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Autoantibody signatures: progress and perspectives for early cancer detection

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- **Introduction**
- **Unknown origins of autoantibody production in cancer**
- **The use of proteomics to identify autoantibodies**
- **Clinical utility of autoantibody signatures for early detection of cancer**
  - **Autoantibodies as potential cancer biomarkers**
  - **Defining autoantibody signatures in cancer: still many challenges**
- **Autoantibody detection to build screening tests in high-risk populations**
- **Conclusion and perspectives**

Abstract

Becoming invasive is a crucial step in cancer development, and the early spread of tumour cells is usually undetected by current imaging technologies. In patients with cancer and no signs of overt metastases, sensitive methods have been developed to identify circulating autoantibodies and their antigen counterparts in several cancers. These technologies are often based on proteomic approaches, and recent advances in protein and antibody microarrays have greatly facilitated the discovery of new antibody biomarkers in sera from cancer patients. Interestingly, in a clinical application setting, combinations of multiple autoantibody reactivities into panel assays have recently been proposed as relevant screening tests and validated in several independent trials. In addition, autoantibody signatures seem to be particularly relevant for early detection of cancer in high-risk cancer patients. In this review, we highlight the concept that immunogenic epitopes associated with the humoural response and key pathogenic pathways elicit serum autoantibodies that can be considered as relevant cancer biomarkers. We outline the proteomic strategies employed to identify and validate their use in clinical practice for cancer screening and diagnosis. We particularly emphasize the clinical utility of autoantibody signatures in several cancers. Finally, we discuss the challenges remaining for clinical validation.

Keywords: autoantibodies • tumour-associated antigen • cancer • proteomics • serum biomarker • signature • early diagnosis

Introduction

Early detection represents one of the most promising approaches to reducing the growing cancer burden [1]. The challenge consists of finding tumours at early stages to enable curative treatment before progression occurs. This goal is particularly important in high-risk populations in which incidence of disease is significantly increased. For early detection to be an effective and practical approach, screening tests must satisfy five basic requirements. First, the test must show a high degree of accuracy with a suitable cut-off level defined and agreed. Secondly, detection should be possible at stages where disease is curable. Thirdly, the test should allow discrimination between aggressive lesions requiring treatment from harmless lesions, avoiding the problem of overdiagnosis. Fourthly, tests should be inexpensive and well accepted by the target population [1]. Finally, the test should be reproducible and correctly calibrated to be relevant. However, currently used markers do not satisfy all these requirements. For example, in breast cancer, CA 15-3 antigen concentrations are increased in 10% of patients with stage I disease, 20% with stage II disease, 40% with stage III disease and 75% with stage IV disease. Thus, lack of sensitivity for early-stage disease combined with a lack of specificity precludes the use of CA 15-3 antigen for the early diagnosis of breast cancer [2].

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Over the past few years, evidence of circulating autoantibodies in the sera of cancer patients has created opportunities for exploiting the immune system as a source of cancer biomarkers. Indeed, the release of proteins from tumours triggers an immune response in cancer patients [3]. Considerable efforts have been made to identify the autoantibodies and their antigen counterparts to detect and/or monitor cancer progression. Over the past 10 years, several articles have demonstrated the potential use of autoantibody detection in early cancer detection. In this review, we highlight the features of serum autoantibody biomarkers and outline the strategies employed to identify them and validate their use in clinical practice in several cancers including breast, lung, ovarian and prostate. These strategies should facilitate the discovery of relevant autoantibody signatures for not only cancer screening and diagnosis, but also prognosis and monitoring of therapy. Particularly, we focus on the clinical impact of the autoantibody signatures that were identified these past few years for early detection in high-risk cancer patients.

Unknown origins of autoantibody production in cancer

In the 1960s, Robert W. Baldwin demonstrated that the immune system could react to a developing tumour [4–6]. Autoantibodies might act to drive an effective response against tumours, following several pathways, including opsonization, enhancement of dendritic cell-mediated antigen presentation to T cells, recruitment of natural killer cells to perform antibody-dependent cell-mediated toxicity, generation of tumour antigen-specific $CD8^{+}$ T cells and complement-dependant cytotoxicity [7]. However, these mechanisms are too often not effective enough to provide sufficient clinical responses [7]. Moreover, how exactly these natural autoantibodies originate remains a mystery [8]. One explanation is that they are secreted from self-reacting B cells that escape deletion [9] or from immature B cells [10].

The magnitude of the immune response to cancer, in general, is lower than the immune response to infectious agents, and the potential number of tumour-associated antigens (TAAs) encompasses the entire tumour proteome in all its variations [3]. During the anti-tumoural response, the immune system performs a very efficient biological amplification, with antigenic tumour proteins as templates, that allows indirect detection of very small amounts of antigen. Indeed, because the immune response is generated locally, tumour-specific or tumour-associated proteins can be concentrated, processed by antigen-presenting cells and displayed by lymphocytes in the lymph node draining the tumour site. For instance, it has been shown that in medullary carcinoma of the breast, tumour cells expose actin on their surface, eliciting a major antibody response by infiltrating B cells and therefore increasing tumour cell apoptosis [11]. Moreover, this amplification begins at an early stage when the tumour is not clinically detectable [12].

Although little is known about the origin of this immune response, it is now largely established that cancer patients produce autoantibodies to mutated tumour proteins [13], misfolded [14], overexpressed [15], aberrantly degraded [16] or aberrantly glycosylated proteins [17, 18], and proteins that are ectopically expressed [11]. These spontaneous responses are frequently found in cancer patients, ranging from 5% to 30% for a single TAA. Therefore, because secreted autoantibodies reflect the presence of a tumoural burden, they represent attractive and suitable biomarkers for cancer detection.

The use of proteomics to identify autoantibodies

The technical difficulties in defining human autoantibody signatures related to the development of tumours, mainly based on the difficult establishment and limited availability of pre-characterized tumours cell cytotoxic T lymphocyte clones for most neoplasms [19], led to the search for strategies that allowed for the exploitation of the autoantibody repertoire by the systematic identification of autoantigens. Two main techniques have allowed identification of many autoantigens: serologic identification of antigens by recombinant expression (SEREX; Fig. 1) and serological proteome analysis (SERPA; Fig. 2). Recent advances in proteomic methods, such as mass spectrometry and protein arrays, have greatly facilitated the discovery of new antibody biomarkers in sera from cancer patients. SEREX and SERPA remain techniques of reference for the study of the humoural response to cancer because they have allowed identification of a great number of TAAs (for review, see Ref. 20). The success of DNA chip approaches for wide genome analysis has inspired research groups interested in the humoural response over the past few years, and protein microarrays have thus renewed the field of autoantibodies in cancer detection. The utility of antigen arrays to profile serum autoantibodies responses in human disease is generating much interest because of their potential for improving early diagnosis, monitoring disease progression and guiding interventions for disease prevention and early treatment (Fig. 3; Ref. 21]. Joos *et al.* began printing TAAs and then showed that this technique could be used for fast and highly sensitive diagnosis of autoimmune diseases [22]. This work was followed by the study of Robinson *et al.*, who spotted 196 known antigens onto a glass slide. They tested over 100 patients' samples from eight different autoimmune diseases and showed that comprehensive autoantigen microarrays could be used to profile autoantibodies in these diseases with high sensitivity and specificity [23]. The development of TAA microarrays for autoantibody profiling in systemic autoimmune diseases has paved the way for the use of this technology in other diseases, including cancer. To improve autoantigen coverage, and to circumvent the limited availability of commercialized or homemade proteins that are to be spotted on the support, Chinnaiyan's team developed a method derived from the SEREX technology based on the use of a phage-display cDNA expression library from isolated prostate tumour mRNA [24]. The study, performed with sera from 119 patients with prostate cancer and 138 healthy controls, allowed

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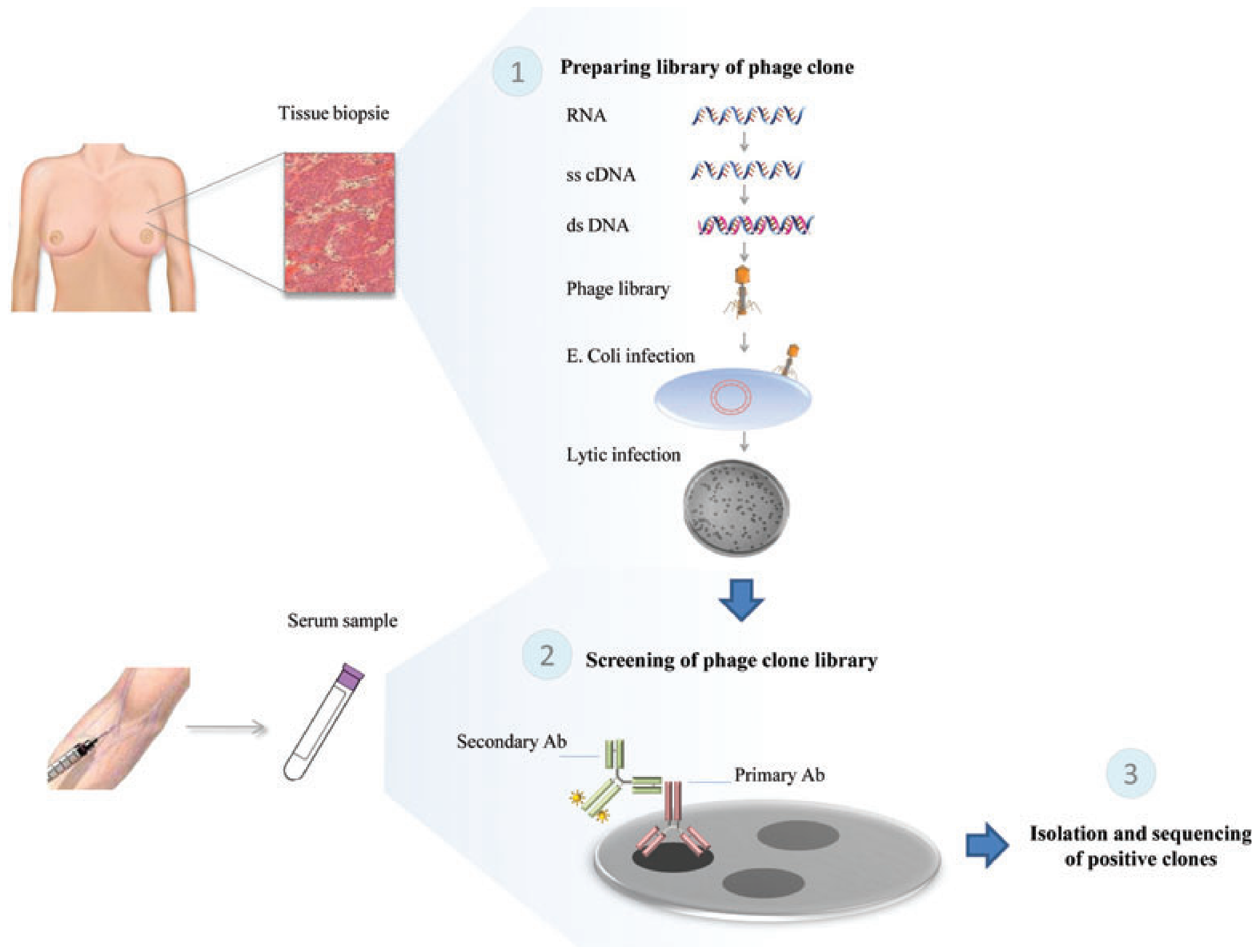

Fig. 1 Screening TAAs expressed in tumours using SEREX. A cDNA expression library is first (step 1) constructed from tumour specimens and cloned into λ-phage expression vectors. The resulting recombinant phages are used to transfect *Escherichia coli*. Recombinant proteins, which are expressed during lytic infection of bacteria, are transferred onto nitrocellulose membranes, which are then incubated with serum from the autologous patient (step 2). Reactive clone with high titres of IgG antibodies are identified using an enzyme-conjugated secondary antibody. The inserted cDNA is then sequenced (step 3). The main advantage of SEREX is that it allows exploration of the humoural response in sera from patients with their own tumour as an antigenic source.

for selection of a panel of 22 peptides that discriminated the two groups with 81.6% sensitivity and 88.2% specificity, thus providing additional discriminative power over prostate-specific antigens. This approach allows for high-throughput analysis and more efficient phage selection, thus making it more powerful than SEREX. However, this technology, such as SEREX, does not allow detection of post-translational modifications (PTMs). Hanash's team then developed a multi-dimensional fractionation technique using liquid chromatography (LC) to isolate a mixture of native proteins extracted from cancer cell lines [25, 26]. This original natural protein microarray-based approach resulted in the identification of autoantibodies directed against C-terminal hydrolase L3 ubiquitin in the sera of patients with colon cancer [27] and autoantibodies directed against PGP9.5 in lung cancer [25]. Finally, protein microarrays that enable large-scale testing on more than 80,000 recombinant antigens have also been developed, which, for instance, include ProtoArray® protein chips (Invitrogen, Brandford, CT, USA) [28]. Recently, this technique has been used to identify 94 autoantibodies exhibiting enhanced reactivity in ovarian cancer patients compared to controls [29]. Reverse capture microarrays (Fig. 3), on the other hand, have the advantage of allowing the antigens to be immobilized in their native configuration, with their PTMs. This recent technique [30] allowed identification of 35 autoantibodies in mucinous ovarian cancer; moreover, a panel of six autoantibodies may segregate between non-smoker and smoker patients, who may be more at risk of developing this type of cancer [31]. However, no further validation has been made in a significant independent cohort concerning this signature.

Protein microarrays, although very promising in biomarker discovery, show some common pitfalls. First, this technique is

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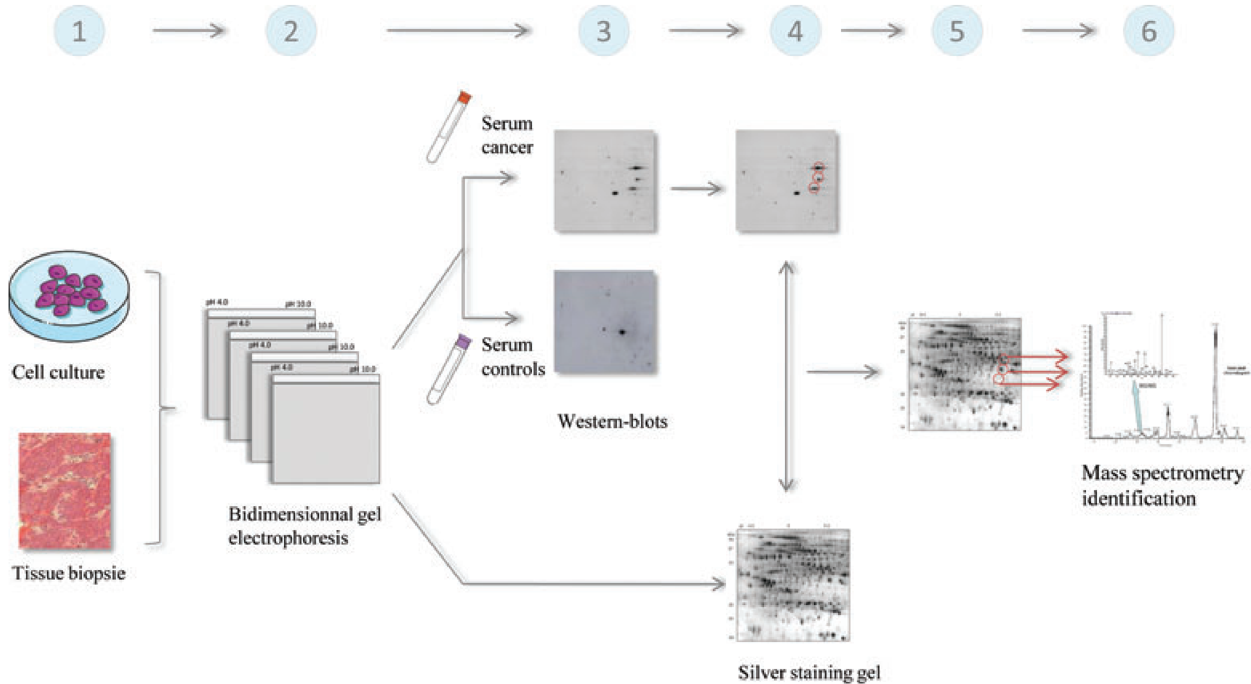

Fig. 2 Screening TAAs expressed in tumours or cell culture using SERPA. A complex mixture of proteins extracted from tumour or cell cultures is first separated by two-dimensional electrophoresis, according to their isoelectric points (p*I*; first dimension) and their molecular weights (second dimension; steps 1 and 2). Proteins are then transferred and immobilized on a membrane (step 3). Sera from cancer patients and controls are screened individually (step 4), allowing immunodetection of relevant antigens among the several thousand individual proteins separated using 2-DE. Comparative probing of blots allows selection of spots specifically reacting with cancer sera (step 5). These spots are then excised from the gel, and the proteins are identified by mass spectrometry (step 6). SERPA allows identification of protein isoforms and PTMs, but it has limitations in its identification of low molecular weight and/or low-abundance proteins, due to the sensitivity of detection.

dependent on the scientific hypothesis stated at the beginning, and some proteins, if not spotted on the array, will consequently be missed. Secondly, high-throughput techniques, such as protein arrays or nano-LC fractionation, are very expensive, and require important expertise. Finally, for the time being, the understanding of protein immobilization is still rudimentary and will require more effort in the coming years [32]. It is essential for building diagnostic tests based on such technologies because orientation of the protein/antibody spotted and spotting conditions determines the performance of the test.

Clinical utility of autoantibody signatures for early detection of cancer

Autoantibodies as potential cancer biomarkers

Autoantibodies are a group of serum biomarkers that show highly interesting properties. Autoantibodies are secreted and are therefore easily accessible. The persistence and stability of autoantibodies in the serum of patients is an advantage over other potential markers currently used. Autoantibodies are present in the sera before TAAs can be detected (if they are). They correspond to an efficient biological amplification of the presence of TAAs, and are secreted in the serum prior to first clinical signs. Moreover, antibodies are highly stable in serum samples and are not subject to proteolysis like other polypeptides, making sample handling much easier. They show a long lifetime ($T_{1/2}$ between 7 and 30 days, depending on the subclass of immunoglobulin) in blood and may persist as long as the corresponding autoantigen elicited a specific humoural response. Finally, antibodies are biochemically well-known molecules, and many available reagents and techniques are available for their detection, simplifying assay development.

Defining autoantibody signatures in cancer: still many challenges

Over the past few years, studies on biomarkers have aimed at identification of a panel of biomarkers because single markers do not display enough sensitivity and specificity to build a reliable assay for early detection. These difficulties are probably due to the heterogeneity of cancer. Indeed, the vast majority of patients are

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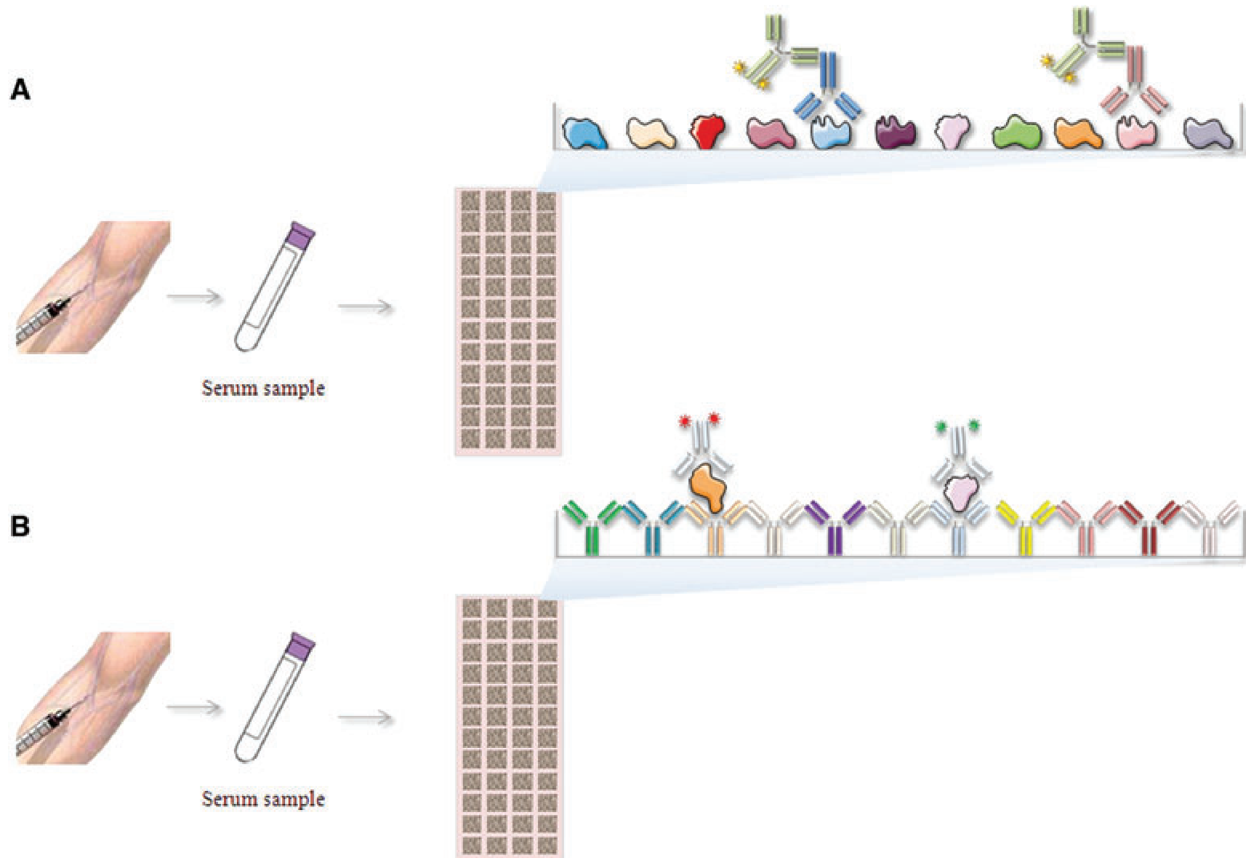

Fig. 3 Screening TAAs expressed using microarrays-based approach. Protein microarrays [22, 23] are based on hundreds to thousands of known antigens immobilized on a glass slide that can be either commercially (*e.g.* Protoarrays®, Invitrogen) or laboratory made (**A**). The arrays are produced either by using on-chip synthesis strategies or with an arrayer based on contact printing or ink jet technology. It is then probed with serum samples from patients and appropriate controls, to isolate antigens that specifically elicit an immune response to cancer. In general, proteins are produced in prokaryotic systems (*e.g. E. coli*), which hampers identification of PTMs. Reverse capture microarrays immobilize well-characterized, highly specific and high affinity antibodies designed to bind native antigens contained in cell extract from tumours or cell lines (**B**). Then, labelled purified autoantibodies from the patient's serum are added. Cancer and control autoantibodies are labelled with different cyanin dyes, and the ratio of fluorescence determines the relative abundance of the autoantibodies in a given serum sample. The identification is direct due to the antibodies, and this technique, contrary to protein microarrays, allows identification of natural tumour epitopes, and PTMs. Microarray-based approach allows for analysis of a great number of targets in one step.

unlikely to respond to the same immunodominant antigens, as is the case in infections. Even cancers of the same type are composed of a mix of different biological subtypes; consequently, patients are likely to develop an immune response to different sets of antigens, and no single antigen is likely to detect all cancers.

The study by Chinnayian's team was the first to show that a specific combination of autoantibodies could detect prostate cancer better than the reference marker, PSA [24]. Megliorino *et al.* have used ELISA to evaluate the detection of a combination of three autoantigens: c-myc, p53 and survivin, in breast, colorectal, oesophageal, gastric, hepatocellular, lung and other carcinomas [33]. They show autoantibody frequencies varying between 9.1% and 38.5% in cancer patients compared to 0–4.9% in controls when the three TAAs were tested together. Several known TAAs were also investigated in 527 patients from six different cancers types by mini-arrays [34]. The authors show an increase of positive antibody reactions from 15–20% for single TAAs to 44–68% for seven TAAs. Therefore, combinations of known TAAs show an increase in the sensitivity, but clearly are not sufficient to build a reliable screening test. Moreover, one can noticed that these studies do not use matched control population neither risk or high-risk control population.

To define relevant combinations of autoantibodies, several points need to be considered. First, adequate statistical methods should be used to define the best signature according to type of cancer. Interestingly, Leidinger *et al.* showed that a 20-antigens signature could achieve 93.1% specificity in normal sera *versus* squamous cell carcinoma (SCC), and an 80-antigen signature was needed to achieve 99.2% specificity in normal *versus* low-grade SCC sera using a standard naïve Bayesian classification method

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combined with a feature subset selection method [35]. Babel *et al.* identified five immunoreactive TAAs in colorectal cancer samples using a commercial protein microarray containing 8000 human proteins [36]. Then, they sought to determine which markers used in combination were more informative and allowed a better discrimination between groups using logistic regression and receiver operating characteristic (ROC) curves. Their final model retained two out of five markers, which gave the highest sensitivity (73.9%) and specificity (83.3%). The study of Wang *et al.* defined a signature based on a phage-display library in prostate cancer using a non-parametric pattern recognition approach that identified a 22 phage-peptide detector [24]. Taylor *et al.* used a supervised analysis to develop a signature most predictive for class distinction across the serum samples [37]. This signature distinguishes benign prostatic hyperplasia from localized prostate cancer with 78% sensitivity and 75% sensitivity. Therefore, various machine-learning algorithms allow establishment of possibly more relevant multi-marker models. The parameters used to create these signatures should be clearly stated so that analyses can be reproduced by other scientists [38]. Secondly, the observation of a significant association does not ensure that the findings can be generalized in other populations or that the association is highly specific for the condition investigated. Therefore, most biomarkers with promising results in a first data set will turn out to have less promising results in independent data sets [38]. In the study of Wang *et al.* [24], phage protein microarrays were used to define autoantibody signature of prostate cancer from 129 serum samples (59 patients with clinically localized prostate cancer and 70 controls without history of cancer). The autoantibody signature was validated with an independent population of 128 serum samples (60 patients with localized prostate cancer and 68 controls without history of cancer). A 22-phage-peptide detector was built allowing to obtain 88.2% specificity and 81.6% sensitivity in discriminating between the group with prostate cancer and the control groups. Interestingly, the validation population included 55 more samples noted ‘other category’, which included 30 patients with lung cancers. Of these 30 samples, nine were classified as having prostate cancer, which suggests some cross-reactivity of autoantibodies across tumour types. These results are not surprising at all, given that several cancer types share a certain number of TAAs. The authors propose that this test may be useful in combination with initial PSA screening because it adds significantly to the diagnostic power of PSA alone and lowers the number of unnecessary biopsies. This study, although very promising for detection of prostate cancer, also highlights the need for an appropriate control population during the training and the validation steps, such as sera from patients with benign diseases (in this case, benign prostatic hyperplasia), autoimmune disorders and other related diseases such as other cancers, to render the signature more specific [39–42]. There is a need for validation in an independent population that was used for the screening set. We can see in Table 1 that only several studies out of 13 [43, 44] were able to validate the results in an independent population. This underscores the difficulty in gaining access to human samples. In addition, the results need to be validated on larger multi-centre cohorts. Only one study was able to do so [45]. The previously identified signatures should be validated in multi-centre appropriated cohorts of patients to evaluate their clinical relevance and diagnostic power.

Autoantibody detection to build screening tests in high-risk populations

Autoantibody signatures might be useful in cancers in which there are high-risk populations and where existing detection methods lack sensitivity and specificity. This is particularly the case in lung and breast cancers (Table 1).

In lung cancer, large clinical studies set up to follow high-risk patients, such as heavy smokers and asbestos-exposed patients (*e.g.* CARET; Ref. 46) and Mayo Clinic Lung Screening Trial (MCLST cohorts; Ref. 47) have rendered available large panels of pre-diagnosis sera, dating from 0 to 5 years before cancer diagnosis, thus allowing studies on early cancer detection. Chest X-rays and computed tomography (CT) are screening methods generally used in high-risk patients groups, such as heavy smokers. However, up to 90% of pulmonary nodules detected are actually benign, resulting in 11.5% false-positive rate because of the high prevalence of non-calcified and ground glass pulmonary nodules in these particular patients [48]. Ugo Pastorino described the result of several observational studies, including 64,475 patients. At baseline, the overall frequency of participants with suspicious non-calcified solid lesions was 20% (range 7–53) and the lung cancer detection rate was 1% (range 0.4–2.7) [49]. Recently, Bach *et al.* reported that screening with CT may increase the rate of lung cancer diagnosis and treatment, but not meaningfully reduce the risk of advanced lung cancer or death from lung cancer [50]. More recently, initial results of the National Lung Screening Trial showed 20% reduction in cancer mortality due to screening and 20–60% abnormalities detected by CT (http://www.cancer.gov/clinicaltrials/noteworthy-trials/nlst/updates). To confirm that these nodules are benign, unnecessary thoracotomy is performed. Finally, the screening process is complex and difficult, creating an urgent need to develop reliable screening tests in these high-risk populations. In lung cancer, autoantibody panels have been developed with sensitivities varying between 36% [45] and 97.9% [51] (Table 1). Improvement of the identification process of TAAs and of the validation step could partially explain this increase in sensitivity. Boyle *et al.* [45] developed an autoantibody panel that shows sensitivities of 36–39% in three different groups of patients and a specificities of 89–91%. The goal is to identify early tumours in high-risk patients (smokers or former smokers). This panel allows identification of approximately 40% of primary lung cancers, with a good specificity against aged-matched, gender-matched and smoking-history–matched controls. In addition, the specificity was similar for patients with benign disease. Very recently, this same team published results obtained on an important cohort of small cell

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Table 1 Identification of autoantibody signatures in lung and breast cancer

Cancer type	Autoantigen signature	Number of sera	AUC	Sensitivity (%)	Specificity (%)	Comments	Reference
Lung	Paxillin, SEC15L2, BAC clone RP11-499F19, XRCC5, MALAT1	*Training set*: 23 risk-matched controls–23 Stage I NSCLC	0.990 (training set)	91.3 (training set)	91.3 (training set)	Retrospective study from the MCLST cohort with 40 sera drawn 1–5 years before diagnosis	[69]
		Validation set: 56 risk-matched controls–46 cancers		87.5 (validation set)	82.6 (validation set)		
	14-3-3θ, annexin 1, PGP 9.5	*Training set 1*: 19 healthy controls (age and gender matched, non-smoker)–19 ADC	0.838 (set 3)	55.0 (set 3)	95.0 (set 3)	Validation set 3: retrospective study from the CARET cohort with sera drawn 1 years before diagnosis	[43]
		Training set 2: 24 risk-matched controls (age and gender similar)–26 ADC					
		Validation set 3: 19 heavy smoker controls–18 pre-diagnosis ADC					
	c-myc, p53, NY-ESO-1, HER2, CAGE, MUC1, GBU4-5	50 non-matched healthy controls–82 NSCLC–22 SCLC	–	78.0 (NSCLC)	–		[70]
				92.0 (SCC)			
				77.0 (ADC)			
				76.0 (all cancer)	92.0 (all cancer)		
	14-3-3θ, annexin 1, LAMR1	85 risk-matched controls–85 pre diagnosis ADC (1 year before detection)	0.730	51.0	82.0	Retrospective study from the CARET cohort with sera drawn 1 years before diagnosis	[54]
	20–80 peptide clones	40 non-matched healthy controls–29 NTLP–39 SCC	0.978 (SCC/Healthy)*	92.9 (SCC/Healthy)*	93.1 (SCC/Healthy)*		[35]
			0.998 (low grade/ Healthy)†	79.0 (low grade/ Healthy)†	99.2 (low grade/ Healthy)†		
			0.892 (SCC/NTLP)‡	75.2 (SCC/NTLP)‡	93.5 (SCC/NTLP)‡		
	1827 peptide clones	80 non-matched healthy controls–26 NTLP-29 NSCLC–18 SCLC	–	97.9 (cancer/ healthy)	97.0 (cancer/ healthy)		[51]
				99.8 (cancer/NTLP)	42.4 (cancer/NTLP)		
				75.9 (Stage IA/IB/Healthy)	97.6 (Stage IA/IB/Healthy)		

Continued

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Table 1 Continued

Cancer type	Autoantigen signature	Number of sera	AUC	Sensitivity (%)	Specificity (%)	Comments	Reference
	c-myc, p53, cyclin B1, p62/IMP2, IMP3/KOC, IMP1, Survivin, Cyclin A, Cyclin D1, CDK2	36 non-matched non-smoking healthy controls–non-matched smoking controls (35 with no nodules, 55 with solid nodules and 46 with GGO, based on CT)–22 lung cancers	0.907 (cancer/smoking controls)	90.9 (cancer/smoking controls)	82.0 (cancer/smoking controls)	High-risk tobacco smokers and asbestos-exposed individuals from the New York University Lung Cancer Biomarker Center	[53]
	IMPDH, phosphoglycerate mutase, ubiquilin, annexins I and II, HSP70-9B	31 non-matched 'cancer free' controls–32 COPD–13NTLP–117 NSCLC	0.934	94.8	91.1		[55]
	p53, NY-ESO-1, CAGE and GBU4-5 for set 1	*Set 1*: 145 healthy controls (gender, age and smoking history matched)–145 stage I/II lung cancer (NSCLC+SCLC)*	0.710 (set 1)	36.0 (set 1)	91.0 (set 1)		[45]
	p53, NY-ESO-1, CAGE, GBU4-5, Annexin I, SOX2 for set 2 and set 3	*Set 2*: 240 healthy controls (gender, age and smoking history matched)– 241 lung cancer (NSCLC+SCLC)	0.630 (set 2)	39.0 (set 2)	89.0 (set 2)		
		Set 3 (validation): 269 healthy controls (gender, age and smoking history matched)–269 lung cancer (NSCLC+SCLC)	0.640 (set 3)	37.0 (set 3)	90.0 (set 3)		
	Six peptide clones	*Training set*: 10 healthy controls (gender and age matched)–10 NSCLC	0.969 (stage I–IV)	95.6 (stages I–IV)	95.6 (stages I–IV)		[44]
		Validation set: 90 healthy controls (gender and age matched)–12 COPD–90 NSCLC	0.962 (stages I and II)	96.7 (stages I and II)	95.2 (stages I and II)		
	p53, NY-ESO-1, CAGE, GBU4-5, SOX2, Hu-D	247 gender, age and smoking history matched controls–243 SCLC	0.760	55.0	90.0		[52]
				42.0	99.0		
Breast	12 phage breast cancer clones	*Training set*: 26 healthy controls (gender, age and race matched)–7 DCIS–38 IDC	–	76.0 (training set)	92.0 (training set)		[63]
		Validation set: 25 healthy controls (gender, age and race matched)–8 DCIS–37 IDC		78.0 (validation set)	84.0 (validation set)		

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Table 1 Continued

Cancer type	Autoantigen signature	Number of sera	AUC	Sensitivity (%)	Specificity (%)	Comments	Reference
	c-myc, p53, NY-ESO-1, BRCA1, BRCA2, HER2, MUC1	94 non-matched healthy controls–40 DCIS–97 IDC	–	45.0 (DCIS/healthy)	85.0		[65]
				64.0 (IDC/healthy)			
	ASB-9, SERAC1, RELT	87 healthy controls (gender and age matched)–87 breast cancer	0.861 (training set)	80.0 (training set)	100.0 (training set)		[64]
				77.0 (leave-one-out cross-validation)	82.8 (leave-one-out cross-validation)		
	FKBP52, PPIA, PRDX2, HSP60, MUC1	*Training set*: 20 healthy control (gender and age matched)–20 autoimmune diseases–20 other cancers–20 IDC	0.800 (validation set, DCIS/Healthy)	72.2 (validation set, DCIS/Healthy)	72.6 (validation set, DCIS/Healthy)		[62]
		Validation set: 93 healthy control (gender and age matched)–82 DCIS–60 IDC	0.730 (validation set, IDC/healthy)	55.2 (validation set, IDC/healthy)	87.9 (validation set, IDC/healthy)		

*Twenty clones signature.
†Eighty clones signature.
‡Sixty-nine clones signature.
MCLST: Mayo Clinic Lung Screening Trial; CARET: Carotene and Retinol Efficacy Trial; NSCLC: non–small cell lung cancer; SCLC: small cell lung cancer; SCC: squamous cell lung cancer; ADC: lung adenocarcinoma; NTLP: non-tumour lung pathologies; DCIS: ductal CIS; IDC: invasive ductal carcinoma; GGO: ground glass opacities; CT: computed tomography.

lung cancer (SCLC) and showed that a panel of six autoantibodies could detect SCLC with a 42% sensitivity and a 99% specificity [52]. In the context of this disease, which has a high mortality rate (85–95%), the authors propose that this test should be complementary with imaging. Leidinger *et al.* show 79% sensitivity when comparing low-grade SCC with healthy controls [35] and 75.9% when comparing stages IA and IB with healthy controls [51]. Wu *et al*. show 96.7% sensitivity when comparing stages I and II to controls [44]. Moreover, specificities are high, decreasing the number of false positives, thus circumventing the overdiagnosis problem. Rom *et al.* propose that a relevant panel of autoantibodies may achieve the necessary sensitivity and specificity for early detection in CT scan screening trials when nodules are discovered in the >8-mm size range [53] to be able to distinguish cancerous from non-cancerous nodules and to avoid unnecessary surgery. Interestingly, Hanash *et al*. show that autoantibodies can be detected before clinical diagnosis in high-risk patients [54]. They identified an autoantibodies signature able to discriminate 85 lung cancer patients from 85 matched controls with an AUC of 0.73, a sensitivity of 51% and a specificity of 82% [54]. All the patients came from the CARET cohort with pre-diagnosis sera (1 year before cancer diagnosis). Finally, studies on lung cancer highlight the difficulty of discriminating high-risk populations without cancer from those with cancer with a high specificity. Several studies also evaluated autoantibody signature in Non-Tumour Lung Pathologies (NTLP) comparatively to lung cancer (Table 1). For example, Leidinger *et al.* achieved 93.5% specificity comparing NTLP *versus* SCC using a combination of 69 antigen clones [35]. The several misclassifications observed in these studies on lung cancer can occur due to inflammatory diseases, such as interstitial lung disease, asthma and chronic obstructive pulmonary disorder (COPD), which induce autoantibody production that may be common to those elicited in non–small cell lung cancer [55]. These signatures also show that it is quite difficult to discriminate low grades from high grades [51] and lung cancer subtypes [45, 51]. This difficulty is probably due to the common subsets of autoantibodies that are elicited in response to cancer. Finally, statistical optimization to define the best signature should be used to decrease the misclassification rates and to determine precisely which subsets of autoantibodies are specific of which subgroups of cancer.

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In breast cancer, the widespread use of screening mammography has resulted in increased detection of early-stage breast disease, particularly for *in situ* carcinoma (CIS) and early-stage breast cancer. However, women with dense breast tissue in 75% or more of the breast have a risk of breast cancer four to six times as great as the risk among women with little or no dense tissue [56, 57]. Moreover, mammographic breast density appears to be a major risk factor for interval cancer [58, 59]. This is probably because mammography lacks sensitivity in women with dense breast tissue and these tumours show an aggressive phenotype. Contrast-enhanced magnetic resonance imaging (CE MRI), although more sensitive than mammography [60], shows insufficient specificity in high-risk populations for a general screening tool [61]. In addition, in other high-risk populations such as women who carry BRCA1 or BRCA2 mutations, MRI is also currently proposed as a screening test. Therefore, there is an important need to improve screening and diagnosis of early-invasive and non-invasive tumours in these high-risk populations. In breast cancer, a few studies have attempted to identify and/or evaluate panels of autoantibodies for early detection of breast cancer. The results are promising, showing in some cases sensitivities above 70% [62–64], and specificities above 70% for most of them. Chapman *et al*. propose the introduction of a test comprising seven autoantibodies for younger women who are at increased risk [65]. These women, who are mammography negative and autoantibody positive, would undergo MRI to detect early tumour lesions.

In conclusion, research on autoantibodies in cancer has recently seen great improvements in sensitivity and specificity and has shown pre-clinical detection is achievable in high-risk groups, such as heavy smokers in lung cancer, if the appropriate signature is designed. The first tests should be used early as complements to CT scans in these patients to reduce false-positive rates. In breast cancer, however, these studies are hampered by several problems despite the excellent prognosis shown by this disease. First, there is a very limited availability of early breast cancer sera (*e.g.* ductal CIS), which prevents studying this particular type of cancer. There are only three studies [62 ,63, 65] that are exploring autoantibodies in ductal CIS cohorts. In addition, there are only a few pre-diagnosis cohorts (*e.g.* high-risk breast cancer patients), and no study has been published yet about the humoural response in these particular populations. The most appropriate antigen source to identify new TAAs and autoantibodies would be the tumours themselves. In the case of breast cancer, CIS are very small tumours and, very often, the whole biopsies are used for histological diagnosis. Therefore, there is no extra tissue left for TAAs and autoantibodies identification. The availability of human samples is thus a very limiting issue for studying the humoural response in breast cancer.

Conclusion and perspectives

Biomarker development consists of several phases including pre-clinical studies, clinical assay development, retrospective studies of stored specimens, prospective screening studies and multi-centred randomised clinical trials [66]. All these phases must allow to develop a reproducible and economically viable test. Current studies investigating autoantibody biomarkers are promising, but still lack important validation steps. Indeed, few studies have validated signatures in the clinical context. In the future, most of these studies will require clinical validation using a broader independent patient population to determine the value of potential biomarkers in terms of sensitivity, specificity and predictive value. The encountered difficulties are mainly sample availability (*i.e.* tumours and sera) and signature definition. This leads to a question not yet answered in the field of autoantibodies and cancer detection: how should we choose the right combination that gives the highest sensitivity and specificity? For the moment, there is no guiding principle. Ideally, tumours, for the discovery step, should be preferred as source of autoantigens and would be fractionated enough to improve the detection of any TAA by autoantibodies. TAAs from appropriate cohorts of cancer patients would then be compared to matched populations, including controls (*e.g.* healthy, related benign diseases, inflammatory diseases), subtype of cancer and other types of cancer. The combinations would then be validated by appropriate high-throughput techniques on independent populations, and the results exploited with highly confident statistical methods. Finally, the signature would be validated in multicentre cohorts before the introduction in the clinical context of autoantibody tests based on ELISA, multiplex immunoassays or protein microarray.

Recent published reports that used protein microarray approaches foretell a promising future for implementation of sensitive and specific tests. Therefore, the establishment of autoantibody signatures specific of cancer will allow for improved diagnosis, monitoring disease progression and response to therapy. Moreover, the hope is that these signatures render possible screening of patients who are likely to develop disease by monitoring their autoantibody profiles before clinical manifestation of symptoms. It has already been proven that autoantibodies can be detected in high-risk patients who lack any clinically detectable cancer [67]. Thus, autoantibody signature represents a non-invasive assay potentially applicable for high-risk population screening, in complement to other screening tests such as mammography for breast cancer detection or CT scan for lung cancer. Finally, we should hope for a transition from the current retrospective studies to prospective analysis of patients' autoantibody responses and an assessment of its efficacy in the clinical setting. We may also be able to design diagnostic tests on the basis of one autoantibody signature and use another autoantibody signature as prognostic marker [68].

Conflict of interest

The authors confirm that there are no conflicts of interest.

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