Chapter 3

Discovery of Antibody Biomarkers Using Protein Microarrays of Tumor Antigens Cloned in High Throughput

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Summary

Development of humoral and cellular immunity against self-cellular proteins in cancer patients is a phenomenal observation. The ability of immune system to sense the presence of the disease and to fight of the disease by generating autoantibodies against tumor antigens makes it a natural biosensor. Several screening technologies have been employed for the identification of tumor-specific antibodies in cancer patients. We have developed a multidimensional approach for the identification of diagnostic antigens that utilizes a combination of high-throughput antigen cloning and protein microarray-based serological detection of complex panels of antigens by exploiting the serum autoantibody repertoire directed toward tumor-associated antigens in cancer patients. Furthermore, validation of these antigens by different bioinformatics and biological approaches will reveal the diagnostic/prognostic utility of these antigens for personalized immunotherapy.

Key words: Immune response, Tumor biomarkers, Autoantibodies, Epitopes, Protein microarrays, Immunotherapy.

1. Introduction

The early diagnosis of cancer has been a challenging field of research for many years. Early-stage diagnosis increases the probability of cure, and therefore diagnostic screening tests that can detect these early stages are crucial. Some cellular proteins elevated in sera from cancer subjects are also found in individuals suffering from other diseases such as inflammatory diseases and autoimmune disorders. Tumor-specific proteins are identified as elevated in primary tumor tissue, metastatic lesions, or ascites fluid are only true tumor biomarkers suitable for early detection

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if they are elevated in blood (plasma, serum), saliva, or urine from cancer patients. These biomarkers are found to be specific and sensitive reporters of disease that are able to indicate a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to various therapeutic interventions. In comparison to secreted tumor proteins that may be present in circulation for a short time because of transient shedding from tumors in the tumor microenvironment or degradation in serum, serum antibodies are stable, present in the serum for a long time, and are readily detectable with an enzyme-conjugated secondary antibody *(1)*. The idea of using serum antibody for diagnosis of cancer is supported by the vast evidence of generation of humoral immune responses against tumor-associated antigens (TAA) in different cancers *(2)*. It has been observed that 30% of patients with DCIS in which the protooncogene HER2/neu was overexpressed had serum autoantibodies specific to this protein *(3, 4)*. In addition, autoantibodies to p53 have been reported in patients with early-stage ovarian and colorectal cancers *(5, 6)*. Autoantibodies against heat shock protein HSP90 were also found to correlate with patient's survival and metastasis *(7, 8)*. Autoantibodies against ribosomal proteins may constitute a novel serological marker. In this regard, autoantibodies to ribosomal protein S3 were detected in 10% of patients with breast cancer, whereas no reactivity was found in normal sera *(9)*. Occurrence of autoantibodies to ubiquitin C-terminal hydrolase L3 in colon cancer had also been reported *(10)*. Changes in the level of gene expression in cancer *(3, 11–13)* and aberrant expression of tissue-restricted gene products in cancer *(14, 15)* are factors in the development of a humoral immune response in cancer patients. Various technologies have been reported for the detection of antigens/TAAs on the basis of large repertoire of serum antibodies that are generated because of humoral immune responses in different cancers.

Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX) was developed by the group of Michael Pfreundschuh at the University of Saarland, Germany, in 1995 *(2)*. A SEREX/Cancer Immunome database (http://www2.licr.org/CancerImmunomeDB) has been organized and lists more than 2,000 antigens *(16)*. The SEREX approach includes the construction of cDNA libraries from fresh tumor specimens and cloning into lambda phage expression vectors. The recombinant phages are used to infect *E. coli* cells. Recombinant proteins expressed during lytic infection of the bacteria are replica-plated onto nitrocellulose membranes and immunoscreened with autologous serum. Clones reactive with high-titer IgG antibodies are identified using enzyme-conjugated secondary antibody specific for human IgG. The identity of the reactive antigens is revealed by sequence results of the cDNA inserts.

The underlying mechanism for immunogenicity of cancer antigens is often overexpression of normal gene products or mutation in cancer. The immune response to cancer testis (CT) antigens is clearly related to the anomalous expression of gene products in cancer tissues that are normally only expressed in primitive germ cells *(17)*. CT antigen expression in cancer has been ascribed to abnormal demethylation *(18)*. Humoral immune response to SEREX-defined CT antigens, MAGE, tyrosinase, and NY-ESO-1 has been detected in melanoma patients *(19)*. Amplified expression appears to be one of the most frequent reasons for the immunogenicity of antigens isolated by SEREX, and many examples of antibodies to overexpressed gene products in cancer have been detected in SEREX analysis, e.g.,, eIF-4 gamma in lung cancer *(20)*; HER-2*/neu* in breast cancer *(13)*. With regard to mutations as a basis for immunogenicity, the tumor suppressor gene p53 is a classic example of mutational antigen that was detected by SEREX analysis of ovarian cancer *(21)*. The CT antigens are immunogenic and highly restricted to tumors. Therefore, they are considered as excellent targets for immunotherapy. Jager *et al.* have reported that immunization with peptides obtained from MAGE-1 and MAGE-3 when used alone or in combination with adjuvants have resulted in regression of tumor in more than 30% of patients diagnosed with melanoma *(22)*. Although the SEREX approach has been successful in identifying tumor antigens from many different cancers, this technology has some limitations. First, this technology is based on screening tumor-derived cDNA libraries made from a particular patient with serum obtained from autologous patient. As a result, the identified TAAs in most cases do not show high reactivity with allogeneic patients' sera. Second, this approach tends to identify antigens that were overexpressed in the tumor and thus highly abundant in tumor-derived cDNA libraries. Thus low abundant messages that may be encoding for tumor relevant antigens are generally missed. Third, as prokaryotic system is used in this biopanning, post-translational modifications such as glycosylation cannot be found as novel cancer antigens. As a result, humoral immune responses directed toward glycosylated residues in TAA can remain unidentified. Thus, several modifications have been introduced to improve this technology. The first variation involves established tumor cell lines instead of fresh tumor specimen as a source of cDNA. Thus, contaminating normal cell types present in tumor specimens will not be included in cDNA preparation, and cDNA cloning of IgG sequences expressed by tumor infiltrating B cells giving rise to false-positives will be prevented *(23)*. Second, the screening of sera from cancer patients on allogeneic cDNA libraries including testicular cDNA libraries may result in identification of TAA and CT antigens that will show higher reactivity with serum IgGs obtained from many different cancers.

1.1. Global Profiling of Cancer Antigen Epitopes (Epitomics)

1.1.1. Protein Microarrays

A new high-throughput technology has been recently developed by our group based on serological identification of tumor antigens by profiling humoral immune responses in cancer patients. We refer to this approach as "Epitomics." Our technology detects antibodies that are produced by cancer patients in reaction to proteins overexpressed or mutated in their tumors and uses them as diagnostic biomarkers. Our multidimensional strategy comprised high-throughput antigen cloning using phage display technology coupled with serological identification of tumor antigens and profiling humoral immune responses in cancer patients on protein microarrays. For high-throughput antigen cloning, our group has developed an undirected approach using T7 phage display techniques ("differential biopanning") to identify the cancer antigen space within the human proteome *(24, 25)*. T7 phages have several advantages over lambda phage in that one can screen 2–3 log more phage clones in a single experiment, several libraries can be pooled and screened in parallel, and the life cycle of the T7 phage is far faster so that the process of cloning is more rapid. Our lab has established a bar coding strategy for the identification of T7 phage cDNA clones from a pool of T7 phage cDNA libraries that are constructed with mRNAs obtained from multiple sources (cancer cell lines or tumor specimens) to maximize representation and heterogeneity of cDNA (**Fig. 1a–d)** also described in **Subheading 3.4**). Differential bioppaning involves immunoscreening of T7 phage tumor-derived cDNA libraries using a two-step process, starting with protein-G beads bound with serum IgGs pooled from different age-matched normal healthy individuals (as described **Subheading 3**). This step helps in the removal of nontumor/common antigens that bind to IgGs in normal sera. Next, serum IgGs from cancer patients also bound to protein-G beads are used as the bait in biopanning in order to enrich for clones of tumor antigens. The bound antigens are eluted and the resulting phage clones are amplified for the next round of biopanning. A graphical representation of output eluant phage enrichments/phage titers after each cycle of biopanning resembles the general shape of an exponential function with a trend to approach saturation curve in the last two stages of biopanning. That may be explained by the nature of T7 phage selection and its enrichment process retaining the diversity of the reactive phage clones (**Fig. 2a)**. However, for certain biopannings, we have observed a variety of possible shapes for the curves. First, this may indicate a kinetic complexity of gradual enrichment of the eluant in specifically bound and selected T7 phage as well as complex equilibrium of reversible adsorption of phage to the beads and antibodies. We postulate that interactions between beads, antibodies, and phage are maintained by complex sets of forces such as hydrophobic and van der Waals bonds that can be formed or broken upon the change of pH, different washing

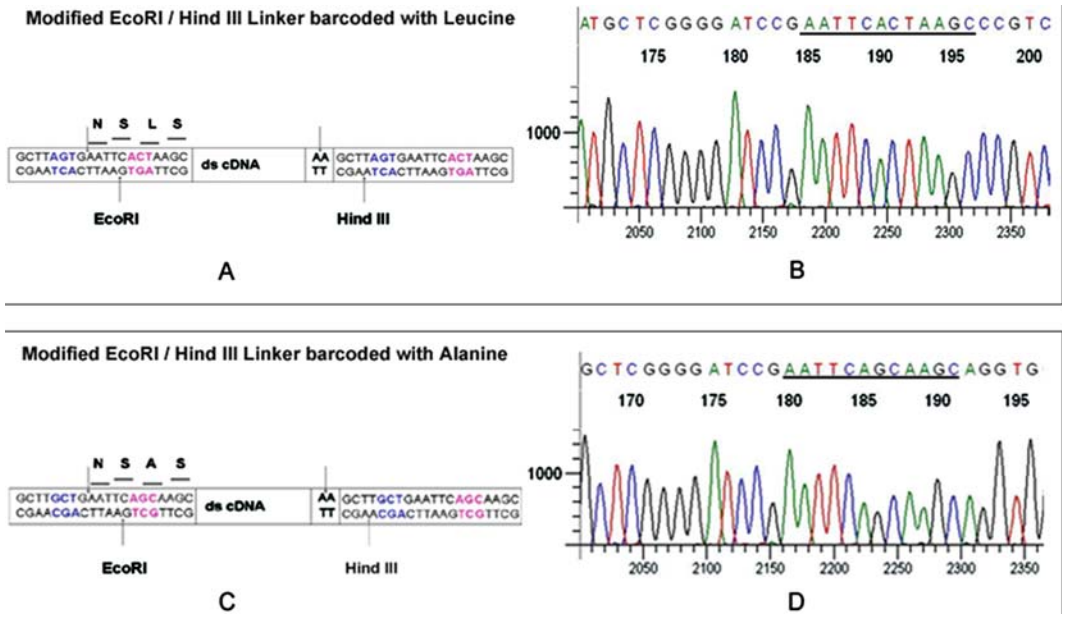

Fig. 1. Bar coded *Eco*RI/*Hind*III directional linkers used for the construction of T7 phage cDNA libraries. (**A**) The modified linker with the bar coding sequence obtained by adding triplet nucleotides in frame (color coded in pink and blue) and it resulted in the addition of amino acid "Leucine" in the linker region of the cDNA. (**B**) Partial sequence result representing the underlined linker sequence of the T7 phage clone obtained from T7 phage cDNA library constructed from OVC0156 ovarian tumor. (**C**) The modified linker with the bar coding sequence that resulted in the addition of amino acid "Alanine" in the linker region of the cDNA. (**D**) Partial sequence result representing the underlined linker sequence of the T7 phage clone obtained from T7 phage cDNA library constructed from OVCAR3 ovarian cancer cell line.

conditions, temperature of the reaction environment *(26, 27)*. Second, any noticeable divergence from a saturated-exponential shape of enrichment (**Fig. 2c**) such as a sudden increase in the titer between two consecutive cycles (magnitude more than 3–6 times) or early plateau of the titer curve might be indicative of an irregular distortion of the enrichment process and, hence, may dramatically diminish the diversity of the clones for a particular fraction. In an extreme situation, the recovered fraction contains a few or just a single clone that binds tightly to the antibodies on the beads which may be considered "overbiopanning." The process of enrichment proceeds at relatively slow degree in the beginning of biopanning (cycles 1 and 2) with rapid progression between cycles 3 and 5 (**Fig. 2a**, **c)**. The extent of clonal diversity can be determined by PCR amplification of the cDNA inserts and comparing the sizes from a group of at least 20 phage clones from each biopanning (**Fig. 2b**, **d)**.

Generally, after four cycles of biopanning, the immunoreactive phage clones are picked from multiple independent patients and are robotically printed on protein microarrays to identify

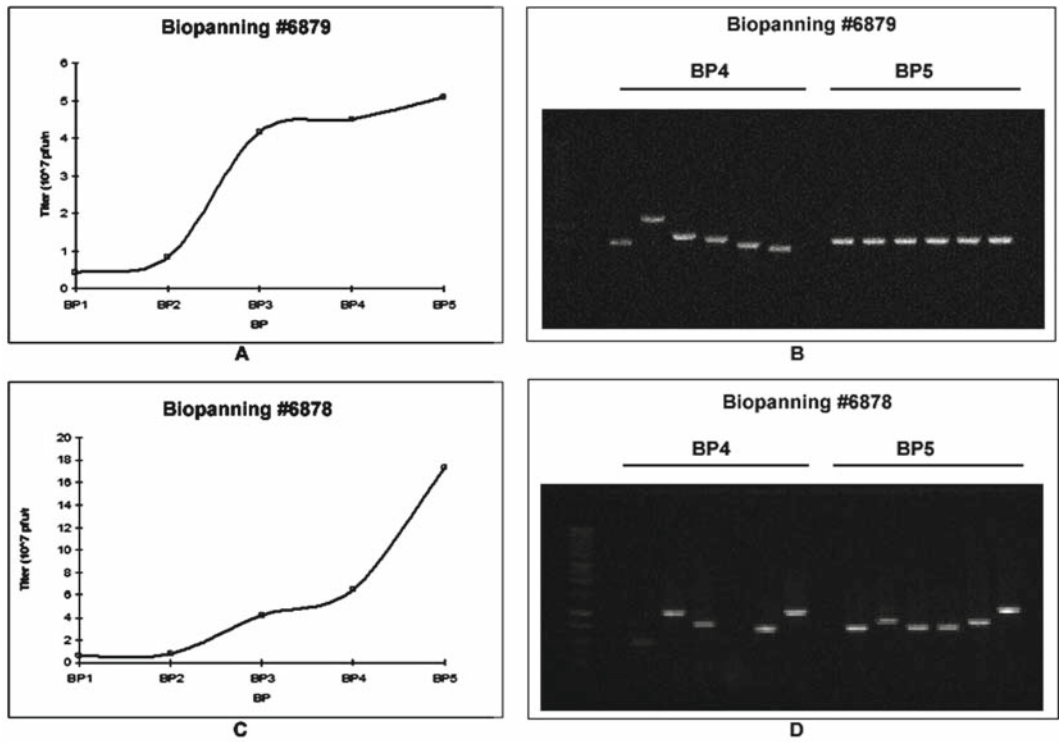

Fig. 2. Typical output eluant phage titers obtained after biopanning (cycles *1–5*) depicting variation in insert size. (**A**) Graphical representation (not the shape of an exponential function) of the output eluant phage titers as a function of biopanning. (**B**) Occurence of over-biopanning in fifth cycle (BP5) resulting in an unexpected jump in the titer value and corresponding lack of diversity of its clones as indicated by PCR amplification of phage clones picked up from biopanning 4 and 5 (BP4 & BP5). (**C**) Graphical representation (shape of an exponential function) of the output eluant phage titers resulting in the enrichment of immuno-reactive phage after the completion of each cycle of biopanning. (**D**) PCR amplification of the phage clones picked up from biopanning 4 and 5 (BP4 & BP5) showing retention of diversity in the pool of immuno-reactive phage as indicated by the range of insert sizes.

circulating serum antibodies produced by the cancer patient presumably to proteins from the cancer cells or tissue. Microarrays are processed with several sera obtained from cancer patients as well as healthy individuals. We previously used Cy3 (green fluorescent dye)-labeled T7 monoclonal antibody, directed against phage capsid protein, and Cy5 (red fluorescent dye)-labeled goat antihuman IgG that recognized the test subject's IgG bound to the antigens on the arrays for processing arrays *(24, 25)*. Recently we have introduced Alexa Fluor-labeled goat antimouse and goat antihuman secondary IgGs as secondary antibodies as described in **Subheading 3**. After processing, arrays are scanned and the ratio of antihuman IgG and anti-T7 capsid is determined by comparing the fluorescence intensities in the Cy5-Cy3 *(24, 25)* or Alexa Fluor-specific channels at each spot using standard image analysis programs. The microarray data are preprocessed and normalized

using local background subtraction of the raw spot signals. The red over green channel intensity ratios are log-transformed and the data are normalized to the print-tip group median within each array. Mean of spot replicates (quadruplicate) is taken for further statistical analysis.

1.1.2. Validation of Biomarkers by Different Approaches

Bioinformatics approach. Initially, selection of clones for a second generation of protein microarray chips is performed by applying two-sided t-test or Bootstrapping ROC method *(28)* on the data set that comprised certain number of cancer patients, patients with benign or other diseases of the homologous tissue and healthy controls. These newly selected clones are printed on a second-generation antigen microarrays. These chips are processed with these and other samples that are now going to constitute the training set. Selection of clones and development of a model are conducted on this training set. For evaluation of the selected clones, validation is conducted on an independent test set that comprised other cancer patients, more healthy controls, and other benign and other diseases. All of the serum samples in these test sets cannot have been previously used in any phase of the study.

Biological Approaches

1. *Immunohistochemistry (IHC).* Statistical analysis of our microarray data revealed a panel of molecular markers (classifier) which showed highest reactivity with sera from epithelial ovarian cancer (EOC) patients and no reactivity with sera obtained from normal healthy individuals or from patients with benign or other gynecological diseases *(24)*. For the assessment of expression levels of these top-ranking biomarkers, like RCAS1, eIF5A, and Nibrin, paraffin-embedded formalin fixed tumor specimens were collected from ovarian cancer patients to test the protein level using immunohistochemical staining with commercially available antibodies to those biomarkers using a standard protocols *(24)*. We found that RCAS1 was highly expressed in 23 out of 30 early-stage and 27 out of 30 late-stage tumors. Likewise the antigen biomarker eIF5A (isolated twice) was also highly expressed in 13 out of 30 early-stage and 27 out of 30 late-stage tumors. The biomarker, p95 Nibrin, was positive in the sera of early- and late-stage EOC patients in our microarray analysis, but the immunohistochemistry showed that elevated expression was specific to late-stage cancers. This marker was expressed in only 1 out of 30 early-stage but 15 out of 30 late-stage tumors. The one early-stage patient who had a positive immunohistochemical reaction was a stage 1C patient who was never off therapy and died 2.5 years after diagnosis. All of these antibodies have no positive staining on normal ovaries or ovarian cysts. These data confirm our

hypothesis that these proteins are overexpressed in ovarian tumors, and it is likely that overexpression is the mechanism by which they become immunogenic. These data also indicate that our approach to identifying serum biomarkers may reveal tissue markers that are stage-specific at the immunohistochemistry level *(24)*.

2. *Quantitative real-time PCR (Q-RT-PCR)*. The expression of immunoreactive antigens at the mRNA level could also be validated by performing Q-RT-PCR. Generally, a correlation between high protein expression (using IHC) as a result of high mRNA expression (using Q-RT-PCR) explains the underlying mechanism of generation of humoral immune response. In cases where high expression level of protein does not correlate with mRNA level, genetic mutation analysis should be pursued because mutations can lead to increased immunogenicity of an epitope thereby eliciting humoral immune response *(29)*.

Thus top-ranking antigens obtained from statistical and biological analyses are readily amenable to reformatting into an immunoassay as a diagnostic predictor of cancer as described earlier. The utilization of this diagnostic test in the clinic would be as a periodic, *in vitro* diagnostic screening immunoassay to detect the presence of cancer. The Epitomics approach has been adopted by others in the field of cancer antigen biomarkers *(30, 31)*. The strength of this approach is the developing of large panels of biomarkers from the antigen space within the human serum proteome that are less sensitive to interpatient variations in the population. This methodology is capable of accurate detection of antibodies in the sera of test subjects. The greater speed and simplicity of this serological approach may also be used for detection of autoantibodies in various autoimmune diseases and possibly even surrogate markers for disease for unknown etiology. A major advantage of our system is that we detect specific IgGs in serum. The IgG is the most stable, functional protein in serum having 5–10 times the half-life of other immunoglobulins as described earlier. To further verify the stability of serum IgGs, we have performed several tests with serum sample such as repeated freezing or left overnight at room temperature with little change in the results at the microarray analysis. Many serum analytes that are labile are generally subjected to interlaboratory variations due to differences in sample handling. In our approach, we identify biomarkers that are IgGs. We have found that leaving the blood overnight at 37°C also does not substantively alter the microarray results. However, the serum cannot be heated to 65°C or dried and rehydrated. Therefore, there should be little interanalyte stability variation among the biomarkers. We have successfully used sera from many other laboratories and serum banks. The only limitation is that the phage display technology cannot detect

post-translational modification such as glycosylation. Therefore, the serum antibody repertoire that this technology utilizes for the detection of TAAs is directed toward amino acid epitopes of TAAs.

2. Materials

2.1. Preparation of Total RNA and mRNA from Fresh Tumor Specimen Obtained from Cancer Patient

1. *RNAlater®(Ambion, Austin, TX)*. Used as stabilizing agent for tumor specimen (*see* **Note 1**).
2. *RNeasy Maxi Kit (Qiagen, Valentia, CA)*. For extraction of total RNA from tumor tissues.
3. Absolutely mRNA Purification Kit (Stratagene, La Jolla, CA) for purification of mRNA from total RNA obtained from tumor tissues.

2.2. Northern Blotting for Quantitation and Quality Control of Tumor Poly(A)-Selected mRNA

1. Agarose (Sigma-Aldrich, St. Louis, MO).
2. Formaldehyde (Sigma-Aldrich, St. Louis, MO).
3. *10× MOPS (Sigma-Aldrich, St. Louis, MO)*. 0.22 M MOPS, 0.030 M NaAc, 0.5 M EDTA. Adjust pH 6.6–7.8 with 5N NaOH.
4. Formamide (Sigma-Aldrich, St. Louis, MO).
5. RNA Marker (0.28–6.58 kb) (Promega Corporation, Madison, WI).
6. *Ethidium Bromide (Sigma-Aldrich, St. Louis, MO)*. Dissolve at 10 mg/mL in water.
7. *20× SCC*. 3.0 M NaCl, 0.3 M sodium citrate. Adjust pH to 7.0 with concentrated HCl.
8. *NaAc/Methylene Blue for staining the RNA marker*. 0.5 M Sodium acetate, pH 5.0, 0.045% (w/v) Methylene Blue (Fisher Scientific, Pittsburgh, PA). Store at room temperature.
9. *RadPrime DNA Labeling Kit (Invitrogen Corporation, Carlsbad, CA)*. Used for labeling cDNA probe for hybridization.
10. Gel blot paper (Schleicher & Schuell, Keene, N.H).
11. Nylon transfer Membrane (Hybond-N+) from GE Healthcare Biosciences Corp, Piscataway, NJ).
12. *Hybridization buffer*. 0.14 M Na_2HPO_4, 0.06 M NaH_2PO_4, 1 mM EDTA, 7% (w/v) SDS, 1% (w/v) BSA.
13. *Wash Buffer 1*. 2× SSC, 0.1% SDS.
14. *Wash Buffer 2*. 0.5× SSC, 0.1% SDS.

2.3 T7. Bacteriophage cDNA Library

1. *Eighty percent glycerol.* 80% (v/v) in water and autoclave.
2. *Fifty percent polyethylene glycol 8000 (PEG). Dissolve 100 g PEG in deionized water with stirring, autoclave, and bring the volume to 200 mL with sterile deionized water.*
3. *Tris-EDTA (TE). 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.*
4. *62.5% CsCl.* (25 g CsCl + 15 mL deionized water).

2.4. Growth Media for Bacteriophage T7

1. *LB.* Add 10 g Bacto peptone (Fisher scientific, Pittsburgh, PA), 5 g Yeast extract (Fisher scientific, Pittsburgh, PA), 10 g NaCl (Fisher scientific, Pittsburgh, PA) per liter. Adjust pH to 7.5 with 1N NaOH. Autoclave for 20 min and store at room temperature.
2. 20× M9 salt solution (Fisher scientific, Pittsburgh, PA). Autoclave and store at room temperature.
3. Twenty percent glucose solution (Fisher scientific, Pittsburgh, PA). Autoclave and store at room temperature.
4. One molar $MgSO_4$ (Fisher scientific, Pittsburgh, PA). Autoclave and store at room temperature.
5. 50 mg/mL (w/v) Carbenicillin (Sigma-Aldrich, St. Louis, MO) (*see* **Note 2**).
6. 0.1 M Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO) (*see* **Note 2**).
7. 3.5 mg/mL (w/v) Phenylmethylsulfonylfluoride (PMSF) (Sigma-Aldrich, St. Louis, MO). This solution has to be freshly prepared before use.
8. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).
9. Two percent (w/v) Gelatin (Sigma-Aldrich, St. Louis, MO). Autoclave and store at room temperature.

2.5. Biopanning of T7 cDNA Phage Displayed Libraries with Human Sera

1. 1× Phosphate-buffered saline (1× PBS) (Sigma-Aldrich, St. Louis, MO).
2. Protein G PLUS-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
3. 1% (w/v) Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, St. Louis, MO) in water.

2.6. Agar Plates for Plaque Assay of Bacteriophage T7

1. 0.7% (w/v) agar in LB and autoclave (for performing plaque assay).
2. 1.5% (w/v) agar in LB and autoclave (for preparing plates).

2.7. Protein Microarrays

1. *1× Phosphate-buffered saline (1× PBS).* 0.136 M NaCl, 0.0026 M KCl, 0.0017 M KH_2PO_4, 0.01 M Na_2HPO_4. Adjust pH to 7.4. Filter PBS through 0.22-μm membrane (Fisher scientific, Pittsburgh, PA) using vacuum.

2. *PBS-T*. 0.1% Tween-20 (v/v) in PBS.
   Four percent milk solution. Add 4 g of powdered milk in 100 mL of 1× PBS. Mix with magnetic stirrer for 30–40 min. Centrifuge for 10 min at 9300 × g and decant the milk solution. Store it at 4°C until use.
3. T7 monoclonal antibody (EMB Chemical Inc., San Diego, CA), Alexa Fluor 532 goat antimouse IgG (H + L) and Alexa fluor 647 goat antihuman IgG (H + L) (Invitrogen Corporation, Carlsbad, CA).

3. Methods

This study describes a novel approach to clone and evaluate utility of tumor antigens in high throughput to detect serum antibodies on protein arrays. Multiple steps of selection of tumor antigens are used in this process. First, a differential biopanning technique is employed to screen a tumor-derived T7 phage display cDNA library so as to isolate cDNAs coding for antigenic proteins binding with antibodies present specifically in the sera of cancer patients but not with antibodies in the sera of healthy women. The selected phage clones are then robotically printed on protein microarrays and processed with several sera (from cancer patients and healthy individuals) and T7 mouse monoclonal antibody (directed toward phage capsid protein). We employ Alexa fluor 647 (red fluorescent dye)-labeled antihuman IgG to detect patient serum IgGs binding to clones on the microarrays. The red fluorescent intensity representing the binding of the test subject's IgG molecules on the same spot of phage proteins is compared with an Alexa flour 532 (green fluorescent dye)-labeled antimouse IgG to detect the T7 phage capsid protein. This latter reagent binds to every T7 phage clone and serves to normalize for variations in the amount of each antigen clone at each spot. We perform these microarray assays at a 1:300 dilution of each patient's serum. The digitized dye ratio data derived from the images are analyzed by IMAGENE™ software. Next, the processed chips are scanned using axon scanner, and the intensity of each spot is quantified using IMAGENE™ software (Biodiscovery). Statistical analyses are performed as described previously.

3.1. Serum Samples

1. Blood samples from cancer patients and healthy individuals are obtained from different institutions. Processing of blood to extract serum is performed in our laboratory. Briefly, blood samples are centrifuged at 150 × g at 4°C for 20 min and supernatants are stored at –80°C until use.

3.2. Isolation of Total RNA and mRNA from Fresh Tumor Specimen

1. *Isolation of mRNA from total RNA*. Total RNA is prepared from fresh tumor specimen (suspended in RNA*later*®) using RNeasy Maxi Kit following manufacturer's instructions.
2. Purification of Poly(A)+ mRNA is performed using Absolutely mRNA Purification Kit by following the instructions as suggested by the manufacturer. Poly(A)+ mRNA is quantitated by UV spectroscopy and the process of poly(A) selection is repeated. Twice poly(A) selected mRNA is stored at –80°C for use in library preparation. For quality control, northern blot is performed with the double poly(A)+ selected mRNA obtained from tumor tissues and comparing the hybridization signal for a GAPDH probe with that detection in 20 times more micrograms of total RNA (*see* **Note 3**).

3.3. Northern Blotting of Double Poly(A)+ mRNA

1. *Preparation of agarose gel*. 1.5% Agarose (w/v), 1× MOPS, 3% Formaldehyde (v/v). 10 μg of total RNA or 0.4 μg of mRNA are mixed separately with RNAase-free water to make the final volume to 5.5 μL. To each add 1.0 μL 10× MOPS, 3.5 μL Formaldehyde, 10 μL Formamide, and 2 μL of 6× loading dye.
2. The RNA samples together with RNA ladder (0.28–6.58 kb) are heated to 65°C for 15 min, chilled in ice and run at 100 V for 1 h.
3. The gel is stained with 0.5 μg/mL ethidium bromide and photograph is taken. The gel is then destained with distilled water and treated with 10× SSC for 15 min (two times).
4. A glass plate is placed on a plastic container containing 10× SSC. A wick (made from Gel blot paper) is placed on the glass plate with two ends touching the solution. Three Gel blot papers (soaked in 10× SSC) are placed on top of the wick. The gel is placed upside down on the wick. The nylon membrane is placed on top of the gel along with three presoaked Gel blot papers. A stack of paper towels are placed on top of the Gel blot papers. Another glass plate (with a heavy weight on top) is placed over the stack of paper towels. This completes the setup for the northern blotting and is kept overnight.
5. After 16–24 h, the setup is disassembled, and the nylon membrane is air-dried for 5–10 min, UV crosslinked on both sides.
6. The portion of the membrane containing the RNA marker is cut off and stained with NaAc/Methylene Blue. The marker is washed in distilled water until the bands become visible.
7. The membrane is prehybridized for 1–2 h in a sealed bag containing 10 mL hybridization buffer.
8. Next, the membrane is allowed to hybridize with the cDNA probe that has been labeled with (α-^{32}P) dCTP using RadPrime DNA Labeling kit (one million counts per mL).

9. After 16 h of hybridization, the membrane is washed with wash buffer 1 at room temp for 10 min (repeated two times).
10. Next, the membrane is washed with wash buffer 2 at 65°C (repeated two times).
11. After the second wash, the membrane is quickly wrapped up in saran wrap and is placed in an X-ray film cassette with film for a suitable exposure of time.

3.4. Construction of the OVCA T7 Phage cDNA Library

1. This is performed using Novagen's OrientExpress cDNA Synthesis (Random primer system) and Cloning system as per manufacturer's suggestions (EMB Chemical Inc., San Diego, CA: cDNA manual, TB247; T7Select System, TB178).
2. The OrientExpress Random Primer System is used to achieve orientation-specific cloning between *Eco*RI and *Hin*dIII sites. Briefly, first- and second-strand cDNA syntheses are sequentially carried out in the presence of 5-methyl dCTP. After second-strand synthesis, the cDNA is treated with T4 DNA polymerase to blunt the ends. Addition of *Eco*RI/*Hin*dIII Directional Linker d(GCTTGAATTCAAGC) at the d(A) n:d(T)n end created a *Hin*dIII site d(AAGCTT) in which the two underlined bases are derived from cDNA. The two dTs are provided on the 5′ end of each first strand by the *Hin*dIII random primer d(TTNNNNNN). In order to differentiate cDNAs from different sources we have introduced bar coding sequences as triplet nucleotide bases at two different locations of the directional *Eco*RI/*Hin*dIII linker as indicated in blue and pink colors without introducing stop signals (**Fig. 1a, c**). The translation start site is located on the T7 vector (not shown), and translation of the three nucleotides (in pink) in the linker region in the correct reading frame has resulted in the addition of an extra amino acid, for example, "L" and "A" as described in **Fig. 1a**, **c**. Three other nucleotide bases (in blue) are added to maintain the palindromic sequence of the linker. Thus, sequence result of the bar coded directional linkers present in each T7 phage clone (**Fig. 1b**, **d**) indicated the source of cDNAs used for the construction of T7 phage cDNA libraries. This approach permits pooling of libraries for each biopanning experiment. Excess linkers and small cDNAs (< 300 bp) are removed by a gel filtration step as described in Novagen's manual TB 247. Digestion of cDNAs with both *Hin*dIII and *Eco*RI thus yielded cDNA molecules ready for directional insertion into *Eco*RI/*Hin*dIII vector T7Select 10-3 arms. After vector ligation and packaging using T7 packaging extracts, the phages are plated to determine the library titer. About 50 phage

clones are randomly picked up and PCR is performed with T7 forward primer (TCTTCGCCCAGAAGC**TGC**AG) and T7 reverse primer (CCTCCTTTCAGCAAAAAACCCC), to determine the insert sizes. The insert size range is found to be between 300 bp and 1.5 kb.

3. *Amplification of packaged libraries by liquid culture method.* Ten milliliters of LB/carbenicillin medium is inoculated with a single colony of BLT5615 from a freshly streaked plate. The mixture is shaken at 37°C overnight. One milliliter of the overnight culture is added to 90 mL of LB/carbenicillin medium and is allowed to grow until OD_{600} reaches 0.4–0.5. IPTG (1 mM), M9 salts (1×), and glucose (0.4%) are added and the cells are allowed to grow for 20 min. An appropriate volume of culture is infected with phage library at MOI of 0.001–0.01 (100–1,000 cells for each pfu). The infected bacterial culture is incubated with shaking at 37°C for 1–2 h until lysis is observed. Glycerol (0.02%) and PMSF (0.02 M) are added to the cell lysate to block proteolysis of the capsid fusion proteins. The phage lysate is centrifuged at 8,000 × *g* for 10 min. The supernatant is collected and is stored at 4°C. The lysate is titered by plaque assay under standard conditions. The libraries are stored at –80°C after purification by polyethylene-glycol precipitation and ultracentrifugation through a stepwise CsCl gradient (please refer to T7Select System Manual TB178 for detail procedure).

3.5. Selection of T7 cDNA Phage Displayed Libraries with Human Sera

1. *Affinity selection with sera from normal individuals.* Twenty-five microliters of Protein G Plus-agarose beads (*see* **Note 4**) is taken in 0.6 µL Eppendorf tube and washed twice with 1× PBS. Washed beads are blocked with 1% BSA at 4°C for 1 h. The beads are then incubated at 4°C for 1 h with 250 µL of pooled sera at a dilution 1:20 from 20 healthy women. After 3 h of incubation, beads are washed three times with 1× PBS and then incubated with tumor-derived phage library (~10^{10} phage particles). After incubation, the mixture is centrifuged at 850 × g for 2 min to remove phage nonspecifically bound to the beads and the supernatant (phage library) is collected for biopanning.
2. *Biopanning of the selected phage mixture with serum from patient with ovarian cancer.* Protein G Plus agarose beads are placed into a 0.6-mL Eppendorf tube and washed two times with 1× PBS. Washed beads are blocked with 1% BSA at 4°C for 1 h. The beads are then incubated at 4°C for 3 h with 250 µL of serum from ovarian cancer patient at a dilution 1:20. After this incubation, the beads are washed three times with 1× PBS and are incubated with phage library supernatant mentioned earlier (termed as Biopanning 1, BP1) col-

lected for biopanning at 4°C for overnight (shorter times of incubation have not proven successful using model antibody systems). After incubation, the mixture is centrifuged at 3,000 rpm for 2 min and supernatant is discarded. Beads are washed three times with 1× PBS. To elute the bound phage 1% SDS is added to the washed beads and the mixture is incubated at room temperature for 10 min (*see* **Note 5**). The bound phage is removed from the beads by centrifugation at 8,000 rpm for 8 min. Eluted phage is transferred to liquid culture for amplification (50 μL elution to 5 mL bacterial culture). Four rounds of affinity selection and biopanning are carried out with amplified phage obtained after each biopanning. The number of biopanning cycles generally determines the extent of the enrichment for phage that binds to the sera of patient with ovarian cancer. This strategy allows for one cycle of biopanning to be performed in a single day. Two biopanning experiments are performed with the library of differentially selecting clones between control and cancer patient sera. The selection is to isolate phage antigen clones that did not bind to control sera pooled from 20 control women but did bind to a patient serum. This set of phage clones represent epitopes that are indicative of the presence of cancer as recognized by the host immune system.

3.6. Protein Microarrays

1. Phage clones are spotted in quintuplicate onto FAST™ slides (Schleicher & Schuell, Keene, N.H) by a robotic microarrayer, Prosys 5510TL (Cartesian, Inc.). The microarrays are blocked in 4% milk for 1 h at room temperature, rinsed in 1× PBS for two times, and incubated with human serum (preadsorbed with 120 μg of bacterial lysate, *see* **Note 6**) at a dilution of 1:300 and T7 antibody, at a concentration of 0.15 μg/mL at room temperature for 1 h. The microarray slides are washed three times in PBS-T three times 4 min each at room temperature and next incubated with Alexa fluor 532 goat antimouse IgG at a dilution of (1:40,000) and Alexa fluor 647 goat antihuman IgG at a dilution of (1:2,000) for 1 h in the dark. The microarrays are washed three times in PBS-T for 4 min each, then two times in PBS for 2 min each. The slides are centrifuged at 150 × g for 3 min, dried with aerosol (pressurized air), and stored in the dark at room temperature.
2. The arrays were scanned in an Axon Laboratories 4100A scanner (Axon Laboratories, Palo Alto, CA) using 532-nm and 635-nm lasers. The ratio of anti-T7 capsid and antihuman IgG was determined by comparing the fluorescence intensities in the Alexa fluor 532 and Alexa fluor 647-specific channels at each spot using ImaGene™ software. (Biodiscovery, Inc., El Segundo, CA).

4. Notes

1. Fresh tumor specimens obtained from surgical resection should be cut into small pieces and suspended in RNAlater®. That way diffusion of RNAlater® into the tissue sections will be easier and it will help in the stabilization of tissues.
2. Carbenicillin and IPTG stock solutions are prepared in water and filter sterilized using 0.2-μm syringe filter. Both the solutions are aliquoted in 1.5-mL tubes and stored at –20°C.
3. There are two purposes for the northern blot analysis of the mRNA preparations. The first is to judge the integrity of the mRNA so that the bands revealed by the GAPDH probe are sharp and there is no hint of ribosomal RNA in the preparation. Secondly the intensity of the bands between the total 20 μg RNA and the 0.4 μg of poly(A) selected mRNA should be similar. If not then there is a problem with the quantitation of the mRNA using spectroscopy and the amount of mRNA used in the preparation of the cDNA library adjusted accordingly.
4. Protein G-PLUS agarose beads should be handled very carefully. The bead solution should never be vortexed. Only gentle mixing by hand is recommended.
5. One percent SDS should be prepared freshly just before use. Often, a variation in the concentration of SDS can interfere with the elution procedure and next round of amplification of the phage library (eluant) may not be successful.
6. Serum obtained from cancer patient also has circulating antibodies directed toward bacterial proteins that can nonspecifically interfere with the binding of other serum IgGs with their cognate antigens. Before processing protein microarrays, the patient's serum is preadsorbed with an optimal amount of bacterial extracts (as mentioned in **Subheading 3**) in order to sequester these antibodies. This results in the reduction of nonspecific binding of these bacterial antibodies on protein microarrays thereby improving the actual signal-to-noise ratio.

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