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Panel of SEREX-defined antigens for breast cancer autoantibodies profile detection

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Abstract

Content: Identification of panel of SEREX-defined antigens for breast cancer autoantibodies profile detection.

Objective: To create panel of antigens that can differentiate breast cancer patients and healthy individuals.

Methods: SEREX (serological analysis of cDNA expression libraries) method, ELISA, qPCR.

Results: In large-scale screening of 16 SEREX-antigens by sera of breast cancer patients and healthy donors a combination of 6 antigens (RAD50, PARD3, SPP1, SAP30BP, NY-BR-62 and NY-CO-58) was identified, which can differentiate breast cancer patients and healthy donors with 70% sensitivity and 91% specificity. Elevated mRNA expression of *SPP1* gene was revealed in breast tumors (2-7 fold), that correlated with SPP1 antigen immunoreactivity in autologous patients' sera.

Conclusions: The new panel of 6 SEREX-antigens was proposed which enables creation of serological assay for breast cancer diagnostics and/or prognosis.

Key words: Tumor-associated antigens, breast cancer, autoantibody, antigenic panel.

Introduction

Breast cancer is the most widespread cancer type in women. Many breast tumors with high histological tumor grade, mitotic index and proliferation rate are overrepresented among the so-called interval breast cancers (e.g. cancers arising between annual mammograms), that cannot be detected by currently existing methods, in particular mammography (Collett et al., 2005). In a view of above mentioned facts an urgent need exists for creation and development of new alternative and/or complementary methods to improve breast cancer diagnostics and prognosis. In this sense, identification of serum biomarkers, that can be detected in patient's blood by minimally invasive methods (i.e., peripheral blood sampling) is an urgent task.

Over the last years emerging number of evidence suggests that each type of cancer might trigger unique autoantibody signatures, that reflect tumor-associated antigens (TAAs) repertoire and the nature of the malignant process in the affected organ. A large number of studies focused on assessment of serum anti-TAAs antibodies reported their as molecular biomarkers for a number of cancers including ovarian (Gnjatic et al., 2010; Kim et al., 2010), breast (Piura et al., 2011; Anderson et. al., 2011), lung (Boyle et al., 2011), hepatocellular (Zhang et al., 2010), colorectal (Pedersen et al., 2011; Chan et al.., 2010), gastric carcinomas (Zayakin et al., 2013) etc. Their advantages over other biomarkers, including the TAAs themselves, are accessibility, persistence and high stability in the serum of cancer patients (Anderson et. al., 2005). Autoantibodies correspond to an efficient biological amplification of the presence of TAAs, and are secreted in the serum prior to first clinical signs. However, most serum autoantibodies, used as stand-alone diagnostic assays, demonstrate low sensitivity and/or specificity to be utilized as diagnostic tools (Desmetz et al., 2011). In this respect proposal has been made to combine several antigens into one panel for specific autoantibodies profiles (signatures) identification in patients with different cancer types, including breast cancer. To date several breast cancer antigenic panels for autoantibody detection (Piura et al., 2011; Lacombe et al., 2014) have been reported and most of them include well-known oncoproteins such as p53, p62, c-myc, MUC1, HER2, BRCA1, survin, cyclin D1 (Koziol et al., 2003; Zhang et al., 2003; Chapman et al., 2007; Lu et al., 2008; Ye et. al., 2013; Yahalom et al., 2013; Liu et al., 2015) etc. Sensitivity and specificity of currently existing breast cancer antigenic panels for serum autoantibodies profiles detection varies from 40% to 92% (Piura et al., 2011; Lacombe et. al., 2014). Recently, some autoantibody panels were created considering lymph node status (Lacombe et al., 2014), age, race and current smoking status (Evans et al., 2014) of breast cancer patients and

some clinicopathological features of their tumors including tumor grade (Mange, 2012). No significant differences were seen when patients were subdivided by age, tumor size, histological grade or lymph node status in previous studies (Chapman et al., 2007).

The aim of this study was to create optimal antigens combination among SEREX (serological analysis of cDNA expression libraries)-defined antigens which could distinguish breast cancer patients and healthy donors. For this purpose allogeneic screening of 16 SEREX- defined antigens has been performed using sera of cancer patients with different histological types and grades as well as status of ER, PR and HER2 receptors of their tumors. To evaluate the impact of TAAs expression level in patient tumor on the frequency of anti-TAAs antibodies in patient sera a paired autologous tumor and serum samples has been analyzed.

During our previous studies focused on medullary breast carcinoma (MBC) investigation by SEREX-approach 41 TAAs have been identified and characterized in preliminary phage-based allogenic screening (Kiyamova et al., 2010; Kostianets et al., 2012). In the current study affinity purified recombinant analogues of 13 the most immunogenic MBC antigens together with 3 previously defined by other researches SEREX-antigens associated with breast cancer NY-BR-62, NY-BR-1 and NY-CO-58 (Jager et al., 2001; Scanlan et al., 2001; Scanlan et al., 2002; Gnjatic et al., 2010) were tested in allogenic screening by ELISA with sera of healthy donors (n=35) and breast cancer patients (n=112) considering histological types and grades of their tumors, as well as status of ER, PR and HER2 receptors. It was shown that 6 TAAs including RAD50, PARD3, SPP1, SAP30BP, NY-BR-62 and NY-CO-58 had higher immunogenicity in breast cancer patient's sera compare with sera of healthy donors regardless their histological types and receptor status. Nevertheless, antibody response to PARD3 and SPP1 antigens was associated with more aggressive breast tumors. Subsequent ROC-analysis allowed to identify the optimal combination of 6 TAAs among 16 antigens tested which can differentiate cancer patients and healthy donors with high sensitivity (70%) and specificity (91%). Analysis of mRNA expression of the most immunogenic antigens SAP30BP, PARD3 and SPP1 in breast tumors by quantitative real-time PCR revealed elevated *SPP1* gene expression in tumors compared with non-cancerous tissue samples that was correlated with SPP1 antigen immunoreactivity in autologous patient's sera.

Materials and Methods

Patients serum and tissue samples

Sera samples of breast cancer patients and healthy donors were collected during routine diagnostic procedures at the Dnipropetrovsk Clinical Oncological Center (Dnipropetrovsk, Ukraine) between 2008 and 2010 and stored with glycerol (50%) at – 20°C. In total, 112 patients with newly diagnosed breast cancer and 35 patients with no neoplasm (control group) were recruited to the study (Table 1). Among them 80 patients had invasive ductal carcinoma (IDC), 23 had invasive lobular carcinoma (ILC) and 8 had medullary breast carcinoma (MBC). Fresh cancer and non-cancerous breast tissue (NCT) samples were obtained from 17 female patients with primary breast carcinomas and 7 patients with fibrocystic disease correspondingly, which undergoing surgery in the Dnipropetrovsk Clinical Oncological Center (Dnipropetrovsk, Ukraine) between 2009 and 2011. Tissue samples were kept frozen in liquid nitrogen after resection. Breast cancer tissue samples (n=17) included invasive ductal carcinomas (n=10), invasive lobular carcinomas (n=2), and medullary breast carcinomas (n=5). In all patients with a clinical diagnosis of breast cancer, histology findings were reviewed to confirm the diagnosis. The diagnosis of cancer was verified on tissue samples by expert pathologist at the Dnipropetrovsk Clinical Oncological Center (Dnipropetrovsk, Ukraine). The study was approved by the Ethics Committee of the Institute of Molecular Biology and Genetics, NASU (Kyiv, Ukraine) and the Clinical Oncological Center (Dnipropetrovsk, Ukraine), and informed consent was obtained from all patients.

Cloning, expression and purification of recombinant proteins

cDNAs of 13 antigens including *ANKRD11, RAD50, FAM50A, LGALS3BP, HMGN2, LRRFIP1, PABPC4, PARD3, PDCL, RBPJ, SAP30BP, SPP1,* and *TOP2B* genes, isolated from MBC cDNA libraries, were cloned in bacteria using pGEX4T3, pET28b or pET42b expression vectors, containing glutation-S-transpherase (GST), 6His and GST-6His sequences, respectively. Recombinant plasmids pET23d/NY-CO-58, pET23d/NY-BR-62, and pET24d/NY-BR-1 were kindly provided by Prof. Dirk Jager (National Center of Tumor Diseases, Heidelberg, Germany) and Dr. Mattew J. Scanlan (Ludwig Institute for Cancer Research, New-York, USA). Expression of fused recombinant proteins was induced by 1 mM IPTG at 37°C in LB-medium for 4h in *E. coli* BL21 (DE3) pLysE cells transformed by correspondent recombinant plasmids. Recombinant proteins were affine purified using GST-sepharose or Ni-NTA-agarose according to manufacturers' protocols. For

purification of RAD50, LRRFIP1, RBPJ, and SPP1 recombinant proteins, which were predominantly in inclusion bodies, the protocol designed by Yang et al. (Yang et al., 2004) was used to increase their purity. Purity of antigens was defined by SDS-PAGE (Coomassie staining).

ELISA

To identify autoantibodies in patients' sera samples ELISA (enzyme-linked immunosorbent assay) was performed using 96-well plates (Sarstedt, USA), which were coated by recombinant GST, GST-6His, 6His-fused antigens (3 μg/ml) in PBS, pH 7.4 (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl) overnight at +4°C. The plates were washed 4 times by PBS containing 0,1% Tween-20, blocked with 5% casein hydrolysate (USB, USA) in PBST 2h at 37°C and 100 μl of sera samples (diluted 1:100) with 0.5% casein hydrolysate in PBS were applied in duplicates for 90 min at 37°C. The plates were washed as described above and processed with 100 μl of anti-human Fc-specific IgG antibodies conjugated with horse radish peroxidase (Jackson Immuno Research, USA) diluted $1:10^4$ with 1% casein hydrolysate in PBS for 1h at 37°C. The plates were washed again and the signal was visualized with 100 μl of ABTS (2,2'azinodietiltiazolilsulfoate acid) substrate (Sigma, USA) for 30 min at 37C. Optical density (OD) of wells was evaluated at A_{410} using spectrophotometer Multiscan (Labsystems, USA).

Real-time quantitative RT-PCR

Total RNA from 17 breast cancer and 7 non-cancerous breast tissue samples was isolated by the guanidinium thiocyanate method (Chomczynski et al., 1987). 3 μg of RNA was converted into cDNA using M-MuLV Reverse Transcriptase (Fermentas, USA) at 37°C for 60 min using oligo (dT)18 primers in 20 μl reaction volume according to the standard protocol of manufacturer (Fermentas, USA). Gene-specific PCR primers were designed using NCBI software Primer-BLAST (URL:http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qPCR reactions were prepared in duplicates using 50 ng of cDNA diluted in SYBR Green I Master Mix ×2 buffer (Fermentas, USA) with 9 pmol of corresponding gene-specific forward and reverse primers. The following thermal conditions were applied: 95°C for initial denaturation (30s) and 40 cycles consisting of 95°C denaturation (10s), 55°C annealing (5s), and 60°C extension (60s). Thermal cycling and fluorescent monitoring

were performed using iCycler iQ5 PCR thermal cycler (Bio-Rad, CA, USA). Cycle threshold (Ct) was determined and normalized against Ct value of the actin (ACTB) endogenous control product (ΔCt= Ct target gene — Ct ACTB). For 7 non-cancerous breast cDNA samples normalized ΔCt was calculated as the mean value. The relative concentrations of gene-specific mRNAs in breast cancer tissues compared to non-cancerous breast tissues were calculated by subtracting the normalized mean ΔCt value obtained for non-cancer breast tissues from those obtained for each of 17 tumor samples (ΔCt = ΔCt of tumor—mean ΔCt for 4 non-cancer breast). The relative concentration was determined as $2^{\Delta\Delta Ct}$ (Kubista et al., 2006).

Statistical analysis

Primary data systematization and analysis was performed using Excel software (Microsoft Office, 2007). Serum samples were considered as positive for the presence of autoantibodies when their optical density (OD) according to ELISA data exceeded the accepted cut-off for an assay to the antigens. The cut-off of the test was considered as the mean plus 3 standard deviations of the OD values of all healthy donors' samples. To determine whether the frequency of autoantibodies to each of 16 TAAs was significantly higher in sera of breast cancer patients compare with sera of healthy donors, data were analyzed using the Fisher two-sided exact test. A p value 0.05 was considered as a statistically significant. SPSS 17.0 (USA) and Graphpad Prism 5.0 (USA) were used to compute sensitivity and specificity, and to generate receiver operator characteristic (ROC) curves. Area under the curve (AUC) for each autoantibody also was tested to determine differences from chance. Spearman rank tests were used for pairwise correlations among immunogenicity and mRNA expression levels of antigens.

Results

Antigens for serological analysis by ELISA

16 potential tumor-associated antigens, previously identified by SEREX analysis of breast and colon cancer cDNA libraries (Kiyamova et al., 2010; Kostianets et al., 2012; Jager et al., 2001; Scanlan et al., 2001; Scanlan et al., 2002) were selected for current study. Twelve out of 13 antigens (RAD50, FAM50A, LGal3SBP, HMGN2, PARD3, SAP30BP, ANKRD11, SPP1, PDCL, PABCP4, RBPJ, LRRFIP1) identified in MBC cDNA library had tumor-associated serological profile of autoantibodies according to the data of phage based

allogeneic screening of all 41 identified medullary breast carcinoma autoantigens (Kostianets et al., 2012). TOP2B MBC antigen was included in this study as potential tumor antigen and target for cancer treatment (Cowell et al., 2000; Wang, 1996), although it had not demonstrated high level of immunoreactivity in cancer patient's sera according our data (Kostianets et al., 2012). In addition, 3 breast cancer associated antigens identified during serological screening of cDNA libraries from breast (NY-BR-1 (Jager et al., 2001), NY-BR-62 (Scanlan et al., 2001) and colorectal (NY-CO-58) (Scanlan et al., 2002; Gnjatic et al., 2010) tumors were kindly provided for analysis by Prof. D. Jager and Dr. M. Scanlan were included in this screening.

cDNAs of all selected antigens were cloned in frame with GST-, GST-6His-, and His-tags in correspondent vectors and expressed in bacteria (Table 2). Purified recombinant TAAs (full-size proteins or their immunogenic fragments) were used as antigens in ELISA screening (Table 2) of breast cancer patients (IDC, ILC, MBC) and healthy donors sera.

Evaluation of the frequency of antibody response in sera of breast cancer patients toward TAAs

A frequency of autoantibodies in breast cancer patients sera directed against each individual recombinant antigen was evaluated by ELISA. Sera of 112 patients with breast tumors of different histological types and grades, and sera of 35 healthy donors, which were obtained from the same geographical region, were included in the analysis. The data of allogenic screening presented in Table 3 demonstrate that the autoantibody frequency in sera of breast cancer patients to individual TAAs varied from 0 to 21.7%. Frequency of autoantibodies in cancer patient sera was significantly higher compare to healthy individuals only for 6 out of 16 antigens (RAD50, NY-CO-58, PARD3, SAP30BP, SPP1 and NY-BR-62) and increased frequency of antibody response to NY-BR-62 antigen was found only in sera of ILC patients, but not in sera of cancer group patients (Table 3). As for individual antigens, the frequency of autoantibodies varied in patients with different histological types of breast tumors. In patients with IDC the highest immune reactivity was detected for RAD50 (18,8%), SPP1 (18.8%), NY-CO-58 (16.3%), PARD3 (15%), and SAP30BP (15%) antigens, while in patients with MBC - for SAP30BP (22.2%) and SPP1 (33.3%) antigens, and in patients with ILC - for NY-BR-62 (26.1%), PARD3 (21.7%) and SAP30BP (13%) antigens. However we did not find statistically significant differences of antibody response between groups of cancer patients with tumors of different histological types.

So, statistically significant difference was found between autoantibodies existence to 6 antigens in sera of cancer patients predominantly with IDC histological type compare with frequency of autoantibody response in sera of healthy donors, but not between groups of patients with different histological types.

Frequency of antibodies to RAD50, NY-BR-62, NY-CO-58, PARD3, SAP30BP, and SPP1 was further assessed considering tumors grade (Table 4). Histological tumor grade is based on the degree of differentiation of the tumor tissue and is determined according to Nottingham histologic score. In breast cancer, it refers to the semi-quantitative evaluation of morphological characteristics of tumor tissue sections. This grading method evaluates three parameters and assigns a score of 1 to 3 for each parameter as follows: tubule formation (> 75%, 1; 10–75%, 2; < 10%, 3), nuclear pleomorphism (none, 1; moderate, 2; pronounced, 3), and number of mitoses/10 high power fields (HPF), based on a HPF size of 0.274 mm2 (< 10 mitoses, 1; 10–19 mitoses, 2; ≥ 20 mitoses, 3). The final grade is based on the sum of the scores of the three parameters: 3, 4, or 5 = grade 1; 6 or 7 = grade 2; and 8 or 9 = grade 3) (Elston et al., 1991; Frkovic-Grazio et al., 2002).

Autoantibodies to NY-CO-58 and SAP30BP were detected only in patients with G2 and G3 tumors, to RAD50, SPP1, and NY-BR-62 in all breast cancer patients irrespectively of tumor grade, and to PARD3 – only in patients with G3 tumors. It should be noted that statistically significant difference between frequencies of autoantibodies in the groups of patients with tumors of different grade was shown only for SPP1 antigen in the groups of patients with G1 and G3 tumors (Table 4).

No correlation between autoantibodies existence to all 16 antigens with molecular (HER2-, ER-, PR-status) variables of tumors of cancer patients was found during this study (data not shown).

Assessment of potential immunodiagnostic value of antigens combination

To assess immunodiagnostic value for each of 16 antigens we detected their predictive accuracies by logistic regression model (ROC-analysis). Predictive accuracies of selected antigens was evaluated in accordance with sensitivity and specificity values as well as AUC scale which can be classified as middle (AUC>0,6) and good (AUC>0,7). Table 5 presents sensitivity and specificity and AUC values of the most immunogenic 6 antigens according to ROC-analysis of allogenic screening data. According to these data all of the TAAs demonstrated quite low sensitivity values when analyzed separately.

Further we analyzed different combinations of all 16 antigens and found optimal antigenic combination, that included all 6 antigens with highest immunoreactivity in breast cancer patient's sera and which could distinguish cancer patients and healthy donors with 70% sensitivity, and specificity to 91%, AUC=0,808 [95% CI, 0.736-0.879] (Figure 1).

Evaluation of SPP1, PARD3, and SAP30BP mRNAs expression in breast carcinomas of different histological type by quantitative real time PCR

Taking into account that autoantibody production in human body may be caused by overexpression of the correspondent proteins in breast tumors, we examined the expression profile of some antigens such as SPP1, PARD3, SAP30BP in 17 breast carcinomas of various histological types (IDC, ILC, MBC) and 7 non-cancerous tissue samples by real-time PCR. These antigens were selected for analysis because of their highest sensitivity compare with other studied antigens according to ROC-analysis data (see above). The results of relative gene expression analysis of 3 genes in breast tumors are shown in Figure 2. Notably that mRNA expression of *SPP1* was 2-7 fold upregulated compared with noncancerous tissue samples in 11 out of 17 breast tumor samples (Figure 2). At the same time *PARD3* and *SAP30BP* mRNA levels in the most tumor samples did not differ from the respective mRNAs levels in non-cancerous tissue samples. The difference between expression values in cancer and normal tissues was statistically significant only in case of SPP1 antigen (13.7±12.55 vs. 2.14±1.57, two-tailed U-test p=0.0051).

Taking into account this finding it was interesting to study whether gene expression of TAAs correlated with immunoreactivity of respective antigen in the sera of the same patient. For this purpose paired autologous tumor tissue and sera samples obtained from 17 breast cancer patients were analyzed in respect of *SPP1*, *PARD3* and *SAP30BP* genes expression in tumors and immunoreactivity of correspondent antigen in sera. The moderate positive correlation (r = 0.393028) between TAA gene expression and its immunoreactivity in autologous sera was detected only for SPP1 (see Figure 3).

Discussion

The identification and study of TAAs and corresponding autoantibodies for cancer diagnostics, monitoring and therapy became the key trend stream of investigations focused on malignant tumors biomarkers research in the last 20 years. The aim of the current study was to characterize SEREX-defined antigens and determine autoantibodies profiles, which could differentiate patients with breast cancer and healthy donors. For this purpose large-scale allogenic screening of previously identified 16 SEREX-antigens was performed by ELISA followed by ROC-analysis of results obtained. The frequency of autoantibodies directed against above mentioned antigens in sera of 112 patients with different clinicophatological characteristics of breast

carcinomas compared with sera of 35 healthy donors was assessed. Autoantibody frequency for individual TAA varied in groups of patients with tumors of different histological types. In IDC patients the most abundant were autoantibodies to RAD50, NY-CO-58, NY-BR-62, PARD3, SAP30BP, SPP1; in MBC patients – to SAP30BP and SPP1; while in the group of ILC patients to PARD3, SAP30BP, and to NY-BR-62. So, 6 antigens (RAD50, NY-CO-58, PARD3, SAP30BP, SPP1 and NY-BR-62) of 16 showed individual significant difference between breast cancer patients and healthy donors demonstrating ability to discriminate these cohorts.

Notably, no significant difference in frequency of autoantibodies between the groups of patients with tumors of different histological types and ER, PR, HER2 status was detected in case of any antigen tested. However, when tumor grade was taken into account antibody to the PARD3 antigen was detected exclusively in sera of patients with more aggressive disease, suggesting that PARD3 as a potential prognostic marker. In addition, significant decrease of autoantibody frequency with increasing of tumors grade was detected for SPP1 antigen. It may be explained in part by existence of anti-SPP1 autoantibody and SPP1 protein immune complexes at the advanced stages of breast tumor development especially that elevated serum level of SPP1 protein has been previously shown in sera of patients with more aggressive breast tumors (Fedarko et al., 2001). The existence of immune complexes in breast cancer patients' sera has been already described for glycolysis and splicesome proteins (Ladd et al., 2013). Authors indicated that immune complex formation with increasing levels of antigen is one possible explanation for the observed decrease of autoantibody signal closer to diagnosis and suggest, that formation of these complexes may explain the observed immune suppression in breast cancer patients (Ladd et al., 2013).

Among these 6 antigens we are the first who described increased frequency of antibody response toward SPP1, RAD50, PARD3 and SAP30BP antigens in sera of breast cancer patients compared with sera of healthy women. This fact may indicate these antigens as potential molecular markers of breast tumors. In this connection, association with breast cancer was already described for SPP1 (Wai et al., 2004; Rodrigues et al., 200;, Tilli et al., 2011) and RAD50 (Tomminska et al., 2006; Bartkova et al., 2008) antigens.

SPP1 (osteopontin) is a secretory protein, which mediates cell adhesion, chemotaxis, contributes to apoptosis avoidance, and is associated with many cancer types (Wai et al., 2004) including breast cancer (Rodrigues et al., 2007; Tuck et al., 2007). It should be noted that elevated level of serum autoantibodies to SPP1 was shown only in patients with prostate cancer (Tilli et al., 2011). RAD50 is a highly conserved DNA

double-strand break repair factor (Dolganov et al., 1996). The mutations which lead to RAD50 gene inactivation, increase predisposition to pancreas and breast cancers development (Wang et al., 2008). PARD3 (Par-3 partitioning defective 3 homolog *C. elegans*) is an adapter protein involved in asymmetrical cell division and cell polarization processes. It was shown that this protein is down regulated in primary esophageal squamous cell carcinoma compare to normal tissues (Zen et al., 2009). Cell functions and expression pattern of SAP30BP (SAP30-binding protein) are still unknown.

Immunoreactivity of the rest two antigens, namely NY-CO-58 (Gnjatic et al., 2010) and NY- and BR-62 (Scanlan et al., 2001), already has been studied in cohorts of patients with different cancer types. NY-CO-58 plays a central role in chromosome segregation during mitosis, proliferation and possibly in tumor growth (Gnjatic et al., 2010). Sequence of gene encoding NY-CO-58 is similar to kinesin family member 2C (KIF2C), which upregulated expression was detected by a genome-wide microarray analysis of breast cancer tissues (Shimo et al., 2008). Of note, antigen NY-BR-62 also is similar to kinesin 2 and can be involved in chromosome segregation during mitosis (Sueishi et al., 2000). Data of immunoreactivity of NY-BR-62 and NY-CO-58 antigens in sera of breast cancer patients received during this study are consistent with those of other authors (Scanlan et al., 2001; Scanlan et al., 2002).

So, large-scale allogenic screening allowed us to characterize immunoreactivity of 16 SEREX-defined antigens in sera of breast cancer patients and select 6 the most immunogenic antigens including RAD50, PARD3, SPP1, SAP30BP, NY-BR-62 and NY-CO-58. ROC-analysis of antibody response toward all 16 antigens led to identification of the optimal combination that included 6 antigens with the highest immunoreactivity in breast cancer patients' sera. This panel of 6 antigens can differentiate breast cancer patients and healthy donors with high sensitivity and specificity (70% and 91% correspondently). Since these antigens and autoantibodies thereto are potential breast cancer biomarkers, they could be used for creation of new and/or improving sensitivity and specificity of already existing antigenic/autoantibody signatures for breast cancer detection. For today several antigenic panels were already proposed for autoantibodies profile identification in breast cancer patients by different researchers with a wide range of sensitivity, specificity and AUC. As it was mentioned above the most of breast cancer antigens panels include widely characterized and well-known antigens, which were identified by different approaches predominantly without preliminary study of immunoreactivity in sera of cancer patients. Notable that breast cancer antigenic panels which were created

on the base of preliminary serological analysis (SEREX, SEPRA or other techniques) possess higher sensitivity compared with other antigenic panels (Zhong et al., 2008; Piura et al., 2010; Lacombe et al., 2013; Dong et al., 2013). Summarizing this fact together with our data we suggest that application of serological approaches for TAAs identification and characterization leads to the creation of the most sensitive combinations of new antigens important for further development of minimally invasive breast cancer detection assays.

To understand a possible mechanism underling the increased immunoreactivity of autologous proteins we examined the gene expression for 3 of the most immunogenic antigens including SPP1, PARD3 and SAP30BP in breast tumors and noncancerous tissue samples. The elevated mRNA expression level in breast tumors was found only for SPP1 antigen which correlates with its increased immunoreactivity in autologous patients' sera samples. One may be supposed that the reason of autoantibody response toward SPP1 in breast cancer patients may be caused by its elevated level in sera of breast cancer patients (Liang et al., 2011; Fedarko et al., 2001), which in turn may be caused by increased *SPP1* gene expression in breast tumors showed during this study. Moreover, taking into account literature data about elevated expression of osteopontin in tumors of high grade (Chakraborty et al., 2008) and our data showing decreased level of autoantibody to SPP1antigen in correspondent patients' sera detected by ELISA, we supposed that immune complexes may be formed at advanced stage of breast cancer disease.

Conclusions

The new combination of 6 antigens including RAD50, NY-CO-58, PARD3, SAP30BP, SPP1 and NY-BR-62 for autoantibodies profile detection, which can distinguish breast cancer patients and healthy individuals with 70% sensitivity and 91% specificity regardless histological types and receptor status of their tumors, was proposed. Antibody response toward PARD3 and SPP1 antigens may be associated with more aggressive breast tumors. Analysis of MBC TAAs and other SEREX-antigens performed in this study provides information that can be used not only for application of potential breast cancer biomarkers, but also for understanding of molecular events involved in breast cancer initiation and progression.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

1. Anderson KS, LaBaer J. (2005). The sentinel within: exploiting the immune system for cancer biomarkers. *J Proteome Res*: 4(4):1123–33.
2. Anderson KS, Sibani S, Wallstrom G, Qiu J, Mendoza EA, Raphael J, Hainsworth E, Montor WR, Wong J, Park JG, Lokko N, Logvinenko T, Ramachandran N, Godwin AK, Marks J, Engstrom P, Labaer J.. (2011). Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. *J Proteome Res*: 10(1):85-96.
3. Bartkova J, Tommiska J, Oplustilova L, Aaltonen K, Tamminen, A, Heikkinen T, MistrikM, Aittomaki K, Blomqvist C, Heikkila, P, Lukas J, Nevanlinna H, Bartek J. (2008). Aberrations of the MRE11-RAD50-NBS1DNA damage sensor complex in human breast cancer: MRE11 as a candidate familial cancer-predisposing gene. *Mol Oncol*: 2:296–316.
4. Boyle P, Chapman CJ, Holdenrieder S, Murray A, Robertson C, Wood WC, Maddison P, Healey G, Fairley GH, Barnes AC, Robertson JF. (2011). Clinical validation of an autoantibody test for lung cancer. *Ann Oncol*: 22(2):383-9.
5. Chakraborty G, Jain S, Patil TV, Kundu GC. (2008). Down-regulation of osteopontin attenuates breast tumour progression in vivo. *J Cell Mol Med*: 12(6A):2305-18.
6. Chan CC, Fan CW, Kuo YB, Chen YH, Chang PY, Chen KT, Hung RP, Chan EC. (2010). Multiple serological biomarkers for colorectal cancer detection. *Int J Cancer*: 126(7):1683-90.
7. Chapman C, Murray A, Chakrabarti J, Thorpe A, Woolston C, Sahin U, Barnes A, Robertson J. (2007). Autoantibodies in breast cancer: their use as an aid to early diagnosis. *Ann Oncol*: 18(5):868-73.

8. Chomczynski P, Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem:* 162(1):156-9.
9. Collett K, Stefansson IM, Eide J, Braaten A, Wang H, Eide GE, Thoresen SO, Foulkes WD, Akslen LA. (2005). A basal epithelial phenotype is more frequent in interval breast cancers compared with screen detected tumors. *Cancer Epidemiol Biomarkers Prev*: 14(5):1108-12.
10. Cowell IG, Okorokov AL, Cutts SA, Padget K, Bell M, Milner J, Austin CA. (2000). Human topoisomerase IIalpha and IIbeta interact with the C-terminal region of p53. *Exp Cell Res*: 255(1):86-94.
11. Desmetz C, Mange A, Maudelonde T, Solassol J. (2011). Autoantibody signatures: progress and perspectives for early cancer detection. *J Cell Mol Med*: 15(10):2013-24.
12. Dolganov GM, Maser RS, Novikov A, Tosto L, Chong S, Bressan, DA, Petrini JH. (1996). Human Rad50 is physically associated with human Mre11: identification of a conserved multiprotein complex implicated in recombinational DNA repair. *Mol Cell Biol*: 16:4832–4841.
13. Dong X, Yang M, Sun H, Lü J, Zheng Z, Li Z, Zhong L. (2013). Combined measurement of CA 15-3 with novel autoantibodies improves diagnostic accuracy for breast cancer. *Onco Targets Ther*: 6:273-9.
14. Elston, CW, Ellis IO. (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*: 19: 403-10.
15. Evans R. L., Pottala J. V., Egland K. A. (2014). Classifying Patients for Breast Cancer by Detection of Autoantibodies against a Panel of Conformation-Carrying Antigens. *Cancer Prev Res (Phila)*: 7(5): 545–555.
16. Frkovic-Grazio S, Bracko M. (2002). Long term prognostic value of Nottingham histological grade and its components in early (pT1N0M0) breast carcinoma. *J Clin Pathol*: 55(2): 88–92.
17. Fedarko NS, Jain A, Karadag A, Van Eman MR, Fisher LW. (2001). Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. *Clin Cancer Res*: 7(12):4060-6.
18. Gnjatic S, Cao Y, Reichelt U, Yekebas EF, Nölker C, Marx AH, Erbersdobler A, Nishikawa H, Hildebrandt Y, Bartels K, Horn C, Stahl T, Gout I, Filonenko V, Ling KL, Cerundolo V, Luetkens T, Ritter G, Friedrichs K, Leuwer R, Hegewisch-Becker S, Izbicki JR, Bokemeyer C, Old LJ, Atanackovic D.. (2010). NY-CO 58/KIF2C is overexpressed in a variety of solid tumors and induces frequent T cell responses in patients with colorectal cancer. *Int J Cancer*: 127(2):381-93.

19. Gnjatic S, Ritter E, Büchler MW, Giese NA, Brors B, Frei C, Murray A, Halama N, Zörnig I, Chen YT, Andrews C, Ritter G, Old LJ, Odunsi K, Jager D. (2010). Seromic profiling of ovarian and pancreatic cancer. *Proc Natl Acad Sci USA*: 107(11):5088-93.
20. Jäger D, Stockert E, Güre AO, Scanlan MJ, Karbach J, Jäger E, Knuth A, Old LJ, Chen YT. (2001). Identification of a tissue-specific putative transcription factor in breast tissue by serological screening of a breast cancer library. *Cancer Res*: 61(5):2055-61.
21. Kim S, Cho H, Nam EJ, Kim SW, Kim YT, Park YW, Kim BW, Kim JH. (2010). Autoantibodies against stress-induced phosphoprotein-1 as a novel biomarker candidate for ovarian cancer. *Genes Chromosomes Cancer*: 49(7):585-95.
22. Kiyamova R, Kostianets O, Malyuchik S, Filonenko V, Usenko V, Gurtovyy V, Khozayenko Y, Antonuk S, Old L, Gout I. (2010). Identification of tumor-associated antigens from medullary breast carcinoma by a modified SEREX approach. *Mol Biotechnol*: 46(2):105-12.
23. Kostianets O, Shyian M, Sergiy D, Antoniuk S, Gout I, Filonenko V, Kiyamova R. (2012). Serological analysis of SEREX-defined medullary breast carcinoma-associated antigens. *Cancer Invest*: 30(7):519-27.
24. Koziol JA, Zhang JY, Casiano CA, Peng XX, Shi FD, Feng AC, Chan EK, Tan EM. (2003). Recursive partitioning as an approach to selection of immune markers for tumor diagnosis. *Clin Cancer Res*: 9(14):5120-6.
25. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonrak J, Lind, K, Sindelka R, SjËoback R, SjËogreen B, StrËombom L, Stahlberg A, Zoric N. (2006). The real-time polymerase chain reaction. *Mol Aspects Med*: 27:95–125.
26. Lacombe J, Mangé A, Bougnoux AC, Prassas I, Solassol J. (2014). A multiparametric serum marker panel as a complementary test to mammography for the diagnosis of node-negative early-stage breast cancer and DCIS in young women. Cancer Epidemiol Biomarkers Prev: 23(9):1834-42.
27. Lacombe J, Mangé A, Jarlier M, Bascoul-Mollevi C. (2013). Identification and validation of new autoantibodies for the diagnosis of DCIS and node negative early-stage breast cancers. Rouanet P, Lamy PJ, Maudelonde T, Solassol J. *Int J Cancer*: 132(5):1105-13.
28. Lacombe J, Mangé A, Solassol J. (2014). Use ofautoantibodies to detect the onset of breast cancer. *J Immunol Res*: 2014:574981.

29. Ladd JJ, Chao T, Johnson MM, Qiu J, Chin A, Israel R, Pitteri SJ, Mao J, Wu M, Amon LM, McIntosh M, Li C, Prentice R, Disis N, Hanash S.. (2013). Autoantibody signatures involving glycolysis and splicesome proteins precede a diagnosis of breast canceramong postmenopausal women. *Cancer Res*: 73(5):1502-13.

30. Liang Y, Li H, Hu B, Chen X, Miao JB, Li T, You B, Chen QR, Fu YL, Wang Y, Hou SC. (2011). Elevated circulating levels of osteopontin are associated with metastasis in advanced non-small cell lung cancer. *Chin J Cancer Res*: 23(1):64-8.

31. Liu W, De La Torre IG, Gutiérrez-Rivera MC, Wang B, Liu Y, Dai L, Qian W, Zhang JY. (2015). Detection of autoantibodies to multiple tumor-associated antigens (TAAs) in the immunodiagnosis of breast cancer. *Tumour Biol*: 36(2):1307-12.

32. Lu H, Goodell V, Disis ML. (2008). Humoral immunity directed against tumor-associated antigens as potential biomarkers for the early diagnosis of cancer. *J Proteome Res*: 7(4):1388-94.

33. Mangé A, Lacombe J, Bascoul-Mollevi C, Jarlier M, Lamy PJ, Rouanet P, Maudelonde T, Solassol J. (2012). Serum autoantibody signature of ductal carcinoma in situ progression to invasive breast cancer. *Clin Cancer Res*: 18(7):1992-2000.

34. Pedersen JW, Blixt O, Bennett EP, Tarp MA, Dar I, Mandel U, Poulsen SS, Pedersen AE, Rasmussen S, Jess P, Clausen H, Wandall HH. (2011). Seromic profiling of colorectal cancer patients with novel glycopeptide microarray. *Int J Cancer*: 128(8):1860-71.

35. Piura E, Piura B. (2010). Autoantibodies to tumor-associated antigens in breast carcinoma. *J Oncol:* 2010:264926.

36. Piura E, Piura B. (2011). Autoantibodies to tailor-made panels of tumor-associated antigens in breast carcinoma. *J Oncol*: 2011:982425.

37. Rodrigues LR, Teixeira JA, Schmitt FL, Paulsson M, Lindmark-Mänsson H. (2007). The role of osteopontin in tumor progression and metastasis in breast cancer. *Cancer Epidemiol Biomarkers Prev*: 16(6):1087-97.

38. Scanlan MJ, Gout I, Gordon CM, Williamson B, Stockert E, Gure AO, Jäger D, Chen YT, Mackay A, O'Hare MJ, Old LJ. (2001). Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. *Cancer Immun*: 1:4.

39. Scanlan MJ, Welt S, Gordon CM, Chen YT, Gure AO, Stockert E, Jungbluth AA, Ritter G, Jäger D, Jäger E, Knuth A, Old LJ. (2002). Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. *Cancer Res*: 62(14):4041-7.

40. Shimo A, Tanikawa C, Nishidate T, Lin ML, Matsuda K, Park JH, Ueki T, Ohta T, Hirata K, Fukuda M, Nakamura Y, Katagiri T. (2008). Involvement of kinesin family member 2C/mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. *Cancer Sci*: 99(1):62-70.

41. Sueishi M, Takagi M, Yoneda Y. (2000). The forkhead-associated domain of Ki-67 antigen interacts with the novel kinesin-like protein Hklp2. *J Biol Chem*: 275(37):28888-92.

42. Tilli TM, Silva EA, Matos LC, Faget DV, Dias BF, Vasconcelos JS, Yokosaki Y, Gimba ER. (2011). Osteopontin is a tumor autoantigen in prostate cancer patients. *Oncol Lett*: 2(1):109-114.

43. Tommiska J, Seal S, RenwickA, Barfoot R, Baskcomb L, Jayatilake, H, Bartkova J, Tallila J, Kaare M, Tamminen A, Heikkilea P, Evans DG, Eccles D, Aittomäki K, Blomqvist C, Bartek J, Stratton MR, Nevanlinna H, Rahman N.. (2006). Evaluation of RAD50 in familial breast cancer predisposition. *Int J Cancer*: 118:2911–2916.

44. Tuck AB, Chambers AF, Allan AL. (2007). Osteopontin overexpression in breast cancer: knowledge gained and possible implications for clinical management. *J Cell Biochem*: 102(4):859-68.

45. Wai PY, Kuo PC. (2004). The role of Osteopontin in tumor metastasis. *J Surg Res*: 121(2):228-41.

46. Wang JC. (1996). DNA topoisomerases. *Annu Rev Biochem*: 65:635-92.

47. Wang X, Szabo C, Qian C, Amadio PG, Thibodeau SN, Cerhan JR, Petersen GM, Liu W, Couch FJ. (2008). Mutational analysis of thirty-two double-strand DNA break repair genes in breast and pancreatic cancers. *Cancer Res*: 68(4):971-5.

48. Yahalom G, Weiss D, Novikov I, Bevers TB, Radvanyi LG, Liu M, Piura B, Iacobelli S, Sandri MT, Cassano E, Allweis TM, Bitterman A, Engelman P, Vence LM, Rosenberg MM.. (2013). An antibody-based blood test utilizing a panel of biomarkers as a new method for improved breast cancer diagnosis. *Biomark Cancer*: 5:71-80.

49. Yang XA, Dong XY, Li Y, Wang YD, Chen WF. (2004). Purification and refolding of a novel cancer/testis antigen BJ-HCC-2 expressed in the inclusion bodies of Escherichia coli. *Protein Expr Purif*: 33(2):332-8.

50. Ye H, Sun C, Ren P, Dai L, Peng B, Wang K, Qian W, Zhang J. (2013). Mini-array of multiple tumor-associated antigens (TAAs) in the immunodiagnosis of breast cancer. *Oncol Lett*: 5(2):663-668.
51. Zayakin P, Ancāns G, Siliņa K, Meistere I, Kalniņa Z, Andrejeva D, Endzeliņš E, Ivanova L, Pismennaja A, Ruskule A, Doniņa S, Wex T, Malfertheiner P, Leja M, Linē A.. (2013) Tumor-associated autoantibody signature for the early detection of gastric cancer. *Int J Cancer*: 132(1):137-47.
52. Zen K, Yasui K, Gen Y, Dohi O, Wakabayashi N, Mitsufuji S, Itoh Y, Zen Y, Nakanuma Y, Taniwaki M, Okanoue T, Yoshikawa T. (2009). Defective expression of polarity protein PAR-3 gene (PARD3) in esophageal squamous cell carcinoma. *Oncogene*: 28(32):2910-8.
53. Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EK, Tan EM. (2003). Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. *Cancer Epidemiol Biomarkers Prev*: 12(2):136-43.
54. Zhang JY, Tan EM. (2010).Autoantibodies to tumor-associated antigens as diagnostic biomarkers in hepatocellular carcinoma and other solid tumors. *Expert Rev Mol Diagn*: 10(3):321-8.
55. Zhong L, Ge K, Zu JC, Zhao LH, Shen WK, Wang JF, Zhang XG, Gao X, Hu W, Yen Y, Kernstine KH. (2008). Autoantibodies as potential biomarkers for breast cancer. *Breast Cancer Res:* 10(3):R40.

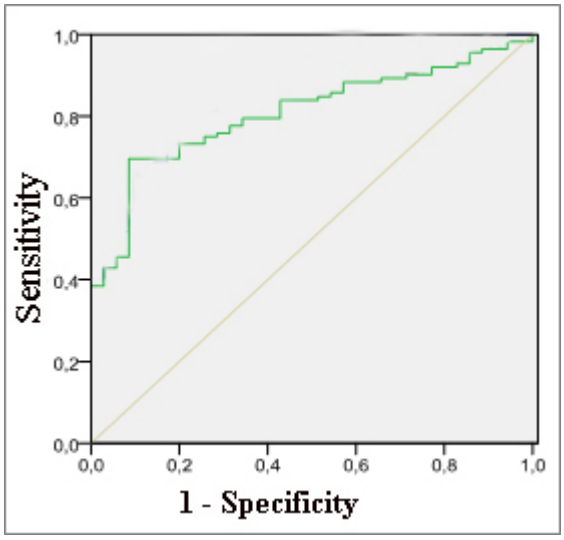

Figure 1. The ROC curve obtained from the analysis of six top TAAs (RAD50, NY-BR-62, NY-CO-58, PARD3, SAP30BP, SPP1). The false positive rate (1-specificity (x-axis)) is plotted against the true positive rate (sensitivity (y-axis)) for each cut-off point applied. AUC = 0.945, 95% confidence interval = 0.895 − 0.996). AUC=0,808 [95% CI, 0.736-0.879].

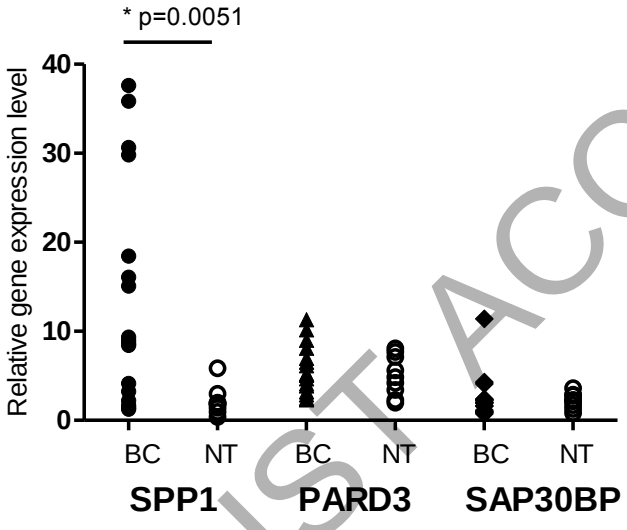

Figure 2. Analysis of *SPP1, PARD3, SAP30BP* mRNAs expression levels in breast cancer (BC) (n=17) and noncancerous tissues (NT) samples (n=9). The value of mRNA levels is expressed in arbitrary units of normalized actin expression level.

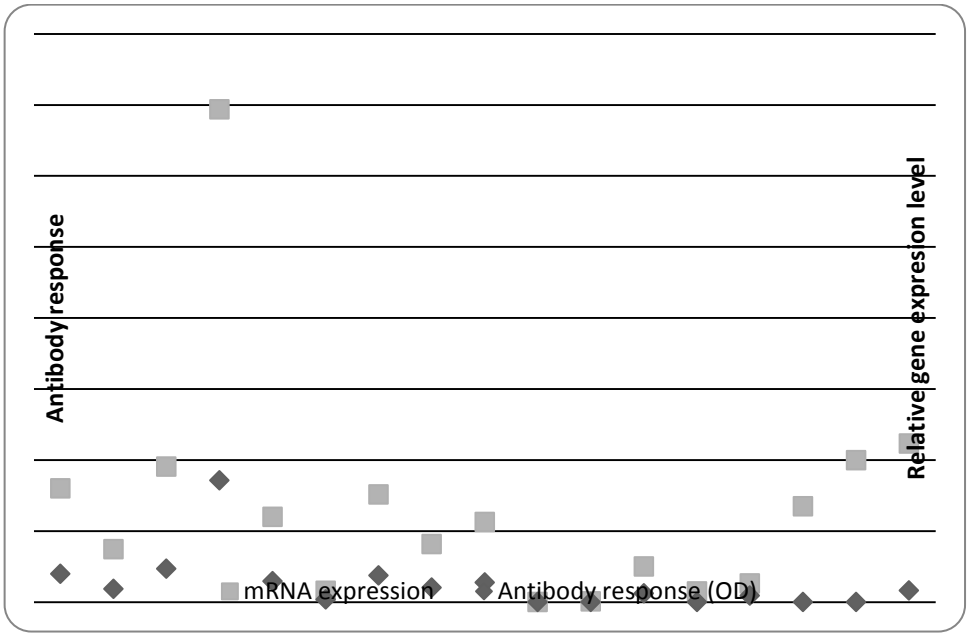

Figure 3. Analysis of *SPP1* gene expression and immunoreactivity of SPP1 antigen in the sera of the 17 breast cancer patients.

Table 1. Molecular and clinicopathological characteristics.

Characteristics	Serum samples	Tissue samples
Control group		
Number of patients	35	7
Age at diagnosis, years (range)	17-60	28-49
Mean ± SD (years)	39.2±12.56	40.2±10.04
Group of cancer patients		
Number of patients	112	17
Age at diagnosis, years (range)	19-82	35-82
Mean ± SD (years)	59.49±17.2	59.22±12.7
Tumor type		
invasive ductal breast carcinoma	80	10
invasive lobular breast carcinoma	23	2
medullary breast cancer	9	5
Tumor grade (%* from general)	82	86
	grade 1 – 7.3	grade 1 – 0
	grade 2 – 33.9	grade 2 – 22.2
	grade 3 – 59.8	grade 3 – 77.8
ER-status (%*)	52	76
Positive	71.1	78.1
Negative	28.9	21.9
PR-status (%*)	52	76
Positive	30.8	62.5
Negative	69.2	37.5
HER-2/neu status (%*)	52	76
Positive	30.8	31.3
Negative	69.2	68.7
Lymphoid nodes status (% of positive)	28.8	20

* Tumor grade, ER-, PR-, HER-2/neu status was known not for all tissue samples, the respective % of tumors with known characteristics is indicated in the table.

Table 2. Antigens selected for serological screening

Antigen	Full name	NCBI reference (sequence number)	cDNA fragment, bp	Vector
ANKRD11	Homo sapiens ankyrin repeat domain 11	NM_013275.4	951-1811	pGEX4T3 (GST-tag)
RAD50	Homo sapiens RAD50 homolog (S. cerevisiae)	NM_005732.2	2552-3374	pET28b (6His-tag)
FAM50A	Homo sapiens family with sequence similarity 50, member A	NM_004699.1	76-1095	pGEX4T3 (GST-tag)
LGALS3BP	Homo sapiens lectin, galactoside-binding, soluble, 3 binding protein	NM_005567.2	1483-1686	pGEX4T3 (GST-tag)
HMGN2	Homo sapiens high-mobility group nucleosomal binding domain 2	NM_005517.3	191-463	pGEX4T3 (GST-tag)
LRRFIP1	Homo sapiens leucine rich repeat (in FLII) interacting protein 1	NM_004735.2	417-1804	pET28b (6His-tag)
PABCP4	Homo sapiens poly(A) binding protein, cytoplasmic 4 (inducible form)	NM_003819.2	927-3052	pET28b (6His-tag)
PARD3	Homo sapiens par-3 partitioning defective 3 homolog (C. elegans)	NM_019619.2	714-1416	pGEX4T3 (GST-tag)
PDCL	Phosducin-like protein	NM_005388.3	97-1002	pET28b (6His-tag)
RBPJ	Homo sapiens recombination signal binding protein for immunoglobulin kappa J region	NM_203284.1	492-1850	pET28b (6His-tag)
SAP30BP	SAP30 binding protein	NM_013260.6	67-981	pGEX4T3 (GST-tag)
SPP1	Homo sapiens secreted phosphor-protein 1 (osteopontin)	NM_000582.2	166-1068	pET42b (GST-6His-tag)
TOP2B	Homo sapiens topoisomerase (DNA) II beta 180 kDa	NM_001068.2	2383-4866	pGEX4T3 (GST-tag)
NY-BR-1	ankyrin repeat domain-containing protein 30A	NM_052997.2	-	pET24d (6His-tag)
NY-BR-62	Kinesin-like protein KIF15	NM_020242.2	1-451	pET23d (6His-tag)
NY-CO-58	Kinesin family member 2C	NM_006845.3	194-648	pET23d (6His-tag)

Table 3. Frequency of autoantibody detection to 16 tumor-associated antigens in patients with different types of breast carcinomas

Antigens	HD[a] (n=35)	C[a] (n =112)	MBC[a] (n=9)	IDC[a] (n=80)	ILC[a] (n=23)
	Number of positive sera[b] n(%)				
ANKRD11	1 (2,9)	3 (2,7)	0	2 (2,5)	1 (4,3)
RAD50	0	18 (16,1)**	1 (11,1)	15 (18,8)**	2 (8,7)
FAM50A	1 (2,9)	4 (3,6)	0	4 (5)	0
LGAL3BP	0	8 (7,1)	0	6 (7,5)	2 (8,7)
HMGN2	1 (2,9)	7 (6,3)	0	5 (6,3)	2 (8,7)
LRRFIP1	1 (2,9)	1 (0,9)	0	1 (1,3)	0
NY-BR-1	0	5 (4,5)	0	4 (5)	1 (4,3)
NY-BR-62	1 (2,9)	14 (12,5)	0	8 (10)	6 (26,1)*
NY-CO-58	1 (2,9)	17 (15,2)*	2 (22,2)	13 (16,3)**	2 (8,7)
PABCP4	1 (2,9)	4 (3,6)	0	3 (3,8)	1 (4,3)
PARD3	1 (2,9)	17 (15,2)**	0	12 (15)**	5 (21,7)*
PDCL	2 (5,7)	7 (6,3)	1 (11,1)	6 (7,5)	0
RBPJ	0	8 (7,1)	0	5 (6,3)	3 (13)
SAP30BP	0	17 (15,2)**	2 (22,2)*	12 (15)**	3 (13)*
SPP1	1 (2,9)	19 (17,1)*	3 (33,3)*	15 (18,8)**	1 (4,3)
TOP2B	0	2 (1,8)	0	2 (2,5)	0

[a]HD – healthy donors; C – all histological types of breast carcinoma, MBC – medullary breast carcinoma, IDC – invasive ductal carcinoma, ILC – invasive lobular carcinoma. * $p<0,05$, ** $p<0,01$, *** $p<0,001$.

[b] Cut-off: mean of healthy donors group plus 3 SD;

Table 4. Frequency of autoantibodies in sera of patients with tumors of different grades [c]

Grade	RAD50	NY-BR-62	NY-CO-58	PARD3	SAP30BP	SPP1
G1,n=6	33,3(2)	22,2(2)	0	0	0	50(3)
G2, n=27	14,8(4)	7,4(2)	18,5(5)	0	11,1(3)	14,8(4)
G3, n=49	12,2(6)	14,3(7)	14,3(7)	14,3(7) **	14,3(7)	14,3(7) *

[c] % of positive sera (n)
* compare with G1 group, $p<0,05$
** compare with G2 group, $p<0,05$

Table 5. Sensitivity and specificity of 6 top individual TAAs detected by ROC-analysis

Antigen	AUC	P-value	Sensitivity (%)	Specificity (%)
RAD50	0,624	0,02666	9,29	100
NY-BR-62	0,647	0,00871	13,39	97,14
NY-CO-58	0,709	0,00019	12,50	97,14
PARD3	0,689	0,00076	32,14	88,57
SAP30BP	0,647	0,00871	33,04	91,43
SPP1	0,744	<0,0001	41,07	85,71