CANCER Letters

Mini-review

High throuput proteomic strategies for identifying tumour-associated antigens

C. Geeth Gunawardana [a,b], Eleftherios P. Diamandis [a,b,*]

[a] *Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ont., Canada*
[b] *Department of Laboratory Medicine and Pathobiology, The University of Toronto, Toronto, Ont., Canada*

Abstract

Tumours elicit an immune response in the host organism and this area has been studied for decades. Initially, tumour-associated antigens were studied by examining a few proteins at a time using techniques such as 1-D SDS–PAGE and sandwich ELISAs. Now, however, with the development of high-throughput strategies, multiple potential antigens in a single experiment could be uncovered. The prevailing view is that these antigens can be used as biosensors for cancers. In addition, some of these antigens may indeed be used as targets for immunotherapy. SEREX, SERPA, and protein microarray technology have been the three dominant strategies employed to identify tumour-associated antigens. In this mini-review, we aim to describe these three techniques and provide their advantages and disadvantages. In addition, we aim to address some of the challenges of cancer immunomics.
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1. Introduction

Cancer continues to be a global problem, taxing societies physically, mentally, and monetarily. In the United States and Canada, cancers of the prostate, lung, breast and colon top the list of new cases and deaths in 2006 [1,2]. Current biomarkers for early detection of cancer have thus far been unsatisfactory due to low specificity and sensitivity. While cliché, one cannot emphasize the need for better diagnostic and prognostic markers of cancer.

In the –omics era, cancer immunomics has become an intense area of research. Robert W. Baldwin pioneered the work on cancer immunology in the 50s and 60s, demonstrating that solid tumours can be identified and destroyed by the host immune system [3–6]. Both the humoral arm and the T-cell arm are activated in response to a tumour [7]. Cancer patients produce autoantibodies to proteins that are mutated, misfolded, improperly glycosylated, overexpressed, truncated, or aberrantly localized in tumour cells. These autoantibodies or their aberrant targets can be utilized as molecular signatures of tumorigenesis, thus being excellent candidates for biomarkers.

* Corresponding author. Tel.: + 416 586 8443; fax: + 416 361 2655.

E-mail addresses: geeth.gunawardana@utoronto.ca (C.G. Gunawardana), ediamandis@mtsinai.on.ca (E.P. Diamandis).

URL: http://www.acdclab.org (E.P. Diamandis).

Table 1
Some established tumour-associated antigens

Tumour-associated antigen	Cancer	Ref.
p53	Ovarian, colon	[27,28]
NY-ESO-1	Lung, melanoma	[29,30]
MUC-1	Breast	[31]
Tyrosinase	Melanoma	[32]

Research on tumour-associated antigens (TAAs) and their cognate autoantibodies have provided an abundance of targets for therapy and uncovered candidate biomarkers for early detection, prognostication, and response to therapy Table 1. provides some well-studied TAAs in cancer. The strategies employed thus far for the discovery of TAAs and autoantibodies in cancer have stemmed from techniques employed in identifying autoantibodies and their targets in autoimmune diseases. In this mini-review, we aim to describe the prominent proteomic approaches utilized thus far in identifying TAAs and autoantibodies, namely serological analysis of recombinant cDNA expression libraries (SEREX), serological proteome analysis (SERPA), and protein microarray technology.

2. Serological analysis of recombinant cdna expression libraries

The development of SEREX [8] in the 90s offered a high-throughput approach to analyze the humoral response against TAAs in cancer patients. The approach involves the construction of a cDNA expression library in phage from a fresh tumour specimen (Fig. 1), the use of which confines the analysis to genes expressed by the tumour *in vivo*. The phages are used to transfect *Escherichia coli*, and the recombinant proteins expressed during the lytic phase of phage infection are blotted onto nitrocellulose membranes. Clones are selected based on their reactivity to autologous patient serum. Positive clones are sub-cloned to colonies containing a unique cDNA, thus allowing molecular characterization by cDNA sequencing.

There are several positive facets in the SEREX methodology. First, since cell lines acquire altered protein expression during short and long-term culture, the use of fresh tumour specimens avoids the artefacts innate to cultured cells. Second, the use of a multi-antigen specific patient serum allows the identification of several TAAs in one experiment. Third, both the TAA and its coding cDNA are pres-

ent in the same plaque when immunoscreening, which allows the subsequent matching and sequencing of the protein's cDNA immediately. The sequence information can then be used to design probes for expression studies by Northern blotting, or design primers for quantitative polymerase chain reaction (QT-PCR).

Indeed, a multitude of TAAs from a variety of cancers have been identified using SEREX. These include the cancer-testis antigens such as NY-ESO-1 [9] and CAGE-1 [10] and breast cancer antigens such NY-BR-1 [11] and ING1 [12]. Despite the success in identifying many potential TAAs using SEREX, the technique has pitfalls. One, the use of tumour tissue from a single cancer followed by screening with autologous patient serum limits the identification of TAAs to that particular patient. Two, prokaryotic systems used for immunoscreening do not produce glycosylated protein products, nor is there certainty that the recombinant proteins are properly folded. Thus, humoral responses to certain epitopes of a TAA or even the entire TAA can remain unidentified. Third, the screening of tumour derived cDNA libraries puts a bias towards TAAs that have high message levels in the tumour. Subsequently, relevant TAAs encoded by low-abundance messages may be missed. Fourth, patients may exhibit autoimmunity to certain proteins, thus the immunoscreening step may be confounded by irrelevant (non-tumour associated) autoantibodies.

The success of the SEREX approach is attributable to its ability to screen a large pool of cDNA clones to identify multiple TAAs. As in any technique, SEREX has its drawbacks, which however, do not preclude it as a viable method for identifying TAAs. Several modifications have been added to the original approach, such as using heterologous cDNA libraries and multiple patient sera [13], to circumvent these drawbacks thus making SEREX a reliable proteomic tool for discovery. A database (http://www2.licr.org/CancerImmunomeDB/), Cancer Immunome Database, contains all the autoantigens identified by SEREX.

3. Serological proteome analysis (SERPA)

Spanning over two decades, two-dimensional gel electrophoresis (2-DE) has been an invaluable tool for biomarker discovery. The technique involves the in-gel separation of proteins according to their isoelectric point (pI), followed by a second
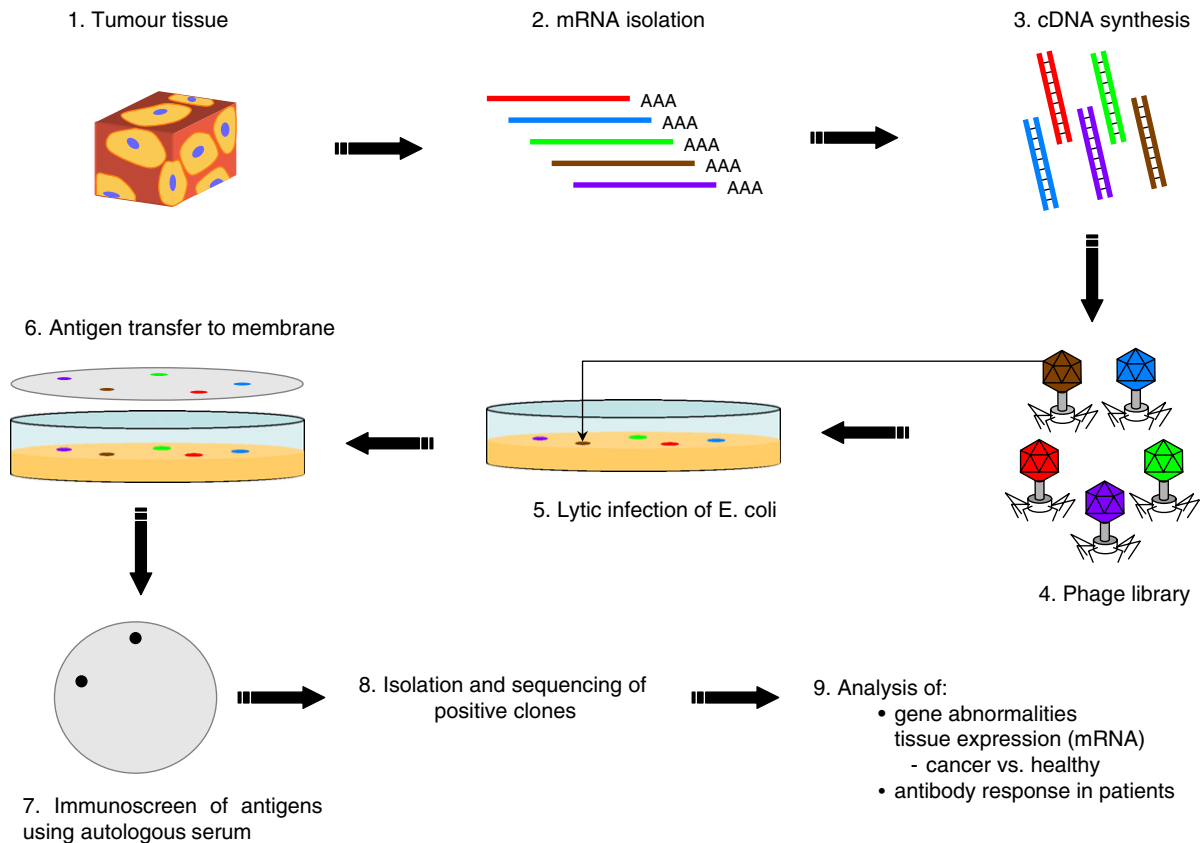
Fig. 1. The strategy followed in serological analysis of recombinant cDNA expression libraries (SEREX).

separation based on molecular mass. This method enables protein separation over a large area, thus providing better resolution of protein components compared to standard 1-D gel electrophoresis.

Combining 2-DE and serological analysis, Klade and colleagues developed the SERPA technique to identify proteins that induce antibody responses in cancer patients (Fig. 2) [14]. In SERPA, three 2-DE gels are run simultaneously under identical conditions, with equal amounts of proteins. Two gels are blotted to nitrocellulose or PVDF membranes and probed with serum from a cancer patient or a normal individual. The third gel (the preparative gel) is stained with Coomassie blue. Immunoreactive spots from the blot of the cancer patient are compared with that of the control. Spots that are either unique to, or are significantly brighter on the blot of the cancer patient, are identified and excised from the preparative gel. Originally, these spots were identified using Edman degradation. However, with the advances in mass-spectrometry, these spots are now identified using MS/MS, a method with high sensitivity and specificity.

In contrast to SEREX, the SERPA method requires less time for a complete experiment. With SEREX, the construction of a representative cDNA library in phage requires at least several days, whereas with SERPA, proteins can be prepared from tumour cryosections within hours. Serological testing with SEREX requires extensive pre-adsorption steps to eliminate non-specific binding artefacts. This is not an issue with SERPA. Parallel analysis of tumour proteins with healthy donor sera as controls can be performed with SERPA, whereas with SEREX, such specificity controls cannot be applied easily. Although the proteins are denatured in SDS/Urea, SERPA utilizes proteins with their potential immunoreactive, post-translational modifications intact, thus offering greater antigenic determinants for serological testing. Although SEREX may be more sensitive since a TAA encoded by a single copy of mRNA may be detected, the use of 2-D immunoblots in SERPA provides a global view of the antibody–TAA interaction.

SERPA, however, is by no means an infallible technique. Its limitations are due primarily to the
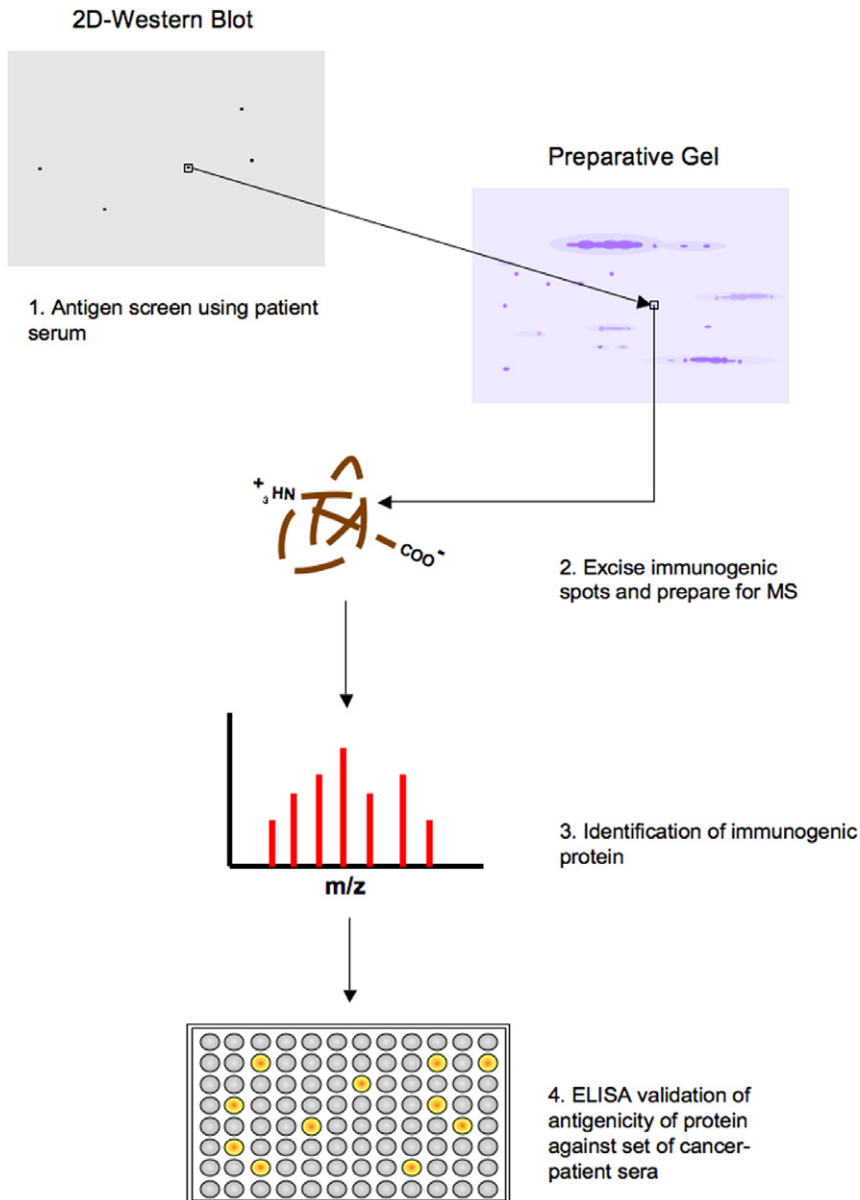
Fig. 2. Serological proteome analysis (SERPA).

analytical limitations inherent in 2-DE. First, 2-DE is limited to identifying relatively abundant proteins due to constraints in sample capacity and detection sensitivity. This has been improved upon slightly by the use of fluorescent labelling methods instead of dye-based methods for protein detection. Second, 2-DE is unable to separate different proteins that, due to post-translational modifications, co-migrate on gels, thus complicating the quantification of visualized spots. Third, although advances have been made with the use of 2-DE compatible detergents,

the separation of cell membrane proteins remains a challenge due to their insoluble nature in aqueous buffers [15]. Four, the method is labour-intensive owing to weaknesses in reproducibility of 2-D gels and the cumbersome task of excising protein spots from gels for identification.

Weaknesses aside, SERPA is a robust technique for identifying TAAs. With emerging advances in robotic automation and mass-spectrometry, it is conceivable that newer TAAs will be found using SERPA. Indeed, multi-dimensional liquid phase

based systems can be used to separate proteins, and serological screening can be performed in a multiplex enzyme-linked immunosorbant assay (ELISA) format [16,17]. The advantage being that liquid-based separations are amenable to automation and the ELISA format can be coupled to mass spectrometric analysis, thereby increasing throughput.

4. Protein microarrays

Microarray technology appears to have the most promise in analyzing the "immunome" of a cancer. Specifically, protein microarrays provide a form of protein analysis at a scale beyond that which is achievable by 2-DE or ELISA. Essentially, there are two approaches for investigating the tumour immunome. One is the biased approach, and the other being the unbiased approach. Table 2 describes some of the biased and unbiased microarray platforms in use.

A biased microarray approach analyzes a large panel of known analytes, usually proteins or antibodies. Such platforms have been used to study autoimmunity in diseases such as Systemic Lupus Erythematosus, Rheumatoid Arthritis, and Sjögren's syndrome [18] and therefore the same approach has been applied to study the immune response in cancer. Indeed, several biased microarray formats have been employed to study prostate cancer. Haab and colleagues screened dye-labeled prostate cancer sera and control sera (non-cancer) against a microarray containing 184 unique antibodies. Five proteins (von Willebrand Factor, IgM, alpha1-antichymotrypsin, villin, and IgG)

showed significantly different levels in the prostate cancer samples compared to controls [19]. In a variation of this approach, Liu and his team developed a "reverse capture" autoantibody microarray to screen prostate cancer sera [20]. Briefly, proteins isolated from prostate cancer cell lines were immobilized on BD Clontech's AB Microarray 500 (containing 500 unique monoclonal antibodies in duplicate). Antibodies were isolated from patients with prostate cancer (test) and benign prostatic hyperplasia (control). Test and control antibodies were labeled with different CyDyes, mixed in an equal ratio, and then used to screen the microarray. Forty-eight proteins were listed as antigenic, including known TAAs such as p53 and Myc.

Biased approaches are not limited to antibody microarrays, as microarrays containing known proteins can uncover potential TAAs. In our laboratory, we utilized Invitrogen's ProtoArray™ containing ≈2000 known and hypothetical proteins, and screened using ascites fluid from ovarian cancer patients. As a control, non-malignant ascites was used. Using Invitrogen's ProtoArray®Prospector software to analyze the antibody–antigen interaction under highly stringent conditions, nine proteins were found to be antigenic with 100% reproducibility in two separate microarray experiments (unpublished data). Presently, Invitrogen has released an 8000 protein microarray thereby increasing the number of targets that can be screened in one experiment. With the increasing number of new proteins being discovered, it is only a question of time before the field develops the entire human proteome on one slide.

Table 2
Protein microarray formats

Microarray formats	Description	Drawbacks	Ref.
Biased			
Autoantibody	High quality antibodies are printed on slides	Only antibodies available commercially or high quality "in-house" antibodies can be tested. Limited number of antigens can be detected	[19]
Autoantigen	Purified proteins known to be part of the tumour proteome are printed on slides.	Only known proteins can be studied. Not suitable for discovery experiments	[33]
Unbiased			
Phage display	Phages, each expressing a specific tumour protein, are printed on slides	Must distinguish between irrelevant autoantibody response and tumour specific response. Phage proteins lack proper PTM[a]	[22]
Cell lysate	Tumour cell lysates are fractionated into multiple fractions and each fraction is then printed on a slide	Reproducibility, enormous data requiring specialized software tools for analysis	[23]

[a] PTM, post-translational modifications.

Screening a microarray containing an "unknown" group of proteins, with antibodies specific to unknown antigens is the basis of an unbiased approach. This type of approach is suitable for discovery experiments due to the potential of identifying novel antibody–antigen interactions. The most promising strategies used in the design of unbiased protein microarrays involve the use of a phage display system to express the tumour protein repertoire on a microarray, or the use of multidimensional fractionation techniques to simplify cell lysates into less complex fractions for the purpose of producing natural protein microarrays.

Invented in 1985 by Smith [21], phage display is a powerful proteomic tool used to express proteins or domains of proteins. The system has played a pivotal role in mapping epitopes of monoclonal and polyclonal antibodies, defining amino acid substrate sequences, and identifying peptide ligands for drug research. Concerning tumour immunology, the most promising usage of phage-display has been in combination with microarray technology in which phages displaying a single protein are printed on a microarray and screened using antibodies isolated from cancer patients (Fig. 3).

Chinnaiyan and his colleagues demonstrated the effectiveness of the use of a phage-protein/microarray hybrid to discriminate prostate cancer from normal [22]. Briefly, a phage-display library is constructed from prostate cancer tissue. Several cycles of biopanning are performed to enrich for phages carrying peptides that are reactive to cancer sera and not control sera. Reactive phage clones are cultured to monoclonality and propagated prior to printing on the microarray. The microarray is incubated with Cy5 labeled antibodies isolated from cancer patients, and a Cy3 labeled antibody specific to a phage capsid protein. The Cy5 signal is normalized to the Cy3 signal (indicator of the quantity of phage per spot). With this approach, 186 proteins were discovered to be antigenic. Of these 186 potential TAAs, 22 were subsequently evaluated on an independent validation set of 128 serum samples (from patients with prostate cancer and from controls). The panel of 22 TAAs performed better than prostate specific antigen (PSA) alone in distinguishing the group with prostate cancer from the control group.

Chinnaiyan's approach in discovering novel TAAs and autoantibody signatures circumvents many of the drawbacks found in the SEREX and SERPA methods. For example, the multiple rounds of biopanning enriches for antibodies that react specifically with tumour proteins, whereas in SEREX, there is only one affinity-screening step. Furthermore, biopanning and microarray incubation is not limited to autologous sera. The labour intensive task of cutting gel bands and analyzing them by mass spectrometry (in SERPA) is avoided. The discriminatory power of this method on a large set of cohorts remains to be further validated.

The production of thousands of recombinant proteins for microarray production is cumbersome and costly. Recombinant proteins produced in systems other than mammalian ones may not have the proper post-translational modifications and may be misfolded after production. However, proteins isolated from mammalian cells using non-denaturing lysis buffers, will retain their native conformation and will possess the correct post-translational modifications. If these proteins are isolated from tumours, then it is likely that aberrant tumour proteins will also be represented in the lysates. With recombinant proteins, sufficient amounts of protein can be produced with excellent purity, and therefore is more suitable for printing on microarrays. With cell lysates, however, it is not possible to divide the constituents into fractions containing a single type of protein and therefore is less amenable for protein microarray production. However, with the advances in automated chromatographic techniques, one can separate a complex mixture, such as cell lysates into smaller, less complex fractions. These fractions can then be used to produce natural protein microarrays, where each fraction is a single feature on the slide (Fig. 4).

Hanash and his colleagues have demonstrated the feasibility of manufacturing natural protein microarrays using advanced chromatographic techniques and microarray printing technology [23]. Briefly, cell lysates from a cancer cell line is first resolved using liquid-based isoelectric focusing into 20 fractions. Each fraction is then separated into 92 fractions using a reverse-phase column HPLC. The fractionated proteins are lyophilized and resuspended in a suitable buffer to be printed on a nitrocellulose-based microarray. The protein microarray is then screened using sera from cancer patients, and sera from healthy individuals are used as controls. Antigenic signals can be traced back to the original fraction from which the spot was created, and using mass spectrometry, the protein content of the fraction can be determined. The reactive antigen can then be determined using a combination of Western

1. Tumour tissue   2. mRNA isolation   3. cDNA production

recombinant protein

capsid protein

6. enrichment of cancer-specific phage peptide   5. elimination of non-cancer specific phage peptides by multiple rounds of biopanning   *4. in vitro* packaging
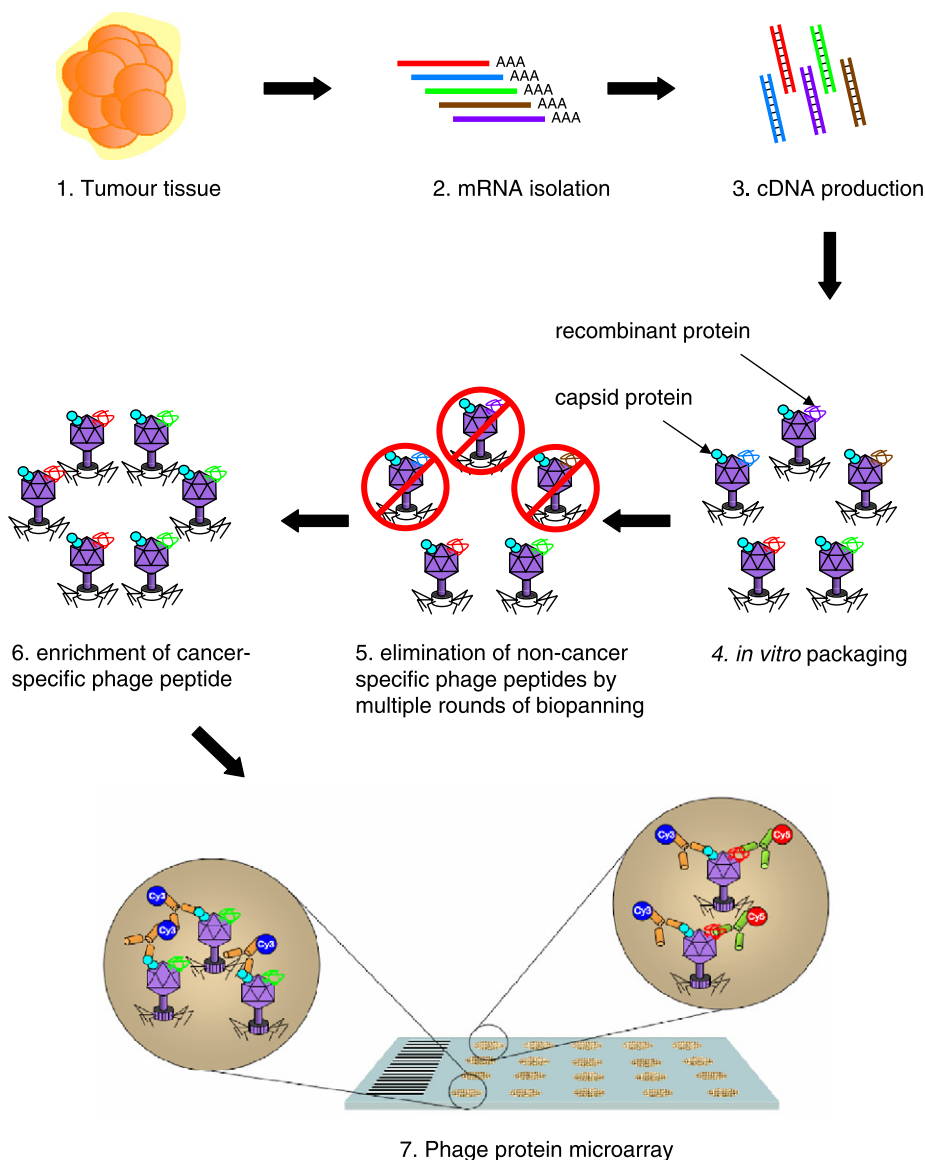
7. Phage protein microarray

Fig. 3. Overview of the phage display protein microarray production strategy [26].

blotting and mass spectrometry. From one fraction, Hanash was able to identify antibodies to ubiquitin C-terminal hydrolase L3 in colon cancer patients [24]. Although, it is unclear how many other putative TAAs were discovered by this group, the approach is indeed promising.

It appears that the protein microarray is the new "flavour of the month" as a discovery tool in tumour immunology. With its ability to screen a multitude of antigens or antibodies in one experiment and the considerable data that can be generated, it is clear to see the reason for its popularity. A caveat, however, is that the enormous amount of data that is generated requires proper bioinformatics and statistics tools for analysis. As with any technique, reproducibility is a concern. Tied in with reproducibility is cost, as the production of a protein microarray is an expensive task, and multiple experiments can cost thousands of dollars.

5. Conclusions

Exploiting the humoral immune response is undoubtedly a powerful strategy to identify candidate biomarkers of cancer. In this review, we presented three robust approaches, namely SEREX,
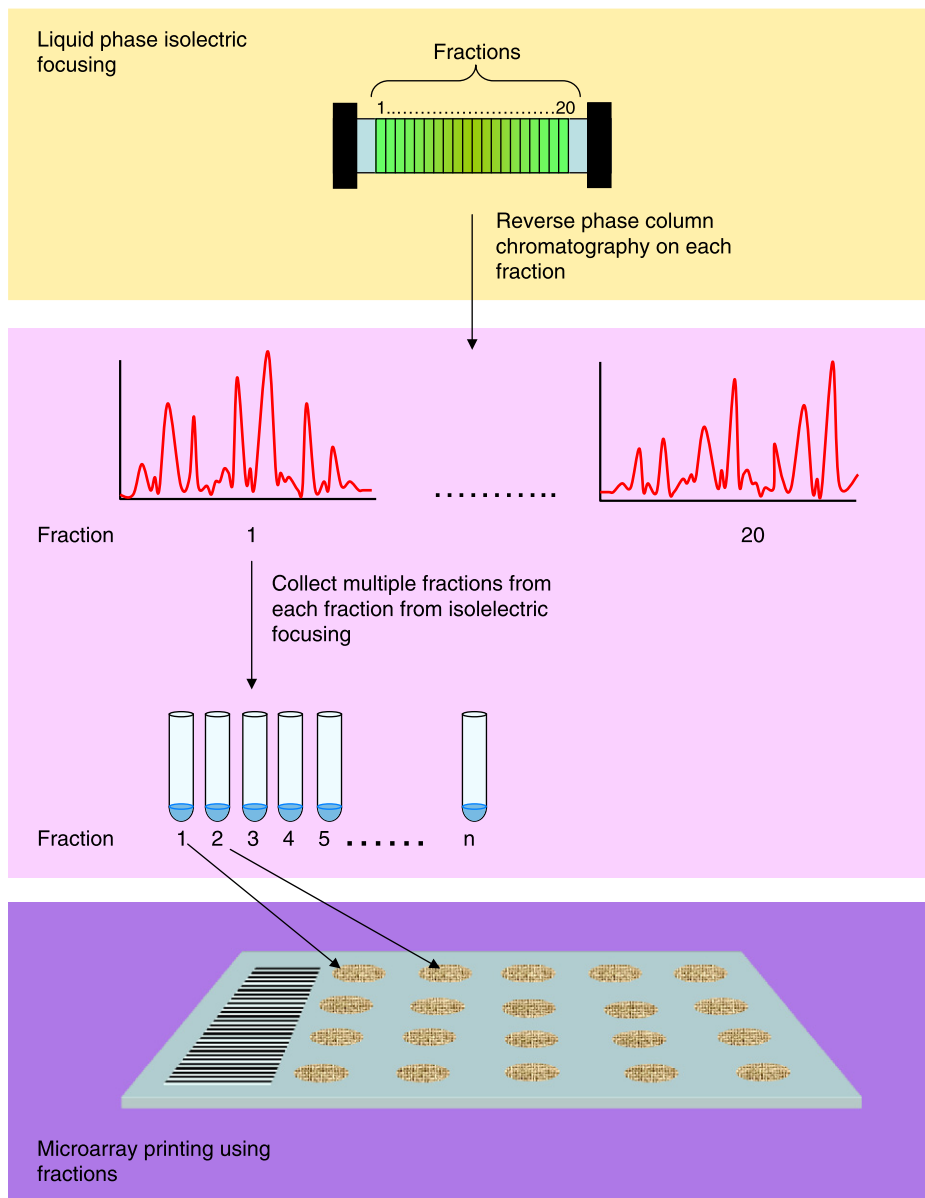
Fig. 4. Overview of the natural protein microarray production strategy.

SERPA, and protein microarray technology, each with their advantages and disadvantages (Table 3). Each technique has revealed multiple novel TAAs. With the advances in protein separation and detection techniques, newer TAAs will be found. Indeed, this has been the case with technologies described in this review.

How do the high-throughput technologies discussed in this mini-review benefit the clinic? Given the issues with specificity and sensitivity, the TAAs discovered thus far are not proficient for the early detection of cancer. For example, in one study,

autoantibodies to p53 were found in ovarian cancer but only 24% of patients were positive [25]. Furthermore, there was no significant association between the presence of p53 antibodies and clinical stage, histological type, or overall patient survival. Thus, TAAs as potential biomarkers, need further validation in the context of early detection, prognostication, and response to therapy.

A good biomarker would be a protein (in the context of this review) that is detectable in body fluids (serum, urine, effusions, etc.) and demonstrates high specificity and sensitivity to a particular disease.

Table 3
Current proteomic strategies for studying TAAs

Technique	Description	Advantages	Drawbacks	Ref.
SEREX	Phage-expressed tumour proteins are screened for antigenicity using autologous patient serum and identified by cDNA sequencing	Identify several TAAs in one experiment. cDNA of TAA can be sequenced and analyzed in same experiment	Phage expressed proteins lack PTM. Biased towards highly expressed proteins	[13]
SERPA	2-DE separation and immunoblotting of tumour proteins followed by MS identification of antigenic proteins	Relatively quicker than SEREX. Global analysis of TAA and antibody interactions	Reproducibility of 2-DE. Not amenable to automation. Sensitivity	[34]
Protein microarray				
Biased	Known subset of proteins or autoantibodies are printed on microarays and probed with patient sera or tumour lysates, respectively	High quality antibodies can be used. Low amounts of material needed. High sensitivity	Only known antibodies can be tested. Not good for discovery experiments	[19]
Unbiased	The "entire" tumour protein is printed on a microarray and probed with patient sera	Large panel of proteins can be tested. Suitable for discovery experiments	Cross reactivity of "irrelevant" antibodies. Analysis of large sets of data	[22,17]

However, it is likely that most TAAs will display low sensitivity and specificity. To circumvent this issue, the prevailing view is to use panels of TAAs in conjunction with powerful bioinformatics tools (e.g. pattern recognition software) to devise diagnostic tests that provide better sensitivity and specificity than single biomarkers alone. Indeed, this has been shown in a study of prostate cancer versus non-cancer, where a validation set of 22 protein peptides in microarray format was able to detect prostate cancer with a specificity of 88.2% and a sensitivity of 81.6% [26]. The results were better than using PSA alone.

In developing a panel of TAAs as a biosensor of cancer, one must keep in mind that such a panel must have sufficient sensitivity and specificity, and be simple to perform, technically. The microarray format is quick, high-throughput, and sensitive, but is also costly and requires proper standardized bioinformatics algorithms to analyze the data. The ELISA has been a time-tested assay for detection of antigens, and with the advances in multiplex ELISA systems, such assays may be suitable for testing multiple antigens. The Luminex xMAP technology appears to be very promising as 100 or more analytes can be studied in one sample alone.

The growth of cancer immunomics is intimately tied to advances in proteomic technologies. SEREX, SERPA, and protein microarray technology have been the most powerful techniques utilized in the discovery phase. The discoveries thus far, however, have not provided the solution to the problem of detecting a malignancy early.

Acknowledgement

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