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RESEARCH

Identification of Tumor-Associated Antigens from Medullary Breast Carcinoma by a Modified SEREX Approach

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Abstract Medullary breast carcinoma (MBC) is a relatively rare malignancy with heavy lymphocytic infiltration that despite cytologically anaplastic features and high mitotic index has more favorable prognosis than other types of breast cancer. Lymphocytic infiltration of tumors reflects ongoing immune response against tumor antigens which could represent a great interest as potential targets for cancer immunotherapy. The search for MBC antigens by SEREX methodology has not been successful due to a very high titer of false positive clones, representing immunoglobulin genes. Here, we describe a novel approach for generating cDNA expression libraries from MBC tumor samples which are depleted of IgG cDNA clones and, therefore, are suitable for the identification of novel tumor-associated antigens (TAA) by SEREX approach. Modified methodology allowed us to isolate a panel of known and novel TAA which are currently under further investigation.

Keywords Medullary breast carcinoma · cDNA expression library · SEREX · Tumor-associated antigens · Cancer immunotherapy and diagnostics

Introduction

The immune system plays a central role not only in fighting infection diseases, but also in many others pathologies, including cancer. It is well established that the immune system responds to malignant tumor by recognizing tumor-associated antigens (TAA) that exhibit aberrant expression

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in cancer [1]. These antigens are potential targets for development of cancer immunotherapy and diagnostics. Different approaches have been developed for identifying TAA, including hybridoma technology, T-cells-based biochemical and genetic techniques, and some high throughput technologies such as SEREX methodology, protein microarray, phage display, and serological proteome analysis [2–7]. A panel of novel and already known TAA has been isolated from various types of human cancer by SEREX (serological identification of recombinantly expressed clones) methodology that was established in Dr. M. Pfreundschuh's laboratory in 1995 [8]. Identification of TAA by SEREX approach is based on the generation of λ phage expressing cDNA libraries from tumor samples, followed by immunoscreening with autologous patient sera. Notably, many SEREX-defined antigens are currently in clinical trials for the development of TAA-based cancer vaccines [9].

Medullary breast carcinoma (MBC) is a very attractive type of cancer for the identification of TAA because it is heavily infiltrated with lymphocytes. To date, the cause of lymphocytic infiltration in MBC is unknown. Studies from different laboratories have indicated that the lymphocytic infiltrates of MBC consist predominantly of cytotoxic $CD8^+$ T-cells, as well as large number of $CD4^+$ T and plasma B-cells (10, 11, and 12). The absence of neutrophils in examined infiltrates indicates that tumor-infiltrating lymphocytes (TIL) in MBC accumulate in response to specific stimuli but not to nonspecific inflammatory response caused by tumor necrosis or bacterial agents [12]. It has been proposed that lymphoid infiltration is caused by the immune response to "strong" tumor antigen(s), expressed on the surface of tumor cells. Therefore, several approaches have been employed with the aim to identify tumor antigens in MBC responsible for overwhelming immune response. The techniques which have been applied for the identification of MBC antigens were predominantly based on screening of frozen sections or membrane fractions of tumor samples with IgG phage display libraries generated from TILs. These investigations resulted in the identification of several potential antigens, including ganglioside D3 (GD3) and cytoskeletal protein actin [12–14]. However, the significance of these findings requires further investigations. Application of SEREX methodology for the identification of TAA from MBC has not been successful so far, mainly due to a very high percentage of IgG positive clones derived from tumor infiltrating lymphocytes. These circumstances have restricted the use of standard SEREX methodology for the search of MBC antigens.

In this study, we present for the first time a novel approach for the production of IgG depleted cDNA expression libraries from MBC which is suitable for the identification of TAA by SEREX methodology. By employing this approach, we have created two cDNA expression libraries efficiently depleted of IgG-specific cDNA clones. The immunoscreening of generated libraries with autologous sera resulted in the isolation of 59 positive cDNA clones corresponding to 41 different genes. These genes represent potential targets for cancer immunotherapy and diagnostics and, therefore, are being the subject of further investigation.

Materials and Methods

Tumor and Serum Samples

Tumor and serum samples were obtained from 37 (Br 499) and 65 (Br 502) years old female patients admitted for tumor resection at the Dnipropetrovsk Municipal Clinical Hospital No.4 (Dnipropetrovsk, Ukraine). The study was approved by the Ethics Committee of the Institute of Molecular Biology and Genetics, and consent forms were obtained from both patients. Tumor samples were kept frozen at −80 °C. Sera from appropriate patients were collected and stored at −20 °C with 50% glycerol.

RNA Extraction and Construction of cDNA Libraries

Total RNA from each tumor samples (no less than 500 mkg) was purified by the TRIsol Reagent (Life Technologies, USA). The depletion of total RNA purified from MBC samples of IgG RNA was performed using specifically designed matrix. For the generation of affinity matrixes, we coupled 20 pmol (100 fold excess) of biotinylated oligonucleotides (corresponding to CH2 and CH3 domains of IgG genes) (Operon, UK) to BioMag streptavidin magnetic beads (QIAGEN, GmbH, Germany) during 15 min at room temperature in BioMag Binding buffer (20 mM Tris HCl pH 8.0, 0.5 M NaCl) as recommended by BioMag streptavidin magnetic beads manual (QIAGEN). Then BioMag Binding buffer was replaced with an appropriate amount of Oligo dT Binding buffer (10 mM Tris HCl pH 7.5, 0.5 M LiCl, 1 mM EDTA) during the washing of the oligonucleotide-bound magnetic particles. Total RNA, purified from MBC samples, was loaded on generated affinity matrixes in the same buffer. Unbound fraction of RNA (depleted) was used for mRNA purification using Dynabeads Oligo $(dT)_{25}$ (Dynal, Oslo, Norway). cDNA expression libraries from purified mRNA samples were constructed using ZAP Express® cDNA Synthesis Kit (Stratagene, USA). An in vitro packaging was performed with ZAP Express® cDNA Gigapack III Gold Cloning Kit (Stratagene, USA) to generate recombinant phages. In total, two cDNA libraries depleted of IgG genes were

constructed from the MBCs (Br499 and Br502) and one control cDNA library was created from undepleted mRNA purified from Br499 tumor sample.

Immunoscreening of cDNA Expression Library

In total, 1×10^6 recombinant phages were screened with autologous sera. For the primary screening, 600 μl of XL-1 Blue MRF cells in 10 mM $MgSO_4$ ($OD_{600} = 0.6–0.7$) were infected with 1×10^4 phage particles, mixed with melted top agarose, containing 1 mM IPTG, plated onto NZY agar covered plate and grown at 37 °C for 5–6 h until phage plaques become visible. The transfer of the expressed proteins to the Hybond-C nitrocellulose membrane was carried out overnight at 37 °C. Membranes were subsequently washed in TBST (0.05% Tween 20, pH 8.0) to remove an excess of agar and blocked with 5% nonfat milk (NFM) for 1 h at room temperature. For the detection of false positive clones of human IgG and their exclusion from further analysis, membranes were initially incubated with HRP-conjugated, Fc-fragment specific goat anti-human IgG (Jackson laboratories, USA) for 1 h at room temperature (1:2000 dilution). After extensive washing in 1× TBS the immune complexes were developed in the presence of HRP substrate—3,3′ diaminobenzidine tetrahydrochloride. IgG-specific clones were marked by piercing with a needle. Then, the membranes were washed in TBS and further incubated for 15 h at room temperature with autologous sera diluted at 1:100 in TBS with 0.2% NFM and 0.02% NaN_3. The serum was preliminary depleted from antibodies that react with bacterial and phage proteins by absorption on BrCN Sepharose coupled with E. coli XL-1 Blue MRF‘ and nonrecombinant phage lambda proteins to minimize nonspecific antibody binding. Pre-absorbed and diluted sera samples were stored at +4 °C. Specific binding of sera antibody to recombinant proteins was detected by incubation with AP-conjugated Fc-fragment specific goat anti-human IgG antibody (Jackson laboratories, USA) diluted at 1:4000 in TBST. Visualization of the antigen–antibody complexes was performed by staining with BCIP/NBT (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolil-phosphate) (Sigma, USA). Positive clones were extracted from agar and stored in 0.5 ml of SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris–HCl, 0.01% gelatine) containing 20 μl of chloroform. Selected phage clones were subjected to sub-cloning for monoclonality in the secondary round of screening.

Plasmid Manipulations

Isolated monoclonal positive phages were converted into pBK-CMV phagemids using an in vivo excision protocol (Stratagene, USA). Rescued plasmid DNAs were purified according to manufacturer’s instruction (Qiagen, USA) and subjected to restriction enzyme analysis and DNA sequencing with T3 forward and T7 reverse primers. The insert sequences were compared to the known ones in the Genbank database using BLASTN alignment algorithm (NCBI database).

Immunohistochemical Analysis

Representative sections of tumor samples were prepared from paraffin blocks and stained with hematoxylin-eosin according to a standard protocol. Endogenous peroxidase was quenched with H_2O_2 (3%) in 0.01% PBS. After blocking of nonspecific binding by avidin–biotin blocking solution (Vector Laboratories, Burlingame, CA, USA), tissue sections were incubated overnight at 4 °C with appropriate mAb (1 μg/ml). Then, sections were incubated with biotinylated secondary antibodies for 2 h at room temperature (1:400, goat anti-mouse biotinylated IgG, Sigma), followed by incubation with avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The immune complexes were developed with diaminobenzidine solution. Hematoxylin was used for counterstaining. Prepared slides were examined with the use of Zeiss Universal microscope (Zeiss, Germany); images were captured using digital Axiocam software.

Results and Discussion

Samples of MBC from 37 and 65 years old female patients were taken for the construction of λ phage cDNA libraries (Br499 and Br502, respectively). Histochemical analysis of both samples of MBC revealed the presence of lymphoplasmacytic infiltrates that surround the interconnecting sheets of epithelial tumor cells with pleomorphic and hypochromic nuclei (Fig. 1a, b).

The further immunohistochemical analysis of both tumor samples showed that they are Ki67 and PCNA positive, indicating high proliferation rate of these poorly differentiated MBCs (Fig. 1c, d). Based on the immunoreactivity of antibodies specific toward CD3 and CD19 antigens, we have concluded that both tumors are infiltrated by T and B lymphocytes (Fig. 1e, f).

The previous attempts of searching of MBC TAAs by a standard SEREX methodology were unsuccessful due to a high level of IgG positive clones being detected at the prescreening stage by Fc-fragment or Fab-fragment specific anti-human IgG secondary antibodies. High background of the IgG clones hampered the identification of true positive clones corresponding to TAA when cDNA libraries were subsequently probed with autologous sera.

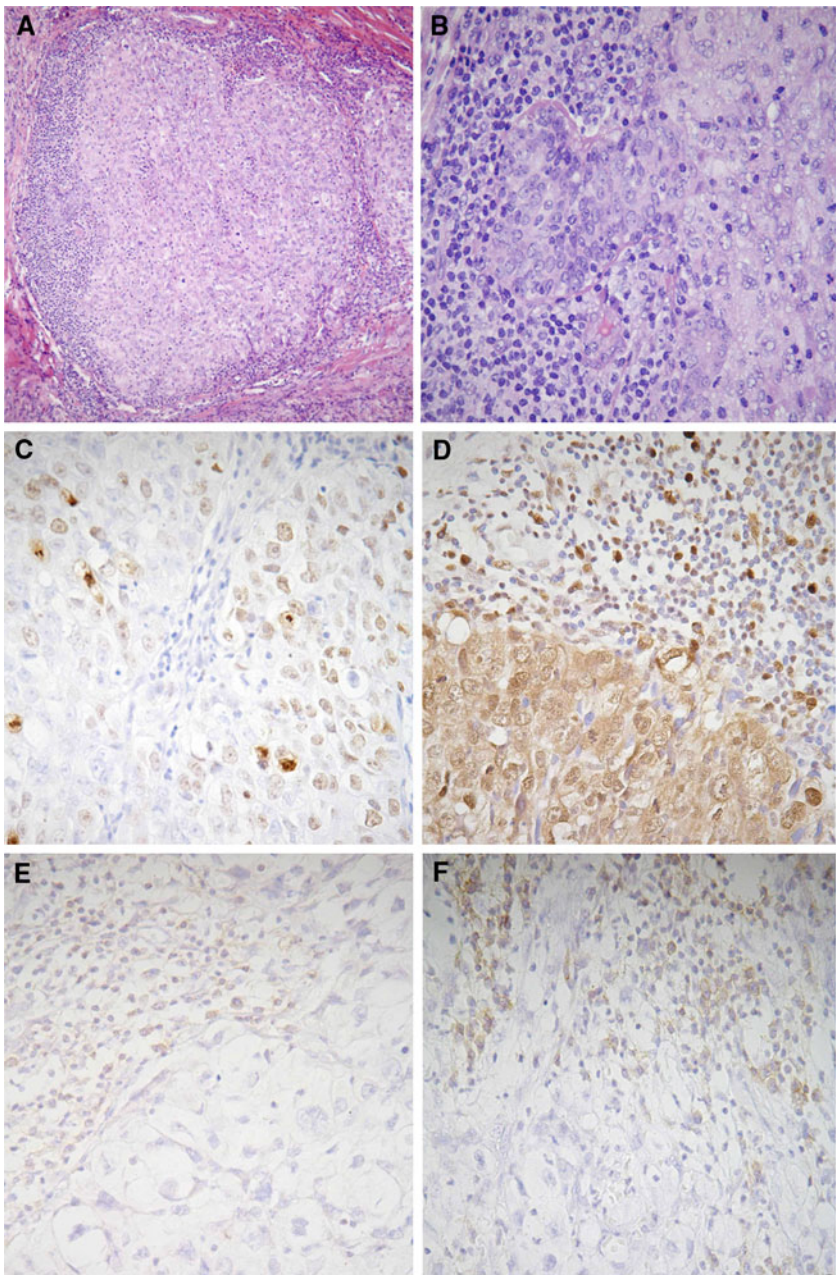

Fig. 1 Histochemical and immunohistochemical analysis of medullary breast carcinomas. Severe lymphoplasmacytic infiltration between medullary breast carcinoma tumor sheets (**a**–**b**). Br499 and Br502 tumor samples were stained with hematoxylin and eosin, and examined microscopically at magnification 200× and 400×, respectively. Immunohistochemical detection of Ki67 (**c**), PCNA (**d**), CD3 (**e**) and CD19 (**f**) expression in Br502 tumor. Magnification 400×

The main focus of this study was on the generation of MBC cDNA expression libraries depleted of IgG clones. Among various potential strategies, we have chosen the option of removing IgG specific transcripts at the stage of RNA purification from selected tumor samples. To do so, we created affinity matrixes (BioMag streptavidin magnetic beads) coupled with biotinylated oligonucleotides, corresponding to the most conserved sequences of human IgG heavy chains genes. The design of corresponding oligonucleotides was performed by multiple sequence alignment of 32 alleles of four IgG genes (IGHG1, IGHG2, IGHG3, IGHG4) using CLUSTALW2 software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The alignment allowed us to identify the highly conservative sequences within the CH2 and CH3 domains of heavy chains of IgG genes corresponding to Fc-fragment of immunoglobulins (Fig. 2a, b). Based on these alignments, two 25 nucleotides long sequences from the CH2 (5′ TGTTGGAGACCTTGCA CTTGTACTC 3′) and CH3 (5′ AGAAGCCTTTGAC-CAGGCAGGTCAG 3′) domains were selected and oligonucleotides in anti-sense orientation (Fig. 2c) have been synthesized and biotinylated.

Biotinylated oligonucleotides (corresponding to CH2 and CH3 domains, 20 pmol of each) were coupled to BioMag streptavidin magnetic beads as described in material and methods. The total RNA purified from MBC

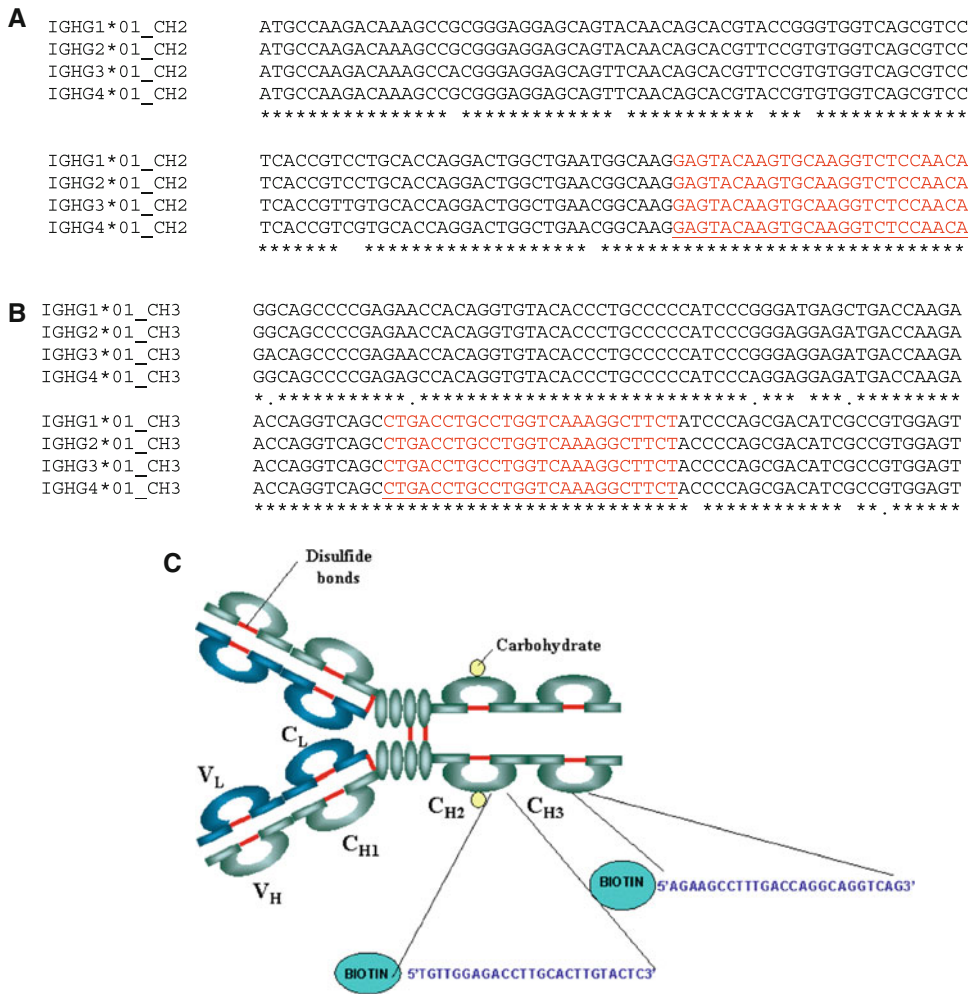

Fig. 2 Bioinformatic analysis and the design of oligonucleotides for affinity depletion of IgG transcripts. Multiple sequence alignment of CH2 domains sequences of the four IGHG genes (Fc-fragment); 25 bp conservative oligonucleotide sequences are marked (**a**). Multiple sequence alignment of CH3 domains sequences of the four IGHG genes (Fc-fragment); 25 bp conservative oligonucleotide sequences are marked (**b**). Schematic structure of the immunoglobulin molecule. The position of selected oligonucleotides (25 bp) in anti-sense orientation, corresponding to the most conserved regions in CH2 and CH3 domains, is shown (**c**)

samples was passed through generated affinity matrix according to QIAGEN BioMag streptavidin magnetic beads manual and unbound (depleted) fraction of the total RNA was collected and used for the mRNA purification (see material and methods). The pool (5 μg) of IgG depleted mRNA from each MBC sample was used for the generating of cDNA expression libraries using ZAP Express® cDNA Synthesis Kit (Stratagene, USA). The strategy of generating IgG depleted and undepleted cDNA libraries from MBC tumor samples is presented in Fig. 3a. The generation of a cDNA expression library from Br499 tumor sample that was not depleted of IgG mRNA was performed to compare the efficacy of the depletion procedure. As shown in Fig. 3b–d, the proposed protocol of depletion allowed us to create two cDNA libraries which have very low level of cDNA clones coding for IgG. The depletion was nearly complete in the case of the Br499 library (titer 1×10^5 pfu; 0.0001% of IgG clones) and only 0.05% of phage clones represented IgG cDNA in the Br502 library (titer 1×10^6 pfu; 0.05% of IgG clones). In contrast, undeleted cDNA library from Br499 tumor generated by a standard protocol contained over 1% of IgG clones (titer 1×10^5 pfu; 1% of IgG clones) (Fig. 3b).

Depleted cDNA libraries from Br502 and Br499 tumor samples were then screened with pre-absorbed autologous sera from corresponding patients. Taking into account that the Br499 depleted cDNA library was not well represented (1×10^5 pfu), we were only able to identify two positive clones by SEREX screening of this library. However, the screening of 10^6 of recombinant phages from the Br 502 depleted library resulted in the isolation of 88 positive clones. The second round of the screening of all clones identified confirmed the specificity of 59 clones (isolated clones were considered as positive when their subclones showed immunoreactivity with autologous serum). In total, 168 subclones were converted to pBK-CMV plasmids by an in vivo excision procedure and subjected to restriction analysis with Xho1 and EcoR1 endonucleases. Subclones with the same size of cDNA inserts were sequenced and their identities were determined by searching NCBI GenBank

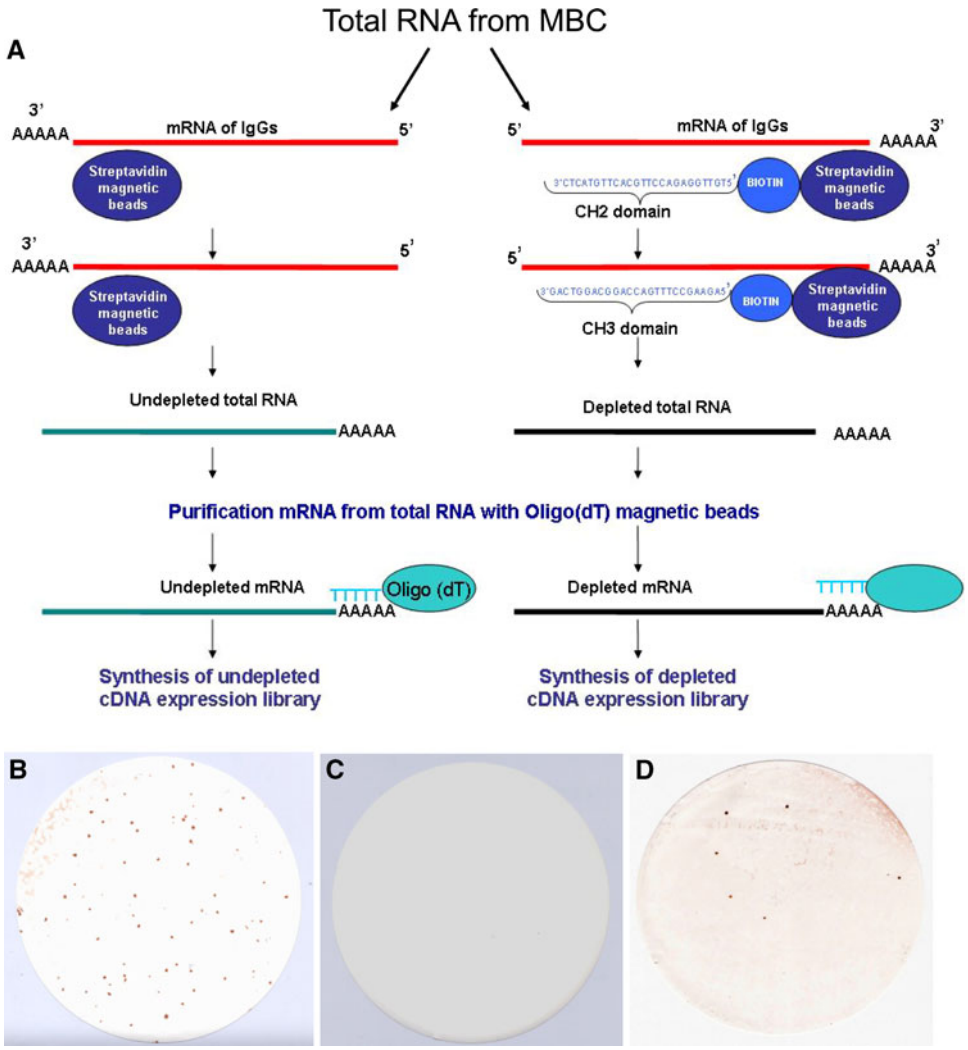

Fig. 3 The strategy of construction and analysis of IgG depleted cDNA libraries. Schematic diagram of the developed protocol for creation of a cDNA library from medullary breast carcinoma depleted of IgG specific mRNAs (**a**). SEREX screening of undeleted library from Br499 tumor (1% of IgG positive clones) (**b**), depleted libraries from Br499 (0.0001% of IgG positive clones) (**c**) and Br502 (0.05% of IgG positive clones) (**d**) tumors

Table 1 Forty one genes (59 clones) were identified by SEREX screening of Br 499 and Br502 IgG depleted cDNA libraries from medullary breast carcinoma

Regulation of	Number of genes	Identified before by SEREX	Genes presented by several clones	Expression in other tumors
Transcription	10	7	5	Hepatocarcinoma, breast and prostate cancers, colon and colorectal adenocarcinomas
Translation	7	4	1	Colon cancer malignant cell line, prostate, pancreas and stomach cancers, melanoma
Cell signaling	4	1	–	Breast, lung and renal cancers
Cell adhesion	3	1	–	Colon and stomach cancers
Protein folding	2	1	–	Leukemia associated, primary head and neck squamous cell tumors, stomach, and breast cancers, osteosarcoma
DNA repair	3	–	1	–
Metabolic pathways	4	–	–	
Cell division	2	1	0	Prostate cancer
Unclear	6	2	2	Breast cancer
Total	41	17	9	

databases. The search showed that 59 cDNA clones correspond to 41 genes (40 and 1 different genes from Br502 and Br499 libraries correspondently) among which 9 are represented by several cDNAs (Table 1). The products of these genes (antigens) are involved in regulation of different cellular processes such as cell signaling, transcription and translation, DNA repair, cell cycle, cell survival, etc. Encouragingly, our screening revealed 17 antigens which have been previously identified by SEREX approach (http://ludwig-sun5.unil.ch/CancerImmunomeDB/) as cancer-associated antigens in different human cancers, including breast cancer. The identity of these antigens is shown in Table 2. These data indicate that some antigens share common immunogenic profile in different types of cancer and could be useful for cancer diagnosis and prognosis as well as for understanding of molecular mechanisms underlying the oncogenic transformation. Among the isolated genes, there are also those which are overexpressed in cancer or show cancer/testes expression profile. Allogenic analysis of identified clones with a panel of allogenic sera from patients with different types of breast cancer and healthy donors for further selecting of cancer-associated antigens as potential targets for cancer immunotherapy and diagnostics is currently in progress.

In conclusion, the proposed protocol provides an efficient way of removing of IgG transcripts from RNA samples of tumors that exhibit a high level of lymphocyte infiltration which is a pre-requisite for generating cDNA expression libraries suitable for successful identification of TAA by SEREX methodology.

Application of this approach enabled generating and screening of two MBC cDNA libraries with corresponding

Table 2 MBC-associated antigens identified previously by SEREX approach in other tumor types and deposited to SEREX data base (http://ludwig-sun5.unil.ch/CancerImmunomeDB/)

	Antigens	SEREX ID number	cDNA library
1	Homo sapiens family with sequence similarity 50, member A (FAM50A)	NGO-Br-10	Breast cancer
2	Homo sapiens poly(A) binding protein, cytoplasmic 4 (inducible form) (PABPC4)	NGO-St-48	Stomach cancer
		NY-TLU-13	Human testis
3	Homo sapiens leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1)	HOM-HCC-2.5.1	Hepatocarcinoma
		NY-TLU-20	Testis
4	Homo sapiens high-mobility group nucleosomal binding domain 2 (HMGN2)	LO-108	Breast cancer
		NY-Co-17	Colorectal adenocarcinoma
5	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subf a, member 5 (SMARCA5)	NGO-Pr-169	Prostate cancer
6	Homo sapiens ribosomal protein L15 (RPL15)	NW-FW-108	Colon cell line (malignant)
7	Homo sapiens zuotin related factor 1 (ZRF1)	NGO-St-48	Stomach cancer
		NY-Br-13	Breast cancer
8	Homo sapiens ankyrin repeat domain 11 (ANKRD11)	TL15-50.2	Testis
9	Homo sapiens MORC family CW-type zinc finger 4 (MORC4)	MO-BC-1113	Breast cancer
10	Homo sapiens par-3 partitioning defective 3 homolog (C. elegans) (PARD3)	NGO-Pr-115	Prostate cancer
		TP6	Testis
11	Homo sapiens mitochondrial ribosomal protein S24 (MRPS24)	NGO-Pr-109 NGO-Pr-65	Prostate cancer
12	Homo sapiens CCAAT/enhancer binding protein zeta (CEBPZ)	NGO-Pr-10	Prostate cancer
13	Homo sapiens Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)	HOM-TS-GLI-68	Testis
		MO-CO-1069	Colon adenocarcinoma
		NGO-Br-4,	Breast cancer
		NGO-Lu-29	Lung cancer
		NW-TK 156	Testis
		NY-REN-35	Renal cancers
		NY-SAR-66	Fibrocarcoma
14	Homo sapiens poly(A) binding protein, cytoplasmic 1 (PABPC1)	KM-PA-9	Pancreas adenocarcinoma
15	Homo sapiens myosin VI (MYO6)	NGO-St-129 NGO-St-154	Stomach cancer
16	Homo sapiens ribosomal protein L32 (RPL32)	Mz19-82	Melanoma
17	Homo sapiens topoisomerase (DNA) II beta 180 kDa (TOP2B)	HOM-TS-GLI-13	Testis
		NGO-Pr-86	Prostate cancer
		NW-CS 59	Colon adenocarcinoma

autologous sera that resulted in the identification of 41 potential MBC-associated antigens. The identification and characteristic of these antigen(s) may provide an important tool not only for the development of novel diagnostic and immunotherapeutic approaches but for further elucidation of immune response in cancer patients.

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Conflicts of Interest Statement The authors declare no competing interests.

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