

Autoantibodies for cancer detection: Still cause for excitement?

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Abstract. The identification of circulating biomarkers of early stage malignancy is a critical component of ongoing efforts aimed at reducing the overall public and personal impact of human cancer through early detection. The human immune system is capable of identifying and reporting the presence of tumor-derived factors appearing during the initial events of tumorigenesis with a sensitivity and specificity far beyond currently developed biochemical assays. Tapping into the process of immune surveillance through the identification and evaluation of autoantibodies against tumor-associated antigens (TAAs) represents a promising avenue of biomarker development. Here we review a diverse series of reports describing the use of TAA-specific autoantibodies for the discrimination of cancer from control groups. A description of the major technical platforms being utilized as well as specific innovations implemented for the detection of autoantibody biomarkers is included. This review provides an objective survey of results obtained using individual TAA-specific autoantibodies as well as multi-autoantibody panels in order to identify and assess emerging trends in this field of research. Such trends provide a basis for the discernment of the specific challenges currently facing autoantibody biomarker development, and lay the groundwork for future innovations aimed at overcoming those challenges.

Keywords: Autoantibodies, tumor-associated antigens, SEREX, SERPA, Luminex, early detection

1. Introduction: Autoantibodies as cancer biomarkers

Despite the intense efforts aimed at the development of early detection strategies for human cancer, all currently utilized methodologies still lack the superior level of sensitivity displayed by our own immune system. The same immune system that stands ready to identify and eliminate foreign pathogens is now well recognized for its role in the first-line identification of endogenous anomalies, such as those appearing during the earliest stages of tumorigenesis [1]. The immuno-

genicity of tumor cells appears to derive in part from the production of neo-antigens unique to the tumor and also, in greater part, to self-antigens that are aberrantly expressed in tumor cells [2–5]. The aberrant expression of self antigens may result from overexpression or altered tissue-specific expression, mutation leading to the production of new antigenic epitopes, deregulated post-translational modifications such as misfolding or aberrant glycosylation, or unregulated degradation [6–11]. Thus, proteomic factors inherent to the process of tumorigenesis lead to a loss of self-tolerance similar to that observed in a number of autoimmune disorders [12]. Such proteomic factors have been termed tumor-associated antigens (TAAs) and the immune response to TAAs can be measured in the form of specific autoantibodies. Although current experimental evidence suggests that TAA-specific autoantibodies func-

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tion predominantly as reporters [13], with very few exhibiting pathogenic effects, their cognate TAA targets function in a variety of critical molecular processes including DNA replication and transcription, RNA processing, protein translation, mitosis, signal transduction, proliferation, and apoptosis [14–16]. Many of the TAAs identified thus far represent intracellular proteins, calling into question the precise mechanism underlying their immune surveillance [17]. Increased rates of tumor cell proliferation and death, damage to tumor cells associated with inflammation, and the aberrant localization and secretion of tumor-derived proteins have been proposed to explain the release of these factors into immune-accessible compartments [18–21].

Investigations into the use of autoantibodies as biomarkers of malignancy are concurrent with those focused on conventional protein-based biomarkers although the latter have proven more fruitful to date, resulting in the emergence of such biomarkers as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), CA-125, CA 19-9, and alphafetoprotein (AFP). Although each of these factors represent a considerable achievement in biomarker discovery and development, clinical applications have thus far yielded disappointing performances characterized by insufficient sensitivity and specificity. Several lines of evidence have indicated that autoantibodies may offer the potential to improve upon conventional biomarkers in several performance aspects. The immune response to TAAs originates locally and can be stimulated by a very small number of tumor cells at an otherwise undetectable early stage [8]. The subsequent systemic B-cell response leads to an amplification of TAA-specific autoantibodies and the presence of high-titer antibodies against TAA during presymptomatic and prediagnostic stages of disease [18,22]. TAA-specific autoantibodies can be readily measured in the sera of cancer patients and such measurements are further facilitated by the inherent stability and long half-life of immunoglobulins in circulation, which promotes improved assay reproducibility [23]. The past few decades have recorded a number of significant advances in analytical techniques aimed at the measurement of TAA-specific autoantibodies for clinical diagnostic applications.

Here we review the diverse landscape of investigation detailing efforts to identify autoantibody biomarkers with diagnostic relevance in cancer. This review will focus predominantly on reports of the past five years, although earlier reports have been included where appropriate. Technological advancements and innovations upon established techniques utilized in au-

toantibody detection will also be discussed in detail. Findings obtained from the analysis of individual autoantibodies and panels of autoantibodies will be reviewed separately and discussed collectively. Research reports included in this review meet the criteria that autoantibody levels were compared between discreet cancer case/control groups of sufficient size to confer statistical significance for the purpose of discriminating between the two. Investigations aimed strictly at autoantibody discovery were excluded.

2. The technological evolution of autoantibody detection in cancer

2.1. Library-based platforms

The hallmark technological innovation associated with autoantibody detection is the serological analysis of tumor antigens by recombinant cDNA expression cloning, termed SEREX. First described in 1995 by Sahin et al. [19], SEREX entails the serological screening of cDNA expression libraries generated from tumor tissues obtained from the same patient. The results of this screening process are two-fold, enabling the identification of autoantibodies in patient sera directed against specific TAA, and also the discovery of novel immunogenic TAAs themselves. This technique has been applied in a variety of malignant settings and has led to the identification of over 2300 autoantigens, all compiled into the Cancer Immunome Database (CIDB) [24], a public access online repository of SEREX findings and related information. Although the contributions to autoantibody discovery using SEREX are well documented and ongoing, several important limitations of this technique have been noted [23]. The use of a bacterial expression system incorporates a bias toward linear epitopes and antigens highly expressed in the tumor tissues, leading to the omission of folded or lower abundance antigens from SEREX findings. Any immunogenicity resulting from post-translational modifications, such as glycosylation, is likewise undetected. SEREX is also a time and labor intensive platform that is not amenable to high-throughput screening and automation.

An improvement upon the throughput capacity of SEREX screening has been achieved through the development of the phage-display method of library-based autoantibody detection. In this method the need for expression cloning of tumor-derived cDNAs has been eliminated as cDNAs are incorporated directly

into phage particles and expressed on the phage surface as fusion proteins [25]. This technique also involves a process known as biopanning, wherein the phage libraries can be enriched of tumor-specific peptides through successive rounds of incubation with normal sera followed by immunoprecipitation of non-tumor-specific antigen/antibody complexes. Several innovations of this technique have extended its utility including the use of randomly assembled peptides in the phage library [26] and the recent development of phage-based protein/peptide microarrays capable of screening large cohorts of patient sera in an automated fashion [27–29]. Throughput aside, the phage-display method shares several limitations with SEREX, including the preclusion of conformational epitopes and immunogenic post-translational modifications [30,31]. Additionally, not all peptide antigens can be effectively displayed on the phage surface [32].

Library-based autoantibody detection methods have been utilized in a wide variety of human cancers. The use of SEREX has enabled the identification of TAAs in lung, breast, prostate, ovarian, head and neck cancer, and esophageal cancer, as well as hepatocellular carcinoma (HCC), renal cell carcinoma (RCC), lymphoma, and leukemia (reviewed in [33]) while phage display has been effectively applied to studies of breast, prostate, ovarian, and colorectal cancer (CRC) (reviewed in [34]), and more recently to gastric [35], nasopharyngeal cancer (NPC) [36], and lung cancer [37–39]. Recent trends in the use of both techniques have included the use of established tumor cell lines for the generation of cDNA libraries in place of tumor tissues. This has come amid observations of patient heterogeneity which may serve to limit the utility of a tissue-based library and the presence of differential cell types within individual patient tissue samples [40].

2.2. Proteomic-based platforms

A significant number of investigators have employed proteomic techniques in efforts to identify autoantibodies associated with malignancy. A combination of techniques, commonly referred to as serological proteome analysis (SERPA), has emerged as a popular platform for TAA and autoantibody discovery [41–44]. In SERPA, proteins from patient tissue or cell line lysates are first separated by 2D electrophoresis (2DE). The separated proteins are then transferred to a membrane and probed with sera obtained from cancer patients or healthy individuals. Upon comparison of the immunoreactive patterns of both the cancer and normal

sera, the tumor-associated spots are further characterized by mass spectrometry (MS). Since its inception, a number of variations to the SERPA platform have been introduced as part of several investigations. Notable among these is the use of 2D immunoaffinity chromatography in place of electrophoresis and blotting followed by tandem MS analysis. This procedure, termed MAPPING, was developed by Hardoin et al. [45] in an effort to screen for autoantibodies associated with CRC.

Recently, SERPA-based approaches have been utilized in the identification of tumor-associated autoantibodies in breast cancer [46–48], HCC [49], lung cancer [50], esophageal cancer [51], pancreatic cancer [52, 53], and leukemia [54]. Proteomic approaches offer the capability to identify antigens resulting from post-translation modifications, an advantage over library-based methods, however several limitations to this strategy have been identified [55]. Chief among these limitations is the low throughput of proteomic techniques given the high time and labor requirements. The initial component of SERPA, 2DE is also hampered by limited detection sensitivity for low abundant species, an inability to discriminate between co-migrating proteins, and the nagging difficulty regarding the solubility of membrane-bound proteins.

2.3. Protein microarrays

Protein microarrays have emerged as popular tools in the identification of tumor-associated autoantibodies. This approach offers several distinct advantages including high throughput capacity, ease of automation, the ability to perform multiplexed analyses, and scalability to greatly reduce reagent and sample volume requirements. Protein microarrays have been utilized for a number of years in the identification of autoantibodies in patients with autoimmune disease and have recently gained wide support as tools for cancer research [23]. In this approach, proteins are spotted systematically onto a solid-phase support and then incubated with specific sera. The nature of the protein probe reflects the diversity of the platform and may include purified or recombinant proteins, synthetic peptides, or native proteins from tumor tissues or cell lysates [6,7]. Often, the array of recombinant proteins chosen reflects results obtained through SEREX or phage-display screening. Each type of protein probe is hampered by unique limitations [56]. Short peptide microarrays are relatively easy to produce and manipulate, however background levels of immunogenicity often lead to difficulty in da-

ta interpretation. Full length recombinant proteins offer a more complete immunoreactive analysis, however post-translational modifications are still missed. Native proteins obtained from tissues or cell lysates better reflect the true immunogenicity of the tumor, however such proteins are difficult to produce and often present challenges in reproducibility.

The recent widespread use of protein microarrays has led a number of groups to develop specific innovations aimed at overcoming the challenges associated with the various types of arrays. Several groups have recently reported significant technological advances which further improved the throughput, adaptability, and overall utility of recombinant protein microarrays. Chatterjee et al. [29] described their method of high throughput antigen cloning performed in concert with microarray analysis while Ramachandran et al. [57] and Anderson et al. [58] reported their development of “self-assembling” and programmable protein microarrays, respectively. Several other groups have made substantial progress regarding the use of native proteins through a series of protein lysate fractionation methods [40,59,60]. The successful use of natural protein microarrays to identify autoantibodies in lung and prostate cancer has also been reported [61,62]. Several other advances related to protein microarrays include a two-color Fab labeling method to reduce assay variability [63], the improvement of assay sensitivity through the use of a preliminary cyanogen bromide protein digestion [64], and the use of glycan arrays to specifically detect autoantibodies directed against post-translational glycosylations [65,66].

2.4. Other platforms

The “reverse-capture” microarray is a novel method developed by Brian Liu and colleagues [67–69] specifically for the identification of autoantibodies from patient sera. In the initial phase of the process, lysates derived from clinical specimens or cell lines are incubated with commercial antibody arrays. Following this step, the antigen-bound arrays are incubated with purified and fluorescently labeled IgG from patient and control sera. Differential labeling of the patient and control IgGs permits subsequent identification of cancer-specific autoantibodies. This method was recently utilized in the identification of 9 immunogenic TAAs involved in the integrin and Wnt signaling pathways in the sera of women diagnosed with mucinous epithelial ovarian cancer [70]. The limitations of this method center around its reliance on commercially available antibody

arrays. Thus, only known proteins and antigens for which specific antibodies have been developed can be detected.

SMARTA, or serological mini-arrays of recombinant tumor antigens, represents another novel development in the field of autoantibody detection. This technique has been presented as an efficient means of allogeneic screening for a small number of candidate TAAs identified through previously completed library-based (i.e. SEREX, phage-display) screening [71]. The method calls for the systematic spotting of selected phages onto LB agar dishes containing host cells. Following amplification, the phage spots are transferred to a nitrocellulose membrane which is subsequently incubated with patient sera and quantified by luminescence staining. This method has been successfully implemented in the evaluation of autoantibody panels for CRC [72] and ovarian cancer [73]. While improving upon the throughput and overall applicability of library-based screening methods, SMARTA remains hampered by the same limitations of SEREX and phage-display inherent to the use of recombinant antigen probes.

The development of bead-based immunoassay platforms has had a significant impact on the field of serum biomarker discovery and development. Such platforms represent a synergistic combination of the reproducibility and diverse utility of solid phase ELISAs with the improved kinetics and flexibility of a liquid-phase assay. Bead-based systems also exhibit a high capacity for multiplexing which greatly reduces sample and reagent volume, conveys high throughput and automation capabilities, and permits the generation of large amounts of biomarker data in a single experiment. The technique was first conceptualized by Streefkerk in 1976 [74] leading to a patent filed in collaboration with Coulter in 1979. Multiplexed assays based on bead size and a flow cytometric analysis platform were introduced and implemented by McHugh [75,76] and Stewart [77] from 1989 through 1994. Commercialization of the platform by Luminex Corporation (Austin, TX) in 1997 has ushered in the widespread usage of bead-based immunoassays for multiplexed biomarker analysis. Current protocols involve the use of a set of 100 distinct 5µm microspheres internally labeled with a combination of two laser-reactive dyes. Each of the dyes can be loaded into the bead at 10 levels of intensity, thus allowing for 100 spectrally-distinct microsphere lots. Following the covalent coupling of each microsphere lot to a different antigen of interest, beads of different spectral lots can be mixed together and utilized in a multiplex format. The bead mixture is then incubated

with patient sera and bound autoantibodies are detected using an anti-human IgG secondary antibody labeled with phycoerythrin (PE). The bead set is analyzed using one of several Luminex[®] analyzers, which incorporates flow cytometry-based fluidics with dual-laser optics. While one laser excites the internal bead dye combination, the other laser simultaneously excites the PE label. The instrument then records and reports the identity of each bead, according to dye composition, along with the intensity of bound antibody, represented by PE fluorescence.

Bead-based immunoassays have been utilized to a large extent for the measurement of conventional circulating biomarkers in a variety of cancers, however the use of this technology for the measurement of autoantibodies has occurred primarily in the setting of autoimmune disease. Several of these investigations have focused on autoantibodies present in patients diagnosed with systemic lupus erythematosus (SLE) [78], Sjogren's syndrome [79], and systemic rheumatoid disease [80,81]. In each of these studies, along with several others [82–84], autoantibodies were readily detectable and concordance with traditional ELISA was found to be high. An innovation upon this platform involving the use beads embedded with semiconductor nanocrystals, known commercially as quantum dots [85], has also been applied to the identification of autoantibodies in autoimmune patients [86]. Recently, the application of Luminex[®] technology has led to the identification of autoantibodies directed against NY-ESO-1 in esophageal cancer [87] and IL-8 in ovarian cancer [88]. In each case the identified autoantibody was found to be useful in discriminating cancer cases from control subjects.

3. Autoantibodies for cancer detection

3.1. Individual autoantibodies

According to our review criteria, we identified 32 reports describing the use of individual autoantibody biomarkers for the discrimination of a variety of cancer cohorts from healthy control groups. An overview of these findings is presented in Table 1. Autoantibodies directed against 32 distinct TAAs were investigated in breast, cervical, esophageal, gastric, ovarian, pancreatic, and prostate cancers as well as CRC, NPC, HCC and melanoma. This set of TAAs included oncoproteins, tumor suppressor proteins, survival factors, cell cycle regulators, mRNA binding proteins, molecu-

lar chaperones, cytokines, cytoskeletal proteins, calcium binding proteins, adhesion molecules, cancer-testis antigens, enzymes, glycoproteins, transcription factors, translation factors, and growth factors. While several of the investigations employed multiple techniques for autoantibody detection, many of them relied on a specific detection platform. ELISA/western blot was the most commonly used technique (41%) followed by SERPA (28%). Four of the 32 studies utilized SEREX, while phage-display, protein microarrays, and Luminex were each used twice, and mass spectrometry once. In five studies ELISA/WB was utilized to confirm results obtained through SEREX or SERPA. Autoantibodies were identified in the cancer cohorts at frequencies ranging from 8–94.7% (median 28%) and were largely absent from controls with frequencies of 0% in half of the studies and < 5% in 75% of the studies.

Several autoantibodies were found to be useful in the detection of breast cancer. Groups utilizing SERPA identified autoantibodies to HSP60 in invasive breast cancer ($n = 40$) [89] and in early stage breast cancer ($n = 107$), including DCIS [46]. In the latter study, increasing titers of anti-HSP60 were associated with high-grade DCIS. In both studies, the frequency of anti-HSP60 found in breast cancer sera (31% and 47.5%, respectively) was significantly higher than that of the control group (4%, $n = 42$ and 5%, $n = 94$, respectively). Two other groups identified high levels of anti- α 2-HS glycoprotein (AHSG) utilizing SERPA of resolved urinary proteins obtained from breast cancer patients ($n = 81$) [48], and moderate levels of anti-Her2 based on ELISA in breast cancer sera ($n = 107$) [22]. The presence of anti-Her2 autoantibodies has been previously linked to overexpression of the Her-2 antigen in breast cancer [90] and CRC [5].

The tumor suppressor protein p53 is among the most highly immunogenic TAAs identified to date, with a variety of malignancies demonstrating associations with elevated levels of anti-p53. Using a combination of phage display and ELISA, Qui et al. [35] identified elevated titers of anti-p53 in gastric cancer patients ($n = 61$) in comparison to controls ($n = 400$), and also noted a correlation of autoantibody titer with tumor size. Discriminatory levels of anti-p53 were also associated with esophageal cancer ($n = 46$) [91] and HCC ($n = 67$) [92], both determined by ELISA, however the latter study based its comparisons on a relatively small control group ($n = 10$). Several SEREX analyses also identified elevated levels of autoantibodies to Cyclin B1 [93], HCC-22-5 [94], and p62 [95] in HCC patient sera ($n = 100, 128, 95$, respectively), although ele-

Table 1
Single autoantibodies capable of discriminating cancer patients from healthy controls and/or benign subjects

Cancer	Platform	TAA	Sample size (N)			AA frequency (%)			Cancer vs. Norm	Ref
			Case	HC	Ben	Case	HC	Ben		
Breast	SERPA	HSP60	40	42		47.5	4.7		79 SN	[89]
	SERPA	AHSG	81	73		79	10			[48]
	SERPA, ELISA	HSP60	107	93		31–33	4.3			[46]
	ELISA, WB	Her-2	107	200		11	0			[22]
Cervical	ELISA	survivin	117	80	209	28	1.2	12		[109]
CRC	MA(f), MS, WB	UCH-L3	43	54		44	0			[59]
Esophageal	ELISA	p53	46	30		39	0		89 SN/100 SP	[91]
	Luminex, MA(p)	NY-ESO-1	18	14						[87]
	SERPA/MA(p)	HSP70	16	13		93.7	0			[51]
Gastric	ELISA, phage	p53	61	400		31	< 1			[35]
HCC	ELISA	p53	67	10	27	73	0	7.4		[92]
	SEREX	HCC-22-5	128	80	99	40	0	22		[94]
	SEREX, ELISA	p62	95	70	77	21	0	0		[95]
	SEREX	Cyclin B1	100	70	140	15	0	1–4		[93]
Lung	WB	recoverin	143	86	50	17	2.3	0		[98]
	WB	Prx-1	53	50		47	8			[97]
	WB	L-myc	70	30		10	0			[104]
	WB	c-myc	68	30		13.2	3.3			[105]
	SERPA	PGP9.5	64	71		14	0			[99]
	SERPA	Annexin I	54	61		30	0			[100]
	SERPA	Annexin II	54	61		33	0			[100]
	SERPA, ELISA	TIM	40	50		27.5	6			[73]
	SERPA, ELISA	MnSOD	40	50		8	4			[73]
	SERPA, ELISA	a-enolase	94	60		28	1.7			[103]
	ELISA	DKK1	93	87					62 SN/84 SP	[102]
	SEREX	eIF-4 G	33	17	17	15	0	0		[101]
Melanoma	ELISA	NY-ESO-1	127	70		9.4	0			[96]
NPC	Phage	MAGE	74	93					0.89 ROC	[36]
	Phage	HSP70	74	93					0.84 ROC	[36]
	Phage	Fibronectin	74	93					0.81 ROC	[36]
	Phage	CD44	74	93					0.79 ROC	[36]
Ovarian	Luminex	IL-8	94	80					66 SN/98 SP	[88]
Pancreatic	SERPA	vimentin	36	15	18	44.4	0	5.5		[52]
	SERPA	calreticulin	36	15	18	58	6.7	5.5		[106]
Prostate	ELISA, WB	AR	105	41		17	0			[107]
	ELISA	LEDGF/p75	207	166		18.4	5.5			[155]
multiple	ELISA	ECPKA	295	155					87 SN/90 SP	[110]

HC – healthy controls, Ben – benign (or unrelated cancer); CRC – colorectal cancer, HCC – hepatocellular carcinoma, NPC, nasopharyngeal cancer; WB – western blot, MA – protein microarray, f – fractionated lysate, p – peptide; SN – sensitivity, SP – specificity, ROC – area under ROC curve; AHSG – alpha-2-HS glycoprotein, UCH-L3 – ubiquitin C-terminal hydrolase L3, TIM – triosephosphate isomerase, MnSOD – manganese superoxide dismutase, AR – androgen receptor.

vated levels of HCC-22-5 were also found in patients with benign liver disease [94], and p62 levels were only tested in a subgroup of HCC patients expressing p62 antigen. In two other investigations concerning esophageal cancer, strong discriminatory values for autoantibodies against HSP70 using combination SERPA/MS approach [51], and NY-ESO-1 using Luminex and protein microarrays [87] were reported, however the small sample sizes utilized in each of these studies ($n < 20$ cases, controls) raises questions regarding the significance of the findings. Moderately elevated

levels of NY-ESO-1 were also observed in melanoma ($n = 127$) and ovarian cancer patients ($n = 94$) using ELISA [96]. In our own work regarding ovarian cancer, we reported circulating levels of anti-IL-8 could discriminate early-stage ovarian cancer ($n = 94$) from controls ($n = 80$) with 66% sensitivity (SN) and 98% specificity (SP) [88].

The most actively studied disease condition identified in our review was lung cancer. We identified 10 separate reports regarding the individual performance of 12 distinct autoantibodies evaluated by a variety of

techniques. The autoantibody with the highest frequency among lung cancer patients was anti-peroxiredoxin-1 (Prx-1) which was found in 47% of NSCLC patients ($n = 53$) according to a western blot analysis [97]. In a separate study, western blotting was also used to identify antibodies against recoverin in 20% of patients diagnosed with NSCLC, and 17% of all evaluated lung cancer patients ($n = 143$) [98]. In several other analyses of lung cancer subtypes, autoantibodies against PGP 9.5 and annexins I and II were associated with lung adenocarcinomas with frequencies ranging from 14–33% determined by SERPA [99,100], while 5 of 33 (15%) lung squamous carcinoma patients demonstrated elevated levels of anti-eEIF-4G measured by SEREX [101] in comparison to 17 controls. Autoantibodies against a number of other TAAs including triosephosphate isomerase (TIM) [73], MnSOD [73], DKK1 [102], and α -enolase [103] were also able to discriminate between lung cancer ($n = 40, 93, 94$, respectively) and control groups ($n = 50, 87, 60$, respectively) using a variety of techniques. The oncogenes c-myc [104] and L-myc [105] were also implicated as immunoreactive markers of lung cancer, although the relatively low frequencies in cancer patients (10%, $n = 70$ and 13%, $n = 68$, respectively) and the limited number of controls utilized ($n = 30$) render these results preliminary.

Proteomic-based methods have been applied to the analysis of autoantibodies in several other human cancers. In a pair of publications, Hong and colleagues reported the use of SERPA in the identification of antibodies directed against vimentin and calreticulin in patients diagnosed with pancreatic cancer [52,106]. Although both antibodies demonstrated elevated frequencies (44–58%) in the cancer cohort ($n = 36$), autoantibodies were also detectable in patients diagnosed with chronic pancreatitis ($n = 18$) and/or healthy controls ($n = 15$). Separately, Nam et al. [59] used a series of techniques including protein microarrays, mass spectrometry, and western blotting to examine levels of antibodies specific for ubiquitin C-terminal hydrolase L3 (UCH-L3) in colon cancer sera. UCH-L3 autoantibodies were identified in 44% of colon cancer patients ($n = 43$) but were absent from the controls ($n = 54$).

Autoantibodies associated with prostate cancer were evaluated by ELISA and western blot in several fairly large patient cohorts with mixed results. Autoantibodies against androgen receptor (AR) were identified in 17% of prostate cancer sera ($n = 105$) and none of the controls ($n = 41$) [107] while autoantibodies directed against LEDGF/p75 were found in 18.4% of cancers ($n = 207$), but also in 5.5% of controls ($n = 166$) [108].

ELISA was also used in an evaluation of cervical cancer in which autoantibodies against survivin were found to be elevated in both cancer patients ($n = 117$) and patients with defined precursor lesions ($n = 209$) in comparison to controls ($n = 80$) [109].

The most highly discriminatory individual autoantibody encountered in our review was directed against extracellular protein kinase A (ECPKA), a secreted c-AMP dependent protein kinase. ELISA measurement of anti-ECPKA levels in a large cohort made up of multiple cancer types ($n = 295$) and healthy controls ($n = 155$) demonstrated 87% SN at 90% SP [110], leading the investigators to propose ECPKA as a universal cancer biomarker. A SEREX analysis of autoantibodies in NPC also revealed several TAAs with high discriminatory ability [36]. Antibodies against MAGE, HSP70, fibronectin, and CD44 demonstrated ROC AUC values ranging from 0.79–0.89 when 74 patients diagnosed with NPC were compared to a group of 93 controls.

3.2. Autoantibody panels

The high level of specificity displayed by the diverse array of individual TAA-specific autoantibodies identified, evidenced by the low or absent levels of detection in normal sera, speaks to their potential as cancer biomarkers. However, no individual autoantibody has yet to reproducibly demonstrate a high enough frequency among patients diagnosed with a specific cancer to warrant clinical advancement. As such, while the search for additional informative autoantibody biomarkers remains ongoing, many groups have applied the techniques and findings related to autoantibody discovery to the evaluation of multi-autoantibody panels, which may offer improved cancer sensitivity while preserving a high level of specificity. Our review identified 30 separate investigations into the use of 31 distinct panels of autoantibodies capable of discriminating cancer from control groups (Table 2). Autoantibody panels were applied to patients diagnosed with breast cancer, CRC, HCC, lung cancer, ovarian cancer, and prostate cancer. The size of each panel, ranging from 2–13 autoantibodies, received considerable attention in our review given the potential for overfitting bias. Overfitting in biomarker analyses occurs when a multimarker panel is inappropriately large with respect to the number of cases being evaluated resulting in out of range predictions based largely on noise [111, 112]. A case to marker ratio less than 10 is indicative of potential overfitting bias. Panels larger than 13 were excluded from Table 2, however several notable exam-

Table 2
Autoantibody panels that discriminate cancers from controls

Cancer	Platform	Panel	Cases	Controls	SN/SP	Ref
Breast	ELISA	p53, c-myc, HER2, NY-ESO-1, BRCA2, MUC1	137	94	64/85	[114]
	ELISA	p53, HER2, TOPO2a, IGFBP2	184	134	0.70 ROC	[56]
	ELISA, WB	p16, p53, c-myc	41	82	44/97.6*	[134]
	Phage, ELISA	ASB-9, SERAC1, RELT	87	87	77/83	[113]
	SERPA, ELISA	HSP60, PPIA, PRDX2, FKBP52, MUC1	142	93	0.7–0.8 ROC	[115]
CRC	ELISA	Imp-1, p62, Koc, p53, c-myc	46	58	61/90	[117]
	ELISA, WB	Calnuc, p53, c-myc, cyclin B1, cyclin D1	69	82	65.4/94*	[118]
	Phage	LGR6, AGBL5, PDE4A	24	24	92/92	[116]
	SEREX, WB	P53, MAGEA3, SSX2, NY-ESO-1, HDAC5, MBD2, TRIP4, NY-CO-45, KNSL6, HIP1R, SEB4D, KIAA1416, LMNA	74	75	46/100*	[120]
	SMARTA	KIAA1864, MOTES-391, CCND1, RGS5, MMP-7	100	100	50/98	[72]
HCC	ELISA	CCCAP, HDAC5, p53, NMDAR, NY-CO-16	94	54	58.5/92.6	[119]
	ELISA	Survivin, p53	49	60	26.6/100*	[124]
	ELISA	Imp-1, p62, Koc, p53, c-myc, cyclin B1, survivin, p16	142	82	60/88	[121]
	SEREX	KRT23, AHSG, FTL	55	30	98.2/30	[156]
	SEREX/ELISA	Imp-1, p62, Koc, p53, cyclin B1, c-myc, p16, survivin, Sui1, RalA	77	82	66/88	[122]
Lung	SERPA	DEAD box p3, eEF2, AIF, hnRNP A2, PBP, TIM	174	71	90/82.6	[49]
	ELISA	livin, survivin	37	7	71/100*	[157]
	ELISA	p53, c-myc, HER2, NY-ESO-1, CAGE, MUC1, GBU4-5	104	50	76/92	[126]
	MA (f)	Annexin I, 14-3-3 theta, LAMR1	85	85	51/82	[128]
	Phage, ELISA	HSP70, HSP90, p130, GAGE, BMI-1	40	49	0.84 ROC	[39]
	Phage, MA(p)	GAGE7, BAC clone RP11-499F19, SEC15L15, PMS2L15, eEF1A	40	41	90/95	[38]
	WB, MS	14-3-3 theta, Annexin I, PGP 9.5	63	62	55/95	[127]
	ELISA	c-myc, cyclin B1, Imp-1, Koc, p53, p62, survivin	56	346	80/90	[125]
	Phage	ROCK1, PRKCB1, KIAA0376	39	40	93% ACC	[158]
	ELISA	survivin, p53	55	60	29.4/100*	[124]
Ovarian	Phage	SEC15L2, RP11-499F19, XRCC5, MALAT1	69	79	91/91	[37]
	MA (p)	Lamin A/C, SSRP1	30	30	95/97.5	[129]
	SEREX, SSH, SMARTA	TM4SF1, C1D, TIZ, BARD1, FXR1, OV-189	96	96	66 SN, 73 ACC	[130]
	Prostate	c-myc, p53, cyclin B1, p62, Koc, Imp-1, survivin	206	264	46/90*	[132]
	ELISA	cyclin B1, p62, Koc, Imp-1, p90, cyclin D1	133	96	92.5/85*	[131]
multiple	ELISA	cyclin B1, p62, Imp-1	206	346	79/86	[125]
	ELISA, WB	Koc, p62	777	82	20.5/97.6*	[123]

CRC – colorectal cancer, HCC – hepatocellular carcinoma; SN – sensitivity, SP – specificity, ACC – accuracy, ROC – area under ROC curve, * – SN/SP tabulated by reviewers based on reported autoantibody frequencies; WB – western blot, MA – microarray, f – fractionated lysate, p – peptide

ples from this category are discussed in the following section. Half (15/30) of the identified reports utilized ELISA as their primary technique with phage-display accounting for an additional 20% (6/30). SEREX was utilized by 4 investigations; SERPA, SMARTA, and protein microarrays were each used twice, and suppression subtraction hybridization (SSH) and MS were each employed once.

Five autoantibody panels, ranging in size from 3 to 6 analytes, were evaluated for breast cancer detection. A panel consisting of 3 phage-identified clones: ASB-9, SERAC1, and RELT provided the highest level of discrimination at 77% SN and 83% SP ($n = 87$ cases (CA), 87 healthy controls (HC)) [113]. Three of the

panels contained p53 and two of these three also contained Her-2. Muc1 was also identified twice. Two panels demonstrated particular utility in the identification of carcinoma in situ (CIS), which represents breast cancer at its earliest stages. The first of these, consisting of p53, c-myc, Her-2, NY-ESO-1, BRCA2, and muc1, was identified by ELISA and identified DCIS at 45% SN and all primary breast cancers at 64% SN at a SP of 85% ($n = 137$ CA, 94 HC) [114]. The second panel, identified by SERPA and ELISA, also contained muc1 along with hsp60, PPIA, PRDX2, and FKBP52 and identified CIS patients with a ROC value of 0.8 and early invasive breast cancer with a ROC value of 0.73 ($n = 142$ CA, 93 HC) [115].

Our review identified seven autoantibody panels evaluated for the detection of CRC. These panels ranged from 2 to 13 analytes in size and were identified using a variety of techniques. The panel with the highest performance consisted of 3 phage-identified clones: LGR6, AGBL5, and PDE4A, and provided 92% SN at 92% SP [116]. It should be noted that the sample size for this investigation was relatively small, consisting of only 24 cases and 24 controls, which may suggest overfitting of the selected panel. Five of the seven panels included p53 and two of these included the combination of p53 and c-myc. Each of these latter two panels consisted of 5 autoantibodies identified by ELISA/WB and performed similarly with SN greater than 60% and SP of at least 90% in similar size cohorts ($n = 46$ CA, 58 HC; 69 CA, 86 HC, respectively) [117, 118]. Two other 5-autoantibody panels identified by SMARTA and ELISA provided a high SP (92.6–98%) with a SN of 50 or greater [72,119]. An additional study utilizing SEREX evaluated a 13-autoantibody panel in a group of 74 CRC cases resulting in a SN/SP of 46%/100% [120]. These latter 3 reports demonstrate case:marker ratios of 5.7–13.8, an indication of overfitting bias. An evaluation of the selected panels in independent validation sets would thus be required to confirm their utility.

Four autoantibody panels were developed for the detection of HCC ranging from 3 to 10 analytes in size and several of these findings are noteworthy. Using ELISA, Zhang and colleagues identified an 8-autoantibody panel consisting of Imp-1, p62, Koc, p53, c-myc, cyclin B1, survivin, and p16 that demonstrated a SN of 60% at 88% SP ($n = 142$ CA, 82 HC) [121]. The same group later utilized SEREX in the discovery of two additional autoantibodies specific for Sui1 and RalA. When these two autoantibodies were added to the previously identified panel of eight, the SN increased to 66% while the SP remained at 88% ($n = 77$ CA, 82 HC) [122] although this large panel may be subject to overfitting bias. In a separate study, two of the TAAs included in these panels, Koc and p62, were evaluated in a large cohort of multiple cancer types ($n = 777$ CA, 82 HC) [123]. Here, this pair demonstrated a relatively low SN (20.5%) at a high level of SP (97.6%). The highest performing panel identified for HCC, which provided 90% SN at 83% SP ($n = 174$ CA, 71 HC), consists of six autoantibodies identified using SERPA including: DEAD box p3, eEF2, AIF, hnRNP A2, prostate binding protein (PBP), and triphosphatase isomerase (TIM) [49].

Similar to our observations regarding individual autoantibody analyses, lung cancer received the highest

level of attention among all malignancies in the development of autoantibody panels. A total of 10 separate panels were identified ranging in size from 2 to 7 analytes. The highest levels of discrimination were achieved by a series of panels identified by Zhong and colleagues using the phage display platform in the setting of NSCLC. Initially using a combination of phage-display and ELISA, Zhong et al. identified a panel of five autoantibodies including hsp70, hsp90, p130, GAGE, and BMI-1 capable of discriminating NSCLC patients ($n = 40$) from healthy controls ($n = 49$) with an ROC AUC value of 0.84 [39]. Later, the same group utilized protein microarrays in concert with their T7 phage system to identify a distinct panel of 5 clones demonstrating a SN of 90% at a SP of 95% ($n = 40$ CA, 41 HC) [38]. It should be noted here that the design of both of the aforementioned studies is permissive of overfitting bias and the identification of two completely distinct panels by the same group using similar methods only reinforces this observation. In their most recent report, Zhong et al. once again utilized their T7 phage system to identify a panel of 4 clones, one of which was retained from the previous analysis, that could identify NSCLC patients ($n = 69$) five years prior to detection by autoradiography from a clinical cohort containing high-risk healthy controls ($n = 79$) with a SN of 91% at a SP of 91% [37]. Our review identified 4 autoantibody panels for the detection of lung cancer identified through the use of ELISA. Among these panels, p53 and survivin were each included three times while c-myc was included twice. The paired combination of p53 and survivin resulted in a SN of only 29.4 in the identification of lung cancer patients ($n = 55$ CA, 60 HC) [124], however in a separate study, when p53, survivin, and c-myc were combined with four other TAA autoantibodies: cyclin B1, Imp-1, Koc, and p62, a SN of 80% at a SP of 90% ($n = 56$ CA, 346 HC) was achieved [125], although this larger panel also may be subject to overfitting. A 7-autoantibody panel containing both p53 and c-myc also demonstrated a high SN/SP of 76%/92% ($n = 104$ CA, 150 HC) for the identification of early stage lung cancer [126]. Using several techniques, Pereira-Faca and colleagues evaluated the use autoantibodies to annexin I and 14-3-3 theta for the early detection of lung cancer. In preliminary findings, this pair of markers, combined with either PGP 9.5 or LAMR1 was able to identify a small subset of prediagnostic lung cancer patients ($n = 18$) with a SN/SP of 55%/95% and 51%/82%, respectively [127,128].

Two autoantibody panels were identified for the detection of ovarian cancer. A 3-autoantibody panel iden-

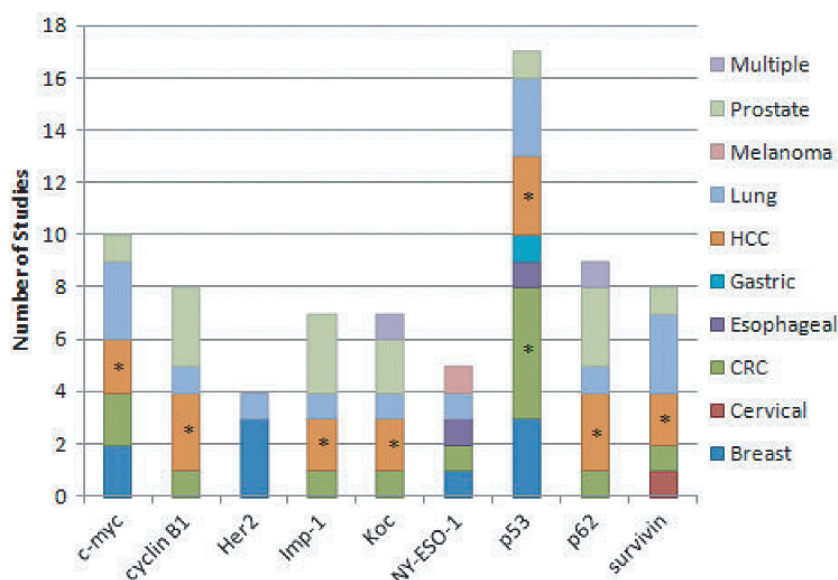


Fig. 1. Cancer distribution of most commonly identified autoantibodies. In each case, autoantibodies against the indicated tumor-associated antigen (TAA) were used to discriminate cancer patients from a control group either singly or as part of a panel. (*) indicates that multiple autoantibody detection platforms were utilized within the cancer-specific subgroup. HCC – hepatocellular carcinoma, CRC – colorectal cancer.

tified by protein microarray consisting of lamin A, lamin C, and SSRP1 provided a very high SN/SP of 95%/97.5%, however the cohort utilized in this study was relatively small ($n = 30$ CA, 30 HC) and the panel was found to react with a number of unrelated malignancies [129]. A 6-autoantibody panel identified through a combination of SEREX, SSH, and SMARTA also demonstrated modest performance for ovarian cancer, providing a SN of 66% and an accuracy of 73% ($n = 96$ CA, 96 HC) [130].

In a series of investigations, Tan and colleagues utilized ELISA to evaluate several combinations of autoantibodies for the detection of prostate cancer [125, 131, 132]. Consistent within their reported panels were the TAAs cyclin B1, p62, and Imp-1. Tan's group achieved their best result by combining these three autoantibodies with Koc, p90 and cyclin D1 to produce a 6-autoantibody panel which demonstrated a SN of 92.5% at a SP of 85% ($n = 133$ CA, 96 HC) [131].

4. Emerging trends in TAA identification and development

Our review revealed several informative trends regarding the distribution of individual TAA findings across and within specific cancers. In the combined set of studies reporting individual and multiplexed autoantibody results, twenty-one TAAs were identified more

than once. Of these twenty-one, nine were identified four or more times and the distribution of findings for these TAAs is presented in Fig. 1. The most commonly identified TAA was p53, consistent with trends present in the literature. Autoantibodies directed against p53 were utilized in 17 separate investigations concerning 7 different cancers. In studies of HCC and CRC, anti-p53 antibodies were identified by multiple detection techniques. The second most commonly identified TAA was c-myc, followed by p62, cyclin B1, survivin, Imp-1, Koc, NY-ESO-1, and Her2. Clearly, these TAAs are among the most promising candidates for biomarker development, however their appearance in multiple malignant settings also presents challenges regarding cancer specificity. The non-specific association of autoantibodies against TAAs such as p53, Her2, and survivin with various human cancers and also autoimmune conditions has been well documented [23, 124, 132, 133]. The exclusion of any of these TAAs from panel development on the basis of cancer specificity is likely to greatly hinder the overall sensitivity of those panels; rather it is essential that cancer-specific combinations of the most informative TAAs be identified. Unfortunately, no such combination has emerged from our review, as the most informative autoantibody panels identified for each cancer type appear to draw from a common TAA pool. Therefore, the discovery of additional cancer-specific autoantibody biomarkers that can be combined with existing immunological biomark-

Table 3
Autoantibodies identified by multiple studies for a specific cancer diagnosis

TAA	Cancer	Platform	Sample size (N)			AA frequency (%)			Ref
			Case	HC	Ben	Case	HC	Ben	
c-myc	Lung	ELISA	104	50		11.5	0		[126]
		WB	68	30		13.2	3.3		[105]
		ELISA, WB	112	82		12.5	0		[134]
Cyclin B1	HCC	SEREX	77	82	60	14.3	2.4	0	[122]
		ELISA	142	82	60	13.4	2.4	0	[121]
		SEREX	100	70	140	15	0	1–4	[93]
Her-2	Breast	ELISA	137	94		18	6		[114]
		ELISA	184	134		13	5		[56]
		ELISA, WB	107	200		11	0		[22]
p62	HCC	SEREX	77	82	60	16.9	1.2	1	[122]
		ELISA	142	82	60	14.1	1.2	1	[121]
		SEREX, ELISA	95	70	77	21	0	0	[95]
p53	Breast	ELISA	137	94		24	4		[114]
		ELISA	184	134		10	1		[56]
		ELISA, WB	41	82		12.2	1.2		[134]
p53	CRC	ELISA	46	58		23.9	2.4		[117]
		ELISA, WB	69	82		9.6	1.2		[118]
		SEREX, WB	74	75		14.9	0		[120]
		ELISA	94	54		24.5	2		[119]
		ELISA	49	60		18.4	0		[124]
p53	HCC	SEREX	77	82	60	18.2	2.4	1.6	[122]
		ELISA	142	82	60	14.1	2.4	1.6	[121]
		ELISA	67	10	27	73	0	7.4	[92]
p53	Lung	ELISA	104	50		11.5	2		[126]
		ELISA	55	60		7.8	0		[124]
		ELISA, WB	112	82		7.1	1.2		[134]

HC – healthy controls, Ben – benign (or unrelated cancer); WB – western blot; HCC – hepatocellular carcinoma, CRC – colorectal cancer.

ers of tumorigenesis to improve upon overall SN and SP will be critical for the development of diagnostic panels.

The findings presented in Tables 1 and 2 were further reviewed to identify those studies which evaluated the same TAA within a single cancer type in order to assess the reproducibility of published results. This analysis was aided by the observation that many of the studies reporting on the performance of autoantibody panels also reported on individual autoantibody frequencies within their experimental cohort. Eight distinct TAA/cancer associations were evaluated in three or more separate investigations and these findings are summarized in Table 3. Results obtained in studies of anti-c-myc in lung cancer [105,126,134] and anti-Cyclin B1 in HCC [93, 121,122] were fairly reproducible with cancer related frequencies of 11.5–13.2% and 13.4–15%, respectively. The frequencies of autoantibodies directed against either of these TAA in control groups was also uniformly low (0–3.3%). In contrast, results obtained for autoantibodies directed against Her-2, p62, and p53 in a number of cancers displayed a high level of variability.

Levels of anti-Her-2 were not only variable in separate analyses of breast cancer patients (11–18%) but also among healthy control subjects (0–6%) [22,56,114]. The successive evaluation of anti-p62 in two HCC cohorts conducted by the same research group yielded a decrease in frequency from 16.9% to 14.1% [121, 122] while a separate group reported a frequency of 21% in their HCC cohort [95]. Levels of anti-p53 were highly variable within each evaluated cancer type ranging from 10–24% in breast cancer [56,114,134], 9.6–24.5% in CRC [117–120,124], 14.1–73% in HCC [92, 121,122], and 7.1–11.5% in lung cancer [124,126,134]. These observations corroborate those made in a recent systematic review of cancer-related autoantibody findings which noted a considerable amount of discrepancy throughout the literature regarding methods of normalization and the establishment of cut-off values [135]. Such methods typically rely on comparisons of autoantibody levels between case and control sets as opposed to the use of standardized calibrators for the generation of quantitative data. Our group has successfully applied quantitative methods to the analysis of cancer-

associated autoantibodies [88], and the expanded adoption of similar methodology should lead to an overall improvement in data reproducibility.

5. Conclusions and future directions

The search for autoantibody biomarkers useful in the early detection of human cancers has been characterized by the continuous incremental advancement of our technological ability to reliably monitor the immunological response to malignancy. Indeed, the development of novel methods and the innovation upon existing platforms has kept pace with the discovery of TAA-specific autoantibodies themselves. However, our review of cancer-specific autoantibody findings revealed some surprising trends in the usage of available technologies. Nearly half of the reports reviewed here utilized ELISA or western blot as the primary detection method. The combined reports of SEREX, SERPA, and phage-display usage accounted for roughly 40% of the primary methodology. While these latter three platforms have been integral in the discovery of the diverse array of autoantibodies currently under investigation, the preclinical and clinical advancement of autoantibody signatures characteristic of specific malignancies will likely rely upon methods more amenable to high-throughput multiplex biomarker analysis, such as protein microarrays and Luminex®. These latter techniques were utilized in less than 10% of the studies under review, indicative of their delayed adoption by research groups. These methods offer dramatic reductions in terms of time, labor, and sample volume requirements, thus enabling the high degree of measurement repetition necessary to fully evaluate biomarker reproducibility. It would seem from these observations that a transition to methodology better suited for biomarker development that incorporates standardized procedures for data analysis will be required in order to advance autoantibody biomarker candidates beyond the basic research setting.

Another trend related to methodology apparent in our review is the high performance of autoantibody panels identified through the use of phage-display systems. Such systems were utilized in studies of breast [113], colorectal [116], and lung [37,38] cancers and the resulting panels outperformed all of those generated using other methods. However, a caveat to this high performance is the observation that none of TAAs identified in the phage-display analyses were also identified by other methods, indicating a disconnect between the

various technological platforms in use. Several other recent reports describing the use of phage-display in the identification of autoantibody signatures for HNSCC [136], lung adenocarcinoma [137], and prostate cancer [138] also fit this trend. While the reported high levels of SN and SP, exceeding 80% in each case, are strengthened by the use of independent validation sets in each of these studies, the identified panels consist of a large number of TAA clones (22–130), only a fraction of which represent known genes. The large size of these panels will likely hinder their clinical development, as will the lack of corroborating results obtained by other methods. It is possible that the integration of protein microarrays into phage-based systems will lead to an improvement in overall reproducibility.

In our review of findings related to the use of autoantibody panels, several significant limitations are evident. Of the 32 panels evaluated, 8 were found to be at a high risk of overfitting bias due to sub-optimal study design. Validation of multimarker panels in independent, blinded sample sets is an integral step in their development. In the case of panels that may potentially result from overfitting, independent validation is necessary for even a preliminary assessment of efficacy. The lack of independent validation in any of the studies listed in Table 2 is indicative of the emergent nature of autoantibody panel development. An additional limitation apparent for the reviewed autoantibody panels is their inability to outperform traditional non-IgG biomarkers currently under investigation. While it remains the case that no single biomarker or biomarker panel has yet achieved the requisite SN and SP to warrant widespread clinical implementation for any of the cancers discussed herein, several recently described examples currently offer a level of cancer discrimination that meets or exceeds that of the best autoantibody panels presented here. Pro-gastrin-releasing peptide (ProGRP) could discriminate both SCLC and NSCLC from benign pulmonary disease with 76–78% SN at 95–98% SP [139], while a combination of TIM and Prx-6 could discriminate lung squamous cell carcinomas from healthy controls with 69% SN at 83% SP [140]. Early prostate cancer antigen (EPCA) recently demonstrated both high SN (92–94%) and SP (92–94%) for prostate cancer in two small cohorts [141,142]. For HCC, several markers have shown promising results including AFP-L3 (75–97% SN, 90–92% SP) [143–145], and des-gamma carboxyprothrombin (DCP) (74% SN, 86% SP) [146]. For the detection of CRC, biomarkers such as C3a-desArg (97% SN, 96% SP) [147] and a family of proteins known as colon-cancer-specific an-

itigens (CCSA) 1–3 (78–89% SN, 82–97% SP) [148] all outperform each identified autoantibody panel. A number of groups have reported considerable progress in the development of biomarker panels for the early detection of ovarian cancer [149]. Several of these currently outperform the panel of six autoantibodies discussed herein [130]. Notable among these is the panel of leptin, prolactin, osteopontin, and IGF-2 reported by Mor et al. [150] (95% SN, 94% SP), the combination of CA-125 and mesothelin reported by McIntosh et al. [151] (86.5% SN, 98% SP), and our own report of a 4-biomarker panel consisting of CA-125, HE4, CEA, and VCAM-1 (86–93% SN, 98% SP) [152].

It is apparent that while much progress has been made in efforts to identify and develop TAA-specific autoantibodies as cancer biomarkers, much still remains to be accomplished. In addition to those obstacles identified in this review, many others must be overcome. Among these is the phenomena of background autoimmunity, well documented in several reports [153, 154], present throughout healthy populations. While poorly understood, such factors may hinder the overall specificity of autoantibody-based screening. Along with these obstacles, there exist avenues of promise, including the combined use of conventional and autoantibody biomarkers. This type of approach was applied in a number of the works reviewed here resulting in an overall improvement in cancer identification [35, 87, 88, 102, 116, 117, 119, 122, 130]. The ongoing refinement of detection techniques and recognition of the specific challenges impeding progress will undoubtedly lead to continued advancement in this emerging area of early detection.

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