4%)	
Mixed Invasive carcinoma	4 (5.2%)	1 (2.4%)	
Medullary carcinoma	0 (0.0%)	1 (2.4%)	
Histologic grade			
G1	12 (15.6%)	3 (7.3%)	$\chi^2=1.962$ p=0.375
G2	51 (66.2%)	28 (68.3%)	
G3	14 (18.2%)	10 (24.4%)	
Lymphovascular invasion			
Detected	42 (54.5%)	34 (82.9%)	$\chi^2=9.526$ p _(MC) =0.005*
Not detected	34 (44.2%)	7 (17.1%)	
Cannot be assessed	1 (1.3%)	0 (0.0%)	
In situ component			
Detected	45 (58.4%)	20 (48.8%)	$\chi^2=2.448$ p=0.379 NS
Not detected	32 (41.6%)	21 (51.2%)	
ER status			
Negative	22 (28.6%)	17 (41.5%)	$\chi^2=8.108$ p=0.044*
Mild positivity	6 (7.8%)	8 (19.5%)	
Moderate positivity	24 (31.2%)	10 (24.4%)	
Intense positivity	25 (32.5%)	6 (14.6%)	
PR status			
Negative	17 (22.1%)	16 (39.0%)	$\chi^2=7.835$ p=0.050
Mild positivity	13 (16.9%)	11 (26.8%)	
Moderate positivity	32 (41.6%)	10 (24.4%)	
Intense positivity	15 (19.5%)	4 (9.8%)	
HER-2 status			
Negative	67 (87.0%)	28 (70.0%)	$\chi^2=4.991$ p=0.025*
Positive	10 (13.0%)	12 (30.0%)	
Molecular classes			
Luminal A	57 (74%)	20 (48.8%)	$\chi^2=13.95$ p _(MC) =0.002*
Luminal B	12 (15.6%)	7 (17.1%)	
Her-2 enriched	2 (2.6%)	9 (22.2%)	
Triple negative/basal-like	6 (7.8%)	5 (12.2%)	

Ki67 LIs

Ki67 LIs values in both study groups using visual counting and ImmunoRatio® are illustrated in Table 2. Comparison between ki67 LIs in both study groups using Mann-Whitney test revealed that ki67 LIs were significantly higher in G2 ($p=0.006^*$). On the other hand, Ki67 LIs obtained by image analysis were not significantly different between both study groups.

The overall agreement between both methods of ki67 LI evaluation was good (Cronbach's $\alpha=0.786$). In the present study, good positive correlation was found between ki67 LIs obtained by both methods ($r=0.638$, $p<0.001$). Agreement between both methods of ki67 LI calculation was very good (Cronbach's $\alpha=0.817$) in G1. While agreement between both methods of ki67 LI calculation was fair (Cronbach's $\alpha=0.652$) in G2. Ki67 immunopositivity is shown in Fig. 1.

Table 2. Comparison between ki67 LIs obtained by visual counting and ImmunoRatio® in both study groups.

	G1 (n=77)	G2 (n=41)	Total (n=118)
Ki67 LIs (visual counting)			
Min-Max	0.00-96.00	10.00-95.00	0.00-96.00
Median	50.00	77.00	61.00
Inter-quartile range	10.00-85.00	50.00-90.00	15.00-85.50
Mann-Whitney U test		Z=2.731 p=0.006*	
Ki67 LIs (ImmunoRatio®)			
Min-Max	0.00-98.50	0.00-98.00	0.00-98.50
Median	32.00	50.00	41.50
Inter-quartile range	10.00-67.00	19.50-80.00	11.00-74.00
Mann-Whitney U test		Z=1.781 p=0.075	

Securin LIs

Securin LIs acquired by visual counting and ImmunoRatio® were significantly higher in G2 compared to G1 (Table 3). Good positive correlation was also found between securin LIs obtained by both methods ($r=0.671$, $p<0.001$). The overall agreement between both methods of securin assessment was fair (Cronbach's α coefficient = 0.637).

In G1, Agreement between both methods of securin scoring was fair (Cronbach's α coefficient = 0.634). In G2, Agreement between both methods of securin expression assessment was poor (Cronbach's α coefficient = 0.571). Securin immunoreactivity is shown in Fig. 2.

Ki67 scoring by the visual counting method was statistically significant when ROC curve was applied. Area under curve (AUC) was 0.653 with confidence

Table 3. Comparison between securin LIs obtained by visual counting and ImmunoRatio® in both study groups.

	G1 (n=77)	G2 (n=41)	Total (n=118)
Securin LIs (visual counting)			
Min-Max	0.00-40.00	0.00-25.00	0.00-40.00
Median	1.00	5.00	2.00
Inter-quartile range	0.00-3.00	3.00-10.00	0.00-5.00
Mann-Whitney U test		p=0.000*	
Securin LIs (ImmunoRatio®)			
Min-Max	0.00-55.00	0.00-58.00	0.00-58.00
Median	4.00	11.00	5.35
Inter-quartile range	1.00-9.35	4.50-19.25	1.00-13.00
Mann-Whitney U test		p=0.000*	

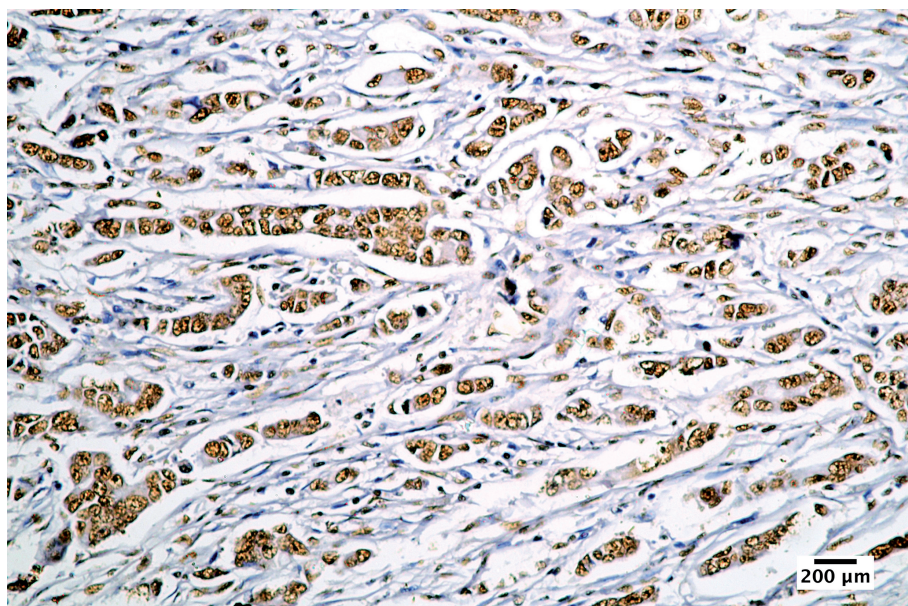


Fig. 1. Invasive Carcinoma, NST, GII showing immunopositivity for ki67 in the form of nuclear staining.

interval (CI) = 0.560 - 0.738 (p=0.0020). The cutoff point with the highest specificity and sensitivity is >18% (sensitivity =95.12% and specificity=38.96%). On the other hand, ki67 scoring by image analysis showed no statistical significance (p= 0.0635).

Global correlation shows 67.1% concordance for securin and 63.8% for ki67 between the visual counting and the ImmunoRatio® (Table 4). Global comparison using Pearson correlation and linear regression between ki67 and securin LIs obtained by the visual counting method showed 33.6% concordance and linear regression showed that there is 1.7 unit increase in

securin for every ki67 positive cell (Table 5).

As regards securin LIs obtained by visual counting assessment and image analysis, ROC curves were also applied. Securin scoring by visual counting assessment showed statistical significance, (p<0.0001, CI=0.689-0.847). AUC was 0.775 denoting good diagnostic accuracy. The optimal cut off point is >1% with 87.80% sensitivity and 64.94% specificity. Securin LIs by image analysis was statistically significant, (p<0.0001, CI=0.604-0.777). AUC was 0.696 indicating fair diagnostic accuracy. The optimal cut off point is >5% with 73.18% sensitivity and 62.34% specificity. Ki67 and securin immunopositivity by the image analysis are shown in Figs. 3, 4 respectively.

Comparison of ROC curves in the form of pairs was performed for both ki67 and securin LIs by visual counting assessment and image analysis (Fig. 5). Pairwise comparison between AUCs of ki67 and securin visual counting assessment was statistically significant (difference between areas =0.0432, p=0.0414). Comparison between AUCs of ROC curves for assessment of securin and ki67 LIs by image analysis

Table 4. Global correlation between ki67 and securin LIs obtained by visual counting and ImmunoRatio® in both study groups.

	Ki67 LIs (ImmunoRatio®)	Securin LIs (ImmunoRatio®)
Ki67 LIs (visual counting)	r=0.638, p<0.001	
Securin LIs (visual counting)		r=0.671, p<0.001

Table 5. Correlation and linear regression analysis between ki67 and securin.

	Ki67 LIs (ImmunoRatio®)	Securin LIs (ImmunoRatio®)
Ki67 LIs (visual counting)	-	B=1.715, p<0.001, r2=0.336. p<0.001
Securin LIs (visual counting)	B=1.715, p<0.001, r2=0.336. p<0.001	-

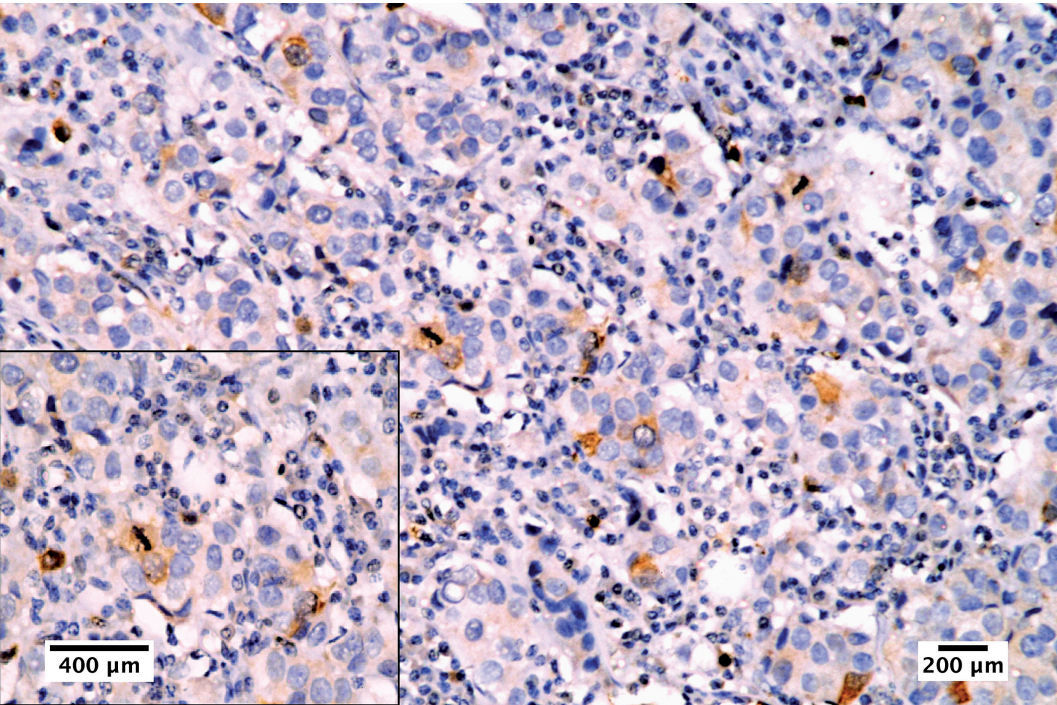


Fig. 2. Invasive breast carcinoma, NST, GIII showing immunopositivity for securin in the form of nuclear staining, the inset in the bottom left shows immunopositivity in mitotic figure.

was statistically significant (difference between areas =0.176, $P = 0.0105$).

Discussion

Breast cancer is the second most common malignancy worldwide responsible for increased morbidity and mortality burden in females (Ferlay et al., 2015). The prevalence of the disease in Egypt is similar to the international trends (Ibrahim et al., 2014). Clinical outcome of breast cancer patients is controlled by a range of clinicopathological parameters with variable levels of evidence supporting its role in the clinical practice (Fitzgibbons et al., 2000; Bradley, 2007).

Tumour size was not significantly different among study groups. However, it represents a well-established

prognosticator especially in node-negative cancer (Johnson et al., 1992; Rosen et al., 1993; Trudeau et al., 2005). Lack of statistically significant association between the tumour size and the poor clinical outcome can likely be attributed to the small sample size ($n=118$) and the short follow-up duration (FU=24 months). Neither histologic type nor histologic grade showed risk association with the clinical outcome. This is attributed to the fact that most of the cases were invasive carcinoma, NST or invasive lobular carcinoma. Both subtypes have a similar outcome in published series (Mersin et al., 2003; Lester, 2009). Histologic grade wasn't statistically significant due to clustering of cases under Grade II category and variability issues including fixation time which affects mitotic figures (Rakha and Ellis, 2012).

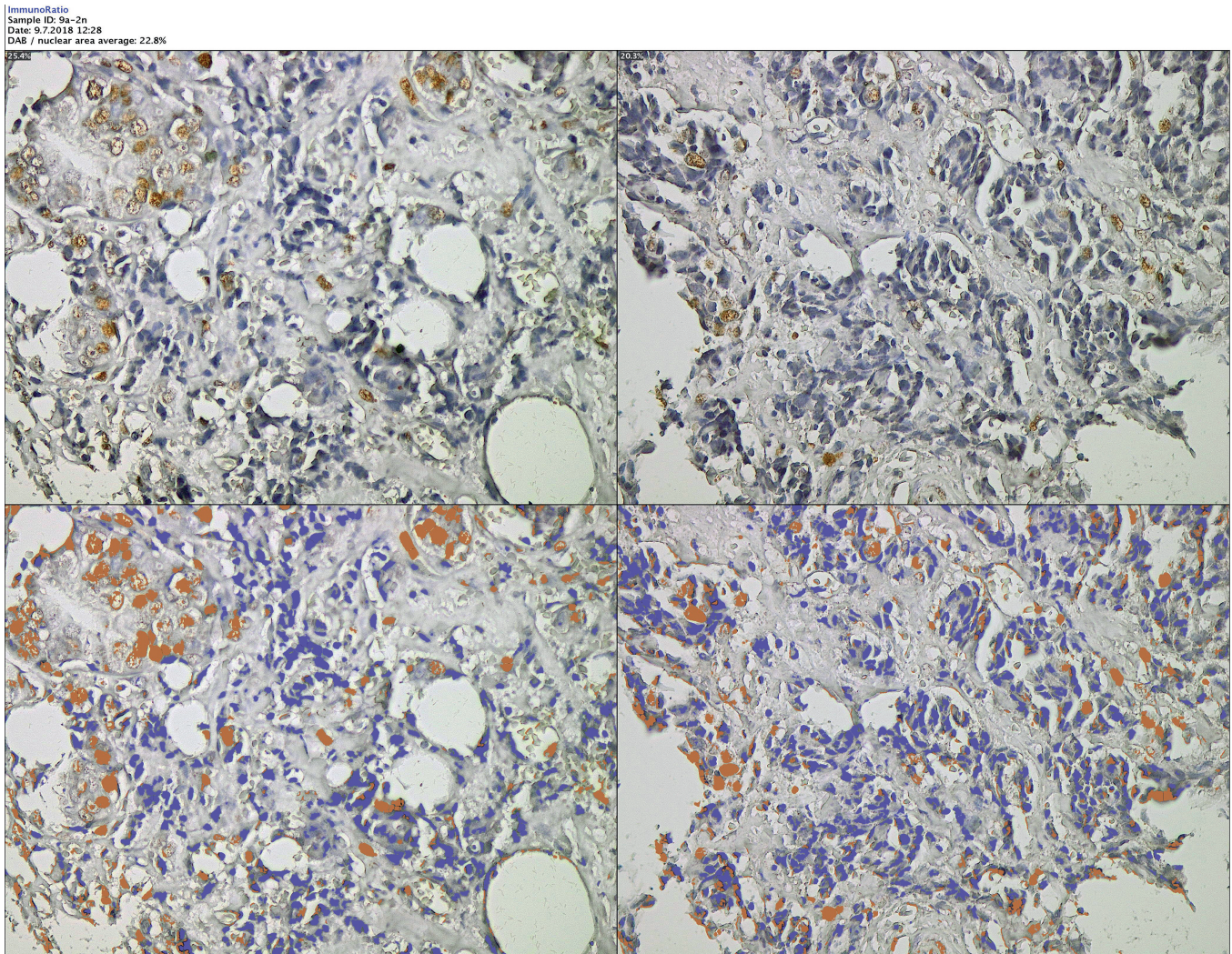


Fig. 3. Montage image showing two images of ki67 immunostaining captured from the same core (**A, B**) with their corresponding matched pseudo-coloured images (**C, D**). The light brown coloured areas in the bottom two images correspond to positively stained nuclei while the violet coloured ones denote haematoxylin counterstained nuclei. The image top shows a bar displaying the sample ID and DAB/nuclear area average (LI) of both images. x 200.

Nodal status was significantly different between the study groups. Axillary lymph node status is considered the most powerful independent prognostic factor in breast cancer (Fisher et al., 1983; Clayton and Hopkins, 1993). In the current work, the extranodal extension showed a significant association with the poor clinical outcome. Fisher et al. (1976a,b) were the first to emphasize extranodal extension association with local recurrence and distant metastasis. This was supported by further studies (Mambo and Gallager, 1977; Clayton and Hopkins, 1993; Ilknur et al., 2004; Neri et al., 2005).

ER status was significantly different between the studied groups. This is concordant with the literature (Bardou et al., 2003; Hameed, 2008). PR on the other hand was not significantly different among study groups.

This is concordant with the published data (Hammond et al., 2010).

HER-2 expression was associated with occurrence of recurrence and distant metastasis. HER-2 expression and gene amplification identify worse prognosis (Slamon et al., 1989; Borg et al., 1990; Battifora et al., 1991; Ross and Fletcher, 1998). Despite extensive research, there is not sufficient evidence to support this finding (Harris et al., 2007). Molecular classes also showed significant association with the clinical outcome. These findings are consistent with data retrieved from different studies (Perou et al., 2000; Carey et al., 2006; Cheang et al., 2009; Park et al., 2012).

Hormonal receptors together with HER-2 expression evaluation is essential for the management of breast

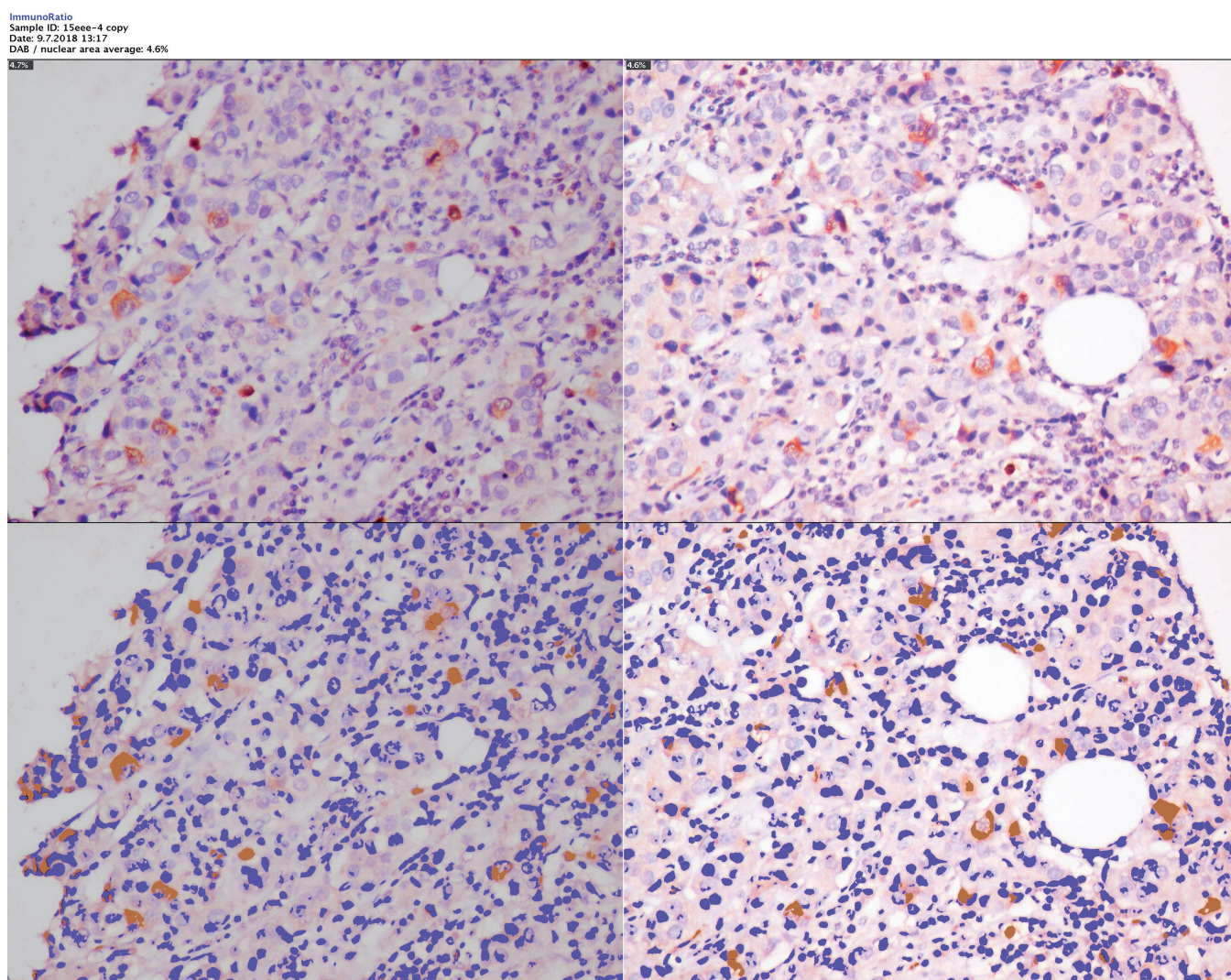


Fig. 4. Montage image showing two images of securin immunostaining captured from the same core (A, B) with their corresponding matched pseudo-coloured images (C, D). A, B. Show black arrows denoting positively stained nuclei. C, D. Feature black arrowheads marking the bright brown coloured areas corresponding to positively stained nuclei. The violet coloured areas in C, D denote haematoxylin counterstained nuclei. The image top shows a bar displaying the sample ID and DAB/nuclear area average (LI) of both images. x 200.

cancer patients. Nevertheless, their value as predictive factors to hormonal and targeted therapy is far superior to their prognostic role, as prognosis is likely controlled by interplay between established clinicopathological parameters and a range of biomarkers including proliferation markers. Ki67 as a marker of tumour proliferation is thoroughly investigated to establish its routine use in predicting the clinical outcome of breast cancer patients (Dowsett et al., 2011; Polley et al., 2015).

In this study, we evaluated ki67 and securin expression in two groups of Egyptian breast cancer patients using both visual counting method and ImmunoRatio®. Ki67 LIs obtained by visual counting were significantly higher in patients who developed recurrence and/or distant metastasis. High ki67 expression, routinely obtained by visual counting, was associated with poor clinical outcome in multiple studies (Brown et al., 1996; Viale et al., 2008; Nishimura et al., 2010; Yerushalmi et al., 2010; Aleskandarany et al., 2012; Joensuu et al., 2013). Other studies performed on ki67 LIs in Egyptian breast cancer patients yielded similar results (Soliman and Yussif, 2016; Ragab et al., 2018). The conclusions of the largest two meta-analyses to date performed on ki67 expression in primary breast carcinoma have supported these studies (De Azambuja et al., 2007; Petrelli et al., 2015).

On the other hand, ki67 LIs obtained by ImmunoRatio® were not significantly different between the study groups. Tuominen and coworkers (Tuominen et al., 2010) showed correlation between labelling indices obtained by ImmunoRatio® and short disease free survival in breast cancer. The use in the clinical

practice has been supported by subsequent studies (Sundara et al., 2014; Yeo et al., 2017). However, a single study has shown that ImmunoRatio® was inferior to visual counting in predicting cancer survival (Mohammed et al., 2012). Good positive correlation between LIs acquired by both methods was detected as well as good agreement between both methods. In accordance with our results, recent studies suggested that there is strong linear correlation between LIs obtained by visual counting and ImmunoRatio® (Tuominen et al., 2010; Fulawka and Halon, 2016).

The cutoff point proposed for ki67 in the current study by the visual counting method using ROC curve is >18%. Cutoff point set at $\geq 20\%$ was endorsed by multiple studies to distinguish patients with higher likelihood of recurrence or metastasis (Yerushalmi et al., 2010; Rossi et al., 2015). Cheang and coworkers (Cheang et al., 2009) set the cutoff value at $\geq 14\%$ in order to distinguish luminal-B cases, who have worse outcome than luminal-A. This cutoff value was endorsed by multiple studies and guidelines (Goldhirsch et al., 2011; Inwald et al., 2013). The mild discrepancy in scores can be explained by the small number of cases, cases selection, the clone of antibody and the use of TMA instead of the conventional sections. Tissue microarrays were reported to have lower ki67 LIs compared to the conventional sections (Muftah et al., 2017). The largest meta-analysis to date on ki67 performed by Petrelli et al (Petrelli et al., 2015) suggested that any ki67 value more than 10% is associated with short disease free survival. Nevertheless, the recommendations from the ki67 working group suggested that the set cutoff points used in practice should not be applied to TMAs (Dowsett et al., 2011).

Securin LIs assessed by both visual counting and ImmunoRatio® were significantly higher in breast cancer patients who developed recurrence or distant metastasis. Talvinen and coworkers (Talvinen et al., 2008) found that securin might be an independent prognostic factor in invasive breast carcinoma. This was supported by subsequent studies by the same research group on different patient groups (Talvinen et al., 2009; Talvinen, 2010; Karra et al., 2012; Gurvits et al., 2016). They also reported that securin low expression in cases of breast cancer with high ki67 LI have better outcome compared to combined overexpression (Talvinen et al., 2009). Only Hatcher and coworkers reported (Hatcher et al., 2014) that securin acts as a tumour suppressor gene and is responsible for branching of mammary epithelia during development. Positive correlation between both methods of assessment of securin LIs was also found. No published data to time support this finding.

The suggested cut off value for securin by visual counting is >1%. This result is in keeping with cutoff value proposed by Talvinen (Talvinen et al., 2009). A cutoff value of more than 5% was detected by ImmunoRatio®. To date, there is no data in literature about the use of ImmunoRatio® in securin scoring. Concordance between securin LIs obtained by visual

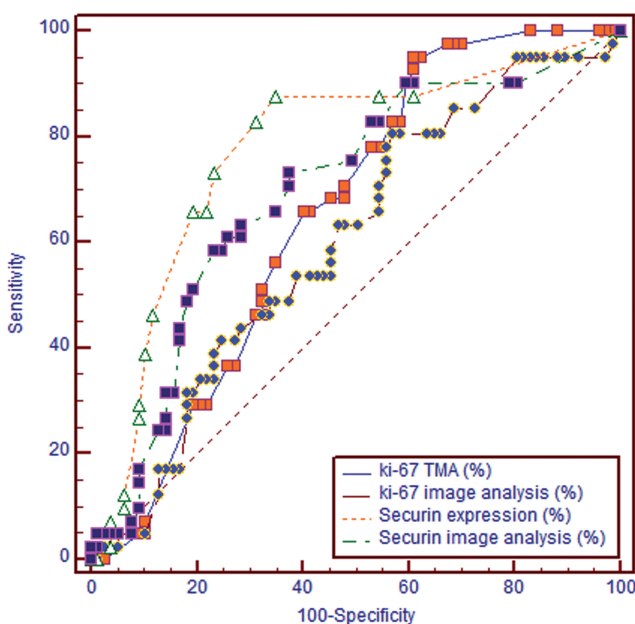


Fig. 5. ROC curves for ki67 and securin LIs by visual counting assessment and image analysis.

counting and ImmunoRatio® was slightly higher than ki67 LIs which may indicate better homogeneity in securin expression compared to ki67. However, ki67 LIs obtained by both methods showed excellent concordance in various studies (Tuominen et al., 2010; Fulawka and Halon, 2016; Yeo et al., 2017). In the current study, increased LIs of ki67 was associated with increased securin LI. This finding is slightly discordant from published data suggesting that low securin expression may be used to detect patients with favorable prognosis among breast carcinoma patients with high ki67 LIs (Talvinen et al., 2008, 2009).

Securin has advantages over ki67 as a proliferative marker. The securin protein is expressed in a cell-cycle-dependent manner. It peaks in G2/M-phase and thereafter is rapidly degraded by ubiquitination, unlike ki67 which is expressed throughout the cell cycle (Talvinen et al., 2009). Another major advantage is the easier method of assessment (perceptible nuclear positivity) compared to ki67 that suffers from variability issues (Dowsett et al., 2011). We also reviewed some cases from the Human Protein Atlas for both ki67 and Securin, and they are in accordance with our results.

Conclusion

In the present work, it could be concluded that securin may add to the prognostic value of Ki67 in highlighting intra-tumoural heterogeneity in invasive breast carcinoma patients with poor clinical outcome. In addition, the study showed that since securin has a visual counting cutoff with more than 1%, making it easier to use as a breast cancer biomarker in conjunction with ki67 to predict the outcome of the cases more accurately than using only ki67. However, a multivariate analysis on a larger cohort of patients is mandatory to test its potential prognostic value.

Conflict of interests. Authors declare no conflict of interests.

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Securin and Ki67 in breast carcinoma

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Securin and Ki67 in breast carcinoma

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