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PRECLINICAL STUDY

Clinical utility of reverse phase protein array for molecular classification of breast cancer

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Abstract Reverse Phase Protein Array (RPPA) represents a sensitive and high-throughput technique allowing simultaneous quantitation of protein expression levels in biological samples. This study aimed to confirm the ability of RPPA to classify archival formalin-fixed paraffin-embedded (FFPE) breast cancer tissues into molecular classes used in the Nottingham prognostic index plus (NPI+) determined by immunohistochemistry (IHC). Proteins were extracted from FFPE breast cancer tissues using three extraction protocols: the Q-proteome FFPE Tissue Kit (Qiagen, Hilden, Germany) and two in-house methods using Laemmli buffer with either incubation for 20 min or 2 h at 105 °C. Two preparation methods, full-face sections and macrodissection, were used to assess the yield and quality of protein extracts. Ten biomarkers used for the NPI+ (ER, PgR, HER2, Cytokeratins 5/6 and 7/8, EGFR, HER3, HER4, p53 and Mucin 1) were quantified using RPPA and compared to results determined by IHC. The Q-proteome FFPE Tissue Kit produced significantly higher protein concentration and signal intensities. The intra- and inter-array reproducibility assessment indicated that RPPA using FFPE lysates was a highly reproducible and robust technique. Expression of the biomarkers individually and in combination using RPPA was highly consistent with IHC results. Macrodissection of the invasive tumour component gave more reliable results with the majority of biomarkers determined by IHC, (80 % concordance) compared with full-face sections (60 % concordance). Our results provide evidence for the technical feasibility of RPPA for high-throughput protein expression profiling of FFPE breast cancer tissues. The sensitivity of the technique is related to the quality of extracted protein and purity of tumour tissue. RPPA could provide a quantitative technique alternative to IHC for the biomarkers used in the NPI+.

Keywords Reverse phase protein array · Formalin-fixed and paraffin-embedded · Protein extraction · Breast cancer · Nottingham prognostic index plus

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Introduction

The use of tissue fixatives followed by paraffin embedding of pathologic specimens represents a cost-effective approach for tissue architecture preservation; a fundamental prerequisite for accurate pathological diagnosis as well as detection/assay of molecular diagnostic, prognostic and predictive biomarkers. Formalin fixation and paraffin embedding of tissue specimen is the most commonly used preservation method in routine clinical pathology practice. In addition, formalin-fixed paraffin-embedded (FFPE) samples are collected and archived, along with patients'

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clinicopathological and outcome data, allowing potential access for further clinical and research purposes. FFPE samples are essential for scrutiny of molecular pathways, discovery and validation of novel prognostic/predictive biomarkers of cancer development and progression [1, 2].

Although very effective in architectural preservation, protein cross-linking may challenge full-length protein extraction from FFPE tissue samples. It has also been reported that FFPE tissue does not adequately preserve the activation states of signalling proteins [3]. The latter is attributed to the relatively slow process formalin fixation which leads to exposure of the living cells to stress and subsequent change of phosphorylation-dependent signalling networks. However, full-length, non-degraded immunoreactive proteins from unstained FFPE tissues have been successfully extracted [4–6].

Immunohistochemistry (IHC) has long been reliably used to assess the expression patterns and status of different prognostic and predictive biomarkers. However, it does not allow the quantification of the subtle changes in protein expression levels. In addition, IHC suffers from subjectivity and intra- and inter-laboratory variability [7, 8].

The Nottingham prognostic index plus (NPI+) is a novel prognostic test incorporating the measurement of biological and clinicopathological variables to stratify patients into distinct prognostic groups [9, 10]. The expression of a panel of 10 biomarkers relevant to breast cancer (BC) as assessed by IHC is used to identify seven core molecular classes of BC including luminal, basal and HER2 [9]. Incorporation of the clinicopathological variables in a second-stage analysis results in the identification of distinct prognostic groups within each molecular class [10].

Reverse phase protein array (RPPA) represents a high-throughput technique that allows simultaneous quantitation of protein expression levels in a large number of biological samples [11]. Minute amounts of proteins extracted from multiple biological samples are robotically spotted on a nitrocellulose-coated microarray slide which is incubated with a single specific primary antibody to detect expression of the target. Detection and quantification of antigen–antibody reaction is performed using either a primary or a secondary fluorescent-labelled antibody [12]. Over the past decade, RPPA has been successfully used for different applications including basic and preclinical research, biomarker discovery and clinical trials [13–17].

It was hypothesised that molecular features of BC determined by the high-throughput RPPA would enable the accurate quantification of protein levels in FFPE breast cancer tissue compared with IHC. Therefore, the aim of this study was to validate the use of RPPA in improving quantification of protein expression in FFPE breast cancer tissues and providing fast, reproducible and reliable molecular classification of BC using the biomarkers incorporated into the NPI+.

Materials and methods

Breast cancer samples

For this study, 25 cases of primary early invasive BC prospectively collected in 2012 from patients treated at Nottingham City Hospital were selected from the 100 well-characterised cases on the basis of the three main biological classes of BC (ER positive, ER negative and HER2 positive), with a pathological tumour size measuring at least 1 cm in diameter. Moreover, for selection of candidate tumour blocks, the invasive component had to constitute at least 50 % of the overall area of the tissue section. Table 1 displays the clinicopathological criteria of the 25 BC cases used in the current study. Samples were provided by the Nottingham Health Science Biobank and the study was approved by the North West 7 Research Ethics Committee—Great Manchester Central (Nottingham Research Biorepository (NRB)—REC Reference number 10/H1008/72).

Optimisation of the protein extraction method from formalin-fixed samples

Three protein extraction protocols were assessed in order to evaluate protein yield from FFPE breast cancer tissues including the Q-proteome FFPE Tissue Kit (Qiagen, Hilden, Germany) and two in-house methods using Laemmli buffer [3, 18]. FFPE tissue sections were cut at 10 µm in thickness, deparaffinised in xylene and dehydrated in a graded ethanol series (100, 96 and 70 %). Excess alcohol was removed by centrifugation at $16,000 \times g$ for two minutes before adding the extraction buffers. The Q-proteome FFPE Tissue Kit was used according to the manufacturer recommendations. Briefly, EXB extraction buffer was added to the tissue pellet and incubated for 5 min on ice. Following heating the tube at 100 °C for 20 min, the tube was subsequently incubated at 80 °C for 2 h with shaking at 750 rpm using a Thermomixer (Eppendorf, Hamburg, Germany). Finally, the tube was centrifuged at $1400 \times g$ for 15 min at 4 °C and the supernatant was collected. The Laemmli buffer method was performed as described [3] where samples were either incubated for 20 min or 2 h at 105 °C.

Validation of the protein extracts

Protein concentrations from the extracted lysates were quantified by Fast Green Stain as described previously [3, 19]. In brief, protein lysates were robotically spotted in triplicate onto nitrocellulose-coated glass slides (Grace Bio-labs, USA) with a microarraying robot (MicroGrid 610, Digilab, Marlborough, MA, USA). In addition, serial

Table 1 The Clinicopathological criteria of the invasive BC cases used in this study

Clinical parameters		Number of cases (%)
Tumour grade	2	5 (20)
	3	20 (80)
Tumour size	≤2 cm	6 (24)
	>2 cm	19 (76)
Axillary lymph node stage	1	16 (64)
	2	6 (24)
	3	3 (12)
Nottingham prognostic index (NPI)	GPG	1 (4)
	MPG	4 (16)
	PPG	20 (80)
NPI+ Cluster membership	Luminal	7 (29.1)
	Basal	14 (58.3)
	HER2+	3 (12.6)
	Unclassified	1

NPI Nottingham prognostic index, *GPG* good prognostic group, NPI < 3.4

MPG: NPI > 3.4–4.4. PPG: NPI > 4.4

two-fold dilutions of 1.0 mg/mL bovine serum albumin (Sigma-Aldrich, UK) were spotted onto the array as a total protein standard. Printed arrays were stained with Fast Green Stain (0.005 % FCF (Sigma-Aldrich, UK) in 30 % ethanol, 10 % acetic acid and 60 % water, V/V/V) for 45 min at room temperature on a rocking platform at 40 rpm. Destaining was performed two times 15 min each with 30 % ethanol, 10 % acetic acid and 60 % water. After washing, the slide was air dried and scanned at 700 nm using an Odyssey scanner (LI-COR, Lincoln, USA).

The integrity and immunoreactivity of the extracted proteins within lysates were evaluated by Western blotting. Lysates were separated on SDS-PAGE using the NuPAGE system (Invitrogen, Paisley, UK), and transferred to nitrocellulose membranes. Primary anti-β-actin antibody (Sigma-Aldrich, UK) was applied diluted 1:1000 in 5 % Bovine Serum Albumin (BSA) in Tris Buffer Saline (TBS) 0.05 % Tween-20, pH 7.5 and incubated overnight at 4 °C. Blots were incubated with swine anti-rabbit immunoglobulin-HRP conjugate (DAKO) diluted 1:2000 in blocking buffer for 1 h at room temperature. After washing, membranes were incubated with Enhanced Chemiluminescence Detection reagent (ECL; Amersham, UK), exposed to film (Kodak, Sigma-Aldrich, UK) and developed.

Macrodissection of invasive BC component

Macrodissection of FFPE tissue sections was performed for invasive tumour component enrichment using the 25 cases of BC. In each case, two serial sections were cut onto glass slides. One serial section 4 μm was stained for H&E and used as a guide for gross manual dissection of the invasive breast carcinoma component using disposable surgical blades in the unstained 10 μm section [20]. The Q-proteome FFPE Tissue Kit was used for protein extraction from the macrodissected tissue.

Reverse phase protein microarray

Protein lysates were robotically spotted in triplicate onto nitrocellulose-coated glass slides with a microarraying robot (MicroGrid 610, Digilab Marlborough, MA, USA). Blocking of slides was performed using Super G blocking buffer (Grace Bio-labs, USA) overnight. Next, slides were incubated with the primary antibodies used in the NPI+ (Table 2) overnight at 4 °C with agitation at 20 rpm. Validation of the specificity of the primary antibodies used was performed using strip Western blotting (Fig. 2) as previously described [21]. Only those antibodies with a single band at the expected molecular weight were included in this study. Detection of the reaction was performed with infrared secondary antibodies (680 CW anti-mouse or 800 CW anti-rabbit antibody) diluted 1:5000 in washing buffer for 30 min at room temperature and visualised using an Odyssey high-resolution scanner (LI-COR, Lincoln, USA) at 21 μm resolution using both channels 700 (red) and 800 (green). Axon Genepix Pro-6 Microarray Image Analysis software was used to determine the average fluorescence pixel intensities of all spots on each array. Protein signals were finally determined with background subtraction and normalisation to the internal housekeeping targets using RPPanalyzer, a module within the R statistical language on the CRAN (http://cran.r-project.org) [22].

Table 2 Antibodies used in IHC and in RPPA, their clones, dilutions, incubation times and antigen retrieval methods used for IHC staining

Antibody	Product code	Clone	RPPA dilution	IHC dilution	Incubation time (min)	Antigen retrieval method, buffer solution & duration
Oestrogen receptor	M3634	SP1	1:200	1:150	30	Microwave (MW), Citrate, pH6.0 for 20 min
CK 7/8	345779	CAM 5.2	1:50	1:125	30	MW, Citrate, pH6.0 for 20 min.
CK5/6	M7237	D5/16B4	1:400	1:750	60	MW, EDTA, pH8.0 for 20 min.
EGFR	Mouse	31G7	1:100	1:50	60	Proteinase K (8 min at 37 °C on cytomation hotplate)
HER 2	A0485	Rabbit polyclonal	1:50	1:1	30	NONE
HER 3	NCL-c-erbB-3	RTJ1	1:200	1:400	15	Hot water bath (95 °C), Leica ER1 buffer, for 25 min
HER 4	RB-9045-P	Rabbit polyclonal	1:50	1:30	30	MW, Citrate, pH6.0 for 20 min.
Mucin 1	NCL-MUC-1	MA695	1:50	1:50	30	MW, Citrate, pH6.0 for 20 min.
p53	NCL-p53-DO7	DO-7	1:25	1:30	30	MW, Citrate, pH6.0 for 20 min.
Progesterone receptor	M3439	PgR 637	1:200	1:100	30	MW, Citrate, pH6.0 for 20 min.

Reproducibility of protein extraction and RPPA

The reproducibility of RPPA using FFPE extracts was evaluated. For intra-slide validations, lysates from five FFPE BC tissues were extracted using the Q-proteome FFPE Tissue kit and printed onto 16-pad nitrocellulose slides in ten replicates.

In addition, the inter-slide reproducibility was assessed by printing the slides at two different days to produce identical RPPA slides required for the profiling of BC samples. To confirm the reproducibility of obtained results, representative slides of each run were incubated with primary antibodies which directed the housekeeping β-actin, and three makers of close relevance to breast cancer (ER, HER2 and HER4).

Immunohistochemistry

The NPI+ biomarker panel (Table 2) was assessed in full-face tissue sections by IHC (Fig. 3) as previously described using the Novocastra Novolink Polymer Detection System (Leica Microsystems, Newcastle, UK). For IHC scoring, the H-score method was used taking into account the staining intensity and the percentage, giving final values ranging between 0 and 300 as previously described [23].

Statistical analysis

Statistical analyses were performed using GraphPad prism version 6.5. Coefficient of variations between signal intensities of different lysates within the same slides or different slides (intra or inter-slides) was used to evaluate the reproducibility of RPPA. Statistical correlation between IHC and RPPA was tested using linear regression test. A

p value < 0.05 was considered significant. Unsupervised one-way hierarchical cluster analysis of the expression of the 10 biomarkers used in NPI+ using RPPA based on Pearson distance combined with complete linkage rule was performed using MeV (Multiple Experiment Viewer) version 4_8.

Results

Protein extraction

The protein yield from FFPE breast tissue using the three different protocols was evaluated in terms of quantity and quality. Protein concentrations showed that the protein yield extracted by the Q-proteome FFPE Tissue Kit was approximately double (2 mg/ml) compared to the other protocols (Fig. 1a). Western blot analysis for β-actin demonstrated preservation of protein extracted from the paraffin sections using any of the three protein extraction protocols where a single band at the correct molecular weight (42 kDa) was detected in all tested samples. However, the signal intensity of β-actin was higher for extracts utilising Q-proteome FFPE Tissue Kit protocol (Fig. 1b). RPPA showed that the level of β-actin was threefold higher in lysates extracted by Q-Proteome FFPE tissue kit compared with the other two protocols (Fig. 1c, d).

Reproducibility

Both intra-slide and inter-slide (Fig. 1e, f) variations of tested antibodies were verified to check the reproducibility of RPPA protein quantitation. The coefficient of variation
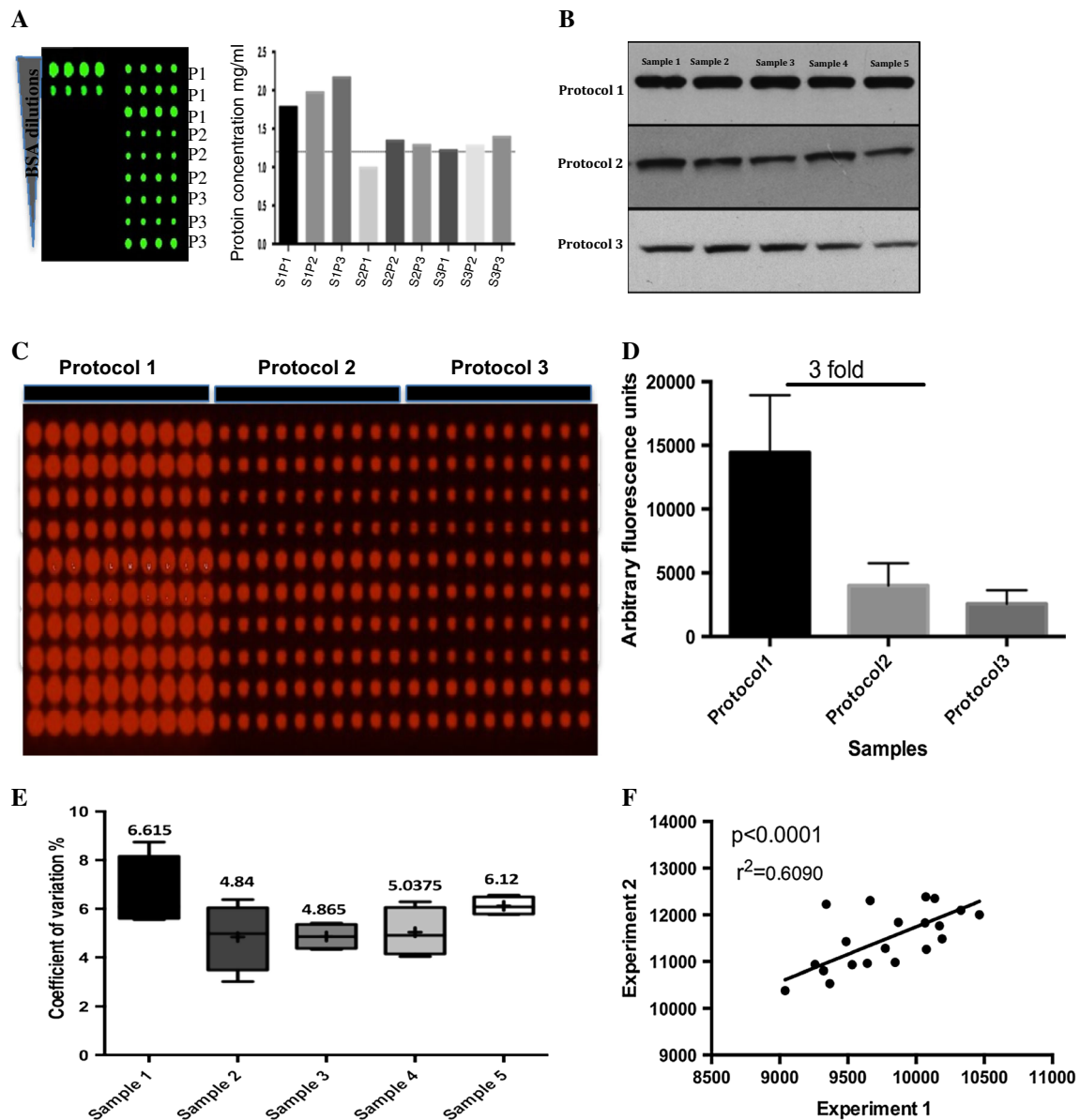
Fig. 1 Optimisation and reproducibility of protein extraction from FFPE samples. **a** and **b** Quantification of β-actin from FFPE lysates prepared by three different protocols Lysates from five FFPE samples were prepared by Q-proteome FFPE Tissue Kit (Protocol 1), Laemmli buffer (Protocol 2) and Laemmli buffer (Protocol 3) with an additional incubation period of 2 h at 105 °C. **a** Protein quantification using Fast Green Stain: Protein quantification was measured using Fast Green Stain. In brief, lysates from three protocols were spotted onto nitrocellulose slides in quadruplicates, and two fold of BSA standard was also spotted on the slide. The slide was stained with Fast Green Stain and the protein concentration was interpolated from the BSA standard curve. Protocol 1 gave the highest concentration of the protein (average 2 mg/ml) compared to the other protocols (average 1 mg/ml). **b** Western blotting image for β-actin detection in lysates prepared from the three protocols. A protein band at 42 kDa is seen in the lanes for all the lysates without any protein degeneration. **c** Representative microarray image of nitrocellulose slide showing higher signals with the Q-proteome kit. **d** Bar graphs show the quantitative representation of the signal intensities of β-actin. **e** Intra-slide reproducibility of RPPA: protein extracts from five FFPE breast cancer tissues (Sample 1 to sample 5) were prepared and were printed (replicate $n = 10$) on 16-pad slides. Slides were assayed for the expression of β-actin, ER, HER2 and HER4 using reverse phase protein microarray. The average of coefficient of variation (%) for all used markers is indicated for each sample as a box and whisker plot with median represented by a black line within the box representing the interquartile range, using Tukey's estimation for whisker length. The CV % for all the samples were less than 7 %, **f** Inter-slide reproducibility: protein extracts from five FFPE breast cancer tissues (Sample 1 to sample 5) were prepared and were printed twice onto 16-pad slide in two independent experiments. Slides were probed twice for β-actin, ER, HER2 and HER4 and the signals from the two different experiments were compared. High correlation between the signals from the two experiments (day 1 and day 2) is seen ($r^2 = 0.6090$)

(CV %) within the 10 intra-slide replicates was less than 7 % for all the analysed samples. A statistically significant positive correlation was also observed between signals of corresponding samples printed on the two different slides ($p < 0.05$, $r^2 = 0.61$).

Biomarker expression by IHC and their NPI+ biological class membership

BC cases were assessed using IHC for the expression of 10 biomarkers, and clustering analysis was carried out to molecularly classify BC into distinct classes as previously published [9, 10]. Regarding the NPI+ biological class membership of these cases, seven cases were luminal; fourteen cases were basal-like whilst the remaining three cases were HER2 positive.

NPI+ Biomarker expression by RPPA

RPPA-derived expression levels of the 10 biomarkers from each dissection method were estimated, and the signals of the IHC positive and negative groups were compared for the respective target proteins (Figs. 2, 3).

In macrodissected samples (Fig. 4a), RPPA-derived data significantly discriminated the IHC positive and negative groups of patients for the following dichotomised markers: ER, EGFR, HER2, HER4, Mucin 1, p53 and PgR ($p < 0.05$). Although there was a difference in CK5/6 expression levels between positive and negative groups, the difference was not statistically significant ($p = 0.06$).

Contrasting this, using protein extracted from full-face samples, the IHC positive and negative groups could be only discriminated using four markers: ER, PgR, HER2 and p53 (Fig. 4b).

In addition, the correlation between the RPPA signals intensities and the IHC scoring for the 25 tumour samples revealed that six out of ten biomarkers (ER, PgR, HER2, cytokeratin 7/8, CK5/6, EGFR, HER4 and Mucin 1) showed significant positive correlation between RPPA and IHC results, in both dissection methods (Table 3). Furthermore, Cytokeratin 5/6 and EGFR were significantly correlated to their respective IHC results only in the macrodissection approach. There was no correlation between RPPA quantification of the expression level for HER3 and P53 and their corresponding IHC scores using either macrodissected or full-face sections.

Although the number of the samples in this study is limited, a hierarchical cluster analysis of the NPI+ biomarkers was performed for RPPA data obtained from macrodissected BC tissue samples to molecularly classify BC into distinct classes. Interestingly, the 25 cases were clustered into six groups. To categorise and characterise each group, we compared these with the NPI+ biological class membership determined using IHC. Figure 5 and Table 4 show that RPPA Group 1 includes five samples in which four belong to the basal class of tumours whilst the remaining case was characterised as HER2+. The second RPPA group contained four samples; three of them were characterised as the luminal biological class with the remaining case characterised as basal. The third group clustered all five samples as members of basal class whilst Group 4 primarily contained HER2+ tumours. All five samples in Group 6 were classified as luminal. The fifth group showed mixed tumour characteristics as either basal or luminal.

Discussion

RPPA represents an emerging high-throughput technology as a powerful tool for protein profiling, signalling pathway analysis and biomarker discovery. FFPE is the preferred method for fixation and preservation of human tissue samples in routine clinical practice and represents a valuable resource for retrospective studies aiming at biomarker discovery and validation. Moreover, FFPE tissues are highly useful for quantitative protein assay on protein extracts using different established protocols for protein extraction which have been successfully analysed using widely used proteomic technologies such as mass spectrometry and RPPA [24–26]. RPPA has been successfully applied in quantifying proteins extracted from FFPE materials [24, 27, 28]. In the current study, we optimised and applied RPPA for quantification of the biomarkers required for NPI+, a novel prognostic assay currently reliant on the semi-quantitative IHC.

Initially, we have systematically evaluated, in terms of both quantity and quality, protein extraction methods from FFPE tissue for optimal performance. This step is a crucial step for proteomic application on protein extracts from FFPE. It is well acknowledged that the amount and the quality of protein yield from FFPE is affected by formalin-fixation and paraffin-embedding process as well as the differences in time taken for preparation compared to frozen equivalents. Herein, we compared the efficiency of protein extraction from BC tissues using three different popular protocols, the commercially available Q-proteome protein extraction Kit and an in-house Laemmli buffer with either incubation for 20 min or 2 h at 105 °C. In this study, the highest protein yield from FFPE samples was achieved using the Q-proteome protein extraction Kit from FFPE tissues as evidenced from Western blotting and RPPA results. This is in line with previously published results [18]. Another technical aspect of RPPA-based tumour profiling is the reproducibility of target protein expression measurements.

Fig. 2 Validation of the primary antibodies by strip Western blotting. Western blotting images for validation of selected antibodies in this study. A single band was obtained at the predicted molecular weight of each protein
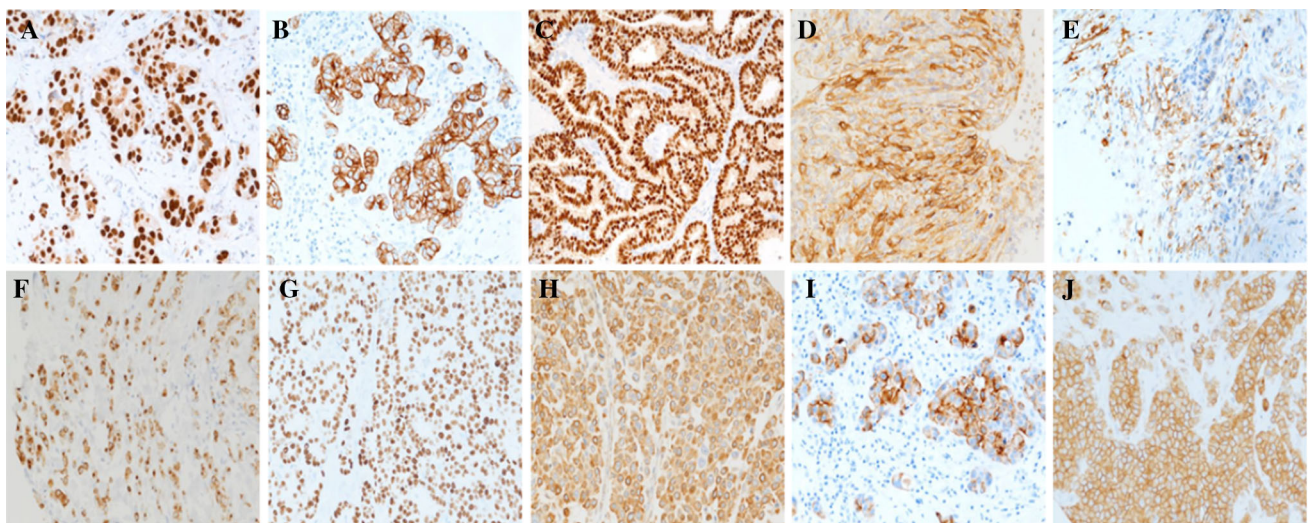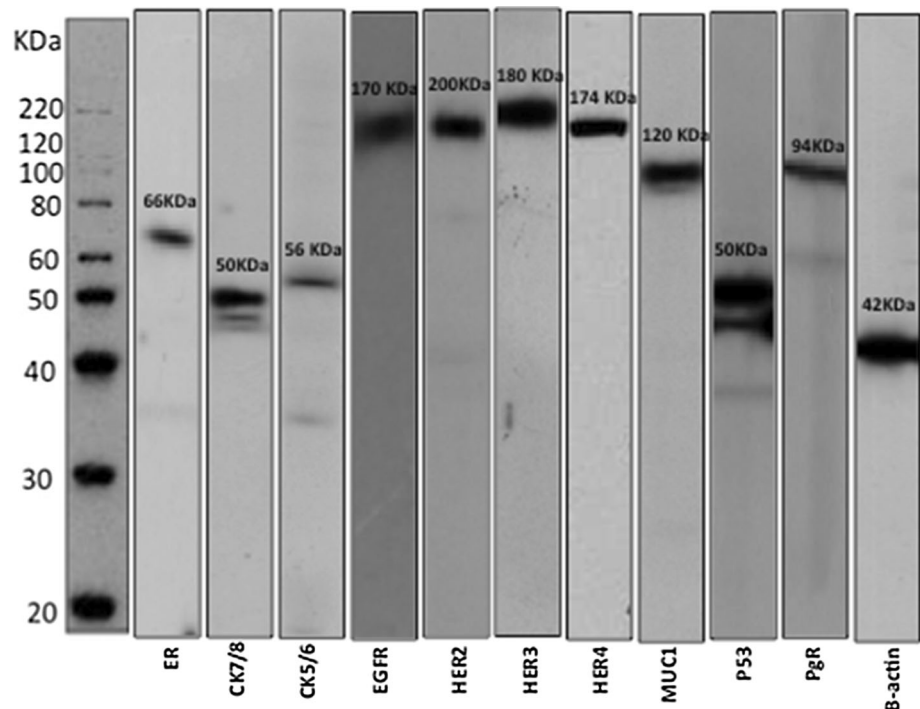

Fig. 3 IHC images for the expression of NPI+ markers. **a** ER show nuclear staining, **b** CK7/8, **c** CK5/6, **d** EGFR show membranous staining, **e** HER2, **f** HER3 show membranous and cytoplasmic staining, **g** HER4, **h** Mucin 1, **i** P53 and **j** PgR show strong and moderate cytoplasmic staining, respectively. All pictures were taken using digital pathology system at ×20

This issue was addressed by assessment of four different proteins (β-actin, ER, HER2 and HER4) on slides printed in replicates and stained on different days. For intra-assay reproducibility the coefficient of variation (CV %) within the replicates printed onto the same slide was less than 7 % for all the analysed samples. Moreover, the one-to-one comparison revealed an excellent correlation for all four tested proteins stained at different days ($r^2 = 0.61$). This result is consistent with our previous study, which demonstrated the reproducibility of RPPA technique using cell lines [21].

In order to evaluate the benefits of applying RPPA to archival FFPE breast cancer tissues for the molecular classification of the NPI+, we performed a prospective study comparing the panel of NPI+ biomarkers determination with IHC and RPPA. In order to assess the effect of tissue heterogeneity, we prepared the lysates from FFPE tumour tissues using either macrodissected or full-face sections.
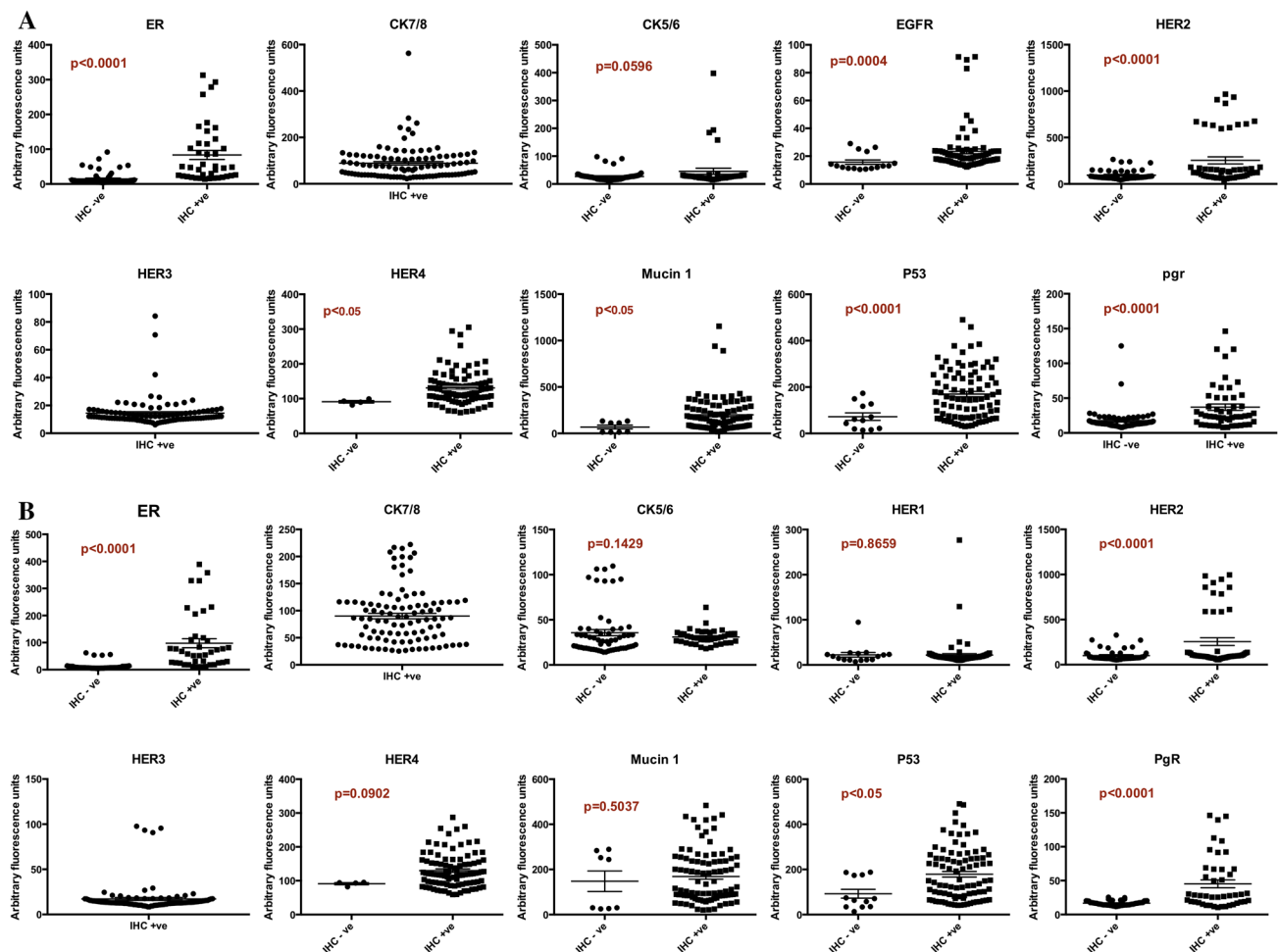
Fig. 4 Comparison of RPPA data with classification of patients based on routine IHC. RPPA-derived ER, EGFR, HER2, HER4, Mucin 1, p53 and PgR, expression levels significantly separated the respective IHC-based groups ($p < 0.05$) in macrodissection (**a**). Whilst (**b**), in full-face samples, the RPPA was discriminative for only ER, PgR, HER2 and p53. All cases were scored positive for HER3 and Ck7/8

Table 3 Correlation between IHC results and RPPA results

Antibody	Macrodissection	Full face
Oestrogen receptor	<0.0001	<0.0001
Cytokeratin CK7/8	0.046	0.0092
Cytokeratin CK5/6	0.0107	0.3738
EGFR	0.0328	0.2761
HER2	<0.0001	<0.0001
HER3	0.8083	0.0813
HER4	0.0456	0.0001
Mucin 1	0.0022	0.0126
P53	0.9607	0.8286
Progesterone receptor	<0.0001	<0.0001

Interestingly, applying RPPA for quantitative measurement of the BC biomarkers could discriminate between positive and negative IHC groups, especially in macrodissected sections. These data indicate the feasibility to use RPPA as an alternative to IHC. This has several advantages including

the detection of more subtle differences in protein expression levels, the ability of simultaneous quantification of multiple proteins in multiple samples and the objectivity of assay as compared to the subjective IHC. It is noteworthy that intra-laboratory and inter-laboratory variability in IHC studies exists. For instance, a quality-control study in UK and EU reported a significant inter-laboratory variability to classify ER positive versus ER negative tumours, especially for tumours with lower ER levels [7, 29].

For the same biomarkers assessed in this study, the concordance of results from RPPA in correlation with those assessed by the routinely used IHC platform was greatly enhanced by macrodissection compared to the results acquired from full-face tissue sections (80–60 % respectively). The protein profiling differences observed are likely to have arisen from the input samples as full-face sections incorporate the tumour microenvironment and other tissues [30]. The discordance of the level of some of these proteins determined by both IHC and RPPA such as HER3 was
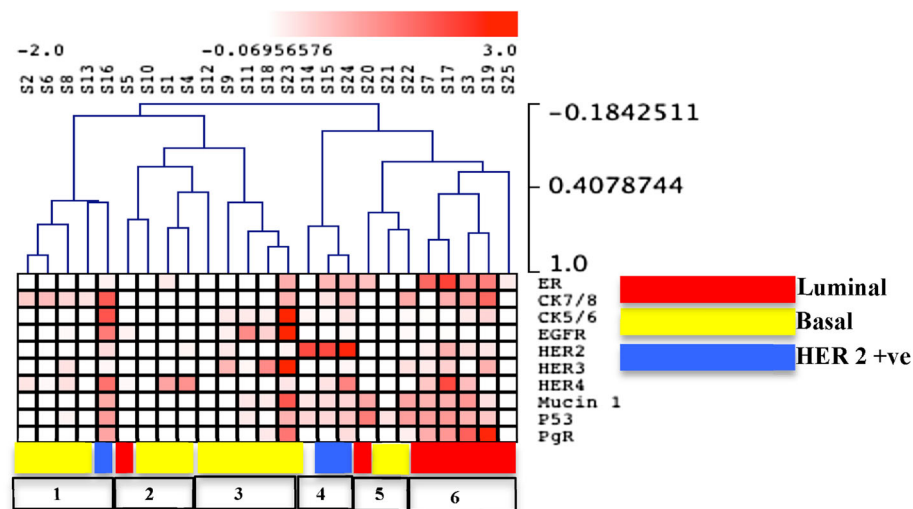
Fig. 5 Hierarchical cluster analysis of NPI+ biomarkers. Heat map illustrating an unsupervised one-way hierarchical cluster analysis of the expression of the 10 biomarkers used in NPI+ using reverse phase protein arrays based on Pearson distance combined with complete linkage rule in macrodissection. Patient samples are oriented on the horizontal axis and the different studied target molecules are oriented on the vertical axis. Clustering of the 25 tumour samples was achieved using RPPA into 6 different group-derived protein expression data of NPI+ biomarkers. Each group is mainly belonging to one NPI+ group (Luminal, Basal or HER2+). Only three out of 25 samples were misclassified. Colour code of the heat map: *white* low expression, *red* high expression

Table 4 RPPA classification of BC according to NPI+

	Number of cases	Luminal	Basal	HER2 +ve	Unclassified
Group 1	5		4	1	
Group 2	4	1	3		
Group 3	5		5		
Group 4	3			2	1
Group 5	3	1	2		
Group 6	5	5			

perhaps due to the heterogeneity of the staining and breast tissue or can be related to the expression and subcellular distribution; as in this study, the membranous IHC staining of HER3 was only considered positive irrespective of cytoplasmic staining, if any. However, RPPA can detect the subtle differences in membranous and cytoplasmic protein expression levels (Fig. 2). In addition, some antibodies failed to detect the expected membranous localisation certain markers by IHC such as HER3 and this is turn will affect the reproducibility [31]. In general, a common issue for all genomic and proteomic studies based on tumour specimens is the sample quality and the invasive tumour burden relative to other non-cancerous cell components as this can make the comparisons of molecular profiles from undissected full-face BC tissue samples inconclusive. Although laser microdissection minimises the percentage of tissue heterogeneity by isolation of pure samples containing only tumour cells, it is a labour-intensive procedure and the quality of the obtained tissue material could challenge to get adequate protein levels [30, 32].

In conclusion, the analysis of FFPE tissue lysates using RPPA is demonstrated as a reliable method for protein quantification. Data generated through this high-throughput technique can be used in simultaneous analysis of protein portraits in a large number of clinical samples. Accordingly, the RPPA could be reliably used in molecular classification of BC, such as the NPI+, through fast and reliable proteomic quantification of multiple proteins in BC samples allowing patient stratification according to BC progression, risk of recurrence and response to therapy. Further validation studies in a larger cohort of patient samples are therefore warranted.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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