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Improved approach to identify cancer-associated autoantigens

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Abstract

The failure to identify biomarkers of clinical significance for cancer diagnosis and prognosis generated a great deal of skepticism in regard to the usefulness of autoantibody-based methods. SEREX was a major advancement in immunoscreening that resulted in the identification of a large group of autoantigens recognized by cancer sera. However, few SEREX-defined autoantigens have proven to have definitive diagnostic value in clinical practice. Often, the identified antigens are patient-specific rather than tumor-specific and many tumor-associated antigens are rare in expression libraries made from non-autologous cells.

Since autoantibodies are part of the normal immune response, it can be difficult to single out tumor-associated antibodies from the scores of irrelevant patient-specific responses. In our view, any practical approach for identifying cancer-related autoantigens must include an integral strategy for demonstrating tumor relevance early in the screening process. Care must also be taken not to exclude potentially important autoantibodies by pre-screening manipulations to patient sera.

We have introduced substantial modifications in SEREX, designed to minimize confounding effects of unrelated autoantibodies and to eliminate steps that preclude the identification of cancer-related autoantigens commonly recognized by cancer sera. In addition, we incorporate methodology to identify candidate antigens that have potential diagnostic or prognostic value prior to their molecular cloning and characterization.

Keywords

Immunoscreening; Autoantibodies; Biomarkers; Breast cancer; Microarrays

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1. Introduction

Immunoscreening cDNA expression libraries with antibodies present in human sera has been done for almost 20 years [1]. The introduction of SEREX [2,3] led to the identification of a large group of candidate cancer biomarkers recognized as autoantigens by cancer patient sera [3]. An important problem of existing autoantibody-based methods of identifying tumor-related antigens, however, is the subsequent need to demonstrate the tumor relevance of the antigens. While SEREX is a powerful tool for identifying autoantigens, it fails to employ criteria to select those that may be recognized by multiple patients. Any protein expressed in the autologous tumor may be identified, including antigens unique to the patient. Because SEREX involves screening tumor-derived autologous cDNA libraries, there is an inherent bias towards selecting antigens that are highly expressed in the tumor, as these are over-represented in such libraries. Although antigen expression levels are important, they are not necessarily related to tumorigenesis, and low abundance messages encoding highly relevant antigens may be missed. Since autoantibodies are part of the normal immune response [4], each SEREX-identified antigen must be validated by subsequent tests of its relatedness to cancer. Because the fraction of these autoantigens directly associated with tumorigenesis is small, we suggest that there may be a more efficient approach to identifying useful clinical markers. We have introduced substantial modifications in the SEREX methodology designed to minimize the confounding effect of unrelated autoantibodies and to eliminate steps that may prevent the identification of cancer-related molecules [5–10].

2. Methods and results

Sera or biological fluids used to identify cancer-related autoantibodies are usually pre-treated to remove interfering non-specific binding partners. These steps are indeed effective, but have also the undesirable effect of non-specifically removing tumor-associated antibodies. A step thought to be crucial [2,3] is to absorb the biological fluid with non-transfected host cells to produce a sample stripped of contaminating antibodies that react with non-transfected host antigens. With the intent of eliminating vector-related reactivity, SEREX also absorbs the fluid sample with lysed host cells transfected with the vector into which the cDNA library is inserted, but without said cDNA library. We suspected that these steps could remove cancer-related antibodies. First, there may be tumor-associated immune responses to proteins that are denatured, localized in the wrong cellular compartment, or overexpressed. Such tumor-expressed antigens are not necessarily mutant proteins, i.e., they may share the primary amino acid sequence, and therefore some of the epitopes present in the protein expressed in non-transfected host cells. Antibodies to such proteins would be eliminated by a pre-screening step. Second, initial autoimmune responses to an epitope on a mutant tumor-expressed protein may be amplified as a polyclonal response directed against the entire protein. Thus, most of the autoantibodies targeted against the altered protein may cross-react with the native protein from non-transfected cells, and again be eliminated by a pre-screening step. In support of this contention, Naftzger et al. showed that under certain circumstances the immune system could actively respond to normal tissue autoantigens [11]. Their studies immunizing mice with a human protein or with altered sources of a syngeneic antigen favored a model of tolerance which can be broken by presenting sources of an altered antigen. Thus, it is conceivable that immune responses induced by altered tumor

proteins may elicit antibodies against a normal protein present in non-transfected cells. Initial experiments indicated that absorption procedures interfere with the cloning of antigens commonly recognized by cancer patient sera and therefore we omit them. Omitting these steps has the minor drawback of allowing the occasional cloning of vector-related antigens, but these can be readily recognized and removed based on vector sequence homology. We also found that another procedure used in immunoscreening cDNA expression libraries [2,3], paradoxically to eliminate irrelevant clones, may also remove cancer-relevant clones. Prescreening cDNA libraries with pools of normal sera is commonly done to remove clones that non-specifically bind IgG. A shortcoming of this technique is that pools of “normal sera” are likely to contain tumor-associated antibodies, because many normal subjects will eventually develop cancer. We and others have found that antibodies recognizing cancer-associated autoantigens develop months or years before the clinical diagnosis of cancer [8,12]. As a consequence, these tumor-relevant autoantigens will be removed and will not be available for specific interactions with cancer sera. It is clear that the larger the pool of “normal” sera used as a control, the more likely will be the removal of tumor-associated clones. We have compared the results of biopanning with and without this step and confirmed that prescreening with non-cancer control sera eliminates some of the autoantigens that react with multiple cancer patient sera.

We also found that the careful selection of the cloning sera is important. Our studies suggest that not every cancer serum is suitable for screening cDNA expression libraries [5–10]. Limitations to detecting tumor-associated autoantibodies can in part be avoided by selecting cancer sera based on reactivity to immunoblots of whole protein extracts from the cell type of the patient’s tumor, and by using a heterologous cDNA library of cancer autoantigens for biopanning. Although this method may miss rare patient-specific autoantibodies, only detectable using autologous tumor libraries, our approach identifies antigens recognized by multiple cancer patient sera. This favors identification of useful cancer biomarkers without resorting to constructing autologous cDNA libraries from each patient’s tumor. Recently, a variation of SEREX involving screening of sera from cancer patients on allogeneic testicular cDNA libraries has validated this approach, and led to the recognition of several novel cancer-testis antigens [3]. Characterization of cancer sera by immunoblots prior to cloning may allow selection of those sera with the highest potential in identifying antigens relevant to the oncogenic process [8,9]. Sera containing high titer antibodies, or those recognizing antigens of identical molecular mass on immunoblots, could be of value in cloning antigens responsible for dominant reactivities. Tan has also suggested that high titer antibodies increase the likelihood of cloning cancer-associated molecules [13]. In our study on breast cancer biomarkers, we selected the cloning sera from women with high titer IgG autoantibodies who had established diagnoses of invasive ductal carcinoma (IDC) of the breast and a known outcome. We assumed that antibodies present at this advanced stage of breast cancer are representative of the autoimmune response mounted in breast cancer, possibly including earlier clinical stages. This assumption was based on our previous finding that autoantibodies with potential predictive value for lung cancer are predominantly of the IgG isotype, indicative of an established immune response at the time of diagnosis [6,7]. We probed the immunoblots of breast cancer cell proteins with multiple sera from women with newly diagnosed breast cancer collected prospectively and followed during a 10-year period

[8,9]. These experiments identified numerous sera showing high titer IgG signals (>1:500 serum dilution) of the same apparent kDa value. While similar bands on immunoblots may correspond to different proteins, pre-selecting sera that recognize proteins of identical molecular mass biases towards identifying antigens that might be commonly recognized, and thus are more likely cancer-associated. Selected sera were used to immunoscreen a T7 phage library of heterologous cDNA [14]. In order to increase the probability of cloning antigens relevant to cancer, we immunoscreened plaques obtained from sequential rounds of immunoprecipitation, and selected a subset of strongly recognized antigens to assemble a limited library [9]. We reasoned that selecting clones with the guidance of antibody recognition might allow us to identify clones corresponding to dominant reactivities. Our work suggested that adoption of these principles in the selection of the cloning sera and in the biopanning of cDNA expression libraries could lead to the discovery of antigens related to the oncogenic process that may have diagnostic and/or prognostic value [6–9].

After the initial selection of antibody-reactive clones, the first step in SEREX analysis is usually to sequence the clone to determine its relationship to known genes and to search for structural or functional modifications in the gene, followed by determination of the expression pattern at the mRNA and protein levels [2,3]. SEREX-defined antigens are then tested against a small panel of sera from normal subjects, and those showing to be cancer-restricted can be tested in a larger-scale serological survey using recombinant protein as a substrate in ELISAs [3]. This approach is very labor intensive and at this time not all SEREX-defined antigens have been through this battery of steps [3]. Our approach further differs from SEREX in that after we clone positive phage, we resort to the use of high-throughput methodology to validate the immunoscreening approach. Our approach seeks to establish the tumor-relatedness of the antigen–antibody systems identified and to differentiate tumorassociated antibodies from irrelevant antigen–antibody systems or from non-specific antibodies found in normal individuals that may be related to systemic autoimmune diseases and/or to the aging process [4]. We further characterize antigens only if our high-throughput screening suggests they may have clinical value. The specifics of our approach for the identification of autoantigens with potential value as biomarkers of cancer diagnosis are summarized in Table 1.

Our approach has the potential to allow the cloning of gene products which may be activated in the oncogenic pathways operating in cancer patients as suggested by the cloning of partial sequences of RPA32 [8] and those of annexin XI-A [15] (CB331917), ribosomal protein S6 [16] (CB331938) (CK235954), ribosomal protein S12 [17] (CF931532), nucleolar protein interacting with the forkhead-associated domain of pK-67 [18] (CB331935), KIAA1671 protein [19] (CK235955) (CB331932), elongation factor-2 [20] (CB331921), peripheral benzodiazepine receptor-associated protein 1 [21] (CB331937), cyclin K [22] (CB331940, CK235956, CF751988), Ku-p80 subunit [23] (CF751978, CB331943), Ca/calm-dp kinase II [20] (CB334782), clone IMAGE:4127835 [20] (CK235958), Grb2 associated protein 2 [24] (CB331927), nucleolar phosphoprotein p130 [20] (CF931533), and 28 unknown ESTs which were recognized by multiple breast cancer sera [9].

3. Discussion

The success of SEREX in identifying candidate autoantigens is likely attributable to enriching for cDNA expressed by the autologous tumor which facilitates the identification of antigens reacting with autoantibodies present in the autologous serum. SEREX usually identifies autoantigens that are overexpressed but otherwise normal antigens rather than mutated in tumor cells [2,3], and although SEREX analysis has identified a series of provocative cancer antigens that probably have relevance to the etiology, diagnosis, and therapy of cancer [2,3], it does not preferentially select for antigens that are oncogenic. Although in aggregate these studies suggest that hundreds of autoantibodies cloned with cancer sera using SEREX [3] have potential as biomarkers of cancer, thus far, they have not resulted in serologic markers with definitive predicting ability in the clinical arena [3,25], and none have proved to be of value for the early diagnosis of cancer. A number of modifications of the original SEREX design have been introduced or are being considered [3]. Here we report on a new approach that facilitates the identification of autoantigens commonly recognized by antibodies present in the sera of multiple cancer patients and minimizes confounding effects of irrelevant antibodies. Using this approach we have identified a collection of breast cancer autoantigens from a T7 phage breast cancer cDNA library. These proteins are recognized by sera from multiple patients with ductal carcinoma in situ (DCIS) and IDC of the breast, but not by non-cancer control sera. Moreover, some of these antigens can distinguish DCIS from IDC of the breast, suggesting that this approach has the potential to recognize different subsets of certain malignancies. The findings of a large number of breast autoantigens exhibiting the ability to distinguish breast cancer sera from normal sera, and others that react preferentially with DCIS of the breast, the earliest clinically recognizable form of breast cancer, suggest that our strategy allows the identification of autoantigens relevant to breast cancer [9]. We showed that the recognition of RPA32 and annexin XI-A by multiple breast cancer sera corresponds with overexpression of these proteins in tumor tissue [8,9].

Our previous work [6–10] and the work of Tan [13] suggested that screening autoantigen expression libraries with cancer sera containing high titer autoantibodies has the potential of revealing a number of proteins that may be involved in cellular functions related to tumorigenesis. Our identification of RPA32, and annexin XI-A, cyclin K, ribosomal protein S6, Grb2-associated protein-2, and elongation factor-2 as autoantigens recognized by breast cancer sera could be the expression of molecular alterations in the signal transduction mechanism in breast cancer [8,9]. The finding that several autoantigens cloned with the same cancer patient serum are also recognized by multiple cancer patient sera suggests that the molecular changes responsible for the autoimmune reaction in cancer sera may be related to the oncogenic process. We suggest that the use of this approach in large cohorts of patients with cancer may allow the molecular characterization of cancer phenotypes [9].

Acknowledgments

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Take-home messages

- Method avoids excluding potentially important tumor-associated antigens by pre-screening manipulations.
- Protocol minimizes confounding effects of unrelated antibodies.
- Stresses the importance of characterization of cloning sera.
- Proposes strategy to demonstrate tumor relevance early in the screening process.
- Approach can identify proteins involved in cellular functions related to tumorigenesis.

Table 1

Characterization of cloning sera

- **a.** Potential cloning sera are selected from patients with tumors with known pathologic diagnosis and outcome.
- **b.** Immunoblots of whole cell extracts of tumor proteins are probed with a large number of potential cloning sera, representative of the cancer being investigated.
- **c.** Sera with high titer (<1:500 dilution) IgG antibodies on immunoblots are pre-selected.
- **d.** Sera exhibiting recurrent IgG bands of identical molecular mass are selected for immunoscreening.

Immunoscreening approach

- **a.** Absorption steps with non-transfected host cells or with vector-related antigens are omitted.
- **b.** Biopanning with pools of “non-cancer” sera to remove irrelevant clones is avoided.
- **c.** Immunoreactivity rather than random selection is used in biopanning as the criterion to select positive clones.
- **d.** The size and the number of the clones in each informative phage are determined by RT-PCR.
- **e.** The nucleotide sequence of each clone shown to be informative by microarray analysis is determined.
- **f.** Homologies of the cloned partial sequences are searched using the GenBank and other databases.

Validation of the immunoscreening method

- **a.** An autoantigen microarray is constructed with the phage antigens identified by immunoscreening.
- **b.** The microarray is probed with a training set of sera from patients with established diagnosis of:
  1. early and advanced stages of cancer,
  2. non-cancer control sera and sera from patients with autoimmune diseases.
- **c.** The microarray is also probed with an independent set of sera from patients with early and advanced forms of cancer and with different sets of subjects without cancer.
- **d.** The probing sera to test the microarray for reactivity are selected randomly from a collection of patients with established pathologic diagnosis of cancer and outcome measures collected during complete follow up.
- **e.** The serum reactivity of positive phages on the microarray is correlated with diagnoses and outcome measures.