Autoantibodies as Biomarkers in Cancer

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Abstract
Circulating autoantibodies produced by the patient’s own immune system after exposure to cancer proteins are emerging as promising biomarkers for the early detection of cancer. An advantage of autoantibodies in cancer detection is their production in large quantities, despite the presence of a relatively small amount of the corresponding antigen. Autoantibodies are also expected to have persistent concentrations and long half-lives due to limited proteolysis and clearance from the circulation. Here, we review current methods for the broad screening of cancer-specific autoantibody targets and the use of such targets to develop clinically relevant assays for the detection of cancer.

Keywords: cancer, glycomics, glycans, glycobiology, tumor antigens, autoantibodies, biomarkers, cancer, glycans, glycoproteins, immunoassays, immunodiagnosis

Glossary
Autoantibody: An antibody formed against one’s own cells or tissues.
Biomarker: Characteristic that can be measured as an indicator of the biological state.
Epitope: The portion of an antigen eliciting an immune response.
Mimotope: Sequences of amino acids (or other chemical entities) that specifically bind to the antigen-binding region of an antibody.
Post-translational modification: Structural or sequence modification of a protein after translation.
Self-tolerance: Tolerance to one’s own antigens.

After reading this article, readers should have a basic knowledge of different methods used for discovery of autoantigens, be able to understand how autoantibodies develop in cancer patients, and recognize the potential of autoantibodies detection in cancer diagnostics.

Immunology exam 41103 questions and corresponding answer form are located after this CE Update on page 629.

More than 1.5 million men and women in the United States were diagnosed with cancer in 2010, causing up to 570,000 cancer deaths.1 Advances in cancer treatment have increased survival for some cancers, but the most important factor for prognosis is early detection reflected in a 30% reduction in colorectal cancer mortality in individuals enrolled in regular screening programs.2-4 The importance of detecting cancer at an early stage is also appreciated by the fact that 90% of patients diagnosed with stage I colorectal cancer survive 5 years, whereas 60%-70% of patients with stage II or III cancer and only 10% with stage IV disease survive 5 years. Similar findings have been reported for other cancers, though the exact benefits of screening are debated. Current cancer screening methods include mammography for breast cancer, fecal occult blood test followed by imaging procedures such as sigmoidoscopy, colonoscopy, or potentially computed tomography (CT) for colon cancer, prostate-specific antigen (PSA) for prostate cancer, and Papanicolaou (Pap) stains for cervical cancer.5 The screening strategies depend on the prevalence of disease in the screened population and, therefore, should accommodate factors such as gender, age, disease-associated behavior, and genetic predispositions. Although current screening methods are helpful, they are not optimal and present major concerns regarding sensitivity, specificity, complexity, cost, and compliance. To address some of these concerns, simple and informative tests must be developed to detect cancer early in as many patients as possible.6

Current Cancer Biomarker Strategy

Only a few FDA-approved serum cancer biomarkers are currently available, including HER2/neu, CA27-29, and CA15-3 (Mucin-1 [MUC1]) for breast cancer; CA125
(MUC16) for ovarian cancer; carcinoembryonic antigen (CEA), which is primarily for colon cancer; CA19-9 for pancreatic cancer; and PSA for prostate cancer. Of these markers, PSA is the only serological biomarker used to screen asymptomatic patients, which is still controversial due to inadequate sensitivity and low specificity (90% and 21%, respectively).7 CA15.3, CEA, and CA125 are not used in the general population but are limited to monitoring disease progression in breast, colorectal, and ovarian cancer patients, respectively. The potential use of CA125 as a primary screening test for ovarian cancer is being investigated and has shown promising results in combination with selective follow-up, though the problems with false-positives have not been solved.8

Most efforts to identify new serum biomarkers are focused on the identification of tumor-specific constituents released into the circulation or body fluids. Unfortunately, current genomic and proteomic approaches have generally failed to develop simple and non-invasive screening tests for the early detection of cancer.9 One of the main problems of the search for cancer-specific proteins in the blood is the vanishing small amount of protein released from early stage tumors. In addition, many of the cancer-associated proteins are shed intermittently and only circulate briefly at low concentrations due to rapid degradation or clearance.10,11

Autoantibodies

Circulating antibodies elicited by the patient’s own immune system after exposure to cancer proteins are emerging as promising biomarkers for the early detection of cancer. An advantage of autoantibodies as biomarkers is their production in large quantities despite the presence of a relatively small amount of corresponding antigen. Autoantibodies are also expected to have persistent concentrations and long half-lives due to limited proteolysis and clearance. The immune system constantly monitors the body for the invasion of microorganisms and foreign molecules. A tightly regulated network of antibodies, T-lymphocytes, antigen-presenting cells, cytokines, and microenvironment signals secures the development of an appropriately targeted immune response to combat infections. Foreign extracellular and surface antigens are recognized by B-lymphocytes, which respond by secreting antibodies. To mount a sustained antibody response, B cells require an additional signal from T helper cells, which present the relevant antigen as peptide fragments 15-25 amino acids in length that are in complex with major histocompatibility complex (MHC) class II. Antigens can also stimulate CD8+ T lymphocytes. These cells are activated by intracellular and membrane proteins that are processed by the endogenous processing pathway and presented as peptides 8-10 amino acids in complex with MHC class I. The 2 systems are highly coordinated, and in most cases, high affinity immunoglobulin G (IgG) antibody responses require recognition of the antigen by both B and T lymphocytes. During the initial development of the immune system, more than half of the newly generated B cell receptors are estimated to be capable of binding autoantigens. Most autoreactive B cells are eliminated during B cell maturation, however, preventing mature B cells from reacting with self-molecules. This selection provides the basis for the development of self-tolerance, the ability of the immune system to recognize and ignore the body’s own cells and tissues. Sometimes this mechanism fails and the immune system reacts with one’s own antigens as a consequence of over-expression, mutations, changes in protein half-lives, misfolding, aberrant degradation of self-proteins, or altered post-translational modifications (eg, glycosylation and phosphorylation) of the protein. Autoantibodies have long been recognized in autoimmune diseases, including systemic lupus erythematosus, myasthenia gravis, and rheumatoid arthritis. In some of the diseases, autoantibodies play a central role in its pathogenesis (eg, myasthenia gravis), whereas their role in others is less clear. Nevertheless, the existence and detection of autoantibodies is an important element in establishing an accurate diagnosis. In rheumatoid arthritis, for example, the test for an anti-IgG antibody, also known as the rheumatoid factor, is useful and has a sensitivity of approximately 80%.

Autoantibodies as Biomarkers in Cancer

Autoantibodies directed against various antigens have also been detected in cancer patients. The antigens are present predominantly in cancer cells while scarcely present in healthy cells. Such cancer-related autoantibodies were first detected more than 40 years ago.12 These studies revealed the presence of autoimmunity against tumor cells, but they provided limited information about target proteins and specific epitopes. Later biochemical characterization provided the identity of some of these autoantibody targets, such as CEA13 and tumor suppressor p53.14,15 With the introduction of enzyme-linked immunosorbent assay (ELISA) methods, the presence of autoantibodies to p53 and other antigens was assessed in elaborate screening programs. These studies suggested that autoantibodies may be of diagnostic use in cancer similar to the use of anti-IgG antibodies for the diagnosis of rheumatoid arthritis.16-18 However, single targets such as p53 do not possess sufficient diagnostic sensitivity and specificity to be used in clinically relevant screening tests. Therefore, multiple markers are clearly necessary if detection of autoantibodies should be used in cancer screening.

High Throughput Methods in Autoantibody Discovery

Several new high-throughput methods have been developed for the discovery of new autoantibody targets, including serological analysis of tumor antigens by phage-display libraries,19 recombinant cDNA expression cloning (SEREX),20 peptide and protein arrays, and self-assembling protein arrays (Figure 1).21 With these methods several new targets have been identified. Among the most frequently identified targets are: HER2,22 carbonic anhydrase XII,23 mutated p53, tyrosinase, SOX2, ZIC2, SSX2, MAGE, and NY-ESO.24,25 A major challenge is to deselect targets identified by antibodies present in healthy individuals or patients with non-malignant disease. At best, studies have collectively demonstrated up to 80% sensitivity when several tumor-associated antigens are combined, whereas single antigens have provided a sensitivity of only 10%-30%. Although the sensitivity and specificity obtained with multiple markers is encouraging, clinically relevant screening methods must have higher sensitivity and specificity. The Phage display strategy has elegantly provided a partial solution to this problem.
Figure 1. Methods for identifying autoantibodies in cancer patients.

Serological proteome analysis (SERPA): Proteins from cell lines or cancer tissue are separated by 2DE, transferred to nitrocellulose membranes, and probed with sera obtained from healthy individuals and cancer patients. Cancer-associated antigens are identified and further analyzed by mass spectrometry (MS). The identified proteins can be validated with purified proteins in ELISA or microarray format.

Protein microarray: Protein is spotted on a microarray, which is then incubated with sera from cancer patients and controls. Autoantibodies are detected with a fluorescent-labeled secondary antibody. An alternative approach is to spot cDNA on slides, followed by in situ translation in a cell-free system before analysis with relevant sera.

Reverse-capture antibody microarray: Antibodies reacting with specific proteins are spotted onto the microarray, which is then incubated with tumor lysate or serum proteins. The microarrays with captured proteins are then incubated with sera from controls or cancer patients. Autoantibodies are detected with fluorescent-labeled secondary antibody.

Phage library: Phage libraries are constructed based on mRNA from cancer cells or tumor tissue. First, clones reacting with sera from healthy individuals are removed by incubating the phage library with beads coated with autoantibodies from controls. Subsequently, the phage library is incubated with beads coated with autoantibodies from cancer patients and the bound phages eluted. The enriched phage libraries are then spotted onto a microarray and incubated with sera from healthy individuals and cancer patients.
Phage-Display Methods

In phage display-based methods, a cDNA library is constructed from a cancer cell line, tumor tissue, or random phage library by expressing the phage proteins fused with the antigens on the surface of the bacteriophages. This approach makes it possible to screen a large number of antigens with serum from cancer patients and includes rational protocols to deplete antigens recognized by autoantibodies from the serum of healthy individuals. In this manner, high throughput screening procedures have been developed facilitating the detection of specific targets discriminating between cancer patients and healthy controls. One study identified a panel of 22 phage-derived peptides and demonstrated their clinical relevance in a screening test for prostate cancer (sensitivity 82%, specificity 88%). Only 4 of the 22 phage-derived peptides were identified as known proteins (BRD2, elf4G1, RPL22, and RPL13a). The remaining peptides most likely represent so-called mimotopes unrelated to the antigen against which the antibody was raised, which is a general finding for many of these methods. A similar strategy was used for ovarian cancer, identifying a panel of 65 antigens that detected ovarian cancer with a sensitivity of 55% and specificity of 98%.27

Protein Arrays

Other high throughput methods are composed of protein arrays presenting peptides or proteins on a microarray format.28 These methods have the advantage of enabling the simultaneous screening of a large number of targets while consuming only a limited amount of patient material. Purified or recombinant proteins, synthetic peptides, or fractionated proteins from tumor or cancer cell lysates are spotted onto microarrays and incubated with sera from patients and controls. Most peptide and protein arrays present recombinant proteins expressed in E. coli lacking post-translational modifications. Alternative host expression systems, including yeast and insect cells, have been used to produce libraries presenting proteins with more correct folding and the inclusion of some degree of post-translational modification. An example is a new commercial proto-array from Invitrogen (Carlsbad, CA) that presents more than 9000 proteins purified from baculovirus-based expression systems. Using this microarray, a panel of 94 antigens was identified aiding in the detection of ovarian cancer.29 An additional use of the protein microarray platform is high throughput screening of thousands of fractionated protein samples in a single test, which presents possible targets in their native form with potential post-translational modifications as expected for the native protein. For example, solubilized proteins from a colon cancer cell line were separated by a liquid-based 2D separation system into 1760 fractions and subsequently printed on a microarray to identify fractions containing proteins discriminating between cancer patients and controls. Using this method, ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCH-L3) was identified as an autoantibody target, detecting 19 of 43 colorectal cancer patients.30 A similar strategy was used to identify targets in patients with lung cancer31 and prostate cancer.32 One important feature of this method is the presentation of native proteins including their post-translational modifications. An alternative approach to present native proteins on microarrays is by reverse-capture antibody microarrays. In this approach, specific antibodies are used to capture native proteins from a cancer cell line or tissue to detect autoantibodies in serum.33

Self-Assembly Microarrays

Self-assembly microarrays were recently introduced as useful tools in autoantibody discovery with the benefit of the simple production of a large number of proteins, limiting the problems with production, purification, and storage. This method uses cDNA vectors printed on the microarray, which are translated into proteins in a cell-free expression system, enabling the expression and subsequent screening of large numbers of antigens.34 Thus, approximately 5000 antigens were expressed and screened against breast cancer and controls in 1 study, identifying a panel of 28 tumor-associated antigens that identified breast cancer patients with a sensitivity of 81% and specificity of 62%.35 However, this method continues to present targets devoid of post-translational modifications.

Inclusion of Post-translational Modifications on Targets Improves Cancer Detection

Although many methods have allowed the testing of thousands of candidate autoantigens with the identification of several informative targets, several limitations still exist for the procedure. Notably, the majority of identified targets are intracellular proteins to which there is limited tolerance. One reason for the limited number of cell surface proteins among identified targets may be that the examined libraries generally lack cancer-related post-translational modifications. The phage display, self-assembly arrays, and random peptide arrays present, for the most part, antigens expressed in bacteria without relevant post-translational modifications. Even methods that do include proteins with post-translational modifications in target screens, such as 2D gel electrophoresis (2DE), liquid-based fractionated protein arrays, and reverse-capture antibody microarray, rarely include cancer-relevant protein modifications in the subsequent validation of targets. The inclusion of post-translational modifications on proteins and peptides could potentially result in the selection of targets with higher specificity. In addition, post-translational modifications are particularly appealing because they expand the number of cancer-associated protein targets for autoantibodies. Only 20 primary amino acids exist, but the diversity created by these amino acids is expanded dramatically by protein modifications, some of which are specifically altered in cancer. Cancer-associated changes in glycosylation36 and phosphorylation37,38 result in the presentation of neo-antigens to the immune system.

A consistent characteristic of cancer cells is aberrant truncation of mucin-type O-glycans to short mono- and disaccharides.39,40 Mucin-type O-glycosylation is 1 of the most abundant post-translational modifications. The glycosylation is initiated by up to 20 different GalNAc transferases, and further elongated in a highly complex manner by a number of other glycosyltransferases. Truncated, immature glycans are recognized by natural anti-carbohydrate immunoglobulin M (IgM) antibodies, as well as lectin receptors on antigen-presenting cells, with subsequent presentation of aberrantly O-glycosylated proteins to the immune system, inducing immunity.31,42 Autoantibodies induced by aberrant O-glycoproteins may be directed to aberrant carbohydrate structures, peptide epitopes in the protein backbone, and newly combined glycopeptide epitopes induced by an aberrant O-glycan structure. The latter O-glycopeptide epitopes are particularly appealing as targets for autoantibodies because they are likely to be cancer-specific.43,44
Mucin-1 is a heavily O-glycosylated molecule known to be over-expressed in most adenocarcinomas. Many studies have reported aberrant glycoforms on MUC1, and low levels of autoantibodies to non-glycosylated MUC1 tandem repeats have been identified. Recently, autoantibodies (IgG) to aberrant O-glycosylated MUC1 were identified in sera from a small series of patients with breast, ovarian, prostate, and colorectal cancer. Importantly, the type of glycan linked to MUC1 appears to discriminate between patients with inflammatory lesions and cancer. Autoantibodies of the immunoglobulin A (IgA) isotype were also identified for glycosylated mucins, increasing the range of antibody isotypes that should be included in future screening. The general finding that autoantibodies in cancer patients can be directed to combined epitopes of glycans and peptide backbone is promising and might allow a search for autoantibodies specifically directed to cancer-associated post-translational modifications. Potentially, this finding may aid in solving some of the problems with the panels of antigens discovered until now: namely the lack of specificity and lack of discrimination between benign disease and malignancy. A combination of high throughput methods and O-glycosylation is currently being investigated as exemplified by the recent development of random O-glycopeptide bead libraries. Similar to O-glycosylation, several reports have shown that autoantibody targets are dependent on N-glycosylation, including annexin and cysteine-rich fibroblast growth factor receptor 1 (CFR-1). However, the general role of N-linked glycosylation in autoantibody targets is less clear.

Finally, other post-translational modifications, such as phosphorylation, acetylation, methylation, citrullination, and O-GlcNAc glycosylation, could be important to include in future screenings of new biomarkers. Comparable to O-linked glycosylation, the phosphorylation pattern can be changed in cancer cells during the disruption of normal signaling pathways; altered phosphorylation of key residues has been observed in cancer and autoimmune diseases. In a recent study, circulating autoantibodies were detected against a phosphorylation-dependant epitope of glycolytic enzyme alpha-enolase in 62% of patients with pancreatic cancer. Using the same concept, O-GlcNAc can be added to the serine and threonine residues of intracellular proteins, creating novel epitopes and potentially inducing cancer-specific glycopeptide autoantibodies. This hypothesis is supported by autoantibodies to citrullinated proteins found in the majority of patients with rheumatoid arthritis. Citrulline is a non-natural amino acid generated by a post-translational modification of the amino acid arginine by the enzyme peptidyl arginine deiminase (PAD), which is only activated during inflammation and cell death due to an increased Ca²⁺ concentration in dying cells. Citrullination changes the positively charged amino acid arginine to a neutral citrulline, which alters the tertiary structure of the protein, and citrullinated peptides are currently used in autoantibody tests for rheumatoid arthritis with high sensitivity and specificity. Because over-expression of PAD has been observed in various malignant tumors, the inclusion of libraries presenting citrullinated peptides might identify novel informative targets.

While the sensitivity and specificity obtained with multiple markers with or without post-translational modifications are very encouraging, only a few examples exist in which the method has been used in clinical practice. In lung cancer there is no usable blood test to screen asymptomatic individuals, but screening of high-risk groups, such as heavy smokers, can be considered. Recently, a larger study validated the use of a panel of 6 tumor-associated antigens to detect lung cancer by the presence of auto-antibodies with a sensitivity of 36%-39% when the specificity was set to 90%. This test is now commercially available.

Conclusion

Autoantibodies to cancer antigens are favorable as biomarkers for the early detection of cancer. Because single autoantibody targets identify only a relatively small proportion of cancer patients, multiple antigens must be used to ensure the required sensitivity and specificity of assays that can be used in the clinic. New methods are expected to aid in the detection of new and more informative targets, including proteins with relevant post-translational modifications, for the development of simple non-invasive screening methods for early stage cancer.

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