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Annexins in human breast cancer: Possible predictors of pathological response to neoadjuvant chemotherapy

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ABSTRACT

Neoadjuvant chemotherapy is used in women who have large or locally advanced breast cancers. However, up to 70% of women who receive neoadjuvant chemotherapy fail to achieve a complete pathological response in their primary tumour (a surrogate marker of long-term survival). Five proteins, previously identified to be linked with chemoresistance in our *in vitro* experiments, were identified histochemically in pre-treatment core needle biopsies from 40 women with large or locally advanced breast cancers. Immunohistochemical staining with the five proteins showed no single protein to be a predictor of response to chemotherapy. However, pre-treatment breast cancer specimens that were annexin-A2 positive but annexin-A1 negative correlated with a poor pathological response ($p = 0.04$, Fisher's exact test). The mechanisms by which annexins confer chemoresistance have not been identified, but may be due to inhibition of apoptosis. Annexin-A1 has been shown to enhance apoptosis, whilst annexin-A2, by contrast, inhibits apoptosis.

1. Introduction

Neoadjuvant chemotherapy (NAC) is being used increasingly in women with breast cancer, particularly in those who have large [(L) 3–5 cm] or locally advanced [(LA) T3, T4, N2] breast cancers (BCs).[1] The advantages of NAC are to downstage the BCs, thereby, increasing the likelihood of breast conserving surgery (up to 50%) and reducing systemic micrometastatic tumour load, and thus possibly improving long-term survival.[2,3] Moreover, NAC can provide an opportunity to assess the likely outcome in any subsequent adjuvant therapeutic setting.[4] NAC treatment with 4 cycles of adriamycin and cyclophosphamide, followed subsequently by 4 cycles of docetaxel, has demonstrated a good overall clinical response (up to 90%).[4] However, the complete pathological response (cPR) rate is far less (up to 30%).[4–6] NAC is a toxic and expensive treatment. Therefore, it would be clinically very beneficial to patients and satisfying to medical personnel if it was possible to predict the likelihood of response to NAC, thereby, selectively targeting treatment to those likely to benefit and preventing those patients unlikely to respond from undergoing expensive, toxic, prolonged and unnecessary treatment.

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However, to date, there is no study that has reported accurate and reliable markers to predict pathological response to NAC.

Previously, we had documented *in vitro* a group of proteins, using two-dimensional gel electrophoresis (2-DE) and MALDI-TOF mass spectrometry, demonstrating differential expression in chemosensitive and chemoresistant MCF-7 breast cancer cell lines.[7] These proteins suggested a possible prediction profile in women with breast cancer, who were about to undergo NAC. In the study reported here, five proteins of interest, which are selected based on the results obtained from our previous proteomic studies and hypothesised to be putative biomarkers of prediction of response to chemotherapy, were investigated using immunohistochemical (IHC) staining of pre-treatment core needle biopsies (CNBs) from human breast cancers. These included proteins showing up- or down-regulation, proteins common to the two breast cancer cell lines (resistant to adriamycin and paclitaxel), proteins to which antibodies were commercially available for the research use in human tissues, proteins associated with different functions, as well as proteins that had not previously been documented in published studies. The results of the IHC staining in the pre-treatment tissues were correlated with the pathological response rates obtained after completion of the NAC.

2. Materials and methods

2.1. *Cell lines and cell clot*

The MCF-7 wild type (MCF-7/WT, human breast adenocarcinoma) cell line was purchased from the European Collection of Cell Cultures (ATCC Number HTB-22). The MCF-7

Table 1 – Clinical details of the 40 patients investigated in the study.

Patient	Age (years)	Tumour grade	Staging (TNM)	Operation	Pathological response in breast	ER	IHC staining	
							Ann-A1	Ann-A2
1	57	2	T_2N_0	Rt WE	Poor	+	–	+
2	49	3	T_3N_1	Rt MT	Poor	–	–	+
3	60	3	T_4N_1	Lt MT	Complete	+	–	–
4	56	3	T_2N_0	Rt WE	good	+	–	–
5	66	3	T_3N_1	Lt MT	Complete	+	–	–
6	59	3	T_3N_1	Lt MT	Poor	–	–	+
7	58	3	T_2N_0	Rt WE	Complete	–	–	–
8	51	3	T_3N_0	Lt MT	Poor	+	–	–
9	52	2	T_4N_2	Rt MT	Complete	+	–	–
10	49	3	T_2N_1	Lt WE	Poor	+	–	–
11	50	3	T_3N_0	Lt MT	Poor	+	–	–
12	47	3	T_2N_0	Rt WE	Complete	+	+	+
13	39	3	T_2N_0	Rt MT	good	+	–	–
14	54	3	T_2N_1	Lt WE	Complete	+	–	–
15	42	3	T_3N_0	Lt MT	Poor	–	+	+
16	42	3	T_2N_1	Rt WE	Complete	+	+	–
17	50	3	T_3N_0	Rt MT	Poor	+	–	+
18	38	3	T_2N_0	Lt WE	Complete	+	+	–
19	60	3	T_3N_0	Lt WE	Complete	+	+	+
20	35	3	T_2N_0	Rt MT	Complete	+	+	+
21	57	3	T_2N_0	Rt MT	Complete	–	+	+
22	37	3	T_2N_0	Rt WE	Poor	+	–	–
23	60	2	T_2N_1	Rt WE	Poor	+	–	+
24	53	3	T_2N_1	Lt WE	Complete	+	+	+
25	48	3	T_2N_1	Lt WE	Complete	–	+	+
26	37	3	T_2N_0	Lt WE	Poor	+	+	+
27	38	3	T_2N_0	Rt WE	Complete	+	–	–
28	50	3	T_2N_0	Rt MT	Complete	+	–	+
29	51	3	T_2N_1	Lt WE	Poor	+	+	–
30	46	3	T_3N_0	Lt WE	Complete	+	+	–
31	47	3	T_2N_1	Rt WE	Complete	+	–	–
32	49	2	T_2N_0	Rt WE	Complete	+	–	–
33	44	3	T_2N_0	Lt WE	Poor	+	–	–
34	59	2	T_3N_0	Lt MT	Poor	+	–	–
35	48	2	T_2N_1	Lt WE	Poor	+	+	–
36	55	3	T_3N_0	Rt WE	Poor	+	+	–
37	45	3	T_2N_0	Lt WE	Complete	–	+	ND
38	37	3	T_2N_0	Rt WE	Poor	–	+	–
39	40	3	T_2N_0	Rt WE	Complete	+	+	+
40	77	3	T_4N_1	Rt MT	Poor	+	+	–

ER = oestrogen receptor; IHC = immunohistochemical; Rt = right; Lt = left; WLE = wide local excision; MT = mastectomy; and ND = no data.

adriamycin-resistant subline (MCF-7/ADR) and the MCF-7 paclitaxel-resistant subline (MCF-7/PAC) were kind gifts from Dr. Timothy Gant (MRC Toxicology Unit, University of Leicester, UK)[8] and Dr. Susan Bates (National Cancer Institute, USA),[9] respectively. All cell lines were grown, subcultured and tested for resistance to chemotherapeutic agents, as described in our previous study.[7]

Cell pellets of MCF-7/WT, MCF-7/ADR and MCF-7/PAC (5 X10^6 cells each) were made to clot, were wax, embed and cut onto the glass slides. Briefly, cell pellets were resuspended with sterile phosphate-buffered saline (PBS), and were mixed with 1000 μl of Cryocheck™ plasma (Precision Biologic, Nova Scotia, Canada) and 1000 μl of thromboplastin (HemosIL™, Instrumentation Laboratory, Lexington, USA). The prepared solution was allowed to clot at room temperature for 30 min. Then, neutral buffered formaldehyde (Genta Medical, York, UK) was added to fix the clotted cells. The clotted cells were subsequently processed on an ASP300 tissue processor™ (Leica Microsystems, Nussloch, Germany). The paraffin-embedded blocks with clotted cells were then cut in serial 5 μm sections, and were mounted onto SuperFrost™ adhesive-coated slides (Menzel Gläser, Braunschweig, Germany).

2.2. Patients and core needle biopsy specimens

Forty women with LLA invasive ductal carcinoma of the breast, diagnosed between 2004 and 2006, were included in the study. All patients had T2 (3–5 cm), T3 or T4 cancers, or had palpable or matted axillary lymph nodes (N1B or N2). Clinical details of all the 40 patients in the study are shown in Table 1. The study had ethical approval from the Local Research Ethics Committee. The study was conducted in accordance with the Helsinki Declaration.

Prior to chemotherapy treatment and at the time of first presentation with suspected breast cancer, CNBs were performed under ultrasonographic guidance, and the specimens were kept fixed in formaldehyde before processing through the paraffin fixation processes. The clinical data relevant to the patients involved in the study were anonymised, the investigator being 'blind' to these characteristics. A third person, who was a research nurse in the study, kept these data. The clinical data were 'unblinded' at the end of the study. The specimens used were from patients who had been assessed as good/complete and poor responders to NAC.

All patients in the study received the same NAC regimen containing four courses of adriamycin (60 mg/m^2) and cyclophosphamide (600 mg/m^2), and followed sequentially by four courses of docetaxel (100 mg/m^2). During the NAC treatment, prior to every cycle of chemotherapy, clinical responses of the tumours were assessed by calliper measurement and these were recorded. The final clinical response was also evaluated by ultrasonographic and mammographic measurements. The final pathological response was assessed microscopically on the surgical specimen of tumour that was removed during the definite surgical procedure by an independent consultant pathologist.

Briefly, the histopathology of the initial diagnostic CNBs was compared with that of the resected tumour specimens. The pathological response was graded as complete (DCIS accepted), good and poor pathological response, depending on the percentage reduction in tumour cell volume. The absence of all viable tumour cells in the surgically removed specimens was considered to be a complete response (cPR); residual DCIS was accepted. Eighty percent or more reduction of tumour cell volume was considered as a good pathological response. A reduction of less than 80% of the tumour cell volume in the primary breast cancer was graded as a poor pathological response (Table 2).

2.3. Immunohistochemical (IHC) staining of cell clots and CNBs of human breast cancers

Evaluation of the expression of proteins of interest in the cell clots and CNBs was assessed using automated IHC staining of the formalin-fixed, processed, paraffin-embedded cell clots and CNBs mounted on the adhesive-coated glass slides. Briefly, the slides were immunohistochemically stained using anti-annexin-A1 (1:800, BD Biosciences, Oxford, UK), anti-annexin-A2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-sorcin (1:600, Zymed Laboratories, San Francisco, CA, USA), anti-stathmin (1:2000, Abcam) and anti-heat shock protein 27 (HSP27, 1:100, Abcam) antibodies. Slides were processed for microwave antigen retrieval (MAR) in 0.01 M, pH 6, citrate buffer solution (Acros, NJ, USA) for 20 min. The staining process was carried out using a Biogenex I6000™ automated staining machine (Launch diagnostics, Kent, UK), using the avidin-biotin method. A second biotinylated antibody (Vector Laboratories, Peterborough, UK) was added to the slides and incubated for 20 min, followed by incubation in horseradish peroxidase substrate (Vector Laboratories) for 20 min, haematoxylin for 1 min and DAB solution (DAKO, Denmark) for 15 min. In each automated run, positive and negative control slides were included.

Table 2 – Definition of pathological response used in the study.

Response	Definitions
Complete pathological response (cPR)	No viable invasive breast cancer cells identifiable in sections from the site of the previous tumour
Good pathological response	Marked disappearance of invasive breast tumour cells (reduction > 80%); small clusters of widely dispersed viable cells detected
Poor pathological response	Moderate reduction of invasive breast tumour cells (reduction < 80%); minor loss of invasive tumour cells, or some alteration to individual malignant cells, but no reduction in overall number of cancer cells

Table 3 – Results of IHC staining of MCF-7 cell clots.

Proteins evaluated	Intensity of IHC staining in cell clot (grades 0-3)		
	MCF-7/WT	MCF-7/ADR	MCF-7/PAC
Annexin-A1	0	3	3
Annexin-A2	0	3	3
Sorcin	1	3	3
Stathmin	2	3	3
HSP27	3	2	2

2.4. *Evaluation of staining*

The IHC staining of the cell clots and CNBs of human breast cancer tissues was read independently by two investigators, without any information as to the clinical data on each of the patients concerned. The assessment was carried out using a scoring system of 0–3 (0: no staining; 1: <10% of cells staining; 2: 10–49% of cells staining; and 3: >50% of tumour cells staining). Any disputed scores were subsequently viewed on a double-headed microscope until a consensus staining score was achieved. Tumours with grades 0 and 1 staining

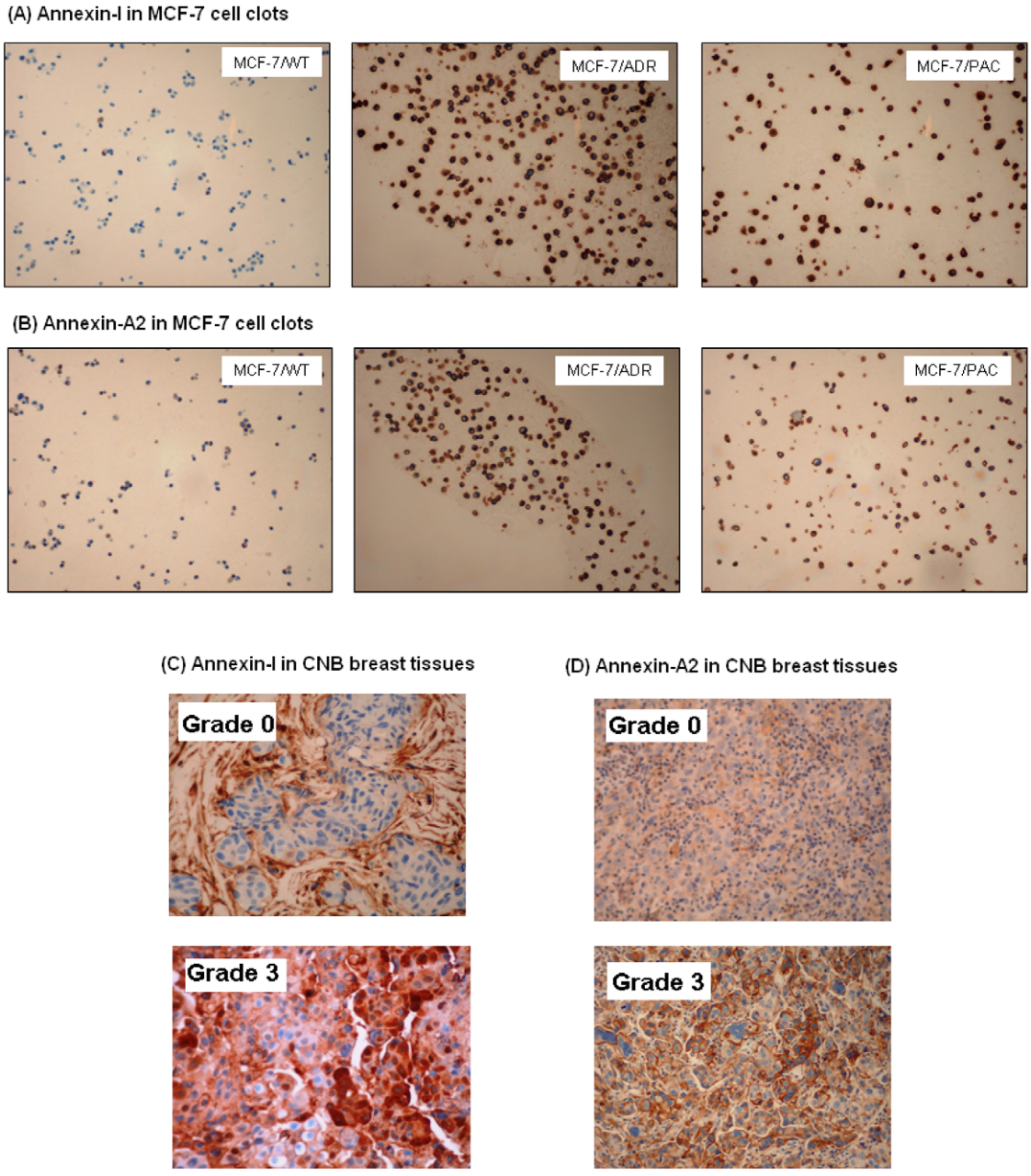

Fig. 1 – Representative images of IHC staining of (A) adriamycin- and (B) paclitaxel-sensitive and resistant MCF-7 cell clots (20×). The intensities of staining with anti-annexin-A1 and anti-annexin-A2 antibodies were of grade 0 in MCF-7/WT and of grade 3 in MCF-7/ADR and MCF-7/PAC. In addition, representative images of IHC staining in CNBs with (C) anti-annexin-A1 and (D) anti-annexin-A2 antibodies are shown; intensities of staining in tumour cells are indicated.

Table 4 – Correlation between various clinical characteristics versus pathological response and expression of annexins.

	p-Value (χ^2 test)		
	Pathological response ($n = 40$)	Annexin-A1 expression ($n = 40$)	Annexin-A2 expression ($n = 39$)
Age (<50 versus >50)	0.53	0.52	0.65
AJCC stage (stage II versus stage III)	0.79	0.13	0.77
Tumour grade (2 versus 3)	0.25	0.11	0.78
Oestrogen receptor status (positive versus negative)	0.75	0.27	0.02

AJCC: American Joint Committee on Cancer.

Table 5 – Results of IHC staining with five specific antibodies in 40 pre-treatment CNBs of breast cancer tissues (22 complete/good responders and 18 poor responders).

Proteins evaluated	Complete/good responders ($n = 22$)		Poor responders ($n = 18$)		p-Value (χ^2 test)
	Positive staining	Negative staining	Positive staining	Negative staining	
Annexin-A1	11	11	7	11	0.43
Annexin-A2[a]	7	14	8	10	0.48
Sorcin	8	14	9	9	0.39
Stathmin[b]	17	4	12	5	0.46
HSP27[a]	10	11	8	10	0.84

a $n = 39$.
b $n = 38$.

were considered as showing negative staining; whereas tumours with grades 2 and 3 staining were regarded as positive staining.

2.5. *Statistical analysis*

Chi square and Fisher's exact tests with SPSS13.0 software (SPSS, Chicago, IL) were used to analyse correlations between positivity of the immunohistochemical staining for the annexins and various clinical characteristics (age, AJCC stage, tumour grade and oestrogen receptor status), as well as any possible correlations between the pathological response documented in the tissue samples and the positivity of immunohistochemical staining for the annexins.

3. Results

3.1. *IHC staining of MCF-7 cell clots*

The results of staining of the MCF-7 cell clots are shown in Table 3, and their representative images (20× microscopic examination) are shown in Fig. 1A and B. Annexin-A1, annexin-A2, sorcin and stathmin were found to be over-expressed in chemoresistant MCF-7 cells than in chemosensitive MCF-7 cells. In contrast, HSP27 was found to be under-expressed. These findings were comparable to our previous proteomic study.[7]

3.2. *Clinical characteristics versus pathological response and expression of annexins*

There was no correlation between the pathological response documented and the various clinical characteristics, including age, AJCC stage, tumour grade and oestrogen receptor status (Table 4). However, enhancement of annexin-A2 was significantly correlated with the absence of oestrogen receptor expression in the tumour samples ($p = 0.02$, χ^2 test, Table 4).

3.3. *IHC staining of CNBs of breast cancers*

Based on the pathological response after NAC treatment, 22 tumours were classified as complete/good responders, and

Table 6 – Analysis focusing on annexin-A2 positive and negative tumours.

Tumour phenotypes		Pathological response		p-Value (Fisher's exact test)
		Complete/good	Poor	
Annexin-A2 positive	Annexin-A1 negative	1	6	0.04
	Annexin-A1 positive	6	2	
Annexin-A2 negative	Annexin-A1 negative	10	5	0.40
	Annexin-A1 positive	4	5	

18 were graded as poor responders. Of the five proteins tested in the pre-treatment CNBs, no single protein demonstrated a statistically significant ability to predict the pathological response to NAC (Table 5). Examples of positive and negative IHC stainings in CNBs of the breast cancer tissues are shown in Fig. 1C and D. However, when the combination of two proteins was analysed, over-expression of annexin-A2 protein in association with a lack of expression of annexin-A1 in CNBs from women with breast cancer correlated significantly with poor pathological responses ($p = 0.04$, Fisher's exact test). Six patients whose tumours were annexin-A2 (+) but annexin-A1 (–) had a poor pathological response, whereas only one patient who had this phenotypic profile had a cPR (Table 6). However, the reverse tumour annexin expression (annexin-A2 negative and annexin-A1 positive) did not correlate with a good pathological response to chemotherapy. Of the nine tumours where annexin-A2 showed a negative staining and annexin-A1 was positive, four were good responders and five were poor responders.

4. Discussion

Five proteins, significantly over- or under-expressed in our previously reported *in vitro* study on breast cancer cell lines resistant to chemotherapy, were selected for IHC staining.[7] There is an ongoing debate about the reliability of using cancer cell lines as a model for biomolecular cancer research. Breast cancer cell lines have been widely used to investigate various biological mechanisms and signalling pathways, including proliferation, apoptosis, invasion and metastasis of breast cancer cells. These have led to a better understanding as to how the cancer cells regulate these processes, and have produced useful information which subsequently has been used to target cancer therapy at the molecular level. However, breast cancer is a heterogeneous disease, and there is an ongoing debate about the appropriateness of cell lines as a model in this type of research. Studies investigating gene expression profiles in breast cancer cell lines and primary breast cancers indicate that no single cell line is truly representative.[10,11] There are a variety of breast cancer cell lines available for study. We believe that the cell lines we have chosen are appropriate models for our study; as adriamycin and a taxane (paclitaxel or docetaxel) are used in the NAC regimens.

The results of the IHC staining with five specific antibodies in 40 pre-treatment CNBs showed that no single protein was a good predictor of response to NAC. However, the enhanced expression of annexin-A2 and the concurrent decreased expression of annexin-A1 in CNBs of breast cancers were associated with a poor response to NAC. Six of seven women who had over-expression of annexin-A2 but lacked annexin-A1 in their tumours had a poor pathological response to NAC. Albeit the samples were small, the findings were statistically significant. The link between both annexins and chemosensitivity has raised the possibility of using annexin-A2 and annexin-A1 as predictors of response to chemotherapy in women scheduled to undergo NAC treatment. If confirmed in a larger cohort of women, it could have a significant impact on the management of a subset of women with clinical breast cancer. Validating the findings by a larger number of samples is urgently required.

Although preliminary analysis in our study found no correlation between various clinical characteristics and pathological response, enhanced levels of annexin-A2 were observed in oestrogen receptor negative tumours (Table 4). Breast cancers lacking expression of oestrogen receptors had previously been linked with a poor response to chemotherapy.[12] In our study, however, only a small number of oestrogen receptor negative tumours were detected ($n = 8$). Validation in a larger samples size is necessary to confirm this observation.

Annexins are a family of calcium-binding proteins found in various organisms, from humans to moulds.[13] Calcium can form specific complexes with various proteins and, thus, functions as an intracellular messenger through regulation of ionic pore function in cellular membranes. Calcium-binding proteins, including annexins, therefore, are key molecules involved in a number of signalling pathways.[13–15]

Annexin-A1, a calcium-binding protein belonging to the annexin family, had been previously linked with various cancers as a tumour suppressor protein.[16] In one study, decreased levels of annexin-A1 have been documented in human breast cancer, compared with normal and benign breast tissues.[16] However, another study has reported contradictory findings.[17] Thus, the role of annexin-A1 in human breast cancer is still unclear and not characterised in breast cancer drug resistance. There is some evidence suggesting that annexin-A1 may be involved in the regulation of the extra cellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK). Annexin-A1 inhibits cell proliferation by ERK-mediated disruption of the actin cytoskeleton and by ablation of cyclin D1 protein expression.[18]

Enhanced expression of annexin-A2 had previously been reported in human pancreatic carcinoma cells and primary pancreatic cancers, but not in normal pancreatic tissues.[19,20] There is also evidence supporting the roles of annexin-A2 in mediating *in situ* tissue cancer invasion and metastatic dissemination in breast, lung and kidney cancers.[21–23] Annexin-A2 can mediate angiogenesis via the plasminogen/plasmin system, thus, increasing tumour aggressiveness.[22] Plasmin is an active enzyme linked to various processes involved in enhancing tumour biological aggressiveness, including the activation of matrix metalloproteases (MMPs) and growth factors, and the proteolysis of membrane glycoproteins.[24] Annexin-A2, protein sited on the cell surface of breast cancer cells, is a receptor for conversion of plasminogen to plasmin, which ultimately may regulate breast cancer angiogenesis and activate tumour invasiveness.[24] Plasmin is also a key enzyme involved in degradation of extracellular matrix and, consequently, switching on various angiogenesis growth factors including the vascular endothelial growth factors (VEGFs).[24]

The mechanisms by which both annexins confer chemotherapy resistance have not been established. However, there is evidence, in *in vitro* experiments with lung cancer cells, showing that over-expression of annexin-A2 promotes cell proliferation rate via the p53 apoptotic pathway[25]; thereby, over-expression of annexin-A2 may interrupt the apoptotic pathway and, thus, induce chemoresistance. On the other

hand, over-expression of annexin-A1 may enhance apoptosis.[26] Annexin-A1 may mediate engulfment of apoptotic cells when binding with endogenous phosphatidylserine on the cell membrane.[26] This was supported by a study carried out by Fan and colleagues, where the use of monoclonal antibodies against annexin-A1 inhibited phagocytosis of apoptotic lymphocytes.[27] Therefore, the over-expression of annexin-A2 and decreased level of annexin-A1 may result in a significant inhibition of apoptosis. Cancer cell death by NAC occurs substantively through apoptotic pathways. Further studies are required to support these postulates of annexins in chemoresistance.

In the present study, on human breast cancer tissues, HSP27 did not show any association with chemotherapy response rate. The expression of HSP27 had previously been shown to be increased in malignant tissue than in non-cancerous tissue.[28] Also, high levels of HSP27 on IHC staining were associated with biologically aggressive tumours and axillary lymph node metastatic disease in the previous studies in women with breast cancer.[29–31] Interestingly, an inverse relationship has been documented between tumour stage and HSP27 expression, in both breast and renal cell carcinomas.[28,32]

Although over-expression of sorcin, a calcium-binding protein, was found in the paclitaxel-resistant breast cancer cell lines, there was no obvious relationship between the expression of sorcin in our 40 breast cancer CNB tissues. To date, there are no published data on the expression of sorcin in human breast cancers; only two have reported the expression of this protein in breast cancer cell lines.[7,33] It is still interesting and worth studying the role(s) of this protein in breast cancer chemoresistance, since the data on human acute myeloid leukaemia (AML) have shown an enhanced expression of this protein in drug resistant AML patients.[34] As well, the presence of this protein was associated with increasing levels of P-glycoprotein (P-gp).[34]

The function of stathmin has raised an interesting issue for further study, particularly with the use of taxanes as anti-cancer drugs. Taxanes (including paclitaxel and docetaxel) are microtubule stabilisers and bind microtubule polymers, thus promoting cell death by suppressing microtubule dynamics.[35] Stathmin is a cytosolic phosphoprotein, and over-expression of this molecule results in a decreased cellular binding of paclitaxel, thereby causing paclitaxel resistance in *in vitro* cell lines.[36] High levels of stathmin (identified by IHC staining) had previously been linked with poor disease-free survival and poor disease-specific survival in women with early breast cancer.[37] However, there have been no publications linking this protein with chemoresistance in human breast cancer tissues. In this study, 71% of poor responders (12 of 17) had enhanced expression of stathmin in their tumours. However, 81% of good responders (17 of 21) also had stathmin over-expressed in their cancers. As a result, the documentation of stathmin in human breast cancer tissues is not a reliable predictor of response to NAC, and was not able to show any significant correlation between this molecule and chemoresistance.

In conclusion, our study has documented that the enhanced presence of annexin-A2 in conjunction with the absence of annexin-A1 may be a potential predictor of pathological response to NAC in human breast cancer. Should this finding be confirmed in a much larger cohort study, it would be of great benefit to women who are undergoing NAC treatment for breast cancer and would prevent unnecessary severe side-effects in women who are unlikely to benefit from NAC.

Conflict of interest statement

None declared.

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