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ORIGINAL ARTICLE

Pattern of serum immunoreactivity against breast cancer cell lysates may predict severity of disease in breast cancer patients

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Abstract Humoral tumor-specific immunity has been investigated as a potential tool to identify tumor-associated antigens and evaluate cancer diagnosis and prognosis. Using SDS-PAGE and western blotting techniques we investigated the humoral immune response against tumor cell antigens in 36 breast cancer patients, 17 node-positive (NP) and 19 node-negative (NN). As a source of antigens, we prepared protein lysates from four breast cancer cell lines (AU565, BT474, MCF-7 and MDA-MB-231) which in vitro exhibit different features of invasion, estrogen receptor/progesterone receptor status and HER2/*neu* expression thereby potentially representing mild to aggressive forms of clinical disease. A higher number of immunocomplexes Ag–Ab were formed when serum from NN patients was immunoreacted against lysates from AU565 and MCF-7 in comparison to serum from NP patients ($P < 0.01$). BT474 cells were not a good antigenic source. MDA-MB-231 cells could not significantly discriminate between NN and NP patients since both groups showed higher amounts of reactivity against the lysate. However, comparative analysis of protein preparations purified from MCF-7 and MDA-MB-231 cells and immunodetected concomitantly with the same serum samples showed that serum from patients with cancers with worse prognosis (stage, nodality, HER2/*neu* and hormonal status) reacted more intensely to proteins purified from the relatively more invasive cell line MDA-MB-231 compared to MCF-7. These findings suggest that the study of serum antibody reactivity to antigens purified from breast cancer cell lines with different invasive properties should be further investigated for its potential in providing beneficial prognostic information in breast cancer.

Keywords Breast cancer · Serum antibody · Immunoreactivity · Tumor cell lysates

Abbreviations

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
NP	Node-positive
NN	Node-negative
HER2/*neu*	Human epidermal growth factor receptor/*neu*
cDNA	Complementary deoxyribonucleic acid
SEREX	Serological expression cloning
SERPA	Serological proteome analysis
IR	Immunoreactivity
ELISA	Enzyme-linked immunosorbent assay
IgG	Immunoglobulin
TI	Total immunoreactivity

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ANOVA	Analysis of variance
SE	Standard error
IP	Immunoreactivity pattern
Ag-Ab	Antigen-antibody
TIP	Total immunoreactivity pattern

Introduction

Serum from cancer patients has been shown to contain antibodies reactive with tumor-associated proteins, thereby holding the potential to identify antigens that may be useful for the treatment, detection, and perhaps the prognosis of the disease. High throughput technologies, such as serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX), serological proteome analysis (SERPA), enzyme-linked immunosorbent assay (ELISA) and protein chips and microarrays, together with artificial intelligence-based pattern recognition algorithms, have been successfully used to generate and analyze complex serum "-omic" data sets [1].

SEREX is a very powerful methodology used to identify tumor-associated antigens capable of eliciting high titer IgG responses using antibodies present in the serum of cancer patients and recombinant proteins [2]. However, the main disadvantage of SEREX is its reliance on recombinant proteins which may not reflect the true content of cancer tissue, where proteins may be over-expressed or undergo post-translational modifications fundamental for the tumorigenic properties of the cancer itself. Indeed, autoantibodies specific to cancer patients are often against proteins that are mutated (MUC1, p53), over-expressed (HER2/*neu*), or translocated (myc). SERPA is based on separation of proteins on two-dimensional electrophoresis followed by identification by mass spectrometry. It has been used to identify proteins present in serum to compare the profiles of diseased individuals to healthy volunteers, as well as to identify novel tumor-associated antigens capable of priming the immune system [3–7]. SERPA has been used to test antibody responses to autologous and heterologous tissues as well as to cell line lysates [8]. The advantage of SERPA over SEREX is the use of antigenic pools that resemble more closely the original protein content in vivo, thus increasing the chances of binding the serum autoantibodies. ELISA and protein chips are fast-screening methodologies used to determine the titer of specific antibodies against known antigens using recombinant as well as native proteins [9, 10]. Despite their limitations and certain disadvantages, all the techniques cited above have made contributions towards the progress in discovering novel tumor-associated antigens as well as specific molecular signatures that can be used for early diagnosis and better prognosis for cancer patients [2, 4, 11, 12].

In the present study, we have used four breast cancer cell lines as the source of proteins for studying humoral immunoreactivity (IR) in breast cancer patients. They represent mild to aggressive forms of breast cancer. Three of the cell lines, MCF-7, AU565 and BT474, are characterized by in vitro assays as being weakly invasive while MDA-MB-231 is considered a highly invasive cell line. Our findings show that not all sources of antigens are equivalent. Depending on the cell line used, sera from NN breast cancer patients could be differentiated from that of NP patients suggesting the absence or presence of stage-specific antigens that engender IR. Moreover, comparative analysis of the IR against a weakly invasive and a highly invasive cell line reacted with the same serum was able to distinguish cancer patients with worse clinical parameters of disease. We show here that the proper source of antigens used for serological evaluation is crucial to the type of analysis to be performed and could potentially have a beneficial impact on the clinical utility of autoantibody screening.

Materials and methods

Patient characteristics and clinical protocols

Serum samples were obtained from 19 NN and 17 NP breast cancer patients who are currently enrolled in clinical trials testing a HER2/*neu* E75-peptide vaccine. All patients were screened for eligibility criteria and given proper counseling prior to consenting and enrollment into the study. Patients' pathological data including age, grade and stage of the tumor, estrogen receptor/progesterone receptor status and HER2/*neu* immunohistochemical stain, lymph node involvement, metastatic sites and recurrence were collected and maintained in a database. All serum samples used for this study were obtained prior to the administration of the vaccine and therefore are representative of serum from breast cancer patients. These patients had all undergone primary surgical and medical therapies and were proven to be without evidence of disease at the time of enrollment into the trial. Patients were on average 2 years post-completion of their standard treatment, thereby strongly reducing the possibility of any remnant effects from their surgery, chemotherapy and/or radiation regimens. Patients were skin tested with a panel of recall antigens (Mantoux test = mumps, tetanus, candida) and all patients in this study were established as being immunocompetent since they had a positive reaction (>5 mm) to $\geq$2 antigens.

Serum collection and preparation

Serum samples were prepared by drawing 10 ml of blood into a Vacutainer Gel and Clot Activator tube (Becton

Dickinson, Franklin Lakes, NJ) that was then centrifuged and the serum aspirated. Sera were aliquoted and stored frozen at −84°C prior to its use with the western blots.

Cell culture

MCF-7, BT474, AU565 and MDA-MB-231 cell lines (ATCC, Manassas, VA) were maintained in IMEM (MCF-7, BT474, and MDA-MB-231) and RPMI-1640 (AU565) (GIBCO, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA). The cells were cultured at 37°C in 5% CO_2 and the medium was changed three times a week.

Preparation of cell lysates

Cells were washed with ice-cold PBS, scraped and lysed in 50 mM Tris–HCL, 150 mM NaCl, 1% IGEPAL CA-630, protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN), 50 mM NaF, 30 mM sodium β-glycerophosphate, and 0.1 mM PMSF. Protein concentration was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA).

SDS electrophoresis and western blotting

Eight hundred micrograms of cell lysate was run on a NuPAGE® Novex 4–12% Bis–Tris Gels 1.5 mm, 2D-well (Invitrogen, Carlsbad, CA) and proteins transferred to nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ). After transfer, membranes were incubated for 2 h in blocking buffer (5% milk in TBS, 0.05% Tween 20) and placed in a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, Hercules, CA) to create 16 leak-proof channels. Each channel was loaded with serum at a 1:100 dilution and incubated overnight at 4°C. For every experiment, four channels in each blot were loaded with a control serum for quality control; membranes showing poor reactivity towards the controls were discarded. Secondary antibodies were goat-anti-human IgG antibodies (CALTAG Laboratories, Invitrogen Corp., Carlsbad, CA) at a dilution of 1:2,000. Immunodetection was accomplished by ECL detection (Amersham Biosciences Corp., Piscataway, NJ). Preliminary negative controls were done to ensure specificity of IgG binding. These controls included omission of sera from the WB protocol, serial dilution of the samples (1:100 to 1:1,000), use of prostate cancer cell line (LnCap) and primary normal breast cells (HMEC) which resulted in no or very limited signal. Other antibodies used were anti-Erb2/c-Neu (Ab-3) monoclonal antibody (EMD Biosciences, San Diego, CA) and rabbit-anti-human actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Software-based analysis of western blots

Following immunodetection, blots were scanned with a HP Scanjet 3570c scanner (Hewlett-Packard, Palo Alto, CA) and the digitalized images analyzed using GeneTools software (Syngene, Frederick, MD). Semi-automated analysis including auto-background correction was performed for each sample loaded on the multi-screen apparatus. For each sample, bands above background were detected and counted. At least one set of three independent blots was run for each sample after which the number of bands detected in each experiment was counted and averaged. To ensure reproducibility and consistency of the analysis among the various runs of experiments, we excluded bands above 191 kDa and below 28 kDa and bands whose intensity was less than 5 pixels.

The average of the number of bands among the replicates of experiments was used for analysis. Measure of total immunoreactivity (TI) was performed as per the manufacturer's instructions. Briefly, the bands in each lane were detected by the software, digitalized, decomposed into a matrix of pixels, and finally reconstructed as peaks. Peaks were characterized by height (average intensity within one row of pixels), volume (sum of the intensity of all the peaks constituting the band) and position along the track. TI was the sum of the volumes of all the bands in each lane and reflected the overall amount of immunocomplexes formed.

Serum IgG titer

Concentration of IgG in serum samples was measured with the Easy-Titer Human IgG Kit (Pierce, Rockford, IL) as per the manufacturer's instructions. Absorbance at 405 nm was read using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical analysis

ANOVA (analysis of variance) was used to decide if there was a difference in the number of immunoreactive bands detected as described above between the two groups (NN and NP). If significant, then the Student-Newman–Keuls test was used to decide if one group differed from the other. Each test was two-sided and a significance level of 5% was used. Mean ± standard error (SE) was used.

Results

Sera from NN and NP breast cancer patients show differential reactivity to cell lysates prepared from AU565, BT474 and MCF-7 breast cancer cell lines

Autoantibodies present in the serum may specifically react with tumor-associated antigens universally present in cancer revealing a signature for the detection of the disease. Therefore, the serum antibody content of breast cancer patients could hold great promise for the identification of biomarkers associated with disease and also be potentially useful for the diagnosis and/or prognosis of breast cancer.

Samples from patients enrolling in a HER2/*neu* E75-peptide vaccine clinical trial at Walter Reed Army Medical Center were employed for this study. As an initial study we tested a set of serum samples from 11 NN and 9 NP breast cancer patients for antibody reactivity against lysates from the cell lines AU565, BT474 and MCF-7, which express varying levels of the proto-oncogene HER2/*neu* (Fig. 1a). The cell line MDA-MB-231 (estrogen receptor/progesterone receptor negative) did not express detectable levels of HER2/*neu*.

Following immunodetection, blots were scanned and subjected to digital image analysis that allowed the samples to be grouped as NN and NP for each cell line and subsequent evaluation (Fig. 1b). Analysis was done on bands between 29 and 191 kDa. Previous studies have reported on the finding of antibodies in the serum of breast cancer patients to a variety of low and high molecular weight proteins [8, 13]. The wide molecular weight range we chose to analyze covers multiple proteins whose over-expression or post-translational modifications are correlated to cancer, such as growth factor receptors (HER2/*neu*), oncogenes, heat shock proteins, protein kinases and phosphatases, as well as known antigenic proteins expressed in breast cancer such as MUC1, annexin XI-A and RPA32, among others [14]. Furthermore, this molecular weight range showed the highest difference among the serum samples that we tested.

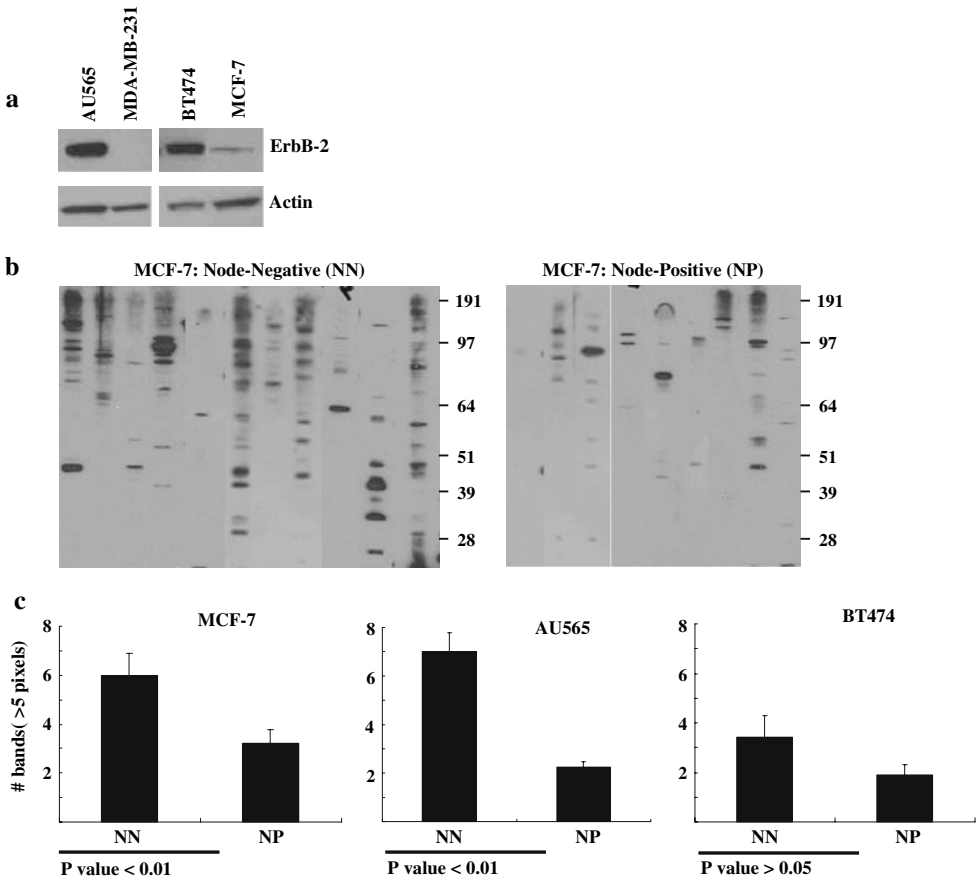


Fig. 1 Immunoreactivity of serum autoantibodies from breast cancer patients to lysate purified from cell lines expressing different amount of HER2/*neu*. **a** Expression of HER2/*neu* was tested on 40 μg of total lysate from AU565, BT474, MCF-7 and MDA-MB-231 cells and probed by western blot with an anti-ErbB2-specific antibody; anti-actin was used as loading control. **b** Example of serum screen using lysate from MCF-7. Total lysate was run on a one-well SDS-PAGE, transferred to nitrocellulose, immunoreacted with serum samples from breast cancer patients (1:100) and immunodetected using chemiluminescence. Digital images show lanes incubated with serum from node-negative (NN) and node-positive (NP) patients reacting to antigens from MCF-7. **c** Blots scanned into digital images and subjected to automated analysis using GeneTools image analysis software. For each cell line and for each sample, the number of immunoreactive bands was calculated. The samples were grouped into NN and NP patients and the average number of bands was quantified for each group and for each cell line (MCF-7, AU565 and BT474). *Bars* represent standard errors

The results from these analyses are shown in Fig. 1c. For MCF-7 cells the number of reactive bands detected for the NN group was significantly different from the NP group. Apart from a few exceptions, for MCF-7 cells the average number of bands was significantly higher in the NN than in the NP group (6.01 ± 0.88, 3.22 ± 0.53, respectively; $P \leq 0.01$). The same was true for AU565 cells (7.02 ± 0.76, 2.25 ± 0.21; $P \leq 0.01$). Finally, for BT474 cells no significant difference was seen in the number of bands detected between the two groups (3.42 ± 0.86, 1.91 ± 0.40, respectively; $P > 0.05$). Taken together, these observations suggest a general pattern where the serum from NP patients was significantly less immunoreactive against the lysates from MCF-7 and AU565 cells compared to the serum from NN breast cancer patients. In comparison, total lysate from the BT474 cell line did not demonstrate any such significant differences in IR against the antibodies in the patients' sera and seemed to be a poorer source (an overall less number of bands) of immunogenic antigens.

IgG level of serum samples from NN and NP patients

Prior to conducting further investigations of the differences seen in the IR between NN and NP patients, we first wanted to exclude the possibility that the differences seen by western blotting were merely a reflection of significant variations in the amount of total IgG present in the sera tested. Therefore, we measured the titer of total IgG in eight NN and seven NP sera randomly chosen among the samples (the choice was based upon sample availability) used for the previously presented experiments. As shown in Fig. 2, the results for serum IgG titer in these sera demonstrated no significant differences between the individual samples.

Screening of NN versus NP sera with lysate prepared from the MDA-MB-231 breast cancer cell line

Encouraged by the results that two out of the three cell lines tested above (Fig. 1c) revealed IR patterns that could discriminate NN versus NP sera, we were interested to examine the serum IR pattern against the relatively aggressive, mesenchymal-type cell line MDA-MB-231 [15]. Therefore, we tested the same nine NP and 11 NN patient samples against cell lysate prepared from the MDA-MB-231 cell line. A representative set of the results from such an experiment is shown in Fig. 3a. Upon analysis of the results, the difference in the mean number of bands for the two groups (NP, 7.7 ± 5.7 vs. NN, 10.7 ± 5.7) (Fig. 3b) did not reach statistical significance ($P > 0.05$). However, a higher number of immunoreactive bands were observed for

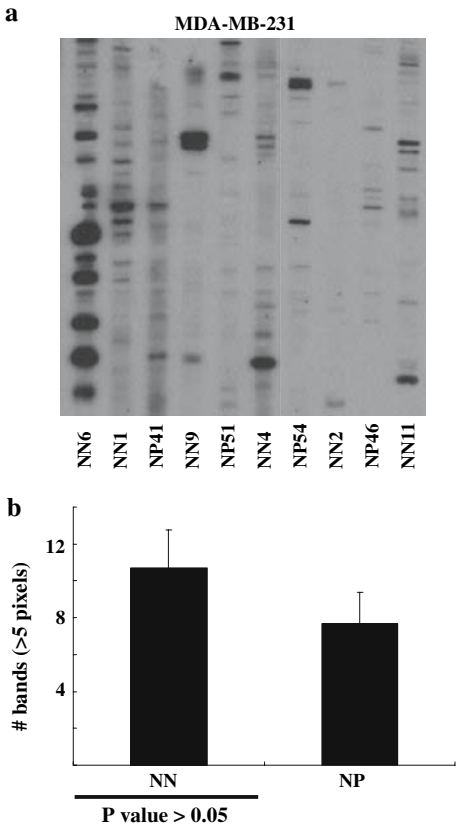


Fig. 3 Immunoreactivity of serum from NN and NP patients to total cell lysate from MDA-MB-231 cells. **a** Serum from NP and NN patients was screened against lysate from MDA-MB-231 cells as described in the Materials and Methods section. **b** Number of immunocomplexes formed with antigens present in the lysates was counted for every sample; samples were divided into NN and NP and the average number of immunoreactive bands was quantified for each group. *Bars* represent standard errors

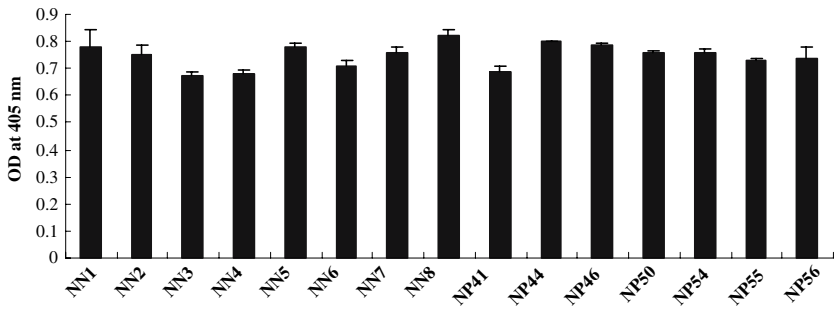


Fig. 2 IgG titer of serum samples from NN and NP patients

both NN and NP samples in comparison to what was seen against lysates from MCF-7, AU565 and BT474 cells (Fig. 1c).

Comparison of IR against lysates from MDA-MB-231 versus MCF-7 cells

In order to avoid biases due to variability among experiments and to further validate our observations, we ran in parallel a set of 19 NN and 17 NP sera against lysates prepared from MDA-MB-231 and MCF-7 cells and compared the immunoreactive profiles side-by-side. A representative set of the samples tested is shown in Fig. 4a and b, reacted respectively against MDA-MB-231 and MCF-7 cell lysates, and their corresponding number of bands is also shown. As displayed in these gels, the NN and NP sera were interspersed on the blots in order to ensure the most accurate interpretation of the observed data. Figure 4c represents the same samples as in Fig. 4a and b but grouped into NN and NP for each of the cell lines. An actin control was performed on each blot to ensure equal loading of the cell lysates. The number of bands detected for each cell line for all of the samples tested is shown in Fig. 4d. Some of the samples tested gave high standard errors. However, variations among experiments did not favor one category of patients versus the other or one cell line versus the other. Variation was found to be evenly spread with both NN and NP showing high and low standard error against both MDA-MB-231 and MCF-7 cell lines.

After individual comparative analysis (Fig. 4d), the mean number of bands immunodetected for the NP sera screened against MDA-MB-231 cell lysate was higher than that obtained for MCF-7 lysate ($NP_{MDA\text{-}MB\text{-}231} = 8.5 \pm 2$ versus $NP_{MCF\text{-}7} = 7.3 \pm 1.7$), while for the NN samples the number of detected bands was approximately the same ($NN_{MDA\text{-}MB\text{-}231} = 10.1 \pm 2.3$ versus $NN_{MCF\text{-}7} = 9.9 \pm 1$) for the two cell lysates. Although the increased number of samples tested yielded a higher overall number of bands in this experiment (compared to the earlier section—Fig. 1c), a statistically significant difference was still present in MCF-7 cell lysate between NN and NP ($P < 0.01$). However, that was not the case for MDA-MB-231 cells, where even though the number of bands derived from the NP population was still lower than the NN, it did not reach significance ($P > 0.05$).

Serum IR patterns against MDA-MB-231 versus MCF-7 proteins can discriminate breast cancer patients with more severe disease

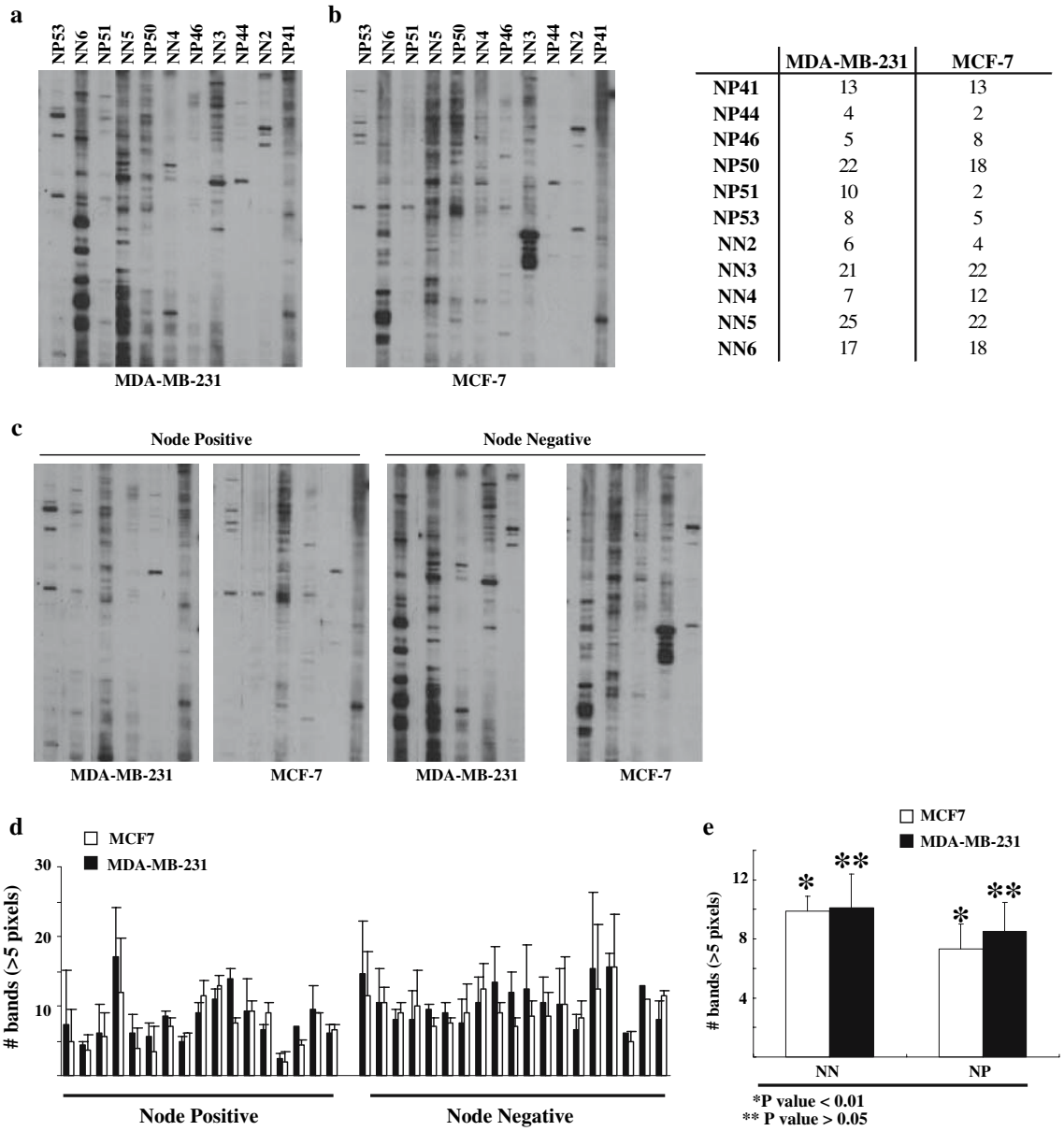
In our experiments studying the reactivity of the 36 serum samples (Fig. 4), we did not limit our analysis to the number of Ag–Ab complexes formed after incubation of the blots with the patients' sera, but we performed a different type of analysis. Blots from MCF-7 and from MDA-MB-231 cell lines were incubated with the same serum sample and then developed and compared using image analysis software (Syngene) (Fig. 5). The software detected and digitalized the bands present in each lane along the blot and the background noise was automatically and equally subtracted from all the samples. The output of the software analysis was in the form of a profile for each sample examined, characterized by height (y-axis), position along the gel (x-axis), and volume (representing the sum of the values of all the pixels constituting each band and being proportional to the amount of signal generated in the blot). An example of the results obtained for a subset of the samples analyzed is shown in Fig. 5. Using this approach, we defined the TI of a sample as the sum of the volumes for each lane on the gel detected and digitally measured for each sample by the software. We then measured the TI of each sample against both the MCF-7 and MDA-MB-231 cell lines. Based on the preferential reactivity of the sample for one cell line versus the other, we divided the patients in two groups. Those patients with serum that demonstrated a higher TI against the MDA-MB-231 versus the MCF-7 cell lines were assigned to group 1. The remaining patients were assigned to group 2.

Once the two groups were established and the results confirmed by repeated runs of western blots, we linked the clinical information to the serum samples. Data on known prognostic factors such as age, race, grade, stage, nodal status, HER2/*neu* and hormonal status, and recurrence were analyzed. Table 1 shows the percentage of patients in group 1 (higher TI towards the MDA-MB-231 cell line) and in group 2 (patients with equal or higher TI towards the MCF-7 cell line) and their correlated clinico-pathologic parameters. These results show that the higher TI against MDA-MB-231 cell lysate was significantly associated with more severe disease status.

In general, the patients in group 1 were younger ($P = 0.02$), had tumors of higher stage ($P = 0.033$), had more hormonally insensitive tumors ($P = 0.05$), were more likely to have HER2/*neu* (3+) ($P = 0.23$) tumors, and most importantly, were more likely to be NP ($P = 0.12$). Additionally, two patients in group 1 had recurred compared to only one patient in group 2; also, one NN patient in group 1 was found to have micrometastasis to one lymph node.

Discussion

While early detection is still the best cure for breast cancer and mammography remains the golden standard for breast cancer detection, there is a critical need for new and sensitive markers that would allow for earlier diagnosis and

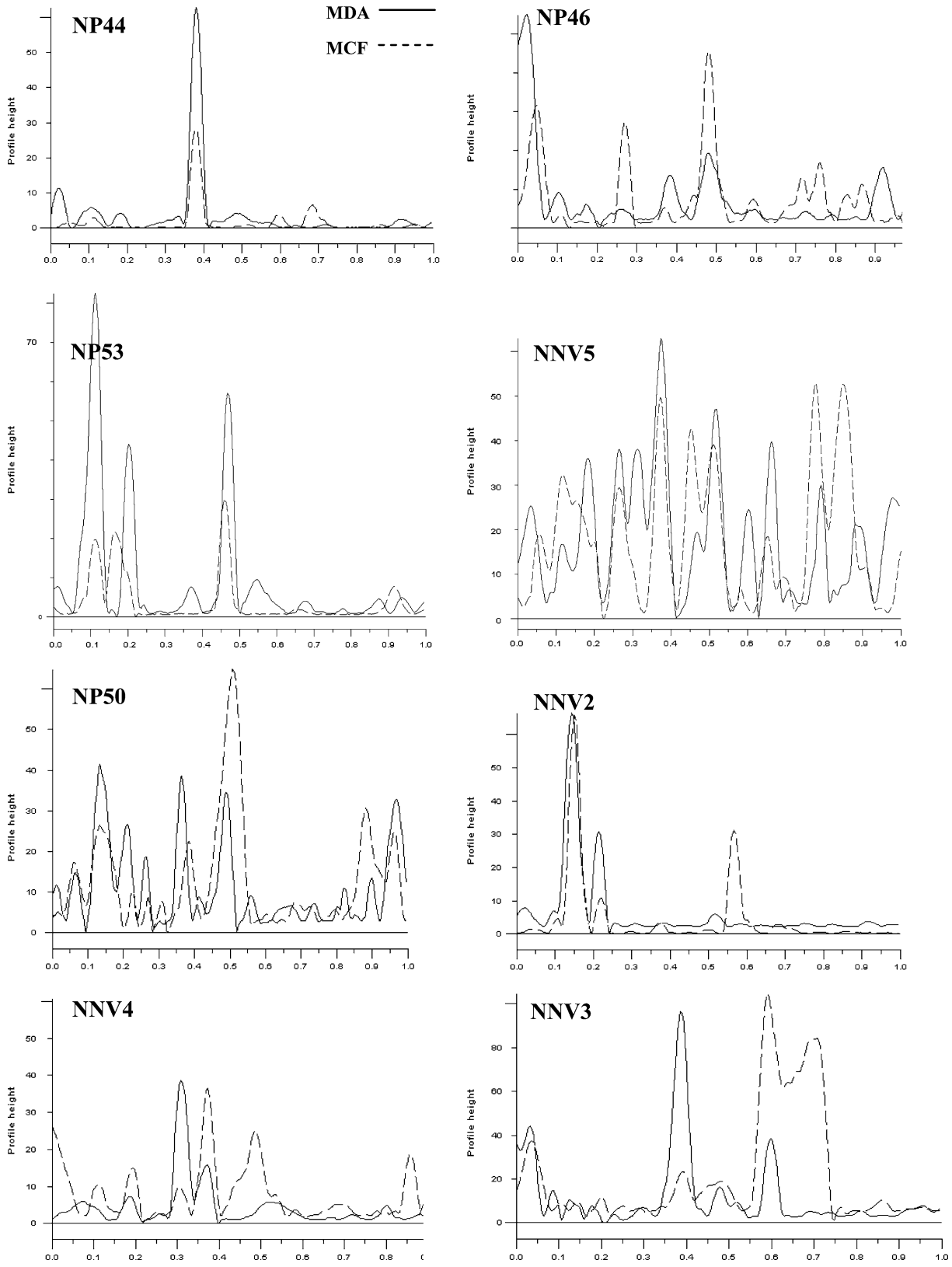


	MDA-MB-231	MCF-7
NP41	13	13
NP44	4	2
NP46	5	8
NP50	22	18
NP51	10	2
NP53	8	5
NN2	6	4
NN3	21	22
NN4	7	12
NN5	25	22
NN6	17	18

Fig. 4 Comparison between number of bands immunodetected after incubation of MCF-7 and MDA-MB-231 cell lysates with sera from 19 NN and 17 NP breast cancer patients. Example of experiment showing the sera screen results against total lysate from MDA-MB-231 (**a**) and MCF-7 cells (**b**) that was performed as described in "Materials and methods". **c** Blots were scanned and the images reconstructed to combine the lanes into groups corresponding to serum from NP and NN patients. **d** Graph showing the comparison between average number of immunocomplexes formed by each individual serum sample (19 NN and 17 NP) with antigens from MDA-MB-231 and MCF-7 cells. Each sample was run at least in triplicate; the number of immunocomplexes formed for each sample and for each cell line was counted and averaged. **e** Graph showing the results after the samples have been grouped into NN and NP and averaged for each cell line separately. =MCF-7 cells; =MDA-MB-231 cells. *Bars* represent standard error

better classification and prognosis. Serum is now being intensely investigated as a new tool for detecting early stages of diseases, for prognosis, and for monitoring response to treatment. Besides the obvious reason that blood is easier to obtain than biopsies, serum also holds fingerprints of the cancer it perfuses in terms of molecular content and antigen-driven humoral immunity.

In this study, we have utilized four breast cancer cell lines with different characteristics and degrees of aggressive growth patterns as the source of total tumor proteins in order to assess the differences in the serum humoral IR in NN and NP breast cancer patients. Through the use of tumor cell lysates from two moderately aggressive breast cancer cell lines such as AU565 and MCF-7, we could



detect a different immunoreactivity pattern (IP) in sera from NP versus NN patients, with the NN group revealing significantly higher reactivity than the NP group towards the antigenic pool derived from these cell lines. The difference seen was not due to variations in the serum content of total IgG. Some exceptions were detected where certain NN

◀ **Fig. 5** Antigenic profiles of identical serum preparations from NP and NN patients incubated with lysates from MDA-MB-231 and MCF-7 cells. Total lysates from MCF-7 and MDA-MB-231 cells were separated on 4–12% SDS-polyacrylamide gels, transferred in parallel to nitrocellulose membranes and incubated under identical conditions with the same preparation of diluted serum sample as described in "Materials and methods". Scanned blots were subjected to digital analysis and the immunological profile of each lane was translated by the software as a profile of peaks whose area was proportional to the amount of Ag–Ab complexes formed. The software algorithm automatically subtracted background noise. *Dashed line* represents MCF-7, *solid line* MDA-MB-231 cells

Table 1 Correlation of clinical parameters with TI against MDA-MB-231 and MCF-7 cells

	Group 1 (n = 20)	Group 2 (n = 16)	P value
Grade 3 or > (%)	44	43	NS
Stage 2B or > (%)	35	0	0.033[a]
Node-positive patients (%)	60	33	0.12
# Nodes (average)	3	1.8	0.37[a]
HER2/*neu* (IHC 3+ or >) (%)	42	20	0.23
ER/PR negative (%)	61	21	0.05
Recurrence (%)	10	7	NS
Age (average)	54	63	0.02[a]

NS not significant

[a] Unpaired T test. All other P values are Chi-square with Yates' correction

sera had fewer bands than some of the NP samples. One explanation may be that the NN samples with low reactivity to weakly invasive cell lines could actually be from high-risk patients, but this possibility can only be validated with the long-term follow up of clinical status for these patients. The higher reactivity of the NN group with respect to the NP may be explained by the fact that serum antibody content is a reflection of the type of cancer from which the serum is derived. AU565, BT474 and MCF-7 cells, despite their differences in expression of HER2/*neu* and estrogen receptor/progesterone receptor status, were characterized by a low proliferation rate and a low invasive nature in vitro, thus originating from a less aggressive phenotype (as in the case of the NN group).

Lysate prepared from BT474 cells was unable to distinguish between the two groups and displayed an overall lower amount of IR with the antibodies in the serum. Similarly, the ability to discriminate between NN and NP was not apparent when lysate prepared from an aggressive cell line (MDA-MB-231 cells) was used as the source of the antigenic pool. However, in this case, increased rather than decreased IR was an issue since the NP serum samples now showed higher reactivity, thus reducing the difference in the number of bands detected by NN and NP groups. Recent findings are beginning to support a central role for antibodies in regulating cancer pathology through their ability to trigger chronic inflammation and therefore stimulate tissue remodeling, cytokines and growth factor release and signal transduction cascades [16, 17]. In this context increased immunoreactivity of NP sera towards a very aggressive cell line such as MDA-MB-231 cells would appear to fit the hypothesis that the cancer generates antibodies to further implement its ability to maintain an inflammatory state where it can grow, transform and expand.

In performing the side-by-side comparison of the IR against MDA-MB-231 and MCF-7 cell lysates, we became acutely aware of another aspect of difference in the observations being made. After comparative analysis, it was clear that not only the number of bands was different between the cell lines, but also the intensity of the signal and the position along the gel could differ among the two cell lines when tested with the same serum (e.g., in Fig. 4: NN2, NN3, NN5, NN6 NP53, NP46). Therefore, the number of immunocomplexes formed did not fully characterize the immunoreactive patterns since every band above background was counted equally regardless of its intensity. Similarly for the same sample, the position of the bands along the blots from MDA-MB-231 and MCF-7 cells was also not necessarily identical. Based on this observation, we formulated a working hypothesis that proteins specific to or more highly expressed in one cell line may be important for the phenotype of that cell line (e.g., invasiveness and proliferative features). The logical assumption of this premise was that if serum had previous exposure to the antigenic pool prepared from a very aggressive cell line then that serum originated from a patient with a more aggressive tumor. Therefore, differences in the immunoreactive affinity towards cell lysates derived from aggressive or non-aggressive cell lines could potentially give information about the status of the disease. With the help of automated image analysis software we compared the computer-generated profiles of antigenic reactivity of MDA-MB-231 and MCF-7 cell lysates. Based on such an approach of combined analysis, a total immunoreactivity pattern (TIP) against the aggressive versus non-aggressive tumor cell lysates emerged that correlated with known poor clinicopathologic parameters for breast cancer. While IR of serum towards MDA-MB-231 cell lysate alone did not provide any insight as to the clinical features of the tumors, comparative analysis of TIP against two cell lines proved to be useful in identifying patients with more severe disease. The cut-off for assigning the samples to group 1 was set to exceed the highest standard deviation for the TIP calculated for the triplicate experiments. The choice of the cut-off was arbitrary, however, our goal was not to establish the standards for clinical application of comparative TIP among cell lines. That will require standardization and validation

using a much larger set of samples and a more sensitive technique. The importance of our study consists in showing the potential of comparative analysis using multiple lysates over a single pool of antigens derived from multiple cell lines, tissues or phage display libraries.

During the initial phase of this investigation we had also screened a set of 17 commercially available serum samples from healthy donors (data not shown). A large degree of variability was observed within such samples. Although samples were described as "cancer free", they were, however, lacking associated clinical information. That made it difficult and perhaps also "incorrect" to include the results obtained into the current analysis. In comparison, a study of two groups of patients afflicted with the same disease, albeit of different stages, with documented clinical correlates seemed to be a scientifically rational approach to take. The large degree of variability among control samples was not completely unexpected, since it is known that healthy individuals possess an enormously diversified array of antibodies [18] and we did not screen serum against purified tumor-associated antigens. Nevertheless, we saw a difference in terms of the number of bands around the 40–60 kDa molecular weight range between cancer patients and healthy donors (not shown), but since variation between NN and NP requires analysis of IR profiles over a larger molecular weight range (28–191 kDa), a different experimental design would be required to further explore the differences between healthy donors and cancer patients and it is not part of this study.

Improvements and optimization of high throughput screening methodologies such as SERPA and SEREX have begun to show some success in being able to identify new antigens that could be potential targets for therapeutic cancer vaccines, as well as proteins or modifications preferentially expressed in tumors versus healthy controls [2, 7, 11, 19]. Autoantibody fingerprints or signatures have recently been shown to diagnose ductal carcinoma in situ of the breast [20]. In a recent study, Todorova et al. [21] reported on the use of lysate from the prostate cancer cell line LnCaP to detect serum IR against the prostate-specific membrane antigen that was being used as a vaccine in a clinical trial for prostate cancer patients. All of these studies indicated that the application of these serum autoantibody IR techniques could be used for screening tests and identification of immunotherapeutic targets. Functional immunomics and peptide microarrays have proven very useful in determining signature for a variety of diseases. Our study shows that an analytical comparison of signatures derived from a panel of properly chosen antigens or total lysates could increase the amount of information that can be collected from this type of methodology.

The novelty of our study was to recognize the heterogeneity among tumors and to address that issue by comparing IR of antibodies to breast cancer cell lines exhibiting various features in terms of invasiveness, hormonal status, growth factor receptors, proliferation and genetic profiling [15]. Instead of using recombinant proteins, we used total cell lysate to maintain as much as possible tumor-specific alterations in protein expression, mutation, degradation, intracellular localization or cellular lysis, and we concentrated on the difference in immunoreactive pattern exhibited by the same serum sample against lysates from cell lines with luminal epithelial-like (MCF-7) and mesenchymal-like (MDA-MB-231) phenotypes. In this case, a signature of TIPs more than unique antigen identification has allowed us to identify patients with worse outcome in terms of age, hormonal and HER2/*neu* status, and lymph node involvement.

Conclusions

An analysis of the humoral immune response to proteins purified and/or generated from tumor cell lines has been shown to be a scientifically proven approach for the identification of immunogenic antigens for vaccine development. In the current study, we explored the approach that a comparative profiling of autoantibody reactivity in the serum of breast cancer patients against an array of antigens from two or more breast cancer cell lines has the added possibility of providing additional information that could be of clinical benefit. Our results presented here suggest a rationale for using unique panels of tumor cell lines that when used in combination have the potential to identify specific patterns of serum IR which may have clinical implications both in the diagnosis and treatment of disease. Therefore, experiments to explore the potential of our technique as a diagnostic tool using a wider spectrum of cell lines and healthy controls as well as to monitor vaccine trials and to detect new target antigens are ongoing in the lab.

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