

Autoantibodies in breast cancer: their use as an aid to early diagnosis

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Background: There is increasing evidence that the immune system produces a humoral response to cancer-derived antigens. This study assessed the diagnostic potential of autoantibodies to multiple known tumour-associated proteins.

Patients and methods: Sera from normal controls ($n = 94$), primary breast cancer patients ($n = 97$) and patients with ductal carcinoma *in situ* (DCIS) ($n = 40$) were investigated for the presence of autoantibodies to p53, c-myc, HER2, NY-ESO-1, BRCA1, BRCA2 and MUC1 antigens by enzyme-linked immunosorbent assay.

Results: Reproducibly elevated levels of autoantibodies were seen in at least one of the six antigens in 64% of primary breast cancer patient sera and 45% of patients with DCIS at a specificity of 85%. No significant differences were seen when patients were subdivided by age, tumour size, histological grade, lymph node status or detection methodology.

Conclusions: Autoantibodies against one or more of these tumour-associated antigens appears to indicate the presence of early-stage breast cancers. Autoantibody assays against a panel of antigens could be used as an aid to mammography in the detection and diagnosis of early primary breast cancer, especially in younger women at increased risk of breast cancer where mammography is known to have reduced sensitivity and specificity.

Key words: antigen, autoantibodies, breast cancer, diagnosis, immunologic tests, tumour markers

introduction

Breast cancer accounts for 22% of all cancers diagnosed in women worldwide [1] with the risk of developing breast cancer increasing with age. About 18% of breast cancer diagnoses are among women in their 40s and ~77% of women with breast cancer are older than 50 when they are diagnosed. While incidence rates have risen considerably over the last 10 years, mortality rates from breast cancer are reducing [2]. This reduction is attributed to a combination of earlier detection through the mass implementation of screening mammography programmes and improved treatment regimes, especially adjuvant therapy. Research has shown that the early detection of small breast cancers, for example by screening mammography, significantly improves a woman's chances of survival [3] and if breast cancer is diagnosed and treated while it is still confined to the breast, the cure rate can approach 100%.

However, screening mammography is far from perfect in terms of uptake by women, sensitivity of cancer detection

(70%–80% across all age groups) and specificity, with a recall rate of ~5%–10%. In only 5%–10% of those women recalled for additional testing will a breast cancer be found, which means that between 4% and 9% of women undergoing screening mammography have a false positive test. Furthermore, while screening mammography results in significantly better breast cancer mortality rates, the relative risk reduction is only ~23% in randomised controlled trials—i.e. >75% of women between 50 and 75 years of age who would have died of breast cancer before screening mammography, will still die of breast cancer, even if they undertake regular screening mammograms. There is therefore a significant clinical need for additional accurate tests for the earlier diagnosis of breast cancer for use as an aid to mammography, particularly for younger women (under the age of 50) in whom mammography is less sensitive [4] and who are at higher risk, for example through a family history of the disease.

Breast cancer is a heterogeneous disease with tumours expressing a variety of aberrant proteins. Current blood tests that identify circulating tumour antigens are elevated most commonly in patients with metastatic disease and appear to reflect tumour bulk. They are too insensitive to be used for the screening and early diagnosis of primary breast cancers [5]. Autoantibodies produced against such tumour-associated

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antigens may provide an *in vivo* amplification of an early carcinogenic signal and therefore may allow earlier detection of cancer than current methods allow. Our group has hypothesised for some years now that the heterogeneity of antigen expression will mean that a panel of assays for autoantibodies of various specificities should provide better sensitivity to breast cancer than a single assay [6].

In this manuscript, the authors have used an enzyme-linked immunosorbent assay (ELISA) to identify the presence of autoantibodies to a panel of cell surface and internal cancer-associated antigens, in patients newly diagnosed with primary invasive breast cancer (PBC) or ductal carcinoma *in situ* (DCIS) and have compared the results to a normal control population. Furthermore, blood samples collected months to years before 'at-risk' women being diagnosed with breast cancer through screening mammograms, have been tested for autoantibodies to assess how early the signal is present in the peripheral blood. The sensitivity, specificity and importantly reproducibility of the assay are reported.

patients and methods

serum samples and patient details

Blood samples were collected from patients attending breast clinics who gave written, informed consent under a study protocol approved by the institutional ethics committee. Blood specimens were allowed to stand before being centrifuged at 1250 g for 5 min, the serum removed and then stored at -20°C . Blood samples were collected preoperatively from 97 patients with newly diagnosed PBC and from 40 patients with DCIS. None of these patients received neo-adjuvant therapy and all had surgery within 4 weeks of initial diagnosis of PBC. Patient details and tumour

Table 1. Patient details and clinical characteristics for invasive breast disease (PBC) and DCIS samples

	PBC	DCIS
Number	97	40
% Female	100	97.5
Mean Age $\pm$ SD (years)	59 $\pm$ 12	59 $\pm$ 11
Age range (years)	30–82	37–86
Mean tumour size $\pm$ SD (mm)	17 $\pm$ 10	27 $\pm$ 23
Tumour size range (mm)	0.5–47	3–110
Size groupings		
<2 cm	64/97	17/40
2–5 cm	33/97	14/40
>5 cm	0/97	9/40
Type		
Invasive ductal cancer	66/97	NA
Other	31/97	NA
Grade (% of total)	1 = 12.6%	Low = 10.0%
	2 = 51.6%	Intermediate = 27.5%
	3 = 35.8%	High = 62.5%
LN status (% positive)	30	0
Vascular invasion (% positive)	26	0
ER status (% positive)	81	NA

PBC, primary invasive breast cancer; DCIS, ductal carcinoma *in situ*; SD, standard deviation; NA, not applicable; LN, lymph node; ER oestrogen receptor.

characteristics are shown in Table 1. *BRCA1* and *BRCA2* gene mutation status were unknown on these patients.

Blood samples were also obtained from 94 normal individuals, 50 were from healthy blood donors with no further clinical information. The remaining samples were gathered from individuals attending the National Health Service Breast Screening Programme who were being returned to three yearly mammography after a routine screen, or were collected from a symptomatic breast clinic from women who were being discharged with no detected abnormality following triple assessment (age range 19–71, mean age 54 ± 11). All PBC and normal samples were collected in the same 12-month period. The DCIS samples were collected over a 5-year period.

antigen production

Specific complementary DNAs for p53, P185^{HER-2} (HER2), c-myc, *BRCA1*, *BRCA2* and NY-ESO-1 were subcloned, along with a small tag, into the Pet21b expression vector (Novagen, Darmstadt, Germany). The recombinant proteins were expressed in BL21(DE3) bacteria (Novagen), grown in CYM media, and purified using His trap affinity columns (Amersham, Uppsala, Sweden) according to manufacturer's protocols. A negative control protein was also produced (small tag alone) under identical conditions. Proteins were refolded according to the manufacturer's protocol and tested for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (silver and coomassie stained [7]) as well as by western blotting with appropriate mouse monoclonal antibodies. Only proteins that were >95% pure were used in the assays. In this study, assessment of MUC1 autoantibodies was made using a MUC1 'VNTR' [8] peptide (Peptide Protein Research Ltd, Fareham, UK) conjugated to bovine serum albumin.

autoantibody detection

Autoantibody detection was by ELISA using microtitre plates coated with recombinant antigen or peptide according to in-house protocols (patent pending). Remaining binding sites were blocked with high salt buffer (HSB) [HSB: phosphate-buffered saline (PBS) + 0.5 M NaCl, 0.2% mass/vol casein]. For all assays, freshly thawed serum samples (diluted 1/100 in HSB) were incubated in triplicate at 50 μl per well for 90 min, as well as appropriate control mouse monoclonal antibodies specific for capture proteins. Horseradish peroxidase (HRP) conjugated rabbit anti-human immunoglobulin (Ig) G/M/A/kappa (Stratech, Soham, UK) or anti-mouse Ig antibodies (Dako, Ely, UK) were used as secondary antibodies at the dilution recommended by the manufacturer. Ready prepared 3,3',5,5'-tetramethylbenzidine (TMB, Chemicon, Chandlers Ford, UK) was used as the chromogenic substrate for HRP and absorbance values were determined after a 10 min period at $A_{650\text{nm}}$. All incubations were carried out with shaking at room temperature and plates were washed four times with PBS containing Tween 20 (0.1% vol) (Sigma, Poole, UK) between each step. All sera were assayed on three to five separate occasions.

statistics

Student *t*-tests were used to identify statistical significant differences between autoantibody measurements in cancers and normals. Chi-squared tests were carried out to identify correlations of positivity with clinical parameters.

results

For each autoantibody assay, positive seroreactivity was defined as an absorbance value greater than the mean + 2 standard deviations (SDs) of the normal cohort. This cut-off level represented a 95% confidence interval and was used in recognition that in any apparently 'normal' population some patients would be harbouring occult malignancies, and

therefore a 95% confidence level might prove to be a clinically more relevant value. Values that were greater than the mean + 5 SDs were removed to produce cut-off values, but were included in analysis of specificity.

All assays were carried out in triplicate on at least three (DCIS) or four (PBC and normal sera) separate occasions. Samples were designated positive for each separate autoantibody assay if there was a reproducible signal above the cut-off level of the normal group. For example, in PBC patients a sample was only deemed reproducibly positive for an autoantibody assay if at least three out of four interassay runs showed an elevated value above the cut-off. Where it was deemed that the result from one of the first four runs was technically unassessable and where only two out of the remaining three assessable runs were elevated, a fifth run was carried out. Assays were deemed to be reproducibly positive if the result agreed in at least three out of four interassay measurements. Reproducibility data for each individual assay are shown in Table 2.

Table 2 also shows levels of detection of autoantibodies against individual antigens in the two disease groups. The normal cohorts were significantly different from the patients with invasive disease for all autoantibody assays except BRCA1 ($P < 0.05$). Individual autoantibodies were raised in between 8% and 34% of PBC sera. Measurement of BRCA1 autoantibodies did not provide any clinically useful information and so this antigen was not included in the panel analyses. Sixty-four per cent of patients with early operable primary breast cancer had elevation in the peripheral blood of at least one of the six remaining autoantibodies (Table 2). These data when subdivided by patient age (Table 3), tumour grade (Table 4), lymph node status or method of presentation (i.e. mammographic screening or symptomatic) (data not shown) showed no significant difference. There was also no significant difference by tumour size, oestrogen receptor status or Nottingham Prognostic index [9] (data not shown).

Within the DCIS cohort, 45% of patients had elevation of one or more autoantibodies (Table 2). Individual autoantibodies were raised in between 3% and 23% of PBC sera. Positivity was again independent of patient age (Table 3), tumour size or grade (Table 4). Interestingly, one of the autoantibody-positive DCIS cases progressed within 2 years to a locally advanced form of the disease.

Specificity was calculated as the number of true negatives expressed as a percentage of the number of true negatives plus false positives in the normal control population. Individual assay specificity for each antigen varied from 91% to 98%. Combination of the assays showed that the overall specificity of the panel was 85% (Table 2).

discussion

The sensitivity of individual assays was between 8% and 34% for PBC and 3% and 23% for DCIS with specificities between 91% and 98%. This is in broad agreement with published data for individual autoantibody assays (reviewed in [10]) and confirms the finding of ours and others that measurement of serum autoantibodies to a single tumour-associated antigen is of little use for screening and early diagnosis of primary breast cancer. Levels of autoantibodies to each of the antigens in the panel were significantly different in PBC compared with normal samples, except in the case of autoantibodies to BRCA1.

The *BRCA1* mutation status of this unselected group of PBC patients were unknown but would be expected to be <2% of the group. It may therefore not be surprising that few patients were found to have autoantibodies to BRCA1. Further research is ongoing assessing the percentage of patients with known *BRCA1* mutations who have developed PBC who express autoantibodies to BRCA1. The low level of BRCA1 autoantibodies emphasises the need for an optimised panel of antigens to maintain high sensitivity and specificity rather than simply screening for seroreactivity against a whole array of proteins.

Sixty-four per cent of the women with PBC had reproducibly elevated autoantibodies to at least one out of six antigens while the corresponding value for patients with DCIS was 45%. The specificity of the panel was 85%. This level of specificity is acceptable when one considers that first, screening mammography can have a similar specificity when applied across all ages of women as the autoantibody test had in this study [11]. Secondly, a large proportion of the normal cohort described in this study were women who had attended routine mammographic screening and were therefore in the ‘at risk’ of breast cancer group by virtue of their age. Not only does mammography not detect all breast cancers but also blood samples taken when women were routinely attending an ‘at-risk’ screening clinic have been reported to show autoantibodies in

Table 2. Reproducibility, sensitivity and specificity of detection of autoantibodies in breast cancer

%	Antigen							Panel ^a
	p53	c-myc	NY-ESO-1	BRCA1 ^a	BRCA2	HER2	MUC1	
Reproducibility	96	97	96	90	90	90	92	93
Sensitivity								
PBC	24	13	26	8	34	18	20	64
DCIS	15	8	8	3	23	13	23	45
Specificity	96	97	94	91	92	94	98	85

Assays were judged to be reproducible if the result (either positive or negative) agreed on at least three separate occasions. Positivity (sensitivity) was defined as a value greater than the mean of the normal population plus 2 SD. Specificity was calculated from the normal group as the number of true negatives expressed as a percentage of the number of false positives plus true negatives.

^aThe detection of autoantibodies to BRCA1 afforded no diagnostic potential so were not included in the panel analysis.

PBC, primary invasive breast cancer; DCIS, ductal carcinoma *in situ*; SD, standard deviation.

the blood months to years before the breast cancer was diagnosed on the screening mammogram. Indeed, using prototype assays for three of the antigen assays reported in this manuscript (i.e. p53, c-myc and MUC1), six of the nine (67%) women were reported to show autoantibodies between 7 and 27 months before the cancer was diagnosed on the screening mammograms [12]. Further follow-up has confirmed that nine of fifteen (60%) patients in a larger cohort showed autoantibodies detectable up to 4 years before mammographic detection [13]. These levels of sensitivity, specificity and lead time have thus far never been reported for a diagnostic blood test for early breast cancer. Previous studies both from our own group [12] and others [14] have shown that autoantibodies' responses to cancer antigens in patients with benign breast and benign autoimmune diseases are similar to, or even lower than, those seen in the normal healthy population.

Although other groups have reported higher specificity and sensitivity levels for autoantibody panels, some of these studies have only used blood donor samples as their normal controls. These samples are generally provided by individuals who have a lower mean age, and therefore lower risk of cancer than their disease groups [15, 16].

The authors are also aware that some of the autoantibodies measured in this study, such as p53 and NY-ESO-1 are not solely detected in breast cancer [17, 18] and therefore the possibility of women harbouring latent cancers of other organs cannot be ruled out. Indeed, this is one reason why it is proposed that the

initial introduction of the autoantibody test should be for younger women at increased risk where: (i) breast $\pm$ ovarian cancer are by far the most likely tumour types (unless there are specific risk factors for other tumour types, e.g. smoking or genetic mutations) and (ii) where mammography is known to be less sensitive and specific as a screening test.

Equally important from a clinical perspective, the present study reports on the reproducibility of the individual results which is essential for a clinical diagnostic test. The reproducibility reported in this manuscript (i.e. >90% for each individual assay and 93% for the panel) is the best data thus far reported in the literature for autoantibody detection. We have attributed the high level of reproducibility demonstrated here to a combination of factors such as the production of antigens with rigorous quality assurance. It also seems likely that specific epitopes of the same antigen will give different levels of immune response. Presentation of the correct epitopes which have induced the humoral immune response would seem to be important with respect to sensitivity and specificity. Being able to reproducibly produce this antigen would seem to be important in terms of interassay reproducibility. The importance of using highly purified antigens has been reported by others [19]. The present authors have ensured strict compliance to detailed standard operating procedures for each assay to eliminate nonspecific binding. In addition, immobilisation of the antigens in a defined and controlled manner (patent pending) has contributed significantly to a level of reproducibility not reported before.

No significant difference was seen in autoantibody detection of cancer when the patients were subdivided by the majority of clinical (e.g. age) and histological (e.g. tumour size, grade and lymph node status) factors. There was no correlation with tumour grade, in either the panel or individual antigens. However, it is interesting to note that there were no patients with low-grade tumours who had autoantibodies against HER2, while the proportion of patients positive for NY-ESO-1 autoantibodies increased with grade. A larger cohort of samples would be needed to confirm correlations between different autoantibodies and tumour heterogeneity.

Given the heterogeneity of breast tumours, these findings are extremely encouraging for the future introduction of such a test for the early detection of primary breast cancer across all

Table 3. Autoantibody assay panel sensitivity by age

Age (years)	PBC (n)	PBC +ve (%)	DCIS (n)	DCIS +ve (%)
<50	21	48	6	33
50–59	30	57	14	43
60–69	27	74	16	50
>70	19	79	4	75
Total	97	64	40	45

Percentage positivity in each patient group is shown. Positivity was defined as a value greater than the mean of the normal population plus 2 SD. n denotes number in each sample set.

PBC, primary invasive breast cancer; DCIS, ductal carcinoma *in situ*; SD, standard deviation.

Table 4. Autoantibody assay panel sensitivity by grade where known

	p53 (%)	c-myc (%)	NY-ESO-1 (%)	BRCA1 ^a (%)	BRCA2 (%)	HER2 (%)	MUC1 (%)	Panel ^a (%)
PBC grade								
Grade 1 n = 11	9	0	18	0	36	0	9	55
Grade 2 n = 50	32	14	20	10	36	20	24	62
Grade 3 n = 33	15	15	39	9	30	18	18	73
DCIS grade								
Low n = 5	0	0	20	20	0	0	0	20
Intermediate n = 13	15	15	8	0	15	23	23	62
High n = 22	18	5	5	0	32	9	30	41

Percentage positivity for each antigen is shown. Positivity was defined as a value greater than the mean of the normal population plus 2 SD.

^aThe detection of autoantibodies to BRCA1 afforded no diagnostic potential so were not included in the panel analysis.

PBC, primary invasive breast cancer; DCIS, ductal carcinoma *in situ*; SD, standard deviation.

patient groups. However, in the first instance the group of women for whom the test would appear to be most useful are younger women (e.g. <50 years) assessed to be at increased risk of developing breast cancer (e.g. with a familial history of breast cancer) and currently offered mammographic screening which is not the ideal test for this group of women. Furthermore, the probability of a younger woman with a positive autoantibody test having a solid tumour type other than breast cancer without known risk factors (e.g. smoking, carcinogen exposure) is very low, increasing the tests specificity for breast cancer. The authors would therefore initially see such a blood test as being useful as an aid to screening mammography [or magnetic resonance imaging (MRI)].

The use of the panel of antigens, reflecting for example tumour suppressor genes (p53), tumour oncogenes (HER2), differentiation markers (MUC1, c-myc) and genetic mutations (e.g. BRCA2) provides a plausible scientific explanation for the similar detection rate across all types of breast cancer represented in this study. Even more significant clinically is the finding that autoantibodies were detected at similar rates irrespective of the method of presentation of breast cancer (i.e. screening or symptomatic). The implications of this would seem firstly to be that autoantibodies would provide a blood test for the earlier diagnosis of patients who currently do not attend for mammographic screening or in whom screening did not detect their cancer (e.g. interval cancers). Secondly, it seems biologically implausible that in patients with a screen-detected primary breast cancer that the autoantibodies were produced at the time of the mammogram being taken. Indeed, our previously reported and presented data from patients attending our 'at-risk' clinic has confirmed that autoantibodies can be measured up to 4 years before mammography imaged the tumour. These striking data imply that the human immune system detects the tumour-derived antigens as 'nonself' and makes a humoral immune response very early in the disease process. The latter appears to provide an '*in vivo* window on early carcinogenesis' which at the same time may be used as an aid to mammography for the early detection of breast cancer. For example, younger women at increased risk who are mammogram negative/autoantibody positive would undergo breast MRI or any other imaging technology which could detect early tumour lesions. If a more sensitive test imaged the tumour then definitive treatment could be carried out earlier.

summary

The real clinical significance of autoantibodies for screening and early diagnosis appears to lie in combining an optimised panel of assays to measure autoantibodies. The advantage of this approach is that it should be possible to detect the full range of heterogeneous breast cancers by increasing the number of autoantibody assays or altering the antigens used in the autoantibody panel. A blood test is noninvasive, cheap relative to imaging tests, carries no side-effects and is acceptable to the vast majority of patients. The sensitivity, specificity and lead time reported in this study are the highest thus far reported for breast cancer. The reproducibility rates are the best thus far reported for any autoantibody study in cancer. More importantly, it is of a level required for a clinical diagnostic test

(e.g. the initial hepatitis B serum tests). We would suggest therefore that the detection of tumour-associated autoantibodies in the serum of individuals with an increased risk of developing cancer could be carried out along with mammography. Initially, the authors see the preferred patient population as being younger women assessed as being at increased risk of breast cancer who currently undergo mammographic screening. Patients found to be antibody positive but mammographically negative would require further investigation such as more intensive clinical follow-up or entering an MRI screening programme which has been proven effective in screening women at high risk of breast cancer (reviewed in [20]).

The impact of the findings of this study lies beyond breast cancer. Autoantibodies have been reported in other solid tumour types and the authors too have data, for example, on colorectal, lung, bladder and prostate cancer where similar autoantibody detection rates to different panels of antigens have been found. This platform technology almost uniquely provides an *in vivo* amplification (i.e. sensitivity) with specificity but can be adapted through changing the antigen panels to detect all solid tumour types.

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