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Review

Molecular profiling of signalling pathways in formalin-fixed and paraffin-embedded cancer tissues

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ABSTRACT

In most hospitals word-wide, histopathological cancer diagnosis is currently based on formalin-fixed and paraffin-embedded (FFPE) tissues. In the last few years new approaches and developments in patient-tailored cancer therapy have raised the need to select more precisely those patients, who will respond to personalised treatments. The most efficient way for optimal therapy and patient selection is probably to provide a tumour-specific protein network portrait prior to treatment. The discovery and characterisation of deregulated signalling molecules (e.g. human epidermal growth factor receptor 2, mitogen-activated protein kinases) are very promising candidates for the identification of new suitable therapy targets and for the selection of those patients who will receive the greatest benefit from individualised treatments. The reverse phase protein array (RPPA) is a promising new technology that allows quick, precise and simultaneous analysis of many components of a network. Importantly it requires only limited amounts of routine clinical material (e.g. FFPE biopsies) and can be used for absolute protein measurements. We and other research groups have described successful protein extraction from routine FFPE tissues. In this manuscript we show how these recent developments might facilitate the implementation of RPPA in clinical trials and routine settings.

1. Introduction

Although cancer is often classified as a genetic disease, at its functional level it is in fact both a genomic and a proteomic disease. Gene mutations in oncogenes or tumour suppressors and associated deregulations in key signalling pathways result in survival advantages for cancer cells and support tumour development and progression.[1] Intracellular signals are transmitted through reversible phosphorylation and dephosphorylation of proteins reflecting their activation status within the protein network. In this regard, some of the best characterised signalling molecules are the mitogen-activated protein kinases (MAPK) located downstream of the *ras* protooncogene. Activation of this molecule is known to promote carcinogenesis.[2,3]

Deregulated signalling pathways can cause enhanced cell growth, proliferation, survival, invasion, and metastasis or reduced apoptosis of cancer cells. Hence, the discovery and characterisation of deregulated signalling pathways are promising candidates for studies of cancer progression, for the identification of suitable therapy targets, and for the selection of those patients who will respond to personalised therapy

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most likely. Many attempts have been made to discover possible connections between activated receptor tyrosine kinases such as kinases of the ErbB receptor family like the human epidermal growth factor receptor 2 (HER2/neu/ErbB2) or the human epidermal growth factor receptor 1 (EGFR/HER1/ErbB1) and downstream signalling molecules to identify potential new therapeutic targets.[4] Nowadays some therapies are already based on protein expression changes (e.g. HER2, EGFR) in tumour tissues. In fact, the first tyrosine kinase that was directly linked to cancer was EGFR. EGFR overexpression due to gene amplification is often found in human cancers. For example in gliomas EGFR gene amplification is often associated with structural rearrangements leading to in frame deletions in the extracellular domain of the receptor.[5] The HER2 status is important to predict a response to chemotherapy and hormonal therapy in breast cancer patients and is also essential to predict a response to the monoclonal anti-HER2 antibody trastuzumab.[6] Trastuzumab is a humanised monoclonal antibody directed against the extracellular domain of HER2. The HER2-receptor is overexpressed in 25–30% of breast cancer patients via HER2 gene amplification or transcriptional activation.[6–9] Currently, the HER2 status is determined by immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH). Pathological characteristics for breast cancer such as tumour size, degree of tumour cell differentiation, presence or absence of metastases, immunohistochemical classification and quantification of relevant proteins (HER2, oestrogen- and progesterone-receptor) have major impacts on therapy decisions.[10] But these parameters are not sufficient to reflect the complexity and heterogeneity of individual tumours regarding for example activation status of receptor tyrosine kinases or deregulated signalling pathway proteins. However, knowledge of those factors would be crucial to assess success or failure of personalised molecular therapies and to conceive, why only a subset of patients responds to individualised treatments.[10] Hence, for optimal patient selection and therapy, new techniques being able to detect the entire spectrum of deregulated pathways in tumours before and during treatment are needed in addition to IHC and FISH. Reverse phase protein array (RPPA) is a very promising new technology that allows the simultaneous analysis of multiple parameters. Since formalin-fixation and paraffin-embedding (FFPE) is the standard method for tissue handling in almost all hospitals world-wide, the focus of this manuscript is on the analysis of FFPE cancer tissues. In fact, it became possible to successfully extract full-length, immunoreactive proteins from FFPE tissues only recently (see below). Subsequent analysis by RPPA allows the quantification of clinical relevant proteins such as HER2.[11,12]

2. Extraction of proteins from FFPE cancer tissues

In most hospitals world-wide formalin-fixation and paraffin-embedding is the standard tissue fixation and storage method. Formalin fixation results in cross-linking of macromolecules and keeps the tissue in an excellent condition for histopathological analysis.[12] Additionally it has been shown in several reports that not only proteins but also protein modifications such as phosphorylations are stabilised during fixation and can be analysed even years later for example by IHC.[13,14] Formalin is 37–40% formaldehyde in water, stabilised by 10% methanol. Tissues are routinely fixed in a phosphate buffered 10% solution of Formalin. Formaldehyde reacts with amino groups of basic amino acids such as lysine, asparagine, arginine, histidine and glutamine, leading to the formation of highly reactive methylol adducts. A subsequent condensation reaction of adducts occurs through Schiff base formation. This results in the formation of methylene bridges with amine, guanidyl, phenol imidazol and indole groups of several other amino acids like arginine, asparagine, glutamine, histidine, tryptophan and tyrosine, leading to inter- and intramolecular cross-linking of proteins.[15]

Many diseases are characterised and diagnosed by the expression of specific proteins, tissue morphology, cellular configuration, pleomorphisms, nuclear shape and contour and staining patterns. Beside the more traditional ways of diagnosing and characterising disease, the analysis of specific proteins becomes more and more crucial. Therefore, protein profiling in FFPE tissues is very important for diagnosis and therapy. The utilisation of FFPE tissues for protein analysis is superior to the use of fresh frozen tissues for numerous reasons such as cost, availability, standardised technique and most importantly the easy storage of tissues for many years.[16] Up to now, IHC is the only routinely used method to analyse proteins in FFPE tissues. Although IHC can give some information about the abundance of several proteins in tissues, this method is not suitable for the analysis of subtle quantitative changes in multiple classes of proteins taking place simultaneously within a cell or tissue.[17] In addition, the quantification of protein expression based on stained slides is difficult and depends at least in part on the observer. The analysis and extraction of full-length and immunoreactive proteins from FFPE tissues was believed to be impossible for a long time due to cross-linking during fixation. Nevertheless, in recent years, several research groups including our own have established protocols to extract proteins from FFPE tissues.[12,18–25] For use in clinical *in vitro* diagnostics the extraction protocol should not be too complex and has to be compatible with downstream molecular analysis such as Western blot, RPPA or mass-spectrometry.

So far, seven groups described different protocols for extraction of full-length proteins from FFPE tissues summarised in Table 1. All protocols listed need high concentrations of SDS and exposure to high temperature. In contrast to other recent studies[26–29] that used proteases to cleave proteins into peptides, the protocols in Table 1 are suitable for extraction of full-length proteins.

3. Reverse phase protein array (RPPA)

Over the last few years, new approaches and developments in patient tailored cancer therapy have generated a need to more precisely define and identify those patients, who will receive the greatest benefit from targeted drugs. The most efficient way to identify patients, who will respond to personalised therapies, is to provide a tumour-specific network portrait in each patient prior to treatment (Fig. 1). Such

Table 1 – Summary of protocols for protein extraction from FFPE tissues.

Extraction buffer	Protocol	Advantages	Disadvantages	Applications	Reference
RIPA plus 2 % SDS	100 °C 20 min 60 °C 120 min –80 °C for storage	• Cheap (not commercial)	• Only proteins up to120 kDa were analyzed • Time consuming extraction protocol	• SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) • Immunoblotting	Ikeda et al.[19]
NDME (BioQuick Inc., Silverspring, MD, USA)	100 °C 20 min –80 °C for storage	• Parallel extraction of protein and nucleic acids • Short extraction protocol • Extraction of proteins up to 188 kDa	• Low yield • Special device needed	• SDS–PAGE • Immunoblotting • RPPA • SELDI-TOF MS	Chu et al.[18]
20 mM Tris–HCl buffer pH 7 or 9 plus 2% SDS	100 °C 20 min 60 °C 120 min –80 °C for storage	• Cheap (not commercial)	• Time consuming extraction protocol	• SDS–PAGE • Chromatography • Immunoblotting	Shi et al.[22]
Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany)	100 °C 20 min 80 °C 120 min –20 °C for storage	• No differences in protein yield and abundances between fresh frozen and FFPE tissues • Analysis of phosphospecific proteins • Extraction of proteins up to 190 kDa • Standardised reagent (commercial product)	• Time consuming extraction protocol	• SDS–PAGE • Immunoblotting • RPPA	Becker et al.[12,23,24]
1× AgR buffer (pH 9.9), 1% NaN_3, 1% SDS, 10% glycerol, protease inhibitor	15 min 115 °C, 10–15 psi –80 °C for storage	• Deparaffinisation not necessary • Fast extraction protocol • Analysis of phosphospecific proteins • Very sensitive	• Smearing in FFPE samples	• SDS–PAGE • Immunoblotting • RPPA	Chung et al.[21]
Laemmli buffer	20 min 105 °C –20 °C for storage	• Cheap (not commercial) • Fast extraction protocol	• Low yield • Several different Laemmli buffer formulation exist	• SDS–PAGE • Immunoblotting	Nirmalan et al.[20]
EB buffer 20 mM Tris–HCl; pH 8.8 plus 2% SDS plus 200 mM dithiothreitol (DTT)	100 °C 20 min 80 °C 2 h –80 °C for storage	• Extraction of proteins up to 130 kDa	• High molecular weight proteins (e.g. myosin) could not be detected	• SDS–PAGE • Immunoblotting • RPPA • ELISA • NanoHPLC-nanoESI-Q-TOF profiling	Addis et al.[25]

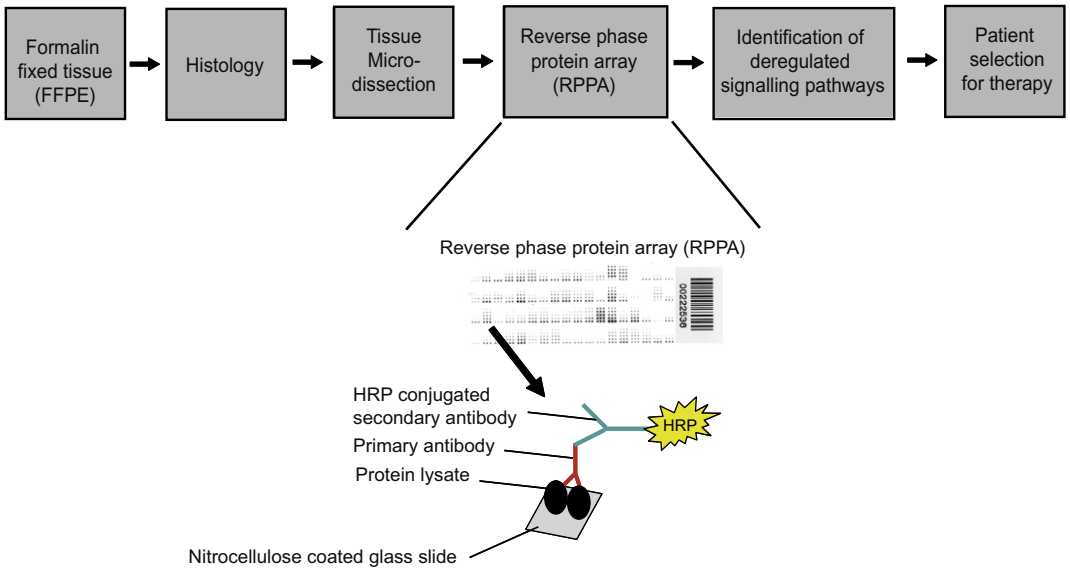


Fig. 1 – Patient selection for individual cancer therapy using reverse phase protein array. Formalin-fixed and paraffin-embedded (FFPE) material is the major source of tissues in hospitals. After histologic inspection and tissue microdissection a tumour-specific network portrait is determined. The identification of deregulated signalling pathways serves as a basis for optimal patient selection and therapy decision. Recently, it became possible to use FFPE tissue samples to identify deregulated signalling pathways by reverse phase protein array (RPPA). Here protein lysates are immobilised on nitrocellulose-coated glass slides and detected with specific antibodies.

a portrait may ideally be prepared by using tissue material taken from each patient by biopsy. The reverse phase protein array (RPPA) represents a new high-throughput technology to monitor changes in protein phosphorylation over time, before and after treatment, between disease and non-disease states and between responders and non-responders.[30] This array format allows analysis of multiple samples for the expression of one protein under the same experimental conditions at the same time. Moreover it is suited for signal transduction profiling of small numbers of cultured cells or cells isolated by laser capture microdissection from human biopsies.[17,31] After protein extraction, each patient sample is arrayed in triplicates on nitrocellulose-coated slides in a miniature dilution curve. Thus, each analyte/antibody combination can be analysed in the linear dynamic range.[30,32]

In our group we established a method for precise protein quantification by RPPA. Purified recombinant proteins of known concentration arrayed together with the patient samples on the same slide serve as internal reference standards and allow absolute sample quantification. We used purified recombinant HER2 as an example (own unpublished data). Since patient samples that consist of a pool of proteins may cause more background than purified proteins alone, 2.5–0.15 pg HER2 protein was mixed with a HER2-negative patient lysate adjusted to 2 mg/ml (Fig. 2A). We generated a signal-intensity–concentration curve to determine the exact receptor abundance in a sample. For instance, in our example the signal-intensity of the unknown patient sample was 39. Using the HER-signal-intensity–concentration curve, it was very easy to determine the HER2 concentration in the unknown sample (0.5 pg HER2/nl, see Fig. 2B). Finally, the HER2 concentration was normalised to total protein. Total protein concentration was determined by Bradford analysis before arraying and was 2 mg/ml in the undiluted first spot. In our example we used the second dilution (1 mg/ml total protein) for HER2 quantification. Consequently, the normalised HER2 concentration in the sample was 0.5 pg/ng total protein. Hence, the RPPA technology offers an attractive method to measure protein expression more precisely than IHC. Therefore, RPPA is expected to bear advantages for patient and therapy selection. However, the correlation between protein abundance and histology is lost.

The RPPA technology offers unique advantages over enzyme-linked immunosorbent assays (ELISA), tissue arrays or antibody arrays: First, RPPA is much more sensitive than the ELISA with detection levels of attogram (1.0×10^{-18} g) amounts of a given protein and the ability to detect variances of less than 10%.[30,33] RPPA can also use denatured lysates so that antigen retrieval and antibody performance, commonly problematic with IHC and tissue arrays are reduced. Second, RPPA does not require direct labelling of patient proteins and utilisation of a two-site antibody sandwich, thus reducing experimental variability.[33,34]

The major limiting factor for the successful implementation of RPPA technology is the lack of availability of specific antibodies particularly for post-translational modified proteins.[30,35] For gene transcript profiling, probes with predictable affinity and specificity can be produced. In contrast, this is not possible for antibodies.[32,36]

Prior to use of an antibody in RPPA, its specificity must be confirmed by Western blot. Preferably the same materials as in the array are used. The optimal evidence for antibody specificity is a single band at the appropriate molecular weight. Phosphospecific antibodies should additionally show different signals between control and treated samples. In our group, we have validated 35 commercially available phospho-

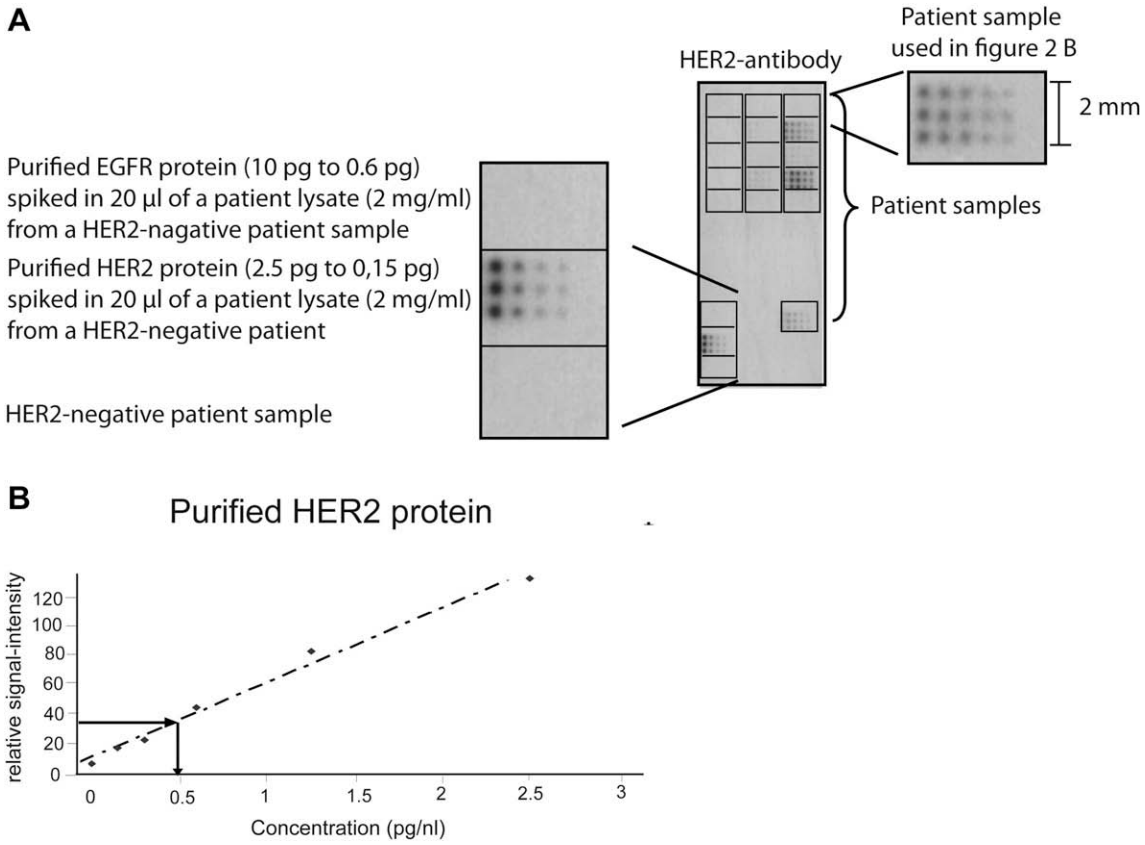


Fig. 2 – Quantification of protein expression by reverse phase protein array. (A) Purified recombinant human epidermal growth factor receptor 2 (HER2) is arrayed together with the patient samples in a dilution curve on nitrocellulose slides as protein reference. The HER2 protein (2.5 pg start concentration) was mixed with 20 µl of a HER2-negative patient sample (2 mg/ml) before spotting to eliminate potential influences of complex protein mixtures on signal-intensity compared to purified proteins. The slides were incubated with a HER2-specific antibody (DAKO; 1:1000 diluted). No HER2 signal was detected in the HER2-negative patient sample and in the patient sample mixed with purified EGFR. (B) The signal-intensity was plotted against the protein concentration generating a signal-intensity–concentration curve. Since the protein concentration of recombinant HER2 is known, the unknown HER2 concentration in a patient sample can be determined according to the HER2 standard curve. The protein concentration was normalised to total protein. In our example the signal-intensity of the patient sample is 39. Hence according to the HER2 standard curve the HER2 concentration in the patient sample is 0.5 pg/nl spot (a spot contains 1 nl protein lysate). Prior to spotting total protein concentration was determined by Bradford assay (2 mg/m in the undiluted first spot). For HER2 quantification we used the first dilution (1 mg/ml). After normalisation the HER2 concentration in the patient sample is 0.5 pg/ng total protein (own unpublished data).

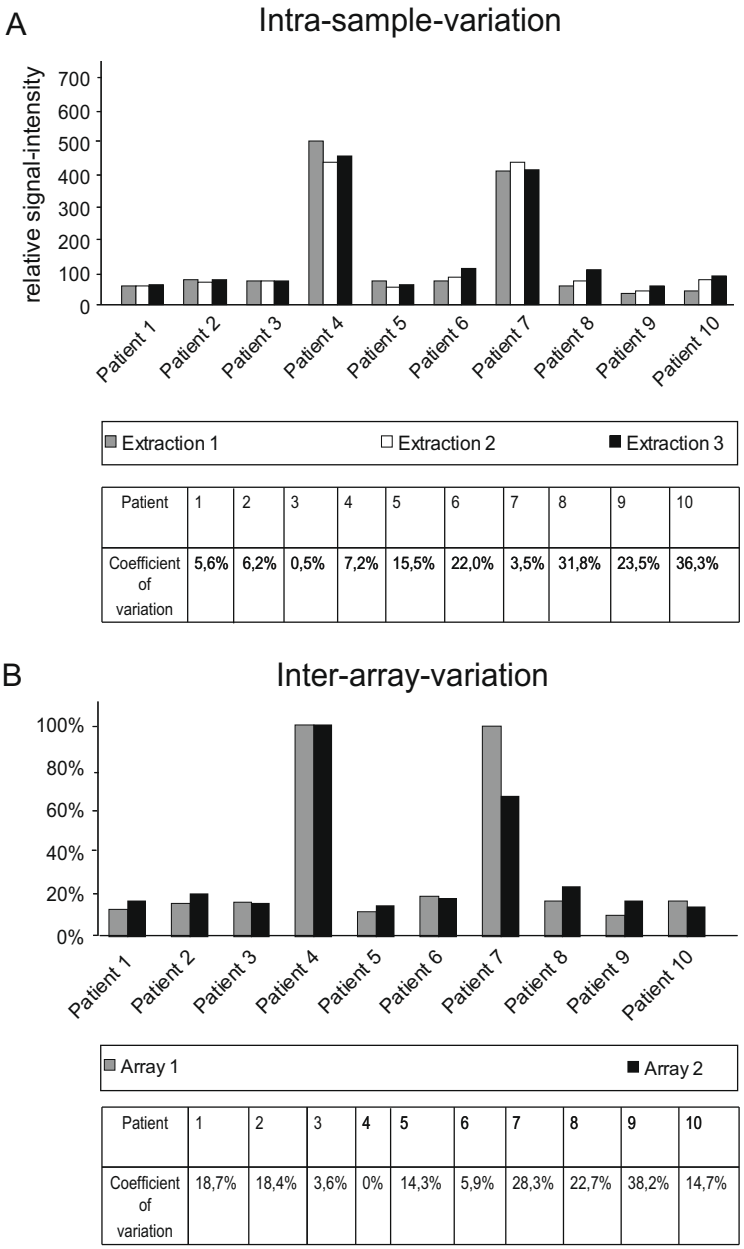
specific and non-phosphospecific antibodies detecting receptor tyrosine kinases and their downstream signalling pathway proteins (Berg et al., in preparation).

Probably the biggest challenge for the widespread use of the RPPA in clinical settings is the variability and comparability of staining between arrays, which often hamper productive data comparisons between different hospitals or experiments. This is a very relevant issue as multiple arrays may be required in clinical studies to consider all samples for a particular study. Ideally, a RPPA reference standard for the comparison of different slides detected with the same antibody should serve as a universal positive control for the staining process and antibody validation and it should also be incorporated into data analysis. Additionally, a satisfying RPPA quality reference should be renewable, reproducible in large scale, successful over a broad range of end points, stable over a long period of time, and, finally, as closely related to the test sample as possible.[33]

It was thought that human tissue extracts highly identical to the test samples were suitable candidates for reference standards. But these lysates are hardly renewable and not available in quantities large enough for large scale analysis. In contrast, extracts from various stimulated and non-stimulated cell lines can be produced in large quantities. Unfortunately, long term reproducibility and stability of cell lines is as difficult as that of tissue extracts.[33]

To analyse the reproducibility and variability of RPPA we evaluated the variation between lysate-preparation and the variation between plate and experiment setups and array runs (intra-sample as well as inter-array variations).

Therefore, we analysed HER2 abundances in 10 FFPE breast cancer tissues and normalised protein expression to total protein. Total protein was determined by Sypro Ruby Protein Blot stain. First, we tested the impact of sample preparation on RPPA variability. Therefore, all 10 patient samples have been extracted independently three times and printed onto the same slide (intra-sample variation). The intra-sample variation ranged from 0.5% to 36.3%. It was shown that the average % CV (coefficient of variation) of three independent protein extractions was about 15% (Fig. 3A; own unpublished data). Second, the samples were spotted onto different slides to determine the inter-array variation. The average % CV between two arrays was about 17% (Fig. 3B; own unpublished data). It is obvious that only samples with low-intensity (relative signal-intensity < 100) show%CV > 10%, except patient 7 who exhibited an apparent inter-array variation.



Patient	1	2	3	4	5	6	7	8	9	10
Coefficient of variation	5,6%	6,2%	0,5%	7,2%	15,5%	22,0%	3,5%	31,8%	23,5%	36,3%

Patient	1	2	3	4	5	6	7	8	9	10
Coefficient of variation	18,7%	18,4%	3,6%	0%	14,3%	5,9%	28,3%	22,7%	38,2%	14,7%

Fig. 3 – Reproducibility and variation of spotting and lysate preparation. Protein extracts from 10 breast cancer patients were prepared and assayed for human epidermal growth factor receptor 2 (HER2) expression using reverse phase protein array (RPPA) technology. Each slide was incubated with a HER2-specific antibody (DAKO; 1:1000 diluted) to determine the HER2 protein expression. Total protein was determined by Sypro Ruby Protein Blot stain. Subsequently protein expression was normalised to total protein. (A) For analysis of intra-sample variation proteins of 10 FFPE breast cancer patient samples were extracted three times independently and arrayed on the same slide (own unpublished data). (B) The inter-array variation is determined by spotting the samples on different slides (own unpublished data).

4. Signalling pathway profiling of human cancer tissues by RPPA

Recently, many studies revealed RPPA technology to be very promising for signalling pathway profiling of human tissues and cell lines, thus producing a valuable information for the development of new therapeutics or patient selection. Feinberg was the first to use a microspot technique to detect antigens in serum.[37] In 2001 RPPA was applied to show activation of pro-survival proteins and pathways during prostate cancer progression.[17] Moreover, it was possible to identify changes in expression or function of many kinases and phosphatases in other settings using RPPA. These are crucial events in carcinogenesis. For example, the phosphatidylinositol 3-kinase (PI3K) pathway is involved in growth and invasion and is activated in a significant number of ovarian and colon tumours.[38,39] Additionally, alterations in mitogenic signalling pathways like the mitogen-activated protein kinase pathway (MAPK) or the overexpression of HER2 are important factors involved in carcinogenesis.[6,31,38–42] Based on those phosphorylation-driven signalling networks it seems to be possible to subdivide breast cancers into several groups. These groups could be defined by EGFR-family signalling, proteinkinase B (Akt)/mammalian target of rapamycin (mTor) pathway activation, c-kit/ableson leukaemia oncogene cellular homologue (abl) growth factor signalling, and extracellular signal-regulated kinase (ERK) pathway activation[10] to name just some possible targets.

Another very promising field for the implementation of RPPA is the comparative analysis of signalling pathways in primary cancer and metastatic lesions. Metastases are the major cause of death in cancer patients. In breast cancer, for instance, most metastases occur in distant organs like lung, bone, brain and liver. Metastasis is a complex multistep process including detachment from the primary tumour, invasion into surrounding connective tissue, intravasation, survival in lymph and blood vessels, extravasation and settlement at target organs.[43,44] This process requires the survival of clones being able to obtain genetic or epigenetic changes necessary for metastasis. It is known that most cancers, due to their genetic instability, produce heterogenous clones while they grow at primary sites. Thus, metastasis competent clones are eventually produced exhibiting growth advantages and therefore may become the dominant clones in primary tumours later. Because metastases are derived from a primary tumour, their molecular profiles were expected to be similar to those of primary cancer. However, this expectation turned out incorrect in several human cancers such as colorectal cancer, gastric cancer and ovarian cancer.[33,45,46] Thus, for example, in metastatic gastric cancer tumour-associated proteins and their prognostic significance differ from those in primary tumours. As tissue environment of metastasis is different from that of the primary tumour, phosphorylation events in metastatic cells can differ significantly from those of primary tumour cells.[30]

Unfortunately, cancer is often diagnosed when metastasis has already occurred. Therefore, it is essential to additionally compare signalling pathway activity between primary tumours and metastases to understand signalling pathways of the tumour within its metastatic microenvironment. Thus, for targeted therapy decisions and optimal therapy selections, it is crucial to include patients' metastatic lesions also.

5. Combining protein and RNA profiles from the same FFPE tissue sample

In the last decade gene expression signatures (e.g. genes regulating cell cycle, invasion or metastasis) have been described exhibiting potential clinical value for prognosis, for predicting response to treatment or for identifying therapeutic targets for drug development.[47] The quantitative protein approach described here allows a combination of clinical sample profiling on the protein and RNA level using the same paraffin blocks. In addition, gene mutations in critical genes can be determined at the DNA level and related to expression profiles. Gene mutations not always result in reduced expression of the encoded proteins. Some genes may be mutated in cancer cells but their encoded proteins may still be expressed at the RNA and protein levels although the proteins are not functionally active. One example is the cell adhesion molecule E-cadherin that is often mutated in diffuse-type gastric cancer although the protein is strongly expressed.[48] Thus, our approach now allows the integration of the entire spectrum of biomarkers.

6. Promises of proteomic profiling of FFPE tissues for diagnosis, therapy selection and response prediction

As many cancers exhibit substantial differences in cell signalling, tissue behaviour and susceptibility to chemotherapeutic agents, proteomic profiling is very promising for efficient patient and therapy selection. Thereby, phosphoprotein driven cellular signalling events represent most of the new molecular targets for cancer treatment. RPPA is suitable to analyse multiple signalling pathways simultaneously and to characterise interconnecting protein pathways as well as different phosphorylation levels in tissues. Additionally, this technology may allow identification of healthy patients being at high risk to develop cancer.[30] For example, the signalling pathway portrait of patients who will develop an aggressive tumour is expected to be different from that of patients with benign tumours. Thus, RPPA is a promising tool for the analysis of cell and tissue physiology. Formerly, the limitation of RPPA technology was its incompatibility with routinely processed FFPE tissues based on the assumption that protein extraction from FFPE tissues is not possible. Yet, many FFPE-protein extraction protocols have been published[18–25] recently which might facilitate the implementation of RPPA in retro- and prospective clinical trials and routine settings.

Conflict of interest statement

K.F.B. is a named inventor of a patent related to protein extraction from FFPE tissues.

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