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# Tumor-associated autoantibodies as diagnostic and prognostic biomarkers

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**In the process of tumorigenesis, normal cells are remodeled to cancer cells and protein expression patterns are changed to those of tumor cells. A newly formed tumor microenvironment elicits the immune system and, as a result, a humoral immune response takes place. Although the tumor antigens are undetectable in sera at the early stage of tumorigenesis, the nature of an antibody amplification response to antigens makes tumor-associated autoantibodies as promising early biomarkers in cancer diagnosis. Moreover, the recent development of proteomic techniques that make neo-epitopes of tumor-associated autoantigens discovered concomitantly has opened a new area of 'immuno-proteomics', which presents tumor-associated autoantibody signatures and confers information to redefine the process of tumorigenesis. In this article, the strategies recently used to identify and validate serum autoantibodies are outlined and tumor-associated antigens suggested until now as diagnostic/prognostic biomarkers in various tumor types are reviewed. Also, the meaning of autoantibody signatures and their clinical utility in personalized medicine are discussed. [BMB Reports 2012; 45(12): 677-685]**

## INTRODUCTION

An immunosurveillance system recognizes the changes in tumor cells and a humoral response to tumor-associated antigens (TAAs) takes place. From the first study on tumor-associated autoantigens in the 1960s by Baldwin (1), hundreds of tumor-associated antibodies have been reported and many studies have been performed on their application to biomarkers. Tumor-associated autoantibodies are a group of serum biomarkers which show highly interesting properties. They are easily accessible in blood samples and have a long half-life, which confer advantages over other protein biomarkers currently used. Moreover, the nature of an antibody amplification response to an antigen means that even relatively small quantity of antigen in the early stage of tumorigenesis can trigger a larger immune response, which makes it useful as an early diagnosis marker. Moreover, the recently improved proteomic technologies have enabled discovery of many autoantigens concomitantly in spite of the limitations in patient sera (2-6), and they can be used for the generation of a panel of TAAs that exhibit better diagnostic value than a single TAA marker (7). Recently, based on the autoantibody profile of cancer patients, studies on the utility of autoantibodies as prognostic biomarkers and anti-cancer vaccine immunotherapy have also been performed (8), although their exact roles in the body or development mechanism are still a matter of controversy. In this article, we will review the issues about tumor-associated autoantibodies encompassing the development and innate functions of tumor-associated autoantibodies, their discovery and validation techniques, and their utilities as diagnosis/ prognosis markers in cancer.

## DEVELOPMENT OF TUMOR-ASSOCIATED AUTOANTIBODIES IN IMMUNE SURVEILLANCE

The immune system, which is composed of a variety of inter-dependent mechanisms, collectively defends the body from external agents such as bacterial and viral infections. The cancer cells, which divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body, are another important target of the immune system, although tumorigenesis is an internal process. Tumor cell remodeling in the process of tumorigenesis causes changes in proteins expression patterns and in tumor microenvironments, accompanied with the secretion of proteins different from those of normal cells. Microvesicles shedding from tumor cells and intracellular proteins released from dead tumor cells also influence the tumor microenvironment, which may be recognized by the defense system as external agents and elicit humoral as well as cellular immune responses (8, 9). In addition to the immune response recognizing and preventing the development of cancer, much evidence now suggests that the immune system interacts with cancer to promote and direct tumor growth

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(10, 11). The interplay between the immune system and pre-cancerous and cancer cells seems to be an inevitable part for tumorigenesis.

The stages and mechanisms of how cancer and the immune system interact have been termed as 'immunosurveillance', which is divided into three phases encompassing elimination, escape and equilibrium, and immunosubversion (12, 13). In the elimination phase, the immune system recognizes pre-cancerous cells and destroys cancer precursors (14). The immune response induced by natural killer group 2D (NKG2D) ligands on cancer cells and its specific receptor on natural killer (NK) cells or subsets of T-cells is a typical type of tumor elimination process (15, 16). NKG2D-deficient mice have been shown to be defective in tumor surveillance (17). After the first elimination of immuno-stimulatory tumor cells, poorly immunogenic tumor cell variants seem to be primed to escape the immune system and to reach a state of equilibrium with the host defense system. In this phase, the robustness of the tumor for continual survival and growth within an immune-competent environment seems to be determined (12). There are evidences supporting the immune surveillance hypothesis in human cancers, although it is difficult to analyze directly. It has been noted that immunosuppressed individuals have high incidences of cancer subtypes (18). In colorectal cancer, the cells expressing NKG2D ligands were decreased with tumor stage progressively (15). Lastly, immunosubversion is a process by which cancer cells actively suppresses the immune response. Dendritic cells (DC), the most important antigen presenting cells, are critical regulators of adaptive T- and B-cell immune responses as well as natural killer cell activation (10). DC differentiation and maturation is shown to be suppressed by high levels of vascular endothelial growth factor (VEGF), which is known to be produced by tumor cells. VEGF also acts as a potent stimulator of immature dendritic cells (iDCs) resulting in recruitment from bone marrow to the tumor site. iDCs further contribute to immunosubversion by inducing T-cell dysfunction (19). Other tumor-derived factors, such as IL-6, M-CSF and IL-1β, also recruit myeloid suppressor cells (MSCs), and act to prevent maturation of the MSCs into dendritic cells. An increased number of MSCs then act upon tumor specific T-cells, inhibiting the T-cell responses through nitric–oxide (NO) synthesis (14), which may tip the balance in favor of a pro-tumor environment (20). Using such mechanisms, tumor cells can have an immunosuppressive effect on the local microenvironment.

As such different mechanisms relating the interplay between the immune system and cancer cells are involved in the course of tumor progression, the immunoproteomics approaches have been performed to identify the different components of these processes for the improvement of understanding, prevention, diagnosis, staging and treatment of cancer. The development pattern of tumor-associated autoantigens and their specific autoantibodies in the process of tumorigenesis is an important aspect of immunoproteomics. The early development of tumor-specific autoantibodies implies their possible roles in the elimination step of immunosurveillance. However, most of the autoantibodies identified in cancer have showed low titer and are ineffective for stimulating effector functions, which seems to be the result of immune suppression or tolerance induced by tumor cells in escape and equilibrium steps of immunosurveillance. Therefore, further understanding of the early processes of tumorigenesis and related autoantibodies might show important implications for tumor biology and, from these results, additional biomarkers that could potentially assist in improved diagnosis or treatment will be identified (8).

## PROFILING OF TUMOR-ASSOCIATED AUTOANTIBODIES

Hundreds of TAAs that elicit autoantibodies have been identified after the first report on the immune response to solid tumors by Baldwin (1), and in recent years, due to the development of new proteomic technologies, there have been more studies profiling cancer patient sera for the detection of tumor associated antigens (21). Earlier studies on TAAs have focused on a few antigens at a time, using techniques such as one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis or enzyme-linked immunosorbent assay (ELISA). Improved technologies using proteomics platforms have enabled many TAAs to be discovered concomitantly (Fig. 1). These approaches are useful to screen large numbers of antigens at once with small numbers of sera and can be used for the generation of a panel of TAAs that exhibit better diagnostic value than a single TAA marker (7), although they are labor and cost intensive. The pros and cons of these methods are as follows.

### Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX)

SEREX involves the identification of TAAs by screening patient sera against a cDNA expression library obtained from the autologous tumor tissues. By using SEREX, Sahin *et al*. (22) first showed that cancer-testis antigens (CTAs) elicited a humoral response in cancer patients. Subsequently, a large number of TAAs associated with numerous cancer types have been identified using this method. The panel of SEREX-defined immunogenic tumor antigens includes CTAs (e.g. NY-ESO-1, SSX2, MAGE), mutational antigens (e.g. p53), differentiation antigens (e.g. tyrosinase, SOX2, ZIC2) and embryonic proteins (23-25). Many of these TAAs are potential serological biomarkers, although several are reported to have low sensitivity. SEREX methods have some limitations to identify autoantigens (9). First, recombinant cDNA expression clones are gene products expressed in bacteria, which present linear epitopes only. In addition, they do not display post-translational modifications (PTMs), such as glycosylation, acetylation, phosphorylation and proteolytic cleavage, which are known to be important for neo-antigenicity of tumor-associated proteins. Second, as the cDNA expression clones are constructed from a tumor tissue

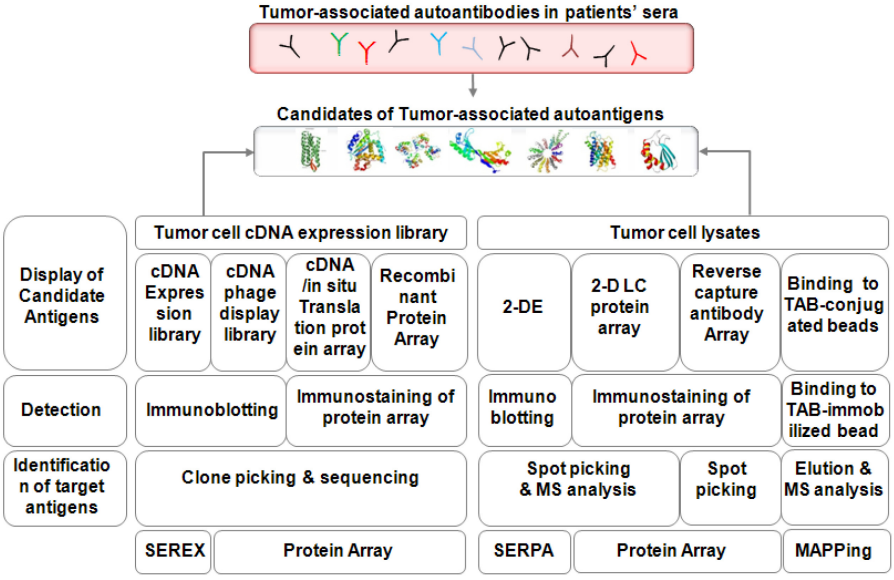


**Fig. 1.** Systematic approaches for identifying tumor-associated autoantibodies.

specimen, SEREX is limited to identifying TAAs from the tumor of one patient. Owing to the heterogeneity of gene expression in the different cell types in tumor tissues, the cDNA expression library derived from one patient is not sufficient to identify TAA candidates. Improvements to the SEREX approach have been made by the combination with solution-based phage-display technologies or by using eukaryotic expression systems (e.g. yeast, baculovirus system) (26), or by enlarging the repertoire of the cDNA expression library.

### Serological proteome analysis (SERPA)

Another commonly used technique for the identification of TAAs is the proteomics-based approach termed 'SERPA' (27), which involves the discovery of TAAs using a combination of two dimensional (2D) electrophoresis, western blotting and mass spectrometry (MS) (2, 9). Proteins from tumor tissues or cell lines are separated by 2D electrophoresis, transferred onto membranes by electro-blotting and subsequently probed with sera from healthy individuals or patients with cancer. Then the respective immunoreactive profiles are compared and the cancer-associated antigenic spots are identified by MS. TAAs firstly identified by SERPA were SM22-alpha and several members of the cytoskeletal family (such as cytokeratin 8, stathmin and vimentin) in kidney cancer patients (27). The drawbacks of SERPA are related to the inherent limitations of 2D electrophoresis. These include bias to abundant proteins, limitations in resolving certain classes of proteins (e.g. membrane proteins) and difficulty in producing reproducible 2D gels (28). In addition, because of the way that western blots are prepared, only sequential epitopes can be detected.

### Multiple affinity protein profiling (MAPPing)

MAPPing involves 2D immunoaffinity chromatography followed by the identification of TAAs by tandem MS (nano-LC MS/MS) (29). In the first phase of immunoaffinity chromatography, nonspecific TAAs in a cancer cell line or tumor tissue lysate bind to immunoglobulin G (IgG) obtained from healthy controls in the immunoaffinity column and are removed from the lysate. The pre-cleared lysate is then subjected to the 2D immunoaffinity column that contains IgG from cancer patients. TAAs which bind at the second phase are likely to be cancer-specific and are eluted for enzymatic digestion and identification by tandem MS. Hardouin *et al.* used this approach to screen sera for autoantibodies from patients with colorectal cancer (29). MAPPing maintains tumor antigen in solution, allowing for the potential identification of conformational epitopes. However, immunoprecipitation using affinity columns often restricted the discovery of TAAs to antibody interactions with low dissociation rate constant.

### Reverse-capture microarray

Ehrlich *et al.* (30) presented a 'reverse-capture microarray' method based on a dual-antibody sandwich ELISA. Cancer cell lysates or tumor lysates are incubated with commercial antibody arrays so that each antigen is immobilized on a different spot in their native configurations. Meanwhile, IgGs from patient and control sera are purified and labeled with different fluorescent dyes and then incubated with the antigen-bound microarrays. This allows the instant identification of cancer-specific autoantibodies using native antigens expressed in tumor cells, which allows for the detection of TAAs presenting post-translational modifications. TAAs encompassing von Willebrand Factor, IgM, alpha1-antichymotrypsin, villin and IgG were identified by screening prostate cancer sera against an array containing 184 antibodies (31). Qin *et al.* (32) also identified 48 TAAs from prostate cancer sera using re-

verse-capture microarray, including p53 and Myc. However, the only known antigens with commercially available antibodies can be analyzed using reverse-capture microarray, which is appropriate for the validation rather than the discovery of biomarkers.

### Protein microarrays

Protein microarrays constitute a quick and convenient technology for characterizing humoral immune response in serum (33, 34). The array platforms can be two dimensional (e.g. glass slides, nitrocellulose membranes and microtiter plates) or three dimensional (e.g. beads and nano-particles) (9). Recombinant proteins, fractionated proteins from cancer cell lysate or synthetic peptides are spotted systematically onto microarrays and then incubated with specific sera (2, 3). Because of its miniature platform, the amount of samples and reagents needed are greatly reduced. Protein array technology enables the identification of antigens in native configuration and especially useful for the discovery of post-translationally modified antigens. Because the microarray technology provides multiplexed analyses of thousands of proteins, this method permits high-throughput identification of TAA signatures for the development of cancer diagnostics (9). Sera autoantibodies against ubiquitin C-terminal hydrolase L3 were identified in colon cancer patients by fractionating cancer cell lysate onto a nitrocellulose-based array (35).

## THE PROPERTIES OF TAAS AND GENERATION OF SPECIFIC HUMORAL IMMUNE RESPONSE

Hundreds of TAAs have been identified in many cancers using systematic profiling methods. The list of TAAs includes oncoproteins (e.g. HER-2/Neu, ras and c-MYC), tumor suppressor proteins (e.g. p53), survival proteins (e.g. surviving), cell cycle regulatory proteins (e.g. cyclin B1), mitosis-associated proteins (e.g. centromere protein F), mRNA-binding proteins (e.g. p62, IMP1, and Koc), and differentiation and CTAs (e.g. tyrosinase and NY-ESO-1)(36, 37: and the latest results in Table 1 and 2). In cancer, TAAs show post-translational modifications, aberrant localization or overexpression, which might confer neo-antigenicity to TAAs (3). However, it is not entirely clear how modifications of antigens trigger the humoral response, especially when TAAs are intracellular proteins which do not elicit the immune defense system at normal states. One hypothesis involves aberrant tumor cell death, when the modified intracellular proteins are released from tumor cells and are presented to the immune system in an inflammatory environment (22, 38). Tumor cell death also releases proteases that would generate cryptic self-epitopes to trigger an immune response. Another hypothesis is based on tumor cell microvesicle (MV) shedding (39). The bioactive cargo of MVs includes growth factors and their receptors, proteases, adhesion molecules, signaling molecules, as well as DNA, mRNA, and micro-RNA (miRs) sequences (40). Tumor cells emit large quantities of MVs containing pro-coagulant, growth regulatory and oncogenic cargo (oncosomes), which can be transferred

**Table 1.** Tumor-associated antigens evaluated as diagnostic marker[a]

| Tumor-associated autoantigens | Patient number | Tumor type | Validation method | Specificity/ Sensitivity (%) | Ref. |
|---|---|---|---|---|---|
| Phage display clones (N = 45) | 235 | Gastric cancer | Microarray | 89.7/58.7 | (61) |
| ABCC3 | 114 | ESCC | ELISA | >95/13.2 | (62) |
| HSP60, p53, Her2-Fc, NY-ESO-1, HSP70 | 29 | Breast cancer | Microarray | 82.7/— | (63) |
| NY-ESO-1, XAGE-1, ADAM29, MAGEC1 | 94 | NSCLC | Microarray | 89/36 | (64) |
| GAL3, PAK2, PHB2, RACK1, RUVBL1 | 182 | Breast cancer | ELISA | 84/66 | (65) |
| A1AT | 25 | Breast cancer | WB | —/96 | (66) |
| NOLC1, MALAT1, HMMR, SMOX | 65 | NSCLC | ELISA | 60/66.7 | (67) |
| GRP78, AFP | 76 | HCC | ELISA | —/71.4 | (68) |
| Ku86 | 58 | HCC | ELISA | 90/60.7 | (69) |
| Lymphocyte antigen 6 complex locus K (LY6K) | 62 | ESCC | ELISA | 78.7/80.6 | (70) |
| p53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD, MAGE A4 | 235 | Lung cancer | ELISA | 91/41 | (71) |
| BMI-1 | 67 | Cervical cancer | ELISA | 76/78 | (72) |
| p53, p16, p62, survivin, Koc, IMP1 | 23 | Pancreatic cancer | ELISA | 87/60.9 | (73) |
| Phage display clones (N = 5) | 60 | Colon cancer | ELISA | 91.7-93.3/90-92.7 | (74) |
| RPH3AL | 84 | Colon cancer | WB | 84.1 /72.6 | (75) |
| NY-ESO-1, SSX-2,4, XAGE-1b, AMACR, p90, LEDGF + PSA | 131 | Prostate cancer | seroMAP | 84/79 | (76) |
| MMP-7 | 50 | ESCC | ELISA | 81/78 | (77) |
| SEC61β | 86 | Colon cancer | WB | 75/79 | (78) |
| STK4/MST1, SULF1, NHSL1, SREBF2, GRN, GTF2 | 50 | Colon cancer | ELISA | 73.9/72 | (79) |
| p53, NY-ESO-1, CAGE, Hu-D, SOX2, Annexin I, GBU4-5 | 243 | SCLC | ELISA | 99/42 | (80) |
| Prgrammable protein clones (N = 28) | 51 | Breast cancer | Microarray | 61.6/80.8 | (81) |

[a]An updated list of the most recent studies (2011-present).

**Table 2** Tumor-associated antigens evaluated as prognostic marker[a]

| Autoantigens | Number of patients | Tumor type | Prognosis | Ref. |
|---|---|---|---|---|
| ENOA 1, 2 | 120 | Pancreatic cancer | Increased survival | (82) |
| MUC1 | 28 | Ovarian | Decreased survival | (83) |
| MUC1 | 395 | Breast | Increased survival | (84) |
| EpCAM | 84 | Ovarian | None | (85) |
| ALK | 95 | Anaplastic large cell lymphoma | Decreased recurrence | (86) |
| CDC25B phosphatase | 134 | Esophageal cancer | Decreased survival | (87) |
| p53 | 120 | Ovarian cancer | Increased survival | (88) |
| Panel of 29 antigens | 60/59 | Ovarian cancer/Pancreatic cancer | Increased survival | (89) |
| MIA | 34 | Pancreatic cancer | Increased survival | (90) |

[a]An updated list of the most recent studies (2010-present).

throughout the cancer cell population and to non-transformed stromal cells. MVs which are not taken up by neighboring cells would disperse into blood and could stimulate humoral immune responses. Oncogene products and RNA binding proteins, which are typical example of TAAs, are suggested as components of MVs. Tissue remodeling at the site of tumourigenesis can be another event conferring TAAs (8). Besides these, innate secretory oncofetal proteins aberrantly expressed in various tumors (e.g. AFP, PSA, CEA, CA 15-3) are also well known TAAs (41). TAAs described above seem to encompass a large portion of tumor proteomes. Therefore, researchers expect that TAA panels would serve early molecular signatures for diagnosis and prognosis of cancer patients. Also the identification and functional characterization of these immunological signatures for cellular mechanisms associated with tumorigenesis would be useful to uncover the early molecular events of carcinogenesis (2, 42).

## CLINICAL UTILITY OF TUMOR-ASSOCIATED AUTOANTIBODIES

As one of adaptive immune response, antibodies serve multiple functions to prevent pathogenic infections. However, the function of tumor-associated autoantibodies, which bind self-antigens and have escaped self-tolerance, is generally unknown. Autoantibodies can mediate antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). They can also enhance antigen cross-presentation and activation of T lymphocytes. Also, autoantibodies against cell surface receptors or growth factors can interfere in receptor/ligand interactions. For example, HER2/neu-specific autoantibodies can interfere in signal transduction via HER-2/neu and its phosphorylation (43). In spite of the fact that innate functions of autoantibodies are undefined, the immune system can act as an extremely sensitive reporter for identification of new altered proteins that are different from self-proteins (44).

### Tumor-associated autoantibodies as diagnosis markers

The ideal target for the early diagnosis and prognosis of cancer is a biomarker detected in blood, which can be utilized in a simple and inexpensive manner. Therefore, many studies to identify molecular changes (especially, elevation of tumor-cell derived proteins) in blood were conducted for several decades. However, despites these efforts, most of protein markers do not pass the clinical trials. There are several reasons for this, including short half-lives and low levels of tumor antigen proteins in blood, as well as heterogeneity of tumor cell proteomes.

Tumor-associated autoantibodies have a number of advantages over traditional protein biomarkers (8). Contrary to the short-lived changes of tumor-antigens in serum, the antibody molecule is stable in the blood and antibody response is enduring. Moreover, the nature of an antibody amplification response to an antigen means that even a relatively small quantity of antigen can trigger a larger immune response that is reflected in relative antibody concentrations (45). Because of these advantages, the autoantibodies are useful as biomarkers and can be applied to cost-efficient and diagnostically and clinically relevant assays, such as ELISAs. Another important aspect of autoantibodies as biomarkers is that assays for TAAs can be easily combined to establish a panel for the multiplex detection of tumor-associated antibody biomarkers, which might suggest a way to overcome the heterogenicity of tumor cell proteomes.

A combined analysis of autoantibodies to p53, HER-2, IGFBP-2, and TOPO2α increased both diagnostic specificity and sensitivity of up to 75% for breast cancer patients (46). Recently, a diagnostic assay using five autoantigens (p53, NY-ESO-1, CAGE, GBU4-5, Annexin 1) was performed over 600 patients with lung cancer, which showed a remarkable 90% specificity, while sensitivity remained relatively low with 40% (47). Now, most studies for the discovery of tumor- associated autoantibodies are performed using proteomics approach and the combined assay of different autoantibodies is suggested as a promising approach for the diagnosis of cancer (Table 1).

### Tumor-associated autoantibodies as prognosis markers

Prognostic biomarkers that predict cancer recurrence and sur-

vival are crucial for targeting therapies to high-risk populations (48). Clinical parameters, such as tumor-node-metastasis staging and tumor grade/differentiation, are routinely used for risk stratification in clinical practice. Molecular prognostic markers that measure gene expression within primary tumor specimens have broad applications for multiple cancer types, and have revealed fundamental differences in tumor biology between cancers with similar histology (49). Although tumor antigen-specific autoantibodies, as described above, have been suggested as a reporter of cancer progression, few autoantibodies have been assessed as prognostic biomarkers of cancer (48, 50).

Autoantibody against tumor suppressor p53 probably has most extensively been studied with regard to its prognostic value. Several small studies yielded variable results, ranging between a missing effect and a negative influence on the patients' outcome (51). Interestingly, one larger study performed in hepatocellular carcinoma patients suggested that the presence of p53-specific autoantibodies might be associated with an increased overall survival (52). On the contrary, a number of other large studies in breast, lung, colon and oral cancer patients clearly highlighted the correlation between the presence of p53-specific autoantibodies and decreased overall and progression-free survival (53). As shown in other reports (48, 50) and in Table 2, the results correlating autoantibodies and clinical prognosis are mixed.

### Tumor-associated autoantibodies in personalized cancer therapy

The immune system can act as an extremely sensitive reporter for identification of new altered proteins that are different from self-proteins. Therefore, this strategy may help to detect proteins that undergo alterations during the tumorigenesis. In addition, these proteins might become interesting therapeutic targets (36, 44, 54, 55). Various anti- tumor vaccination strategies that involve humoral and cellular immune responses to TAAs have been studied. These cancer immunotherapies have been shown to target tumors without affecting normal tissues or resulting in adverse side-effects (56, 57). However, patient heterogeneity often results in a contradictory response to immunotherapy (58, 90). Thus, personalized profiles of TAAs and autoantibodies should be used to identify therapeutic targets to develop vaccines for targeted immunotherapy against cancer.

## CONCLUSION & PROSPECTIVES

The mechanisms underlying the emergence of tumor-associated autoantibodies and the regulation of their production are not completely understood. However, accumulating evidence about TAAs and characterization of TAAs would lead us to understand the process of tumorigenesis and interaction between tumor cells and immune system more precisely. In spite of the incomplete understanding of autoimmune response against tumor, autoantibodies can be used as a reporter identifying aberrant de novo or dysregulated cellular mechanisms (59). The establishment of more precise time lines to determine when autoantibodies to these TAAs appear as early predictors of cancer and whether anti-TAA antibody expression varies with progression or response to treatment is also necessary in further study. In addition to its use for early diagnosis of tumor, the potential utility of TAA-autoantibody systems as biomarker tools to monitor therapeutic outcomes or as indicators of disease prognosis has been explored. Moreover, the association of individual mutations with antibody responses might be used to tailor treatment according to individual variations (44). The use of autoantibody profiles has already demonstrated the capacity to classify clinically challenging cohorts of prostate cancer patients (60). This classification can be useful for personalized medicine.

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