Pathol. Oncol. Res. (2016) 22:461–470
DOI 10.1007/s12253-015-0018-5

ORIGINAL ARTICLE

Runx2 Expression as a Potential Prognostic Marker in Invasive Ductal Breast Carcinoma

Saba Mohamed El-Gendi[1] · **Mohamed Farouk Mostafa**[2]

Received: 18 October 2014 / Accepted: 16 November 2015 / Published online: 23 November 2015

Abstract The Runx family of transcription factors has been implicated in cancer progression, both positively and negatively. Recent studies assigned a role for Runx2 in promoting breast cancer metastasis. However, the role of Runx2 during the early stage of breast carcinoma and its association with clinical outcomes remain unknown. Assessing the clinicopathological significance of Runx2 expression in a cohort of breast invasive ductal carcinomas (IDC). The correlation of nuclear Runx2 LI with clinicopathological parameters was assessed in 84 IDCs. To study the association of Runx2 with patient outcomes, in addition to treating it as a continuous variable, Runx2 was categorized by its median value (65) and by an additional two cut-off points determined by ROC curve analyses, at 45 for disease free survival (DFS) and 40 for overall survival (OS). Multivariate Cox regression models were also constructed. We used the best subset regression to identify models that predict DFS and OS with as few predictors as possible, and validation was performed. Based on the "Predicted R^2", the three best models were identified. Using Cox-regression, the interaction between Runx2 and other clinicopathological terms was tested. Runx2 LI was significantly associated only with positive Her-2 status, and did not correlate significantly with other clinicopathological parameters. Although Runx2 LI, in the continuous form and when categorized by the median, did not correlate significantly with DFS and OS; after it was categorized using the optimal cut-off points determined using ROC curve analysis, the patients with Runx2 LI >45 % showed a significantly higher event rate and shorter DFS ($P = 0.047$), whereas patients with Runx2 LI >40 % showed a significantly shorter OS ($P = 0.050$). Moreover, Runx2 LI contributed significantly in the models built to predict DFS and OS. For DFS, no interaction terms contributed significantly to the models. However, among stage IV cases, the interaction term between centred Runx2 and ER significantly contributed to the prediction of OS. Runx2 was a significant predictor of OS in this model. Runx2 has a role in biological behaviour and affects the outcome of IDC; therefore, its inhibition may be a new therapeutic strategy. The predictability of Runx2 for OS in stage IV tumours differs with different ER states. The pattern of this difference was not determined because the sample size was not sufficient to allow pattern testing.

Keywords Runx2 · Breast carcinoma · Prognostic factor · Clinical outcome · Survival

Introduction

Breast cancer is one of the most common malignancies in women worldwide [1, 2]. Bone metastasis is a frequent complication of breast cancer, with distinct gene signatures defining bone-seeking tumours [3–7]. Prolonged exposure to oestradiol (E2) is associated with an increased risk of breast carcinoma [8–12]. The mechanisms by which oestrogens contribute to breast carcinoma initiation and progression are complex and implicate oestrogen receptor (ER)-mediated genomic and nongenomic signalling, as well as the action of genotoxic

Electronic supplementary material The online version of this article (doi:10.1007/s12253-015-0018-5) contains supplementary material, which is available to authorized users.

✉ Saba Mohamed El-Gendi
sabaelgendi@yahoo.com

[1] Department of Pathology, Alexandria Faculty of Medicine, Alexandria, Egypt

[2] Department of Clinical Oncology and Nuclear Medicine, Alexandria Faculty of Medicine, Alexandria, Egypt

oestrogen metabolites [10]. In contrast to E2-mediated carcinogenesis, the presence of ERα is a favourable prognostic marker that is associated with less invasive tumours, and tumours negative for ERα are more aggressive [13].

The Runx family of mammalian transcription factors plays fundamental roles in the differentiation of osteoblasts and chondrocytes (Runx2) [14, 15], hematopoietic cells (Runx1) [16, 17] and neurons (Runx3) [18]. Runx proteins have also increasingly been implicated in cancer progression, both positively and negatively [19, 20].

Runx2 is a lineage-specific transcription factor with crucial roles in both bone biology and carcinogenesis [1, 20, 21]. Although Runx proteins have tumour suppressor properties [20], recent studies assigned a role for Runx2 in promoting breast and prostate cancer metastasis [22–27]. Thus, Runx2 and E2 signalling play dual roles in breast carcinoma, with each functioning to either promote or suppress tumour progression. The mechanisms underlying these contrasting manifestations in cancer are poorly understood [3].

With regard to its potential role at the sites of breast carcinoma metastasis to the bone, Runx2 was reported to regulate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone metastasis [24]. Runx2 was also shown to modulate several factors that contribute to metastasis, including vascular endothelial growth factor (VEGF) [28], several matrix metalloproteinases (MMP) [29]and bone sialoprotein [30]. However, the roles of Runx2 during the early stage of breast cancer have not been established. Moreover, the correlation of Runx2 nuclear immunoreactivity in breast carcinoma cells and the histopathological features of breast cancer were previously reported [31]; however, the correlation between Runx2 expression and prognosis remains unknown [1].

In the present study, we assessed the clinicopathological significance of the status of nuclear Runx2 immunoreactivity in a cohort of invasive ductal breast carcinomas. We then correlated the nuclear Runx2 labelling index (LI) in breast carcinoma cells with the histopathological features of the studied breast carcinomas, including tumour histological grade, stage, hormone receptor status, and HER2 expression, with patient clinical outcomes.

Material and Methods

Eighty-four cases of invasive ductal breast carcinomas were retrieved from the files of the Department of Pathology, Alexandria Faculty of Medicine, Egypt. Breast tissue specimens were obtained from Egyptian female patients who underwent a mastectomy between January 2007 and July 2009 at the Department of Surgery, Main University Hospital, Alexandria Faculty of Medicine, Egypt. All patients did not receive preoperative chemo, radio or hormonal therapy. The follow-up information for the patients was retrieved from the archives of the Clinical Oncology Department, Alexandria Faculty of Medicine, Main University Hospital, Egypt. The Ethics Committee at Faculty of Medicine, University of Alexandria, Egypt, approved the research protocol for the study.

The histological type of primary breast tumour was classified based on Page et al. [32] and the College of American Pathologists recommendations [33]. All invasive carcinomas were graded according to the method described by Ellis and Elston [34].

Immunohistochemical Staining

Sequential sections from each case were stained with four antibodies, Runx2, Her2, ER, and PR, to compare the staining characteristics of the same group of tumour cells. Immunostaining was performed on 5-μm thick sections of formalin fixed paraffin embedded (FFPE) tissue mounted on polylysine-coated microslides, which were dewaxed and rehydrated. Endogenous tissue peroxidase was blocked by incubating the tissue sections in 3 % hydrogen peroxide for 15 min. Heat induced antigen retrieval was performed for all antibodies in a microwave oven in 10 mM citrate buffer, pH 6.0. Then, the tissue sections were incubated with the primary antibodies. The primary antibodies used were mouse monoclonals for Runx2 and Her2 and rabbit monoclonals for ER and PR. Runx2 (Clone (27-K): sc-101145Santa Cruz Biotechnology, Inc., Europe) was used at a dilution of 1:50, Her2 (Clone (e2-4001 + 3B5) Thermoscientific, NeoMarkers, Fremont, USA) was used at a dilution of 1:200, ER (Clone SP1, Thermoscientific, NeoMarkers, Fremont, USA) was used at a dilution of 1:100, and PR (Clone SP2, Thermoscientific, NeoMarkers, Fremont, USA) was used at a dilution of 1:200. The antigen-antibody reaction was visualized with the Thermo scientific UltraVision LP Detection System. Immunohistochemical reactions were developed with diaminobenzidine, and the sections were counterstained with Harrris haematoxylin. Immunostaining was manually processed, with the appropriate external positive and negative controls included for each immunohistochemical run. Furthermore, all of the sections had an internal positive control for hormone receptors and Runx2 (normal breast tissue adjacent to the tumour).

Scoring of Immunostained Slides

Scoring the immunostained slides was performed semiquantitatively. Runx2 immunoreactivity was detected in the nuclei of breast carcinoma cells and evaluated in the nuclei of over 1000 carcinoma cells for each case. The percentage of positive nuclear immunoreactivity (labelling index (LI)) was subsequently calculated [1].

Her2 immunohistochemical staining was scored according to the guidelines published by Ellis et al. [35]. Tumours that showed strong complete membrane staining in >10 % of the tumour cells were considered positive.

The Allred score [36] was utilized to semiquantify the ER and PR immunostaining, in which both the proportion of positive cells and the intensity of staining were considered. The proportion of positive cells was scored on a scale of 0–5 (0 = no nuclear staining, 1 = <1 % nuclear staining, 2 = 1–10 % nuclear staining, 3 = 11–33 % nuclear staining, 4 = 34–66 % nuclear staining and 5 = 67–100 % nuclear staining), and the staining intensity was scored on a scale of 0–3 (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining). The proportion and intensity were then summed to produce total scores of 0 or 2 through 8. A score of 0–2 was regarded as negative, whereas 3–8 was regarded as positive.

Statistical Analysis

Statistical analyses were performed using SPSS® Statistics 21. Quantitative data were described using the median (Mdn), minimum and maximum as well as the mean (M) and standard deviation (SD). Qualitative data were described using the number and percentage. Correlations with qualitative ordinal variables were tested using Spearman's rho. The Mann-Whitney U test was used to compare variables between two groups because the assumption of normality was violated in all cases. The log-rank test was used to compare the patient outcomes between different groups using the test for trend for ordered categories with more than two groups. Univariate Cox regression was used to evaluate the association between continuous covariates and patient outcomes. To study the association of Runx2 with the outcome, in addition to treating it as a continuous variable, it was categorized by its median value (65) or by an additional two cut-off points determined by ROC curve analyses, at 45 for disease free survival and 40 for overall survival.

Multivariate Cox regression models were constructed, and the proportional hazards assumption was assessed by plots of the log (−log survival time). We used the best subset regression to identify models that predict disease free survival and overall survival with as few predictors as possible. Subset models estimate the regression coefficients and predict future responses with a smaller variance than the model containing all predictors. Using the best subset regression, we examined all of the possible subsets of the predictors, beginning with all of the models containing one predictor, then all of the models containing two predictors, and so on.

With multicollinearity, *p*-values for one predictor may be insignificant (>0.05), although this predictor remains valuable for the overall prediction of the outcome. Because the parameters included in the model were highly correlated with each other, we did not use the *p*-value to determine whether we retained a predictor in the model. Instead of using *p*-values, we used R^2, adjusted R^2, MAL PC and S (square root of the mean square error) for selecting the parsimonious model, which is the model that accomplishes a desired level of explanation or prediction with as few predictor variables as possible.

Finally, the program listed the baseline cumulative hazard H0 (t) at the mean of all of the covariates in the selected models. The hazard rate H (t) for any case at time (t) was calculated as follows:

$$H(t) = H_0(t) * e^{PI}$$

where H0 (t) is the baseline cumulative hazard at time (t), k is the number of covariates, and PI is a prognostic index:

$$PI = x_1b_1 + x_2b_2 + x_3b_3 \ldots\ldots + x_kb_k$$

Validation for each of the three models was conducted by systematically removing each observation from the data set, estimating the regression equation, and computing the "Predicted R^2" to determine how well the model predicts the removed observation.

Using Cox-regression, the interaction between Runx2 and other clinicopathological terms was tested. We centred Runx2 first to prevent the multicollinearity of Runx2, clinicopathological parameters, and interaction terms from disturbing the estimated *p*-values in the models.

Results

Clinicopathological Features

The current study included 84 female patients with a mean age of 50.2 (SD = 11.6) years, ranging from 27 to 76 years. Runx2 immunoreactivity was detected in the nuclei of breast carcinoma cells (Fig. 1A, 1B). The mean Runx2 labelling index was 54.9 (SD = 37.2), ranging from 0 to 100, and its distribution had two peaks, at 0 and 100 (Fig. 2), with a median of 65. Runx2 immunoreactivity was also detected in non-pathological myoepithelial and ductal cells (Fig. 3). Sixty-three cases (75 %) were ER positive, and 21 cases (25 %) were ER negative. Fifty-eight cases (69 %) were PR positive, and 26 cases (31 %) were PR negative. Only thirty-five cases (41.7 %) were Her2 positive.

Correlation of the Nuclear Runx2 LI with the Clinicopathological Factors

The mean Runx2 LI was not significantly correlated with patient age ($\rho = 0.209$, $p = 0.056$) and was significantly associated only with positive HER2 status ($P = 0.047$).

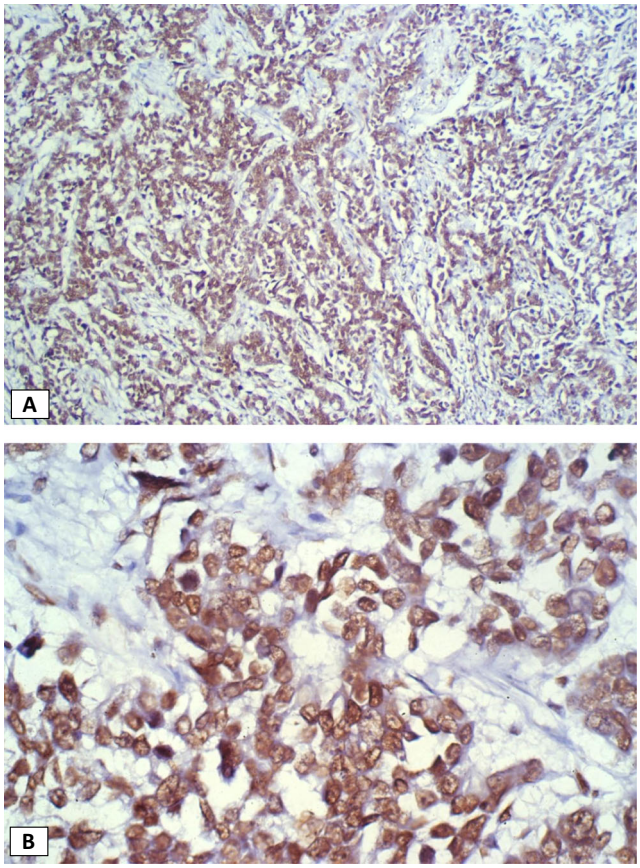

Fig. 1 Runx2 immunostaining in breast carcinoma cells. **a** Diffuse strong nuclear immunoreactivity (Runx2 LI = 100 %), (IHC, X40); **b** Higher power view demonstrating the positive nuclear stain in all tumor cells, (IHC, X400)

Supplemental Table 1 summarizes the correlation of the nuclear Runx2 LI in breast carcinoma cells with the clinicopathological parameters in the studied cohort of breast carcinoma cases.

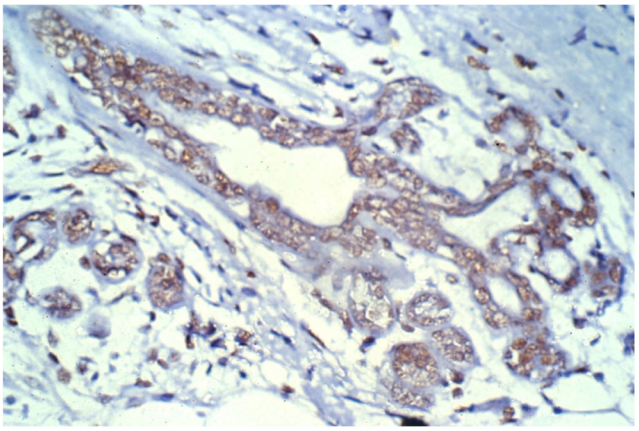

Fig. 3 Runx2 positive immunostaining in the non-pathological myoepithelial and ductal cells adjacent to the tumor; (IHC, X100)

Correlation Between the Runx2 LI and Patient Clinical Outcomes

Runx2 LI in the continuous form did not show any statistically significant association with the occurrence of any disease related event (HR = 1.008, 95 % CI = 0.997, 1.020) or with death (HR = 1.008, 95 % CI = 0.997, 1.019). This result did not change after the categorization of Runx2 by the median (Fig 4A, B). However, after Runx2 was categorized using the optimal cut-off point (45) determined by ROC curve analysis, the higher group (patients with Runx2 LI >45 %) showed a significantly higher event rate and shorter disease free survival time (P = 0.047) (Fig 5A). Moreover, the categorization of cases using the optimal cut-off point (40) determined by ROC curve analysis revealed that the higher group (patients with Runx2 LI >40 %) had a significantly shorter OS (P = 0.050) (Fig 5B).

Bivariate analysis was performed to detect the other significant predictors of DFS and OS. Tumour size (T-stage),

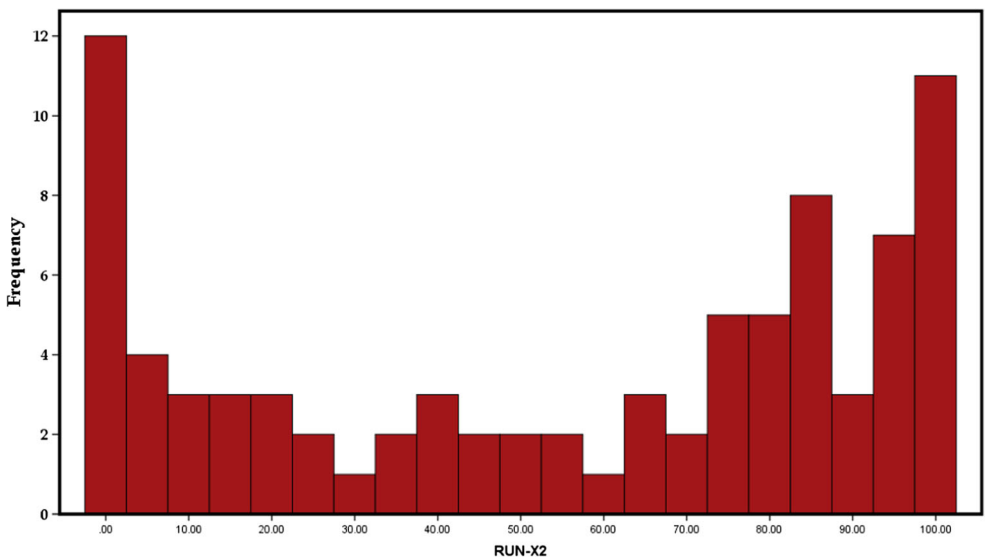

Fig. 2 Histogram showing the distribution of Runx2 in the studied breast carcinoma cases

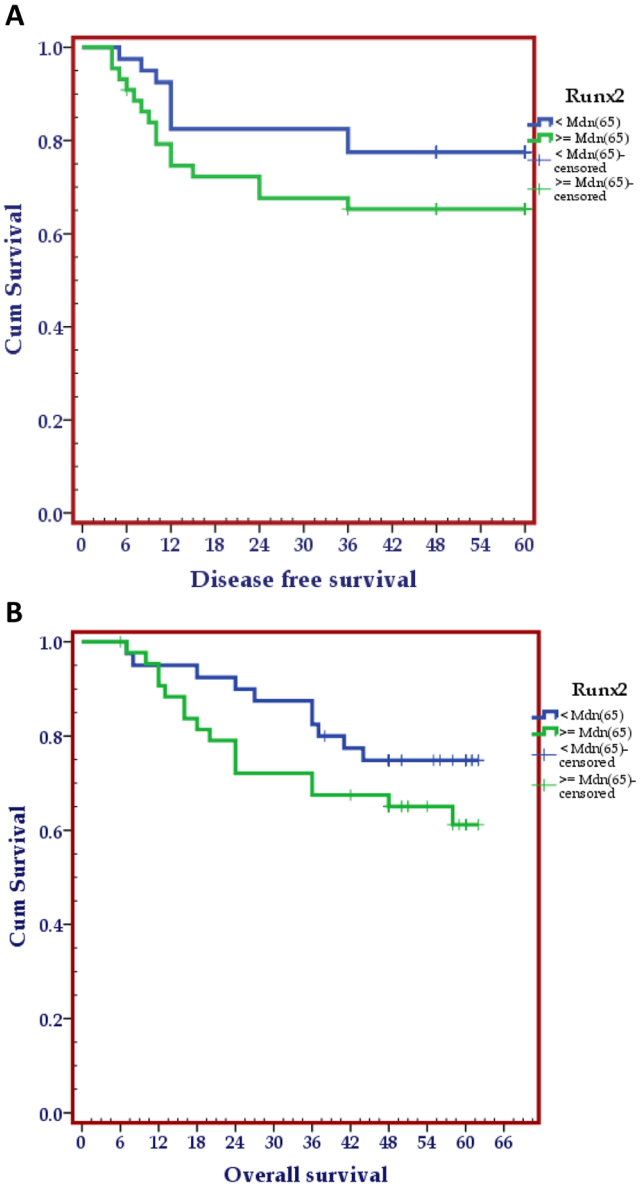

Fig. 4 **a** Kaplan Meier plot showing how the disease free survival curves of the 84 breast carcinoma patients differed according to the Runx2 LI categorized by its median value (65). **b** Kaplan Meier plot showing how the overall survival curves of the 84 breast carcinoma patients differed according to the Runx2 LI categorized by its median value (65)

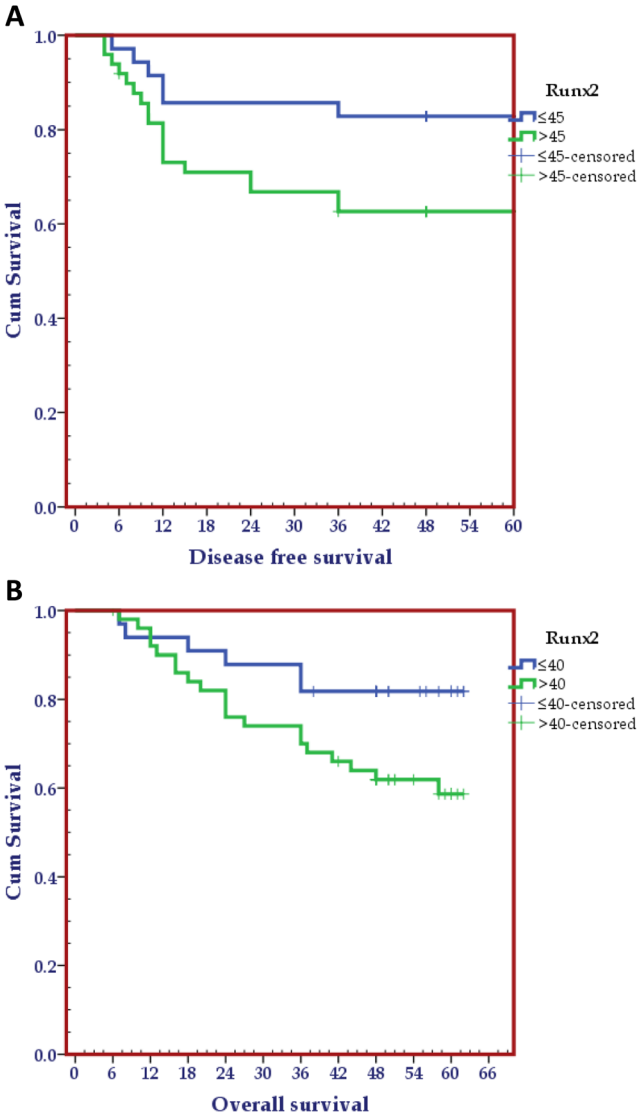

Fig. 5 **a** Kaplan Meier plot showing how the disease free survival curves of the 84 breast carcinoma patients differed according to the Runx2 LI categorized according to cut-off point (45) determined by ROC curve analysis. **b** Kaplan Meier plot showing how the overall survival curves of the 84 breast carcinoma patients differed according to the Runx2 LI categorized according to cut-off point (40) determined by ROC curve analysis

clinical stage, and hormone receptor status were significant predictors of both DFS and OS. Age did not show any statistically significant association with death (HR = 1.010, 95%CI = 0.976, 1.045) or with any disease related events (HR = 1.012, 95%CI = 0.977, 1.048). Tumour grade and Her2 status were insignificant predictors of DFS and OS. Nodal stage was a significant predictor of OS but showed no significance as a predictor of DFS, **(**Table 1**).**

We constructed several regression models to predict disease free survival, which in addition to Runx2 (continuous form), included the parameters significantly associated with DFS in bivariate analysis, including the stage of tumour (all vs. IV), T-stage (T1, 2 vs. T3, 4), ER (positive vs. negative) and PR (positive vs. negative). We used both ER and PR as an ordinal variable (negative, +, ++, and +++). Unexpectedly, the models containing ER and PR as ordinal variables had less prediction power, as measured by R^2, than the models containing ER and PR as dichotomous variables (+ve and -ve). As shown in Table 2, each row represents a different model "M". "Par" is the number of predictors in the model in addition to the constant. R^2 and the adjusted R^2 are presented as percentages. The predictors present in the model are indicated by an X.

For each number of predictors, only the two best models based on the size of the R^2-value are displayed. The first two

Table 1 Predictors of disease free survival and overall survival among the studied cohort of breast carcinoma patients

Predictor		Disease Free survival					Overall survival				
	Total	Events		Survival time		Log-rank test	Events		Survival time		Log-rank test
	N	n	%	Mean	SE	*p*-value	n	%	Mean	SE	*p*-value
T											
T1 or T2	29	3	(10.3)	54.6	(3.0)	6.2 **(0.013)**	3	(10)	57.1	(2.7)	7.296 **(0.007)**
T3 or T4	55	21	(38.2)	42.6	(3.1)		23	(42)	47.0	(2.7)	
Grade											
I or II	62	16	(25.8)	48.5	(2.6)	1.1 (0.284)	18	(29)	52.5	(2.1)	0.960 (0.327)
III	22	8	(36.4)	41.5	(5.2)		8	(36)	43.3	(4.8)	
Stage											
I, II or III	72	14	(19.4)	51.5	(2.1)	32.0 **(<0.001)**	15	(21)	54.2	(1.9)	33.662 **(<0.001)**
IV	12	10	(83.3)	18.1	(5.5)		11	(92)	27.8	(4.7)	
LN											
Negative	15	4	(26.7)	48.5	(5.1)	0.02 (0.884)	4	(27)	53.1	(3.8)	0.121 (0.728)
Positive	69	20	(29.0)	46.3	(2.6)		22	(32)	49.7	(2.3)	
LN stage											
N0, N1 or N2	68	17	(25.0)	48.4	(2.5)	2.4 (0.121)	18	(26)	52.5	(2.1)	4.277 **(0.039)**
N3	16	7	(43.8)	39.4	(6.1)		8	(50)	41.4	(5.6)	
ER											
Negative	21	12	(57.1)	35.2	(5.1)	11.2 **(0.001)**	12	(57)	41.1	(4.2)	8.899 **(0.003)**
Positive	63	12	(19.0)	50.5	(2.5)		14	(22)	53.2	(2.2)	
PR											
Negative	26	13	(50.0)	37.4	(4.7)	8.8 **(0.003)**	14	(54)	41.9	(3.8)	9.420 **(0.002)**
Positive	58	11	(19.0)	50.6	(2.6)		12	(21)	53.7	(2.3)	
HER2											
Negative	49	13	(26.5)	47.3	(3.1)	0.2 (0.692)	13	(27)	51.5	(2.7)	0.721 (0.396)
Positive	35	11	(31.4)	45.7	(3.7)		13	(37)	48.8	(3.2)	
Runx2_median											
<65	40	9	(22.5)	50.1	3.0	1.8 (0.179)	40	10	25.0	53.4	1.6 (0.207)
≥ 65	44	15	(34.1)	43.5	3.5		44	16	36.4	47.6	
≤40	33	6	(18.2)	51.6	(3.2)	3.0 (0.085)	6	(18)	54.6	(2.9)	3.833 **(0.050)**
>40	51	18	(35.3)	43.4	(3.2)		20	(39)	47.6	(2.8)	
≤45	35	6	(17.1)	52.1	(3.0)	3.9 **(0.047)**	7	(20)	54.4	(2.7)	3.289 (0.070)
>45	49	18	(36.7)	42.7	(3.3)		19	(39)	47.4	(2.9)	

Significant data (P<005) is shown in bold

models, A and B, were the best one-predictor models, followed by the best and second best two-predictor models, C and D, and so on. The last model, I, is the full model containing all parameters. As shown in Table 2, Runx2 is retained in two models, "F" and "G", in addition to the full model, "I".

Different "best" models were selected based on the different criteria. Based on the R^2-value criterion, the "best" models were "I", followed by "G" and "H". According to the adjusted R^2-value criterion, the best regression models were "E", followed by "G". Based on the Cp value, the two best models were "G", followed by "H". Based on the S value, the two best models were "E", followed by "G". Therefore, the three best models were "G", "H" and "E", Supplemental Table 2.

The disease free probability S (t) for any case at time t was calculated as follows:

$$S(t) = \exp(-H_0(t) \times PI)$$

where H0 (t) is the baseline cumulative hazard and PI is a prognostic index:

$$PI = x_1 b_1 + x_2 b_2 + x_3 b_3 + \ldots + x_k b_k$$

Based on the predicted R^2-value, the "best" model was "E" (32.9 %), followed by "H" (31.8 %). However, the predicted R^2 for model "G", containing Runx2, (31.6 %) was not significantly lower.

Several regression models to predict the overall survival were also constructed from Runx2 (continuous form) and

Table 2 Best subset regression models for predicting DFS and OS among the 84 studied breast carcinoma patients

Outcome	M	Par	Criteria for selecting the best model				Variables in the model						PredR^2	Prognostic index (PI)
			R^2	AdjR^2	Cp	S	*Runx2*	*Stage*	*T*	*ER*	*PR*	LN		
Disease-free survival	A	2	24.5	23.6	18.4	1.0000		X						
	B	2	13.3	12.3	33	0.4257				X				
	C	3	37.8	36.3	3.1	0.3628		X		X				
	D	3	31.9	30.2	10.8	0.3797		X			X			
	E	4	39.8	37.5	2.5	0.3593		X	X	X			32.9	(1.935)*$Stage^a$+(0.86)*T^b+(−1.339)*ER^c
	F	4	38.2	35.8	4.6	0.3640	X	X		X				
	G	5	40	37	4.2	0.3608	X	X	X	X			31.6	(1.930)*$Stage^a$+(0.816)*T^b+(−1.112)*ER^c+(−0.301)*PR^d
	H	5	40	36.9	4.3	0.3610		X	X	X	X		31.8	(1.898)*$Stage^a$+(0.817)*T^b+(−1.457)*ER^c+(0.007)*$Runx2^e$
	I	6	40.2	36.3	6	0.3626	X	X	X	X	X			
Overall survival	A	2	28.7	27.9	20.1	0.3950		X						
	B	2	11	9.9	45	0.4414					X			
	C	3	39.4	38	7.1	0.3664		X		X				
	D	3	36.7	35.1	10.9	0.3745		X			X			
	E	4	42.2	40.1	5.1	0.3600		X		X		X		
	F	4	42.2	40	5.2	0.3601		X	X	X				
	G	5	44	41.2	4.7	0.3567		X	X	X		X	34.4	(0.855)*T^b+(1.733)*$Stage^a$+(−1.203)*ER^c+(0.618)*LN^f
	H	5	43.5	40.7	5.3	0.3582		X		X	X	X		
	I	6	45	41.5	5.2	0.3557		X	X	X	X	X	34.1	(0.776)*T^b+(1.732)*$Stage^a$+(−0.837)*ER^c+(0.677)*LN^f+(−0.550)*PR^d
	J	6	44.3	40.7	6.3	0.3582	X	X	X	X		X		
	K	7	45.2	40.9	7	0.3575	X	X	X	X	X	X	32.7	(0.776)*T^b+(1.672)*$Stage^a$+(−0.880)*ER^c+(0.621)*LN^f+(−0.559)*PR^d+(0.004)*$Runx2^e$

x^a Stage = 0 if tumor stage is I, II or III, Stage = 1 if tumor stage is IV

x^b T = 0 if the tumor size is T1 or T2, T = 1 if the tumor size is T3 or T4

x^c ER = 0, if tumor ER is negative, ER = 1 if tumor ER is positive

x^d PR = 0 if tumor PR is negative, PR = 1 if tumor PR is positive

x^e Runx2 is the Runx2 labeling index

x^f LN = 0 if N is 0, 1, or 2, LN = 1 if N = 3

the parameters that were significantly associated with OS using a bi-variate analysis, including the stage of the tumour (IV vs. all), T-stage (T1, 2 vs. T3, 4), ER (positive vs. negative), PR (positive vs. negative), and N-stage (LN3 vs. all). We used the ER, PR, and N-stage as the ordinal variables (negative, +, ++, and +++). Unexpectedly, the models containing those predictors as ordinal variables had less prediction power as measured by the R^2 than the models containing these variables as dichotomous variables (+ve and –ve for ER and PR, and all vs. +++ for N-stage) As shown in Table 2, Runx2 is retained in only one model, "J", in addition to the full model, "K".

Different "best" models were selected based on the different criteria. Based on the R^2-value criterion, the "best" models were "K", followed by "I". According to the adjusted R^2-value criterion, the best regression models were "K", followed by "I". Based on the Cp value, the two best models were "G", followed by "I". Based on the S value, the two best models were "I", followed by "G". Therefore, the three best models were "G", "I" and "K", Supplemental Table 3.

Validation was performed, and according to the "Predicted R^2", the "best" models were "G" (34.4 %) and "I" (34.1 %). However, the predicted R^2 for model "K", containing Runx2, was lower (32.7 %).

For example, the characteristics of three breast carcinoma patients at 12, 15 and 48 months of follow up are shown in Supplemental Table 4. Using the developed model "G" for

predicting the hazard rate, the estimated hazard rate for the first case was the highest (0.78), whereas for the third case, it was the lowest (0.15).

Regarding disease free survival, no interaction term significantly contributed to the models ($p > 0.05$). Among stage IV cases, the interaction term between centred Runx2 and ER significantly contributed to the prediction of overall survival ($p = 0.033$). In this model, Runx2 was a significant predictor of overall survival (HR = 1.047, 95%CI = 1.004, 1.092, $p = 0.027$).

Another interesting finding was detected in the models that assessed the interaction between Her-2 and Runx2. Although Her-2 and the terms of its interaction with centred Runx2 were not significant predictors of disease free survival or overall survival, Runx2 did show a statistically significant association with disease free survival (HR = 1.024, 95 % CI = 1.001, 1.048, $p = 0.042$) and overall survival (HR = 1.021, 95CI% = 1.001, 1.042, $p = 0.027$).

Discussion

In the studied cohort of breast carcinomas, the mean Runx2 LI was 54.9 (SD = 37.2), ranging from 0 to 100. Its distribution revealed two peaks at 0 and 100. This mean, similar to that reported by Onodera et al. [1], was significantly associated with positive Her-2 status ($P = 0.047$). Conversely, Das et al. [31] demonstrated that Her-2 showed a negative correlation with Runx2 expression in grade 2 and grade 3 breast tumours. This contradiction could be explained by differences in the interpretation and scoring of Runx2 immunostaining. Based on the known role of Runx2 as a nuclear transcription factor, Das et al. [31] proposed that nuclear expression of Runx2 reflects a functionally active form of the protein, whereas its cytoplasmic localization indicates a loss of function in gene regulation. They hypothesized that cytoplasmic Runx2 provides a reservoir of sequestered Runx2 that translocates to the nucleus only upon stimulation or simply represents an inactive bystander molecule. Therefore, they interpreted the concurrent expression of Runx2 in both the nucleus and cytoplasm as indicating that the protein has retained its transcriptional function, and is, at least in part, active in gene regulation. Whether Runx2 contributes to the aggressive biological behaviour of Her-2 positive breast carcinomas must be verified.

In our study, Runx2 LI tended to correlate with the clinical stage ($P = 0.072$) but the level did not reach statistical significance. Runx2 LI was not significantly associated with patient age, tumour grade, T-stage, N-stage, or hormone receptor status. These findings oppose those of Onodera et al. [1], who reported that Runx2 LI, categorized by the median, was significantly associated with tumour stage and histologic grade.

Breast cancer development consists of many sequential steps, including primary tumour growth, neovascularization around the tumour, invasion, extravasation and subsequent formation of bone metastasis [37]. Many in vitro studies demonstrated that Runx2 may participate in these steps in multiple modes. Regulation or modification of VEGF secretion by Runx2 was reported in neovascularization [38]. Regulation of the secretion of several MMPs by Runx2 was also postulated as being linked with the subsequent invasion of carcinoma cells [39, 40]. Runx2 was proposed to subsequently mediate PTHrP expression of metastatic breast carcinoma cells in the microenvironment of bone [41]. These may all be related to an adverse clinical outcome for patients, but little has actually been demonstrated in clinical cases of human breast carcinoma.

In our studied breast carcinoma cohort, Runx2 LI in the continuous form, and when categorized by its median, was neither significantly associated with DFS nor with OS. However, at a cut off of >45, Runx2 LI significantly predicted a higher event rate and shorter disease free survival time, ($P = 0.047$), and at a cut off of >40, Runx2 LI predicted a significantly shorter OS, ($P = 0.050$). Similarly, Ondera et al. [1] demonstrated that the prognosis or clinical outcome of breast carcinoma cases with a high Runx2 LI was generally poor, and Chang et al. [42] reported that both relapse free survival (RFS) and OS were significantly lower in breast carcinoma patients with overexpressed Runx2.

Despite this, in the regression models built to assess the predictability of Runx2 for DFS and OS, we used Runx2 LI in the continuous form to avoid bias. Based on the predicted R^2-value, the "best" model for the prediction of DFS was model "E" (32.9 %), which included the stage, T-stage and ER, followed by model "H" (31.8 %), which included the stage, T-stage, ER and PR. However, the predicted R^2 for model "G", containing Runx2 in addition to the stage, T-stage, and ER, was not significantly lower (31.6 %) than model "G". This suggests that the role of Runx2 may be related to or equivalent to the role played by PR. Based on the predicted R^2-value, the "best" three models for the prediction of OS were models "G", including the stage, T-stage, ER, and N-stage (34.4 %), and "I", including the stage, T-stage, ER, PR and N-stage (34.1 %). The predicted R^2 for model "K", containing Runx2, was lower (32.7 %).

The finding that for all models, both for DFS and OS, the predicted R^2 was near 30 %, demonstrates that nearly 70 % of the variation in disease progression remains unexplained. Moreover, no significant difference was observed between the R^2 and predicted R^2. These findings indicate that we need to identify additional variables other than those addressed in the models to improve the predictive performance of the models.

We then tested the interaction between Runx2 and clinicopathological parameters using Cox-regression to assess whether Runx2 differs with different grades, T-stage, and N-stage. No interaction term contributed significantly to their

models regarding DFS. Therefore, the predictive performance of Runx2 did not differ significantly with the change of grade or stage. However, among stage IV cases, the interaction term between centred Runx2 and ER significantly contributed to the prediction of overall survival. This highlights that the predictability of Runx-2 for OS differs between stage IV ER positive and stage IV ER negative patients. The pattern of this difference was not assessed because of the small sample size, which was not sufficient to allow further pattern testing.

The variation of Runx2 expression with different tumour grades and stages was previously reported by Das et al. [31], who showed that the nuclear expression of Runx2 varied significantly across the three grades of breast cancer, with the highest expression in G2 tumours. Furthermore, there were no significant differences between Runx2 expression and different breast cancer stages or with axillary lymph node metastasis.

The potential involvement of Runx2 in earlier phases of breast cancer development was raised by Onodera et al. [1]. In that study [1], in particular, among the non-metastatic breast carcinomas, the groups of patients with elevated Runx2 expression were significantly associated with a poor prognosis in the ER-negative group of patients, whereas this association was not detected in the ER-positive carcinoma patients. Moreover, they demonstrated that the Runx2 LI evaluated as a continuous variable was also a significant prognostic factor of disease-free survival and overall survival. Chang et al. [42] also reported that the expression of Runx2 in ER negative and triple negative breast cancer was higher than in ER positive breast cancer, and related this to the intricate interactions among oestrogen, oestrogen receptor alpha (ERα) and Runx2 to mutually regulate their expression and activation.

An interesting finding in our study was that in the models assessing the interaction between Her-2 and Runx2, although Her-2 and the terms of its interaction with centred Runx2 were not significant predictors of disease free survival or overall survival, surprisingly, Runx2 did show a statistically significant association with disease free and overall survival.

In this study, we evaluated Runx2 expression in human IDC immunohistochemically. The nuclear Runx2 LI in carcinoma cells was correlated with positive Her-2 status. Runx2 LI, in continuous form and when categorized by the median, did not significantly correlate with DFS and OS; however, at a cut-off of 45 % and 40 %, as determined by ROC curve analysis, it was a significant predictor of DFS and OS, respectively. In addition, Runx2 LI effectively contributed to the models created to predict DFS and, to a lesser extent, OS. Because the predicted R^2, which did not differ from that of model R^2, was low, further research should be directed at identifying new markers and disease features, other than those included in the models, rather than increasing the sample size. The predictability of Runx2 LI for OS in stage IV, ER positive patients was significantly different from that of stage IV ER negative patients. This finding needs further assessment to determine the potential roles of Runx2 in these interactions. Moreover, because Runx2 shows a statistically significant association with disease free and overall survival in the models assessing the interaction between Her-2 and Runx2, there is the possibility of an unknown role for Runx2 in the biological behaviour of breast carcinomas. Therefore, inhibition of Runx2 may be a new therapy strategy for these cases.

Acknowledgments The authors appreciate all technicians at Department of Pathology, Alexandria University, Egypt.

Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest.

References

1. Onodera Y, Miki Y, Suzuki T, Takagi K, Akahira J, Sakyu T, Watanabe M, Inoue S, Ishida T, Ohuchi N, Sasano H (2010) Runx2 in human breast carcinoma: its potential roles in cancer progression. Cancer Sci 101:2670–2675
2. Gnant M, Dubsky P, Fitzal F, Blaha P, Schoppmann S, Steger G, Marth C, Samonigg H, Hüttner K, Fohler H, Ruecklinger E, Jakesz R, Greil R, Austrian Breast and Colorectal Cancer Study Group (2009) Maintaining bone density in patients undergoing treatment for breast cancer: is there an adjuvant Benefit? Clin Breast Cancer 9(Suppl 1):S18–S27
3. Chimge NO, Baniwal SK, Little GH, Chen YB, Kahn M, Tripathy D, Borok Z, Frenkel B (2011) Regulation of breast cancer metastasis by Runx2 and estrogen signaling: the role of SNAI2. Breast Cancer Res 13(6):R127
4. Roodman GD (2004) Mechanisms of bone metastasis. N Engl J Med 350:1655–1664
5. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ (2009) Cancer statistics. CA Cancer J Clin 59(4):225–249
6. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA, Massagué J (2003) A multigenic program mediating breast cancermetastasis to bone. Cancer Cell 3(6):537–549
7. Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, Foekens JA, Massagué J (2009) Latent bone metastasis in breast cancer tied to Src dependent survival signals. Cancer Cell 16(1):67–78
8. Henderson IC (1993) Risk factors for breast cancer development. Cancer Supplement 71:2127–2140
9. Ma H, Bernstein L, Pike MC, Ursin G (2006) Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. Breast Cancer Res 8:R43. doi:10.1186/bcr1525
10. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354(3):270–282
11. Chlebowski RT, Hendrix SL, Langer RD, Stefanick ML, Gass M, Lane D, Rodabough RJ, Gilligan MA, Cyr MG, Thomson CA, Khandekar J, Petrovitch H, McTiernan A; WHI Investigators (2003) Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA (24) 289:3243–3253.

12. Pike MC, Krailo MD, Henderson BE, Casagrande JT, Hoel DG (1983) Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer. Nature 303:767–770
13. Sotiriou C, Piccart MJ (2007) Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care? Nat Rev Cancer 7:545–553
14. Chimge NO, Baniwal SK, Luo J, Coetzee S, Omar Khalid O, Berman B, Tripathy D, Ellis M, Frenkel B (2012) Opposing effects of Runx2 and estradiol on breast cancer cell proliferation: in vitro identification of reciprocally-regulated gene signature related to clinical letrozole responsiveness. Clin Cancer Res 18(3):901–911
15. Komori T (2010) Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res 339:189–195
16. Lo Coco F, Pisegna S, Diverio D (1997) The AML1 gene: a transcription factor involved in the pathogenesis of myeloid and lymphoid leukemias. Haematologica 82:364–370
17. Woolf E, Xiao C, Fainaru O, Lotem J, Rosen D, Negreanu V, Bernstein Y, Goldenberg D, Brenner O, Berke G, Levanon D, Groner Y (2003) Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc Natl Acad Sci U S A 100: 7731–7736
18. Levanon D, Glusman G, Bettoun D, Ben-Asher E, Negreanu V, Bernstein Y, Harris-Cerruti C, Brenner O, Eilam R, Lotem J, Fainaru O, Goldenberg D, Pozner A, Woolf E, Xiao C, Yarmus M, Groner Y (2003) Phylogenesis and regulated expression of the RUNT domain transcription factors RUNX1 and RUNX3. Blood Cells Mol Dis 30(2):161–163
19. Zaidi SK, Pande S, Pratap J, Gaur T, Grigoriu S, Ali SA, Stein JL, Lian JB, van Wijnen AJ, Stein GS (2007) Runx2 deficiency and defective subnuclear targeting bypass senescence to promote immortalization and tumorigenic potential. Proc Natl Acad Sci U S A 104:19861–19866
20. . Blyth K, Vaillant F, Jenkins A, McDonald L, Pringle MA, Huser C, Stein T, Neil J, Cameron ER (2010) Runx2 in normal tissues and cancer cells: a developing story. Blood Cells, Molecules & Diseases. 45 (2):117–123.
21. Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, Gabet Y (2010) Regulation of adult bone turnover by sex steroids. J Cell Physiol 224:305–310
22. Javed A, Barnes GL, Pratap J, Antkowiak T, Gerstenfeld LC, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2005) Impaired intranuclear trafficking of Runx2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis in vivo. Proc Natl Acad Sci U S A 102:1454–1459
23. Pratap J, Imbalzano KM, Underwood JM, Cohet N, Gokul K, Akech J, van Wijnen AJ, Stein JL, Imbalzano AN, Nickerson JA, Lian JB, Stein GS (2009) Ectopic runx2 expression in mammary epithelial cells disrupts formation of normal acini structure: implications for breast cancer progression. Cancer Res 69:6807–6814
24. Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, Hussain S, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2008) Runx2 transcriptional activation of Indian hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer Res 68:7795–7802
25. Baniwal SK, Khalid O, Gabet Y, Shah RR, Purcell DJ, Mav D, Kohn-Gabet AE, Shi Y, Coetzee GA, Frenkel B (2010) Runx2 transcriptome of prostate cancer cells: insights into invasiveness and bone metastasis. Mol Cancer 9:258
26. Akech J, Wixted JJ, Bedard K, van der Deen M, Hussain S, Guise TA, van Wijnen AJ, Stein JL, Languino LR, Altieri DC, Pratap J, Keller E, Stein GS, Lian JB (2010) Runx2 association with progression of prostate cancer in patients: mechanisms mediating bone osteolysis and osteoblastic metastatic lesions. Oncogene 29: 811–821
27. Pratap J, Lian JB, Stein GS (2011) Metastatic bone disease: role of transcription factors and future targets. Bone 48:30–36
28. Zelzer E, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, Olsen BR (2001) Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Dev 106 (1–2): 97–106.
29. Selvamurugan N, Kwok S, Partridge NC (2004) Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. J Biol Chem 279:27764–27773
30. Barnes GL, Javed A, Waller SM, Kamal MH, Hebert KE, Hassan MQ, Bellahcene A, Van Wijnen AJ, Young MF, Lian JB, Stein GS, Gerstenfeld LC (2003) Osteoblast-related transcription factors Runx2 (Cbfa1/AML3) and MSX2 mediate the expression of bone sialoprotein in human metastatic breast cancer cells. Cancer Res 63(10):2631–2637
31. Das K, Leong DT, Gupta A, Shen L, Putti T, Stein GS, van Wijnen AJ, Salto-Tellez M (2009) Positive association between nuclear Runx2 and oestrogen-progesterone receptor gene expression characterises a biological subtype of breast cancer. Eur J Cancer 45(13):2239–2248
32. Page DL, Jensen RA, Simpson JF (1998) Routinely available indicators of prognosis in breast cancer. Breast Cancer Res Treat 51: 195–208
33. Fitzgibbons PL1, Page DL, Weaver D, Thor AD, Allred DC, Clark GM, Ruby SG, O'Malley F, Simpson JF, Connolly JL, Hayes DF, Edge SB, Lichter A, Schnitt SJ (2000) Prognostic factors in breast cancer. College of American pathologists consensus statement 1999. Arch Pathol Lab Med 124 (7):966–978.
34. Ellis IO, Elston CW (2006) Histologic grade (chapter 19). In: O'Malley FP, Pinder SE (eds) Breast pathology. Elsevier, Philadelphia, PA, pp. 225–233
35. Ellis IO, Bartlett J, Dowsett M, Humphreys S, Jasani B, Miller K, Pinder SE, Rhodes A, Walker R (2004) Best practice no. 176: updated recommendation for HER-2 testing in the UK. J Clin Pathol 57(3):322–327.
36. Allred DC, Harvey JM, Berardo M, Clarck GM (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 11:155–168
37. Pratap J, Javed A, Languino LR, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) The Runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. Mol Cell Biol 25:8581–8591
38. Risau W (1997) Mechanisms of angiogenesis. Nature 386:671–674
39. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2:737–744
40. Itoh T, Tanioka M, Matsuda H, Nishimoto H, Yoshioka T, Suzuki R, Uehira M (1999) Experimental metastasis is suppressed in MMP-9-deficient mice. Clin Exp Metastasis 17:177–181
41. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR (1996) Evidence for a causal role of parathyroid hormonerelated protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 98:1544–1549
42. Chang CH, Fan TC, Yu JC, Liao GS, Lin YC, Shih A, Li WH, Yu A (2014) The prognostic significance of RUNX2 and miR-10a/10b and their inter-relationship in breast cancer. J Transl Med 12:257