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REVIEW

Affinity-based microarrays for proteomic analysis of cancer tissues

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Based on about a decade of technical developments in analysing the human proteome with antibody microarrays and experience in performing such analyses, now there are the means at hand for detailed and simultaneously global investigations of this kind. Many technical aspects have been dealt with of both the microarray format itself – such as overcoming kinetic and mass transport limitations and thus achieving accurate measurements – and ancillary processes – such as extraction procedures that provide good protein solubilisation, produce reproducible yields and preserve the native protein conformation as much as possible. The overall analysis process is robust and reproducible, highly sensitive down to the level of single-molecule detection and permits an analysis of several parameters on many molecules at a time. While the study of body liquids is widely applied, analyses of tissue proteomes are still scarce. However, conditions do exist to perform the latter at a quality level that meets the standards for clinical applications. This review highlights methodological aspects relevant for a biomedically useful analysis of cellular samples and discusses the potential of such studies, in particular, in view of personalised medicine approaches.

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1 Introduction

During the past decade, the field of molecular biology has witnessed yet another leap forward. Genomic techniques have been developed that permit whole genome sequencing of individuals (e.g. The International Cancer Genome Consortium; ICGC) [1] that will very soon be applied routinely in clinical settings. However, despite the remarkable progress of the understanding of complex biological processes involved in disease pathogenesis at the level of nucleic acids, insights into the biochemistry of diseases remain often preliminary and incomplete. Already the, initially surprisingly small number of protein-encoding genes indicates and emphasises the fact that much regulation and activity occurs at the protein level. Proteins are involved in basically all vital biological processes and about 97% of all current therapeutic agents target proteins. Consequently, the scope of globally oriented analysis is expanding beyond the merely genomic and transcriptomic approaches to address also events at the proteome level [2, 3].

Proteome analyses face the challenge of detecting and analysing many molecules and all their variations. As opposed to nucleic acids, proteins are molecules of very different structures and biophysical and biochemical characteristics. Also, the proteome is continuously undergoing dynamic changes not just in the abundance of each particular molecule but also in its degree and kind of substitution. The proportion and importance of protein modification is reflected by the fact that nearly 10% of mammalian genes encode for proteins that modify other proteins. Also, the range of protein concentration is huge and varies across cellular locations. In consequence, many approaches to determine protein biomarkers out of complex proteomic samples have been successful to a lesser extent only. Also, many studies are only aiming at one major type of objective, such as functional [4] or structural aspects [5], deal with particular protein modifications, for example, phosphoproteomics [6] or glycoproteomics [7], or concentrate on a physiologically or biologically defined sub-proteome.

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Abbreviation: scFv, single chain variable fragment

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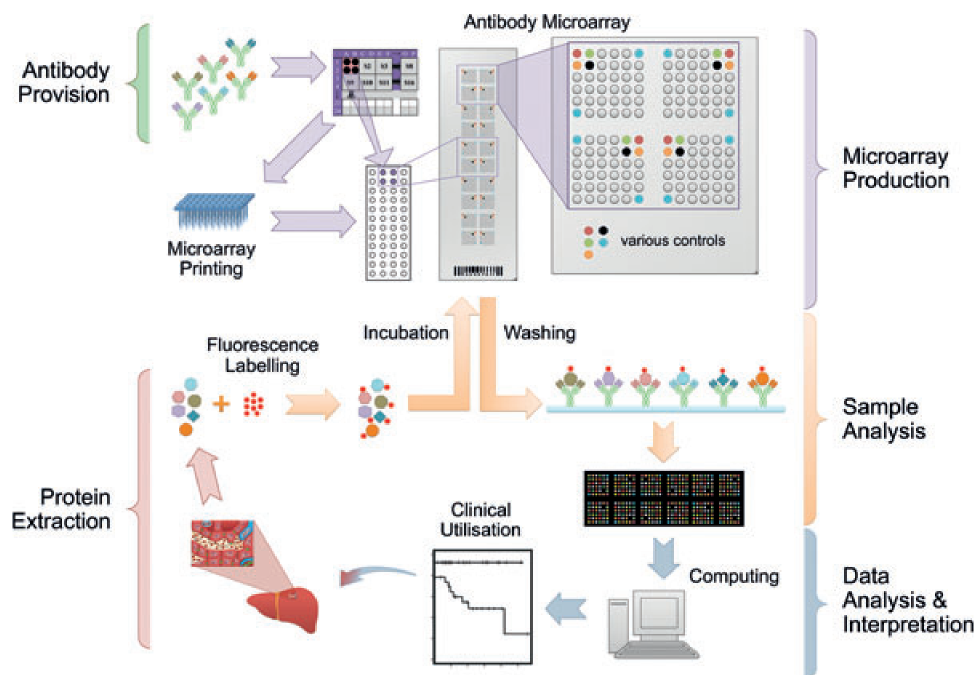

Figure 1. Schematic presentation of the steps involved in performing protein analyses with antibody microarrays.

Technically, MS, either coupled to other, preparative or analytical methods or on its own, is currently the main method in proteomic research achieving in a few applications a data quality and reproducibility that is sufficient for use in a clinical setting. Issues such as sample complexity, detection sensitivity and the method's resolution in whole proteome analyses are limiting, however. Also, structural variations can only be predicted or elucidated indirectly. Antibody microarray analyses could act both complementary and supplemental to MS techniques [8]. In principle, they are an equivalent to chip-based transcriptional profiling (Fig. 1). Well-characterised antibodies (or other appropriate binder molecules) are arrayed on a solid support. The relevant protein mixture of interest is isolated, labelled–usually with a fluorescence dye–and applied to the array. Signal intensities obtained at the various binder molecules provide the basic information. In most antibody microarray analyses reported to date, one sample labelled with one dye was incubated on the array, which according to one study [9] produces better results than a simultaneous incubation of two samples that were labelled with different dyes. Two other studies, however, came to a different conclusion; here, the latter process yielded better reproducibility and differentiation power, if one of the two samples continuingly consisted of a common control [10, 11].

Eventually, affinity microarrays could combine in a single format most if not all aspects that are required for a thorough understanding of protein content and function; it is mainly a matter of having available the appropriate binder molecules. The process has the big advantage that different kinds of data can be obtained in parallel in a single assay. Aspects that can be studied include variations in the abundance of proteins, the occurrence of structural and thus functional differences in the form of protein isoforms or protein modifications and the definition of biochemical activities and regulative processes by virtue of detecting interaction partners. Another advantage is the combination of high throughput, with basically no upper limit to the number of antibodies that can be used in an assay, and the various degrees of specificity that can be applied, thereby generating broad but nevertheless detailed information simultaneously. While currently not being the best methodology in any particular technical aspect, the technology is overall very complete and comprehensive (Fig. 2). In addition, the process is amendable to current immunoassay formats used in many diagnostics laboratories. The factors of user acquaintance and acceptance, although more a matter of human nature, should not be underestimated when it comes to translating a technology to (clinical) practice.

2 Binders

The affinity reagents are a critical aspect of the technology [12]. Currently, still missing is even a broadly available, comprehensive set of binders of sufficient specificity and affinity that cover all human proteins, although more than 500 000 antibodies are currently listed in *Antibodypedia* (www.antibodypedia.com). The type of epitope that should be detected is another matter that is important for binder selection. The identification of structural variations, for example, depends on both the structural intactness of the protein subsequent to the extraction process and the specificity of the antibody. Worldwide, efforts are ongoing towards the provision of a global resource of well-characterised affinity reagents that target the entire basic protein set

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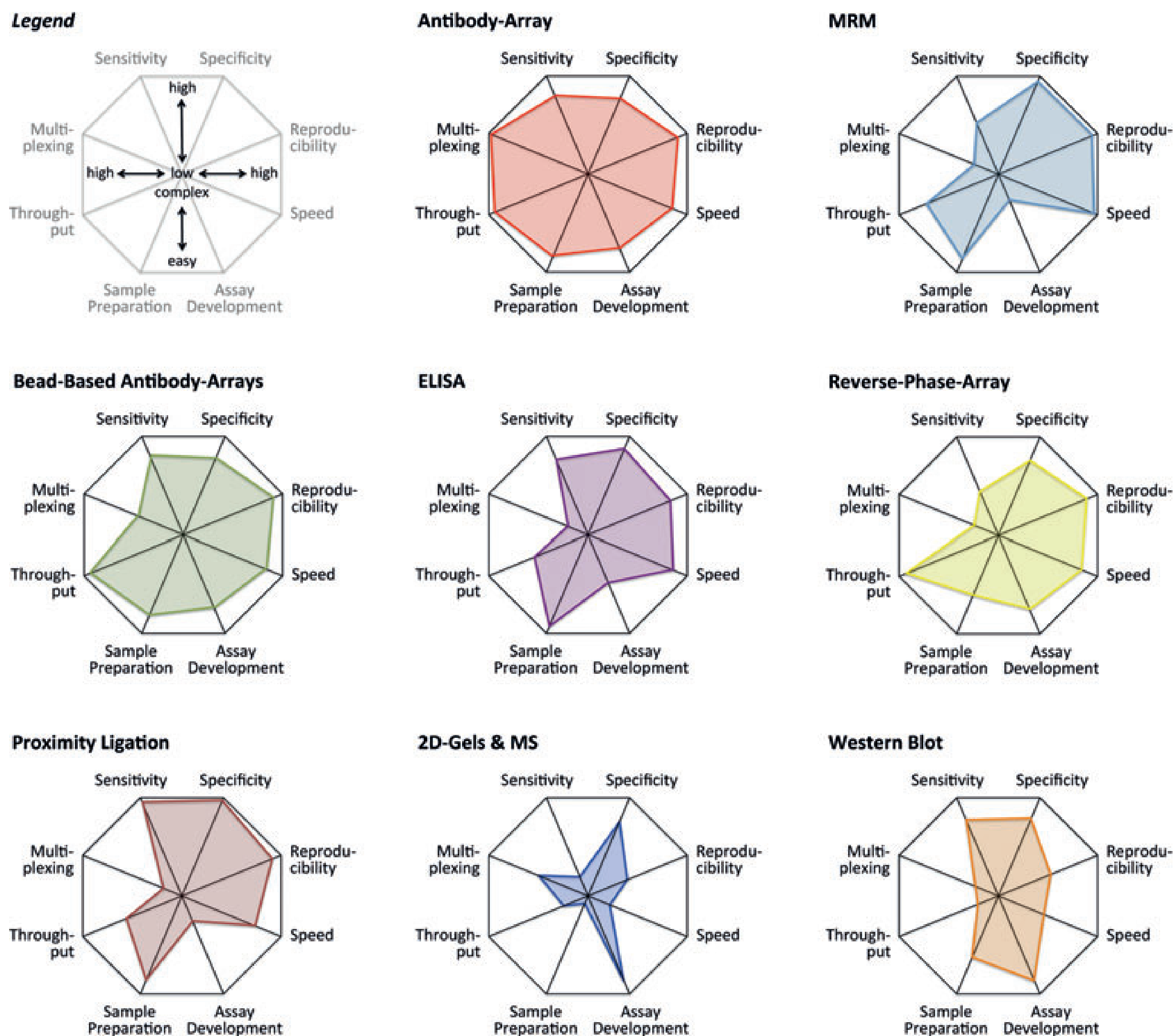

Figure 2. Comparison of performance parameters of different techniques used for proteome analyses; MRM: Multiple Reaction Monitoring by Tandem Mass Spectrometry; 2D-Gels: two-dimensional gel electrophoresis; MS: mass spectrometry. The evaluation represents a personal reflection of the authors. It is also based on the assumption that the degree of automation would be similar, which is not (yet) true. Next to the criteria shown, others do exist, which could be critical, when selecting the process fitting best to a particular task. In particular, a comparison of the aspects of applicability and accuracy is not shown, although of critical importance, since such an assessment would be difficult to make without detailed information on sample type and assay objective.

(e.g. *Affinomics*, www.affinomics.org; *Clinical Proteomic Technologies Initiative*, www.proteomics.cancer.gov; *Antibody Factory* www.antibody-factory.de). The *Human Proteome Atlas* (www.proteinatlas.org) is currently the most advanced initiative in this respect [13]. While the availability of binders for all kinds of purposes and applications will still take quite some time to be established, considering that there are probably more than a million of protein isoforms and modifications, an initial set of polyclonal molecules for the detection of a "basic human proteome" of some 20 000 proteins, assuming that one gene encodes for one protein, will be available in about a year's time (www.proteinatlas.org/about/releases), and there is more to come.

Next to antibodies, single-chain variable fragment (scFv) binders [14], molecules with alternative scaffold structures, such as affibodies, or even biochemically unrelated molecules such as aptamers [15] have been reported to perform well. As with DNA microarrays, the distance to the solid support is a critical performance factor. Consequently, the use of linker molecules is preferential, if small molecules are being applied, although scFv molecules attached directly to the surface have been used successfully [9, 16]. Overall, full antibodies have a better stability during storage. An additional advantage of larger molecules is the fact that undirected attachment to the solid support is less likely to occur accidentally at the antigen-binding site, thus blocking it from

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interacting with the epitope. Despite the various alternatives, the use of antibodies raised by immunisation of rabbits, mice or other animals, such as camels or sharks [17], is currently still the most widely used kind of reagent in immuno-based assays.

Another matter of discussion is the question, if monoclonal or polyclonal antibodies are better for array-based assays. The most frequent arguments in favour of monoclonal binders are the uniqueness of the respective epitope and the unlimited supply of antibody from hybridoma cells. Polyclonal binders, on the other hand, are much cheaper to be produced, and the amounts obtained are still relatively large, considering that 10 μg of an immunoglobulin G antibody are enough for the production of more than 1000 microarrays [11]. For a sandwich approach, polyclonal antibodies could act as both the catching and the detection reagent, while two different monoclonal binders are needed for each protein. However, sandwich assays are not really suitable for the types of analysis, for which complex antibody microarrays are used (see section 2). In terms of performance, the difference in specificity between mono- and polyclonal binders is of only limited relevance for antibody microarray experiments in our experience. For the detection of protein isoforms, for example, we generated pairs of polyclonal antibodies for the detection of different splice-variant conformations using linear targets (unpublished results). About 20% of the antibody pairs were of a quality that both binders discriminated well between the protein types. For a third of the pairs, only one binder worked at that quality level. Therefore, even for this rather demanding purpose, about a third of the polyclonal binders generated in mice yielded highly specific antibodies. Using other targets, higher percentages have been achieved. The continuously increasing availability of recombinant or fully synthetically designed binder molecules [e.g., [18] could make redundant altogether the need of deciding between mono- and polyclonal binders, since one could shift between the two binder formats relatively easily.

Infrastructure has been put in place in the form of databases (e.g. *Antibodypedia*; http://www.antibodypedia.com/) or actual binder resources (e.g. *The Developmental Studies Hybridoma Bank* of the University of Iowa; http://dshb.biology.uiowa.edu/) that provide information or material of binders that meet defined quality criteria.

3 Technical aspects

Established assay processes [11, 19, 20] produce antibody microarrays of intra- and inter-array variation that are similar or even superior to the quality reported for commercial DNA-microarray platforms that have been approved for clinical applications [21]. Also, various auxiliary facets have been dealt with successfully, such as establishing appropriate protocols for protein extraction from tissues [22]. They are integral for the overall success as can be seen in Fig. 3, for instance, exemplifying the effect and importance of preparative processes. With the aspects of kinetics and mass transport being addressed [23], the amount of material needed in an assay–about 5 μg of total protein–is well within a range that can be extracted from tissue samples of the kind available in clinical diagnostics; even fine needle biopsies provide enough cellular material. It is critical for a successful analysis, however, to check the tissue quality. Especially in routine applications, it may occur that fat or other unsuitable tissue components make up the majority of a specimen. As with other assay types, sample composition has an immediate influence on the results, although lipid removal, for example, should be an integral part of protein preparation protocols. In agreement with other immuno-based assay formats, depletion of highly abundant proteins was found to be unnecessary for antibody microarray analyses with specific binders, even though this has only been demonstrated thoroughly with serum samples [11]. However, with albumin making up about 60% of the overall protein content in serum and still not having any measurable effect on the detection of other, far less abundant proteins, one would not expect a much different result concerning this aspect in tissue analyses.

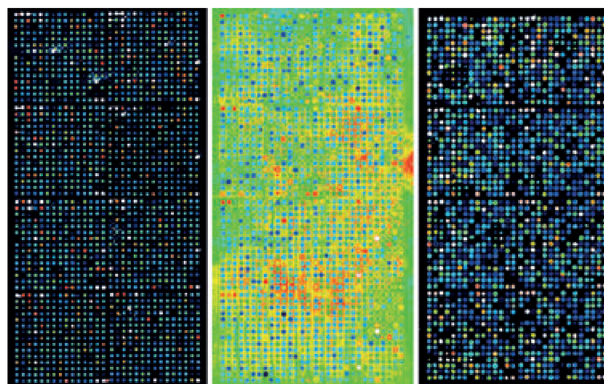

Figure 3. Example of a technical parameter that is crucial for analysis quality. Protein samples were isolated, labelled with fluorescent dye and incubated on complex antibody microarrays. False colour images of the resulting patterns are shown. The left panel shows the quality of an analysis of a protein extraction from serum. In centre, a protein extract was incubated that had been isolated from tissue by the same protocol used for the serum sample. On the right, a result is presented that was obtained with a tissue sample but using another extraction and handling protocol. The enormous difference in background signal intensity is obvious.

Many immunoassays are based on a detection mode with at least two different antibodies binding to a target molecule, the most prominent example being the sandwich approach of ELISAs. In other systems, a combination of three or up to four binders has been reported for the detection of protein interactions [24]. For complex microarray analyses, however, a sandwich assay format is less desirable since it restricts the multiplex factor to less than 50. Directly labelling the target proteins permits a basically unlimited multiplexing

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that is defined solely by the number of antibodies displayed on the array platform. If studying proteins with an array of 20 000 binders, for example, more than 400 sandwich analyses instead of one experiment with a directly labelled protein extract would be required, pushing the cost to unacceptable heights. Also, twice the number of specific binders would be required–unless polyclonal antibodies were used–adding even more constrain to the limiting issue of binder provision.

As a matter of course, the average specificity of detection is bound to be better if two or more binders identify a particular protein. In this respect, the use of polyclonal antibodies as the catching reagent attached to the microarray surface may actually be advantageous, since it is likely that the recognition of different but related epitopes by the individual antibodies in a polyclonal pool creates a cooperative effect, thus improving the selectivity and strength of a binding event. From other assay formats, such as Western blotting [25] or immunoprecipitation [26], it is well documented that antibody binding could occur to several proteins via a similarity in epitopes or by virtue of binding a protein complex. The latter can largely be circumvented by appropriate assay conditions [19], if required. The former does rely mostly on the frequency of particular epitopes in the proteome and the quality of the binder molecules.

The quality of binders used on an array platform should be assessed stringently, particularly if functional conclusions or diagnostic information should be drawn from the analyses. In short, the more quality controls are applied to the binders the better. To date, there is no overall accepted set of criteria for this. One reason is that these criteria could differ widely dependent on the actual analysis purpose. Nevertheless, an overall set of validation recommendations for antibodies does exist, which does not consider the actual application, however (e.g. www.antibodypedia.com/text.php#validation_criteria). In an optimal case, there should be several binders for each protein, since then the specificity of each can be investigated by a comparison to the others.

In terms of sensitivity, detection limits in the low attomolar range have been reported [23, 27]. In order to achieve this, especially the aspects of labelling, kinetics and mass transport need to be taken care of. Under appropriate conditions, even single-molecule detection is possible with the help of adapted detection hardware that nevertheless exhibits a detection speed that would be acceptable even in a diagnostic setting [28]. Such technology could make the analysis really quantitative, since the number of individual binding events on each array feature is counted, thereby producing absolute numbers instead of relative data.

4 Tissue studies

While analysis of serum samples and other body fluids offers the opportunity to identify biomarkers that can help in diagnosis and disease monitoring, a proteomic investigation of tissues will also provide data that reveal the pathophysiological background of a disease and identify potential targets for new treatment modalities. To date, only a very limited number of analyses were performed on human tissues (Table 1). Few additional reports have been published on cells [e.g., 45, 46] and mouse tissues [e.g. 47, 48]. Besides the relatively small number of studies overall, the number of samples analysed in each was usually small and did not permit a validation of the results with an independent set of specimens. Access to tissues samples is frequently still limited, similar to the bottleneck for many, particularly early studies of transcriptional variations. As a matter of fact, the competition of RNA profiling has made the problem worse in some cases; tissues were used for transcript or DNA analyses without performing the more complex extraction of proteins from the same source. With respect to availability, paraffin blocks would be the best source of patient material. Although the successful isolation of proteins from paraffin blocks has been published [e.g. 49], the resulting protein quality varies. The molecules are denatured by definition, thereby limiting the analysis to a measurement of protein abundance and variations in the linear amino acid composition. Also, the overall yield is low, restricting the sensitivity of an assay. However, also preparations from frozen or fresh tissues do not all work. In a study on pancreatic cancer (unpublished results), we started off with 1031 samples. Only 650 of them yielded protein of sufficient quality to pursue subsequent analyses. Although still a substantial number, it was further subdivided by the fact that both a training and test set had to be defined. In addition, the specimens represented ten different tumour and control types. But even the samples of a particular tumour subtype were not homogenous, but differed strongly in their cell composition. For pancreatic ductal adenocarcinoma tissues, for example, we found anything between 5 and 95% of actual tumour cell content.

In all studies published to date (Table 1), few proteins were repeatedly found to be differentially abundant in tumours and controls in more than one study. An unbiased analysis that takes into account the complete data rather than an arbitrarily selected portion has not been done as yet. At current, such a comparison is also relatively impractical, since different antibody sets were used in most studies. In addition, the results were not reproduced independently, mostly for the lack of a similar analysis of the same sample type with the same or a comparable analysis platform. A correlation with published RNA data does not help either in this respect. As long as RNA profiles frequently do not fit to each other well, if done on different samples, it does not make sense to look at protein and RNA expression data originating from independent experiments. A comparison of the various genomic, transcriptomic and proteomic methods with the same set of tumour (or other) samples would be advantageous for a comparative evaluation of the different techniques.

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Table 1. Use of antibody microarrays in profiling human tissue samples

Disease	Type of antibody microarray	Number of antibodies	Number of tissue samples	PubMed reference	Reference
Breast cancer	Panorama XPRESS Profiler725[a)]	725	Five tumours, five controls	22115752	[29]
Breast cancer	Panorama XPRESS Profiler725[a)]	725	Five tumours, five controls four cell lines	21338725	[30]
Breast cancer	BD antibody microarray 380[a)]	378	One sample, one control	15567944	[31]
Breast cancer	Monoclonal antibodies	368	Ten samples of microdissected cells	11721638	[32]
Chronic rhinosinusitis	Panorama Cell Signalling Antibody Microarray[a)]	224	Nine nasal polyps	19490800	[33]
Colorectal cancer	Clontech AB 500 array[a)]	500	16 tumours and adjacent normal tissue	20164542	[34]
Colorectal cancer	Panorama Cell Signalling Antibody Microarray[a)]	224	16 tumours and adjacent normal tissue	17848589	[35]
Congenital nephrotic syndrome	Cytokine antibody microarray[a)]	12	43 tumours, ten controls	18048423	[36]
Gastric adenocarcinoma	scFv antibody microarray	127	15 tumours and adjacent normal tissue	16844680	[37]
Gastric cancer	Proteome Profiler Human Phosphokinase Array[a)]	46	Seven benign tumours, three cancers, two controls	20953656	[38]
Human Aqueous Humor	RayBiotech Array[a)]	507	21 samples	20463327	[39]
Interstitial cystitis /painful bladder syndrome	Proteome Profiler Human Apoptosis Array[a)]	35	29 tumours, five controls	22310775	[40]
Lung cancer	Monoclonal antibodies	378	12 tumours, four controls made of three samples each	16022908	[41]
Melanoma-infiltrated lymph nodes	Clontech AB 500 array[a)]	500	11 melanoma-positive lymph nodes	17297476	[42]
Osteoarthritis	Cytokine antibody microarray[a)]	40	Unknown	18565249	[43]
Radiation proctitis	Proteome Profiler Array, Human Angiogenesis Array[a)]	55	Eight tumours, eight controls	22081051	[44]

a) Commercial array. Information not provided.

5 Towards personalised proteomics and therapy

In the current early stages of antibody microarray screening analyses, the purpose of studying tissues is mostly the identification of biomarkers. However, the biggest gains are likely to be made in understanding intracellular processes at the level of protein expression variations as well as structural and consequently functional alterations. Eventually, this information may be utilised for predicting disease progress, performing patient stratification, identifying pathogenic mechanisms and discovering novel target molecules for therapy.

With the number of new drugs that are broadly applicable to many patients continuously decreasing but with a concurrently steadily improving understanding of the complexity of many biological processes, it will become both necessary for better health care and possible because of improved technology to gather comprehensive and simultaneously detailed molecular information about individual persons for a targeted treatment. In particular, information on the occurrence and distribution of protein isoforms (Fig. 4) and their correlation with functional aspects and disease are crucial for an understanding of cellular activity. Many illnesses are based on or defined by protein isoforms that should be the target of and affected by therapeutic agents, while the protein conformation(s) occurring in healthy people should preferably remain unaffected. Several reports showed, for example, that solid tumours contain up to 100 protein-encoding genes that are mutated, although this is still based on a relatively small sample number [e.g. 50–52]. While only some of these mutations are probably acting as "drivers"–responsible for the initiation or progression of tumours–also many "passenger" variations could be important for maintaining a tumour and thus be of relevance to therapeutic approaches [53, 54]. In addition, the risk associated with many individual gene polymorphisms in complex diseases is so small that one cannot base any

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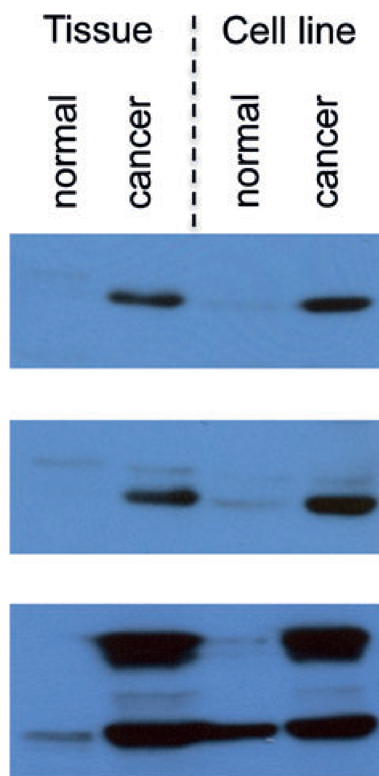

Figure 4. Protein isoform variations in cancer. The protein content of tissues and related cell lines were studied by Western blot analysis using antibodies raised against protein isoforms. In the two upper panels, the respective binder identified the conformation mainly expressed in cancer. In the bottom panel, the result with another binder is shown that identifies two protein conformations, of which one is tumour specific.

therapeutic approach on such data. This could change by analysing the consequences of a combination of risk alleles at the protein level. Here, polymorphic risks could be substantial, since cooperative in their effect, if the functions of the proteins are an integral part of a common pathway, cellular activity or protein complex. The comprehensive DNA sequence information already available from many tens of thousands of healthy and diseased individuals–with much more to come–will provide a basis for a detailed investigation of disease-defining mechanisms in the form of isoforms at the protein level.

In addition, future cancer treatment is likely to be based on the combination of therapies, overcoming the evasive processes that lead to resistance to a particular cure. This will require detailed information on personal protein variations, since the disease-causing variations are likely to occur in different combinations in each patient. Since companion diagnostics will be also required for most if not all drugs used in a personalised approach, the availability of relevant systems for detecting protein variants will be essential for such developments.

6 Conclusions

Already the still relatively few successful projects reported to date indicate the usefulness of tissue analyses for an understanding of cellular biology and the utility for disease diagnosis. With many more specific binder molecules becoming available in the next few years, the width and thereby the importance of the approach will increase substantially towards a really comprehensive proteome analysis; access to good affinity reagents is the most limiting factor to application. Comprehensive studies will also permit to compare data between different tissue types and identify more easily molecules that are relevant for disease development and simultaneously appear in body liquids as marker molecules for disease diagnostics. By extending protein expression studies towards a simultaneous analysis of structural and thereby intrinsically functional variations, also the identification of factors that are important for pathogenesis and their personal combination in individuals will be prerequisite towards the establishment of combination therapies.

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Christoph Schröder and Jörg D. Hoheisel are in the process of setting up a spin-off company based on results produced during academic antibody microarray analyses on tissue, blood and urine samples from cancer patients, with a focus on diagnosis and prognosis.

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