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REVIEWS

Antibody-based proteomics: fast-tracking molecular diagnostics in oncology

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Abstract | The effective implementation of personalized cancer therapeutic regimens depends on the successful identification and translation of informative biomarkers to aid clinical decision making. Antibody-based proteomics occupies a pivotal space in the cancer biomarker discovery and validation pipeline, facilitating the high-throughput evaluation of candidate markers. Although the clinical utility of these emerging technologies remains to be established, the traditional use of antibodies as affinity reagents in clinical diagnostic and predictive assays suggests that the rapid translation of such approaches is an achievable goal. Furthermore, in combination with, or as alternatives to, genomic and transcriptomic methods for patient stratification, antibody-based proteomics approaches offer the promise of additional insight into cancer disease states. In this Review, we discuss the current status of antibody-based proteomics and its contribution to the development of new assays that are crucial for the realization of individualized cancer therapy.

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Personalized medicine requires the discovery and application of unambiguous prognostic, predictive and pharmacodynamic biomarkers to inform therapeutic decisions. High-throughput screening methods, particularly genomic and transcriptomic profiling, have vastly improved knowledge of the molecular basis of tumorigenesis, disease progression and therapeutic response[1,2]. As a result, individualized treatment regimens are now seen as an achievable goal. The role of antibodies in this arena is most likely to involve predictive biomarker development, as highlighted by the success of detecting both oestrogen receptor (ER) and ERBB2 (also known as HER2) expression in breast cancer. In the post-genomic era, however, progress in the development of clinically implemented assays has not kept pace with the rate of biomarker discovery[3]. As such, a pressing need exists for improved and innovative strategies to expedite the translation of cancer biomarkers into the clinical arena.

Antibody-based proteomics provides a logical strategy for the systematic generation and use of specific antibodies to explore the proteome[4,5]. The human proteome consists of approximately 20,500 non-redundant proteins[6], which are defined as a representative isoform from each gene locus[5]. Unlike DNA, which is subject to one major form of modification (methylation), proteins can be post-translationally altered in a myriad of ways; for example, by phosphorylation, acetylation and glycosylation — each of which is capable of producing a functional shift that potentially affects disease development, progression and therapeutic response. Despite this instrinic complexity, endeavours to describe the proteome and provide a comprehensive map of protein expression patterns in cells and tissues, such as the Human Protein Atlas (see the Human Protein Atlas (HPA) website; Further information) (BOX 1) and the complementary resource, Clinical Proteomic Technologies for Cancer (CPTAC; see the CPTAC website; Further information), are ongoing[7]. The improved understanding of antibody–antigen relationships provided by such efforts[8] will greatly expedite the development of new assays using affinity reagents to profile cancer proteomes and enable the exploitation of the specificity and sensitivity that is afforded by antibody-based approaches.

In this Review, we describe current antibody-based methods to identify and validate new cancer biomarkers and therapeutic targets, and discuss the systematic generation and evaluation of specific antibodies for the functional exploration of the cancer proteome. The integration of antibody-based approaches with existing

At a glance

- Personalization of cancer therapy requires the identification of unambiguous diagnostic, prognostic and predictive biomarkers to facilitate the accurate stratification of patients and the monitoring of responses to targeted therapies.
- The systematic generation and validation of specific antibodies offers a high-throughput mechanism for the functional exploration of the proteome and a logical approach for fast-tracking the translation of identified biomarkers.
- Multiple approaches exist, each with specific characteristics and advantages that are suitable for a wide range of applications, which capitalize on the inherent specificity and sensitivity of antibodies as affinity reagents.
- The integration of antibody-based approaches with existing genomic and transcriptomic methods offers huge potential, and the clinical implementation of new high-throughput antibody-based approaches will depend on the integration of data across various platforms.
- The clinical application of new antibody-based assays demonstrates their utility as accurate, sensitive and robust diagnostic and prognostic tests and has led to the development of a new approach, known as pathway diagnostics, which is likely to have a crucial role in the design of future molecular therapeutic trials.

genomic and transcriptomic methods is considered and the clinical implementation of new high-throughput antibody-based approaches is examined, particularly in the context of enabling the personalization of cancer therapy.

Enabling technologies

The generation and use of antibodies for protein profiling on a global scale is an intuitive approach that enables the systematic examination of the human proteome using a wide range of high-throughput assays, including immunohistochemistry (IHC) on tissue microarrays (TMAs), pathway analysis using reverse phase protein arrays (RPPAs) and serum-based diagnostic assays using antibody arrays (BOX 2). Founded on the ability to systematically generate and validate specific and sensitive antibodies (BOX 3), these versatile assays are at the forefront of efforts to generate the molecular diagnostic and predictive assays that are required to facilitate the personalization of cancer therapy[9,10].

Antibody specificity is the foundation of antibody-based proteomics, and although the ideal approach to confirm specificity is the high-throughput production of paired antibodies directed towards separate and non-overlapping target protein epitopes (BOX 3), alternative approaches are also required. Several laboratories combine western blotting and IHC on identical cell lines (ideally using a non-expressing cell line as a negative control[11,12]) that are formatted as cell line microarrays to facilitate high-throughput validation when used in tandem with automated image analysis solutions. If a negative control cell line is not available, small interfering RNA (siRNA)-mediated knock down followed by both western blotting and IHC can also be used[11], and staining reproducibility should be confirmed using a TMA that is stained with each new antibody[12].

Automated analysis of protein expression in tissue. TMAs developed by Kononen *et al.* are a high-throughput platform for the simultaneous investigation of protein expression in multiple tissue specimens, principally using IHC[13] (BOX 2). TMAs were preceded by a related technology known as a 'sausage block', which was constructed by the assembly of larger tissue fragments in a recipient block in a less organized manner[14], an approach that was subsequently modified by Wan *et al.*[15].

Over the past decade, TMAs have become an established and crucial component of the cancer biomarker discovery and validation pipeline[16,17]. Although TMAs have undoubtedly enabled the acceleration of translational pathology, new demands have been placed on the quality, reproducibility and accuracy of IHC assays. Variability in tissue collection, fixation and processing, antigen retrieval, titration of the antibody, application of secondary antibodies and multiple detection systems results in a multi-parameter assay that requires thorough optimization[18].

Historically, IHC assay development has been hampered by the lack of specific antibodies; however, the development of comprehensive antibody resources (BOX 1) promises to help overcome this obstacle. Traditional IHC, although ubiquitous in both clinical and research settings, has been criticized as a semi-quantitative approach. In particular, attention has centred on the intrinsic lack of reproducibility of manual IHC scoring, which remains a time-consuming and subjective process to which only limited statistical confidence can be assigned owing to inherent inter-observer and intra-observer variability and the semi-quantitative nature of the data[19,20]. For example, a large study of inter-laboratory variance in the IHC-based detection of ER in breast cancer samples across 200 laboratories in 26 countries demonstrated a false-negative rate of 30–60%[21]. As ER is routinely used to determine the need for adjuvant hormonal therapy in patients with breast cancer, this level of discordance is all the more alarming and highlights the urgent need for the standardization of molecular diagnostic approaches.

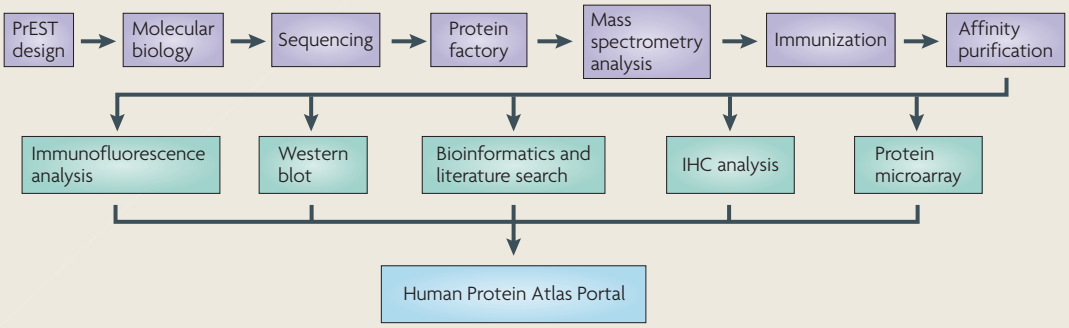
Automated IHC scoring systems offer the opportunity to further advance a well-established and clinically useful assay to accurately quantify both staining intensity and the subcellular localization of protein expression in a reproducible fashion[22,23]. In addition, automated analytical approaches provide quantitative data that can be subjected to more robust statistical analysis than the qualitative or semi-quantitative data that are produced from manual analysis[22,24]. Various automated image analysis solutions are currently available, some of which have received US Food and Drug Administration clearance for cancer-specific biomarker applications (regulatory issues are outlined in BOX 4). Most platforms comprise both image analysis software and scanning hardware[23,25], and focus on the quantification and subcellular localization (cytoplasmic, nuclear or membranous) of the relevant antigen (FIGS 1a,b). Several groups, including our own, have demonstrated that automated algorithms can be used to accurately quantify IHC staining in cell lines[26] and have also linked automated analysis of IHC to clinical outcome in a selection of different tumour types[22,24,27–30]. Although various automated image analysis approaches have been approved at a regulatory level, as indicated above, issues still remain regarding the integration of image analysis technologies into routine clinical practice.

Reverse phase protein array
RPPA. Protein lysate dot blot in a high-density format on a solid surface that allows for multiple samples to be probed with the same antibody, or other affinity reagent, simultaneously.

Box 1 | **The Human Protein Atlas Program: a multidisciplinary antibody-based proteomics initiative**

The Human Protein Atlas (HPA) is a gene-centric database[126] using the human genome sequence (Ensembl) as a template to select coding sequences corresponding to 50–150 amino acids, denoted Protein Epitope Signature Tags (PrESTs; see the figure). Based on the selection of regions with low similarity to other human genes, up to four different PrESTs are defined for each gene to enhance the probability of generating unique, specific antibodies[127]. Recombinant PrEST protein fragments are produced and then used as an antigen to develop polyclonal antibodies, which are affinity purified to generate unique, oligoclonal monospecific antibodies[128]. Binding specificity is tested on protein arrays containing various PrESTs, and all approved monospecific antibodies are subsequently used for western blotting using a standardized protocol. Protein expression patterns are visualized using immunohistochemistry (IHC) on tissue microarrays, representing 48 types of normal tissues and 216 human tumours corresponding to the 20 most common forms of human cancer and 47 cell lines[129,130]. High-resolution images are acquired from all immunostained tissue and cell microarray sections. All histology images are manually annotated and curated by certified pathologists, and cell images are analysed using image analysis-based algorithms[26]. Furthermore, all antibodies are applied to three human cancer cell lines using immunofluorescence and confocal microscopy to determine the subcellular localization of each protein[131].

Of the 20 new antibodies that are processed every day approximately 50% are approved for IHC. Therefore, protein expression data are generated for approximately 3,000 new antibodies per year. Specific emphasis is placed on the confirmation of antibody specificity, which is a key issue for any antibody-based proteomics initiative (BOX 3). All protein expression data, including images, antibody validation and immunized PrEST sequences, are published on the HPA web portal (see Further information), to provide a knowledge base for functional studies and biomarker discovery efforts. The portal contains data and images from both in-house generated monospecific antibodies and external, commercially available antibodies. To allow for searches and queries regarding protein expression profiles in normal tissues and cancer, a web-based analysis tool is also available[132]. The database is updated annually and the current release (version 6.0) contains more than 9 million images and protein profiles based on greater than 11,200 antibodies directed towards 8,489 unique proteins corresponding to 42% of all human protein-encoding genes.



A key potential advantage of such approaches is the provision of an unbiased and cost-effective method of data standardization, which is often a difficult issue with current pathological assessment. The technology itself is perhaps better seen as a complement to routine pathological assessment rather than as a replacement.

Although IHC is a well-validated, clinically applicable assay, immunofluorescence provides a complementary approach (FIG. 1c), particularly for the identification and quantification of co-localized proteins. Immunofluorescence also demonstrates a greater dynamic range than colorimetric IHC-based detection. Given the ongoing trend towards multiplex biomarker assay development, immunofluorescence may gain greater clinical use as a routine diagnostic and prognostic assay. In this context, immunofluorescence quantification is supported by various digital pathology solutions, allowing for the automated definition of regions of interest, cellular compartmentalization and fluorescent signal co-localization.

In translational medicine, the fluorescent-based AQUA (HistoRx) platform is one of the more established automated solutions for cancer biomarker assessment[31]. However, despite a large body of literature describing the approach, doubts remain about its reproducibility and clinical applicability, principally owing to a lack of independent validation studies. The AQUA platform identifies tumour cells using cytokeratin expression, and so creates a region of interest that allows for the definition of subcellular compartments and the accurate quantification of protein expression in formalin-fixed paraffin-embedded (FFPE) tissue samples. Several studies have demonstrated that AQUA can measure protein expression on histological specimens from various tumour types with good accuracy and reproducibility, and this can then be linked to clinical outcome[32–36]. As such, both colorimetric and fluorescent-based detection approaches, coupled with associated automated image analysis solutions, are viable, complementary techniques to accurately and reproducibly evaluate tissue-based cancer biomarkers.

Antibody arrays. Multiplex protein analysis, particularly of the serum proteome, offers great promise for the development of less invasive and more cost-effective diagnostic assays. However, the complexity of the serum proteome, which contains approximately 10,000 proteins with a dynamic range of at least 10 orders of magnitude, presents several technical challenges[37]. The serum

Two-dimensional electrophoresis
2DE. Gel-based technique for the separation of proteins by isoelectric point in the first dimension (achieved by isoelectric focusing), followed by mass in the second dimension (achieved by SDS–PAGE). A higher resolution of protein separation is achieved compared with single dimension approaches.

Multi-dimensional liquid chromatography
Chomatographic separation in at least two dimensions, for example, reverse-phase chromatography followed by ion-exchange chromatography. Using additional dimensions increases the resolution of separation.

Tandem mass spectrometry
Often referred to as MS/MS, it uses two linked mass spectrometers to measure small amounts of proteins. Analytes are separated according to their mass and charge, with samples sorted and weighed in the first mass spectrometer, then fragmented in a collision cell, and fragments sorted and weighed in the second mass spectrometer.

proteome is dominated by 22 high-abundance proteins that constitute 99% of the total protein mass of serum (for example, albumin, immunoglobulins and transferrin) and effectively mask lower abundance proteins. This is one of the principle obstacles to the identification of new biomarkers, which are frequently present at low concentrations (pg per ml or lower compared with upper limits of mg per ml for high-abundance proteins)[37,38].

Several approaches have been used to examine the serum proteome, including classic separation techniques, such as two-dimensional electrophoresis (2DE) and multi-dimensional liquid chromatography, coupled with single or tandem mass spectrometry (MS/MS)[37–39]. Although these approaches have certain advantages, antibody arrays and RPPAs (BOX 2) have emerged as versatile platforms in serum-based proteomics, offering the opportunity to carry out multiplexed, rapid and sensitive (fM range) profiling of samples (reviewed in REFS 40–42) without fractionation or the depletion of high-abundance proteins. Multiple formats exist, facilitating multiplexed analysis of samples with differential labelling. Further modifications involve the selective identification of post-translational modifications, such as the use of lectin-antibody arrays to profile glycan variation on cancer-associated antigens (reviewed in REFS 43,44).

ELISA. Enzyme-linked immunosorbent assay (ELISA) remains the gold standard for measuring protein concentration in human body fluids, particularly blood. Such an approach allows for the accurate and sensitive detection of the antigen of interest; however, it is limited by the fact that classic approaches allow for single antigen detection only and often require relatively large volumes of sample material compared with newer methods.

Multiplex assays have been developed from traditional ELISA assays to quantify multiple antigens in a single sample simultaneously (BOX 2). Multiple proteins in a biological fluid sample can therefore be measured, and although commercial kits are limited to approximately 25 capture antibodies, custom design approaches could potentially scale up to 100 (REF. 45).

Variations on the ELISA approach, such as the Meso-Scale Discovery (MSD) platform that uses electrochemiluminescent detection on patterned arrays to quantify multiplexed biomarkers, offer further promise for patient stratification and monitoring of therapeutic responses. Preclinical studies examining the effect of the PI3K inhibitor LY294002 on human xenografts demonstrated the effectiveness of using multiplexed MSD assays to measure pharmacodynamic responses to accurately monitor the provision of therapy[46].

Compared with traditional ELISA, multiplex arrays have several advantages, including their high-throughput nature, requirement for smaller sample volume, the ability to evaluate one antigen in the context of multiple others and the ability to reliably detect different proteins across a broad dynamic range[47,48]. Although good correlations between ELISA and multiplex assays have been

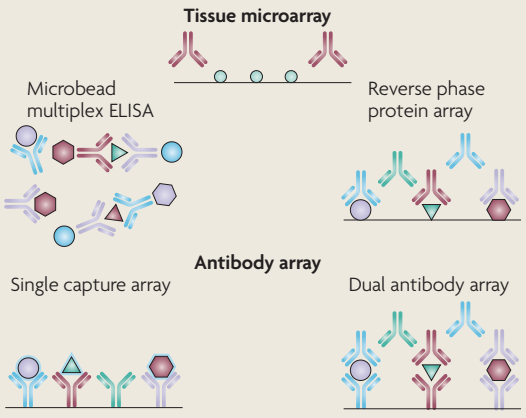
Box 2 | **Antibody-based proteomics technology formats**

Developed by Kononen *et al.*[13], tissue microarrays (TMAs) are a high-throughput platform for the simultaneous investigation of biomarkers in multiple tissue specimens. TMAs are assembled by acquiring cylindrical cores (0.6–2.0 mm in diameter) from donor paraffin-embedded tissues and re-embedding them in a single recipient block. The resultant TMA block is then sectioned, and immunohistochemistry and other assays such as immunoblotting[133] are carried out on individual sections (see the figure).

In the typical double antibody sandwich enzyme-linked immunosorbent assay (ELISA), antibody attached to the bottom of a well provides both antigen capture and immune specificity, and another antibody linked to an enzyme provides detection and an amplification factor. Several different multiplex formats are available. Bead-based multiplex assays are probably the most commonly used format[47,48]. Each bead set is coated with a specific capture antibody, and fluorescence- or streptavidin-labelled detection antibodies bind to the specific capture antibody complex on the bead set, which can be detected using flow cytometry.

Reverse phase protein array (RPPA) technology is a microproteomic approach in which protein lysate is immobilized on a solid surface and subsequently probed with antibodies. RPPAs allow the examination of the activation state of crucial cellular pathways using antibodies directed against total and phosphorylated protein[134,135]. In contrast to TMA or antibody array-based methods, RPPAs can use denatured protein lysates, thus removing the need for antigen retrieval, and the use of non-denatured protein lysates allows for protein–protein or protein–DNA interactions to be probed[135].

Antibody arrays are produced by printing antibodies onto a solid surface that is analogous to a DNA microarray. Two categories of antibody microarray formats have been described, namely direct labelling single-capture antibody arrays[136] and dual antibody (capture and read-out antibody) sandwich arrays[137]. In the direct labelling method, all proteins in a sample are tagged, so that bound proteins can be identified following incubation on a microarray. In the dual antibody format, proteins captured on the microarray are detected using a cocktail of labelled detection antibodies, with each antibody matched to one of the spotted antibodies[41].



Box 3 | **Antibody specificity**

A key issue for all antibody-based assays is the confirmation of antibody specificity towards its antigen. Validation approaches include not only generic binding assays such as protein arrays, which use the antigen as a ligand for the assay, but also various kinetic binding assays that establish affinity and determine binding sites using epitope mapping[138]. Epitope mapping using X-ray crystallography or NMR, which can determine the three-dimensional structure of the binding complex, can be comprehensive approaches but are laborious. Other approaches for epitope mapping include scanning with peptides[139], which are either chemically synthesized or expressed on the surface of microorganisms[140,141]; however, these approaches can also be cumbersome.

One of the major challenges in generating reliable antibodies is high-throughput validation of protein-specific binding in different antibody-based assays[9,10]. This becomes particularly important when generating antibodies to proteins lacking independent experimental validation. Western blotting is often regarded as a gold standard for antibody specificity; however, post-translational modifications can make the interpretation of experimental results ambiguous. Likewise, antibodies that function well in western blotting using denatured proteins might not function in another assay, such as immunohistochemistry or immunofluorescence, in which proteins retain a degree of native conformation[142,143]. Validation of antibodies, therefore, remains a challenge, in particular for antibodies directed towards uncharacterized proteins.

The ideal approach to confirming antibody specificity is the high-throughput production of paired antibodies directed towards separate and non-overlapping target protein epitopes to allow sandwich-based assays and to facilitate the validation of the affinity reagents across various assay platforms, including immunofluorescence, immunohistochemistry and western blotting[138].

reported[49,50], experience with multiplex arrays remains limited and careful side-by-side comparisons are rare. Although concordance between ELISA and the multiplex assay is generally good when using tissue culture supernatant samples, it is much less robust when using serum or plasma samples[51]. Therefore, further direct comparisons in the clinical trial setting are warranted.

RPPAs. Cell signalling pathways are regulated in many instances by the post-translational modification of proteins, and in-depth analysis of deregulated cellular circuitry in cancer requires specialized technologies. As a result, innovative high-throughput proteomic approaches such as RPPAs (BOX 2) have been developed to examine pathway activation using phospho-specific antibodies in large panels of patient samples simultaneously[52]. One of the key advantages of RPPAs is the discrete amounts of patient material required, traditionally using frozen specimens, but more recently successfully demonstrated using FFPE samples[53]. This approach is dependent on the standardization of tissue collection to preserve the state of post-translational modifications[54]. In this context, pre-analytical fluctuations in phosphoproteins have been noted, reflecting the dynamic nature of kinase and phosphatase activity in excised tissue[54]; this reiterates the requirement for standardized sample procurement procedures to allow accurate inter-institutional data comparison.

Additional factors influencing the use of RPPAs focus on the sensitivity and dynamic range of detection of the assay. Traditional colorimetric and fluorimetric detection strategies offer excellent sensitivity, although they are hampered by poor dynamic range, requiring the serial dilution of samples. However, the sensitivity of the assay can be extended to the aM and zM range using electro-chemiluminescent[55] and evanescent field fluorescent detection[56] with a dynamic range of up to 5–6 logs. Similar dynamic ranges and sensitivity can also be achieved using near infrared-based detection, which removes the need for specialized, proprietary detection systems[57].

Clinical applications

In the past decade, vast amounts of data accrued from various molecular profiling platforms have facilitated a shift away from conventional broad therapeutic approaches to cancer, towards more tailored strategies. The expansion of personalized treatment protocols now depends on the development of robust, well-validated, informative predictive and pharmacodynamic assays. ER and ERRB2 were instrumental as early examples of predictive biomarkers in breast cancer and epitomize personalized medicine; however, more recently, transcriptomic approaches have led the way in the advancement towards individualized therapeutic protocols[2,58]. Although the ongoing trials will determine the clinical applicability of these gene expression assays, other reservations have been expressed regarding their prohibitive cost, reliance on frozen tissue and the advanced technical expertise required to use the technology[59].

The most clinically advanced gene expression signatures are MammaPrint[60,61] and OncotypeDx[62], which are currently the subject of large-scale prospective randomized control trials to assess their utility for the stratification of patients with breast cancer to determine the appropriate treatment approach[2,63,64]. Although several DNA microarray and reverse transcription (RT)-PCR-based prognostic and predictive assays have been proposed in other tumour types, such as lung[65–67], colon[68–70] and prostate[71,72] cancer, and lymphoma[73–75], these seem to be further from clinical application at present, mainly owing to a paucity of comprehensive validation studies. Here, we discuss clinically applicable antibody-based assays for diagnostics, incorporating serum- and tissue-based assays, prognostics, using IHC-based signatures, and molecular therapeutics, with a particular emphasis on pathway diagnostics using tissue-based assays (FIG. 2).

Diagnostics. Although tissue-based diagnosis ultimately remains the remit of the histopathologist, in the absence of screening programmes, a considerable number of tumours are diagnosed at an advanced stage, and so efforts have focused on developing more sensitive diagnostic assays. Such an approach should have a significant impact on cancer-related mortality rates.

Serum-based proteomics offer great hope, particularly in the development of more sensitive diagnostic assays. The current gold standard for validating putative biomarkers is ELISA. A high-throughput ELISA has extraordinary sensitivity and specificity for quantifying target analytes. However, ELISA development is costly (typically, US$100,000–US$2 million per biomarker candidate) and is associated with a long development lead time (>1 year) and a high failure rate[76], although the

Epitope mapping
Systematic identification and characterization of the minimum recognition domain for antibodies.

Sandwich-based assay
Antigen detection using surface-bound capture antibodies, followed by the application of the sample and subsequent detection using a second antibody raised against an alternative epitope on the same target protein.

multiplexed approaches discussed above are likely to bring ELISA back to the forefront of biomarker identification and validation.

An important focus of serum-based proteomics has been placed on MS-based approaches; however, antibody-based approaches are viable alternatives and, in combination with MS-based techniques, form a highly complementary strategy for cancer biomarker enrichment to push MS-based detection into the ranges commonly achieved by ELISA. Given the long lead-time and high cost of ELISA development, an assay cannot be developed for every putative biomarker, and more affordable technologies with a shorter lead-time are required for biomarker validation. To this end, the combination of peptide enrichment with antibodies that are immobilised on affinity columns and MS offers great promise, particularly as antibody-based proteomic resources generate larger numbers of validated antibodies. This technology, known as stable isotope standards with capture by anti-peptide antibodies (SISCAPA)[77], has been used to quantify proteins in the physiologically relevant range (ng per ml)[76] and has recently been implemented in an automated multiplex (nine targets in one assay) format.

Serum-based proteomic screens can also be carried out using high-throughput antibody-based platforms, such as antibody arrays and RPPAs, both of which offer the opportunity of reduction to clinical utility in a timely fashion. Antibody arrays have been used to develop potential diagnostic assays for several different tumour types, although the focus has generally been on tumours with an insidious onset that are often diagnosed at a late stage, such as pancreatic cancer. Two groups have published serum signatures developed from antibody arrays, which seem to distinguish patients with pancreatic cancer from healthy controls[78,79]. Although such an assay would be hugely beneficial to the clinical arena, these studies have been hampered by low-density antibody arrays and small cohorts. Additionally, the simple comparison of patients with malignant pancreatic cancer to normal controls is probably an overly simplistic approach, and comparison between normal controls, those with a premalignant condition (such as chronic pancreatitis) and invasive disease may unearth more informative data.

The use of serum proteomics to monitor patients to detect early local recurrence or metastatic deposits and the application of antibody arrays to identify metastatic breast cancer[80] further illustrates the clinical potential of these approaches. The development of higher density antibody arrays, such as the recently published 810 'cancer related' antibody array, may also considerably advance this technology in the translational arena[81].

In addition to serum-based assays, tissue proteomics offer the opportunity to develop IHC-based assays to improve diagnostic sensitivity and disease classification. Kashani-Sabet *et al.*[82] combined gene expression data and IHC profiling using automated analysis to develop a highly sensitive (91%) and specific (95%) five marker diagnostic assay (comprised of actin-related protein 2/3 complex, subunit 2 (ARPC2), fibronectin 1 (FN1), regulator of G protein signalling 1 (RGS1), secreted phosphoprotein 1 (SPP1; also known as osteopontin) and WNT2) that distinguishes benign nevi from melanoma. The same group used a similar approach to develop a three marker prognostic assay (comprised of nuclear receptor coactivator 3 (NCOA3), SPP1 and RGS1) for melanoma[82]. This assay, initially developed in a cohort of 395 patients, and subsequently validated in an independent cohort of 141 patients, was an independent predictor of disease-specific survival in both cohorts. The integration of gene expression analysis and high-throughput IHC profiling using automated analysis offers great potential for the development of similar assays in other tumour types, particularly as more advanced mathematical models can be applied to quantitative automated IHC data.

Box 4 | **Regulatory issues pertaining to quantitative IHC and immunofluorescence analysis**

Within the US market, digital pathology and automated analysis algorithms for clinical use are regulated by the Center for Devices and Radiological Health of the Food and Drug Administration (FDA). There are two major processes by which medical devices come to the US market, namely FDA Clearance (sometimes referred to as 510(k) process) and FDA Approval. There are three FDA regulatory classifications of medical devices: Class I, Class II and Class III.

Class I medical devices present minimal potential harm to the user and are generally exempt from the pre-market notification process. Class II medical devices are devices for which existing methods, standards and guidance documents are available to provide assurances of safety and effectiveness. Class II devices typically require pre-market notification through the FDA Clearance process. Class III devices usually support or sustain human life, are of substantial importance in preventing the impairment of human health, and present a potential unreasonable risk of illness or injury to the patient. Typically, a Pre-Market Approval (PMA) submission to the FDA is required to allow marketing of a Class III medical device.

Currently, the FDA have classified whole slide imaging systems for viewing IHC within clinical context, as well as automated algorithms for IHC analysis, as Class II devices that require pre-market notification through FDA Clearance. Pre-market notification requires a new device to be compared for safety and effectiveness with another lawfully marketed model. In the case of an image analysis solution for a new biomarker, this would require a substantial equivalence study based on comparison of image analysis to conventional manual microscopy. In the case of a new approach to quantification of a marker such as ERBB2 (also known as HER2), for which several algorithms have been cleared, an equivalence study based on comparison to FDA-cleared algorithms would also be required.

Regulation of digital pathology and image analysis for primary diagnosis remains unclear, and the FDA has not yet decided whether a diagnostic application would be classified as a Class II or Class III device. This decision will have a major influence on the industry as the FDA charges a company with more than US$100 million in sales $4,000 to review a FDA Clearance application compared with $217,787 to review a PMA submission.

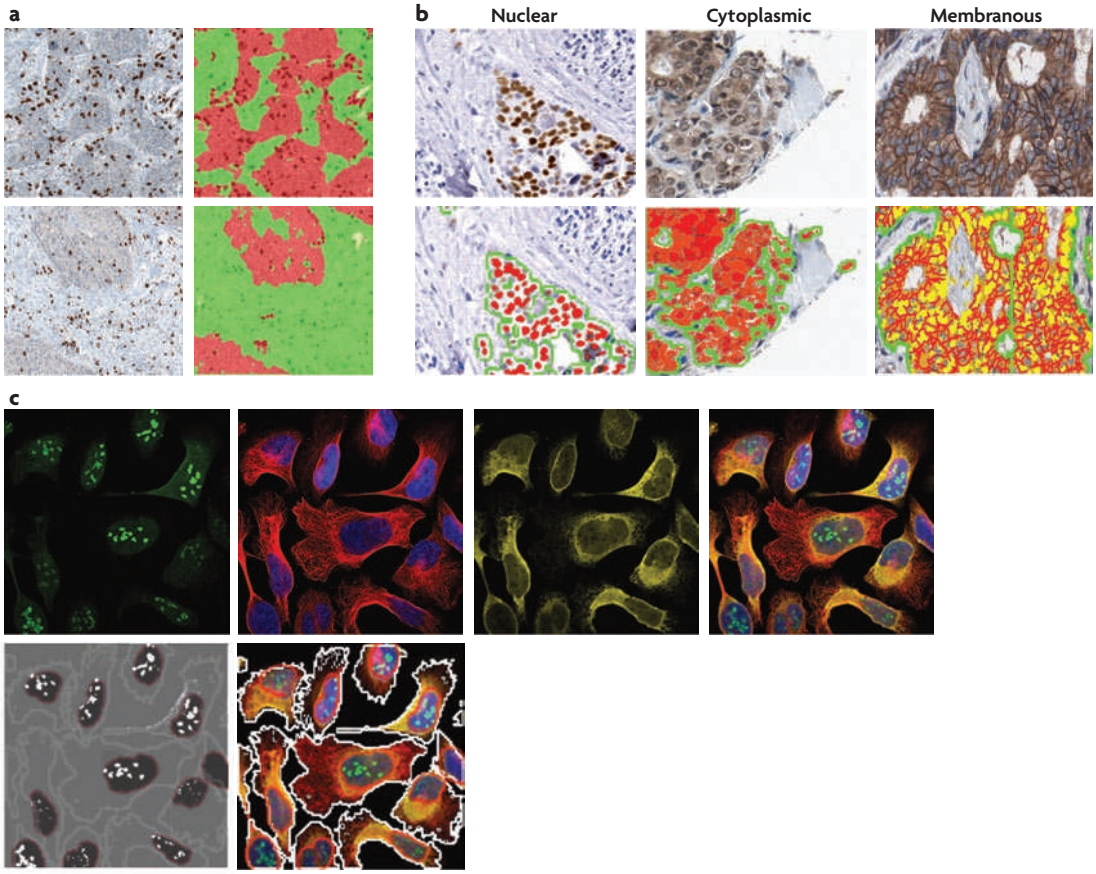


Figure 1 | **Automated quantification of protein expression using immunohistochemistry and immunofluorescence.** **a** | One of the major challenges facing immunohistochemistry (IHC) automated analysis software is distinguishing tumour epithelium from stromal cells, such as fibroblasts and lymphocytes. Advanced pattern recognition software can be used in a supervised manner to train representative images of different tissue patterns such as tumour and stroma, and once trained the algorithm can automatically distinguish between the two compartments. In this example, Genie (Aperio) was trained to distinguish between tumour epithelial cells (shown in red) and stroma (shown in green). **b** | Other automated IHC quantification packages can be used to quantify protein expression in different subcellular compartments in tissues (that is, the membrane, cytoplasm and nuclear compartments). These packages, such as the example provided here through the use of *IHC-MARK* (patent pending; Oncomark Ltd), provide quantitative data, such as positive and negative cell counts and staining intensity. **c** | Alternatively, immunofluorescence microscopy can provide accurate information about subcellular localization in terms of antigen expression, as demonstrated by Ki67 staining of tumour cells (green), the nucleus (blue), microtubules (red) and endoplasmic reticulum (yellow). Immunofluorescence-based image analysis packages, such as Definiens Tissue Recognition technology, can be used to subtract the subcellular compartment signal and provide a read out of the overall staining for the total number of cells (dark black shaded areas in final output), the number of nucleoli (white spots in final output), as well as the boundaries of each subcellular area.

This approach was recently highlighted by Gould Rothberg *et al.*[36] who used a fully automated quantitative immunofluorescence approach combined with genetic algorithms to identify a five marker prognostic assay for melanoma.

In another example, Ring *et al.*[83] recently described a five marker diagnostic assay (comprised of tripartite motif-containing 29 (TRIM29), carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), SLC7A5, mucin 1 (MUC1), cytokeratin 5 (CK5) and CK6)) called PulmoStrat that distinguishes between squamous cell carcinoma and adenocarcinoma of the lung. The assay uses a weighted algorithm trained to discriminate adenocarcinoma from squamous cell carcinoma and was assessed in three independent cohorts comprising more than 1,000 patients. PulmoStrat could have considerable clinical ramifications, as epidermal growth factor receptor (EGFR) mutations, which predict response to EGFR inhibitors such as gefitinib and erlotinib are much more prevalent in adenocarcinoma[84,85].

Prognostics. The advent of TMAs and high-throughput pathology has provided an ideal platform for the development of IHC-based surrogates of gene expression profiles and has enabled the production of simple, effective and reproducible assays that are readily translatable to the clinic[59]. Several groups have used IHC-based surrogates to validate breast cancer molecular subtypes, including Neilson *et al.*[86] and Carey *et al.*[87] who demonstrated that predefined breast cancer molecular subtypes could be identified using a small number of IHC markers. Additionally, it has been demonstrated that several

Unsupervised analysis
A form of gene expression analysis that involves the discovery of empirical structure (patterns) in a given data set without taking into account any prior knowledge of the underlying biology. Gene expression patterns that are discovered in this manner should be unbiased.

different prognostic gene expression signatures developed for breast cancer over the past decade show significant agreement in outcome prediction in a single cohort of patients[88]. As these signatures demonstrated minimal overlap of genes, these findings suggest that they are all tracking a similar phenotype, which may be identifiable in the future by using a panel of IHC markers.

Another approach is to integrate gene expression data and antibody-based tissue profiling to generate new IHC-based signatures. Ring *et al.*[89] generated polyclonal affinity-purified antibodies against 700 targets that were identified from a range of published breast cancer gene expression data sets and developed a five marker signature that measures p53, N-myc downstream regulated 1 (NDRG1), CEACAM5, SLC7A5 and HTF9C (also known as TRMT2A), and predicts disease-free and overall survival in ER-positive patients with breast cancer. Although the five markers are associated with various intracellular pathways, all can be linked to cellular proliferation and differentiation[89]. A subsequent validation study suggested that MammaStrat may predict outcome in ER-positive, lymph node-negative breast tumours and so identify a group of patients who would benefit from adjuvant chemotherapy[90].

Although several other IHC signatures have been published in breast[91,92], colorectal[68] and renal cell[93,94] carcinomas, these have generally been studied in small patient cohorts and have been generated from a limited number of preselected proteins. Such studies require extensive validation and the preselection of antibodies based on expression patterns in a single cohort may hamper future validation studies. The integration of gene expression and proteomic data, combined with the high-throughput generation and/or screening of comprehensive antibody panels, allows for unsupervised analysis of protein expression in large patient cohorts, which is more likely to produce more robust assays, as demonstrated by Ring *et al.*[89].

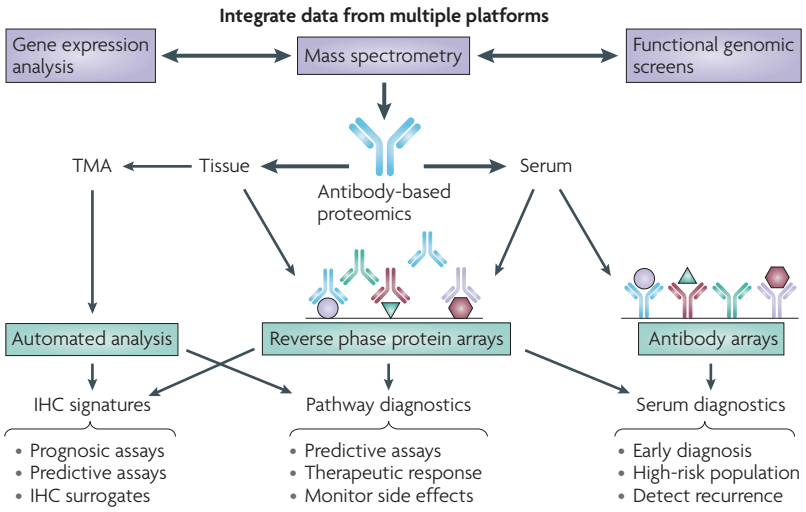


Figure 2 | **Antibody-based proteomics and personalized cancer medicine.** The integration of data from high-throughput screening methodologies, such as DNA microarrays, mass spectrometry and functional genomic screens, with antibody-based proteomics, offers a great opportunity to identify new, robust cancer biomarkers. Clinically applicable assays may be developed using various approaches, including immunohistochemistry (IHC)-based prognostic and predictive signatures and pathway analysis using reverse phase protein arrays (RPPAs) and/or IHC. Immunohistochemical analysis offers the potential to identify patient subgroups for targeted therapy while also monitoring therapeutic response. Serum-based proteomics using antibody arrays and/or RPPAs offer less invasive approaches for diagnosis, particularly for screening high-risk populations and the early detection of recurrence, although molecular imaging has the potential to become the major platform for diagnostic, prognostic and predictive tests in the future. TMA, tissue microarray.

Molecular therapeutics. The advent of molecularly targeted therapy has led to a shift of emphasis away from prognostic signatures. Although intrinsic signatures provide a global view of tumour phenotype, the heterogeneous nature of cancer suggests that more subtle approaches, which are based on the profiling of specific intracellular pathways to personalize treatment regimens, could ultimately prove to be more beneficial. This approach, known as pathway diagnostics, is already in practice, as demonstrated by the use of *KRAS* mutation status (assessed using various assays, although nested PCR followed by direct sequencing and allele-specific real-time PCR is most widely used at present[95]) to predict response to therapeutic EGFR-specific antibodies in metastatic colorectal cancer[96,97].

Clinical application of pathway diagnostics involves a key shift towards monitoring upstream and downstream indicators of pathway function before, during and following treatment. Lessons learned from the first decade of molecular therapeutics suggest that two key elements will predict their successful translation into the clinic. First, the identification of the correct patient subgroup is paramount, as demonstrated by the aforementioned examples of EGFR inhibitors in lung cancer[84,85]. Present understanding suggests that the identification of the correct patient cohort depends on two important factors: namely, the activity of the pathway being targeted and the molecular lesion leading to target activation[98]. Imatinib, which inhibits the constitutive kinase activity of the breakpoint cluster region (BCR)–ABL1 oncogenic fusion protein, which is the product of a chromosomal translocation in patients with chronic myeloid leukaemia[99], is the most obvious example of this phenomenon. Second, measuring inhibition of the targeted signalling pathway is required to guide dose selection and scheduling, and may also monitor off-target effects of potential drugs, thus predicting side effect profiles at an earlier stage[100]. Such an approach also offers the opportunity to identify patterns of both intrinsic and acquired resistance to therapy.

Another example of the importance of identification of the correct patient subgroup is the use of Raf inhibitors in melanomas that harbour a BRAF mutation, which is present in approximately 50% of cases[101]. BRAF mutations (particularly the V600E mutation) result in increased basal kinase activity and hyperactivity of the MAPK pathway, thus promoting tumorigenesis[102]. Targeting BRAF or its downstream effectors may have potential benefits, and early clinical trials examining non-selective Raf kinase inhibitors, such as sorafenib[103–105], and selective compounds, such as PLX4032 (REF. 106), which specifically targets BRAF-V600E, have

shown promise. Such optimism should be tempered by recent findings that Raf inhibitors promote tumour growth and MAPK activation in tumours expressing wild-type Ras and Raf as well as mutant KRAS[107], which further highlights the requirement for strict patient selection and the development of companion molecular diagnostics for the future trials of such agents.

Such assays are most likely to focus on monitoring pathway activity by either definitive quantification of phosphorylated proteins or the identification of surrogate markers of pathway activity. Antibody-based proteomics offers two approaches for monitoring such pathway activity in tumour samples, namely multi-marker IHC-based assays and RPPAs. Both require a large library of phospho-specific antibodies, and although RPPA may be an excellent platform in the discovery phase, particularly in clinical trials in which small amounts of tissue can be profiled with a large number of antibodies, an IHC-based assay may arguably be a more clinically applicable assay. To date, the optimization of phospho-specific antibodies for IHC has been difficult, suggesting that standardization of such an assay is challenging and that surrogate markers of pathway activation could provide an alternative and more robust approach.

Several groups have published IHC-based studies outlining the activation of different intracellular pathways in breast cancer, oesophageal cancer, renal cell carcinoma, soft tissue sarcoma and prostate cancer[100,108–113]. In general, these studies have been limited by small patient cohorts; however, Dahinden *et al.*[114] recently used 15 antibodies to examine both the von Hippel-Lindau tumour suppressor (VHL) and PTEN pathways in 800 clear cell renal cell carcinomas and were able to refine tumour grading and staging accordingly. Likewise, Yoshizawa *et al.*[111] demonstrated that the activation of the AKT pathway was associated with a poor prognosis in 300 cases of non-small-cell lung cancer. Although these studies used large cohorts, they will require validation in independent cohorts from multiple institutions. Both studies used manual IHC analysis, and the development of robust algorithms for IHC quantification will potentially allow the application of more complex mathematical models to IHC data, thus allowing more complex pathway analysis in large cohorts of patients.

The above-mentioned studies were carried out using retrospective cohorts; however, it is likely that reduction to clinical utility will depend on using samples from prospective trials of molecularly targeted agents. This idea is now being applied to colorectal cancer, and two large Phase III trials examining the addition of EGFR monoclonal antibodies to current therapy regimens have now finished the recruitment of more than 2,000 patients[96]. All samples will be profiled in an attempt to prospectively identify predictive biomarkers. An appropriate approach that could be used in this setting would be to use RPPAs to profile a larger number of proteins both upstream and downstream of the inhibitor target to identify potential predictive markers and to then reduce the assay to a clinically applicable multiplex IHC assay or ELISA. Pernas *et al.*[115] recently used RPPAs to profile head and neck squamous cell carcinoma cell lines to pathways downstream of EGFR, such as ERK, AKT, signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB), to identify markers of response to the EGFR inhibitor gefitinib. Interestingly, both STAT3 and phospho-STAT3 were associated with gefitinib response in cell lines and tumour samples using both IHC and RPPAs[115]. Although this study included a small number of patients, and the role of STAT3 as a predictive biomarker requires further validation, it highlights the approach that could be used in the translational group of larger clinical trials in which pretreatment and post-treatment analysis of protein expression can be used to identify predictive biomaker panels (FIG. 3). Given the precious nature of the material available from prospective trials and the small size of pretreatment biopsy samples, RPPAs may be an ideal platform for initial discovery with predictive assays that are later reduced in complexity to IHC or ELISA to ensure clinical applicability.

Such an approach would be a field shift from current practice, in which biomarker discovery is predominantly based on retrospective cohorts, and prospective cohorts are generally used for validation. It is plausible that this could be one of the major reasons why so few biomarkers survive independent validation[3,116]. A need to fast-track predictive biomarker development has been highlighted by several groups[2,117], and it is likely that the combination of high-throughput *in vitro* assays, combining gene expression analysis and functional genomic screens, and proteomic profiling in smaller numbers of patients participating in prospective randomized control trials may be a more successful strategy (FIG. 3).

Although this approach is suitable for newly identified compounds, it is also important to focus on established molecular therapeutics. Anti-angiogenic therapy using monoclonal antibodies and tyrosine kinase inhibitors targeting vascular endothelial growth factor (VEGF) has become the standard of care in several solid tumours, including colorectal cancer, renal cell carcinoma, breast cancer, non-small-cell lung cancer and glioblastoma either alone or in combination with chemotherapy[118]. Despite promising results in various tumour types, anti-angiogenic therapy is still limited by a lack of predictive biomarkers, particularly as innate and acquired resistance is an ever increasing clinical dilemma[119,120]. To examine this, several groups have recently used multiplex bead assays to profile various circulating cytokines and angiogenic factors (CAFs) in patients treated with anti-angiogenic agents[121–123].

In a Phase II trial of untreated metastatic colorectal cancer comparing the addition of the monoclonal VEGF-specific antibody bevacizumab to a chemotherapeutic regimen combining fluorouracil, leucovorin and irinotecan, Kopetz *et al.*[122] used a multiplex bead assay to demonstrate that several CAFs, including basic fibroblast growth factor (also known as FGF2), hepatocyte growth factor (HGF), placental growth factor (PGF), stromal-derived factor 1 (SDF1) and monocyte chemoattractant protein 3 (MCP3), were significantly increased from baseline pretreatment levels before any radiological evidence of progressive disease. These results highlight how this approach can be used to monitor

Retrospective cohort
A study in which the medical records and possibly also the previous tissue specimens of groups of patients with a specific diagnosis (for example, breast cancer) are collected.

Prospective trial
A trial in which the participants or patients are identified, followed over time and the effects of different conditions on their eventual outcome are measured.

patients on therapy. Using a complementary approach, Nikolinakos *et al.*[123] used multiplex bead assay profiling to identify a CAF signature consisting of HGF and interleukin-12 (IL-12), which predicts response to the anti-angiogenic tyrosine kinase inhibitor pazopanib in early-stage non-small-cell lung cancer. Although these data require validation, they demonstrate the application of multiplex bead assays to identify new predictive biomarkers for angiogenesis inhibitors.

Translating assays to the clinic

Despite great advances in the preclinical arena, the translation of new assays to the clinic has been slow. This problem is likely to be multifactorial; however, several issues have come to the fore over the past decade. It is now widely accepted that predictive, rather than prognostic, markers will maximally benefit personalized therapeutic regimens. This poses a considerable problem for solid tissue malignancies.

As technology platforms continue to improve it is likely that high-throughput clinical translation will require a substantial change in the scientific and clinical approach to diagnostic and prognostic assay development. Although descriptive studies demonstrating a technology are helpful, they rarely answer a pertinent clinical question, and it is therefore imperative that translational oncology moves back towards a hypothesis-driven approach in which studies are designed to answer specific, predefined clinical questions similar to those addressed above with respect to angiogenesis inhibitors[122,123].

As mentioned previously, serum-based diagnostics have not delivered on their initial promise, which might be due to poor study design, particularly as most studies have simply compared disease to normal controls in small, underpowered cohorts. A move away from population-based assays to specific tests for high-risk groups could allow for easier clinical translation. A diagnostic serum test for screening high-risk individuals with premalignant conditions such as Barrett's oesophagus, ulcerative colitis, ductal carcinoma *in situ* and atypical endometrial hyperplasia would be particularly beneficial, as patients would avoid recurrent invasive investigations such as endoscopy. Diagnostic serum assays for high-risk individuals are likely to involve different proteins and pathways from those that could be developed for population-based screening, as the underlying biology is likely to be distinct and this group would require a highly specific test, compared with a sensitive test that is required for population-based screening.

Although predictive biomakers are increasingly used in leukaemia trials[117], efforts to apply such markers in clinical trials for treating solid tumours have not been particularly successful, as it is challenging to gain access to tumour tissue during treatment so that predictive biomarkers can be measured. Unlike leukaemia, in which large numbers of tumour cells are present in the peripheral blood, solid tumour tissue is usually only accessed at diagnosis, by either biopsy or resection. Although this approach might be sufficient to study prognostic biomarkers, it severely limits the application of predictive and pharmacodynamic biomarkers because these measurements are ideally carried out concurrently with treatment[117]. In addition, experimental drugs are typically evaluated in patients with late-stage disease who do not routinely undergo additional tumour biopsies. It is hoped that serum-based proteomics, or proteomic strategies that evaluate predictive markers in circulating

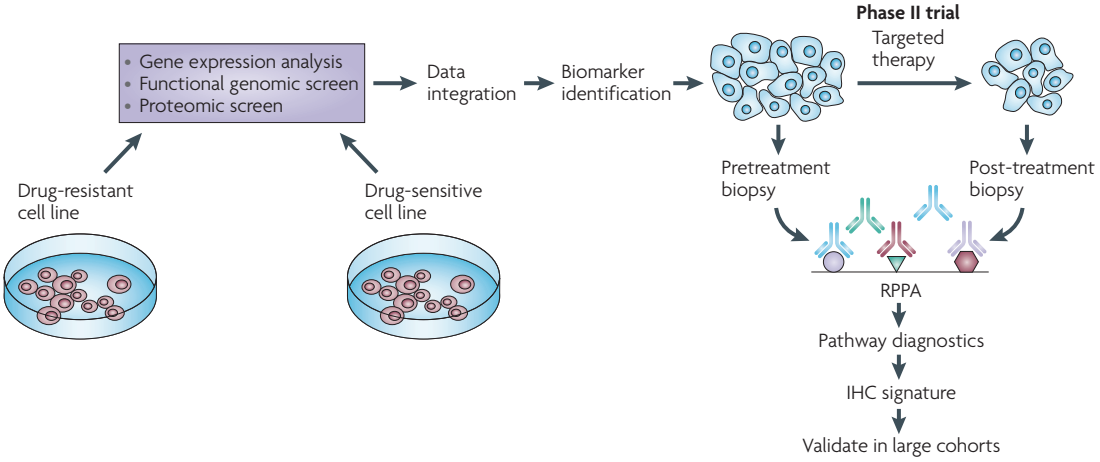


Figure 3 | **Translating antibody-based assays into the clinic.** Predictive biomarkers for targeted therapies will be crucial for identifying correct patient subgroups. The development of such biomarker panels is likely to be based on a systems biology approach, in which high-throughput screening using various methodologies can be carried out *in vitro*. The integration of gene expression and proteomic data will be crucial to the identification of new biomarkers. It will also be necessary to institute biomarker testing in patients at an earlier stage than is currently practised, particularly in Phase I and II trials. Analysis of pathway activation is likely to be a key predictor of response. Reverse phase protein arrays (RPPAs) offer the opportunity to institute pathway diagnostics in early trials, particularly if pretreatment and post-treatment tissue samples can be obtained. Given the precious nature of the material available from prospective studies and the small size of pretreatment biopsy samples, RPPAs may be an ideal platform for initial discovery with predictive assays reduced to clinical applicability through immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA) over time.

tumour cells, might help overcome this hurdle; however, trial designs incorporating multiple biopsies during treatment could also be necessary.

In addition, it is now obvious that extensive heterogeneity exists between patients and tumour samples, and investigations of biomarkers may need to be evaluated in the context of specific tumour subtypes. This was demonstrated by Kobel *et al.*[124] who examined 20 tissue markers in a population-based cohort of 500 epithelial ovarian carcinomas, and demonstrated that the association between biomarker expression and survival varied substantially between histological subtypes and it could be easily overlooked in whole-cohort analyses. As the prevalence of certain tumour subtypes is low (such as clear cell carcinoma of the ovary), it is likely that large research consortia will be required to build collaborative efforts in this field. Such consortia will rely on standardization of tissue fixation and processing, as well as standardized protocols for the collection, processing and storage of serum samples. Although this has been highlighted as a particularly important issue with regard to the reproducibility of various assays, it remains an important consideration when developing a research consortium.

The other obvious bottleneck in transfer from biomarker discovery to clinical application is primarily related to a lack of rigorous validation of emerging biomarkers. In 2004, a standards template was developed: the standards for reporting of diagnostic accuracy (STARD) initiative[125]. Unfortunately, many of the studies published regarding new candidate biomarkers fail to meet these standards. In particular, the studies are often carried out on small retrospective cohorts and lack statistical power. Additionally, many biomarker studies fail to include an independent validation stage, whereby the biomarker is evaluated using a second independent cohort of patients[59].

Conclusion

The past decade has witnessed considerable progress in the development and advancement of affinity techniques, methodologies and concepts. Using the technologies described above, antibody-based proteomics offers the opportunity to exploit the specificity and sensitivity associated with antibody-based assays to functionally interrogate tumour biology on a proteome-wide level. Such an approach has the potential to identify new cancer biomarker panels, which can be reduced to clinically applicable assays, including IHC and ELISA, thus providing a high-throughput approach for biomarker development, validation and clinical implementation. It is hoped that such an approach will accelerate the development of personalized therapeutic regimens for cancer patients.

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Competing interests statement
The authors declare competing financial interests; see web version for details.

DATABASES
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