Targeting serum antibody for cancer diagnosis: a focus on colorectal cancer

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The ability of the immune system to magnify the appearance of disease by generating relatively large amounts of antibody in response to small amounts of disease makes it a natural biosensor, and serum antibodies have emerged as promising biomarkers for the detection of cancer. This review summarizes recent progress in targeting serum antibodies for cancer diagnosis, with a particular focus on colorectal cancer (CRC). Several serum antibodies have been detected at increased levels in CRC patients, including p53, carcinoembryonic antigen, Ras, topoisomerase IIα, histone deacetylase 3 and 5, ubiquitin C-terminal hydrolase L3, tropomyosin and cyclin B1. As each antibody is only present in a limited proportion of patients (usually < 40%), a combination of serum antibodies that defines the ‘immunological signature’ of cancer needs to be developed. High-throughput methods to identify new serum antibodies for cancer diagnosis are also reviewed.

Keywords: biomarker, colorectal cancer, serum antibody

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1. Introduction

For decades, serum antibodies have been used as markers of past exposure to infection. The possibility of using serum antibodies as biomarkers of exposure to tumor has been pursued actively in recent years. Tumors can express an aberrant level or mutated form of specific proteins. Such proteins may be immunogenic; they can be detected by the immune system and elicit subsequent cellular and humoral immune responses. A body of evidence has shown that serum antibodies recognizing tumor-associated antigens (TAAs) can be detected in cancer patients [1-14]. Preliminary work has shown that a serum antibody-based test could be used to differentiate cancer patient sera from normal donor sera [15-17]. With the development of high-throughput technologies to screen for serum antibodies specific to cancer patients, the idea of using serum antibodies to detect cancer is coming closer to reality.

2. Serum antibodies for the diagnosis of cancer

The use of serum antibodies for diagnosis has been the basis of identifying individuals with infectious disease for many years. Antibodies are reliable markers for current or past exposure to pathogens. The advantage of using serum antibodies compared to other serum-derived proteins is that the immune system can respond to immunogenic proteins when those proteins are present at low levels not detectable by direct protein screening [18]. The amplification of signal by the immune system is the equivalent of a polymerase chain reaction using antigenic tumor proteins as templates [19]. Furthermore, antibodies are stable, present in the serum for a long time and can be readily detected with an enzyme-conjugated secondary antibody [20].
The idea of using a serum antibody for the diagnosis of cancer is supported by the evidence that tumor is immunogenic, and a multitude of TAAs have been identified [21-26]. These TAAs have been divided into different categories, including mutated antigens (p53, K-ras), overexpressed antigens (HER2/neu) and cancer/testis antigens (MAGE, NY-ESO-1) [27]. The discovery of many TAAs has not only fueled the interest to perform antigen-specific immunotherapy, but also to exploit the immune system as a biosensor to detect the presence of cancer. Serum antibodies to TAAs have been detected at high titers in patient sera, sometimes even before the clinical appearance of cancer. For example, antibodies to the tumor antigen HER2/neu were measured in the sera of early stage cancer patients at titers > 1:5000, suggesting a strong immune response [28]. Tumor antigen/antibody immune complexes have been detected up to 19 months prior to the development of disease relapse and correlate with survival in patients with early-stage melanoma [29]. Anti-p53 antibodies have been detected in heavy smokers before the clinical detection of lung cancer [30]. Similarly, antibody immunity has been detected in workers exposed to vinyl chloride before the clinical appearance of angiosarcoma [31], and in patients with Barrett’s esophagus before the diagnosis of esophageal carcinoma [32]. Therefore, serum antibodies appear to be a promising biomarker for the detection of cancer. This review focuses on the candidate serum antibody biomarkers for colorectal cancer (CRC).

3. Serum antibodies as biomarkers for colorectal cancer

CRC is the second leading cause of cancer-related death in the US, striking 140,000 people annually and causing 50,000 deaths [33]. Although the use of endoscopic screening is increasing, overall compliance in undergoing the procedure is poor [34]. It is critical to develop a non-invasive test for the early detection of CRC. The available non-invasive screening methods for CRC include fecal occult blood test (FOBT), stool DNA and protein test, and rectal mucosal tests [34,35]. FOBT is the only form of non-invasive testing that has been shown in large, randomized clinical trials to decrease mortality [35-38]. However, the spot sensitivity of FOBT is poor because cancers may bleed intermittently. The specificity of the assay is also limited because the majority of gastrointestinal bleeding is from causes other than cancer [34]. Thus, a new test with better sensitivity and specificity remains to be developed. Furthermore, a serum-based test would be ideal for screening, as compliance with a serum test would likely be better than any test involving feces and stool handling [34]. Serum antibodies that recognize colon cancer-specific antigens may be used to develop a serum-based screening test.

Colon cancer, like most epithelial solid tumors, was once considered poorly immunogenic. Continuous progress in the characterization of tumor-specific antigens and in the study of intratumor T cell infiltrates in CRC patients has suggested that an intratumor immune response may take place in colon cancer patients and positively influence prognosis [25,39,40]. T cell infiltration at the tumor site has been associated with decreased metastatic invasion and prolonged survival [41]. Furthermore, the immunological data (the type, density and location of immune cells within the tumor samples) were found to be a better predictor of patients’ survival than the existing histopathological methods used to stage CRC [42]. These data support the hypothesis that an adaptive immune response exists in CRC patients. Indeed, a number of colon cancer antigens have been discovered using antibody or T cell-based approaches, including p53, histone deacetylase 3 and 5 (HDAC3, HDAC5) and ubiquitin C-terminal hydrolase L3 (UCH-L3) [25,39,43-45]. The following protein antigens are reviewed because they have been reported to elicit increased antibody responses in CRC patients. The incidence of positive antibody responses to each of the TAAs is listed in Table 1.

3.1 p53

p53 is one of the most extensively studied tumor antigens. Antibodies against the p53 protein have been detected in the serum of patients with breast cancer, Burkitt’s lymphoma, lung cancer and pancreatic cancer [22]. Angelopoulos et al. analyzed 1392 sera from patients with various malignancies and found that the highest prevalence of anti-p53 antibodies was associated with ovarian and colon cancer (15%), followed by lung (8%) and breast (5%) cancers [46]. In a study by Zhong et al., the positive rate for anti-p53 serum antibody in CRC patients was 17.8% and was also the highest among the 6 different types of cancers evaluated (breast, gastric, liver, lung, prostate and CRC) [19]. Broll et al. also reported that Anti-p53 antibody was detected in 15% of CRC patients and none of the controls. Furthermore, these investigators reported a significant correlation between p53-Ab production and positive immunostaining of p53 protein concentration in tumor tissue [47]. Anti-p53 antibody was detected at higher percentage in patients with large tumor volume [47]. In a study by Hammi et al., 26% of the CRC patients had anti-p53 antibodies, and 58% of the patients demonstrated significant p53 protein accumulation in the tumor [48]. In a compilation of anti-p53 studies, the positive rate was 24.6% for CRC patients [49]. A significant correlation between the disappearance of anti-p53 antibody and cure following surgical resection has been observed, suggesting that p53 antibody immunity may have use as a marker for postoperative monitoring of CRC [50]. The presence of the antibody has also been associated with good prognosis in ovarian cancer patients [51]. As a candidate biomarker for CRC diagnosis, anti-p53 antibody has a high specificity (100%) but low sensitivity, most likely related to low incidence of positive antibodies. A recent publication reported that testing for anti-p53 antibody can increase the diagnostic sensitivity when used in combination with conventional markers without a parallel decrease in specificity [52], suggesting that the combination of markers may have improved diagnostic value.
### Table 1. Serum antibody responses to TAA detected in CRC patients.

<table>
<thead>
<tr>
<th>Tumor antigen</th>
<th>Identification methods</th>
<th>CRC serum antibody positivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>SEREX [23]</td>
<td>15%</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18%</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26%</td>
<td>[48]</td>
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<tr>
<td></td>
<td></td>
<td>15%</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25%</td>
<td>[49]</td>
</tr>
<tr>
<td>CEA</td>
<td>ELISA</td>
<td>79%</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51%</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20%</td>
<td>Goodell, unpublished data</td>
</tr>
<tr>
<td>RAS</td>
<td>ELISA</td>
<td>32%</td>
<td>[61]</td>
</tr>
<tr>
<td>TOP02α</td>
<td>ELISA</td>
<td>25%</td>
<td>[68]</td>
</tr>
<tr>
<td>UCH-L3</td>
<td>Protein microarray</td>
<td>44%</td>
<td>[45]</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>ELISA</td>
<td>36%</td>
<td>[76]</td>
</tr>
<tr>
<td>HDAC3</td>
<td>SEREX</td>
<td>5%</td>
<td>[44]</td>
</tr>
<tr>
<td>HDAC5</td>
<td>SEREX</td>
<td>17%</td>
<td>[69]</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>ELISA</td>
<td>22%</td>
<td>[81]</td>
</tr>
</tbody>
</table>

CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; ELISA: Enzyme-linked immunosorbent assay; HDAC: Histone deacetylase; SEREX: Serological analysis of recombinant cDNA expression libraries; TAA: Tumor-associated antigen; TOP02α: Topoisomerase II-α; UCH-L3: Ubiquitin C-terminal hydrolase L3.

#### 3.2 Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a membrane glycoprotein involved in intercellular adhesion and overexpressed in a variety of extra-intestinal tumors, including lung, breast, ovarian, bladder and colon cancers [22]. CEA is expressed in > 90% of CRC and contributes to the malignant phenotype of the tumor. Immunotherapy based on CEA is a rapidly advancing area, as summarized in a review by Dalerba et al. [43]. Both serum CEA and serum anti-CEA antibodies have been pursued as markers for diagnosis and prognosis. Level of circulating CEA was the most frequently used marker for CRC, and it has been shown to predict recurrence, sensitivity to chemotherapeutic agents, and overall survival after surgical resection of colorectal carcinoma [53-55]. A study performed by Roberts reported that the sensitivity and specificity for distant metastases was 85% when CEA was used in postoperative monitoring of surgically treated patients [56]. Another recent study showed that CEA is more sensitive than p53 to detect CRC, and that the overexpression of p53 and CEA protein is an indicator of poor prognosis for CRC [57]. The presence of serum antibodies to CEA has been described in a large number of CRC patients (51%) and to be of high titer [58]. Albanopoulos et al. reported that CEA-specific IgM and/or IgG antibodies are detectable in 79.1% of CRC patients (46 out of 58), although serum CEA was elevated in only 20.6% of patients. The presence of circulating anti-CEA IgM antibody was associated with a significantly longer survival in colon cancer patients [59]. Studies from the authors' lab have shown that 20% of CRC patients and 5% of healthy donors have the anti-CEA antibody in sera (Goodell, unpublished data). A previous study by Albanopoulos et al. also reported that 10.5% (3 out of 28) of healthy individuals have the anti-CEA antibody, which may affect its use as a diagnostic marker [59].

#### 3.3 Ras

The Ras gene product is a monomeric membrane-localized G-protein of 21 kDa that functions as a molecular switch linking receptor and nonreceptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Three ras genes, H-ras, K-ras, and N-ras, have been defined, and these genes encode closely related but distinct proteins [50,61]. The ras proto-oncogenes are activated by point mutation in ~ 20% of human malignancies. Mutations in colon cancer are only found in the K-ras gene [60]. The mutations occur primarily at codons 12 or 61 and result in the expression of p21 ras proteins with single substituted amino acids [62]. Antibodies to p21 ras protein have been detected in patients with colon cancer. Takahashi et al. examined the sera from 150 colon cancer patients and 60 normal controls to determine whether antibodies to mutated p21 ras were present. They found that antibodies against the mutated p21 ras-D12 were detected in 51 out of 160 (32%) colon cancer patients and in 1 out of 40 (2.5%) normal individuals [61]. The antibodies in the majority of colon cancer patients also recognized normal p21 ras-C12 protein. Antibody responses to the non-mutated p21 ras may provide a method to identify individuals who have been exposed to mutated p21 ras and have yet to show an early indication of incipient or occult malignancy undetectable by existing conventional methods. In this study, the anti-ras antibody response did not correlate with patient age, sex, histology, stage or serum CEA, but did correlate with lymphocyte count prior to surgery [61].

#### 3.4 Topoisomerase II-α

Topoisomerase II is an essential nuclear enzyme that modulates DNA topology by generating double stranded
breaks, allowing the passage of 1 double strand of DNA through another via an ATP-dependent mechanism. Two topoisomerase II isozymes have been identified in humans: topoisomerase II-a (TOPO2a) and II-b. The two isozymes differ in their cell cycle expression, nuclear localization and tissue-specific expression. As type II topoisomerases play such an important role within dividing cells, they are highly attractive targets for chemotherapeutic agents, especially in human cancers [63]. TOPO2a is associated with resistance to chemotherapy in colon cancers and is consistently found at elevated levels in metastatic tumor cells [64]. In addition, TOPO2a is the primary target of chemotherapeutic agents currently used to treat colon cancers. Anti-TOPO antibodies have been most commonly reported in patients with autoimmune disease [65,66]. Anti-TOPO2 antibodies have also been reported in the sera of liver cancer patients [67]. Recent work from the authors’ lab has shown that anti-TOPO2a antibody immunity was increased in colon and ovarian cancer patients [51,68]. Goodell et al. reported that ovarian cancer patients with advanced-stage (III/IV) disease were more likely to have antibody response to p53, HER2 and TOPO2a than patients with limited-stage (I/II) disease. The serum antibody response to TOPO2a increased from 3 to 8% from limited-stage to advanced-stage disease. Serum anti-TOPO2a antibody was detected in 25% of CRC patients [68]. Whether the presence of the antibody is associated with disease stage, surgical status or prognosis in CRC patients remains to be investigated.

3.5 Histone deacetylase
Histone deacetylases (HDACs) represent the family of enzymes involved in dynamic regulation of chromatin structure during transcription and have been shown to play an important role in carcinogenesis, particularly in CRC [44]. More than one member of the HDAC family has been found to be immunogenic in colon cancer patients. Shezhukhov et al. reported that the C-terminal region of HDAC3 protein contains at least three distinct B cell epitopes that are recognized by the serum antibodies [44]. Serum anti-HDAC3 antibody was detected in 10 out of 194 colon cancer patients, but in 0 out of 90 normal donors. SEREX screening done by Scanlan et al. has identified HDAC5 as a colon cancer antigen [23,69], indicating that multiple members of the HDAC family are immunogenic and may be exploited as potential diagnostic markers.

3.6 Tropomyosin
Tropomyosin (TM) is an intrinsic component of most actin filaments, and > 40 isoforms have been identified in non-muscle cells. TM isoforms are spatially segregated, and existing evidence suggests that they can specify the functional capacity of the actin microfilaments. TM has become a new potential target for anticancer therapy [70]. The TM molecule is a two-chain, α-helical coiled coil, binding along the grooves of actin filaments. These features seem to be associated with many known autoantibody epitopes and may contribute to autoregulatory potential [71]. Several observations suggest that TM is an autoantigen for ulcerative colitis [72-74]. TC22, a novel spliced variant from the human γ-TM gene, is strongly associated with colonic neoplasia and carcinoma and may provide a useful biomarker for surveillance of colon cancer [75]. Using ELISA, Syrigos et al. found that anti-TM antibody was detectable in 36% (20/55) of CRC patients and was associated with a good prognosis [76].

3.7 Ubiquitin C-terminal hydrolase L3
Ubiquitin carboxy-terminal hydrolases (UCH) catalyze the removal of adducts from the C-terminus of ubiquitin and play an important role in protein degradation through recycling free ubiquitin. Ubiquitylation of cellular proteins and targeting them for subsequent degradation via ubiquitin-mediated proteolysis is an important mechanism that regulates cell cycle genes. In tumors, increased de-ubiquitylation of cyclins could contribute to the uncontrolled growth of cells [77]. Both UCH-L1 and UCH-L3 have been shown to be upregulated in tumor tissue. Upregulation of UCH-L1 was found in leukemia, esophageal cancer, CRC and pancreatic cancer [77-79]. UCH-L3 was found to be upregulated in invasive breast cancer tissue and was associated with a poor prognosis [80]. Nam et al. discovered the presence of anti-UCH-L3 antibody in CRC patient sera using protein array [45]. Anti-UCH-L3 antibody was detected in 19 out of 43 patients with colon cancer, and 0 out of 54 sera from subjects with lung cancer, colon adenoma or otherwise healthy individuals [45]. DNA microarray analysis revealed that UCH-L3 was highly expressed in colon tumors [45]. The diagnostic value of this antibody remains to be tested in large-scale studies.

3.8 Cyclin B1
Cyclin B1 is a member of the cyclin family that controls the progression through the cell cycle. Cyclin B1 has been found to be overexpressed in a variety of cancers including colon cancer. Anti-cyclin B1 antibody was detected in 22% of colon cancer patients [81]. Notably, the antibody was also detected in sera from breast cancer patients (43%) and pancreatic cancer patients (18%) [81].

4. The limit of a single biomarker-based assay
The use of a single antibody as a predictor of disease exposure has obvious limitations because most of the known tumor antigens are only present in a limited proportion of patients. For example, HER2, a well-studied immunogenic protein, is only overexpressed in 20 – 30% of breast cancer patients [82], and anti-HER2 antibody has been observed in only 11% of cases [26]. Similarly, the antibody to p53 is only present in 20 – 40% of patients whose cancers have the p53 mutations [83]. As listed in Table 1, the prevalence of antibody response to most colon cancer antigens is < 40%. This suggests that a panel of serum antibodies to colon cancer...
antigens may need to be developed to discriminate cancer patients efficaciously. Zhang et al. have shown that the likelihood of detecting antibodies in any cancer serum increased from ~15% to 26% when one antibody was used to 44% - 68% when 7 antibodies were used [15]. Scanlan et al. reported that most of the SEREX-identified antigens were of low frequency, ranging 3% - 8%, except p53, which was detected in 15% of patients. However, when the antigens were evaluated together, 34 out of 74 serum samples (46%) from colon cancer patients were positive for at least one antigen, whereas none of the control normal sera was positive [23]. Recent studies using protein array have further demonstrated that a serum test using the immunological signature of cancer (i.e., a combination of antibodies) shows promise for cancer diagnosis [11,12,84].

5. Immunologic signature for cancer diagnosis

An immunologic signature of cancer is defined as a panel of antibodies whose presence in serum is associated with the development of cancer. The diagnostic power of evaluating a panel of tumor antigens has been shown in recent publications. Using protein array, Wang et al. identified an antibody signature composed of a 22-phage-peptide detector for prostate cancer patients. An assay based on this antibody panel had 88.2% specificity and 81.6% sensitivity in discriminating between subjects with prostate cancer and a control group [11]. Antibody immuno to this panel of peptides performed better than did prostate-specific antigen, which is the currently used screening test for prostate cancer [11]. Another study of antibodies against defined tumor antigens in serum specimens from 527 patients with cancer and 346 controls found that a panel of 7 antigens was sufficient for the diagnosis of cancer [17]. In addition, Zhong et al. have identified a panel of the 5 most predictive phage proteins that can be used to discriminate non-small cell lung cancer patients from a control group with a sensitivity of 90% and a specificity of 95% [12]. Chatterjee et al. reported a protein array-based assay using a panel of 65 antigens on protein array to detect serum IgG from ovarian cancer patients and healthy women. The average sensitivity and specificity were 55% and 98%, respectively [14]. Using tumor antigen-coated microspheres, Erkani et al. evaluated serum antibody response to a panel of antigens, including p53, NY-ESO-1 and HOXB7, in parallel. They found that Bayesian modeling of these TAA-specific serum antibody responses exhibits similar discrimination of patients with early-stage and advanced-stage ovarian cancer from women with nonmalignant gynecologic conditions and may be complementary to CA125 [15]. These data demonstrate that an assay developed to detect a panel of serum antibodies may be of potential diagnostic value. Although a few tumor antibodies have been found in the sera of colon cancer patients as reviewed in the previous section, no antibody panel has been developed that has been shown to be of diagnostic value. New antigens remain to be developed to increase the sensitivity of any diagnostic assay. Methods available to identify new serum antibodies associated with cancer are reviewed in the next section.

6. Methods to identify serum antibodies associated with cancer

Critical to the discovery of new antibody biomarkers are the availability of high-throughput screening technologies. Several methods have been used to identify tumor-specific antibodies in the serum of cancer patients, all of which depend on using patient sera as probes against candidate antigens derived from tumor cells.

6.1 Two dimensional polyacrylamide gel electrophoresis and western blot

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) can be used to separate thousands of individual cellular proteins from tumor tissue or cell lines. The separated proteins are transferred onto membranes. The membranes are probed with sera from cancer patients or normal donors. The proteins that only react with sera from cancer patients will be identified by mass spectrometric analysis and/or amino acid sequencing. Using this method, antibody responses to annexins I and IV, thymidine phosphorylase, carbonic anhydrase I, Mn-superoxide dismutase and major vault protein were detected in the sera from renal cell carcinoma patients [82]. Antibodies to cytokeratin 8, cytoskeletal tropomyosin and other cytoskeletal proteins were detected in the sera from renal cancer patients [83]. Antibodies to RS/DJ-1 were also identified in the sera from breast cancer patients using this method [87]. The use of this method has also allowed the identification of tumor antigens in gastrointestinal cancer [88], pancreatic cancer [89], hepatocellular carcinoma [90] and lung cancer [91]. A limitation of this strategy is its relatively low throughput, as it is based on 2D PAGE, which is a laborious procedure [92].

6.2 Serological screening of cDNA expression library

In the serological screening of cDNA expression library (SEREX) approach, a cDNA library is constructed using RNA from tumor specimens packaged into lambda-phage vectors and expressed recombinantly in Escherichia coli. Recombinant proteins are transferred onto nitrocellulose membranes and identified as antigens by their reactivity with high-titer IgG antibodies present in the patient serum detected using an enzyme-conjugated secondary antibody specific for human IgG. Positive clones are subcloned to monoclonality, and the nucleotide sequence of the inserted cDNA is determined [27,29]. This method was first developed by Sahin et al. ~10 years ago [94]. The common use of this method has resulted in the identification of >2000 tumor antigens recognized by cancer patient sera as deposited in the human cancer immunome database [901]. Using SEREX to analyse sera from colon cancer patients, Scanlan et al. [23] identified 77 distinct antigens, and 13 of them reacted only with sera from colon cancer patients, including p53,
MAGEA3, SSX2, NY-ESO-1, HDAC5, MBD2, TRIP4, NY-CO-45, KNSL6, HIP1R, Seb4D, KIAA1416 and LMNA. Analysis of antibody frequency showed that 5 out of 7 (71%) stage I colon cancer patients, 4 out of 11 (36%) stage II patients, 2 out of 14 (14%) stage III patients, and 11 out of 21 (52%) stage IV patients had serum IgG antibody that reacted with ≥ 1 of the 13 antigens [23]. Line et al. also used SEREX to identify tumor antigens associated with colon cancer. Among the eight candidate antigens, NAP1L1, RHAMM and AD034 are overexpressed in tumors in comparison with the adjacent non-cancerous tissues. Although these SEREX-identified antigens could be of potential diagnostic value, they remain to be tested in large scale studies. The major bottleneck for the development of large-scale assays is the cloning, expression and the purification of each of the respective antigens [93]. To overcome this barrier, Tureci et al. have developed a crude lysate ELISA assay that permits sensitive and specific autoantibody seroscreening without the need of laborious and time-consuming cloning, expression and purification of recombinant proteins [93]. This assay uses bacteria-expressed recombinant protein as source of protein antigen and therefore requires pretreating the patient sera to remove anti-Escherichia coli antibody. The robustness of this assay remains to be tested. Clearly, the breakthrough in biomarker discovery will not only rely on the high-throughput screening technology but also on the development of subsequent robust validation assays.

6.3 Protein array
Protein array allows the simultaneous evaluation of thousands of proteins in parallel. Several protein microarray platforms have been developed for high-throughput analysis. The recombinant protein arrays use clones from cDNA expression libraries or phage display libraries [11,99,100]; the native protein arrays use proteins derived from tumor tissue or cell lines [97,98]. Phage display libraries can be enriched with peptides specifically recognized by patient serum using a process called biopanning before they are spotted on the array. Biopanning entails successive rounds of immunoprecipitation of phage libraries using patient serum to select the peptides recognized by antibodies in patient serum and using normal donor serum to remove the peptides recognized by antibodies in normal serum [11,99,100]. Robotic microarray spoters that allow grouping of thousands of proteins, in replicate, onto a single glass slide make it possible to evaluate the presence of serum antibody to hundreds of phage-expressed proteins simultaneously, thus making the screening process efficient and reproducible. The use of this new technology has facilitated the successful identification of immunological signatures for prostate, lung and ovarian cancer [11,12,14].

A limitation of these methodologies is that post-translational modifications, such as phosphorylations and glycosylations, which may be essential to the immunogenicity of the proteins, are not captured on the recombinant protein array. In that respect, arraying proteins isolated from tumors or tumor cell lines may be better suited for uncovering immunogenic proteins. Fractionated proteins from a tumor cell lysate can be used to spot the array [97,98]. For example, Nam et al. used a liquid-based 2D separation system to obtain fractions of native protein derived from the LOVO colon adenocarcinoma cell line and arrayed individual fractions onto nitrocellulose slides, which were probed with colon cancer patient sera. This method allowed the identification of autoantibodies to UCH-L3 in colon cancer patient sera [95]. The disadvantage of this method is that each spot on the array may have multiple proteins, and subsequent identification of the individual immunogenic protein can be challenging. The inability to control protein orientation during immobilization also remains a limitation [101]. Similar to SEREX, the candidate markers that emerged from protein array screening remain to be validated by ELISA assay.

7. Conclusion
Antibody immunity to a number of oncofetal antigens has been identified in CRC patients. The use of any single antibody as a marker for diagnosis is limited because most antibodies are present in only a small percentage of patients. Additional markers need to be identified to improve the performance of a serum antibody-based assay. This may be accomplished using high-throughput screening technologies, which identify disease-specific antibody responses by profiling antibody response in patient and normal donor sera. With the continuing effort to identify novel antibody markers, a serum-based test against a panel of tumor antigens may eventually open a new gateway for cancer diagnosis.

8. Expert opinion
The ideal screening test should be highly sensitive, non-invasive, cost-effective and easy to implement across a large population. Serum antibody-based tests for cancer diagnosis exploit the immune system as a biosensor to diagnose the presence of cancer. The immune response represents a form of biological amplification of signals that are otherwise weak due to very low concentrations of antigen, especially in the early stages of cancers. Although the direct detection of serum antigens may be challenging due to the presence of a high abundance of serum proteins, such as albumin, the detection of serum antibodies is relatively easy using well-established secondary antibodies. As each known antibody marker for CRC is only present in a limited percentage of patients, the inclusion of additional markers to an antibody panel may increase population coverage and improve sensitivity. However, the combination of markers may also increase false positivity and thus lower specificity of the assay. False positive results may place a heavy burden on the healthcare system, as they lead to expensive colonoscopies. Therefore, caution needs to be taken in combining markers. Another challenge in developing a serum
antibody-based test for CRC diagnosis is to identify antibody markers specific to colon cancer. As discussed above, most of the serum antibodies, although elevated in cancer patient sera compared to controls, are elevated in more than one type of cancer. This is probably due to the fact that cancers of different tissue origin may share the same mechanism of malignant transformation in terms of gene mutation, cancer initiation and progression. Therefore, the same group of tumor-associated proteins may be immunogenic. This assumption suggests that results from a serum antibody-based test need to be interpreted together with other clinical measures and may never be a standalone diagnostic for a discrete tumor.

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Website

201. http://www2.isr.org/CancerImmunomeDB/
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