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Research paper

Seromic analysis of antibody responses in non-small cell lung cancer patients and healthy donors using conformational protein arrays

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ABSTRACT

Analysis of antibody responses to self-antigens has driven the development of the field of tumor immunology, with the identification of many protein targets found in cancer but with limited expression in normal tissues. Protein microarray technologies offer an unprecedented platform to assay the serological response of cancer patients to tumor antigens in a comprehensive fashion, against many proteins simultaneously. We developed an array containing 329 full-length proteins, originally identified as antigenic in various cancer patients by serological expression cloning (SEREX), that were immobilized as folded, functional products accessible for antibody binding. To validate the use of these microarrays, we selected 31 sera from non-small cell lung cancer patients previously known to react to the following antigens by ELISA: LAGE-1/CTAG2, MAGEA4, TP53, SSX and SOX2. These sera were compared with 22 sera from healthy donors for reactivity against a series of antigens present on microarrays. The sensitivity and specificity of the arrays compared favorably with standard ELISA techniques (94% concordance). We present here a stringent strategy for data analysis and normalization that is applicable to protein arrays in general, and describe findings suggesting that this approach is suitable for defining potential antigenic targets for cancer vaccine development, serum antibody signatures with clinical value, characterization of predictive serum markers for experimental therapeutics, and eventually for the serological definition of the cancer proteome (seromics).

1. Introduction

For over a century, there has been an intense search for human tumor antigens that could be used in cancer diagnosis and therapy (Old and Chen, 1998; Wang and Rosenberg, 1999). Initial efforts involved immunization of heterologous species with human cancer cells or cancer extracts and analyzing the resulting antisera for antibodies with specificity for cancer. The association of α-fetoprotein with hepatocellular cancer and CEA with GI malignancies are two notable successes of this approach (Uriel et al., 1967; Masopust et al., 1968; Collins and Black, 1971; Collins and Black, 1973). Technologies for generating monoclonal antibodies revolutionized the discovery process for cell surface and intracellular antigens of human cancer cells, inaugurating a new era in the clinical application of antibodies (Yeh et al., 1979; Eisenbarth et al., 1980; Oldham and Dillman, 2008).

A persistent question in the field of human cancer immunology is whether humans have the capacity to recognize human cancer antigens. Although a vast literature has accumulated on this subject, a definitive answer to this issue came from the development of a test system called

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autologous typing (Carey et al., 1976), where tumor cells, lymphocytes, antibody, and control cells were all derived from the same patient, thus eliminating the contribution of alloreactivity in the observed results. Autologous typing and the development of antibody recognized antigens have resulted in a growing list of human cancer antigens that are immunogenic in the host of origin (DeLeo et al., 1979; Boon and Old, 1997; Chen et al., 1997; Chen et al., 2000). With regard to cancer antigens recognized by the humoral immune system, over 2700 sequences have been identified by SEREX analysis of sera from patients with a wide range of cancers (see SEREX database, http://ludwig-sun5.unil.ch/CancerImmunomeDB/); these antigens include mutational, overexpressed, oncogenic viral, differentiation, and cancer-testis (CT) antigens (Chen et al., 2000; Scanlan et al., 2004; Chen et al., 2005).

With the sequencing of the human genome and rapid and effective protein expression systems, it is now possible to envisage screening the human proteome with the human antibody repertoire, a process we refer to as Seromics. To this end, we have constructed human protein arrays using a novel method for identifying folded recombinant proteins and attaching them in a stable and oriented manner to the glass surface (Boutell et al., 2004; Blackburn and Hart, 2005) (manuscript submitted). Arrayed proteins were chosen from the SEREX database (Chen et al., 2000) which collates data for antigenic proteins, as the next step in defining the cancer immunome. In addition, we have developed arrays of protein kinases and other proteins involved in signal transduction and used these to screen the sera of cancer patients and to compare to commercially available ones.

To evaluate the sensitivity and specificity of serological analysis using the arrays, we have first compared sera with known activity in ELISA with reactivity for the same antigens on arrays.

2. Materials and methods

2.1. Patient samples

Plasma or serum samples were obtained with approved consent from 31 NSCLC (non-small cell lung cancer) patients at the time of surgery at the Presbyterian New York Hospital under an Institutional Review Board approved protocol and from 22 healthy donors from the New York blood bank. Patient and donor characteristics are described in Supplementary Table 1.

2.2. Elisa

Patient plasma or donor serum samples were analyzed by ELISA for seroreactivity to bacterially-produced recombinant proteins LAGE-1/CTAG2, MAGEA4, SOX2, SSX1, SSX2, SSX4, and p53/TP53 (Stockert et al., 1998). Plasma or serum was diluted serially from 1/100 to 1/100,000 and added to low-volume 96-well plates (Corning, NY) coated overnight at 4 °C with 1 μg/ml antigen in 25 μl volume and blocked for 2 h at RT with PBS containing 5% non-fat milk. After overnight incubation, plates were extensively washed with PBS containing 0.2% Tween 20 and rinsed with PBS (BioTek ELx405 automated washer, Winooski, VT). Plasma IgG (total or subclasses) bound to antigens was detected with specific monoclonal antibodies conjugated with alkaline-phosphatase (Southern Biotech, Birmingham, AL). Following addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a fluorescence reader Cytofluor Series 4000 (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each plasma sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by comparing seroreactivity among various antigens tested. In each assay, sera of patients with known presence or absence of specific reactivity were used as controls. A positive result was defined as extrapolated reciprocal titers >100.

2.3. Protein microarray construction

A general approach for expression and fabrication of functional protein arrays has been described elsewhere (Blackburn and Hart, 2005). Briefly, the SEREX database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/) was used to identify corresponding cDNA clones, primarily from the Mammalian Gene Collection. Full-length open reading frames for 329 target genes were cloned in-frame with a sequence encoding a C-terminal *E. coli* biotin carboxyl carrier protein fused to the myc epitope (BCCP-myc tag; 23, manuscript submitted) in a baculovirus transfer vector and sequence-verified. Recombinant baculoviruses were generated, amplified and expressed in *Sf*9 cells grown in suspension using standard methods adapted for 24-well deep well plates (Whatman, Maidstone, UK; (Zhao et al., 2003; Chambers et al., 2004)). Recombinant protein expression was analyzed for protein integrity and biotinylation by Western blotting. Cells harboring recombinant protein were lysed and lysates were spotted in quadruplicate using a QArray2 Microarrayer (Genetix, New Milton, UK) equipped with 300 μm solid pins on to streptavidin-coated glass slides (Schott Nexterion, Jena, Germany). Following 2 h incubation to allow binding of biotinylated protein to the streptavidin, non-bound proteins were washed away and the slides blocked with BSA. The resulting arrays were referred to as cancer antigen arrays and stored at −20 °C. A range of protein activities have been demonstrated to be stable under these conditions for over 12 months. Batches of arrays were subjected to rigorous quality control to ensure reproducibility between and within array batches prior to use in this study.

In addition to the 329 proteins, four control proteins for the BCCP-myc tag (BCCP, BCCP-myc, β-galactosidase-BCCP-myc and β-galactosidase-BCCP) were arrayed, along with Cy3/Cy5-labeled biotinylated BSA marker, biotinylated IgG, IgM dilution series, unlabeled biotinylated BSA, and buffer only spots. A series of phosphorylated and non-phosphorylated kinase substrate peptide spots were also included for detection of phosphorylated residues and a dilution series of c-myc peptide useful for relative quantitation of proteins on the array via the myc tag with an anti-myc antibody.

ProtoArrays® microarrays (v4.0; Invitrogen, Carlsbad) were purchased and used according the manufacturer's instructions.

2.4. Array profiling assay

Arrays were incubated in Quadriperm dishes (Greiner BioOne, Stonehouse, UK) placed on a horizontal shaker (50 rpm) for 2 h at RT with individual sera diluted 1:200 in

2 ml buffer (0.1% Triton X100 (v/v), 0.1% BSA (w/v) in PBS). After washes, binding of IgG was detected by incubation with Cy3-rabbit anti-human IgG (Dako Cytomation) (labeled according to the manufacturer's recommended protocols; GE Healthcare) diluted 1:1000 in assay buffer for 2 h at RT. Arrays were washed again and dried by centrifugation. Arrays were scanned at 10 μm resolution using a microarray scanner (Axon 4200AL with GenePix Pro Software, Molecular Devices, Sunnyvale, CA 94089) and fluorescence detected according to the manufacturer's instructions. Images were saved as 16-bit tif files and analysis was performed using GenePix. Local backgrounds were subtracted automatically and the median net intensity in relative fluorescence units (rfu) was reported for each spot.

2.5. Calculations

Data from arrays were adjusted and normalized in the following sequence. First, median rfu values of quadruplicate spots were averaged for each antigen within each array. Second, average rfu values for all antigens on each array were proportionally adjusted and normalized according to the mean of values obtained from IgG controls and standard fluorescence markers on all arrays. Next, adjusted values for each antigen were averaged across replicate arrays and expressed as a percentage of interquartile differences per array using the following formula: $\frac{(\text{Observed value})-(25^{\text{th}}\ \text{percentile})}{(75^{\text{th}}\ \text{percentile})-(25^{\text{th}}\ \text{percentile})}$. Finally, all percentage values were normalized using a standard quantile normalization matrix (Bolstad et al., 2003), in which all percentage values of each array were ranked and replaced by the average of percentages for antigens with the same rank on all arrays. These successive transformations resulted in a data distribution with identical median and quartile values, to allow interarray comparisons.

Once data on arrays was normalized, the interquartile difference was calculated for each antigen across all arrays. These interquartile values were used to establish a cutoff and determine antigens showing significant seroreactivity: to be defined as positive for a given antigen, a serum had to react to the antigen with values greater than 2.5× the interquartile difference above the 75th percentile (See Supplementary Fig. 1 for example). These stringent criteria were used to minimize false positive data while providing increased specificity and sensitivity. If the reaction of a serum to an antigen is greater than the cutoff, the ratio between signal and cutoff (S/C ratio) is calculated. The more reactive the serum, the higher the ratio of signal to cutoff is for each antigen.

Finally, a score was assigned taking into account the strength and frequency of signal in healthy donor sera compared to cancer patient sera. To this end, the frequency of sera responding to each antigen was determined by counting how many samples displayed signals above cutoff, independently for healthy donors and lung cancer patients. Then, an average of S/C ratio was calculated per antigen for each cohort, to provide an overall estimate of the strength of reactivity observed. In order to avoid overemphasizing individual rare sera with very high responses, the contribution of the signal strength (S/C ratio) score was reduced by taking its cubic root. Finally, for each antigen, the frequency of responders in each cohort was multiplied by the cubic root of S/C ratio averages. Therefore, emphasis was placed on the frequency in each cohort, while still considering strength of signal. For example, if 10% of healthy donors have a response to an antigen, but if most of these responses are only barely higher than the cutoff and therefore weak, they will have a lower score compared to 10% of lung cancer patient sera with high S/C ratios for the same antigen. The final ranking of reactive antigens was determined by the difference between the scores in each cohort (patient − healthy). A high score (>5) indicates that most seroreactivity is attributable to cancer patients.

3. Results

3.1. Screening samples by ELISA

We analyzed a large series of sera from 75 healthy donors and plasma from 900 patients with non-small cell lung cancer for the presence of antigen-specific antibodies by ELISA (manuscript in preparation). Samples were screened for reactivity to various tumor antigens known to spontaneously elicit antibody responses in a proportion of cancer patients, including LAGE-1/CTAG2, MAGEA4, SOX2, SSX1-4, TP53. Based on ELISA results, we selected 22 representative healthy donor sera, mostly negative for all antigens tested, and 31 NSCLC plasma samples, selected for their observed specificity to each antigen. Specific titers were defined in several titration experiments, based on a cutoff determined by dilution curves of negative control sera. Results are shown in Table 1. Sera were considered to be strongly reactive if their reciprocal titer was greater than 1000, weakly reactive if between 100 and 1000, and not reactive if less than 100.

We then asked if antibody responses seen in ELISA could be used to validate a screening approach using protein array technologies, since the antigens tested by ELISA were also present on the cancer antigen microarray described below.

3.2. Initial normalization and array reproducibility

To fabricate cancer antigen arrays, a set of 329 genes identified from the SEREX database (Chen et al., 2000) were cloned in-frame with a sequence encoding a C-terminal *E. coli* BCCP-myc tag (Cronan, 1990) (manuscript submitted), expressed and analyzed for biotinylation. Site-specific biotinylation of the BCCP tag in the cell requires the native tertiary structure (Cronan, 1990; Reed and Cronan, 1991) which is indicative that the entire recombinant protein has adopted a folded and stable conformation (Blackburn and Hart, 2005) (manuscript submitted). This increases the probability of identifying discontinuous epitopes when screening the arrays with sera. Lysates were printed on streptavidin-coated glass slides and recombinant protein affinity-purified directly on the surface.

Arrays were analyzed for seroreactivity with serum or plasma samples from 22 healthy donors and 31 NSCLC patients. Samples were tested on duplicate arrays. The background reactivity of the sera on the arrays was remarkably low, with a median value of 50 rfu, noting that the detection range of the assay is 1–65,000 (2^{16}) rfu. We did not observe any significant difference between serum and plasma samples in background or in overall reactivity to

Table 1
Validation of protein microarrays in comparison to ELISA results for serum reactivity to known immunogenic targets

	CTAG2		MAGEA4		SOX2		SSX1		SSX2		SSX4		TP53	
	ELISA	Seromics	ELISA	Seromics	ELISA	Seromics	ELISA	Seromics	ELISA	Seromics	ELISA	Seromics	ELISA	Seromics
Healthy Pool														
Con-1														
Con-4														
Con-5			181							1.04				
Con-7														
Con-11														
Con-12														
Con-16														
Con-17														
Con-20														
Con-34														
Con-35														
Con-36														
Con-38														
Con-39														
Con-40										1.91				
Con-41														
Con-45			**1162**	**3.11**										
Con-46														
Con-50				1.96						1.39				
Con-52														
Con-54	**114**	**7.65**			**4124**	**4.55**								
Lu-106														
Lu-135														
Lu-175														
Lu-186	**48,843**	**18.17**			**1054**	**3.15**								
Lu-206														
Lu-217	**21,828**	**18.17**												
Lu-224			**3846**	**62.28**										
Lu-255	**18,563**	**7.65**											825	
Lu-295	**4531**	**3.06**		1.36			**1348**	**1.76**	**6849**	**17.62**	**3659**	**5.24**		
Lu-304														
Lu-331	**1150**	**1.29**												
Lu-335	**2921**	**18.17**			868									
Lu-358			147											
Lu-360													**413,635**	**13.58**
Lu-363					211									
Lu-366														13.58
Lu-368					**1687**	**6.28**				4.25				
Lu-380														
Lu-383			302		192									
Lu-411	200		174						140		260			
Lu-458									**3860**	**17.62**	**2448**	**2.10**		
Lu-473			**6981**	**62.28**									13,690	
Lu-515	**4498**	**18.17**	**171**	**26.21**									**1302**	**4.63**
Lu-537														
Lu-563	**21,685**	**18.17**											1950	
Lu-641			274								234			
Lu-650														
Lu-653					**6914**	**5.29**								
Lu-745	**23,639**	**4.39**	**2128**	**2.04**	105									
Lu-762			**4249**	**26.21**										
Lu-786			**9723**	**62.28**									**1123**	**7.12**

Healthy donor sera (top) or NSCLC sera (bottom) were tested against 7 antigens (CTAG2, MAGEA4, SOX2, SSX1, SSX2, SSX4, TP53) by ELISA (results expressed in reciprocal titers) or by Seromics (results expressed as the ratio of observed reactivity to cutoff). Bold characters indicate concordance.

control antigens, indicating that either sample source is suitable for analysis, similarly to what had been observed for ELISA.

Standard markers on the arrays, i.e., Cy3-biotin-BSA and IgG/IgM dilutions for titrating reactivity of anti-human secondary detection antibody, were used for quality control as well as for initial normalization. Results from these control markers and antigens showed very good reproducibility within quadruplicates, with an average CV of 5.6%. Furthermore, differences between duplicate arrays tested with the same serum were minor, showing 93.3% concordance on average in raw rfu (CV=9.3%). Out of 116 arrays used in this study, only one did not pass the quality criteria and results were discarded due to uneven reactivity across the slide surface.

A first normalization step was applied to minimize differences due to secondary antibody detection: the average of values corresponding to reactivity against IgG and IgM heavy chains, Cy3 fluorescence markers and buffers, was calculated, and all remaining values within each array were

proportionally adjusted to match this average value. This normalization resulted in an average overall uniform adjustment of only −0.4% (CV=6.3%). This indicated excellent reproducibility of secondary antibody reactivity with various sera across arrays.

3.3. Array normalization and specificity

In order to establish the specificity and cancer preference of serum antibody reactivity to an individual antigen, two criteria are usually applied in ELISA: 1) the specific response of a serum should be greater for the antigen of interest compared to other control antigens, and 2) the response of a serum for the antigen of interest should be greater than that of other control sera. We wanted to apply similar criteria for the arrays, which required additional normalization steps.

One of the major hurdles to overcome when defining specific seroreactivity was to account for biological differences from serum to serum, in terms of individual reactivity to the overall antigenic content of arrays. Indeed, some sera appeared to react strongly only to a minority of antigens on the array, while others had broader and more intense levels of fluorescence at baseline to most antigens (*independently of their reactivity in ELISA*), even after adjusting for marker and secondary antibody fluorescence levels. One explanation for such individual differences may be the inherent seroreactivity to BCCP, the molecule fused to each antigen that enables specific, oriented attachment of proteins to the slide surface. While not strong enough to obscure specific responses within an array, such heightened baseline reactivity for individual sera may artificially create a positive signal when comparing the rfu values from one antigen with those of sera with lower overall baseline reactivity. A major adjustment was therefore necessary in order to compare serological responses for a specific antigen across different patient sera.

Several approaches were tested to normalize serum-specific variations. One was to examine reactivity of sera against BCCP itself, and adjust relative values within an array to match values better across the sera tested. With this method, all values of antigen reactivity with sera are proportionally modified to match an average reactivity to BCCP. While BCCP normalization was successful, another approach was eventually chosen because of its universal applicability: we transformed the data for each array as the percentage of the interquartile difference and then applied a quantile normalization, which does not depend on individual antigens but takes into account the overall reactivity within each slide. In this method, ranking of antigen reactivity per array remains the same but relative ratios among antigens are adjusted so that median and quartile values are identical across arrays.

3.4. Determining positive results

Once results were normalized, the next challenge was to define a cutoff value on which to base the positivity of samples to an antigen on the array. We were guided by our experience with ELISA and applied similar criteria for arrays: we had to determine the level of reactivity for a negative control serum and compare it to a positive reaction. Since it is not possible to predict which sera would be negative or positive for each antigen on the array, we decided to use the lower quartile (25th percentile) of all values obtained for a particular antigen with all sera, as our "baseline" reactivity to this antigen, and the higher quartile (75th percentile) for the representative extent of reactivity observed. This was possible due to the normalization steps that allowed inter-serum comparisons. Considering that most samples will be within a similar low range with few outliers reacting strongly, we used a stringent cutoff based on interquartile differences. For an antigen to be regarded as positive, a serum had to react more than 2.5× the interquartile difference above the 75th quartile. This cutoff definition assumes that there should be a large proportion of sera that do not react within each group, to provide a baseline of reactivity, but it adapts to other scenarios too. Specifically, in cases where a majority of samples react to an antigen equally highly, such as against IGHG1 (heavy chain from IgG, which reacts directly with the secondary detection antibody), the interquartile value of all samples is elevated, and thus none of the samples appear positive with a cutoff. Results should therefore highlight only samples with differential specificity in comparison to other sera.

3.5. Validation and sensitivity

For validation purposes, we first analyzed a serum from representative healthy donor Con-35 without reactivity to any of the antigens tested by ELISA, and a plasma sample from NSCLC patient LU-217 with a strong response to antigen CTAG2 (also known as LAGE-1 protein). Comparing the raw rfu data from the arrays, there was already a clear stronger signal for CTAG2 on arrays incubated with LU-217 plasma than on arrays incubated with Con-35 serum (Fig. 1). After applying all normalization steps, this was confirmed, and ELISA and array results correlated for these two samples.

We similarly confirmed a correlation between ELISA and array results with the majority of samples tested. Out of 7 antigens tested with the 53 samples analyzed, results correlated in 94% of cases (Table 1). Most of the discrepancies were observed with low-titered sera (from 1/100 to 1/1000 by ELISA) that failed to reach the cutoff in arrays.

We also attempted to determine the sensitivity of the array profiling using plasma from NSCLC patient LU-331, who had a low titer in ELISA against LAGE-1/CTAG2 (~1/1000) compared to patient LU-295 with a stronger titer against LAGE-1/CTAG2 (~1/4500). Two serum dilutions were used for these two samples, the standard 1/200 as well as 1/1000. We were able to detect a significant response at both dilutions for LU-295 but only at 1/200 for serum LU-331, still indicating a high degree of correlation between ELISA and array results (not shown).

3.6. Scoring of samples for cancer specificity

Once the assay with arrays was validated based on the comparison with ELISA, we determined the number of positive responses for all plasma and sera against all other antigens. A score was assigned to each antigen based on the difference between NSCLC patients and healthy donors. This score took into account the frequency of response in each cohort as well as the intensity of the signal observed: the

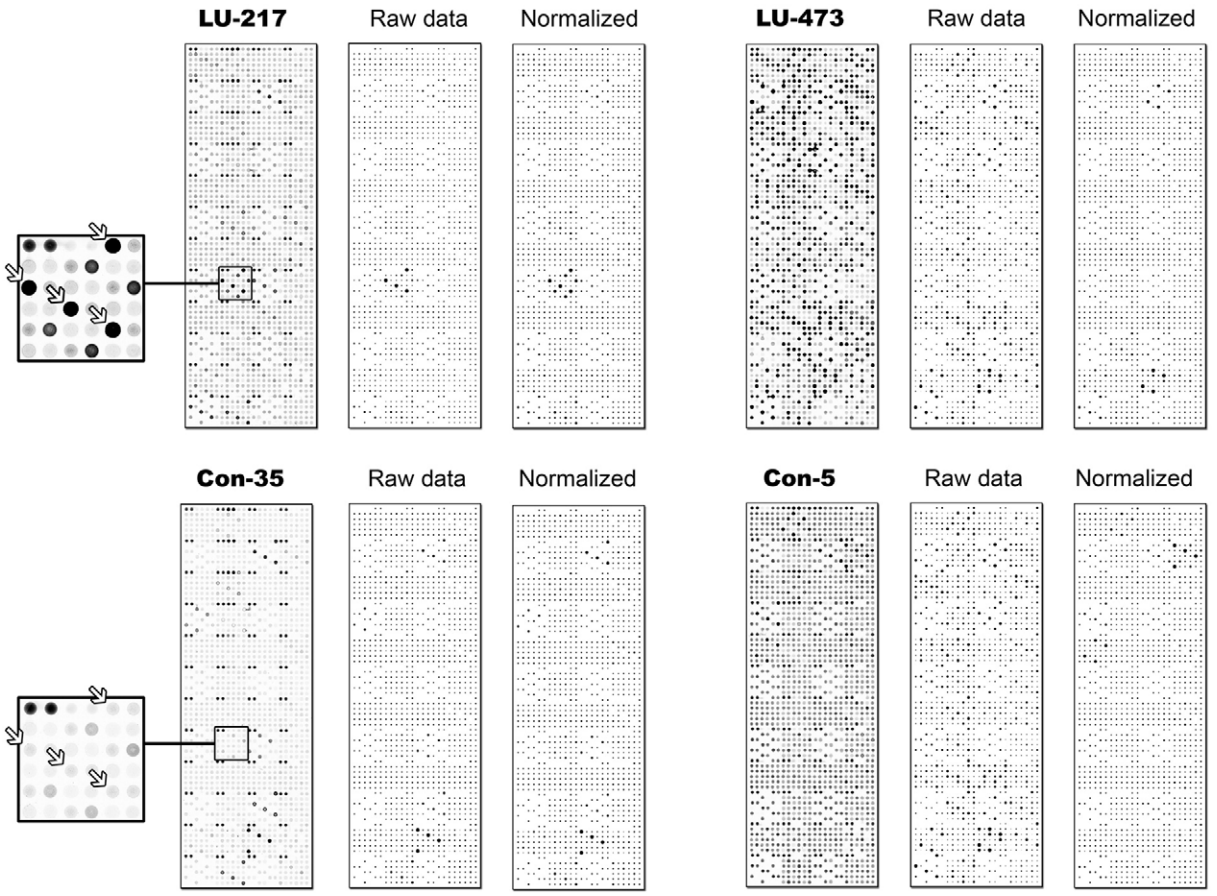

Fig. 1. Examples of normalization procedure. Sera from NSCLC patient LU-217 and healthy donor Con-35 show low overall reactivity to the array while LU-473 and Con-5 have higher overall reactivity. Each patient or donor is represented by 3 panels: the left panel shows scanned image of array at fixed settings, the middle panel ("Raw data") is a visual rendering of the array in which the surface area of each spot is proportional to the intensity observed (only shown for antigens, not controls), and the right panel ("Normalized") is the same rendering following normalization steps. After normalization, spots for each antigen become more comparable to each other across arrays while retaining specific reactivities. Inset with arrows indicates quadruplicate spots corresponding to seroreactivity for antigen CTAG2 in LU-217 and Con-35.

relative contribution of each reactive sera for a given antigen (expressed as the average number of fold over cutoff) was compounded in relation to frequency in healthy donor and cancer patient sera respectively. To avoid overemphasizing the contribution of signal strength alone to the score, the cubic root of this value was used and multiplied by the frequency of response in each cohort. The final score reflected the extent of differential reactivity of cancer and normal sera for a given antigen. A positive score indicated that most seroreactivity was attributable to cancer patients, while a negative score indicated that most seroreactivity was attributable to healthy donors. A score of 0 either meant that no serum reacted in either cohort or that sera from both healthy and cancer cohorts reacted with equal intensity and frequency.

The scores of the antigens selected based on ELISA reactivity were among the highest, confirming that these were found more frequently and more intensely in NSCLC patients, as we expected within our selected cohort (Table 2). The scoring was designed to allow some permissiveness for low titered antibody responses, expected to give a low score over cutoff, while still ranking antigens based on most frequent and strongest antibody recognition in a given cohort. As an example in Table 2, even though the top immunogenic antigen in the list, MAGEA4, was found reactive in 9% (2/22) of healthy donors, these responses averaged a much lower score compared to NSCLC patients (3 vs. 35 fold over cutoff respectively) and were also less frequent (23%, 7/31 NSLCLC patients).

3.7. Extension of analysis to other protein arrays

Given the encouraging data obtained with the cancer antigen arrays, we wanted to determine if this method was applicable to additional antigens using alternative protein array platforms. We also asked whether we could extend our observations of immunogenicity to other cancer types. We used a set of arrays developed for other applications, focusing on functional families of proteins: a kinase array (manuscript submitted), a signal transduction array, a transcription factor array, a cancer-related array (Futreal et al., 2004), and an array with various p53 mutant proteins (Boutell et al., 2004). Together with the cancer antigen arrays, the total number of proteins on all these arrays is 1327. We also compared these

Table 2
List of antigens most associated with serum reactivity from NSCLC patients or healthy donors

Symbol	Average score (ratio over cutoff)	Frequency of response (%)	$\sqrt[3]{\text{Ave Score}} \times$ Freq Resp (%)	Difference Patient–Healthy
	Healthy/Patient	Healthy/Patient	Healthy/Patient	Score
MAGEA4	3/35	9/23	12/74	61
CTAG2	8/12	5/29	9/66	57
TP53	1/10	0/13	0/28	28
SSX3	1/14	0/6	0/16	16
SSX4	1/4	0/6	0/10	10
SOX2	5/5	5/10	8/16	9
SSX2	1/13	14/10	15/23	7

First column of data represents average of reactivity (number of fold over cutoff) for each cohort, second column represents the frequency (freq) of responders in each cohort out of 22 healthy donors and 31 NSCLC patients, the third column represents the product of the cubic root of average reactivity (to attenuate impact of signal strength) and of frequency of responses per cohort, and the final column represents the difference in these products between the two cohorts.
Abbreviation: Ave score = average score from first column; Freq Resp = Frequency of response from second column.

series of arrays with commercially available protein arrays (ProtoArrays® v4.0, Invitrogen).

For this purpose, we used serum pools to represent various cancer types: melanoma, ovarian, prostate, NSCLC, esophageal cancer as well as healthy status. Each cancer serum pool contained 5 sera selected for known immunogenicity by ELISA, to ascertain immunocompetence and have internal validation controls. The five pools were then tested for seroreactivity against the entire range of different arrays. Following the same standardization developed above using quantile normalization, we scored the reactivity on arrays by comparing cancer serum pools with the healthy donor pool.

We could confirm that the use of these arrays was extendable to different serum and plasma samples from patients with various cancers. Once again, antigens expected from ELISA data to react with cancer serum pools gave some of the greatest scores with the multiple array seromic analysis (Table 3). Many of these antigens were present on different arrays (such as TP53, present on cancer antigen arrays, signal transduction arrays, cancer-related arrays, and ProtoArrays®) and still scored with remarkable similarity in their specificity, though with sometimes different sensitivity, further confirming the reproducibility of the technology using our data analysis calculations.

Table 3
Validation of different protein microarrays with pools from various cancer patient sera indicated or healthy sera

Antigen	Array type	Healthy	NSCLC	Melanoma	Ovarian	Prostate
MAGEA4	Cancer Ag	1	59	75	13	0
	ProtoArray®	0	48	64	19	1
SSX2	Cancer Ag	2	2	101	135	2
	Cancer related	1	1	68	29	0
SSX3	Cancer Ag	0	0	13	101	0
	ProtoArray®	0	0	2	12	0
SSX4	Cancer Ag	1	0	2	68	1
	Cancer related	0	0	2	30	0
	Cancer Ag	0	3	0	12	5
TP53	Cancer related	2	49	1	32	101
	ProtoArray®	0	0	0	2	3
	Transcription	4	68	2	59	135

Pools of 5 sera were tested against different type of arrays (Cancer antigen (Ag); Cancer-related; Signaling; Transcription; Invitrogen ProtoArrays®) and compared for reactivity to identical antigens present on more than 1 array. Scores indicate the relative strength of the signal of each serum pool for each antigen in relation to interquartile values per array, after normalization.

4. Discussion

We have designed protein microarrays for the concurrent detection of serum antibody reactivity to multiple SEREX-defined full-length proteins. We used this technology to probe the reactivity of sera from non-small cell lung cancer patients and healthy donors and were able to validate the specific recognition of individual antigens, by comparing it to standard serological methods such as ELISA. We stress here the importance of data normalization and describe a data analysis strategy applicable to any type of protein array for the detection and discovery of immunogenic antigens with cancer specificity.

There are many available methods described for analysis of data generated from both protein and DNA microarrays, including standard and modified *t*-tests (Dudoit et al., 2002; Zhong et al., 2006), empirical methods (Smyth, 2004) and multivariate methods (Ooi et al., 2006). These are typically designed with a proteomic or genomic discovery approach in mind, where the endpoint is to identify targets displaying small-scale changes occurring frequently when two groups are compared. In addition, methods have been published on classification or prediction of samples for diagnostic and prognostic purposes based on data of panels of markers identified, including covariance based methods where the signals of multiple analytes are combined mathematically as opposed to utilizing signals of multiple analytes separately (Golub et al., 1999; Liu et al., 2005; Wang et al., 2005; Zhong et al., 2006). We used a very different approach, based on a serological paradigm, where we wish to identify targets occurring in a limited number of samples with high reactivity, which may be considered outliers in classic statistical models. The variable nature of the biological reagents for antibody profiling, i.e., plasma or serum samples with different intrinsic components, also dictated the use of a strong normalization strategy to allow comparisons across individuals, and stringent cutoff determination. By developing an approach with the goal of identifying rare sera with high reactivity to individual antigens, we introduce a novel data analysis strategy enabling robust measurements of serum reactivity validated by independent methods.

The applications of seromic analysis using protein arrays are multiple and far-reaching, with the potential to redefine the field of cancer immunology. Discovery of immunogenic targets with preferred recognition by cancer patient sera should provide new opportunities for immunotherapeutic interventions, such as vaccine or monoclonal antibody development. From a clinical aspect, the definition of antigen signatures of cancer serum reactivity may be useful for early diagnosis or prove to be of prognostic value for various tumor types. Applied to therapeutic approaches, from chemotherapy to anti-CTLA-4 treatment, seromics can help measure serum-associated changes occurring with treatment, and eventually lead to predictive markers of favorable clinical outcome. Protein arrays may therefore provide an application to guide drug development with physiological rationale. On a molecular level, a humoral response may highlight molecules that underwent mutations triggering an immunogenic event, such as in the case of TP53, and could therefore be used to functionally confirm the accumulating evidence of genomic alterations in tumors.

Our analytical approach was designed to lead to the characterization of multiple previously undescribed antigens recognized more frequently by cancer sera than normal donors, albeit only in a minority of individuals. The hope is that these promising antigens could eventually have an impact on clinical aspects of NSCLC management, something that other serum proteomics approaches have also recognized as sorely needed (Gao et al., 2005; Zhang et al., 2005; Chatterjee et al., 2006; Chen et al., 2007; He et al., 2007; Chapman et al., 2008; Madoz-Gurpide et al., 2008). However, since the cohorts used in the current study were relatively small and selected for presence or lack of immunogenicity to known tumor antigens (for validation purposes), identifying a reliable immunogenic signature of NSCLC will require much larger numbers of sera. Eventually, each antigenic target discovered by seromics should undergo revalidation, back to standard ELISA methods, that are also more suitable for large-scale analyses.

Much as the cancer field embraced the advent of genomic analyses with gene microarrays, we and others (Wang et al., 2005; Casiano et al., 2006; Hudson et al., 2007) are now embarking on defining the cancer immunome using serum profiling, and this will only be achievable if stringent and validated data analysis standards are applied.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jim.2008.10.016.

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