RESEARCH PAPER

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Research Paper

Utilisation of antibody microarrays for the selection of specific and informative antibodies from recombinant library binders of unknown quality

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Many diagnostic and therapeutic concepts require antibodies of high specificity. Recombinant binder libraries and related selection approaches allow the efficient isolation of antibodies against almost every target of interest. Nevertheless, it cannot be guaranteed that selected antibodies perform well and interact specifically enough with analytes unless an elaborate characterisation is performed. Here, we present an approach to shorten this process by combining the selection of suitable antibodies with the identification of informative target molecules by means of antibody microarrays, thereby reducing the effort of antibody characterisation by concentrating on relevant molecules. In a pilot scheme, a library of 456 single-chain variable fragment (scFv) binders to 134 antigens was used. They were arranged in a microarray format and incubated with the protein content of clinical tissue samples isolated from pancreatic ductal adenocarcinoma and healthy pancreas, as well as recurrent and non-recurrent bladder tumours. We observed significant variation in the expression of the E3 ubiquitin-protein ligase (CHFR) as well as the glutamate receptor interacting protein 2 (GRIP2), for example, always with more than one of the scFvs binding to these targets. Only the relevant antibodies were then characterised further on antigen microarrays and by surface plasmon resonance experiments so as to select the most specific and highest affinity antibodies. These binders were in turn used to confirm a microarray result by immunohistochemistry analysis.

Introduction

The demand for highly specific and high-affinity antibodies is continuously and rapidly growing [1]. The advances in genomic sequencing, for example, are uncovering more and more disease-related changes, which frequently need to be elucidated in protein-based assays since they exhibit their functional effect at this molecular level. Companion diagnostics, in which the application of a drug is directly coupled to a patient stratification process, is

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also of increasing importance. Since most drugs affect proteins, most assays will probably analyse this molecular class, too. Access to antibodies is crucial for such ends. Currently, the antibody field is still dominated by monoclonal antibodies isolated from hybridoma cells [2]. While they represent the gold standard at present, binders that originate from recombinant selection systems are catching up quickly and are expected to become the norm sooner rather than later [3,4]. Based on libraries that can match the antibody diversity of the human immune system, they allow the isolation of binders to almost any desired target. They also permit flexibility with respect to the actual application profile. Binders against linear epitopes, for example, may not be suitable for recognition of three-dimensional structures and vice versa. By adaptation of the selection parameters, antibodies from a single library can meet varying requirements. However, similar to antibodies from hybridomas, the production of recombinant binders faces the common problem that individual molecules resulting from the initial selection screens differ tremendously in quality, in particular with respect to specificity and affinity. The majority of generated molecules will eventually fail quality requirements at some stage, making the effort already invested on characterisation useless and void.

In an effort to shorten the usually successively executed processes of antibody production, characterisation, application for antigen identification and selection of binders that exhibit appropriate performance parameters and are of informative value, we propose to combine the last three steps, at least partially, by means of binder microarrays. After an initial selection process – panning from a library by removing the antibodies that do not bind the targeted antigens at all – the remaining antibodies will be arrayed and incubated with protein samples of interest. For lack of any extensive characterisation, a large percentage of the primary antibody hits will be identified immediately as being of inadequate quality. At the same time, however, molecules will be found that exhibit at least some degree of specificity and yield reproducible results that could be of biological or biomedical relevance. Only antibodies that meet sufficiently both requirements – binding specificity and informative value – will be characterised further in order to determine their quality. Once quality is established, the antibodies can then be used to confirm in other analysis formats the biological finding made in the microarray experiment.

In a pilot experiment, we have investigated a set of 456 recombinant antibodies. They were made within the framework of the Affinomics consortium, the most recent of three European networks for binder production and characterisation [5], and were designed to bind 134 tumour-associated antigens. Instead of selecting the best candidates in a one-by-one analysis of specificity and affinity, they were arranged as an antibody microarray without knowing about their quality. By incubation with clinical protein samples, we could combine the identification of proteins, which exhibit differential expression, with an identification of related antibodies with apparently appropriate performance. These antibodies were then subjected to further characterisation. Molecules that passed the process were subsequently used to validate the original microarray result by immunohistochemistry.

In the analysis, protein extracts from pancreatic and bladder cancer were used as well as appropriate controls. Pancreatic cancer is currently the most lethal cancer entity in the Western world [6]. Most patients die within a year of diagnosis, with mean survival being about five months; mortality is basically identical to incidence. In comparison, bladder cancer is far less lethal and frequently diagnosed relatively early. Low-stage, non muscle-invasive bladder cancer can usually be resected successfully. However, 60% of the tumours recur and then often become invasive [7].

Materials and methods

Antibody microarray production

The antibodies were part of the human single-chain variable fragment (scFv) gene libraries HAL4/7/8 [8] and HAL9/10 [9]. Initial selections against 134 antigens (132 proteins and 2 peptides; Supplementary Tab. 1) of oncological context and poor coverage by commercial monoclonal antibodies were performed by library panning as described [10] leading to a set of 456 scFvs. Concentration dependent binding to the respective target molecules was confirmed by an ELISA using dilution series of the antibodies. The scFvs were then re-cloned so as to link them to a human IgG1 Fc part as described in detail [8]. The fusion proteins have superior stability. Experimentally, they behave very similarly to IgG antibodies and can be used with them in immunoassays without changing the underlying protocols. The antibodies were expressed in HEK293 6E cells and purified by affinity chromatography on protein A [11]. Antibody microarrays were prepared as described in detail previously [12,13]. In brief, the antibodies were spotted onto epoxysilane-coated slides (25 mm × 75 mm, Nexterion-E; Schott, Jena, Germany) using the contact printer MicroGrid II 610 (Apogent Discoveries, Mittenwald, Germany) and SMP6B pins (Telechem, Sunnyvale, USA) at a humidity of 55–65%. The printing buffer was composed of 50 mM sodium bicarbonate, pH 9.0, 100 mM NaCl, 50 mM $MgCl_2$, 0.005% (w/v) sodium azide, 0.25% (w/v) dextran, 0.006% (w/v) Igepal CA-630 (Sigma–Aldrich, St. Louis, USA) and 1 mg/ml of the respective antibody. All antibodies were spotted in quadruplicate on each microarray slide. The spots featured a diameter of about 250 μm. After the actual printing process, the slides were allowed to equilibrate at room temperature and 55–65% humidity overnight. They were then stored in dry and dark conditions at 4°C.

Preparation of protein samples

For all tissue samples, written informed consent was obtained from the patients or donors. The analyses performed were approved by the local ethics committee. As a matter of fact, tumour specimens were used, which had been studied before in a different setting. The bladder cancer samples had been isolated from patients with and without tumour recurrence after five years [14]. Also, we used nine tissue samples of patients with pancreatic adenocarcinoma (PDAC) and seven pancreas samples of patients, who had no cancer but had their pancreas removed for other reasons [15; unpublished results]. The protein extracts had been prepared as described in detail previously [13]. Total protein concentration was measured by the BCA assay (Thermo Fisher Scientific, Waltham, USA) and adjusted to 1 mg/ml. The protein was labelled with 0.4 mg/ml of the NHS-ester of the fluorescent dye Dy-549 (Dyomics, Jena, Germany) in 100 mM sodium bicarbonate buffer, pH 8.5, 1% (w/v) Triton-100 on a shaker at 4°C. After 1 h, the reaction was stopped by addition of 10% glycine. Unreacted dye was removed 30 min later and the buffer changed to phosphate-buffered saline (PBS) using Zeba Desalt

columns (Thermo Fisher Scientific). While stoichiometric labelling is possible [16], it is not used routinely. Variations in labelling efficiency are normalised during the data analysis process. Subsequently, the Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) was added as recommended by the manufacturer. All labelled protein samples were stored in aliquots at −20°C until use. For competitive dual-colour incubation [17], a common reference sample was produced by pooling identical amounts of all protein samples and labelling the pool with the dye Dy-649 (Dyomics). The same reference sample was used throughout the analyses.

Antibody microarray analysis

Surface blocking and sample incubation were performed using a Tecan HS 4800 hybridisation system (Tecan, Männedorf, Switzerland). Slides were blocked with the Candor Blocking Solution (Candor Biosciences, Wangen, Germany) for 1 h and incubated with labelled protein for 3 h. Subsequently, the slides were scanned with a Tecan Powerscanner and images were analysed using GenePix 6.0 microarray analysis software (Axon Instruments, Union City, USA). Chipster software version 3.3 (http://chipster.csc.fi/) was applied for loess normalisation and statistical analysis.

Antigen microarray analysis

Full-length antigens were produced at the Københavns Universitet NNF Center for Protein Research (UCPH) by expression in *E. coli* and purified via a polyhistidine tag. To assure their purity, all proteins were tested by SDS–PAGE prior to immobilisation. The list of the 54 antigens used in the analysis is shown in Supplementary Tab. 2. The antigen microarrays were prepared as described for the antibody microarrays. For spotting, however, a glycerol-based buffer (0.4 M $NaHPO_4$, pH 8.6, 10% glycerol, 0.01% (w/v) sarkosyl) was used. Each antigen was spotted in triplicate within each of eight identical subarrays placed on one microarray slide. Incubation with antibodies was performed within 24 h after spotting. After an initial blocking with the Candor Blocking Solution, 250 μl 33 nM recombinant antibody was incubated on the arrays for 1 h. After washing, DY-649-P1-labelled protein A/G (Biotrend, Cologne, Germany) was added for detection to a concentration of 20 nM and incubated for 30 min. Experiments were carried out in triplicate.

Affinity measurements by surface plasmon resonance (SPR)

A Sierra Sensors SPR-4 instrument (Sierra Sensors, Hamburg, Germany) was used to acquire binding kinetics of selected scFv-Fc:antigen pairs. Antigens were immobilised covalently to a flat amine sensor chip to a level of 350 RU (protein CHFR) or 300 RU (GRIP2). Interaction analysis was performed by injecting several concentrations of the corresponding scFv-Fc antibody diluted in PBS buffer at a flow rate of 30 μl/min. After each injection the surface was regenerated using 10 mM glycine (pH 1.9) for 30 s. All measurements were performed at 25°C. Non-specific binding, monitored on a blank flow cell, was subtracted from the SPR signals. Rate constants were determined using the SPR-4 analysis software (Sierra Sensors) assuming a 1:1 Langmuir binding model.

Immunohistochemistry (IHC)

For immunohistochemistry experiments, frozen tissue sections were produced. They were fixed with -20°C cold acetone for 5 min. Peroxidases were quenched with a 3% (v/v) H_2O_2 solution in methanol. Slides and tissue sections were first blocked with avidin (Dako, Kyoto, Japan) for 10 min, second with biotin (Dako) for 10 min and finally with Power Block (BioGenex, San Ramon, USA) for 1 h. Recombinant antibodies were biotinylated with EZ-Link NHS-PEG4-Biotin according to the manufacturer's protocol, diluted in antibody diluent (Dako S3022) to a concentration of 5–10 μg/ml and incubated on the sections overnight at 4°C. Prior to the incubation with streptavidin peroxidase (Kirkegaard & Perry Laboratories) for 30 min, slides were washed with Tris-buffer supplemented with 0.05% Tween-20. The colour reaction was carried out by incubation for 2 min with liquid DAB+ substrate (Dako) and counterstaining by Mayer's hematoxylin solution (Merck, Darmstadt, Germany). Images were captured using a Zeiss Cell Observer Z1 equipped with an AxioCam (Zeiss, Jena, Germany).

Results

Comparative antibody microarray analysis

The binder set used in this pilot study consisted of phage display selected single chain variable fragments (scFvs) isolated from universal phage display libraries, which allow the high-throughput selection of human antibody fragments. After initial selection by panning against 134 proteins or peptides (Supplementary Tab. 1), a set of 456 scFvs was picked from the binding molecules. Each scFv was fused to a human Fc-fragment in order to create scFv-Fc fusion antibodies. This binder format can be used equivalent to human IgGs [18,19]. In order to investigate which of the constructs should be studied in more detail, the scFv-Fc antibodies were spotted onto microarrays and incubated with clinical samples (Fig. 1a). Incubation was with fluorescently labelled protein lysates obtained from pancreatic adenocarcinoma (PDAC) and healthy pancreas tissues as well as resected bladder tumours from patients, who subsequently had either suffered tumour recurrence within five years of tumour resection or had no relapse. All samples had been studied before [14; unpublished results] and were known to exhibit expression differences for proteins other than those represented by the scFv-Fc fusion antibodies used here.

Sample labelling and incubation as well as data analysis were performed according to well-established procedures [12,17]. Volcano plots (Fig. 2) present the degree and significance of differential protein expression. In total, antibodies to 31 proteins exhibited a potentially significant up- or down-regulation in PDAC compared to normal tissues, and antibodies to 38 proteins showed such changes when comparing non-recurrent and recurrent bladder cancer tissues. Since 9 proteins were shared by both analyses, an overall total of 60 proteins was found to be potentially differentially expressed in the initial analysis. For the other 84 of the 134 proteins for which antibodies had been isolated, no variation was identified. This could be due either to the lack of any such difference in the studied protein samples or the absence of an antibody of sufficient sensitivity or specificity. For the majority of the 60 proteins that yielded varying signals, there was only one antibody that showed this variation, while the other binders that were intended to bind to the same target did not produce such a result. Four proteins – CHFR, ITCH, GORASP2 and GRIP2 – exhibited significant variations on more than one of the antibodies that had been produced against them (Fig. 2). The consistent results made it likely that the relevant scFv-Fc antibodies actually bound to the

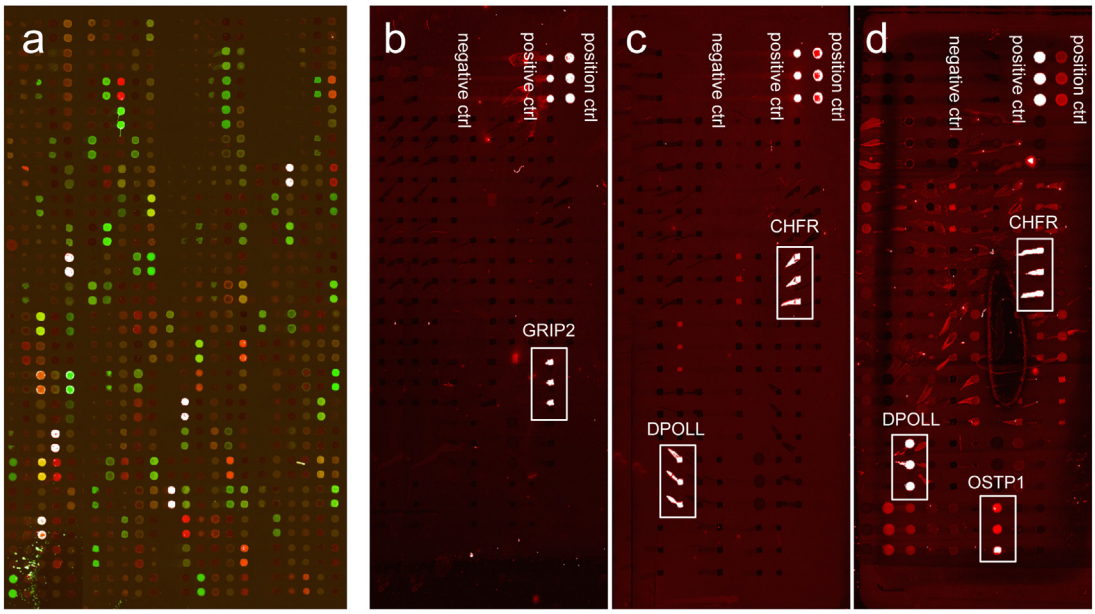

FIGURE 1

Typical results of microarray analyses. **(a)** Overlay-image of the scFv-Fc antibody microarray incubated with protein samples from PDAC tissue (green signal) and the common reference (red signal). Depending on the amount of protein binding to each spot in comparison to the common reference, colours vary from green, via yellow and orange to red. **(b)–(d)** Images are shown of incubations on the antigen microarrays of antibodies DM380-JA70-F6 (b), DM319-JA37-E9 (c) and DM319-JA37-B11 (d). Bound scFv-Fc antibody was detected with fluorescently labelled protein A/G. White spots indicate a signal that is too intense to be presented in colour. Spot streaking is an experimental artefact introduced during drying the arrays with pressurised air.

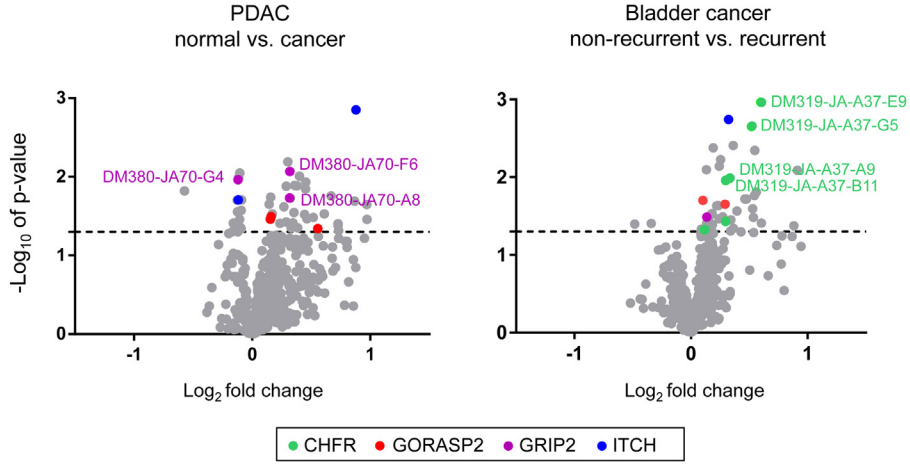

FIGURE 2

Visualisation of the antibody microarray analysis results as volcano plots. (Left panel) Relative variation of protein abundance in lysates of normal pancreatic and PDAC tissues, respectively. A negative $\log_2$ fold change indicates down-regulation, while a positive change indicates overexpression of proteins in PDAC tissue compared to normal tissue. (Right panel) The same is shown for proteins in non-recurrent versus recurrent bladder cancer tissues. Dots of the same colour represent antibodies which were meant to recognize the same antigen; the colour code is given. The names of some relevant antibodies are shown. The dotted horizontal line represents a *p*-value of 0.05.

same target and that this target was differentially expressed in the investigated sample cohorts. These antibodies (Table 1) were selected for further characterisation.

Quality control of selected antibodies

After their initial isolation from the recombinant library, all scFv-Fc antibodies had been used for ELISAs, testing if they recognized the antigen by which they were selected in the panning process. However, these tests only determined if an antibody was able to bind its target, but did not provide any information about specificity. In order to investigate the degree of cross-reactivity of the candidate antibodies selected by the microarray analysis, they were incubated individually on an antigen array onto which full-length proteins had been spotted (Fig. 1b–d). In our pilot

TABLE 1

List of antibodies selected for further characterisation. '+' indicates the detection of a variation in the abundance of the assumed target protein upon incubation of the antibody microarray with protein extracts isolated from clinical tissue samples.

Antibody name	Antigen specificity (presumed)	PDAC versus normal	Bladder cancer recurrent versus non-recurrent
DM319-JA37-E9	CHFR Uniprot ID: Q96EP1		+
DM319-JA37-G5	CHFR		+
DM319-JA37-A9	CHFR		+
DM319-JA37-B11	CHFR		+
DM319-JA37-H7	CHFR		+
DM319-JA37-D4	CHFR		+
DM379-JA69-F11	GORASP2 Uniprot ID: Q9H8Y8	+	
DM379-JA69-C9	GORASP2		+
DM379-JA69-G7	GORASP2	+	
DM379-JA69-H3	GORASP2	+	
DM379-JA69-E4	GORASP2		+
DM380-JA70-F6	GRIP2 Uniprot ID: Q9C0E4	+	
DM380-JA70-A8	GRIP2	+	
DM380-JA70-G4	GRIP2		
DM380-JA70-B1	GRIP2		+
DM382-JA72-B7	ITCH Uniprot ID: Q96J02	+	+
DM382-JA72-H1	ITCH		

scheme, 54 proteins were presented on the antigen array, including CHFR, ITCH, GORASP2 and GRIP2 (Supplementary Tab. 2). Only the anti-CHFR antibody DM319-JA37-G5 (Fig. 3a) and the anti-GRIP2 antibodies DM380-JA70-F6 and -A8 (Fig. 4a) recognized their target proteins with good specificity. The other candidates were either cross-reactive (anti-CHFR antibodies DM319-JA37-A9, -B11 and-E9) or did not bind to the antigen array at all (anti-GRIP2 antibody DM380-JA70-G4 as well as all anti-ITCH and anti-GROASP2 antibodies). The cross-reactive anti-CHRF antibodies produced very similar binding patterns, also recognizing the proteins DPOLL, OSTP1, OTUB1 and PRDM8, but with varying intensities (Fig. 3a). For confirmation that ITCH and GROASP2 did not interact with their antibodies, the proteins were labelled fluorescently and individually incubated on

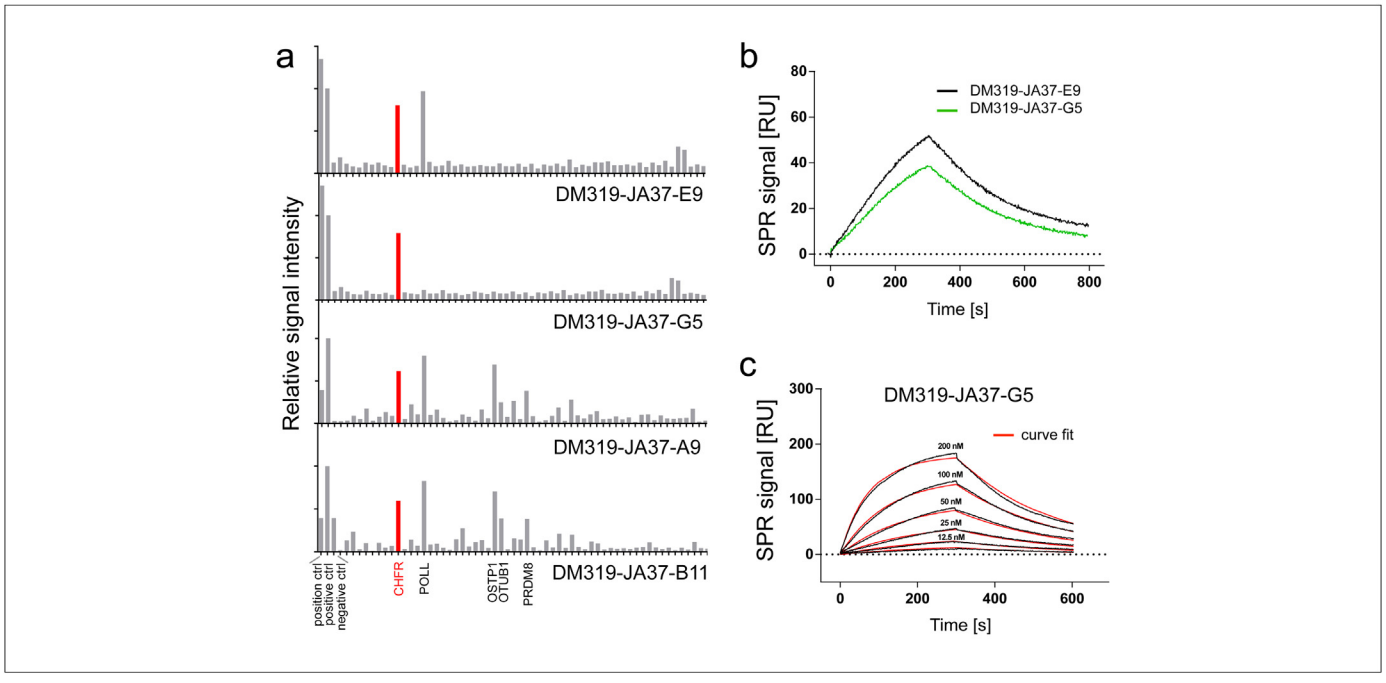

FIGURE 3

Quality control of anti-CHFR antibodies. **(a)** Binding intensities on the antigen arrays are shown. Signal intensities were normalised to the value achieved with an immobilised control antibody. The desired target antigen is marked in red. For better visualisation, no error bars are shown. However, the data is available as Supplementary Tab. 3. **(b)** SPR binding curves of two anti-CHFR scFv-Fc antibodies are shown. **(c)** SPR binding curves are presented of the best performing antibody (DM319-JA37-G5) at different concentrations; from this, a K_D of 82.2 nM was calculated.

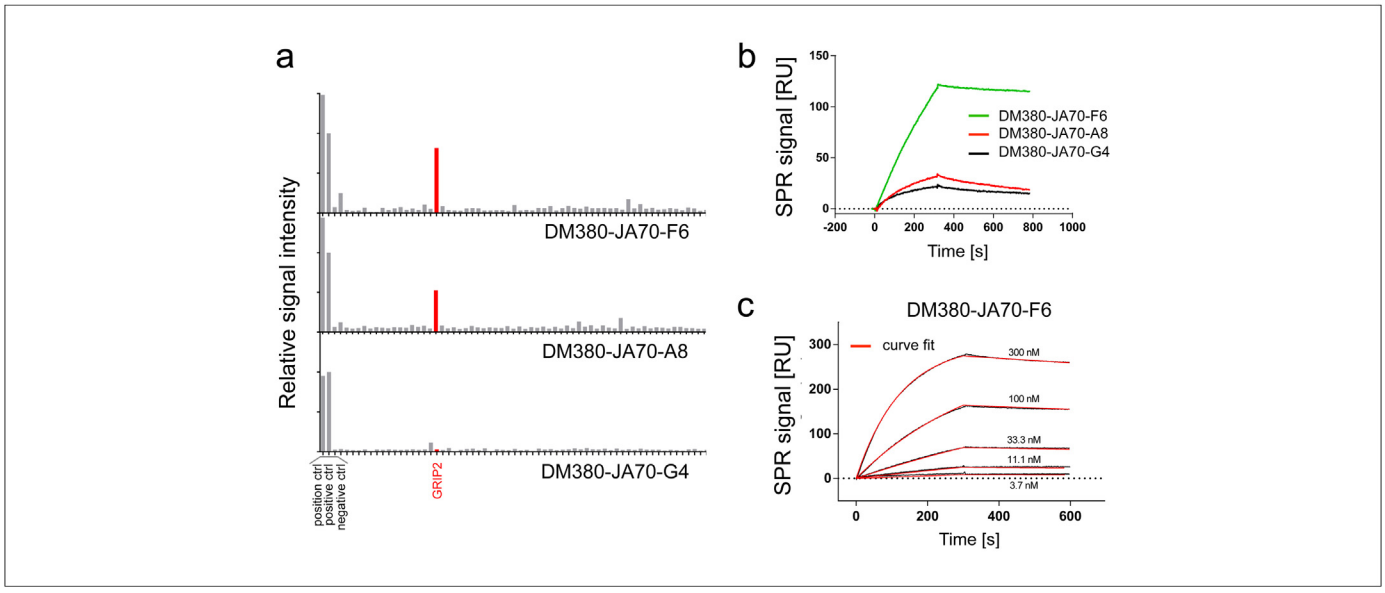

FIGURE 4

Quality control of anti-GRP2 antibodies. **(a)** Binding intensities on the antigen array are shown. Signal intensities were normalised to that of an immobilised control antibody. The desired target antigen is marked in red. **(b)** SPR binding curves of three anti-GRIP2 scFv-Fc antibodies are shown. **(c)** SPR binding curves are presented of the best performing antibody at different concentrations; from this, a K_D of 6.5 nM was calculated.

antibody microarrays. In this inverse system too, there was no binding to the respective antibodies (data not shown), which was surprising as they had exhibited binding in ELISA tests previously.

To qualify the array-based results further, the affinities of the anti-CHFR and anti-GRIP2 antibodies were measured quantitatively with SPR. For comparison to the antigen array data, the antigens were immobilised and the antibodies were added in different concentrations. Anti-CHFR antibodies DM319-JA37-G5 (specific) and DM319-JA37-E9 (cross-reactive) showed comparable binding kinetics with moderate dissociation rates (Fig. 3b). Assuming a 1:1 Langmuir binding model, a K_D of 82.2 nM could be calculated for anti-CHFR antibody DM319-JA37-G5 (Fig. 3c). For the anti-GRIP2 antibodies (Fig. 4), there was strong binding of the specific binder DM380-JA70-F6, which was also characterised by its slow dissociation rate, whereas for the antibodies DM380-JA70-A8 (specific) and DM380-JA70-G4 (cross-reacting) binding was significantly weaker. A K_D of 6.5 nM was calculated for DM380-JA70-F6.

Confirmation of GRIP2 overexpression by immunohistochemistry

Some of the antibodies that had yielded apparently interesting data in the microarray analysis could be confirmed by the quality control experiments as binders of good specificity and affinity. In turn, we used one of these good performers for validating the differential protein abundance in the tumour samples observed in the microarray analysis. To this end, immunohistochemistry was carried out on frozen tissue sections (Fig. 5). Besides confirming

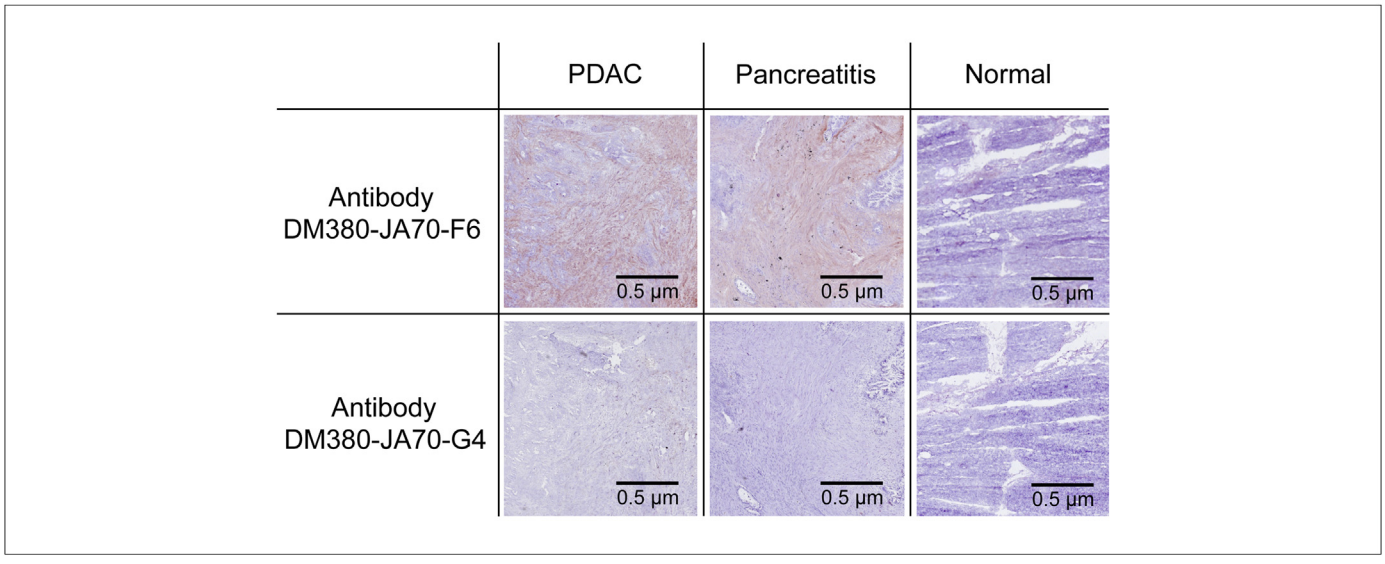

FIGURE 5

Immunohistochemistry analysis of GRIP2 abundance in pancreatic tissues. Tissues isolated from patients with PDAC or chronic pancreatitis and from donors with healthy pancreatic tissue (normal) were stained with two antibodies. The antibody DM380-JA70-F6 clearly showed a higher abundance of GRIP2 in diseased pancreas.

the biological variation seen in the microarray assay, the immunohistochemistry assay allowed simultaneous testing of the binder performance. Analysing pancreatic tissue sections with the specific and high-affinity anti-GRIP2 antibody DM380-JA70-F6 confirmed that GRIP2 was highly abundant in PDAC and chronic pancreatitis compared to normal tissue. As expected, antibody DM380-JA70-G4 produced inconclusive results.

Discussion

Although only performed on a small-scale, these pilot experiments demonstrated the feasibility of screening, by means of antibody microarrays, a preselected antibody library containing binders of unknown quality for the isolation of useful antibodies, without testing the performance parameters of every single candidate beforehand. Simultaneously, utilising representative samples, the process specifically selected antibodies that could be informative for a given application, such as the discrimination of pancreatic tumour from healthy tissue. Actually, there could well be more 'good' antibodies in the set of 456 molecules, useful for analyses other than that of pancreatic and bladder tissues. The strategy is most suited for a targeted binder selection process. The array format permits flexibility with respect to the number of molecules that could be studied. This number and the degree of selectivity of the microarray assay can be adapted to both the actual needs and the capacity for the subsequent, much more laborious binder characterisation. Similarly, at the other end, the stringency of the pre-selection process, through panning the original binder library against the protein(s) of interest, can be tailored, depending on the capacity of the array analysis.

Our analysis also demonstrated, unsurprisingly, that further characterisation of the selected antibodies is essential; the antibody microarray analysis on its own is clearly insufficient when dealing with molecules of unknown quality. Although there were several antibodies that indicated abundance variations of proteins ITCH and GROASP2, for instance, this result could not be confirmed during the subsequent antibody characterisation. It is likely that the relevant antibodies bind to other proteins exhibiting reproducible abundance variation. Neither when antibodies were applied to the antigen array nor in the reverse experiment, when the individual proteins were incubated on the antibody microarrays, could specific binding be detected. In our pilot scheme, only a small antigen array was used for identification of binding specificity. The use of more comprehensive antigen microarrays would lead to a much better proof of the accurate binding of an antibody to the intended target or the identification of any cross-binding activity. In vitro production of proteins, and in particular an in situ synthesis directly on the microarray surface [20–22] permits the production of such complex analysis platforms.

The objective of our study was a demonstration of the utility of the microarray-based selection process. During the course of this, disease related differences in protein abundance were identified and related antibodies of nanomolar affinity were isolated. However, the biological information gained from this is too preliminary to draw any conclusions on the value of GRIP2 or CHFR for diagnosis, for instance. Firstly, the sample number was too small. Secondly, for a comprehensive analysis, further controls would be essential, such as samples from chronically inflamed pancreatic tissue. Nevertheless, even in the very limited setting of the pilot scheme, we succeeded in isolating antibodies that could well be useful for biologically or biomedically motivated studies. Antibody DM380-JA70-F6, for example, is the first monoclonal antibody against GRIP2 according to the Antibodypedia data repository (version 9 of June 2015; http://www.antibodypedia.com) with information on 1 768 896 antibodies.

In conclusion, an efficient strategy was established that combines the identification of protein variations of informative value with the selection of related antibodies from a set of uncharacterised molecules derived from human antibody gene libraries. Only after the application of relevant protein samples to the antibody microarray are binders of potential interest characterised further, so as to isolate appropriately performing antibodies for further utilisation. This limits the task of antibody characterisation – a bottleneck in current antibody production – to antibodies that are informative in a particular biological context of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2015.12.003.

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