

Serum autoantibodies as biomarkers for early cancer detection

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Autoantibodies against autologous tumor-associated antigens have been detected in the asymptomatic stage of cancer and can thus serve as biomarkers for early cancer diagnosis. Moreover, because autoantibodies are found in sera, they can be screened easily using a noninvasive approach. Consequently, many studies have been initiated to identify novel autoantibodies relevant to various cancer types. To facilitate autoantibody discovery, approaches that allow the simultaneous identification of multiple autoantibodies are preferred. Five such techniques – SEREX, phage display, protein microarray, SERPA and MAPPING – are discussed here. In the second part of this review, we discussed autoantibodies found in the five most common cancers (lung, breast, colorectal, stomach and liver). The discovery of panels of tumor-associated antigens and autoantibody signatures with high sensitivity and specificity would aid in the development of diagnostics, prognostics and therapeutics for cancer patients.

Introduction

Cancer is the second leading cause of death worldwide [1]. In 2002, there were reportedly 11 million new cases of cancer and 7 million cancer-related deaths, leaving approximately 25 million people alive with cancer [2]. To date, despite multimodal intervention strategies initiated to reduce cancer-related mortality, many nations, including the USA and the UK, still grapple with significant cancer mortality rates [3,4]. To overcome this challenge, the current medical focus has been centred on early cancer detection that enables curative treatment to be administered before cancer progresses to late (and most often incurable) stages [5].

Consequently, serum biomarkers that manifest prior to the onset of cancer are highly sought after [6]. One potential group of serum biomarkers are autoantibodies that target specific tumor-associated antigens (TAAs). Since the first serological identifications of tumor antigens from the sera of melanoma patients [7], there has been an increase in the number of reports of TAAs and autoantibodies in patients with cancer [8]. The immune response to TAAs functions to remove precancerous lesions during the early events of carcinogenesis [9,10]. Hence, the production of autoantibodies as a result of cancer immunosurveillance has been

Abbreviations

AFP, alpha-fetoprotein; CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTAs, cancer-testis antigens; DCIS, ductal carcinoma *in situ*; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSP, heat shock protein; MAPPING, multiple affinity protein profiling; PGP9.5, protein gene product 9.5; PKA, cAMP-dependent protein kinase; PTMs, post-translational modifications; SEREX, serological analysis of tumor antigens by recombinant cDNA expression cloning; SERPA, serological proteome analysis; TAAs, tumor-associated antigens.

found to precede manifestations of clinical signs of tumorigenesis by several months to years [11–14]. These serological biomarkers would thus serve as early reporters for aberrant cellular processes in tumorigenesis [9].

In this review, we will discuss the discovery of TAAs and autoantibodies as biomarkers for early cancer detection. Furthermore, the identification of a panel of TAA signatures would increase the sensitivity and specificity of such diagnostic markers for cancer patients. Herein, the utility of five different approaches (SEREX, phage display, protein microarray, SERPA and MAPPING), which allow simultaneous identification of multiple autoantibodies, was also discussed. Subsequently, we reviewed TAAs and autoantibodies found in the five most common cancers (liver, lung, breast, colorectal and stomach). Lastly, we commented on the challenges encountered and solutions proposed in their clinical applications for cancer patients.

The humoral response to cancer

Production of autoantibodies

Robert W. Baldwin was the first to establish the presence of an immune response to solid tumors [15]. Immunosurveillance to cancer cells is triggered to initiate antigen-specific tumor destruction [16,17]. The autologous proteins of tumor cells, commonly referred to as TAAs, are thought to be altered in a way that renders these proteins immunogenic [8,11]. These self-proteins could be overexpressed, mutated, misfolded, or aberrantly degraded such that autoreactive immune responses in cancer patients are induced.

TAAs that have undergone post-translational modifications (PTMs) may be perceived as foreign by the immune system [8,11,18]. The presence of PTMs (e.g. glycosylation, phosphorylation, oxidation and proteolytic cleavage) could induce an immune response by generating a neo-epitope or by enhancing self-epitope presentation and affinity to the major histocompatibility complex or the T-cell receptor. The immune response against such immunogenic epitopes of TAAs induces the production of autoantibodies as serological biomarkers for cancers [19]. In addition, proteins that are aberrantly localized during malignant transformation can also provoke a humoral response. For example, cAMP-dependent protein kinase (PKA), an intracellular protein, is secreted by cancer cells. This extracellular PKA (ECPKA) is upregulated in the serum of cancer patients [20,21], and this correlates with the higher titers of autoantibodies against ECPKA in cancer patient sera compared with control

sera [22]. Another example is cyclin B1, which was found to be overexpressed and localized to the cytosol instead of to the nucleus in cancer cells [23–26].

Although some of the immune responses in cancer patients recognize neo-antigens that are found only in tumors, most tumor-associated autoantibodies are directed against self-antigens that are aberrantly expressed (e.g. HER2/neu, p53 and ras) [27–30]. The immunogenicity of p53 was believed to be initiated by its overexpression, missense point mutation and accumulation in the cytosol and nucleus of cancer cells [18,31–36]. The overexpressed proteins appear to increase the antigenic load and prime antibody production in cancer patients. Cancer-testis antigens (CTAs) that are normally only found in germline cells (e.g. testis and embryonic ovaries), and oncofetal proteins that are aberrantly expressed in various tumors (e.g. MAGE, SSX2, NY-ESO-1 and p62) are also well-known TAAs [37–39]. CTAs or overexpressed proteins may conceivably overcome the immune tolerance towards self-proteins [9,38]. More than 40 CTA gene families were found to be expressed in many tumor types [40]. Many of these aberrantly expressed proteins that trigger an immune response in cancer patients contribute to carcinogenesis processes and are therefore potential candidates in clinical trials for cancer vaccines.

It is not entirely clear how modifications of antigens trigger the humoral response, especially as many TAAs discovered thus far are intracellular proteins [41]. 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 [38,42–44]. Aberrant tumor cell death can refer to defective apoptosis, ineffective clearance of apoptotic cells or other forms of cell death, such as necrosis [45]. Repeated cycles of such aberrant tumor cell death can lead to persistent exposure of the modified intracellular proteins. Tumour cell death also releases proteases that would generate cryptic self-epitopes to trigger an autoimmune response. Another hypothesis is based on the discovery that when released upon apoptosis, some TAAs can initiate the migration of leukocytes and immature dendritic cells by interacting with specific G-protein-coupled receptors on these cells [46]. This chemotactic activity of tissue-specific TAAs may alert the immune system to danger signals from damaged tissues and promotes tissue repair. TAAs that interact with immature dendritic cells are immunogenic because they are liable to be sequestered and, subsequently, aberrantly presented to the cellular immune system.

Other hypotheses have been proposed with respect to specific immunogenic modifications. TAAs that bear

structural similarity to cross-reacting foreign antigens may elicit a humoral response as a result of structural mimicry. TAAs that bind to heat shock proteins may be immunogenic as a result of the immunomodulatory properties of the heat shock proteins [47,48]. Intracellular proteins that are relocalized to the tumor cell surface may appear unfamiliar, thereby triggering an immune response. Tumor-associated peptides that are found in blood may also serve as potential antigens. These peptides could originate from tumor intracellular proteins, as exemplified by the presence of calreticulin fragments in the sera of liver cancer patients [49], or from endogenous circulating proteins [50]. In the latter case, Villanueva *et al.* [50] discovered that tumors secrete exoproteases that cleave products of the *ex vivo* coagulation and complement degradation pathways, generating tumor-specific peptides. The immunogenicity of such peptides remains to be verified.

The generated sera autoantibodies targeting these TAAs could serve as early molecular signatures for diagnostics and prognostics of cancer patients. Furthermore, most autoantibodies found in the sera of cancer patients target cellular proteins with modifications, aberrant localization or expression that are associated with processes involved in carcinogenesis such as cell cycle progression, signal transduction, proliferation and apoptosis [51]. The identification and functional characterization of these immunological 'reporters' or 'sentinels' for cellular mechanisms associated with tumorigenesis would help to uncover the early molecular events of carcinogenesis [8,9].

Early cancer detection

The ultimate utility of autoantibodies lies in early cancer detection. Many of the well-known available tumor-associated serum biomarkers, such as carcinoembryonic antigen (CEA) for colon cancer, alpha-fetoprotein (AFP) for liver cancer, prostate-specific antigen for prostate cancer, cancer antigen CA19-9 for gastrointestinal cancer and CA-125 for ovarian cancer, lack sufficient specificity and sensitivity for use in early cancer diagnosis. The immune response to TAAs occurs at an early stage during tumorigenesis, as illustrated by the detection of high titers of autoantibodies in patients with early stage cancer [52]. The immune response to TAAs has also been shown to correlate with the progression of malignant transformation [53,54]. Thus, the production of autoantibodies can be detected before any other biomarkers or phenotypic aberrations are observed, rendering such autoantibodies indispensable as biomarkers for early cancer detection [43,55].

In addition, autoantibodies possess various characteristics that enable them to be valuable early cancer biomarkers [8,11,18,56]. First, autoantibodies can be detected in the asymptomatic stage of cancer, and in some cases, may be detectable as early as 5 years before the onset of disease [43]. Second, autoantibodies against TAAs are found in the sera of cancer patients where they are easily accessible to screening. Third, autoantibodies are inherently stable and persist in the serum for a relatively long period of time because they are generally not subjected to the types of proteolysis observed in other polypeptides. The persistence and stability of the autoantibodies give them an advantage over other biomarkers, including the TAAs themselves, which are transiently secreted and may be rapidly degraded or cleared. Moreover, the autoantibodies are present in considerably higher concentrations than their respective TAAs; many autoantibodies are amplified by the immune system in response to a single autoantigen. Consequently, autoantibodies may be more readily detectable than their corresponding TAAs. Lastly, sample collection is simplified as a result of the long half-life (7 days) of the autoantibodies, which minimizes hourly fluctuations. Moreover, the variety of reagents and techniques available for antibody detection facilitates the development of assays for these autoantibodies.

Nonetheless, autoantibodies do have their limitations. A single autoantibody test lacks the sensitivity and specificity required for cancer screening and diagnosis. Typically, autoantibodies against a particular TAA are found in only 10–30% of patients [56]. The reason for this low sensitivity lies in the heterogenic nature of cancer, whereby different proteins are aberrantly processed or regulated in patients with the same type of cancer. Hence, no protein is likely to be commonly perturbed or immunogenic across a particular cancer type. Moreover, some TAAs, for instance p53, are present in different cancer types and so lack discrimination power in diagnosing a specific cancer. Certain TAAs may also be nonspecific, as they arise both in cancer and in other diseases, particularly those with an autoimmune background such as systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis, type 1 diabetes mellitus and autoimmune thyroid disease [8,57,58]. Moreover, in some circumstances, autoantibodies may be detected in normal individuals.

TAA panels

As stated above, although a single autoantigen would lack adequate sensitivity and specificity, a panel of TAAs may overcome this problem by enabling

multiple autoantibodies to be detected simultaneously [56,59,60]. For example, autoantibodies to a panel of two TAAs (Koc and p62) have been shown to differentiate patients with 10 different cancer types, and autoimmune diseases, from normal subjects [59,61]. Using a panel of seven TAAs (c-myc, p53, cyclin B, p62, Koc, IMP1 and survivin), Koziol *et al.* [62] were able to identify normal individuals and discriminate among patients with breast, colon, gastric, liver, lung or prostate cancers, with sensitivities ranging from 77 to 92% and specificities ranging from 85 to 91%. Zhang *et al.* [63] analyzed 527 sera from six different cancer types [breast, lung, prostate, gastric, colorectal and hepatocellular carcinoma (HCC)], and demonstrated that successive addition of antigen to the same panel of seven TAAs increased the immunoreactivity in cancer patients to 44–68%, but did not increase the immunoreactivity in healthy individuals. Several other studies have reported similar findings, which demonstrated the high sensitivity and specificity that a panel of carefully selected TAAs can achieve in cancer diagnosis [60,64–67].

Although the application of several antibodies or autoantigens would detect cancer with higher efficiency than a single biomarker [11,62,68–72], it should be emphasized that the inclusion of antigens in a panel of TAAs has to be selective for optimization of sensitivity and specificity because not all antigens targeted by antibodies are cancer-specific [56]. The discovery of panels of TAAs that are immunoreactive and have high specificity and sensitivity at the early cancer stage could thus aid in the identification of autoantibody signatures that may represent novel diagnostic biomarkers. The repertoire of TAAs can also be used as markers for monitoring disease progression or therapy efficacy, or as potential therapeutic targets [8,9,60,63,66,68,73,74].

Methods for identifying autoantibodies

Initial studies of TAAs have focused on a few antigens at a time, using techniques such as 1D SDS/PAGE or ELISA. Improvements in technologies such as proteomics platforms have enabled the generation of a panel of TAAs that exhibit better diagnostic value than a single TAA marker [63]. With advances in the development of technologies for autoantibody identification, several high-throughput methods available for uncovering autoantibodies have become increasingly well defined.

Five main techniques, encompassing serological screening of cDNA expression libraries, phage-display libraries, protein microarrays, 2D western blots and

2D immunoaffinity chromatography, can be utilized in this area of research (summarized in Fig. 1). In contrast to the conventional one-TAA-at-a-time approach, the common characteristic of these methods is that many TAAs can be discovered concomitantly [8,11,75,76]. Thus, these strategies can potentially identify panels of TAAs with high diagnostic value.

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

Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX) was first developed in 1995 [38]. SEREX involves the identification of TAAs by screening patient sera against a cDNA expression library obtained from the autologous tumor tissues [16] (Fig. 1A). By using SEREX, Sahin *et al.* [38] showed that 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. More than 2300 of these autoantigens are documented in a public access online database known as the Cancer Immunome Database (CID) <http://ludwig-sun5.unil.ch/CancerImmunomeDB/> [77–80].

The application of SEREX has facilitated the identification of TAAs as potential cancer biomarkers [81,82] in various types of cancer, including lung, liver, breast, prostate, ovarian, renal, head and neck, and esophageal cancers, and in leukemia and melanoma [83–91]. The panel of SEREX-defined immunogenic tumor antigens include CTAs (e.g. NY-ESO-1, SSX2, MAGE), mutational antigens (e.g. p53), differentiation antigens (e.g. tyrosinase, SOX2, ZIC2) and embryonic proteins [39,83,87,92]. Although many of these TAAs are potential serological biomarkers, several are reported to have low sensitivity. As discussed earlier, the combination of several antigens in the panel would greatly increase the sensitivity [93].

There are, however, some limitations to the SEREX approach [29,30]. First, TAAs identified by SEREX are mainly linear epitopes and tend to be gene products that can be expressed in bacteria. Second, there is a bias towards antigens that are highly expressed in the tumor tissues used to generate cDNA libraries [94]. Thus, overexpression of the antigens is often responsible for their immunogenicity detected by SEREX. For example, autoantibodies to CTAs, which are normally restricted to primitive germ cells but are overexpressed in tumor tissues, have often been detected by SEREX [95]. However, TAAs that are of low abundance are missed by SEREX. Third, because of the need to construct cDNA libraries to clone into expression vectors

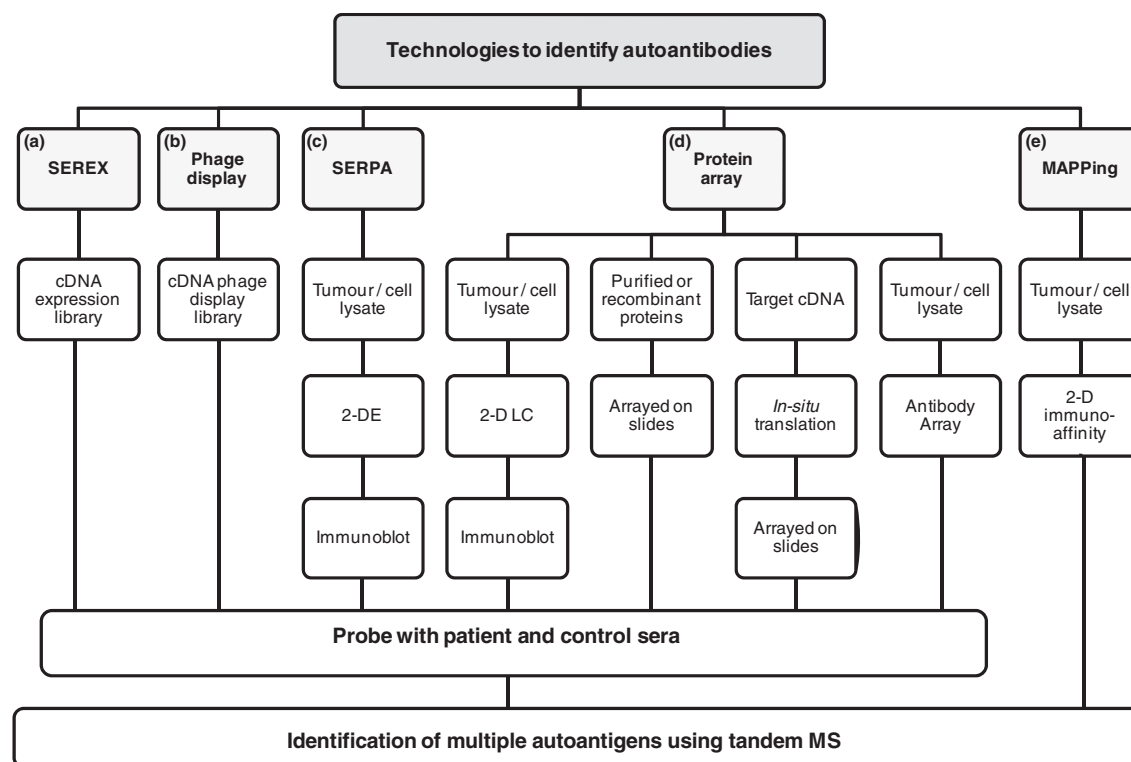


Fig. 1. Overview of five different approaches that enable identification of multiple autoantibodies simultaneously.

and the subsequent need to screen a large pool of cDNA clones, SEREX is time-consuming, labour-intensive and not amenable to automation. Thus, this approach is not applicable for analyzing a large number of patient serum samples with high throughput. Lastly, post-translational modifications cannot be detected by SEREX.

Improvements to the SEREX approach have been made to improve the identification of TAAs [96–99]. One improvement involves the screening of cDNA libraries with allogenic sera and autologous sera to eliminate false-positive results caused by noncancer-specific and patient-specific antigens. Krause *et al.* [100] evaluated reactive phage clones using panels of allogenic sera from cancer patients and control individuals to identify antigens associated with tumorigenesis. As the cDNA expression libraries are constructed from a tumor tissue specimen, SEREX is limited to identifying TAAs from the tumor of one patient. Owing to the heterogeneity of genes in the different cell types in tumor tissues, some groups have used established cancer cell lines as a source of cDNA for SEREX in cancers [101,102]. Phage display and eukaryotic expression systems have also been used to construct cDNA expression libraries in some studies [56,72,79,94,103–110].

Phage display

In the phage display method, a cDNA phage display library is constructed using a tumor tissue or cancer cell line [111] (Fig. 1B). Peptides from the tumor or cell line are expressed as fusions with phage proteins and are displayed on the phage surface. This feature of the method allows cost-effective and labour-effective screening during biopanning. Autoantibodies in patient serum are captured by the phage display library through successive rounds of immunoprecipitation and the corresponding antigens are sequenced for identification. TAAs for prostate and ovarian cancers, amongst others, have been identified using this approach [106,112]. Some caveats associated with this technique include the need to sequence each immuno-reactive phage clone and the preclusion of conformational epitopes of native antigens [68,111]. This method also excludes proteins that cannot be displayed on the surface of the phage species [113]. Although this method is of higher throughput than SEREX, antigens with post-translational modifications (e.g. glycosylated cancer antigens) cannot also be detected [8,106].

Phage clones that bind specifically to cancer sera are selected using a differential biopanning approach [114]. In the first phase of biopanning, protein-G beads are

incubated with pooled normal sera. Protein-G beads with bound IgGs are then incubated with a phage tumor/cancer cell line-derived cDNA library. Phage clones that bind are precluded from the next round of biopanning because they react with normal sera. In the second phase of biopanning, protein-IgG beads are incubated with cancer sera. Protein-IgG beads with bound IgGs are incubated with the same phage cDNA library, with the exception of noncancer specific phage clones that were excluded in the first phase. Phage clones that bind to the bound IgGs are eluted and amplified for the next round of biopanning with cancer sera. After iterative rounds of biopanning, phage clones that bind specifically to cancer sera are obtained. These clones are then arrayed onto glass slides [114] or nitrocellulose membranes [110] and subjected to further serological screenings. Panels of TAAs that yield reasonable sensitivities and specificities for ovarian cancer [110], prostate cancer [68,106], non-small cell lung cancer (NSCLC) [115], breast cancer [104,116] and colorectal cancer (CRC) [105] have been identified in this way.

Recent improvements in technology have enabled the generation of phage-based protein/peptide microarrays, containing thousands of phages, for high-throughput serological screening to identify TAAs in large cohorts of cancer patients [68,73,110,114,116–118]. For example, Wang *et al.* [68] analysed sera from 119 prostate cancer patients and 138 healthy individuals using an array of a phage-display library. A panel of 22 peptide antigens was identified with sensitivity (81.6%) and specificity (88.2%) that were better than for prostate-specific antigen. Similarly, Chatterjee *et al.* [110] employed protein microarrays containing 480 antigen clones from a phage display cDNA library of an ovarian cancer cell line. Autoantibodies specific to 62 antigens were identified in patients with ovarian cancer.

Protein microarray

Protein microarrays enable high-throughput and scalable analyses and are powerful tools for screening the immune response in cancer patients to elucidate autoantibodies and TAAs [67,69]. Purified or recombinant proteins, synthetic peptides, or fractionated proteins from tumor or cancer cell lysates are spotted systematically onto microarrays and then incubated with specific sera [8,11] (Fig. 1D). The array platform can be two dimensional (such as glass slides, nitrocellulose membranes and microtitre plates) or three dimensional (such as beads and nanoparticles). Because of its miniature platform, the amount of samples and

reagents needed are greatly reduced [119]. Protein array technology enables the identification of antigens with PTMs (e.g. glycosylated TAAs have been detected using glycan arrays) [120]. Moreover, this method has the potential to detect unknown proteins as novel TAAs.

In this method, antibody–antigen interactions have been studied to identify autoantibodies from patients with autoimmune diseases and cancers such as colorectal, breast, ovarian, stomach, lung, and prostate cancer, and HCC [56,60,62,93,121–125]. 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 and vaccines [126,127]. However, studies using protein microarrays are hampered by the short shelf-life of arrayed proteins and difficulties in purifying or producing native protein targets [8,128]. To circumvent this, natural protein microarrays are prepared in which liquid-based fractionated proteins from cancer cell lysates, instead of purified proteins, are spotted [66,129]. Sera antibodies against ubiquitin C-terminal hydrolase L3 were identified in colon cancer patients by fractionating cancer cell lysate onto a nitrocellulose-based array [14]. Similarly, Hanash's team fractionated protein lysates from a lung adenocarcinoma cell line using multidimensional liquid chromatography onto a nitrocellulose-coated microarray [66]. Madoz-Gurpide *et al.* [129] also combined liquid phase separations with microarray technology to detect autoantibodies to tumor antigens. Recently, similar natural protein microarrays have been generated to identify autoantibodies of lung and prostate cancer [130,131]. Nonetheless, further steps are necessary to identify specific immune-reactive proteins in the respective protein fractions.

In an attempt to combat the protein amplification problem, Ramachandran *et al.* [128] devised self-assembling protein microarrays that effectively obviated the need for purified proteins and side-stepped protein storage problems. Target cDNAs are printed onto glass slides, and transcribed and translated *in situ* in a cell-free expression system. The resultant proteins can then be screened accordingly. This self-assembling protein microarray technology yields an advantage over the natural protein microarray in that it allows TAAs to be identified readily. Using a similar approach, Anderson *et al.* [125] developed programmable protein microarrays ELISA that, when probed with breast cancer sera, showed reactivity against known autoantigens such as p53.

With progress in technology, the difficulties associated with protein production have slowly been over-

come. This has led to the production of commercial human protein arrays. One such example is the Proto-Array human protein microarray from Invitrogen that is able to analyze more than 80 000 recombinant antigens [124]. Hudson *et al.* [124] recently demonstrated the use of this protein microarray in elucidating 94 autoantigens present in ovarian cancer patients. Other challenges that need to be overcome include the requirement for sophisticated bioinformatics and statistical software, optimization of conditions for antigen spotting and eliminating modifications of antigenic epitopes on the array surface [123,132]. The high-throughput utility of protein microarrays has accelerated the discovery of the autoantibody signature to identify novel cancer biomarkers for early diagnosis, monitoring of disease progression and response to treatment, and development of individualized therapies [123,131].

Reverse-capture microarray

A research group headed by Brian Liu presented a “reverse-capture” microarray method that is based on a dual-antibody sandwich ELISA [133–135]. 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 configuration. Meanwhile, IgGs from patient and control sera are purified and labeled with different fluorescent dyes and then incubated with the antigen-bound microarrays (Fig. 1D). Consequently, autoantibodies that are cancer-specific can be identified. The reverse-capture microarray removes the need for recombinant proteins and allows the instant identification of cancer-specific autoantibodies. More significantly, this platform enables the analysis of native antigens. Previously, five TAAs (von Willebrand Factor, IgM, alpha1-antichymotrypsin, villin and IgG) were identified by screening prostate cancer sera against an array containing 184 antibodies [136]. Application of the ‘reverse-capture’ microarray technology by Qin *et al.* [133] identified 48 TAAs from prostate cancer sera, including p53 and Myc. However, only known antigens with commercially available antibodies can be analyzed. Furthermore, immunoreactivity with post-translationally modified antigens cannot be differentiated unless antibodies that can specifically and exclusively bind to such antigens are commercially available.

Serological proteome analysis (SERPA)

Another commonly used technique is the proteomics-based approach termed SERPA [137] or Proteomex [138]. It involves the discovery of TAAs using a combi-

nation of 2D electrophoresis, western blotting and MS [8,139,140]. Proteins from tumor tissues or cell lines are separated by 2D electrophoresis, transferred onto membranes by electroblotting and subsequently probed with sera from healthy individuals or patients with cancer. The respective immunoreactive profiles are compared and the cancer-associated antigenic spots are identified by MS (Fig. 1C). Klade *et al.* [137] developed SERPA, and identified two TAAs (SM22-alpha and CAI) in kidney cancer patients. Kellner *et al.* [138] showed that several members of the cytoskeletal family (such as cytokeratin 8, stathmin and vimentin) are potential TAAs that could distinguish different renal cell carcinoma subtypes from the normal renal epithelium tissues.

2D electrophoresis is indisputably the classical technique for proteome analysis. Proteins are first separated according to their isoelectric points and then according to their molecular weights [141]. Despite some limitations, 2D electrophoresis is still the best method for the high-resolution separation of a complex mixture of proteins, and its efficacy in distinguishing post-translationally modified proteins and protein isoforms is unparalleled. Consequently, when coupled with western blotting for serological screening, autoantibodies can be used to detect TAAs that have undergone post-translational modifications. Most of these antigens can be subsequently identified with the aid of MS. SERPA avoids the time-consuming construction of cDNA libraries that are required in SEREX or phage-display technology. 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 and difficulty in producing reproducible 2D gels [123,142]. Because of the way that western blots are prepared, only linear epitopes can be detected [56].

SERPA has been applied in the study of many cancers, such as neuroblastoma, lung carcinoma, breast carcinoma, renal cell carcinoma, HCC and ovarian cancer [142–146] to detect novel autoantibodies and autoantigens as early indicators of tumorigenesis [10,68,147]. For example, the use of SERPA has identified calreticulin and DEAD-box protein 48 (DDX48) in pancreatic cancer [148–150]; Rho GDP dissociation inhibitor 2 in leukemia [151]; and peroxiredoxin 6, triphosphatase isomerase (Tim) and manganese superoxide dismutase (MnSOD) in squamous cell carcinoma [152,153].

Multiple affinity protein profiling (MAPPING)

MAPPING involves 2D immunoaffinity chromatography followed by the identification of TAAs by tandem

MS (nano LC MS/MS) [154]. In the first phase of immunoaffinity chromatography, nonspecific TAAs in a cancer cell line or tumor tissue lysate bind to IgG obtained from healthy controls in the immunoaffinity column and are removed from the lysate. The 'flow-through fraction' of the lysate is then subjected to the 2D immunoaffinity column that contains IgG from cancer patients (Fig.1E) [155]. TAAs that bind at that time are likely to be cancer-specific and are eluted for enzymatic digestion and identification by tandem MS. Hardouin *et al.* [154] used this approach to screen sera for autoantibodies from patients with CRC. The 2D immunoaffinity chromatography described here is similar to that used in the differential biopanning phase of the phage display method discussed earlier. In the former, cell or tissue lysates are added to immunoaffinity columns, whereas in the latter, cDNA phage display libraries are added to protein-G beads bound with IgG.

Cancer-associated autoantibodies

The hunt for relevant autoantibodies has intensified in recent years, as evidenced by a search for 'autoantibodies and cancer' on PubMed. Autoantibodies and TAAs have been found many cancers such as HCC, and in lung, colorectal, breast, stomach, prostate and pancreatic cancers [25,42,43,68,84,148,149,151,156–159]. The growing list of TAAs identified in cancers include oncoproteins (e.g. HER-2/Neu, ras and c-MYC) [27,52,160–163], tumor suppressor proteins (e.g. p53) [31], survival proteins (e.g. survivin) [93,157,164,165], cell cycle regulatory proteins (e.g. cyclin B1) [25], mitosis-associated proteins (e.g. centromere protein F) [166], mRNA-binding proteins (e.g. p62, IMP1, and Koc) [61,167–169], and differentiation and CTAs (e.g. tyrosinase and NY-ESO-1) [39,83,170–172]. The following section shall discuss studies of autoantibodies in the five major cancers.

1.3.1 Liver cancer

HCC, the predominant form of primary liver cancer, is the fifth most common malignancy in the world [2,173]. More significantly, it is the third leading cause of cancer-related death worldwide, with a mortality rate comparable to its incidence rate. The survival rate after the onset of symptoms is generally less than one year [174]. Two main factors contribute to the high mortality of HCC. One is the late presentation of HCC, as the dearth of symptoms at the early stages of the disease results in detection of this cancer only when it is at an advanced stage. Another is the paucity of

curative treatments for late-stage HCC. Consequently, in most cases, by the time diagnosis is made, no curative treatment is available [174].

Historically, HCC has been more prevalent in developing countries such as Asia. While this heterogeneous geographical distribution persists, formerly low-incidence areas, particularly Europe and the USA, have witnessed a rising incidence of HCC in the past decade [175]. The incidence and mortality rates of HCC in these areas are expected to double over the next two decades. As a result, much interest in the study of this malignancy has been generated [176].

The gold standard for HCC diagnosis is the histological examination of the hepatic mass [177]. Although ultrasound fares better with a sensitivity of 100%, a specificity of 98% and a positive predictive value of 78% [178], the efficacy of ultrasound is operator-dependent, and, against a cirrhotic background, small tumors cannot easily be detected [176]. In terms of serum biomarkers, AFP is still the best available for HCC diagnosis.

AFP is a normal serum protein that is synthesized primarily during embryonic development but is maintained at a low concentration ($< 20 \text{ ng}\cdot\text{mL}^{-1}$) in healthy adult men and nonpregnant women. Elevated serum AFP levels are observed in pregnant women and in patients with chronic liver disease. Consequently, AFP is sufficiently specific for HCC only when its serum levels rise above $500 \text{ ng}\cdot\text{mL}^{-1}$. This implies that AFP cannot be used as a marker for small HCC tumors and also indicates that AFP is a fairly specific, but insensitive, marker for HCC [179]. AFP has a low sensitivity (40–65%), a variable specificity (75–90%) and a low positive predictive value (12%) [180]. To counteract this, des-gamma-carboxy prothrombin (DCP), a serum protein that has 50–60% positivity in HCC, is sometimes used in combination with AFP for HCC diagnosis, a method that is deemed by some clinicians to be superior to the use of a single biomarker test. A glycoform (AFP-L3) and an isoform (Band + II) of AFP, demonstrating higher specificities, have also been recommended as diagnostic tools [181]. Nonetheless, there is an impetus to find new biomarkers that are more sensitive and specific for HCC and that can detect HCC in its early stages.

Autoantibodies to TAAs have been identified in HCC serum samples at the early stage of liver disease [182,183]. These TAAs are potential biomarkers that allow the early diagnosis of HCC because their autoantibodies are detectable before the development of HCC malignancy. The progression from chronic liver disease to HCC is also associated with the detection of increasing titers of autoantibodies to

specific antigens that are over-expressed in the tumors [183–185]. Two of the more established HCC-associated TAAs are p53 [31,186] and p62 [37,169]. Autoantibodies against p62 were found in 21% of HCC patients who re-expressed this oncofetal protein but were not found in healthy individuals or in patients with noncancerous liver diseases [168]. In a later study by Lu *et al.*, [37] the aberrant expression of p62 was found to contribute to abnormal cellular proliferation in HCC and cirrhosis by regulating growth factors. The potential for autoantibodies to p53 to be early diagnostic biomarkers for HCC has also been demonstrated by their presence in individuals who have a high risk of developing HCC, as exemplified in individuals with chronic liver disease [186].

Many other TAAs immunoreactive in HCC sera have been discovered [187–191]. Takashima *et al.* [189] employed SEREX and identified heat shock 70 kDa protein 1 (HSP70), glyceraldehyde-3-phosphate dehydrogenase, peroxiredoxin and MnSOD as candidate diagnostic biomarkers for HCC. SEREX-identified autoantibody reactivity to HCC-22-5 was as high as 78.9% in AFP-negative HCC patients but was not detected in the sera of lung or gastrointestinal cancer patients, or in normal controls [188]. Stenner-Liewen *et al.* [192] found 19 distinct antigens that were associated with HCC, of which three were novel. Wang *et al.* [85] identified 55 cDNA sequences that could code for HCC-associated antigens. Uemura *et al.* [193] found 27 TAAs. Le Naour *et al.* [194] identified eight TAAs, but only one (an autoantibody against a novel truncated form of calreticulin) was commonly induced in HCC.

Chronic hepatitis B virus (HBV) infection and cirrhosis are well-known major risk factors for HCC [195]. In fact, persistent infection with HBV is one of the most important risk factors for HCC. A 1988 study estimated that chronic HBV infection accounted for 75–90% of HCC cases worldwide [196], while a recent report attributed 53% of global HCC cases to HBV infection [197]. Any form of cirrhosis can lead to HCC, but HBV and hepatitis C virus (HCV) infection, alcoholic liver disease and hereditary hemochromatosis are the most frequent antecedents [173]. Independently of other risk factors, cirrhosis is the single most significant risk factor for the development of HCC [198]. Indeed, cirrhosis is described as a preneoplastic stage that often precedes HCC. Reportedly, 80–90% of HCC cases develop against a cirrhotic background, and cirrhotic patients have an annual HCC incidence of 2.0–6.6%, as opposed to noncirrhotic patients, whose HCC incidence is 0.4% [176]. In particular, a study by Perz *et al.* [197] attributed 30% of cirrhosis cases to HBV. Cirrhosis and HBV infection are proba-

bly synergistic risk factors for HCC. In fact, chronic HBV-infected patients with cirrhosis are more prone to HCC than their counterparts without cirrhosis. In countries with high HBV endemicity, patients with HBV infection and cirrhosis have a three-fold higher risk of developing HCC than those with HBV infection but no cirrhosis, and a 16-fold higher risk of developing HCC than inactive carriers [199]. Autoantibodies against TAAs can be found in HBV-associated HCC patients and those that can be detected in the early stage of the disease can thus facilitate early diagnosis.

In some of these HCC patients, the production of autoantibodies correlates with the transition from chronic liver disease to HCC [182,183]. Autoantibodies that are found in cirrhosis patients are of particular interest because cirrhosis generally precedes HBV-associated HCC development. Cirrhosis-associated autoantibodies can thus highlight individuals at risk of developing HCC and aid risk stratification for early HCC detection. For example, the antibody titers to DNA topoisomerase II were shown to increase in patients during the progression from HCV-related chronic hepatitis to liver cancer [200]. These TAAs were found to participate in the malignant transformation of HCC. The use of SERPA by Le Naour *et al.* [194] showed that autoantibodies against b-tubulin, creatine kinase-B, heat shock protein 60 (HSP60) and cytokeratin 18 are present in the sera of patients chronically infected with HBV and/or HCV. However, autoantibodies against calreticulin, cytokeratin 8, F1-ATP synthase b subunit and NDPKA are restricted to patients with HCC [194].

A panel of TAAs would certainly enhance the ability to detect autoantibodies in HCC patients. Using SERPA and protein microarrays, humoral responses to DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, eukaryotic translation elongation factor 2 (eEF2), apoptosis-inducing factor (AIF), heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2), prostatic binding protein, and triosephosphate isomerase (TIM), were found to be significantly higher in patients with HCC than in patients with chronic hepatitis or normal individuals. Immunoreactivity to four of these antigens (DEAD box polypeptide 3, eEF2, AIF and prostatic binding protein) was shown to be significantly more common in HCC than in other cancer types. The sensitivity of any of these antigens in patients with stage I HCC ranged from 50 to 85%. When these four antigens were analyzed as a panel, the sensitivity increased to 90%. Hence, autoantibodies against this panel of six antigens may be used as early diagnostic biomarkers of HCC [190]. Likewise, using a panel of TAAs, Zhang *et al.* demonstrated a significantly higher frequency of

autoantibody-positive liver cancer patients (58.9%) compared to patients with chronic hepatitis (20%) or cirrhosis (30%), or to normal individuals (12.2%). In contrast, the antibody frequency to any one TAA in the panel was low, varying from 9.9 to 21.8% in liver cancer patients [201]. Recently, the frequency of autoantibodies to five HCC-associated antigens was found to be higher in sera from patients with HCC than in sera from patients with chronic hepatitis and normal sera. The sensitivity and specificity of three of the antigens (KRT23, AHSB and FTL) was up to 98.2% in a joint test and 90.0% in series test separately [202].

Lung cancer

Lung cancer is responsible for the largest number of cancer-related deaths worldwide [2,203]. This high mortality rate can be accounted for partly by the late diagnosis of the disease. To add to the problem, there is no established diagnostic test for early detection because the cancer is notoriously heterogeneous [204]. The search for a suitable panel of TAAs is ongoing and the results are promising.

With the use of SERPA in two separate studies, Brichory *et al.* reported the discovery of sera autoantibodies against protein gene product 9.5 (PGP 9.5) and annexins I and II in patients with adenocarcinoma of the lung, with a sensitivity of 14%, 30% and 33% respectively [13,145]. Although 60% of these patients exhibited reactivity against glycosylated annexin I and II, and none of the healthy controls showed such immunoreactivity, autoantibodies against annexin II were also found in patients with other cancers. Nevertheless, autoantibodies directed to annexin I were found only in lung cancer patients [13]. In a later study, Pereira-Faca *et al.* [205] performed western blotting of chromatographic fractionated protein extracts from lung cancer cell lines, and identified autoantibodies against 14-3-3 theta. They also tested sera against a panel of three proteins – 14-3-3 theta and two previously identified antigens, annexin I and PGP 9.5. This panel gave a sensitivity of 55% and specificity of 95% in identifying lung cancer at the preclinical stage [205]. After further validation, it was discovered that reactivity against PGP 9.5 was not as significant. Instead, annexin I, 14-3-3 theta and a novel lung cancer antigen, LAMR1, demonstrated significant reactivity to prediagnostic sera [206].

Nakanishi *et al.* [139] probed A549 lung adenocarcinoma cell lysate with patient sera and found eight autoantibodies that were reactive with lung cancer sera but not with lung tuberculosis sera or with healthy sera. Yang *et al.* [153] reported reactivity against

triosephosphate isomerase and MnSOD with approximately 20% sensitivity. He *et al.* [207] found autoantibodies against α -enolase in 28% of 94 lung cancer patients. From these three studies, autoantibodies against two proteins, triosephosphate isomerase and α -enolase, were commonly observed in patients with lung cancer.

As demonstrated by the increased sensitivity and specificity when analyzing all five phage-expressed proteins for nonsmall cell lung cancer, a panel of multiple antigens has a higher predictive value than a single marker [108]. Likewise, Chapman *et al.* [71] tested a panel of seven TAAs comprising c-myc, p53, HER-2, MUC1, NY-ESO-1, CAGE and GBU4-5, against 104 patients and 50 noncancer individuals, and achieved a panel sensitivity of 76% and specificity of 92% for detecting lung cancer at an early stage.

Many studies have uncovered potentially useful autoantibodies that might aid early lung cancer detection. Antibodies against p53 were found in heavy smokers, in individuals with chronic obstructive pulmonary disease, or in individuals as a result of occupational hazards (e.g. exposure to vinyl chloride and uranium) before apparent clinical signs of lung cancer were evident [31,59]. The decrease of antibodies against p53 was found to correlate with a good response to early therapy in lung cancer patients [208,209]. Zhong *et al.* [115] has identified tumor-associated autoantibodies for nonsmall cell lung cancer that could detect the cancer 5 years before it could be detected using autoradiography. However, while the autoantibodies can discriminate between lung cancer and healthy individuals, they are seldom able to distinguish between lung cancer subtypes, for example, between small cell lung cancer and nonsmall cell lung cancer [13,71,145,207]. Recently, Türeci *et al.* [172] demonstrated that NY-ESO-1 autoantibodies may be used to distinguish between patients with small cell lung cancer and nonsmall cell lung cancer. Nagashio *et al.* [210] screened sera from patients with adenocarcinoma and small cell lung carcinoma by 2D immunoblotting with cell lysates of four cell lines. Cytokeratin 18 and villin1 were identified as TAAs, and this was validated using an immunohistochemistry study of pulmonary carcinomas of various histologic types. The authors demonstrated that cytokeratin 18 and villin1 could be used to differentiate adenocarcinoma from small cell lung cancer.

Breast cancer

After lung cancer, breast cancer is the second most common cancer in the world, and is the most common

cancer in women [2]. To facilitate early detection, mammography is recommended for women over 40 years of age, and this screening approach has been shown to reduce cancer mortality rates [211,212]. However, even with mammography, fewer than 50% of breast cancers were localized when detected [55]. Moreover, smaller tumors tend to be missed with mammography [213]. Biomarkers accepted for clinical use, such as CA 15-3, CEA and CA 27-29, have low sensitivity and specificity, and are thus more useful for patients at an advanced stage of breast cancer rather than for early cancer diagnosis [211]. Consequently, autoantibodies that can be found in the sera of prediagnostic women with breast cancer are highly sought after [43,213,214].

Autoantibodies against p53 [215], HER2 [216], MUC1 [217] and NY-ESO-1 [83] were some of the first to be discovered in patients with breast cancer. Although some of the autoantibodies, such as anti-HER2/*neu* antibodies [216], have been detected in patients with early stage breast cancer, these autoantibodies tend to be ubiquitous in other cancers and are thus not unique to breast cancer [43,83,213,218]. In 30% of patients with ductal carcinoma *in situ* (DCIS), over-expression of HER2/*neu* and serum antibodies directed against HER2/*neu* was found [52,160]. However, antibodies to HER-2/*neu* were also found in 46% of CRC patients with over-expressed HER-2/*neu* protein, but only in 5% of patients without HER-2/*neu* over-expression [28]. Looi *et al.* showed that successive addition of three TAAs (p16, p53 and c-myc) resulted in a stepwise increase in the sensitivity of the autoantibodies in cancer patients, such as 44% in breast cancer [65]. The focus then shifted to developing TAA panels that gave better sensitivity and specificity [43,214]. Hence, methods that allowed the simultaneous detection of multiple autoantibodies were utilized. For instance, the applications of SEREX have found autoantibodies against annexin XI-A, p80, S6, RPA32 [97] and NY-BR-1 [98,211,213,219]. A recent study also uncovered autoantibodies against three proteins; ankyrin repeat and SOCS box protein 9 (ASB-9), serine active site containing 1 (SERAC1) and receptor expressed in lymphoid tissues (RELT) [116]. Using SERPA, autoantibodies against RNA-binding protein regulatory subunit (RS), DJ-1 oncogene, glucose-6-phosphate dehydrogenase, heat shock 70-kDa protein 1 (HS71) and dihydrolipoamide dehydrogenase have been identified [12,43,155].

Ultimately, TAA panels consisting of multiple autoantigens, rather than single autoantigens, are of clinical use. Chapman *et al.* [70] tested 94 normal sera, 97 primary breast cancer sera and 40 DCIS sera

against a TAA panel comprising seven antigens (p53, c-myc, HER2, NY-ESO-1, BRCA1, BRCA2 and MUC1). Upon statistical analysis, BRCA1 was found to have no diagnostic potential and was excluded from the panel. For individual autoantigen assays, the sensitivity was between 8% and 34% for primary breast cancer and between 3% and 23% for DCIS. The specificity was between 91% and 98% for both types of breast cancer. However, as a panel comprising six autoantigens, the sensitivity increased to 64% for primary breast cancer and to 45% for DCIS. The specificity was 85%. This improvement in sensitivity is of significance to aid mammography in detecting early breast cancer.

Recently, using the SERPA approach, Desmetz *et al.* [220] reported autoantibodies against HSP60 in breast cancer patients. Furthermore, using ELISA, they tested 107 breast cancer sera (49 from patients with DCIS and 58 from patients with early stage breast cancer), 20 sera of other cancers, 20 autoimmune sera and 93 healthy sera for reactivity against HSP60. Autoantibodies against HSP60 were found in 31% of patients with early breast cancer and in 32.6% of patients with DCIS, but in only 4.3% of healthy individuals and in no patients in other control groups. Hamrita *et al.*, [221] also using the SERPA method, analyzed sera from patients with invasive breast cancer. Interestingly, autoantibodies against HSP60 were detected in 19 out of 40 breast cancer patients (47.5%) but in only 2 out of 42 healthy individuals (4.7%). Both of these studies indicate that HSP60 can serve as a potential TAA for diagnosis of noninvasive and invasive ductal carcinoma. Subsequently, Desmetz *et al.* [222] showed that autoantibodies against a combination of HSP60 with four other TAAs (PPIA, PRDX2, FKBP52 and MUC1) could be used for early diagnosis, as they were associated with DCIS and early invasive breast cancer.

The conventional SERPA approach probes sera against immunoblots of resolved tumor/cancer cell lysates. Yi *et al.* [218] screened sera against immunoblots of resolved urinary proteins from breast cancer patients and found reactivity against alpha 2-HS glycoprotein. Upon validation with a commercial antigen, they detected autoantibodies against alpha 2-HS glycoprotein in 79.1% of breast cancer patients (64 out of 81) and in 9.6% of controls (7 out of 73). Cancer-associated autoantibodies against urinary proteins have not been well characterized and the utility of alpha 2-HS glycoprotein remains to be validated.

Several of the autoantibodies found in breast cancer patients target proteins involved in pathways that play crucial roles in breast cancer tumorigenesis [43]. For example, proteins involved in the rapamycin-sensitive

mammalian target of rapamycin (mTOR) phosphorylation pathway, such as ribosomal protein S6, eukaryotic elongation factor 2, eukaryotic elongation factor 2 kinase and heat shock protein 90 (HSP90) have been identified as TAAs in patients with breast cancer. Autoantibodies in breast cancer patients are also directed against components of the DNA repair pathways, such as Ku protein, topoisomerase I and the 32-kDa subunit of replication protein A. These studies demonstrated that the identification of autoantibodies and TAAs has the potential to elucidate novel molecular mechanisms of tumorigenesis.

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related deaths in developed countries [2]. In terms of serum biomarkers, CEA is the only biomarker that has clinical use [223], but it suffers from poor sensitivity and specificity. Numerous autoantibodies associated with CRC have been reported. These include GA-733-2 [224,225], p53 [226], Fas/CD95 [227], MUC5AC [228] and p16 [229].

Seroreactivity to p53 has been detected in patients with precancerous lesions and in individuals with high risk for CRC, such as those with ulcerative colitis. However, screening for antibodies to p53 in CRC patients has been suggested only as a supplement to colonoscopy [93,229,230]. This is a result of its low specificity because antibodies against p53 have also been found in individuals at risk of other cancers, such as in heavy smokers who are at high risk for lung cancer. Recently, He *et al.* [231] detected higher titers of autoantibodies to HSP60 in the sera of patients with CRC than in healthy individuals. However, autoantibodies to HSP60 have also been reported in other patients, such as those with breast cancer.

To evaluate the efficacy of TAA panels in CRC diagnosis, Chen *et al.* probed test and control sera against the following five TAAs: p53, c-myc, cyclin B1, cyclin D1 and Calnuc. The results showed that 65.4% of 52 patients with CRC and 6.1% of 82 normal individuals reacted with at least one TAA [232]. Liu *et al.* also used a five-antigen panel comprising p53, p62, c-myc, Imp 1 and Koc. Their TAA panel achieved 60.9% sensitivity and 89.7% specificity. By the inclusion of CEA in the panel of autoantibodies, the diagnostic sensitivity could be increased to 82.6% [223]. As with other studies involving TAA panels, the sensitivity of any one TAA is inferior to the sensitivity of the panel in its entirety [223]. Similarly, after the addition

of CEA to a panel of six antigens identified from a phage cDNA expression library of colon cancer, the sensitivity and specificity were increased to 91.7% and 95.8% respectively [233].

Stomach cancer

Stomach cancer is the fourth most common cancer and the second most common cause of cancer-related death worldwide [2,203]. This high mortality rate is caused by the asymptomatic nature of the cancer and also by the lack of reliable biomarkers for early cancer detection [234].

Most of the biomarkers of interest tend to be associated with gastritis or other gastric mucosa alterations [234]. Examples include serum pepsinogens I and II, gastrin-17 and antibodies against *H. pylori*. These four biomarkers have been packaged into a GastroPanel™, which is used to detect gastric mucosa alterations such as atrophic gastritis. While such biomarkers are not specific to stomach cancer, they may have utility in early cancer detection because most stomach cancers are known to arise from a chronic inflammatory background. Hence, the presence of such biomarkers may indicate patients at higher risk for stomach cancer.

The humoral response to stomach cancer is not well defined, although p53 autoantibodies have been found to be associated with the cancer [235,236]. Further work involving the elucidation of autoantibodies against gastritis and stomach cancer should aid in early cancer detection as well as improve our understanding of the cancer.

TAAs and autoantibodies for development of immunotherapeutics for cancer patients

Changes in specific antibody titers according to cancer type, tumor status or response to therapy have been demonstrated. The elucidation of the mechanism of humoral response that triggers the production of autoantibodies in cancer patients would help in the development of novel immunotherapeutics for incurable cancers. The TAAs can be potential targets for immunotherapy [59,237,238]. Various antitumor vaccination strategies that involve humoral and cellular immune responses to TAAs have been studied. These cancer immunotherapies target tumors without affecting normal tissues or resulting in adverse side-effects [239–242].

However, patient heterogeneity often results in a contradictory response to immunotherapy [243]. Thus, personalized profiles of TAAs and autoantibodies

could be used to identify therapeutic targets to develop novel vaccines for targeted immunotherapy against cancer. Based on the autoantibody profile of the cancer patient, individualized anti-cancer vaccine immunotherapy can be developed from the antigenic epitopes of TAAs that evoke an immune response [73,244]. Most often, these antigens are TAAs that are essential for tumor growth and have homogenous expression in the tumor tissues [245]. On a similar note, the antigenic peptides could also be used to design drugs to block the destruction of tissues in patients with autoimmune diseases.

Tumor-associated carbohydrate antigens, which are highly expressed in metastatic tumors and in patients with poor prognosis, are excellent clinical targets for humoral immunotherapy [246]. Constant and strong expression of neural cell adhesion molecule (NCAM) in cancers such as small cell lung cancer, neuroblastoma, brain tumors, multiple myelomas and acute myeloid leukemia supports the development of anti-NCAM immunotherapy for these cancers [247]. CTAs are another group of proteins that are currently used for vaccination in cancer patients. Jäger *et al.* [248] have reported that immunization with peptides of CTAs (MAGE-1 and MAGE-3) resulted in regression of tumors in more than 30% of patients with melanoma. Antibody titers to NY-ESO-1 have also been shown to associate with the progression of malignancy in the patients and were proposed as a candidate for immunotherapy [244].

Since the approval of the CD20 mAb (rituximab) by the Food and Drug Administration (FDA) for CD20-positive B-cell malignancies, several antibody-based immunotherapies have been developed for cancers. Antibodies against CD20 exhibit significant anti-tumor activity and greatly improve the survival of patients with B-cell non-Hodgkin's lymphoma [249]. In antibody-based therapy, cell-surface proteins or secreted proteins are targets for such treatment, for example, HER2/*neu* for breast carcinoma, CD20 for B-cell lymphoma, vascular endothelial growth factor for renal cell carcinoma, and epidermal growth factor receptor for CRC. Monoclonal antibodies are one of the largest classes of new therapeutic agents approved for human cancers. For example, antibody against HER2/*neu* is widely used as therapy against breast cancer [250]. This antibody was shown to improve relapse-free survival when used as a component of adjuvant therapy in patients with HER2/*neu*-expressing tumors [251]. Antibodies directed against epidermal growth factor receptor are currently used for patients with advanced CRC [252]. Antibody targeting vascular endothelial growth factor improves the survival rates of patients

with metastatic CRC [253]. Currently, there are many other such immunotherapeutics in clinical trials for cancer patients.

Conclusions

Hundreds of TAAs have been identified in various tumors, and some are commonly found in many different cancers. Indeed, TAAs are often proteins that play crucial roles in carcinogenesis, such as self-sufficiency in growth signals (e.g. epidermal growth factor receptor), insensitivity to growth signals (e.g. MDM2), evasion of apoptosis (e.g. survivin), limitless replicative potential (e.g. hTERT), sustained angiogenesis (e.g. vascular endothelial growth factor), tissue invasion and metastasis (e.g. MMP2) and cancer initiation (e.g. SOX2) [245]. Many such TAAs (e.g. p53, survivin and HSPs) lack specificity as they are involved in malignant transformation and are targeted by the immune system [31,164]. For example, autoantibodies against many HSPs, such as heat shock protein 27 (HSP27) and HSP60, are detected in various cancers [231,254]. This is because HSPs play important roles in cancer initiation and progression, and are often over-expressed in cancers [255–257]. Autoantibodies to p53 have been detected at the early stage of many cancers, such as lung, gastric, colorectal, ovarian, esophageal and oral cancers and in HCC [31,208,258,259]. Autoantibodies to p53 are also associated with high-grade tumors and poor survival [31]. Hence, TAAs with high diagnostic value would commonly be composed of such proteins and cancer-specific antigens.

Currently, clinical application of cancer autoantibodies has been hindered by their low specificities and sensitivities. It is also not always true that the same autoantibodies would be identified when the same experimental technique was employed. This was borne out by several recent reports [12,151,188–194,201]. Similarly, the percentage of autoantibodies detected in patient sera was found to vary in different studies [260]. Many reasons have been proposed to explain these observations. One of the main reasons appears to be the inherent heterogeneity of patients and tumor tissue samples. Thus, Zolg [261] showed that a minimum of 15 samples is necessary to reduce intersample variations. Another reason may lie with the screening method for detecting the autoantibodies from sera. This is because autoantibodies have also been reported to be present in healthy individuals. For example, Li *et al.* [262] showed that > 50% of the sera of healthy subjects contain autoantibodies to α -enolase and heterogeneous nuclear ribonucleoprotein L. More than 20% of the sera also contained antibodies against annexin

II, F-actin capping protein beta and calreticulin. Nolen *et al.* [263] have also identified a subset of healthy individuals with high levels of autoantibodies against several antigens. They proposed that autoantibodies are produced in response to nonmalignant autoimmune disorders. Indeed, autoantigens are found in individuals with benign, inflammatory and autoimmune diseases. It has been demonstrated that there are pre-existing antibodies which are not associated with the tumors in cancer patients [182,200]. This limitation can probably be resolved by the use of the MAPPING strategy (discussed earlier) for the identification of cancer-specific TAAs. Briefly, in this method, nonspecific TAAs that bind to IgGs present in the sera of healthy individuals and/or patients suffering from other disorders (such as autoimmune diseases) are first eliminated from the tumor tissue or cell lysates. This will ensure that TAAs which subsequently bind to IgGs from the sera of cancer patients are truly cancer-specific [154]. Hence, samples of sera from a large cohort of patients, including those with different types of cancer, with inflammatory and autoimmune diseases, and healthy subjects, have to be included to validate the sensitivity and specificity of promising cancer-diagnostic candidates for clinical and epidemiological applications. To overcome challenges associated with sample scarcity, multicentre collaborations are necessary to overcome some of the logistical and statistical challenges that impede validation studies.

As discussed earlier, several independent studies have suggested that a panel of TAAs may exhibit superior sensitivity and specificity compared with a single autoantigen when they are used for clinical diagnosis. This is well illustrated by the study of Lu *et al.* [214] on the seroreactivity of p53, HER2, IGFBP-2 and TOPO2 α in breast cancer patients. Using receiver operation characteristic curve analyses they showed that the response to p53 alone was not a significant predictor of breast cancer [area under curve (AUC) = 0.48, P = 0.538], but by combining responses to p53 and HER-2/*neu*, an AUC of 0.61 (P = 0.006) was obtained. Furthermore, combining responses to a panel of four antigens (p53, HER2, IGFBP-2 and TOPO2 α) resulted in an AUC of 0.63 (P = 0.001). A recent study carried out by Chen *et al.* [264] also demonstrated that the addition of two newly identified TAAs (Sui1 and RalA) to a panel of eight TAAs (IMP1, p62, Koc, p53, c-myc, cyclin B1, survivin and p16) resulted in an increase of sensitivity from 59.7 to 66.2% as a diagnostic modality for HCC patients. In addition, the authors showed that the positive reaction to this panel of 10 TAAs is higher in HCC patients than in patients with liver cirrhosis (33.3%) or chronic hepatitis (20%), or in normal controls (12.2%).

Interestingly, 70.8% of HCC sera with a normal range of serum AFP were immunoreactive to the panel of TAAs. If AFP and anti-TAA were used in combination as diagnostic markers, 88.7% of the HCC patients could be identified. These and other studies have shown that a panel of autoantibodies is better in discerning cancer patients from healthy individuals than the use of a single autoantibody.

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