EUROPEAN JOURNAL OF CANCER 45 (2009) 2239–2248

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Short Communication

Positive association between nuclear *Runx2* and oestrogen-progesterone receptor gene expression characterises a biological subtype of breast cancer

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ARTICLE INFO

Article history:
Received 19 February 2009
Received in revised form 19 June 2009
Accepted 24 June 2009
Available online 24 July 2009

Keywords:
Runx2
Breast cancer
Oestrogen receptor
Progesterone receptor
CerbB2

ABSTRACT

Purpose: The runt-related transcription factor, *Runx2* may have an oncogenic role in mediating metastatic events in breast cancer, but whether *Runx2* has a role in the early phases of breast cancer development is not clear. We examined the expression of *Runx2* and its relationship with oestrogen receptor (*ER*) and progesterone receptor (*PR*) in breast cancer cell lines and tissues.

Methods: Two human breast cancer cell lines, MCF-7 and MDA-MB-231 were transiently transfected with vectors expressing either *Runx2* or *ER* and the levels of both proteins and mRNA were examined by Western blot analysis and quantitative real-time PCR, respectively. *Runx2* expression was also examined in tissue microarray sections of 123 breast cancer patients by immunohistochemistry and results were correlated with clinicopathological characteristics.

Results: Expression of *Runx2* and *ER* was reciprocal in the breast cell culture models and *Runx2* suppressed ERβ but not *ERα* mRNA levels. In contrast, functional expression of *Runx2* was evident in the nucleus in 28% of the breast cancer tissues and in both early and late stages of tumour growth. Importantly, *Runx2* expression was significantly more frequent in Grade 2 compared to Grade 1 and Grade 3 tumours (48% versus 39% versus 13%) and the expression was significantly associated with *ER* ($p = 0.005$), *PR* ($p = 0.008$) expressions in Grade 2 & Grade 3 tumours than Grade 1 tumours.

Conclusion: We propose that *Runx2*, *ER* and *PR* triple positivity in Grades 2 and 3 defines a biological subtype in breast cancer.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.06.021

1. Introduction

Breast cancer occurs due to the action of the two main female steroid hormones, oestrogen and progesterone and their cognate nuclear receptors (respectively, *ER* and *PR*) that are known to be potent mitogenic factors. The pathological roles of both hormone receptors in the aetiology of breast cancer have been demonstrated by *in vitro* and *in vivo* studies.[1] Both *ER* and *PR* have been implicated in the initiation or promotion of breast cancer by stimulating breast cell proliferation while bypassing cell cycle checkpoints that normally prevent DNA damage in response to genotoxic insult and permitting the incorporation of random genetic mutations.[2]

The oestrogen receptor (*ER*) exists in two isoforms, *ERα* and *ERβ* that are encoded by two distinct genes, ESR1 and ESR2, located in chromosomal loci at 6q25 and 14q23, respectively.[3] The ratio of *ERα* and *ERβ* varies between tissues and results in different physiological responses to oestrogen. Breast cancer shows mostly higher levels of *ERα* and its expression is associated with more differentiated tumours and a more favourable prognosis, although the potential role of *ERβ* as a favourable prognostic indicator has also been suggested.[4] Progesterone binds to the progesterone receptor and also has two isoforms, *PR*-A and *PR*-B that differ in protein sequence and function but are encoded by the same gene. *PR*-A lacks the N-terminal 164 amino acids of *PR*-B and has only two of the three activation function domains that are present in *PR*-B. Thus, *PR*-B shows a higher transcriptional activity and acts as a more potent transcriptional activator of target genes than *PR*-A.[5,6] Imbalance in the ratio of these two forms of *PR* may be associated with the development and progression of breast cancer.[6–8]

The majority of breast cancers are either positive for *ER* alone or positive for both *ER* and *PR*. Patients that are both *ER* and *PR* positive have a good prognosis[9] and are most likely to benefit from endocrine therapy.[10] Other forms of breast cancer such as *ER* negative/*PR* negative or triple negative breast cancers that lack *ER*, *PR* and human epidermal growth factor receptor 2 (*HER-2*) also occur, though less frequently. *HER-2* is encoded by the *CerbB2* gene (abbreviation for 'v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2') and is also known as the neuro/glioblastoma (c-neu) proto-oncogene that is located on chromosome 17 and encodes a 185 kDa transmembrane tyrosine kinase receptor. Mutations in the *CerbB2/HER-2* gene predict poor outcome in breast cancer and the encoded protein is over expressed in at least 30% of breast cancers diagnosed.[11]

Steroid hormones including oestrogen action can enhance bone formation by blocking bone resorption by osteoclasts and/or promoting the bone anabolic functions of osteoblasts. Breast cancer that metastasises to bone is predominantly osteolytic.[12–16] The Runt-related transcription factor *Runx2*[17] is known to mediate the expression of proteins that supports breast cancer metastasis.[18–22] *Runx2* is essential for osteogenesis and differentiation of osteoblasts.[23,24] Oncogenic properties of *Runx2* have also been demonstrated in T-cell lymphomas[25] and prostate cancer.[26] Apart from oncogenic functions upon ectopic expression, endogenous expression of *Runx2* in osteoblasts is linked to control of cell growth[27–30] and its expression in mammary epithelial cells may support normal breast development.[31,32] Other reports have suggested links between steroids and *Runx2*.[25,33,34] Steroids such as glucocorticoids alter the expression of genes associated with osteoblast function at low doses,[35] while high doses of this adrenal hormone cause bone loss and fractures.[36,37] Glucocorticoids have inhibitory effects on *Runx2* function by depleting its nuclear accumulation.[38] *Runx2* has also been linked with *ERα* and both were shown to interact through the *ERα* DNA binding domain. Oestrogen enhances *Runx2* activity in dose and oestrogen receptor-dependent ways. Notably, deletion of the DNA binding domain of *ER*, eliminates the stimulatory effects of oestrogen on *Runx2* activity.[39] *ER* and *PR* serve as the basis for many therapeutic interventions in breast cancer and *Runx2* has been implicated as a regulator of early metastatic events in this cancer. However, links between steroid hormone signalling and pathological functions of *Runx2* in breast tumourigenesis remain to be explored.

In this study, we investigate the relationship between *Runx2* and the two main female hormone receptors, *ER* and *PR*, as well as the oncogene *CerbB2* (*HER-2/c-neu*), in a panel of breast cancer clinical samples. Our results show that nuclear presence of *Runx2* is significantly and positively correlated with *ER-PR* positive breast cancer tissues in Grade 2 and Grade 3 tumours. Our results provide direct clinical evidence indicating a biological role of *Runx2* in the pathology of steroid hormone-related breast cancers.

2. Materials and methods

2.1. Cell culture and transfections

Two breast cancer cell lines, MDA-MB-231 and MCF-7 cells and an osteosarcoma cell line, Saos2 (ATCC, Manassas,VA,USA) were cultured in DMEM supplemented with 10% foetal bovine serum. MDA-MB-231 and MCF-7 cells were transfected with either *ER* or *Runx2* pcDNA3 expression vector using Fugene 6 (Roche, Basel, Switzerland) and Lipofectamine 2000 transfection reagent (Invitrogen Corporation, CA, USA), respectively. The empty vector (pcDNA3) was used as a control in both cases. Three days post transfection, protein and RNA were extracted for Western blot analysis and quantitative real-time PCR, respectively.

2.2. Western blotting

All the three cell lines were first checked for the endogenous levels of *ER*, *PR* and *Runx2*. Osteosarcoma cells were used as *Runx2* positive controls. Cells were lysed with lysis buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 62.6 mM Tris HCl, pH 6.8), briefly sonicated and pre-cleared with high speed centrifugation. Supernatants were loaded and proteins were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane. Proteins were probed with anti-*Runx2* (MBL International, Woburn, MA, USA), anti-*ER*, anti-*PR* and anti-β-actin (Santa Cruz Biotechnology Inc., CA, USA) primary antibodies followed by appropriate horse radish peroxidase conjugated to anti-rabbit or

anti-mouse secondary antibodies and detected with luminol on exposure to X-ray film.

2.3. Quantitative PCR

For quantitative PCR measurements, total RNA from the transfected cells (MCF-7 and MDA-MB-231) was extracted and reverse transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems, USA). The cDNA (15 ng) from these cells was amplified in an ABI 7300 Prism system using primers of target genes and Fermentas Maxima SYBR Green qPCR Master Mix. Cycling conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 20 s at 72 °C. GAPDH was used as an internal control. The sequences of the forward and reverse primers of the genes were as follows:

GAPDH_Fwd:5′ GAGTCCACTGGCGTCTTCA 3′
GAPDH_Rev: 5′ GTTCACACCCATGACGAACA 3′
*ERα*_Fwd: 5′ CCTATCTCAGGGAGGGAAGG 3′
*ERα*_Rev: 5′ TCTCCAAGTCCCACTCTGCT 3′
*ERβ*_Fwd: 5′ CACCAACGAGTGCGAGATCA 3′
*ERβ*_Rev: 5′ TCCCCACTTTGAGGCATTTC 3′
*PR*_Fwd: 5′ GATTCAGAAGCCAGCCAGAG 3′
*PR*_Rev: 5′ GACCTTACAGCTCCCACAGG 3′
*Runx2*_Fwd: 5′ GGAGTGGACGAGGCAAGAGTTT 3′
*Runx2*_Rev: 5′ AGCTTCTGTCTGTGCCTTCTGG 3′

2.4. Patient samples

Tissue microarray (TMA) sections from 123 breast cancer patients (invasive ductal carcinomas representing all grades and stages) and 2 normal breast tissues (controls) were obtained from the department of pathology, National University Hospital, Singapore upon ethics approval (B06/006) by the institutional review board. These were constructed according to previously reported methods,[40] representing all grades and stages of the commonest breast cancer (the invasive ductal carcinoma type). The median age of patients was 52 years (range 29–86). The distribution of patients according to the three most common ethnic groups in Singapore showed that they were of Chinese (81%), Malay (15%) or Indian (4%) descent. Histopathological staging of BC was based on the TNM staging system[41] and grading[42] of tumours. Patients that were alive and being followed up in the hospital after initial diagnosis and surgery and at the time of our data analyses were 91/123 (74%), those that did not follow up were 14/123 (11%) and those that died of the disease were 18/123 (15%).

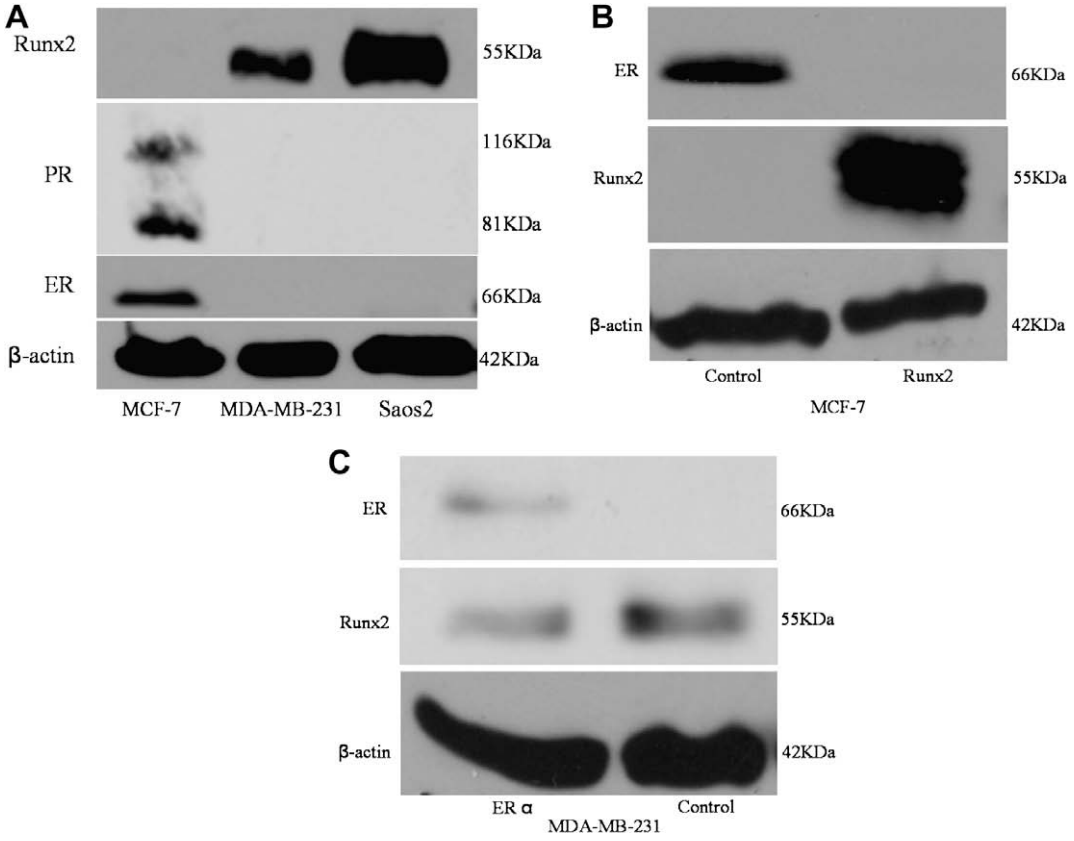

Fig. 1 – (A) Endogenous protein expression levels of ER and *Runx2* in breast cancer cell lines, MCF-7 and MDA-MB-231 [A less aggressive cell line, MCF-7 expressed ER but much lower levels of *Runx2*. Conversely, a highly metastatic, MDA-MB-231 was ER negative but showed an elevated level of *Runx2* expression. Saos2 was included as a positive control for *Runx2* expression. β-Actin protein was used as a loading control]. (B) Transfection of *Runx2* in the *Runx2* negative MCF-7 cell line, suppressed expression of ER. (C) Exogenous expression of *ER* in *ER* negative MDA-MB-231 cells, downregulated *Runx2* expression level.

2.5. Immunohistochemistry

Immunohistochemistry was performed on breast cancer TMAs constructed and stained according to our protocol described previously with minor modifications.[40] Briefly, sections were microwaved at 98 °C for 20 min in citrate buffer (pH 6.0) (DAKO, Denmark) followed by peroxidase blocking for 1 h at room temperature. Anti-*Runx2* mouse mAb[43] at 1:500 dilution, *ER*1D5 antibody at 1:1000, for *ER* detection, NCL-PGR1A6 at 1:1000 for *PR* and Hercep Test K5204 antibody at 1:500 for *CerbB2*, (DAKO, Denmark)[44] were used as primary antibodies, incubated for 2 h at room temperature followed by diaminobenzidine (DAKO, Denmark) staining and detection.

2.6. Statistical analysis

Runx2, *ER*, *PR* and *CerbB2* immunostained TMA sections were visually scored by two independent observers (AG and MST) that were blinded to clinical outcome of the patients. The staining was semi-quantitatively assessed on a scale ranging from 0 to 3 (0-no staining; 1-weak staining, 2-moderate staining and 3-strong staining) following our previously published protocols for Runx family of proteins[43] and also *ER*, *PR* and *CerbB2* proteins.[44] The status *CerbB2* positive and *CerbB2* negative in this study is scored by immunohistochemistry following standard practice, with +0 and +1 representing negative staining and +2 and +3 representing positive. In our hands, this scoring method in TMA is equivalent to the scoring in full sections (see Ref. [44]) and also to HER2 status by FISH.

For statistical analyses negative and weak nuclear or cytoplasmic staining were grouped together and termed as 'negative expression' of the proteins and moderate and strong nuclear or cytoplasmic staining was termed as 'positive expression' of *Runx2*. Fisher's exact test was used to test the association between *Runx2* expression and the clinico-pathological parameters of breast cancer. Survival analysis was performed according to Kaplan and Meier method. All statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago IL). The p values were from two-sided tests and $p < 0.05$ was considered significant.

3. Results

3.1. *Runx2* is inversely correlated to ER status in breast cancer cell lines

We analysed the relationship between *Runx2*, *ER* and *PR* genes *in vitro* by selecting two breast cancer cell lines, MCF-7 and the

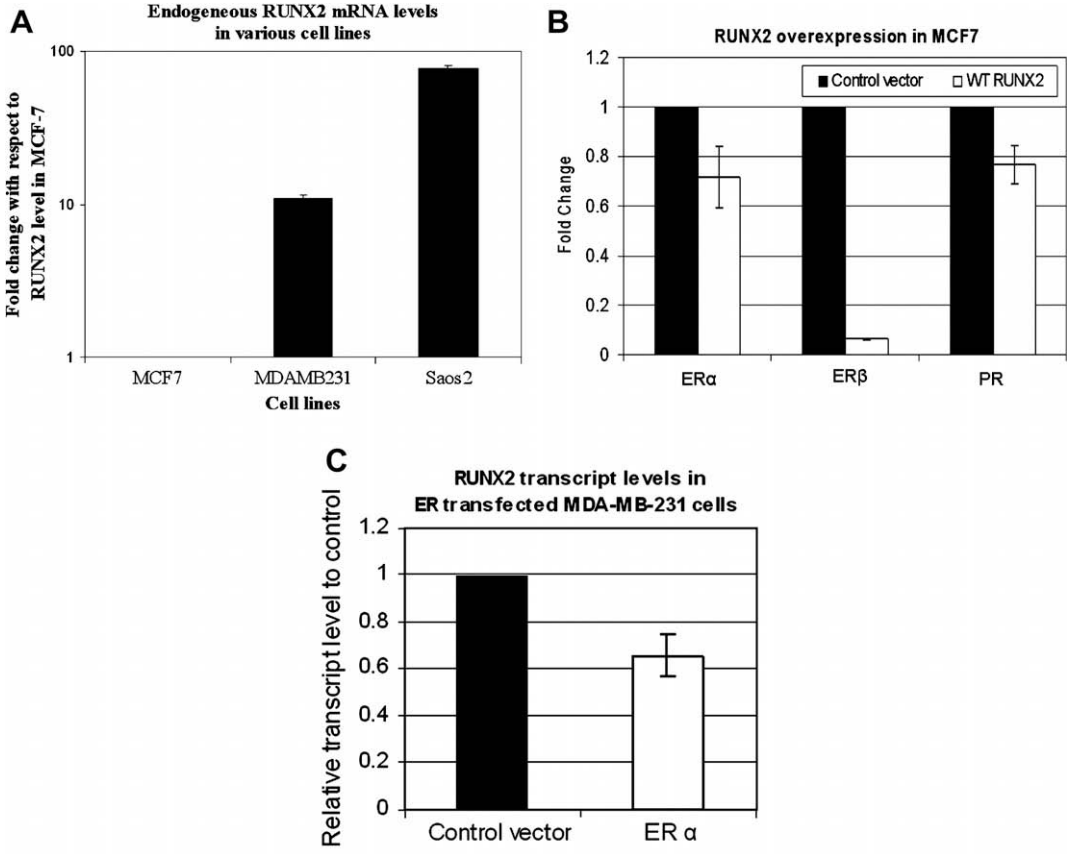

Fig. 2 – (A) Endogenous levels of *Runx2* mRNA in breast cancer cell lines, MCF-7, MDA-MB-231 [Osteosarcoma cell line, Saos2 was used as a positive control] (B) Quantitative RT-PCR graph functional consequences of *Runx2* up and downregulation in MCF-7 cells [*Runx2* over expression in MCF-7 cells showed a similar trend at the transcript level as observed in the protein levels in Fig. 1B] (C) Quantitative RT-PCR graph showing the *Runx2* mRNA level in MDA-MB-231 cells. [Exogenous over expression of ERα in MDA-MB-231 cells downregulate transcript levels of *Runx2*].

highly metastatic MDA-MB-231. The cell lines were examined for endogenous levels of *Runx2* and *ER* and it was observed that MCF-7 was *ER* positive and negative for *Runx2* while MDA-MB-231 was *ER* negative and positive for *Runx2* expression level (Fig. 1A). This finding is consistent with a requirement for *ER* in early stages of breast cancer[2,4] and the metastatic potential of *Runx2* in later stages.[14,18,19]

To assess whether the relationship between *ER* and *Runx2* reflects regulatory coupling, the cells were then transiently transfected with vectors expressing either *Runx2* or *ER* and the levels of both proteins and mRNA were examined by Western blot analysis and quantitative real-time PCR, respectively. In the *Runx2* negative MCF-7 cell line, transfection of *Runx2* suppressed expression of *ER* (Fig. 1B). In contrast, exogenous expression of *ER* in negative MDA-MB-231 cells appreciably altered the levels of *Runx2* protein (Fig. 1C). The protein data for *Runx2* and *ER* were confirmed by examination of transcripts level. The endogenous *Runx2* mRNA level was low or diminished in *ER* positive cell lines (MCF-7). In contrast, the *ER*-negative cell lines (MDA-MB-231) showed high levels of *Runx2* mRNA (Fig. 2A). Interestingly, exogenous expression of *Runx2* in MCF-7 cells exhibited low levels of *ERα*, *ERβ* and *PR* (Fig. 2B) indicating that *Runx2* effects the expression of the two *ER* isoforms. Similarly, when MDA-MB-231 cells were over expressed with *ERα*, the *Runx2* mRNA level was suppressed (Fig. 2C). Thus, *ER* and *Runx2* expressions are inversely correlated in these cell lines, consistent with their respective roles in early and late stages of breast cancer, and *Runx2* is capable of attenuating *ER* levels.

3.2. *Runx2 is localised in the nucleus and cytoplasm of breast tissues*

To understand the clinical relationships between steroid hormone responsive breast tumours and *Runx2*, we performed *Runx2* immunostaining and analysed the localisation of the protein in a spectrum of breast tumour clinical samples. Of the 123 tumours, *Runx2* nuclear expression was detected in 14 (11%) cases (Fig. 3A), cytoplasmic expression in 24 (19%) cases (Fig. 3B) and concurrent nuclear and cytoplasmic expressions were observed in 20 (16%) cases (Fig. 3C). There was no expression of *Runx2* in 65 (53%) cases. Two normal tissues that were considered as controls showed weak nuclear expression only, consistent with previous data suggesting a role for *Runx2* in specific stages of normal breast epithelial development.[31]

Based on the known role of *Runx2* as a nuclear transcription factor, we propose that nuclear expression of *Runx2* reflects a functionally active form of the protein, whereas its cytoplasmic localisation indicates loss of function in gene regulation at least in a basal state. We do not exclude the possibilities that cytoplasmic *Runx2* may provide a reservoir of sequestered *Runx2* that might translocate to the nucleus only upon stimulation, or simply represents an inactive bystander molecule. We interpreted concurrent expression of *Runx2* in both nucleus and cytoplasm indicating that the protein has retained its transcriptional function, and thus it is at least in part active in gene regulation.

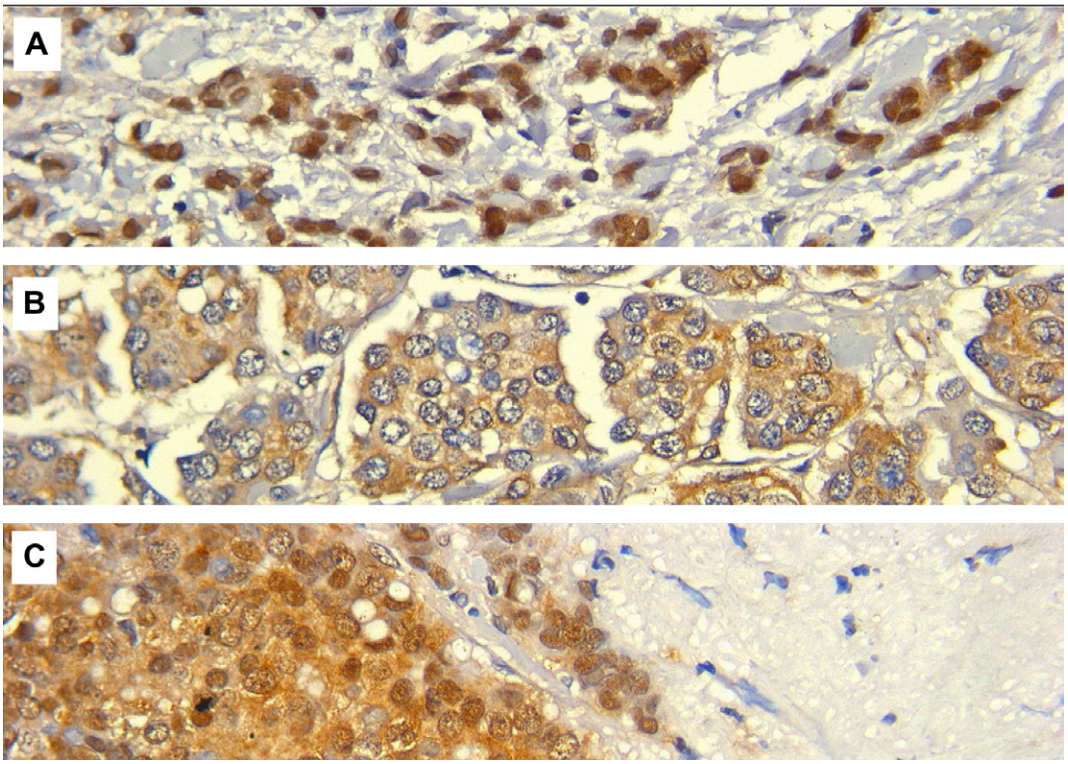

Fig. 3 – *Runx2* immunostaining in breast cancer tissues showing, (A) nuclear staining, (B) cytoplasmic staining and (C) concurrent nuclear and cytoplasmic stainings.

3.3. *Runx2 nuclear protein is strongly associated with ER–PR expression in Grade 2 and Grade 3 breast cancers*

Table 1 shows the correlation of *Runx2* nuclear expression with the pathological parameters in the breast tumours. There was no significant correlation between *ER*, *PR* and *Runx2* nuclear expressions in the breast tumours although *Runx2* nuclear protein was more evident in *CerbB2* negative tumours than in *CerbB2* positive tumours (70% versus 30%) that was nearly statistically significant ($p = 0.092$).

The *ER* and *PR* statuses in the tumours, when compared with pathological grade showed that *ER* was positive for (95%) grade 1 tumours (G1), positivity was 81% in grade 2 (G2) tumours and 47% in grade 3 (G3) tumours that was statis-

Table 1 – Relationship of Runx2 nuclear expression and pathologic features of breast cancer.

Pathologic features of breast cancer	Total cases N = 123	*Runx2* positive	*Runx2* negative	*p* Value
Tumour size		N = 33	N = 90	0.532
<=2 cm	46	15	31	
>2–5 cm	69	16	53	
>5 cm	8	2	6	
Axillary metastasis		N = 33	N = 86	0.521
Positive	45	14	31	
Negative	74	19	55	
Indeterminate	4			
ER		N = 34	N = 89	0.101
Positive	88	28	60	
Negative	35	6	29	
PR		N = 34	N = 89	0.188
Positive	83	26	57	
Negative	40	8	32	
CerbB2		N = 30	N = 75	0.092
Positive	45	9	36	
Negative	60	21	39	
Indeterminate	18			
Histologic Grade		N = 31	N = 81	0.005*
G1	42	12	30	
G2	33	15	18	
G3	37	4	33	
NA	11			
Stage		N = 34	N = 88	0.758
I	36	11	25	
II	60	15	45	
III	19	5	14	
IV	7	3	4	
Indeterminate	1			

* *p* value <0.05 is statistically significant.

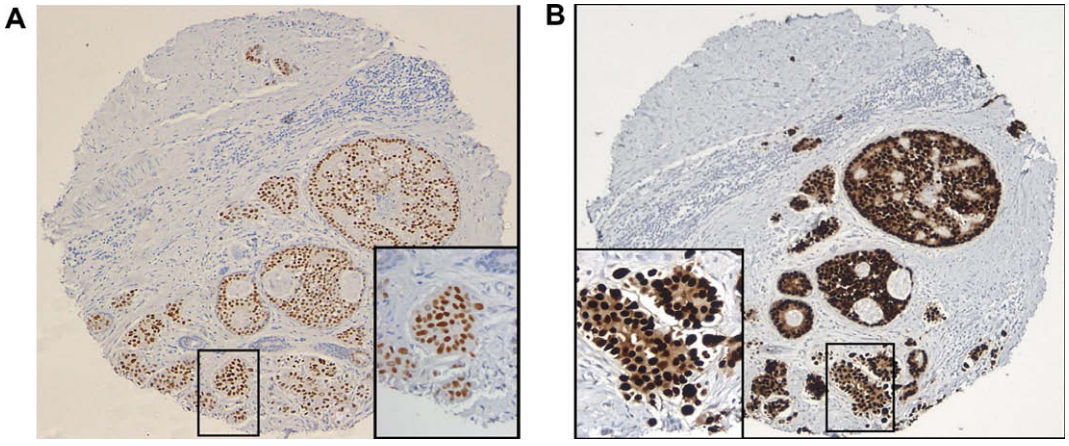

Fig. 4 – ER and PR immunostainings in G2 breast cancer tissues. (A) ER positive staining and (B) PR positive staining.

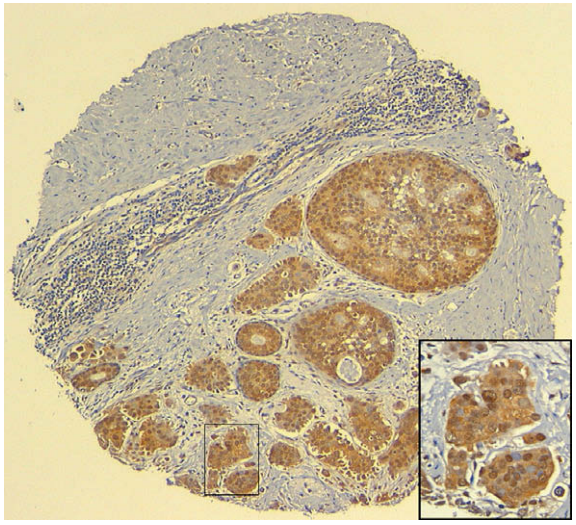

Fig. 5 – *Runx2* positive expression in G2 breast cancer tissue.

tically significant ($p < 0.0005$) (Fig. 4A). Similar relation was also observed between *PR* and tumour grades (91% versus 76% versus 37%; $p < 0.0005$) (Fig. 4B). This inverse relation is consistent with previous studies and consistent with loss of oestrogen-responsiveness in late stage breast tumours (44). Expression of *CerbB2* (HER-2/c-neu) is elevated in tumours of higher grades (15% versus 40% versus 68%; $p < 0.0005$).

We next examined the expression of *Runx2* in different grades of breast cancer. The nuclear expression of *Runx2* varied significantly across the three grades of breast cancer with the highest expression in G2 tumours (Fig. 5). Of the 31 tumours that showed *Runx2* nuclear expression, 48% were G2, 13% were G3 and the remaining 39% represented G1 cancer (Table 1). The *Runx2* expression was then correlated with *ER* and *PR* positivity in different tumour grade and a statistically significant association was observed between them in G2 and G3 tumours compared to G1 tumours (Table 2). However, *CerbB2* showed a negative correlation with *Runx2* expression in G2 and G3 tumours which was also significant (Table 2). In other words, *Runx2* was more frequently expressed in *CerbB2* negative tumours than the positive ones (39% versus 17%). There were no significant differences between *Runx2* expression and different breast cancer stages or with axillary lymphnode metastasis. *Runx2* cytoplasmic expression also did not show any significant difference when compared with the pathological parameters. Our statistical analyses did not show any correlation with patient survival. This is fully consistent with the predominant intermediate grade of *Runx2* expression in breast cancer.

4. Discussion

There are breast cancer conditions as reflected by cell lines in our study that are *ER* positive and *Runx2* negative as observed in MCF-7 and those that are *ER* negative and *Runx2* positive in MDA-MB-231 cell lines. Reciprocal relationship between *Runx2* and *ER* expressions was observed at both the mRNA and protein levels suggesting that high levels of *Runx2* may be effecting the expression of *ER* in MDA-MB-231 cell lines. Because *Runx2* and *ER* can synergise in biological processes (e.g. they are both bone anabolic), one key question is whether there is an *in vivo* transition in breast cancer in which both

Table 2 – Correlation between ER, PR and Runx2 nuclear expressions in breast tumour grades.

Tumour grade type	*Runx2* positive (%)	*Runx2* negative (%)	*p* Value
G1	N = 12	N = 30	
ER			
Positive	27	73	0.492
Negative	50	50	
G2&G3	N = 19	N = 51	
ER			
Positive	39	61	0.005*
Negative	8	92	
G1	N = 12	N = 30	
PR			
Positive	26	74	0.319
Negative	50	50	
G2&G3	N = 19	N = 51	
PR			
Positive	39	61	0.008*
Negative	10	90	
G1			
CerbB2	N = 8	N = 19	
Positive	25	75	0.826
Negative	30	70	
G2&G3			
CerbB2	N = 19	N = 49	0.041*
Positive	17	83	
Negative	39	61	

* *p* value <0.05 is statistically significant.

factors can co-exist. We tested this by co-staining breast cancer clinical samples for both *ER* and *Runx2* and assessed the biological role of *Runx2* in all grades of this disease together with the hormone receptors.

Runx2 and *ER* expression results were similar to those of the cell lines in G1 tumours and G3 tumours while in G2 tumours, *Runx2*, *ER* and *PR* expressions were increased significantly and there was a significant positive association between *Runx2* nuclear protein and *ER* and *PR* expressions. These data suggest that *Runx2* is expressed early on during tumour growth and development.

A link between oestrogen and *Runx2* has been reported through an increase in the number of *Runx2* expressing cells.[45] Selective *ER* modulators, raloxifene having different modes of action from oestrogen enhanced *Runx2* promoter activity.[46] To bind to the target gene promoters, nuclear import of transcription factors is an essential element of the molecular pathways and when these proteins are present in the cytoplasm, they are functionally inactive and may act as bystanders or as a reservoir of unstimulated forms. Runx activity is regulated by tissue-specific expression and also by those elements that control its subcellular localisation. Cytoplasmic and nuclear localisations have been shown for Runx3 in gastric cancer[47] and colon cancer[48] and *Runx2* in osteosarcoma cells.[49] Besides nuclear and cytoplasmic expressions of *Runx2*, our study also showed concurrent expression of this protein in the two subcellular components in 16% of the tumours and more frequently in the G2 tumours. This reflects ectopic induction of *Runx2* expression during breast cancer progression. Although *Runx2* expression was prevalent during tumourigenesis of mammary epithelial cells as evidenced by our breast cancer tissues, only a subset of tumours showed nuclear localisation that would enable it to regulate its target genes.

Strikingly, the nuclear staining correlates with '*ER* positivity' suggesting that both factors synergise to stimulate tumour growth and development at one or more specific stages. Our observation that 32% of *ER*+ breast cancer clinical samples express *Runx2* ($n = 88$) is consistent with a pathophysiological role of ectopic *Runx2* expression. However, because only 28% of breast cancer tissue exhibits nuclear localisation of *Runx2* ($n = 123$), it appears that *Runx2* is not actively participating in gene regulatory events, although we cannot exclude the possibility that *Runx2* may perturb gene regulatory events by its cytoplasmic localisation (e.g. sequestering of key co-factors like Smad and Yap that transduces TGF-beta and Src/Yes-related signals, respectively).

ER and *Runx2* are both known to contribute to carcinogenesis independently. However, both proteins can also synergise at the molecular level in a manner that may reinforce their biological functions in the pathology of breast cancer. The co-expression of *ER* and *Runx2* that we have established in this study suggests that both proteins may have biological synergism that may be further supported by molecular synergy. *In vitro* studies have shown that the expressions of *ER* versus *Runx2* are inversely correlated,[50] parallel to what we have observed. But the novelty of our study is that we also found a subset of breast cancer that showed co-existence of *ER* and *Runx2* suggesting the transition from positive to negative or *vice versa* as a gradual process.

Our results show that the *in vivo* disease is much more heterogeneous than the perceptions based on cell culture studies, highlighting the importance of analysing clinical samples to understand the pathological linkages between different classes of transcription factors such as *ER* and *Runx2*. Indeed, our full analysis showed the cases with this reciprocal relation but, in addition, there were three important subclasses of breast cancer: *ER+/PR+/Runx2-*, *ER+/PR+/Runx2+* and *ER-/PR-/Runx2+* (in order of disease progression). We propose that *ER+/PR+/Runx2+*, triple positivity defines a biological subtype in breast cancer.

In summary, our results stress the idea that solid tumours including breast cancer, are not biologically homogenous disease and defining the biological nature of potential subgroups allows a better understanding of the disease. Finally, it is interesting to note that a defining biomarker of metastasis such as *Runx2* is predominantly expressed in G2 breast cancers. Further investigation of its relationship with *ER* and *PR* may lead to a better understanding of the molecular processes related to breast cancer metastasis.

Conflict of interest statement

None declared.

Acknowledgements

We would like to thank Dr. Doris Mayer, German Cancer Research Center, Germany for the kind gift of ER expression plasmid. Our study was supported by SCS Grants MN05 and MN077 (to MST), NIH Grants P01CA82834 (to G.S.S.) and AR049069 (to AJvW.) and NUS ARF R-364-000-089-112 (to DTL).

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