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The sentinel within: exploiting the immune system for cancer biomarkers

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Abstract

The release of proteins from tumors triggers an immune response in cancer patients. These tumor antigens arise from several mechanisms including tumor-specific alterations in protein expression, mutation, folding, degradation, or intracellular localization. Responses to most tumor antigens are rarely observed in healthy individuals, making the response itself a biomarker that betrays the presence of underlying cancer. Antibody immune responses show promise as clinical biomarkers because antibodies have long half lives in serum, are easy to measure, and are stable in blood samples. However, our understanding of the specificity and the impact of the immune response in early stages of cancer is limited. The immune response to cancer, whether endogenous or driven by vaccines, involves highly specific T lymphocytes (which target tumor-derived peptides bound to self-MHC proteins) and B lymphocytes (which generate antibodies to tumor-derived proteins). T cell target antigens have been identified either by expression cloning from tumor cDNA libraries, or by prediction based on patterns of antigen expression (“reverse immunology”). B cell targets have been similarly identified using the antibodies in patient sera to screen cDNA libraries derived from tumor cell lines. This review focuses on the application of recent advances in proteomics for the identification of tumor antigens. These advances are opening the door for targeted vaccine development, and for using immune response signatures as biomarkers for cancer diagnosis and monitoring.

Keywords

Tumor antigen; antibody; protein array; proteomics; tumor immunology; biomarkers

Introduction

The challenge faced by our immune system resembles that of an intelligent security system, which must continually monitor for the presence of foreign invaders, while recognizing and disregarding normal self. Like a vigilant sentry, immunologic memory persists long after exposure to the threat has abated. Recognizing the value of this persistent response, clinicians have exploited it for years to test individuals for current or past exposure to a wide variety of infections. Compared to other serum-derived proteins, antibodies are stable, highly specific, and readily detected with well-validated secondary reagents, making them ideal for such tests. Indeed, the traditional “blood test” required of couples before obtaining a marriage license is nothing more than a test for antibodies to the spirochete *T. pallidum* that causes syphilis. Thus, assessing immune responses is one of the oldest and most successful forms of biomarkers in medicine.

The immune system employs complex mechanisms to distinguish between self and non-self. It deletes or renders tolerant any cells which react to the constant stream of benign

macromolecules in routine circulation. The system is not foolproof, however, and in certain diseases, the immune system responds to self-derived antigens, perhaps because their location, abundance, modified form or other features appear unfamiliar. Cancer patients often produce responses to self-proteins that are expressed by their tumors, called tumor antigens, most of which are altered in some form that renders them immunogenic. These proteins may be unique to cancer and germ cells (the “cancer-testis” antigens), found only in specific tumors (prostate-specific antigen)¹ or in most tumors (telomerase)².

They may be mutated (p53)³, misfolded⁴, overexpressed (NY-ESO-1)⁵, aberrantly degraded⁶ or aberrantly glycosylated (MUC-1)⁷. The magnitude of the immune response to cancer, in general, is lower than the immune response to infectious agents and the potential number of tumor antigens encompasses the entire tumor proteome in all its variations. At present, we have a limited understanding of the breadth, extent, impact, and dynamic variation of the immune response to cancer (the “cancer immunome”). Identifying the specific targets of B- and T- lymphocyte immunity to cancer may 1) identify potential biomarkers for cancer diagnosis, classification, and monitoring of response, 2) determine the impact of immune regulation on cancer progression, and 3) identify potential antigens and mechanisms for immunotherapy development.

The natural immune response is achieved through a tightly regulated, yet flexible network including antibodies, antigen presenting cells, T lymphocytes, cytokines, chemokines, regulatory systems, as well as microenvironmental signals (Figure 1). Of these responses, the targeted responses to protein (and carbohydrate) antigens relies on the development of antibodies and/or T lymphocytes to target epitopes. T lymphocytes can respond to antigens derived from within cells and without. They primarily recognize short peptides (8-22mer) derived from intracellular proteins (i.e., viral antigens) bound to self-MHC molecules for presentation to CD8⁺ T lymphocytes. Exogenous antigens are endocytosed, degraded, and presented to CD4⁺ lymphocytes (Figure 2). Antibody responses increase antigen presentation by enhancing uptake through the Fcγ receptors on antigen presenting cells. As a result, antibody targets may contain epitopes that are also recognized by T lymphocytes. This has formed the basis for using antibody responses to identify T cell antigens for immunotherapy.

Lessons from Autoimmune Diseases

Spontaneous autoantibodies were first identified in a series of clinical disorders in which the patients' immune systems mount a vigorous response to self antigens, in some cases leading to debilitating symptoms. Systemic lupus, myasthenia gravis, rheumatoid arthritis and others all involve this process, called autoimmunity. In these illnesses, the titers of autoantibodies often track with the severity of the illness and thus have long been used as serum biomarkers (reviewed in^{8, 9}).

Although post hoc arguments can usually be constructed explaining why proven autoantigens may have appeared unfamiliar to the immune system; in general, we do not understand enough about the characteristics that determine whether a protein will act as an autoantigen to predict them *a priori*. To date, only one or two percent of proteins have been identified as autoantibody targets¹⁰. In autoimmune diseases, the autoantigens identified, such as Ro/SSA, La/SSB, Sm, RNP, Scl-70 and Jo-1 are primarily intracellular antigens that function in large complexes with nucleic acids involved in protein synthesis⁸, and are thought to arise as a result of overexpression, apoptosis, reduced degradation, or similarity to cross-reacting foreign antigens (mimicry). It is often not clear whether these autoantibodies have any direct role in disease pathophysiology. In contrast, antibodies directed at membrane proteins, such as the target antigens of hemolytic anemia or the acetylcholine receptor in myasthenia gravis may contribute to the disease process through complement activation, antibody-dependent cytotoxicity, or interference with receptor/ligand interactions. Certain structural motifs enhance antigenicity,

such as carbohydrates, multivalency, epitope repetition, and coiled-coils¹¹. Some of these antigens are linked to degradation during apoptosis¹², or are targets of granzyme B cleavage during T lymphocyte-induced cytotoxicity^{13, 14}. Further elucidation of the mechanisms underlying autoantibody generation will assist in predicting and identifying target antigens.

p53 as a Model Tumor Autoantigen

Perhaps the most well-studied autoantigen in cancer is the p53 protein (reviewed in³). In 1979, DeLeo et al¹⁵ demonstrated that autoantibodies to some tumor cells in mice were directed against the p53 protein. Subsequent studies have demonstrated that the half-life of mutated p53 (several hours) was markedly increased compared to wild-type (several minutes) resulting in accumulation in the cell nucleus. p53 autoantibodies are dependent on the type of p53 mutation¹⁶⁻¹⁸. Notably, the immunogenic epitopes have been mapped primarily to both the N- and C-terminal portions of the molecule, which are heavily glycosylated, but not to the central portion of the molecule, which harbors the mutations, suggesting that the accumulation of protein, rather than the mutations per se, results in autoantibody generation. Multiple assays using recombinant antigens and p53-derived peptides have shown that some antigenic determinants are dependent on full-length antigen, conformation, or phosphorylation^{3, 19}.

The detection of p53 autoantibodies in serum correlates strongly with cancer ($p < 10^{-4}$). p53 autoantibodies have been studied in over 9489 patients with a wide variety of tumors³. Despite the strong specificity of the response, only 20-40% of patients with cancers harboring p53 missense mutations will have p53 antibodies in their sera³. Therefore, there are additional unknown factors beyond antigen accumulation that impact the development of autoantibodies. This illustrates one of the major challenges facing the use of single autoantibody tests for cancer detection. Although the appearance of such antibodies is highly suggestive of cancer, many patients will be missed because they do not respond to p53. p53 autoantibodies have been detected in body fluids, such as ascites, pleural effusions, and saliva, and these correlate with serum autoantibodies¹⁹⁻²¹. Interestingly, gliomas, which are associated with p53 mutations, are not associated with the development of p53 antibodies^{22, 23}, suggesting either that development of tumors in the CNS leads to immunologic privilege, or the use of steroids in this patient population could dampen the serologic immune response.

Anti-tumor Effects of Autoantibodies in Cancer

It is not known if autoantibodies reflect underlying immunosurveillance of cancer or have an impact on the clinical outcome of the disease. For example, multiple studies have attempted to correlate p53 autoantibodies with prognosis showing mixed results³. As the development of B cell immunity often depends on concordant T cell immunity, autoantibody identification can lead to the identification of relevant T cell antigens²⁴⁻²⁶. In patients who have undergone autologous tumor vaccination, certain autoantibodies correlate with tumor response to treatment^{25, 27}. In bone marrow transplantation, the development of autoantibodies may identify minor histocompatibility antigens associated with graft versus host disease²⁸ or tumor antigens associated with the graft-vs-leukemia (GVL) effects of donor lymphocyte infusion^{29, 30}. In murine tumor models, it is the coordinated activities of CD4⁺ and CD8⁺ T cells, CD1d-restricted invariant NKT cells, and antibodies that accomplish protective immunity^{31, 32, 33, 34}.

The serologic identification of tumor antigens has directly led to the development of a number of cancer vaccines currently in clinical trials. The proteinase-3 antigen, first identified as the target antigen of the ANCA antibody assay for Wegener's granulomatosis, is being tested as a target for vaccination in CML³⁵. NY-ESO-1 has been used as an immunogen in multiple clinical trials³⁶. Antibody response to tumor vaccines have been shown to correlate with improved disease-free and overall survival in stage II melanoma patients vaccinated with a

polyvalent vaccine³⁷. Melanoma patients vaccinated with autologous tumor cells may develop highly-individualized antibody responses³⁸. The identification of these autoantibodies has directly led to mapping of CD4⁺ and CD8⁺ T lymphocyte epitopes. The clinical benefit of antigen-specific tumor vaccination strategies is just beginning to emerge, with the recent demonstration of a survival benefit of a dendritic cell-based vaccine in hormone-refractory prostate cancer³⁹.

Autoantibodies as Biomarkers of Cancer

In 2003, it is estimated that 1,334,100 people in the US were newly diagnosed with cancer, with an estimated 556,500 patients dying of the disease⁴⁰. In this setting, there is intense effort in the search for biomarkers that can predict disease, identify biologic subtypes of disease, track response to therapeutic interventions, predict side effect profiles, and monitor for disease progression and recurrence⁴¹.

There are a limited number of serum protein biomarkers that are widely used in clinical oncology for prognosis and treatment monitoring, and their use in early cancer diagnosis has been hampered by false-positive rates in the normal population. The identification and development of these biomarkers took decades of research and required large prospective trials. Carcinoembryonic antigen (CEA), widely used for the monitoring of adenocarcinomas, was originally identified from tumor lysates because of its immunogenicity in rabbits⁴². The subsequent identification of the prostate specific antigen (PSA)⁴³ and the ovarian cancer biomarker CA-125⁴⁴, among others, have demonstrated both the validity and limitations of serum biomarkers in the diagnosis of cancer. Although PSA is now routinely used for screening healthy populations for prostate cancer diagnosis, most serum biomarkers are used for monitoring treatment or for screening highly selected high-risk populations. More recent developments in serum proteomics have been reviewed in this issue and elsewhere⁴⁵.

Compared with other polypeptides, autoantibodies have many appealing features as biomarkers. First, although tumor antigens may circulate only briefly or in low concentration, perhaps due to transient shedding by tumors, rapid degradation in the serum, or rapid clearance, the corresponding antibody response is likely to be persistent. Second, antibodies are highly stable in serum samples and are not subject to the types of proteolysis that are commonly observed for other polypeptides (discussed elsewhere in this issue), making sample handling much easier. Third, the $t_{1/2}$ of antibodies in circulation is >7 days, so hourly or daily fluctuations are expected to be minimal, simplifying sample collection. Finally, the biochemical properties of antibodies are well understood and there are many available reagents for their detection, simplifying assay development. Given these advantages, the challenge that remains is demonstrating that antibodies can be sufficiently informative to reliably detect cancer.

Early Disease Diagnosis—Very limited data are available on the use of autoantibodies to reveal early disease. Antibodies have been detected as early as several years before the clinical appearance of cancer^{46, 47} and in patients with preneoplastic disease⁴⁸. Anecdotal studies have detected p53 autoantibodies in heavy smokers prior to the diagnosis of lung cancer⁴⁷. Another study showed p53 autoantibodies in individuals exposed to vinyl chloride, a risk factor for the development of angiosarcoma of the liver⁴⁶. The p53 autoantibodies predated the diagnosis of angiosarcoma by several years. Although encouraging, these few studies are not yet sufficient to endorse the value of antibodies in the early detection setting.

Monitoring treatment response and predicting recurrence—Antibodies to tumor antigens have been detected in early stages of disease and fluctuate with tumor response. In patients with both stage III and stage IV neuroblastoma, 10% have evidence of antibodies to the NY-ESO-1 antigen, but the antibody is not seen in the sera of patients in clinical remission

or in earlier stages of disease⁴⁹. In melanoma, antibodies to TA90 antigen have been detected in only 12% of patients with 1-2mm primary, node negative melanoma who subsequently relapsed, but 62% of case-controlled patients who did not⁵⁰.

Antibodies to the her2/neu tumor antigen have been detected in the sera of 20% of patients with her2+ early-stage breast cancer⁵¹ but only 7% of late-stage breast and ovarian cancer patients⁵². Some of the early-stage patients had titers >1:5000, suggesting a strong, possibly protective immune response. In patients with both stage III and stage IV neuroblastoma, 10% have evidence of antibodies to the NY-ESO-1 antigen, but the antibody is not seen in the sera of patients in clinical remission or in earlier stages of disease⁴⁹.

The titer of antibodies to NY-ESO-1 have been shown to correlate with disease progression, as well as with disease response/resection⁵³. The titer of anti-p53 antibodies have been shown to increase in a subset of patients with early-stage breast cancer prior to disease relapse⁵⁴, and disappearance of anti-p53 antibodies has been observed in 27 patients after resection of colorectal cancer⁵⁵. Tumor antigen/antibody immune complexes have been detected up to 19 months prior to the development of clinical relapse and correlate with survival in patients with early-stage melanoma⁵⁶.

Methods for assessing autoantibodies

The development process for autoantibodies as biomarkers is outlined in Figure 3. Historically, this cycle begins with identifying antigens that are detected by the sera of cancer patients. Antigens must then be selected and tested by comparing sera from both patients and healthy donors to determine if the antigens are “informative”, i.e., responses are limited to patients. As part of this process, the sensitivity and specificity of the antigens should be determined. Promising antigens are then tested on a set of samples separate from the training set to validate their usefulness. Ultimately prospective trials will establish their value as biomarkers. The cycle is completed by using the sera from patients not detected by the test to screen for new antigens. In some of the newer proteomic approaches, two of these steps may combine into a single step.

Identification of novel tumor antigens

SEREX: A variety of techniques have been developed that use patient sera as probes against candidate antigens derived from tumor cells and tumor cell lysates to find novel autoantigens. Prominent among these, serologic expression cloning (SEREX) was developed ten years ago (Figure 4). This method uses patient sera to probe blotted phage expression libraries derived from tumor cells and has resulted in the identification of over 2000 autoantigens recognized by patient sera^{5, 57-79}. Through a database established by the SEREX collaborative group, sequences from over 1390 genes have been deposited⁸⁰ and <http://www2.licr.org/CancerImmunomeDB/>. As SEREX relies on immunoblotting, these antigens are limited to linear epitopes and those gene products that can be expressed in bacteria.

As with the autoimmune targets, most of the antigens identified with SEREX are nuclear or intracellular antigens, many of which are upregulated, mutated, or specifically expressed in tumors. However, altered degradative pathways, such as sensitivity to granzyme B cleavage, has been shown to have a marked impact on the immunogenicity of a hepatocellular tumor antigen⁶. In addition, frameshifts and alternative reading frames, can result in autoantibodies. Using colon cancer sera, the antigen ADO34 was identified with a frameshift insertion⁸¹, and CDX2 had a frameshift mutation⁸². Antibodies to the OGF_r protein can recognize an alternative-reading frame of the molecule⁷⁹.

The SEREX method identifies autoantigens whether or not they are informative. At present only a handful of antigens have undergone comparison testing, in part because of the technical challenges associated with historical assays for validating autoantigens.

Phage display: Combinatorial phage display is a new and attractive approach for the identification of tumor antigens. By expressing antigens as fusions with phage proteins, antigens can be detected more rapidly and with less serum. Rather than immunoblotting as with SEREX, phage display relies on successive rounds of immunoprecipitation of phage libraries using patient serum. This powerful approach has been used in the field of rheumatology to identify autoantigens in multiple sclerosis, lupus, rheumatoid arthritis and others⁸³⁻⁸⁵. For tumor antigens, one study showed that only 4 of 13 antigens identified with prostate cancer sera had already been identified by SEREX technologies, demonstrating that novel tumor antigens may be identified with different screening approaches⁸⁶. Phage display has been successfully used to identify tumor autoantigens in cancer⁸⁶⁻⁸⁹. However, antigens expressed by phage display may not be in native conformation, do not have mammalian post-translational modifications, and each positive phage clone must be individually sequenced. Yeast display systems are also being investigated⁹⁰.

Validating autoantibodies—To be useful as biomarkers, the tumor antigens must distinguish between individuals with and without cancer. Thus once a candidate autoantigen is identified, comparisons must be made regarding the responses of individuals from both groups. The measurement of an autoantibody response is most often accomplished using the enzyme linked immunosorbent assay (ELISA), in which purified protein (the autoantigen) is immobilized in the wells of a microtiter dish and exposed to serum samples (Fig. 4). After adequate washing, any bound antibody is revealed using standard anti-human antibody reagents linked to enzymatic markers. If the response is very strong, it may be useful to perform serial dilutions of the serum to determine the titer of the antibody. By comparing multiple patient and normal donor samples it is possible to determine the frequency of response. For example, antibodies to dsDNA are present in only 40-60% of SLE patients, but antibodies to histones are present in >95% of patients with drug-induced SLE. To compute the sensitivity and specificity of the test, a threshold value must be set, such that the test is considered positive if the antibody response achieves that value or above. Typically, this threshold is either 2 or 3 standard deviations above the average response in the normal population.

The development of clinical ELISAs require the use of recombinant proteins or peptides, which may be complicated by batch-to-batch variations, loss of conformational epitopes or lack of mammalian post-translational processing^{91, 92}. Antigenic epitopes from proteins such as Ro, La, SmB, SmD, Scl-70, can be linear epitopes of 10-22 amino acids conformational, or cryptic. Furthermore, epitopes may be naturally post-translationally modified by multiple means, including phosphorylation, glycosylation, acetylation, and methylation (SmD1, SmD3, fibrillarin, nucleolin)^{93, 94}, all of which must be considered prior to the development of a clinical diagnostic assay. Of the many candidate antigens identified so far, only a handful have gone through the validation process to determine their sensitivity and specificity as cancer predictors, in part because of the challenges associated with setting up ELISAs.

Proteomic Approaches for the Identification of Tumor Antigens

The Power of Multiplexing—With all of their potential advantages, the Achilles heel of autoantibodies as biomarkers is their sensitivity, i.e., the fraction of true positives that have a positive test. In large part, this may reflect the nature of cancer. Unlike infections in which the vast majority of patients respond to the same immunodominant antigens, even cancers of the same type represent a mix of different biological subtypes. Thus, patients are likely to mount immune responses to different tumor antigens, and no single antigen is likely to detect all

cancers. Typically, only 15-20% of patients demonstrate a response to any given antigen. However, proteomics may hold the key to success because it provides the means to multiplex. By linking the responses to several antigens together, the sensitivity and specificity of the test increases considerably, presumably because the chance that a patient will respond to at least one of the antigens is increased^{76, 95-102}. Koziol et al demonstrated that a panel of just seven tumor-associated antigens (myc, cyclin B1, p62, IMP-1, Koc, p53, and survivin) could be used to segregate sera from patients with cancers from healthy donor sera⁹⁵. In that study, sera from breast, colorectal, gastric, hepatocellular, lung, and prostate cancers were distinguished with sensitivities from 0.77-0.92 and specificities from 0.85-0.91 using no more than 3 tumor antigens for any cancer cohort. Scanlan et. al. studied the serologic responses to 13 defined tumor antigens in sera from colon cancer patients. Of these, 46% of patients, but not healthy donors, had antibodies to at least 1 of these antigens¹⁰³.

These results demonstrate the potential power of simultaneously analyzing multiple autoantigens. It provides a greater likelihood of detecting and diagnosing the appropriate cancer presumably because the sensitivity will improve (increased chance that the patient will have a response to at least one of the tested antigens) and the specificity improves (positive responses on multiple antigens increases certainty). By coupling this with the appropriate statistical modeling, it is likely that *patterns* or weighted schemes of antibody responses, rather than individual responses, will have the greatest utility in clinical assays. The early experiments above were performed by using recursive partitioning. Given the technical challenges inherent in that method for large numbers of antigens, newer technologies are needed for high throughput analysis. To this end, recent development of several proteomic technologies have been adapted for tumor antigen identification and biomarker development. These include probing fractionated tumor cell lysates, phage display, and protein microarrays (See Table 1).

Probing fractionated tumor cell lysate blots with serum

In this approach, tumor cell lysates are fractionated to separate the various protein species and blotted onto a membrane or microarray and then probed with patient or control sera. Response patterns are then analyzed to differentiate between the two (Fig. 4). This has been used to demonstrate antibodies in the sera of lung cancer patients^{96, 104}. A similar approach was used to distinguish sera from prostate cancer patients from healthy donor sera with 98% accuracy¹⁰⁵. The development of automated separation systems using liquid-based chromatography with subsequent microarray spotting of lysate fractions enhances the reproducibility and speed of immunogenic fraction identification. Advantages of this approach include the ability to query a large fraction of the tumor cell proteome and the preservation of the post translational modifications of proteins. Moreover, when this is executed using a microarray format, only small amounts of serum are required. However, the reproducibility demanded by a clinical assay requires an identified and validated antigen. Thus, as with other chromatographic separation systems, the identity of the proteins in the fraction must eventually be determined, presumably using sensitive mass spectrometric analysis. As each lysate fraction may contain many proteins at very different concentrations, of which the minor component may be the immunogen, antigen identification can be difficult.

Probing Candidate Antigen Arrays

Known or predicted tumor antigens may be directly spotted on microarrays and probed with human sera, with the advantage of reproducibility and more rapid screening of small amounts of sera (Figure 4). In this case, the identity of a single protein at each feature on the array is known *a priori*. This approach has been successfully used to screen autoimmune patient sera^{100, 101}. A powerful advantage of this approach is that it offers the ability to screen for informative autoantigens by comparing responses of patients to controls. Because each feature of the array represents a single protein, whose identity is known, it is possible to calculate

sensitivity and specificity values for the response to each candidate antigen during the screening phase. This allows the rapid determination of which antigens are informative for cancer detection. Ultimately, focused arrays of tumor antigens previously identified by other means may have the greatest utility for immunodiagnostics.

Their theoretical advantages notwithstanding, protein microarrays have still not found widespread use, in part because producing them is challenging. Historically it has required the high-throughput production and purification of protein, which then must be spotted on the arrays. Once printed, concerns remain about the shelf life of proteins on the arrays. Recently, programmable protein microarrays that consist of anchored cDNA's and *in situ* transcription and translation of tagged proteins have been shown to result in highly reproducible protein arrays without the requirement of heterologous protein expression and purification¹⁰⁶. As the proteins are synthesized at the time of the assay, shelf life is not an issue and the proteins are translated with a mammalian reticulocyte lysate, which reproduces the folding and some of the post-translational processing of antigenic epitopes. Unlike tumor lysate fractionation, antigen identification is automatic, although epitopes that depend on abnormal processing by tumor cells would not be identified, and transmembrane proteins have not yet been tested in this system. Like most antigen presentation methods (SEREX, ELISA, phage display, etc.), these programmable arrays require access to cDNAs to express the antigens. Fortunately, nearly all known tumor antigen genes are available because they were identified with methods that involved cDNA identification. Moreover, libraries of cDNA clones representing most of the human proteome are increasingly available.

Proteomics and the Identification of T cell Antigens

In contrast to the rapid identification of B cell antigens, the identification of T cell antigens remains much more difficult. Since T lymphocytes specifically recognize peptides derived from protein antigens, the isolation and confirmation of these antigens have traditionally relied on laborious T cell isolation and cloning. Alternatively, direct isolation and sequencing of MHC-associated peptides from cells has been limited by the overall low concentration of specific peptide bound to MHC molecules, and the highly polymorphic nature of the MHC molecules themselves. These challenges are being overcome by advances in high-throughput MHC-peptide binding studies, the bioinformatics of epitope prediction, and in particular, mass spectrometry.

With high-throughput peptide synthesis, direct MHC-peptide binding assays may now be performed on peptides that span target antigens, or using combinatorial peptide libraries¹⁰⁷. Detailed analysis of peptide binding to common HLA alleles has resulted in the development of bioinformatics tools for epitope prediction^{108, 109}. Further limitations imposed by proteasomal cleavage patterns can enhance prediction of peptide epitopes. Multiple prediction models are available, primarily for MHC Class I-binding peptides, due to the more restrictive nature of the peptide binding groove. These systems include BIMAS¹¹⁰, SYFPEITHI¹¹¹, MHCPEP¹¹², and RANKPEP¹¹³, among others.

Advances in mass spectrometry have also greatly aided antigen identification. Original studies of direct peptide sequencing from purified MHC molecules from cell lines¹¹⁴⁻¹¹⁶ demonstrated that the majority of peptides within the MHC grooves are derived from self-proteins, but required billions of cells for the source of antigen. More recent approaches to identify tumor-specific T cell epitopes have used capillary-scale chromatography and tandem mass spectrometry (Figure 5) (nanoLC-MS/MS)¹¹⁷⁻¹²⁰. To enhance sensitivity, nanospray quadrupole-TOF combined with Poisson algorithms¹²¹ to specifically evaluate the likelihood of predicted peptides in unfractionated mixtures has markedly increased the sensitivity of the assay to the range of 1 copy number of peptide/cell in a murine influenza viral model, but is limited to predicted MHC-binding peptide epitopes.

Summary

The development of proteomic-based methods of cell lysate fractionation, phage display, protein microarrays, bioinformatics, and mass spectrometry is resulting in the rapid identification of both B and T cell tumor antigens. These have the potential for clinical diagnosis, identifying targets for immunotherapies, monitoring disease response, and to understand the breadth, scope, and impact of the immune response to cancer. As each new technology leads to the discovery of novel antigenic targets, systematic approaches of target validation and assessment of clinical applications, especially in the area of biomarkers and diagnostic testing, will need to be developed.

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Figure 1. The application of cancer proteomics to tumor immunology

The immune response to cancer, whether endogenous or driven by immunotherapy, involves a complex array of interactions between the tumor, host microenvironment, lymphocytes, antigen presenting cells, antibodies, cytokines and chemokines. Major arms of the immune system are shown in blue, and selected techniques for evaluation are shown in green. The identification of target antigens of B- and T-lymphocyte recognition may be identified on a proteome-wide basis, through cell fractionation, protein microarrays, nanospray mass spectrometry, and protein expression profiling of tumors *in vivo*. Cellular assays may use protein microarrays, microfluidics, and nanotechnology to identify target antigens as well as host responses. Microenvironmental interactions can be analyzed, as well as signal transduction pathways and costimulatory/regulatory molecules. This review will focus on B-cell and T-cell target antigen identification.

Figure 2. Classical model of antigen presentation

Intracellular antigens (i.e., viral) are cleaved into peptides by the proteasome and bound to MHC Class I molecules at the surface of cells for presentation to CD8⁺ T lymphocytes. Exogenous antigens are endocytosed by antigen presenting cells (APC), which is markedly enhanced by binding of serum antibodies both to antigen and to the Fc γ receptor on the APC, with subsequent degradation and binding of peptides to MHC Class II molecules for stimulation of CD4⁺ T lymphocytes. Because of this coordinated immune response, tumor antigens that cause antibody responses in patient sera may contain immunogenic T cell peptide epitopes as well (modified from *D. Miklos*).

Figure 3. The development of autoantibody biomarkers in cancer

The identification of target antigens using antibodies derived from patient sera is the first step in identification of potential biomarkers for cancer diagnosis or immune monitoring. In the field of autoimmunity, detailed evaluation of the autoantibody quantity (titration) and specificity has required development of a confirmatory ELISA using heterologous recombinant protein. Antigens that segregate patient sera from control sera ("informative antigens") would be tested further. Analogous to DNA microarrays, well-annotated and blinded test sets and validation sets of sera (such as sets of sera from different stages or types of cancers) are required to identify areas of potential clinical applications. Finally, prospective clinical trial analysis is required to further validate the sensitivity, specificity, and predictive value of the assay. For tumor antigens, gap analysis that identifies false-negative sera ("missed cancers") would then be further screened for additional autoantibody biomarkers. The potential clinical applications of these biomarkers include diagnosis, immune monitoring, vaccine development, and endpoint analysis of cancer therapeutics.

Figure 4. Methods of antigen identification

In traditional library screening (top row), cDNA libraries from tumor cells are expressed by phage, blotted on a membrane, and probed with patient sera. Confirmation of sensitivity and specificity requires recombinant expression of protein for ELISA analysis. Similar approaches of library screening may use phage display, yeast display or peptide libraries. Cell fractionation approaches (middle row) are a modification of the traditional western blot. Tumor cell lysates may be fractionated using microfluidic 2-dimensional or 3-dimensional separations, followed by printing of fractions on microarrays for probing with patient sera. Fractions identified in patient sera but not normal sera (“informative fractions”) are then further evaluated by mass spectrometry to identify target antigens, which then must be confirmed for sensitivity and specificity using ELISA or similar methods. In the bottom row, protein microarrays can be printed using recombinant proteins or proteins translated in situ, then probed with patient sera. Because the protein microarrays are addressable, no further antigen identification is required, and the sensitivity and specificity of multiple antigens may be evaluated in parallel with screening.

Figure 5. Identification of HLA-binding peptides by mass spectrometry

Peptide-MHC complexes are immunopurified from tumor cell (or antigen presenting cell, APC) lysates using antibodies directed against HLA molecules. Bound peptide mixtures are eluted in acid, and separated by nano-LC followed by MS/MS identification (top arrow). Alternatively, peptides may be directed applied to nanospray quadrupole TOF (bottom arrow), and predicted epitopes identified by fragmentation patterns.

Table 1
Current Methodologies for the Detection of Autoantigens

Method	Advantages	Disadvantages	Application
SEREX	Many clones, clone for gene automatically available	Denatured epitopes, laborious, large amounts of serum, requires separate validation step	Antigen discovery
Western Blotting	Reproducible	Large amounts of serum, requires separate steps to identify and validate antigens	Antigen Discovery
Phage display	Large number of clones, little serum required, ability to refine selection with multiple rounds, clone for gene automatically available	Partial gene products, gene overrepresentation, requires separate validation step	Antigen discovery
Cell Fractionation	Retains post translational modification, multiple arrays allow for screening for fractions that are informative for cancer, do not need to have cloned gene or library for screening	Tedious, must identify the relevant antigen in potentially complex fraction, may need to clone gene for identified protein, requires separate validation step, post translational modification may not be preserved in the validation step	Antigen discovery
ELISA	Reproducible, useful for comparing many serum samples	Tedious to produce protein for the assay.	Validation
Candidate antigen protein microarrays	Screen many candidate antigens, validation and antigen discovery can be done simultaneously, very little serum required, clone for gene automatically available	Arrays can be challenging to produce, novel technology	Antigen discovery and validation