Evaluation of Protein Profiles From Treated Xenograft Tumor Models Identifies an Antibody Panel for Formalin-fixed and Paraffin-embedded (FFPE) Tissue Analysis by Reverse Phase Protein Arrays (RPPA)*

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Reverse phase protein arrays (RPPA) are an established tool for measuring the expression and activation status of multiple proteins in parallel using only very small amounts of tissue. Several studies have demonstrated the value of this technique for signaling pathway analysis using proteins extracted from fresh frozen (FF) tissue in line with validated antibodies for this tissue type; however, formalin fixation and paraffin embedding (FFPE) is the standard method for tissue preservation in the clinical setting. Hence, we performed RPPA to measure profiles for a set of 300 protein markers using matched FF and FFPE tissue specimens to identify which markers performed similarly using the RPPA technique in fixed and unfixed tissues. Protein lysates were prepared from matched FF and FFPE tissue specimens of individual tumors taken from three different xenograft models of human cancer. Materials from both untreated mice and mice treated with either anti-HER3 or bispecific anti-IGF-1R/EGFR monoclonal antibodies were analyzed. Correlations between signals from FF and FFPE tissue samples were investigated. Overall, 60 markers were identified that produced comparable profiles between FF and FFPE tissues, demonstrating significant correlation between the two sample types. The top 25 markers also showed significance after correction for multiple testing. The panel of markers covered several clinically relevant tumor signaling pathways and both phosphorylated and nonphosphorylated proteins were represented. Biologically relevant changes in marker expression were noted when RPPA profiles from treated and untreated xenografts were compared. These data demonstrate that, using appropriately selected antibodies, RPPA analysis from FFPE tissue is well feasible and generates biologically meaningful information. The identified panel of markers that generate similar profiles in matched fixed and unfixed tissue samples may be clinically useful for pharmacodynamic studies of drug effect using FFPE tissues. Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.045542, 2775–2785, 2015.

Many human diseases are characterized by abnormalities in complex signaling pathways (1). The expression and activation status of proteins from these deregulated pathways has traditionally been analyzed using single marker techniques such as immunohistochemistry and Western blotting. Although these techniques have provided valuable information on the molecular abnormalities underlying human disease, they are labor intensive, have a low throughput, and often require high sample volume. Furthermore, techniques such as Western blotting are not applicable in the routine clinical setting. Miniaturized parallel immunoassay techniques have been developed in recent years and have played a pivotal role in biomarker discovery (2). Antibody arrays enable multiple potential disease markers to be investigated in a single sample in parallel (3). Beyond this,
Reverse Phase Protein Arrays (RPPA) are sensitive high throughput tools that can quantify protein expression levels and activation status (posttranslational modifications such as phosphorylation) in multiple experimental samples simultaneously. The technique requires only minute amounts of samples, printed as lysate arrays onto slides, and hundreds of markers of interest can be investigated, array by array, in a miniaturized dot blot manner. Numerous reports have demonstrated that RPPA can be applied to various sources of cells and tissues to analyze protein profiles, signaling pathway networks, and for the identification of biomarkers (4–13). A recently published workshop report reviews the full potential and advances of RPPA for use in clinical, translational, and basic research (11).

In oncology, the parallel profiling of multiple protein markers is particularly desirable to study tumor initiation and progression, to classify tumor disease states on the molecular level, and to discover and monitor biomarkers that can predict therapeutic response or tumor recurrence (14–16). The study of signaling response and analysis of pharmacodynamic (PD) markers upon treatment using in vitro and in vivo test systems (e.g. cell line or patient derived xenograft tumor models) is an established component of preclinical and early clinical drug development. These techniques can provide evidence of target pathway modulation for new therapeutic candidate compounds and provide valuable information on the drug mode of action (17), especially in the translational phase. Multiplex analyses of PD biomarkers by RPPA have been performed in vitro using cancer cell lines (18, 19) as well as in patient-derived tumor tissue and blood samples (20, 21) to assess response to treatment and target inhibition. A combination of RPPA signaling pathway mapping and functional PET imaging has recently been successfully evaluated in xenograft models as an early response PD marker for anticancer drug efficacy (13).

Translating miniaturized multiple protein analysis platforms such as RPPA - from preclinical to clinical applicability is highly desirable; however, issues such as the limited amount of available clinical samples and tumor heterogeneity must first be addressed. Furthermore, most studies of RPPA in tumor tissue to date have been conducted using proteins extracted from fresh-frozen (FF) tissue specimens, whereas, formalin fixation and paraffin embedding (FFPE) is the standard method for tissue preservation used in clinical pathology laboratories. FFPE yields excellent tissue architecture for histological assessment and enables analysis of individual proteins in situ by techniques such as immunohistochemistry. However, formalin leads to extensive protein–protein and protein–nucleic acid cross-linking (22), which can hamper protein extraction and reduce both the overall yield of extracted protein and the profile of proteins detectable by proteomic techniques (23, 24). Furthermore, formalin-induced cross-linking induces conformational changes in protein structure that can alter the immunoreactivity of some proteins in situ by hiding or altering peptide epitopes (25, 26). Such artifacts are absent from snap-frozen tissue; therefore, protein profiles obtained from FF tissue are likely to reflect the in vivo biology of the tumor more closely. However, FF tumor tissue is not widely available because it is costly to collect and maintain in the clinical setting. FFPE tissue samples are routinely archived by nearly every hospital and offer a unique opportunity to study thousands of samples retrospectively with extensive clinical records and follow-up information.

Several groups have now established protocols for retrieving cross-linked proteins from fixed tissues (27–33). These methods are mainly based on the use of concentrated ionic detergents and high temperature protocols closely related to the antigen retrieval methods developed for immunohistochemistry. These studies show that obtaining nondegraded, full-length proteins from FFPE tissues for multiplex analyses is feasible (27–33). More recently, protein extraction techniques optimized for fixed samples have been used to successfully conduct RPPA using FFPE tissue biopsies from different cancer types (34–40). Guo et al. systematically investigated several protein extraction methods and demonstrated that RPPA of FFPE materials is feasible, reproducible and can generate biologically relevant protein profiles (41). Other studies have confirmed the validity of this approach and shown that data generated from RPPA analyses of FFPE tissue demonstrate good concordance with traditional immunohistochemistry markers such as HER2 protein in breast cancer (34, 40). However, to date, analyses have been performed only for a limited set of protein markers.

To evaluate whether analysis of a broader panel of protein markers is feasible and generates meaningful data from FFPE tumor tissue sections, we conducted RPPA on matched samples of FF and FFPE tissues using a set of 300 markers, the largest panel reported to date. Our aim was to identify markers that performed similarly when comparing the protein profiles measured in protein extracts from matched FF and FFPE tissue, using RPPA assays established for use in frozen tissues. Correlating selected markers and assays in such a way should qualify RPPA for further use with FFPE tissues of clinical relevance, e.g. in PD marker studies. In this paper, we have specifically focused on the technical issues relevant for using the RPPA platform in a clinical setting, and did not address the biology of the test systems used in detail. However, the models used have been pre-characterized to identify

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1 The abbreviations used are: RPPA, reverse phase protein arrays; CLB1, RPPA cell lysis buffer; CSBL1, RPPA spotting buffer for lysates; CV, coefficient of variation; EGFR, epidermal growth factor receptor; EXB, protein extraction buffer for FFPE tissue; FF, fresh-frozen; FFPE, formalin-fixed and paraffin-embedded; HER, human epidermal growth factor receptor; IGFR-1R, insulin-like growth factor 1 receptor; IHC, immunohistochemistry; MFI, mean fluorescence intensity; NFI, normalized fluorescence intensity; PD, pharmacodynamic; SCID, severe combined immunodeficient; TCR, treatment to control ratio.
key signaling parameters in context of targeted drug treatment (42). We conducted a systematic comparison of RPPA protein profiles in matched FF and FFPE tumor tissues resected from three different xenograft models of human cancer, each treated with targeted therapeutic antibodies that have previously been shown to achieve tumor growth inhibition. Furthermore, we investigated the effect of targeted drug treatment on protein expression and activation status, and the concordance of matched FF and FFPE tissue RPPA profiles. Finally, with one of the applied tumor models, we compared a set of protein profiles measured with two different multiple assay platforms - the RPPA and the Luminex Bio-Plex system, and discuss their relevance with respect to the analysis of FFPE tissue.

EXPERIMENTAL PROCEDURES

Xenograft Models and Tissue Samples—The human cell lines PK-45P (pancreatic, ductal carcinoma), FaDu (pharyngeal epithelial squamous cell carcinoma), and BxPC-3 (pancreatic epithelial adenocarcinoma) were obtained from the American Type Culture Collection and routinely cultured using methods described previously (43). The treatment and sampling schedule of each xenograft model is depicted in supplemental Table S1.

The preparation and treatment of the FaDu and BxPC-3 xenograft models have been described in detail previously (43). Briefly, 5–10 × 10^6 cells were injected subcutaneously into female severe combined immunodeficient (SCID)-beige mice. On day 21 (FaDu-bearing mice) or 56 (BxPC-3-bearing mice) after inoculation, mice (n = 3 per treatment group) were randomized to receive intravenous (iv) injections of 10 mg/kg of a therapeutic antibody against human epidermal growth factor receptor 3 (HER3; RG7116, Roche) or vehicle control. Treatment group) were randomized to receive intravenous (iv) injection of 100, 56, or 56 ((FaDu)-bearing mice) after inoculation, mice (n = 3 per treatment group) were randomized to receive intravenous (iv) injections of 10 mg/kg of a therapeutic antibody against human epidermal growth factor receptor 3 (HER3; RG7116, Roche) or vehicle control. Treatment was performed according to the methods described by Assadi et al. using the Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany) (34). Briefly, sections were dewaxed in xylene, rehydrated in a series of 100%, 96 and 70% (v/v) ethanol and pelleted by centrifugation. Pellets were resuspended in 100 μl of EXB extraction buffer (Qiagen), incubated on ice for 5 min, heat-dried to 100 °C for 2 h, and then incubated at 80 °C for 2 h with agitation. Samples were then centrifuged to remove cell debris and the protein-containing tissue lysates were stored at −80 °C.

Array Printing—Printing of the RPPA arrays, experimental conditions for immunoassays and methodology for data analysis were performed as described previously (34). The protein content of the tissue lysates was first determined using a Coomassie (Bradford) protein assay (FF samples; Thermo Fisher Scientific, Waltham, MA) or a bichinonic acid (BCA) protein assay (FFPE samples; Thermo Fisher Scientific, Waltham, MA) and samples were adjusted to uniform concentration using the respective lysate buffers. Subsequently, all samples were diluted 10-fold in CSBL1 Reverse Array Spotting Buffer (Bayer Technology Services GmbH, Leverkusen, Germany) to standardize protein content to a final print concentration of 0.3 mg/ml for FF samples and 0.2 mg/ml for FFPE samples. The lysates (matched 20 FF and 20 FFPE tissue samples) were then printed onto hydrophobic protein microarray chips (Bayer Technology Services GmbH, Leverkusen, Germany) using a piezo-electric noncontact printer (NanoPlotter 2, GeSiM, Grosserkmannsdorf Germany). More than 400 replicate arrays were produced, with each array comprising the complete set of all 40 samples, each printed at four serial dilutions (1.6-fold), each in duplicate (0.4 nL per spot, eight spots per sample). Fluorescence-labeled reference material (albumin) was coprinted with the experimental samples to allow for correction of array illumination. Once printed, arrays were blocked with 3% w/v albumin, washed in distilled water, dried, and stored at 4 °C in the dark until use.

Protein Array Immunoassays—Protein levels were measured using a direct two-step sequential immunoassay using a sensitive and quantitative fluorescence read-out. A single array was probed for FF and FFPE tissue samples and cell lines, and selected for specificity and band position at the correct molecular weight. The antibodies had been verified extensively in other studies in FF material prior to implementing in this study. For each marker assay, primary antibody at respective dilution was incubated in Zeptosens assay buffer overnight (15 h) at room temperature. Arrays were washed once in assay buffer and incubated for 45 min with Alexa647-labeled anti-species antibody (Invitrogen, Paisley, UK). Arrays were then washed as before and imaged using a ZeptoREADER instrument (Bayer Technology Services GmbH, Leverkusen, Germany) using the red laser.
channel. Typically, six fluorescence images were recorded for each array at exposure times of between 0.5 and 16 s. Negative control assays incubated in the absence of primary antibody (blank assays) were also performed to measure the nonspecific signal contributions of the secondary antibody. In addition, one chip out of the print series was stained to measure the relative amount of immobilized protein per spot (protein stain assay).

Image Capture and Analysis—For each array/antibody, the image taken at the longest exposure time without showing any saturation effect was analyzed using the ZeptoVIEW™ Pro 3.1 array analysis software (Bayer Technology Services GmbH, Leverkusen, Germany) with the spot diameters set to 160 μm. Mean fluorescence signal intensity (MFI) of each sample was calculated from referenced, back-ground-corrected mean intensities of the single spots (eight spots per sample) applying a linear fit and interpolating to the mean of the four printed protein concentrations. Blank-corrected MFI signals of the samples were normalized for the relative protein concentration printed on the chip to obtain normalized fluorescence intensity signals (NFI). Log2-transformed NFI signals were used for all subsequent statistical analysis.

Luminex Assay—For the analysis of downstream signaling kinases, 10 µg of protein from each sample (FF tissue only; five replicates per group) was analyzed using Bio-Plex® antibody-coated capture beads (BioRad, Germany) specific for total AKT, total ERK1/2, phosphorylated AKT (Ser473) and phosphorylated ERK1/2 (Thr202/Tyr204; Thr185/Tyr186). Samples were analyzed using the Luminex® xMAP® System according to manufacturers’ protocol and results expressed as MFI and standard deviation.

Statistical Analysis—A hierarchical clustering using Ward’s method was performed to gain first insights into the data structure. Mean comparisons of the matched samples of FF and FFPE tumor tissue were performed using paired t-tests to assess absolute differences between the two sample types. For correlation analysis, Pearson’s correlation coefficient was used to assess relative agreement between FF and FFPE samples. For both correlation and paired t test p values, a Bonferroni correction was applied to deal with the multiple testing problem occurring because of the number of markers (n = 300). Treatment-to-control ratios were calculated as $T_{CR} = \frac{V_{out}}{V_{in}}$.

Chi square tests were performed to determine whether the distribution of a factor is the same across the levels of a second factor (e.g. for effect direction across phospho/total proteins). For assessment of the rating agreement of the samples from FF versus FFPE considering the TCR magnitude, the agreement statistic Kappa was calculated for binned categories of the effect size (an absolute log2 TCR ≥ 1 was defined as “high,” ≤0.585 to <1 as “moderate,” ≥0.263 to <0.585 as “low,” and <0.263 as “no effect”).

RESULTS

In total, protein extracts from matched FF and FFPE tissues derived from 20 xenograft individual tumors (40 samples) were evaluated by RPPA using a panel of 300 markers (see supplemental Table S2 for more information). The panel included antibodies against membrane, nuclear, and cytoplasmic proteins and proteins covering a wide range of biochemical pathways including various signaling pathways, apoptosis markers, receptors, extracellular matrix proteins, tumor markers, and histones. In addition, antibodies against both total protein and specifically phosphorylated protein were included. For the BxPC-3 and FaDu-bearing xenograft tumors, protein extracts were prepared from untreated mice (n = 3 biological replicates each) and from mice treated with the anti-HER3 antibody (n = 3 each). For the PK-45P-bearing mice, extracts were prepared from untreated mice (n = 3) and from tissue obtained 30 min (n = 2) and 4 h (n = 3) after treatment with an antibody bispecific for IGF-1R and EGFR.

Overview of RPPA Data in FF and FFPE Tissues—First of all, the measured signals for all individual tumor samples and markers were visualized in a heat map format to better identify similarities or differences of signals with regard to the two different sample subsets (FF and FFPE). Hierarchical clustering of all analyzed individual specimens is shown in Fig. 1. The FF and FFPE samples were clearly separated into two distinct clusters, with a considerable fraction of protein markers detected at higher signal levels in the FF tissue compared with the FFPE and vice versa. Overall, the clustering of the individual biological replicate tumors in the FF tissue set was more pronounced than for the FFPE tissue set. Data from each individual xenograft model clustered for the FF samples, with biological replicate tumor data from different treatment conditions in the FaDu and PK-45P xenograft models forming distinct sub-clusters. Clustering of biological replicate tumors in the FFPE tissue set was less distinct, indicating better reproducibility of biological replicate samples using FF material. In order to illustrate the reproducibility of biological replicates in the matched FF and FFPE sample sets more quantitatively, the coefficient of variation (CV) was calculated for all analytes (n = 300) and within each biological sample group (n = 14 groups including 7 conditions of different xenograft models, treatments and time points × 2 subsets of FF and FFPE). CV values were summarized to a median CV of 11% for FF samples and a median CV of 18% for FFPE samples (calculations performed with NFI values before log transform). To provide a more comprehensive picture of the reproducibility of biological replicates, additional statistical analysis was performed, calculating the following quantities of the CV values: 25% quantile = 6% and 75% quantile = 20% for the FF subset; 25% quantile = 9% and 75% quantile = 33% for the FFPE subset. This performance includes several factors (sample preparation, printing, antibody quality, assay performance) and represents a good reproducibility for FF samples and somewhat lower reproducibility for FFPE samples (as expected because these include a larger number of sample preparation steps). Still, median CVs for both subsets were clearly well below an acceptable limit of 20%.

Comparison of Marker Profiles in FF and FFPE Tissues—Next, we had a closer look at the comparison of signal intensities of the marker profiles measured in the two FF and FFPE sample subsets. First, we investigated the relationship between absolute levels of marker signals in FF and FFPE tissues. Across all tested specimens, a total of 179 (60%) markers exhibited significantly different distribution between log2-transformed NFI values of FF and FFPE tissues after Bonferroni correction for multiple testing (p < 0.00017) in a
paired t-test (Table I). Altogether, 109 markers showed significantly higher signal values in FF than FFPE (145 were significant without Bonferroni correction) and 70 markers showed significantly higher signal values in FFPE than FF (96 significant without Bonferroni correction). 121 (40%) markers showed similar signal levels in FFPE and FF (59 showed no significant difference without Bonferroni correction).

No significant difference of the distribution of phosphoproteins was observed considering effect direction between FF and FFPE samples (Chi-square test $p$ value = 0.1642) or the number of significant markers (Chi-square test $p$ value = 0.2774).

Pearson’s correlation coefficients were then calculated to identify those markers that exhibited agreement in relative signal profiles between matched FF and FFPE tissues. Considering all 20 tumor samples of the different biological treatment conditions together, 25 markers demonstrated a significant correlation of RPPA signal profiles between the FF and FFPE tissues after correcting for multiple testing ($p < 0.002$) (Table II). A further 74 markers demonstrated significant correlation at the uncorrected level; however, some of these correlations were negative, which was assumed to indicate a chance occurrence. Overall, a list of top 60 markers was identified that significantly correlated ($p < 0.01$) between FF and FFPE tissues (the top 25 showing significance at the corrected level and the following first 35 showing significance at the uncorrected level, prior to the first observed negative correlation). This list was used for further analysis. Importantly, no relationship between the cellular location (membrane, cytoplasm or nucleus) of the top 60 correlating markers was apparent, and again both phosphorylated and nonphosphorylated proteins were represented in this list. Seventeen of the 60 top correlated markers identified by Pearson’s correlation coefficient were also in the panel of 59 markers that...
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List of markers demonstrating good correlation between FF and FFPE tumor tissues. Significant correlations between RPPA signals from FF and FFPE tumor tissue samples were seen for 25 markers after multiple testing correction (Bonferroni corrected alpha = 0.00017) and for an additional 35 markers at the uncorrected level.

| Analyte Correlation | p value |
|---------------------|---------|
| Collagen Iα2        | 0.953   | <0.0001 |
| p70S6 kinase-P-Thr389| 0.936   | <0.0001 |
| c-Jun-P-Ser63       | 0.925   | <0.0001 |
| Cyp2e1              | 0.925   | <0.0001 |
| CEA                 | 0.888   | <0.0001 |
| ikappaB alpha       | 0.888   | <0.0001 |
| HSF1-P-Ser326       | 0.873   | <0.0001 |
| Vimentin            | 0.871   | <0.0001 |
| BCRP (ABCG2)        | 0.864   | <0.0001 |
| Bim                 | 0.862   | <0.0001 |
| DUSP4               | 0.858   | <0.0001 |
| p53                 | 0.853   | <0.0001 |
| Survivin            | 0.838   | <0.0001 |
| c-Met               | 0.838   | <0.0001 |
| Connexin43          | 0.837   | <0.0001 |
| Bad-P-Ser112        | 0.836   | <0.0001 |
| Cytokeratin5        | 0.835   | <0.0001 |
| Akt-P-Ser473        | 0.833   | <0.0001 |
| beta-Actin          | 0.831   | <0.0001 |
| Bcl-xl              | 0.808   | <0.0001 |
| Rb                  | 0.803   | <0.0001 |
| HSP27               | 0.796   | <0.0001 |
| CD44variant (Exon v6)| 0.778  | <0.0001 |
| STAT1               | 0.777   | <0.0001 |
| Caseinkinase1 delta | 0.748   | <0.0002 |

B. 35 additional markers showing significance at the uncorrected level

| Analyte Correlation | p value |
|---------------------|---------|
| Caspase9            | 0.741   | 0.0002 |
| alpha1-Actin        | 0.738   | 0.0002 |
| Aurora A            | 0.732   | 0.0002 |
| Jak1                | 0.722   | 0.0003 |
| Dematin             | 0.718   | 0.0004 |
| Aib1A               | 0.716   | 0.0004 |
| alpha-Tubulin       | 0.711   | 0.0004 |
| Integrin alpha5     | 0.706   | 0.0005 |
| MKK4                | 0.702   | 0.0006 |
| Connexin26          | 0.696   | 0.0006 |
| S6 Ribosomal Protein-P-Ser240/244| 0.687 | 0.0008 |
| Okt-4               | 0.687   | 0.0008 |
| Cytokeratin16       | 0.681   | 0.0010 |
| elf2alpha           | 0.675   | 0.0011 |
| CyclinD1            | 0.670   | 0.0012 |
| LAMPI               | 0.665   | 0.0014 |
| HDAC6               | 0.652   | 0.0019 |
| 4E-BP1              | 0.643   | 0.0022 |
| Erk1/2-P-Thr202/Tyr204| 0.641  | 0.0023 |
| Her2-P-Tyr1248      | 0.639   | 0.0024 |
| Cyp3a4, 3a1, 3a11   | 0.637   | 0.0025 |
| MAP2-B-250          | 0.623   | 0.0034 |
| PAK2                | 0.617   | 0.0037 |
| P-Glycoprotein (MDR1, ABCB1) | 0.606 | 0.0046 |
| IGF1 Receptor       | 0.599   | 0.0052 |
| RSK1/2/3            | 0.598   | 0.0053 |
| Tubulin-ac          | 0.596   | 0.0055 |
| MKK6                | 0.591   | 0.0060 |
| DUSP16              | 0.584   | 0.0069 |
| MDM2                | 0.583   | 0.0070 |
| p27                 | 0.582   | 0.0070 |

showed no significant difference in absolute signal between FF and FFPE samples (i.e. these markers showed both absolute and relative agreement).

Influence of antibody dilution and clone on RPPA data—The influence of antibody dilution on the RPPA analysis was investigated for two markers: two antibodies (against integrin β1 and integrin β3) were tested at two different dilutions in two separate RPPA assays/arrays. Good correlation was observed for the signals obtained, with Pearson correlation coefficient values in FF and FFPE tissue, respectively, of 0.7580 and 0.7267 for integrin β1 and 0.6858 and 0.8088 for integrin β3, indicating that RPPA results were insensitive to dilution level.

One protein marker was analyzed twice using two different antibodies in two separate RPPA assays/arrays, allowing us to investigate the influence of antibody selection and quality on the reproducibility of RPPA data in FF and FFPE tissue (two anti-c-Met-P-Tyr1349 antibodies targeting the same epitope but purchased from two different suppliers). Interestingly, no significant correlation was observed between these two antibodies in either FF specimens (r = 0.37) or FFPE specimens (r = 0.40).

Effect of Treatment on the Markers Detected by RPPA in FF and FFPE Tissues—The availability of xenograft tumors from untreated mice and those treated with anti-HER3 or anti-IGF-1R/EGFR monoclonal antibodies allowed us to further investigate the effect of treatment on protein profiles. As monoclonal antibodies are specific to their target (unlike small-molecule tyrosine kinase inhibitors for example), the signaling changes observed are highly likely to be related to HER3 or IGF-1R/EGFR inhibition. The ratio of log2-transformed NFI values in treated animals versus untreated control animals (treatment-to-control ratio; TCR) was calculated for each marker in three tumor models (at different time points where available). Overall, there was limited correlation between TCRs calculated in FF and FFPE tissues when all 300 markers were analyzed together. When analyses were limited to the top 60 markers that demonstrated the strongest correlation between FF and FFPE tissues as measured with Pearson’s correlation coefficient, significant correlations were seen between TCRs measured in FF and FFPE tissue (Fig. 2) for the FaDu model (r = 0.5543; p < 0.001) and in the PK-45P xenografts obtained 4 h after treatment (r = 0.6175; p = 0.0006). These correlations further strengthened when only the top 25 markers were considered (FaDu: r = 0.6450; p = 0.0005 and PK-45P: r = 0.6354; p = 0.0006). However, the TCR effects showed only low agreement between FF and
FFPE samples when comparing the absolute effect levels (Kappa between 0.08 and 0.14, i.e. differences only slightly higher than to be expected by chance).

Biologically relevant changes of marker expression in the RPPA data were consistent with the expected mode of action of the treatments applied in the treated xenografts (47). For example, an antibody specific for AKT kinase phosphorylated at Serine 473 was among the list of top 25 best correlating markers. For this marker (pAKT (Ser473)), we observed the highest NFI signal in tumors from untreated PK-45P xenografts (Fig. 3). Upon treatment with anti-IGF-1R/EGFR a down-regulation in pAKT (Ser473) signal was apparent in FF tumors at 30 min post-treatment and drop was enhanced at 4 h post-treatment. This reduction was also observed with good correlation in FFPE tumor tissue at 4 h post-treatment. A down-regulation in pAKT (Ser473) signal was also observed in FaDu xenograft tumors treated with an anti-HER3 antibody, although this xenograft model expressed clearly lower basal levels of pAKT (Ser473) (42).

Comparison of RPPA Data With Luminex Data—In an independent study, downstream signaling kinases in the FF tissue lysates from untreated PK-45P xenografts (Fig. 3) were analyzed using Luminex assays. This allowed us to compare the RPPA data with that of a different methodology for measuring protein levels. Lysates for the Luminex and RPPA studies were prepared separately and in different buffers from xenografts obtained from identically generated animal models. Luminex data were available for four protein markers represented in the RPPA analysis: total AKT, total ERK1/2, pAKT (Ser473), and pERK1/2 (Thr202/Tyr204; Thr185/Tyr187). On-treatment changes in these phosphorylated versions of these proteins were expected based on the mode of action of this therapeutic antibody (47).

A good correlation was observed between the TCRs calculated from the Luminex and RPPA data both obtained in the FF specimens taken 30 min post-treatment (Fig. 4A; r = 0.86); however, this correlation was less strong for the respective RPPA data in the FFPE specimen (r = 0.71). A clear reduction in pERK (Thr202/Tyr204; Thr185/Tyr187) signal was visible in both data sets. At 4 h post-treatment (Fig. 4B), a very strong correlation was seen between the Luminex TCRs in FF tissue and the RPPA TCRs in both FF (r = 0.96) and FFPE tissue (r = 0.99). At this time point, the reduction in pERK (Thr202/Tyr204; Thr185/Tyr187) was more pronounced, and a clear reduction in pAKT (Ser473) was also apparent.

DISCUSSION

The application of proteomic technologies to the retro- and prospective analysis of pathology specimens has lagged be-
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hind that of genomic analyses because of problems in overcoming the effect of tissue fixation and subsequent recovery and access of proteins for their detection. Our study has demonstrated that RPPA is a valuable method for the multiplex immunoanalysis of large numbers of proteins in fixed tissue using only very small tissue samples, e.g., tissue sections. We identified a substantial number of protein analytes that gave comparable results regardless of starting material (FF or FFPE tissue), and we demonstrated biologically meaningful changes in comparisons of treated and untreated xenografts.

The heatmap analysis of our data set showed that protein profiles from FF and FFPE tissues formed distinct clusters (as opposed to matched samples clustering, regardless of tissue type), which is however unsurprising, as the matched tissue specimens were processed using different buffers and extraction methods. Within each tissue type, subclustering of xenograft model and treatment conditions was observed between replicate samples, particularly for FF specimens, because of differences in the absolute levels of analytes in the different biological subgroups. As FF and FFPE samples were obtained from adjacent halves of the tumor specimen, it is unlikely that tissue heterogeneity influenced our results.

A comparison of the RPPA signals in the two distinct sample sets (FF and FFPE) indicated a slight preference for higher levels in the FF sample set (109/300 [36%] markers significant after multiple testing correction), whereas 40% (121/300) of markers revealed comparable levels. This distribution did not change remarkably when phosphorylated markers were analyzed separately. Furthermore, no preference was observed for markers regarding their different origin and localization in the cell (membrane, cytosol, nucleus). In addition, marker signals obtained from biological triplicate tumor preparations (individual tumors from three different mice) demonstrated a good assay reproducibility (somewhat better for FF than for FFPE samples, CV less than 20% for both cases), which supports the notion that certain protein markers in FFPE tissue can be robustly extracted and reproducibly presented for subsequent immunodetection by RPPA. Differences of observed marker levels in FF and FFPE samples probably represent different presentation and recognition of marker epitopes by the applied immunoassay reagents. Selection of appropriate antibodies is therefore key to conducting a meaningful multi-marker analysis by RPPA in FFPE samples.

The study was set up to investigate 300 protein markers in matched FF and FFPE tumor samples under most comparable assay conditions. Selection of antibodies was driven by the availability of high quality antibodies. The used antibodies had been upfront characterized by Western blot for their specificity in FF tissues and cell lines. In the study, the chosen antibodies have been tested to identify those well-applicable for use with FFPE tissue, with FF tissue as a reference. From the starting set of 300 proteins we identified a total of 60 markers that showed comparable signal profiles with significant correlation (p < 0.01) in the matched FF and FFPE xenograft tumor tissues (not all of the 300 proteins did show noticeable changes upon treatment, which would be required to pick up correlations). We hypothesize that this panel of markers—although limited—will be valuable for conducting reliable PD analyses in drug development programs using...
fixed tissue specimens, as the data generated will be representative of unprocessed tissues. This panel covers markers from a diverse range of pathways representing relevant biological functions and mechanisms of human cancers, including ErbB2 and MAPK signaling (e.g., Her2-P-Tyr1248, Erk1/2-P-Thr202/Tyr204, RSK1/2/3, MKK4), PI3K/AKT/mTOR signaling (e.g., Akt-P-S473, 4E-BP1, p70S6K4, P-Thr202/Tyr204, RSK1/2/3, MKK4), PTEN signaling (IGF1R, MECR, Bcl-xL, Bad-P-S112, Bim, p53). Supplemental Table S3 lists the top ten pathological pathways that cover a very good representation with at least 10 out of the panel of 60 markers that were identified following analysis using the Ingenuity Canonical Pathways database (www.ingenuity.com). Detection of phosphoproteins in archival FFPE tissues by immunohistochemistry is notoriously challenging, mainly because of pre-analytical factors sometimes hard to control (48); however, detection of protein phosphorylation is vital for monitoring changes of activation of proteins and their potential inhibition on treatment in the different cellular signaling pathways. The panel of 60 markers identified included several antibodies specific for phosphorylated forms of target proteins, indicating that accurate assessment of protein activation status as well as expression level is possible in FFPE tissues for certain antibodies, given the tissue is processed appropriately.

The fact that only a limited fraction (60/300, 20%) of all measured marker signal profiles significantly correlated between fixed and unprocessed tumor tissue indicates that several assay factors must be aligned for a robust and meaningful assay performance. Sample preparation of FF and FFPE tissue used different lysis buffers and processes (heat treatment to extract protein from fixed tissue), which might result in a different antigen availability, epitope size, and presentation of marker epitopes to chosen assay reagents. Guo et al. reported that only a low fraction (23 out of 169) of markers gave comparable results in three different sample sets (cell lines, breast and renal cancer tissues) (41), concluding that different tumor type and nature of tissue may also have an impact on antibody selection to be used for FFPE T analysis.

The intention of this study was not to start with a wider set of overlapping antibodies per antigen and cross-compare their performance. The latter approach will be necessary to expand the set of antibodies for FFPE samples and to deliver reproducible and high quality results for further antigens of interest where no antibody had been identified so far. Nevertheless, one protein analyte on our arrays was represented by two antibodies from different suppliers targeting the same epitope. The data from this analyte indicate that RPPA results are critically antibody dependent. This also highlights the importance of understanding and validating antibody clones when comparing RPPA data from different groups. It remains to be determined whether technological differences between different RPPA platforms also affect the comparability of data obtained using different RPPA methods. Antibody dilution did not significantly affect results in our RPPA analysis.

A low comparability was observed between TCRs calculated from RPPA data from FF and FFPE samples when all 300 markers/antibodies were included in the analysis. This may in part be explained by the fact that not all markers are expected to change upon treatments, but also because only a subset of markers showed comparability on the individual samples level. When restricting the analysis to the selected 60 markers, correlations between analytes in FF and FFPE tissues were observed in two of the xenograft models. The identification of on-treatment changes in proteins consistent with the mode of action of the treatments investigated, such as pAKT, provides further evidence that RPPA analysis can generate biologically meaningful data from FFPE tissue samples. This down-regulation of pAKT seen in FaDu xenografts at 168 h after treatment with an anti-HER3 antibody—RG7116 at 10 mg/kg—is in line with recent data from Meneses-Lorente et al. (42).

RPPA data measured in FF tissue were confirmed using a different methodology for the multiplex analysis of proteins: The Luminex system, which is based on antibody-coated capture beads. Although data from only four markers common to both systems were available, the two technologies demonstrated a good correlation and biologically meaningful information consistent with the mode of action of the monoclonal antibody therapy investigated was seen. The Luminex technology has previously been applied to FFPE tissue to investigate small peptide hormones (49); however, to our knowledge, no experience or assays are available for the analysis of larger proteins with this sample type. Luminex technology is based around a sandwich-type immunoassay principle, necessitating whole proteins, or reproducibly available longer protein fragments, that present two epitopes at distinct sites. Application of the Lumex technique to FFPE tissue specimens would therefore require reproducible extraction of full length protein sequences; however, proteins extracted from fixed tissues are often fragmented (41). This may be particularly true for larger proteins (>100 kDa), such as growth factor receptors. Protein fragmentation is also likely dependent on both fixation and extraction conditions. RPPA can detect proteins even when fragmented and requires only the presence of a single intact epitope. Furthermore, RPPA antibodies recognize epitopes under denaturing conditions whereas Lumex assays recognize the native form, which is dependent on the three-dimensional structure of the protein. As demonstrated here and elsewhere (41), RPPA can readily detect partially degraded proteins with little variation compared with intact proteins. Consequently, this technology may allow multiplex analysis of larger antibody panels in fixed samples.
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tissues compared with sandwich immunoassay-based techniques. Developing individual assays for measuring PD biomarkers in clinical studies requires considerable effort; thus, RPPA appears to be an attractive option for conducting multiple analyses of biomarkers using such clinical specimens.

Tissue availability is often another limiting factor for techniques such as immunohistochemistry that analyze proteins individually. By using RPPA, we were able to analyze hundreds of proteins in tandem using a small amount of starting tissue, typical of the amount of material available during preclinical experiments and from clinical biopsies. Although this study wasn’t specifically designed to determine the smallest amount of starting material required for RPPA analysis, we were able to analyze all 300 markers using only 10–20 μg of protein extracted from a few micrograms of tissue (less than before turning to clinical materials. Studies using complementary techniques such as immunohistochemistry that analyze proteins in unprocessed tissues. Verification of the suitability of the identified markers for measuring drug effect in preclinical PD studies using complementary techniques such as immunohistochemistry (that can provide further information including tissue distribution of marker expression) is recommended before turning to clinical materials.

In conclusion, our data indicate that antibody selection is critical when conducting RPPA analyses on archival tissue samples. Using appropriate assay reagents, RPPA can provide biologically significant information for a large panel of proteins using minute amounts of FFPE tissues. We identified a panel of antibodies that result in comparable RPPA profiles in FFPE and FF tissues. The availability of such a validated panel is vital for conducting RPPA analyses on clinical materials, as these antibodies should provide data from fixed tissue specimens that closely reflect the clinical situation in unprocessed tissues. Verification of the suitability of the identified markers for measuring drug effect in preclinical PD studies using complementary techniques such as immunohistochemistry (that can provide further information including tissue distribution of marker expression) is recommended before turning to clinical materials.

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[S] This article contains supplemental Tables S1 to S3.
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