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Humoral response to cancer as a tool for biomarker discovery

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ABSTRACT

There is an important need to find relevant biomarkers that show high sensitivity and specificity for early diagnosis and prognosis of cancer. An immune response to cancer is elicited in humans, as demonstrated in part by the identification of autoantibodies against a number of tumor-associated antigens in sera from patients with different types of cancer. Identification of tumor-associated antigens and their cognate autoantibodies is a promising strategy for the discovery of relevant biomarkers. During the past few years, proteomic approaches, including SEREX, SERPA and, more recently, protein microarrays, have been the dominant strategies used to identify tumor-associated antigens and their cognate autoantibodies. In this review, we aim to describe advantages, drawbacks, and recent improvements of these approaches for the study of humoral responses.

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1. Introduction

The discovery of new serum biomarkers that exhibit increased sensitivity and specificity compared to current biomarkers appears to be an essential field of research in cancer. With the exception of human chorionic gonadotropin hormone, there is no specific tumor marker but rather molecules whose presence in high concentration is associated with cancer. Moreover, most biological markers are not organ-specific. Finally, they show insufficient diagnostic sensitivity and, for the majority of them, their concentrations are elevated only in advanced forms of the disease. For this reason, they are usually employed to monitor therapeutic efficacy during advanced (i.e., metastatic) forms of cancer. It is therefore essential to identify clinically reliable biomarkers and develop effective approaches for cancer diagnosis and prognosis. One promising approach in this field is study of the immune

response to cancer and identification of antibodies and tumor-associated antigens (TAAs).

In the 1960s, Robert W. Baldwin, who was a pioneer in this field, demonstrated that the immune system could react to a developing tumor [1–3]. This immune response is composed of simultaneous cellular and humoral responses [4]. Although little is known about the origin of this immune response, it is now largely established that cancer patients produce autoantibodies to mutated tumor proteins, truncated, misfolded, or over-expressed proteins and proteins that are ectopically expressed. Thus, identification of antigenic proteins and their associated antibodies appears to be a good strategy for the discovery of new tumor biomarkers [5].

Recent advances in proteomic methods, such as mass spectrometry and protein arrays, have greatly facilitated the discovery of new antibody biomarkers and their associated proteins in sera from cancer patients [6,7]. In this review, we

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aim to present the most important proteomic approaches developed in this field.

2. Serological analysis of cDNA expression banks

Serological identification of antigens by recombinant expression cloning (SEREX) was the first technique developed for analysis of the humoral response to cancer. This approach consists of analysing antigens from a cDNA expression library obtained from tumor-extracted mRNA [8] (Fig. 1). The success of this approach for identification of TAAs is probably related to the choice of mRNA source, namely the autologous tumor, which greatly facilitates identification of antigens that react with autoantibodies present in the autologous patient serum. This powerful technique allowed for identification of several autoantibodies and their cognate TAAs. An improved method was developed by Krause et al. [9], consisting in selecting reactive phage clones and evaluate them rapidly using panels of allogeneic sera from cancer patients as well as appropriate control groups, thus circumventing non specific reactivity (related to the patient, but not to the tumor process) that could happen during the first selection (SEREX).

One of the first identified TAAs using the SEREX technology was the NY-ESO-1 protein in squamous cell carcinoma [10]. Antibodies directed against NY-ESO-1 were found with a fre-

quency varying between 20 and 50% in other cancers, such as non-small-cell lung cancer [11], prostate cancer [12], thyroid cancer [13], and melanoma [14]. Thereafter, other TAAs were identified, including CAGE-1 in lung cancer [15]; NY-BR-1, ING1 and hMena in breast cancer [15–17]; and GLEA1, GLEA2 and PHF3 in glioblastoma [18]. Interestingly, some of the identified autoantibodies may be of interest as tools for an early diagnosis or as predictive markers. For instance, anti-NY-ESO-1 antibodies are detected in early forms of lung cancer [11], and anti-GLEA2 antibodies and anti-PHF3 are associated with a good prognosis in glioblastoma compared to the patients lacking this specific humoral reaction [18]. In spite of these very promising results, SEREX suffers some drawbacks due to technical considerations. Indeed, at its inception, this approach was developed to explore the humoral response in sera from patients when their own tumor was used as the source of antigens. On the one hand, construction of a cDNA expression library for every analyzed autologous serum sample is very complex and time consuming. On the other hand, numerous false positives are often detected due to the identification of a humoral response that is specific to the patient but unrelated to the tumor process. Furthermore, the use of a prokaryotic system for the expression of recombinant proteins does not allow for the detection of immunogenic proteins with specific post-translational or conformational modifications, whereas these mechanisms are often implicated in autoimmune reactions or cancers. In order to circumvent these

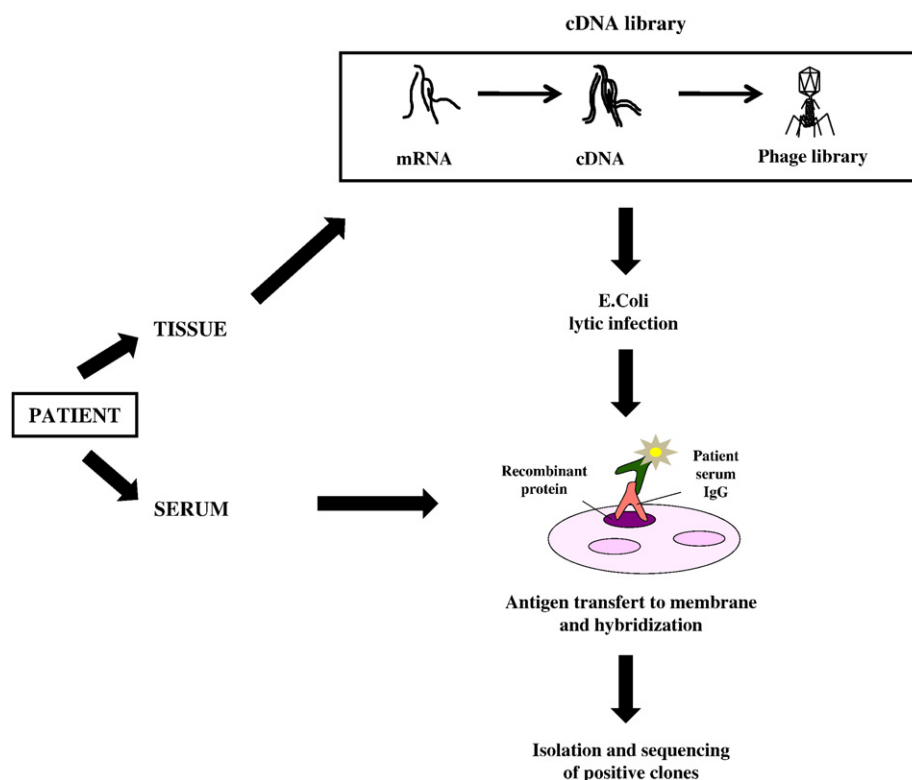


Fig. 1 – Methodology for identification of TAAs using SEREX. Messenger RNAs (mRNAs) are extracted from a tumor. Corresponding double-stranded cDNAs are then synthesized and inserted into the λ phage genome. *E. Coli* bacteria are then infected with amplified recombinant virions. Recombinant proteins expressed during the lytic phase of infection are transferred onto a nitrocellulose membrane, which is then incubated with autologous serum (from the same patient). Reactive clones are then isolated and corresponding cDNAs are sequenced.

problems, several modifications of the original technique have been proposed. Indeed, post-translational modifications, glycosylation in particular, can be evaluated by the expression of recombinant proteins in the yeast eukaryotic model [19–21].

Thanks to these improvements, SEREX remains a technique of reference for study of the humoral response to cancer. As such, it has allowed for the identification of a great number of TAAs whose clinical interest remains to be further investigated. TAAs identified by SEREX are regularly updated in a database (<http://www2.licr.org/CancerImmunomeDB/>).

3. Serological analysis using conventional proteomic techniques

For several decades, two-dimensional gel electrophoresis (2-DE) has remained one of the best tools for proteomic analysis. This technique allows for the separation of a complex mixture of proteins extracted from tumor or cell cultures, according to their isoelectric points (pI) (first dimension) and their molecular weights (second dimension). By combining 2-DE and western blotting, Klade's team developed the SERPA approach (serological proteome analysis) in order to identify antigens associated with the humoral response in kidney cancer [22] (Fig. 2). Two

antigens (SM22-a and CAI) were identified as eliciting a humoral response in 27 to 45% of the tested patients by the use of SERPA [23]. This approach was then successfully used to characterize the humoral responses in several other types of cancers. Indeed, peroxiredoxin 6, triphosphatase isomerase (Tim), superoxide dismutase (MnSOD) were identified as TAAs in squamous cell carcinoma [24,25]; calreticulin in pancreatic cancer [17]; and RS/DJ1 in breast cancer [26]. Interestingly, our team observed that autoantibodies directed against the chaperone protein HSP60 were present in 32.6% of ductal carcinomas *in situ* of the breast [27]. Moreover, a combination of HSP60 with 4 other TAAs, including PPIA, PRDX2, FKBP52, and MUC1 was associated with the presence of ductal carcinoma *in situ* and early invasive breast cancer [28], showing that autoantibodies directed against TAAs constitute a very promising tool for early diagnosis.

Compared with SEREX, SERPA has several advantages. This approach, for a complete experiment, is less time consuming, particularly concerning the preparation of protein extracts and serological analysis. Moreover, it allows for identification of possible post-translational modifications and protein isoforms. Its drawbacks are associated with limits of 2-DE. Indeed, identification of low abundance proteins remains limited by the sensitivity of staining methods, in spite of the development of fluorescent molecules [29]. Furthermore,

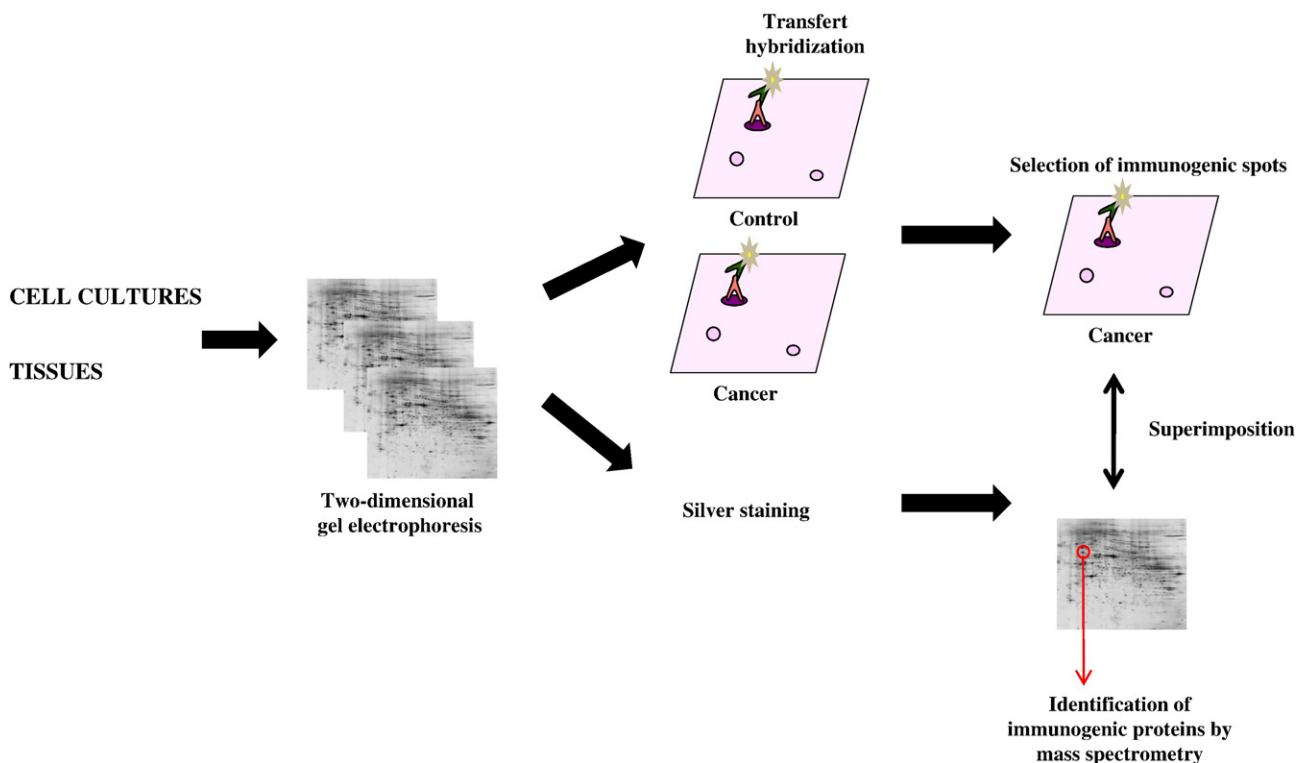


Fig. 2 – Methodology for identification of TAAs using SERPA. This approach consists of concomitant separation of equivalent quantities of protein extracted from cell cultures or tumors on three 2-DE gels. The proteins from the first two gels are transferred onto membranes (nitrocellulose or PVDF) before hybridization with sera from patients with cancer or healthy controls. The third gel is silver- or Coomassie stained in order to establish the tumor protein map. Pictures obtained from the western blots using sera from the two populations (cancer/healthy) are compared in order to locate immunogenic proteins specific for the tumor humoral reaction. Second, western blot pictures are superimposed on the silver stained gel in order to isolate and identify the proteins of interest. To that end, proteins are extracted from the gel, digested with trypsin and identified by mass spectrometry.

transmembrane proteins, which are hydrophobic and insoluble, are difficult to detect. This is also the case for low molecular weight proteins (lower than 10,000 Da) and proteins with extreme isoelectric points. Nevertheless, SERPA remains a very robust method for evaluation of the humoral response to cancer. Moreover, many technological improvements have been reported over the last few years. More specifically, this approach has been advanced by automation of 2-DE techniques and utilization of highly sensitive mass spectrometry, which allows TAA identification.

4. Serological analysis by protein microarrays

The success of DNA chip approaches for analysis of the genome has inspired research groups interested in the humoral response over the past few years. The newly developed protein microarray approaches allow for analysis of a great number of targets in only one-step, which is not possible with SEREX or SERPA. These microarrays derive from a method developed by the team of Fen et al., whose aim was to study humoral responses associated with different autoimmune diseases [30]. There are 2 types of microarrays to study the humoral response. Protein microarrays are based on antigen immobilization on a support (glass) and differential analysis of antigen–antibody interactions (Figs. 3 and 4). They are generated by spotting recombinant, synthetic, or native antigens onto a microarray surface. Protein microarrays that enable large-scale testing on more than 80,000 recombinant antigens have been developed, which, for instance, include ProtoArray® protein chips (Invitrogen) [31]. Recently, this technique has been used to identify 94 autoantigens exhibiting enhanced reactivity in ovarian cancer patients compared to controls [32].

To improve autoantigen coverage, and to circumvent the limited availability of commercialized or home made proteins that are to be spotted on the support, Chinnaiyan's team developed a method derived from the SEREX technology based on the use of a phage-display cDNA expression library produced from isolated tumor mRNA from prostate cancer [33] (Fig. 3). The study, performed with sera from 119 patients with prostate cancer and 138 healthy controls, allowed for the selection of a panel of 22 peptides that discriminated the two groups with 81.6% sensitivity and 88.2% specificity; thus, providing additional discriminative power over prostate-specific antigens. This approach allows for high throughput analysis and more efficient phage selection, thus making it more powerful than SEREX. However, this technology, like SEREX, does not allow detection of post-translational modifications. In order to circumvent this problem, Hanash's team developed a multidimensional fractionation technique using liquid chromatography (LC) to isolate a mixture of native proteins extracted from cancer cell lines [34] (Fig. 4). This original natural protein microarray-based approach allowed for the identification of antibodies directed against C-terminal hydrolase L3 ubiquitin in the sera of patients with colon cancer [35].

Antibody chips are based on a reverse capture system [36]. This technique immobilize well-characterized, highly specific and high affinity antibodies designed to bind native antigens

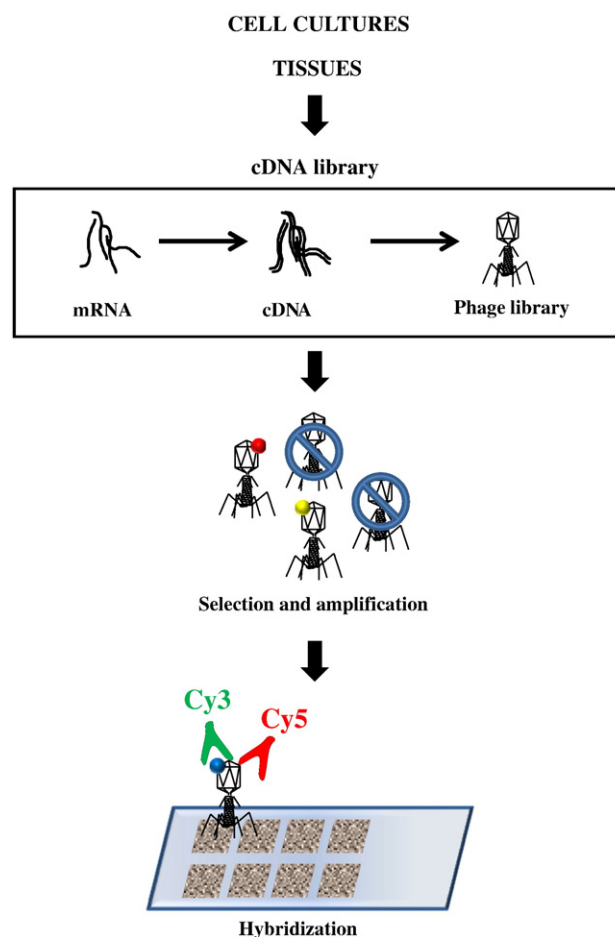


Fig. 3 – Principle of the phage protein microarray-based approach. Proteins expressed from a cDNA library are immobilized on a slide. As with SEREX, cDNAs are synthesized from mRNA that is extracted from tumors or cell cultures. Once inserted into phage genomes, this expression bank is enriched in phages that specifically react with sera from patients with cancer by repeated cycles of purification. The reactive phages are then amplified and spotted on slides, which are hybridized with Cy5-labeled antibodies from patients with cancer. Labeling intensity is normalized using a Cy3-labeled antibody directed against a phage capsid protein. After the slide is scanned, reactive phages are isolated and the corresponding cDNA is sequenced, allowing for antigen identification.

contained in cell extract from tumors or cell lines. Then, labeled purified autoantibodies from a patient's serum is added [36,37]. Cancer and control autoantibodies are labeled with different cyanin dyes, and the ratio of fluorescence determines the relative abundance of the autoantibodies in a given serum sample. In a recent study, antigens extracted from prostate cancer cell lines were captured by 500 monoclonal antibodies immobilized on a glass support. Antibodies from patients with prostate cancer or benign hyperplasia (controls) were preliminarily marked with two different fluorophores (Cy3 and Cy5) and hybridized onto chips, thus enabling identification of 48 TAAs [38]. This technology has

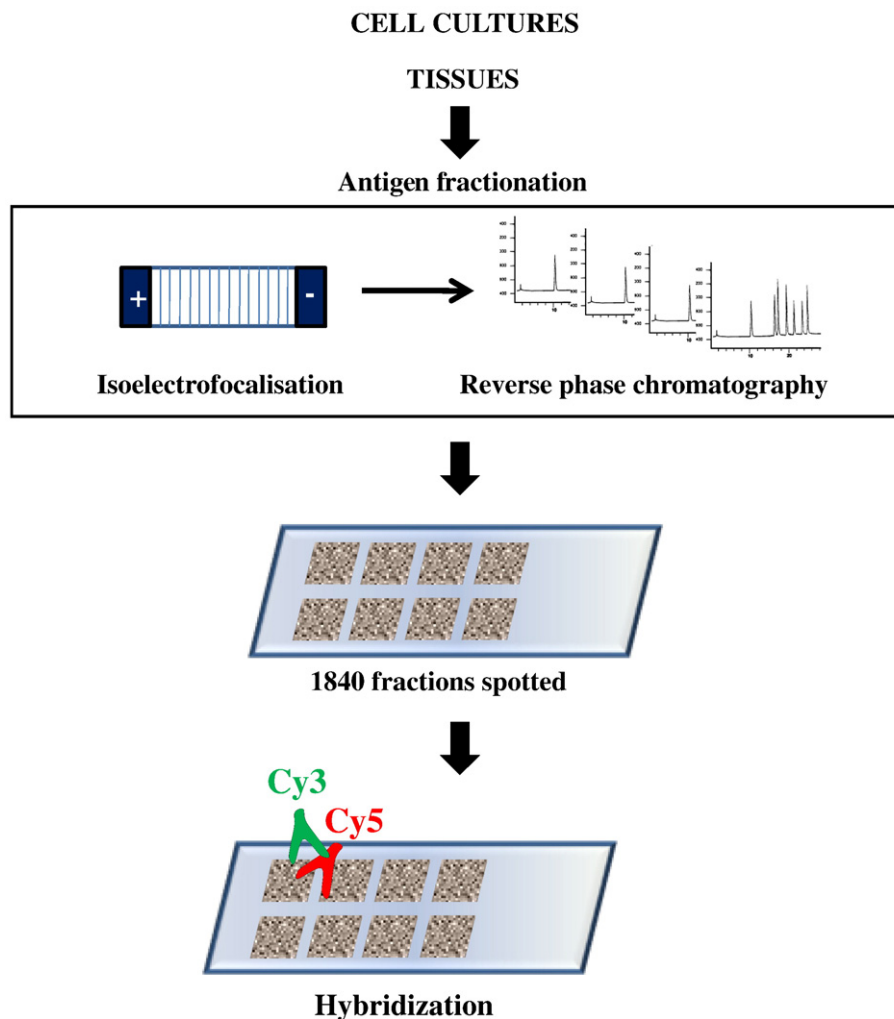


Fig. 4 – The principle of the natural protein microarray-based approach. Tumors or cell cultures are used as antigen sources. The proteins are fractionated by liquid isoelectrofocusing (20 fractions). Every fraction is then separated into 92 new fractions by reverse phase liquid chromatography. A total of 1840 fractions are spotted on a solid support before hybridization, as was described above, with sera from patients with cancer or healthy controls. The reactive antigens are obtained by isolation of the corresponding fraction. The antigens are then identified by mass spectrometry.

the advantage of allowing the antigens to be immobilized in their native configuration, with their post-translational modifications. Moreover, it provides direct information on these antigens. However, like protein microarrays, availability of commercialized antibodies limits analysis by antibody arrays.

In summary, these innovative protein microarray approaches have many advantages for the identification of new tumor biomarkers associated with the humoral response, in particular due to their capacity for high throughput analysis [39].

5. Conclusion

The technological developments presented in this review illustrate the importance of the humoral response for identification of new biomarkers in the study of cancer. These technologies, which are in constant evolution, allow for identification of antibodies and their TAAs. However, the

clinical impact of these potential biomarkers remains to be evaluated using complementary analysis. Indeed, published results demonstrate that most antibodies and antigens that have been identified until now show weak frequency (20%–30% of patients) and are weakly or not correlated with classical clinical parameters (histological type, tumor stage, survival, etc.). As a result, in the future, most of these studies will require clinical validation using a broader independent patient population in order to determine the value of potential biomarkers in terms of sensitivity, specificity and predictive value. Recent published reports that used protein microarray approaches foretell a promising future for the implementation of sensitive and specific tests. Indeed, in order to palliate the lack of sensitivity and specificity of individual biomarkers, it is possible to combine several markers, which, associated with adapted statistical analysis, may be clinically relevant. Thus, the association of several antibodies or antigens seems to allow for the detection of tumors with higher efficiency than isolated biomarkers [6,33,39–43]. These results predict

many upcoming developments in the field of cancer diagnostics. We suggest that molecular signatures, associated with clinical and anatomopathological data, will facilitate improved diagnosis and prognosis in cancer.

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