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CELLULAR AND MOLECULAR BIOLOGY

Serological Analysis of SEREX-Defined Medullary Breast Carcinoma-Associated Antigens

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Medullary breast carcinoma (MBC) despite anaplastic features and high grade has a good prognosis that can be related to prominent lymphocytic infiltration. We analyzed the frequency of antibody response toward 41 SEREX (serological recombinant expression cloning)-defined MBC antigens in sera of allogeneic MBC patients using serological plaque-spot assay and examined the mRNA expression profile of some antigens in MBC tissues. This preliminary study allowed us to select 18 autoantigens as potential MBC-associated antigens. Further studies in large cohorts of breast cancer patients will be performed for their evaluation as targets for cancer diagnostics or therapy.

Keywords Medullary breast carcinoma, SEREX, Tumor-associated antigens, Autoantibodies, mRNA expression

INTRODUCTION

The search for clinically relevant cancer biomarkers that can act as surrogate indicators of disease stages and prognosis is a main goal of many studies, number of which is rapidly growing. Medullary breast carcinoma (MBC) is a promising object for identification of such markers since it phenotipically and immunophenotipically is associated with basal-like breast carcinomas that are known to be resistant to existing treatment modalities (1–3). Today there is no effective therapy of basal-like breast cancer, except cytotoxic chemotherapy (4). Despite the fact that basal-like breast cancer is an aggressive form of carcinoma with poor prognosis and a preference for younger women (5, 6), MBC paradoxically has a better prognosis for patients, than other carcinomas (7, 8). MBC is a grade 3 invasive malignancy, predominantly negative for the expression of ER and PR, HER2 amplification and expresses basal-associated markers, such as basal cytokeratins (CK5/6, CK17, and CK14) and EGFR (9). MBC is characterized by large sheets of poorly differentiated tumor cells, rare lymph node metastasis, and a prominent lymphoplasmacytic infiltration (7, 8). Correlation of increased plasmacytic infiltration with better survival in MBC has been shown more than 30 years ago (7). In immunocytic infiltrates of MBC, T-lymphocytes are more numerous than B-lymphocytes. Moreover, CD8+ cells are the major component of the T-lymphocytes, which have strong expressions of perforin, granzyme B and FasL (10). The prominence of IgG versus the usual IgA plasma cells in MBC together with the confinement of reactions to the tumor beds, suggest a specific response to antigens (11). Thus, tumor-infiltrating lymphocytes are believed to represent the local immune response directed to one or more tumor-derived neoantigens. Attempts to identify these antigens through the use of libraries containing variable regions of immunoglobulin genes from MBC infiltrating B cells led to the identification of ganglioside D3 (GD3) and cytoskeletal protein actin (12,13). The identification of novel MBC antigens may be important not only for developing of novel diagnostics and immunotherapy modalities for MBC, but also other types of breast carcinomas.

SEREX (Serological identification of antigens by recombinant expression cloning) is a high-throughput approach for the identification of TAAs in tumor samples by screening corresponding cDNA expression libraries with autologous sera (14). However, previous attempts to identify MBC antigens by SEREX approach were unsuccessful due to a very high percentage of IgG clones in generated cDNA libraries. To overcome this problem, we proposed and successfully applied the protocol that provided an efficient way of removing mRNAs encoding IgGs, so the depleted MBC cDNA library was free of IgG clones and suitable for serological screening as result of which 41 potential MBC antigens were found (15). In this study, we describe for the first time 24 from 41 potential MBC-associated antigens that were identified by modified SEREX approach. To evaluate the immunogenicity of all identified MBC antigens preliminary immunoscreening with sera from allogenic MBC patients and healthy donors was performed using a plaque-spot serological assay. In the

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course of this analysis, we found that 18 of 41 antigens reacted exclusively with sera from MBC patients and are of interest for further analysis in large-scale allogenic screening. In addition, reversed-transcription followed by quantitative PCR was performed for 6 potential MBC antigens that showed the differential expression profile in MBC tissue samples.

MATERIALS AND METHODS

Tissue and serum samples

Medullary breast cancer (Br 502, Br 505, Br 506, Br 508, and Br 509) and nontumor breast samples from patients with fibrocystic disease were obtained from female patients (46–69 years) undergoing surgery at the Dnipropetrovs'k Municipal Clinical Hospital No. 4 (Dnipropetrovs'k, Ukraine). Tissue samples were kept frozen at −70°C after resection. Sera from cancer patients (36–68 years) were collected during routine diagnostic procedures at the Dnipropetrovs'k Municipal Clinical Hospital No. 4 (Dnipropetrovs'k, Ukraine) and stored at −20°C with 50% glycerol. Autologous sera samples were available for 3 of 5 MBC tissue samples (Br 502, Br 505, and Br 506). The study was approved by the Ethics Committee of the Institute of Molecular Biology and Genetics, and informed consent was obtained from patients.

Construction and immunoscreening of cDNA phage library

Total RNA from tumor sample (no less than 500 mkg) was purified by the TRIsol Reagent (LifeTechnologies, USA). The depletion of total MBC RNA purified from samples of IgG mRNA was performed using specifically designed affinity matrix as previously published by Kiyamova *et al*. (15). Depleted RNA was used for mRNA purification using Dynabeads Oligo(dT)25 (Dynal, Oslo, Norway) and subsequently 5 mkg of mRNA was used for the construction of the λ-ZAP expression library according to the recommendations of manufacturer (ZAP-cDNA synthesis kit, ZAP-cDNA Gigapack III gold cloning kit; Stratagene, USA). The cDNA library consisted of 1×10^6 primary recombinants with insert sizes from 0.2 to 2 kb was screened with autologous serum of cancer patient as described previously (15).

Sequence analysis

Isolated monoclonal positive phages were converted into pBK-CMV phagemids using an *in vivo* excision procedure according to the "Stratagene" protocol (USA). The identification of isolated cDNA clones was carried out by automated DNA sequencing and screening various databases using BLASTN alignment algorithm.

Detection of autoantibodies in allogeneic sera

To assess frequencies of antibody responses to the SEREX-defined antigens in allogeneic immunoscreening procedure, we have used a modified protocol. *E. coli* cells were infected directly on gridded agar plates, by spotting 2 mkl (500 pfu/mkl) of monoclonal phages encoding individual serologically-defined medullary breast cancer antigens. Following a 6 hr amplification phase, plaque-derived proteins were blotted on the nitrocellulose membrane and screened with individual serum samples (dilution 1:100) as described above. In total, 41 MBC antigens were spotted per nitrocellulose membrane. Serum reactivity detected by the spot assay was verified in the conventional plaque assay. Both assays were found to have comparable specificity and sensitivity.

Real-time quantitative RT-PCR

Total RNA from 5 MBC and 5 nontumor breast tissue samples was isolated by the guanidinium thiocyanate method (16) and (3 μg) was reverse transcribed into cDNA using M-MuLV Reverse Transcriptase (Fermentas, Lithuania) at 37°C for 60 min using oligo(dT)18 primers in 20 μl reaction volume according to the standard protocol of manufacturer (Fermentas, Lithuania).

Gene-specific PCR primers were designed using NCBI software Primer-BLAST (URL: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1).

The qPCR reactions were prepared in duplicates using 50 ng of cDNA diluted in SYBR Green I Master Mix ×2 buffer (Fermentas, Lithuania) with 9 pmol of corresponding gene-specific forward and reverse primers. The following thermal conditions were applied: 95°C for initial denaturation (30 s) and 40 cycles consisting of 95°C denaturation (10 s), 55°C annealing (5 s), and 60°C extension (60 s). 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 4 nontumor breast cDNA samples normalized ΔCt was calculated as the mean value. The relative concentrations of gene-specific mRNAs in breast cancer tissues compared to nontumor breast tissues were calculated by subtracting the normalized mean ΔCt value obtained for nontumor breast tissues from those obtained for each of 5 MBC samples (ΔCt = ΔCt of tumor—mean ΔCt for 4 nontumor breast). The relative concentration was determined as $2^{\wedge}\Delta\Delta Ct$ (17).

RESULTS

Identification of serum-reactive MBC cDNA clones

In our previous study, 59 serum-reactive clones from approximately 5×10^5 pfu of MBC cDNA-expression library were identified using autologous patient serum by SEREX methodology (15). Bioinformatic analysis revealed that isolated clones represent 41 known gene products with ubiquitous expression pattern in normal human tissues. In our previous study, we have described 17 (Table 2) from 41 antigens that already have been reported in Cancer Immunome Data Base (http://ludwig-sun5.unil.ch/CancerImmunomeDB/SEREX) as SEREX antigens identified by screening of cDNA libraries from different tumor types (renal, stomach, prostate, lung, colorectal, colon carcinomas, and leukemia) including breast carcinoma (FAM50A, HMGN2, ZRF1, MORC4, and ROCK1) (15).

In this paper, we describe for the first time 24 from 41 antigens (indicated by asterisk in Table 2) that were identified during immunoscreening of cDNA libarary from MBC

Table 1. Real-Time PCR Primers

Gen	Primer	Nucleotide Sequence (5′–3′)	Length (bp)	T_m (°C)
RAD50	F	CAGACCAGGGACAGACTTGCCAA	23	59
	R	GAACTGGGAGTAAACTGCTGTGGCT	25	59
FAM50A	F	AGGAGTGGGAAGCCAAGCAGGA	22	60
	R	GGTGCCCAGAGCCATCCCAGTA	22	60
HMGN2	F	GGTTGTCTGCTAAACCTGCTCCTCCA	26	60
	R	TGCCTGGTCTGTTTTGGCATCTCCAT	26	61
RBPJ	F	TTTGGTGAGCGGCCTCCAC	19	57
	R	GACTTCTGTGCAACTTTTGCATGAAGA	27	57
PABPC4	F	AAATACGCCTCCAGTGTCCGCA	22	59
	R	AGCGTTCTCCCAGCATCTGCTT	22	59
DEK	F	TACAATTGCACAAGGAAAGGGGC	23	63
	R	CTGGCCTGTTGTAAAGCAGTTTG	23	63
ACTB	F	CTGGAACGGTGAAGGTGACA	20	61
	R	AAGGGACTTCCTGTAACAATGCA	23	61

Table 2. Antigens Identified by SEREX Analysis of Medullary Breast Carcinoma and Their Reference Sequences

	Gene Product	NCBI Reference Sequence Number
1	Homo sapiens family with sequence similarity 50, member A (FAM50A)	NM_004699.1
2	Homo sapiens poly(A) binding protein, cytoplasmic 4 (inducible form) (PABPC4)	NM_003819.2
3	Homo sapiens leucine rich repeat (inFLII) interacting protein 1 (LRRFIP1)	NM_004735.2
4	Homo sapiens high-mobility group nucleosomal binding domain2 (HMGN2)	NM_005517.3
5	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5 (SMARCA5)	NM_003601.2
6	Homo sapiens ribosomal protein L15 (RPL15)	NM_002948.2
7	Homo sapiens zuotin related factor 1 (ZRF1)	NM_014377.1
8	Homo sapiens ankyrin repeat domain 11 (ANKRD11)	NM_013275.4
9	Homo sapiens MORC family CW-type zinc finger 4 (MORC4)	NM_024657.3
10	Homo sapiens par-3 partitioning defective 3 homolog (C. elegans) (PARD3)	NM_019619.2
11	Homo sapiens mitochondrial ribosomal protein S24 (MRPS24)	NM_032014.2
12	Homo sapiens CCAAT/enhancer binding protein zeta (CEBPZ)	NM_005760.2
13	Homo sapiens Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)	NM_005406.2
14	Homo sapiens poly (A) binding protein, cytoplasmic 1 (PABPC1)	NM_002568.3
15	Homo sapiens myosin VI (MYO6)	NM_004999.3
16	Homo sapiens ribosomal protein L32 (RPL32)	NM_000994.3
17	Homo sapiens topoisomerase (DNA) II beta 180 kDa (TOP2B)	NM_001068.2
18	*Homo sapiens RAD50 homolog (RAD50)	NM_005732.2
19	*Recombination signal binding protein for immunoglobulin kappa J region (RBPJ)	NM_203284.1
20	*Phosducin-like protein (PDCL)	NM_005388.3
21	*Coiled-coil domain containing 99 (CCDC99)	NM_017785.4
22	*Cathepsin L1 (CTSL1)	NM_001912.3
23	*Lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP)	NM_005567.2
24	*SAP30 binding protein (SAP30BP)	NM_013260.6
25	*DnaJ (Hsp40) homolog, subfamily C, member 7 (DNAJC7)	NM 0033151
26	*Ankyrin repeat domain 36B (ANKRD36B)	NM_025190.2
27	*La ribonucleoprotein domain family, member 7 (LARP7)	NM_016648.2
28	*HAUS augmin-like complex, subunit 3	NM_024511.5
29	*DEK oncogene (DEK)	NM_005406.2
30	*Ribosomal protein S8 (RPS8)	NM_001012.1
31	*Proteasome (prosome, macropain) 26S subunit, ATPase, 1 (PSMC1)	NM_002802.2
32	*Human small subunit (SSU) processome component, homolog (yeast) (KRR1)	NM_007043.6
33	*Genomic contig SCAF_1103279184089	NW_001838769.1| Hs2_WGA162_36
34	*Homo sapiens TWIST neighbor (TWISTNB)	NM_001002926.1
35	*Yes-associated protein 1 (YAP1)	NM_006106.2
36	*Homo sapiens RAD54 homolog	NM_138270.2
37	*Homo sapiens secreted phosphoprotein 1 (osteopontin) (SPP1)	NM_000582.2
38	*EIF3B translation factor (EIF3B)	NM_001037283.1
39	*Destrin (actin depolymerizing factor) (DSTN)	NM_001011546.1
40	*Homo sapiens PRELI domain containing 1 (PRELID1)	NM_013237.2
41	*Caspase recruitment domain family, member 6 (CARD6)	NM_032587.3

*Antigens identified by SEREX approach and present here as potential MBC antigens for the first time.

tumor (Br 502) and never have been detected before by SEREX approach. All identified antigens were grouped on the basis of their function and subcellular localization. Fifteen antigens are classified as predominantly nuclear proteins implicated in DNA repair (e.g., RAD50, RAD54) and transcription (e.g., LRRIP1, ANKRD11, RBPJ). Twenty antigens are known as predominantly cytoplasmic proteins, including ribosomal proteins RPL15 and RPL32, cytoskeletal protein MYO6, proteins of the ubiquitin-proteasome pathway (e.g., PSMC1) and proteins involved in signal transduction pathways (e.g., PDCL, ROCK1). Two antigens are exclusively localized to mitochondria (MRPS24, PRELID1) while one antigen (MORC) is found in both mitochondria and nucleus. Among identified antigens, there are two that exhibit extracellular localization (SPP1, Gal3BP). Finally, one clone corresponded to uncharacterized protein product (Genomic contig SCAF_1103279184089). Notably, some antigens (RAD50, FAM50A, PABPC4, LRRFIP1, HMGN2, PDCL, SMARCA5, CCDC99, and RBPJ) were represented by more than one independent cDNA clone (up to 6) that might reflect their elevated expression at mRNA level.

Allogeneic screening of SEREX-defined MBC antigens with sera of cancer patients and healthy donors

The frequency of autoantibody response against the 59 isolated clones was determined by the serological plaque-spot assay in sera from 10 healthy donors and 6 patients with MBC (Figure 1).

In this analysis, we found that 13 antigens (32%) exhibited serological profile restricted only for autologous serum (Figure 2). Eight antigens (19%) showed a serological profile that was restricted not only to cancer patients, as evidenced by their reactivity with sera from both MBC patients and healthy donors with frequency ranging from 1/10 to 4/10. Autoantibodies to two antigens (5%) were identified in autologous and healthy donors' sera. The remaining 18 antigens reacted specifically with sera from MBC patients and included proteins with diverse cellular functions: a DNA repair protein (RAD50), stabilizer of labile mRNA (PABPC4), a candidate breast cancer tumor suppressor gene (ANKRD11), proteins with unknown function (ANKRD36B, FAM50A), subunit of human augmin complex (HAUS3), nuclear factor (DEK), transcription factor (LRRFIP1), nucleosomal factor (HMGN2), regulatory proteins (PDCL, SAP30BP), two secretory proteins involved in tumorogenesis (Gal3BP, SPP1), H2A-ubiquitin binding protein (ZRF1), protein essential for asymmetric cell division and polarized growth (PARD3), ribosomal protein (RPS8), and two proteasome components (PSMC1, KRR1). Moreover, RBPJ protein also may have a potential diagnostic value, since it demonstrates greater reactivity with sera from MBC patients than with that of healthy donors (Table 3). Notably, among the group of 18 antigens that showed cancer-restricted serological profile in allogeneic screening, 6 proteins were previously identified by SEREX in different types of tumors and three of them (FAM50A, HMGN2, and ZRF1) were detected in breast cancer [http://ludwig-sun5.unil.ch/CancerImmunomeDB/SEREX].

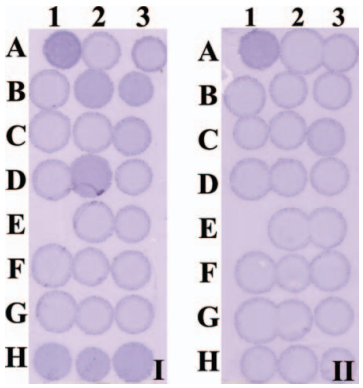

Figure 1. A representative example of a serological plaque-spot assay. SEREX-identified antigens from medullary breast carcinoma cDNA library were exposed to sera from cancer patient (I) and healthy donor sera (II) using plaque-spot assay: A/1—positive control (IgG clone), A/2—negative control (nonrecombinant phage), B/2—RPS8, B/3—ANKRD11, D/2—RBPJ, H/1—PDCL, H/2—RAD50, H/3—DEK.

Among 18 antigens that exhibited cancer-related autoantibody profile, 6 antigens (RAD50, PABPC4, ANKRD11, ANKRD36B, HAUS3, and DEK) showed the reactivity with 3 (43%) or more patient sera (Table 3). Autoantibodies against FAM50A, LRRFIP1, PDCL, HMGN2, Gal3BP, SAP30BP, ZRF1, PARD3, KRR1, RPS8, PSMC1, and SPP1 were found in sera of 2 out of 7 cancer patients. Different frequency of immunological sera reactivity could be explained in part by the heterogenity of cancer cells, whereby different proteins could be aberrantly processed or regulated in patients with the same type of cancer (18). However, limitations of applied experimental approach should be taken into account as well. It would be important to verify obtained results in a large scale plague-spot assay screening as well as other approaches, such as ELISA with purified recombinant antigens.

mRNA expression profile of 6 MBC antigens in MBC tissues

Analysis of the mRNA expression in MBC tissues and nontumor breast tissues using quantitative RT-PCR was performed for 6 potential MBC-associated antigens that showed cancer-related serological profile (RAD50, FAM50A, PABPC4, HMGN2, RBPJ, and DEK) and were represented by several cDNA clones (except DEK) (Table 3). Differences in relative gene expression were calculated as fold changes in gene expression in MBC compared to the mean of expression level in 4 nontumor breast tissue samples of patients with fibrocystic disease. Altered mRNA expression (up- and/or down-regulated) was defined as threefold differences in the expression level in MBC tumors relative to the mean of 4 nontumor breast tissues as described by Scanlan *et al.* (19).

According to the presented data heterogeneous mRNA expression profile in MBC tumors was observed for all antigens analyzed (Table 4). Altered (up- and/or down-regulated) mRNA expression was shown for RAD50, HMGN2, and RBPJ antigens; and up-regulation in the most of MBC

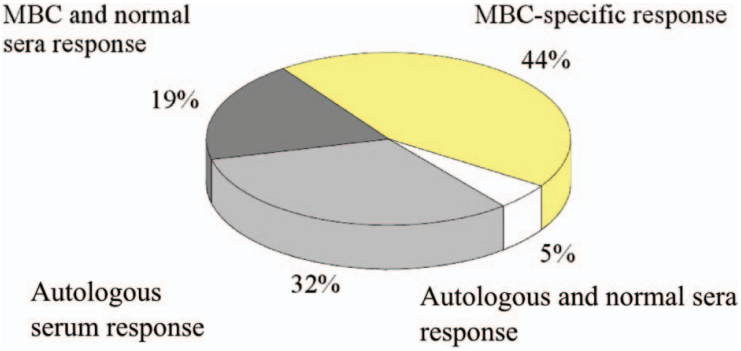

Figure 2. Representation of antigen reactivity with sera of patients with MBC and healthy donors.

tumors was detected for FAM50A and DEK antigens. mRNA expression of PABPC4 antigen was not altered in MBC tissues compared to nontumor breast tissues. Notably, that expression of RAD50 gene was down-regulated in tumor sample (Br502) used for creation of cDNA library.

We also analyzed the role of altered expression levels in inducing humoral immunity by testing serum reactivity and mRNA expression levels in matched pairs of autologous serum and tumor specimens (Table 5). Among 10 cases of up-regulated mRNA expression in seven cases autoantibody in patient sera have been detected toward their correspondent protein products. Two cases from three of down-regulated mRNA expression were accompanied with antibody response in sera of corresponding patients. In four cases from five of not altered mRNA expression we also detected humoral immune response toward correspondent protein products.

DISCUSSION

Identification of TAAs with cancer-restricted serological reactivity is very important for cancer diagnostics, disease monitoring and prognosis, and for developing novel immunotherapeutic approaches. SEREX methodology is a powerful tool for the identification of TAAs, especially for tumors that induce strong immune response, such as MBC. In previous study, we described a novel methodology for the generation of cDNA expression libraries from MBC suitable for serological screening (15). Extensive SEREX screening allowed us to isolate 59 positive clones representing 41 genes. Among these potential MBC-associated antigens we already described 17 antigens that have been previously detected by SEREX screening of a diverse range of human tumors by other researches (15), and remaining 24 antigens are presented in this study for the first time. All MBC-derived antigens were tested for their reactivity with sera from MBC patients and healthy donors and for six of them analysis of mRNA expression was performed.

According to the data presented 18 antigens exhibited cancer-related autoantibody profile and reacted only with sera from cancer patients. Moreover, six antigens from this group namely RAD50 protein, PABPC4, ANKRD11, ANKRD36B, HAUS3, and DEK demonstrated the highest serological reactivity in MBC patients (>40% response).

Table 3. Reactivity of SEREX-Defined Medullary Breast Carcinoma Antigens with Sera from Healthy Donors and Cancer Patients

Name	Number of Clones in cDNA Library	Medullary Breast Cancer Patient*	Healthy Donor	Previously Identified by SEREX in Other Tumors
RAD50	4	4/7	0/10	
PABPC4	3	3/7	0/10	Stomach cancer, testis
ANKRD11	1	3/7	0/10	
ANKRD36B	1	3/7	0/10	
HAUS3	1	3/7	0/10	
DEK	1	3/7	0/10	
FAM50A	6	2/7	0/10	Breast cancer
LRRFIP1	3	2/7	0/10	Hepatocarcinoma,
HMGN2	2	2/7	0/10	Breast cancer, colorectal adenocarcinoma
PDCL	2	2/7	0/10	
LGal3BP	1	2/7	0/10	
SAP30BP	1	2/7	0/10	
ZRF1	1	2/7	0/10	Stomach and breast cancer
PARD3	1	2/7	0/10	Prostate cancer, testis
RPS8	1	2/7	0/10	
PSMC1	1	2/7	0/10	
KRR1	1	2/7	0/10	
SPP1	1	2/7	0/10	
RBPJ	3	4/7	1/10	

*Includes the reactivity with serum used for immunoscreening of MBC cDNA library.

Table 4. Quantitative Analysis of mRNA Encoding 6 Seroreactive MBC Antigens in Nontumor and Medullary Breast Carcinoma Tissues

	Ratio of mRNA in MBC to mRNA in Nontumor Breast Tissue				
Antigen	Br502	Br505	Br506	Br508	Br509
RAD50	0.24**	24.60*	95.05*	0.04**	0.18**
FAM50A	7.01*	3.57*	9.03*	0.22**	4.36*
HMGN2	1.04	3.96*	7.07*	0.30**	0.41
RBPJ	0.55	13.61*	0.04**	1.36	0.74
PABPC4	1.63	1.89	2.02	0.63	1.31
DEK	29.35*	25.71*	0.27**	64.28*	3.12*

Differences in relative gene expression were calculated as fold changes in gene expression in MBC compared to mean of expression level in four nontumor breast tissue samples. The mRNA up-regulation (*) and down-regulation (**) was defined as threefold differences in the expression level of six antigens in five MBC tumors relative to the mean of four nontumor breast tissues.

RAD50, a highly conserved DNA double-strand break repair factor (20), has been already considered as a breast cancer related protein (21). The expression of this protein is aberrantly reduced in ER/PR/ERBB2 triple-negative and higher-grade familial breast tumors (21). Recently, breast cancer associated 687delT mutation in RAD50 was described in two families from Finland (22).

PABPC4 is a poly(A) tail binding protein associated with the 3-prime ends of most eukaryotic mRNAs and implicated in the initiation of translation (23).

ANKRD11 was initially proposed as a candidate breast cancer tumor suppressor gene, because of its location within the 16q24.3 breast cancer LOH (loss of heterozygosity) region (24). LOH of human chromosome 16q occurs in at least half of all breast tumors (25). ANKRD11 also interacts with and suppresses the function of the p160 coactivator family members, including the oncogene AIB1 (amplified in breast cancer-1) (26), which enhances ligand-dependent transactivation of steroid nuclear receptors, including estrogen receptor (27).

The function of ANKRD36B is unknown. HAUS3 is one of eight subunits of the 390-kD human augmin complex (HAUS)—a microtubule-binding complex involved in microtubule assembly within the mitotic spindle and is vital to mitotic spindle formation (28). This was found to have different heterozygous truncating variants in lobular breast cinomas (29).

DEK is a highly conserved nuclear factor that shows preferential expression in actively proliferating and malignant cells (30). DEK has been shown to promote tumoroginesis in a variety of cancers, affecting division and DNA repair, inhibiting cell differentiation, senescence and apoptosis. It has been also found to cooperate with transforming oncogenes in promoting oncogenic transformation (31). DEK is one of the few human nuclear DNA-binding proteins known to be actively secreted and the first transcription-modulating factor to be secreted within exosomes. Autoantibodies to DEK are detected during development of inflammatory diseases, particularly in patients with juvenile rheumatoid arthritis (32).

Other MBC-associated antigens including FAM50A, LRRFIP1, PDCL, HMGN2, Gal3BP, SAP30BP, ZRF1, PARD3, RPS8, PSMC1, KRR1, and SPP1 showed lower frequency of response with sera from cancer patients when compared with earlier-mentioned antigens. However, these antigens should not be dismissed from further investigation since they may be implicated in tumor genesis, in particular HMGN2 serves as regulatory factor in Stat5a-driven gene expression and may contribute to a tumorogenic phenotype (33), ZRF1 and SPP1 have been shown to be involved in the regulation of cell proliferation (34, 35). Notably, ZRF1 was previously tested in allogeneic screening with breast cancer patients' sera by Scanlan *et al.*, which showed that immunoreactivity of this antigen was not cancer-restricted (36). This discrepancy could be explained in part by small sampling of cancer patients' sera taken for evaluation in both studies or by the limitation of the serological plaque-spot assay. Also, it is important to note that in both studies sera of patients with different types of breast cancer were taken for analysis.

Using quantitative real-time PCR, we revealed altered (up- and/or down-regulated) expression profile for three antigens including RAD50, HMGN2, and RBPJ, and up-regulation of FAM50A and DEK antigens in the most MBC tumors compared with nontumor breast tissues. In case of DEK antigen, our results partially agreed with the data of other researches, since DEK has been shown to be up-regulated in different tumor types including breast cancer (37, 38–41). FAM50A is a nuclear protein with unknown function, its gene located at human chromosome Xq28 region (42). Interestingly, that overexpression of genes localized at Xq26-28 region was shown in basal-like breast cancers (43).

Table 5. Analysis of Serum Reactivity and mRNA Expression Levels in Three Matched Pairs of Autologous Serum and Tumor Samples

Tumor/Serum	mRNA Expression in MBC Tumors/Antibody Response to MBC Antigens					
Sample Number	RAD50	FAM50A	HMGN2	RBPJ	PABPC4	DEK
Br502/444	D/+	U/+	NA/+	NA/+	NA/+	U/+
Br505/449	U/+	U/+	U/+	U/+	NA/+	U/+
Br506/452	U/−	U/−	U/−	D/+	NA/−	D/−

D—down-regulated, U—up-regulated, NA—not altered mRNA expression level compared with control tissue samples; "+"—positive antibody response, "−"—negative antibody response.

Also significantly higher expression of FAM50A in t(12;21)-positive acute lymphoblastic leukemia patients compared to t(12; 21)-negative patients was observed (44). These findings suggest that FAM50A is a promising object for further investigation as potential cancer biomarker. Notably, that FAM50A was presented by six cDNA clones in Br502 cDNA libarary whereas DEK was presented by only one clone. So, our suggestion about mRNA up-regulation of some antigens that were represented by several cDNA clones in cDNA library from MBC tumor was not confirmed by qPCR analysis. Moreover, we found that mRNA expression of RAD50, which was represented by four clones, was down-regulated in Br502 tumor compared to control tissue. Taking together our data show that the number of immunoreactive cDNA clones of individual gene in cDNA library does not indicate its elevated mRNA level in each particular tumor. Comparative analysis of mRNA expression level of these six antigens in three MBC tumors and serological response toward these antigens in sera of autologous MBC patients also did not reveal clear correlation. Based on this data we could not confirm generally accepted assumption that overexpression or down-regulation of autologous tumor antigens might lead to breaking of immune tolerance and as a consequence might induce a humoral autoimmune response in cancer patients. In any case, obtained results should be confirmed in larger number of tumor tissue sets and statistically verified.

Among identified MBC-associated antigens, there are also those that have been implicated in the modulation of immune response, including PABPC4 (24), RBPJ (45), LRRFIP1 (46–48), Gal3BP (49, 50), HMGN2 (51), and DEK (33). For example, RBPJ is a critical in the mediation of the canonical Notch signaling pathway that attenuates the dendritic cells dependent antitumor immunity, and the ability of these cells to activate B-cells and NK cells (52). We can not exclude that the earlier-mentioned antigens with immunomodulatory functions may originate from immunocytes, but not cancer cells, since we did not eliminate immune cells from tumor sample during purification of total RNA from MBC.

It is important to note that the majority of identified antigens that elicit immune response in patients with MBC have intracellular localization. It is known that mutations, aberrant expression, and modifications of antigens can trigger humoral immune response via aberrant tumor cell death (defective apoptosis, ineffective clearance of apoptotic cells, necrosis), when this intracellular proteins are released from tumor cells and being presented to the immune system in an inflammatory environment (53). The role of humoral immune response directed toward intracellular tumor antigens is not completely understood, but apparently autoantibodies can serve as reporters of molecular changes associated with continuous remodeling of cells or tissues caused by a cell transformation process and could be used for cancer diagnostic purposes.

In the current study, we described for the first time 24 of 41 potential MBC antigens that were identified by SEREX-approach. Preliminary phage-based serological allogenic screening with sera from MBC patients allowed us to select 18 of 41 antigens that were exclusively reactive with sera of MBC patients. We showed heterogeneous mRNA expression profile for RAD50, HMGN2, RBPJ, FAM50A, and DEK antigens in MBC tissues with no obvious correlation with correspondent serological profile. Antigens that showed cancer-related serological profile will be further investigated in large cohorts of patients with different types of breast cancer using ELISA assay with recombinant analogues of identified antigens for their evaluation as targets for cancer diagnostics or therapy.

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DECLARATION OF INTEREST

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by the National Academy of Sciences of Ukraine and State Fund of Fundamental Research of Ukraine (grant No. F46/457-2011).

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