



Research paper

Longitudinal serum autoantibody repertoire profiling identifies surgery-associated biomarkers in lung adenocarcinoma

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ABSTRACT

Background: Autoantibodies against tumor associated antigens are highly related to cancer progression. Autoantibodies could serve as indicators of tumor burden, and have the potential to monitor the response of treatment and tumor recurrence. However, how the autoantibody repertoire changes in response to cancer treatment are largely unknown.

Methods: Sera of five lung adenocarcinoma patients before and after surgery, were collected longitudinally. These sera were analyzed on a human proteome microarray of 20,240 recombinant proteins to acquire dynamic autoantibody repertoire in response to surgery, as well as to identify the antigens with decreased antibody response after tumor excision or surgery, named as surgery-associated antigens. The identified candidate antigens were then used to construct focused microarray and validated by longitudinal sera collected from a variety of time points of the same patient and a larger cohort of 45 sera from lung adenocarcinoma patients.

Findings: The autoantibody profiles are highly variable among patients. Meanwhile, the autoantibody profiles of the sera from the same patient were surprisingly stable for at least 3 months after surgery. Six surgery-associated antigens were identified and validated. All the five patients have at least one surgery-associated antigen, demonstrating this type of biomarkers is prevalent, while specific antigens are poorly shared among individuals. The prevalence of each antigen is 2%–14% according to the test with a larger cohort.

Interpretation: To our knowledge, this is the first study of dynamically profiling of autoantibody repertoires before/after surgery of cancer patients. The high prevalence of surgery-associated antigens implies the possible broad application for monitoring of tumor recurrence in population, while the low prevalence of specific antigens allows personalized medicine. After the accumulation and analysis of more longitudinal samples, the surgery-associated serum biomarkers, combined as a panel, may be applied to alarm the recurrence of tumor in a personalized manner.

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1. Introduction

B cell-mediated humoral immunity gains increasing attention because of the significant effects in cancer progression and the potential in cancer therapy [1,2]. Autoantibodies that against tumor associated antigens (TAAs), are usually associated with cancer progression

Research in context

Evidence before this study

Autoantibodies against tumor-associated antigens could be used for cancer diagnosis and have potential for minorning of treatment response and tumor recurrence. A few TAAs, e.g., p53, NY-ESO-1, and etc., were identified as treatment-associated antigens. The levels of the corresponding antibodies usually decrease in response to cancer treatment and, in most cases, arise back when tumor recurs. For one particular TAA, it only covers a small portion of patients.

Added value of this study

We found, through longitudinal serum autoantibody repertoire profiling at a proteome level, that surgery-associated antigens are prevalent in lung adenocarcinoma patients. The identified six antigens are shown at a relatively low prevalence but could be combined as a panel, covering ~35% of lung adenocarcinoma patients. In addition, the autoantibody repertoire is surprisingly stable for at least three months after surgery.

Implications of all the available evidence

Surgery-associated autoantibodies are probably prevalent in cancer patients with high variation among individuals. These findings combined with other evidences implicate the potential of autoantibodies for personalized monitoring of tumor recurrence. In addition, autoantibody repertoires are extremely stable in response to surgery and of high variation among individuals indicating high physical background of autoantibodies in cancer patients. This could help understand the challenge to identify autoantibody biomarkers with high sensitivities and specificities.

antibody shows up in only ~20% of lung cancer patients [24]. Thus, it is difficult to use one or a small panel of autoantibody based biomarkers for all the patients [7]. However, it may enable the possibility of precision medicine for a patient through analyzing and comparing the autoantibodies longitudinally.

Moreover, the effect of B cells on tumor progression is still controversial [1,2,25]. They have multifaceted roles in both promoting and inhibiting tumor progression largely depending on the subsets of the B cells and tumor microenvironment [1,26–28]. Due to the protective effect, autoantibodies provide promising strategies for cancer therapy [27,29–32]. For instance, one study showed that the binding of antibodies to tumor antigens with adjuvants induced dendritic cells to engulf portions of the tumor cells, and eventually activate the T cells to kill the cancer cells [27]. Systematical investigation of the alteration of autoantibody repertoires, and the identification of specific tumor antigens associated with the tumor course may facilitate further characterization of the effect of humoral immunity in cancer progress.

Systematical study of the autoantibody repertoire alteration in response to treatment has been barely reported, and to what extent or how prevalent of the autoantibody repertoire alteration is largely unknown. We hypothesize that many more TAAs besides p53 may exist with low prevalence, and one of the best strategies to identify TAAs for recurrence monitoring is to analyze and compare longitudinal samples collected from the same patient. To prove this concept, we collected longitudinal (start from September, 2017, we are still collecting) serum samples from 5 patients of lung adenocarcinoma who received surgery. All the samples were then probed on a human proteome microarray that contains 20,240 recombinant human proteins [33,34], and the autoantibody profiles were collected for each sample. After comparing the profiles of the samples collected at different time points, especially before and after surgery, from the same individual, we identified several antigens with patterned changes along the time period of serum sample collection. After further study, these antigens may serve as potential markers for alarming the tumor recurrence.

2. Methods and materials

2.1. Patients and samples

The Institutional Ethics Review Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China approved this study, and written informed consent was obtained from each patient and healthy control regarding the usage of sera. Five patients recruited for longitudinal study were subjected to surgery in Department of Thoracic Surgery, Ruijin Hospital. Most of the sera were collected during September 2017–January 2018, some were extended to July 2019. Other lung cancer patients, non-cancer patients and healthy controls were from the same hospital. All sera were stored at -80°C until use.

2.2. Human proteome microarray profiling

The HuProt human proteome microarrays (V3.1) from the same batch were purchased from CDI Laboratories, USA. The microarray was used for serum profiling as described previously [33] with minor modifications. Briefly, the arrays stored at -80°C were warmed to room temperature and then incubated in blocking buffer (3% BSA in PBS buffer with 0.1% Tween 20) for 3 h. Serum samples were diluted 1:200 in PBS containing 0.1% Tween 20. A total of 3 mL of diluted serum or buffer only was incubated with the array overnight at 4°C . The arrays were washed with PBS-T and bound autoantibodies were detected by incubating with Cy3-conjugated goat anti-human IgG and Alexa Fluor 647-conjugated donkey anti-human IgM (Jackson ImmunoResearch, PA, USA), the antibodies were diluted 1:1000 in PBS-T, and incubated at room temperature for 1 h. The HuProt arrays were then washed with PBS-T and dried by

and could be applied for cancer diagnosis, monitoring of treatment and immunotherapy [3–5]. Although, the mechanism of autoantibody generation is not fully understood, cancer patients do produce antibodies against proteins that are either with mutations, misfolded, abnormal expression or with altered post-translational modifications [3,6]. A set of TAAs have been identified, accordingly, autoantibodies widely exist in a variety of types of cancers, and are fully appreciated as biomarkers for early cancer diagnosis [3,6–9].

In addition, serum autoantibodies have potential to indicate tumor burden, and also to monitor treatment response and recurrence. Studies have shown that the levels of serum antibodies against p53, the most famous and thoroughly studied TAA, usually decrease in response to cancer treatment in a wide range of cancers [10–15], although there are some exceptions [16]. The antibodies against NY-ESO-1 [17], MAGE-B2 [18] and some other TAAs [19] are also similar to that of p53. The change of autoantibody level has also been observed in response to immunotherapy [5,20]. More importantly, the levels of autoantibodies which decreased in response to treatment usually arise back when tumor recurs [11–13,18,21], as indicated the great potential of serum antibodies for tumor recurrence monitoring. However, possibly because of the difficulty for long-term collecting longitudinal samples, and the inefficiency of traditional strategies for biomarker discovery, extensive identification of treatment-associated autoantibodies and thorough investigation of this potential have not been performed yet. It is essential to notice that one remarkable characteristic of tumor-associated autoantibodies is of high variation from one individual to another. Hundreds of TAAs have been identified, however, the prevalence of most of them are relatively low. Additionally, there are huge differences of autoantibody profiles among patients [4,6,22,23]. For instance, anti-p53

centrifugation at room temperature. The arrays were scanned by Lux-Scan 10K-A (CapitalBio Corporation, Beijing, China), and the fluorescent intensity data was extracted by GenePix Pro 6.0 software (Molecular Devices, CA, USA).

2.3. Data analysis of HuProt microarray results

Signal Intensity was defined as median of foreground subtracted by median of background for each spot and then averaged of the duplicate spots for each protein. IgG and IgM data were normalized and analyzed separately. Normalization between arrays was performed using Loess method by the software InfernoRDN [35] following exclusion of the data of positive (Human IgG, IgM and Cy3/Cy5) and negative controls (GST, BSA and printing buffer). The data of several proteins that directly bind with secondary antibodies detected through buffer incubation without any serum were also excluded (such as IGHG1, IGHG3 and so on). Pearson correlation coefficient between two samples was calculated with the command CORREL of Excel software.

On the microarray, serially diluted GST proteins are immobilized as controls, and the fluorescent intensity of GST is linearly correlated with the concentration when probed with anti-GST (data not shown). Since all the proteins immobilized on the microarray are tagged with GST, we used anti-GST signal intensity to correct the influence of the concentration difference among proteins. The cutoff was set as mean +3SD of background of all spots multiplied by a protein concentration correction factor $(1+Si/\bar{S})$. Si is signal intensity of the protein i for anti-GST, and \bar{S} is mean signal intensity of all proteins for anti-GST.

The criteria to call a candidate antigen to which varied levels of antibody after surgery are as follows: For antibodies of continuous decrease, both Pxp/PxA1 and PxA1/PxA3 are over 1.25 or the ratio of Pxp/PxA3 is over 2 either for IgG or IgM, then the microarray images were further manually inspected, to exclude ones with dirty or missed spots or the spots with abnormally high background.

2.4. Fabrication of focused proteins array and serum profiling assays

A total of 76 proteins were selected to fabricate the focused microarray. Recombinant proteins, which are the same as that on the HuProt array, were purchased from CDI laboratories. The proteins, along with negative (BSA) and positive controls (anti-Human IgG and IgM antibody), were printed in triplicate or quintuplicate on PATH substrate slide (Grace Bio-Labs, Oregon, USA) to generate identical protein arrays in a 2×7 subarray format using Super Marathon printer (Arrayjet, UK). Protein arrays were stored at -80°C until use. A 14-chamber rubber gasket was mounted onto each slide to create individual chambers for the 14 identical subarrays. The subsequent assay process was identical to that described for HuProt array assay

with an exception that the incubation volume was reduced to $200\ \mu\text{L}$ per subarray and the dilution of serum was 1:80 or 1:20. For the longitudinal sera, the assays were repeated at least twice under the dilution of 1:20. For cohort 2 and the control group, the serum dilution was 1:80.

2.5. Data analysis for assays performed on focused microarray

IgM and IgG data were analyzed separately. First, the median values of the foreground intensity were extracted from the replicated spots and averaged for each protein. Data of the assays under the same condition was normalized by a linear algorithm, in which the signal intensity for each protein was divided by the mean value of all proteins for the same subarray and then multiplied by the mean value of all proteins from all subarrays.

When investigate autoantibody prevalence, threshold was set based on Z scores that are commonly used in DNA [36] and protein microarray [34] related studies. For a given protein, Z-score in the serum i of cohort 2 was calculated as: $Z_i = (Si - \bar{S})/\sigma$, where Si is the signal intensity of the protein in serum i , \bar{S} and σ are mean signal intensity and standard deviation of the protein across all serums from cohort 2, respectively. In most cases, potential surgery-associated autoantibody for a given patient of cohort 2 was called out when Z score >2 . For anti-PVALB IgM, cutoff was set as $Z > 1.6$.

3. Results

3.1. Serum sample collection and study design

Five lung adenocarcinoma patients (P1, P2, P3, P4 and P5) were recruited (Table 1). From the preoperative computerized tomography (CT) image and blood test for each patient presented in Fig. S1, no obvious bronchial obstruction and infection was observed. Longitudinal serum samples were collected for these patients. The pre-operative serum was collected before or on the day of surgery for each patient. The sera of follow-ups were collected from the day after surgery to about 3 months later with one (for P1, P2 and P3) or two (for P4 and P5) weeks' interval (Table S1). Extended serum samples were also collected for P3, P4 and P5. It is worth noting that P4 and P5 are identical twins with very similar disease histories.

To identify autoantibodies/ autoantigens which are associated with surgery, for each patient, three sera, *i.e.*, pre-operative (Pxp), ~ 1 (PxA1) and ~ 3 months (PxA3) after surgery were profiled by HuProt human proteome microarray (Fig. 1), since the altered levels of antibodies are usually observed within 3 months [12,21]. The signatures of the longitudinal sera were analyzed to demonstrate the extent of change of autoantibody repertoires in response to surgery, and to identify specific antigens to which the serum autoantibody levels

Table 1
Characteristics of the five lung adenocarcinoma patients.

	P1	P2	P4	P5*	P6*
Gender	Female	Female	Female	Female	Female
Age	63	63	56	47	47
Occupational exposure	None				
Smoking status	No smoking history				
Stage	T1aN0M0	T1bN0M0	T2N0M0	T1aN0M0	T1aN0M0
adjuvant therapy after surgery	None	None	chemotherapy started 1 month after surgery	None	None
Comorbidities	None	High blood temperature, HAV	None	surgery for PCT in 2009; excision of mastofibroma in 2015;with uterine leiomyoma	surgery for PCT in 2009 and 2011; excision of mastofibroma for six times and Bilateral mastectomy from 2015 to 2017;with uterine leiomyoma

*P5 and P6 are identical twins; PCT: Papillary Thyroid Carcinoma.

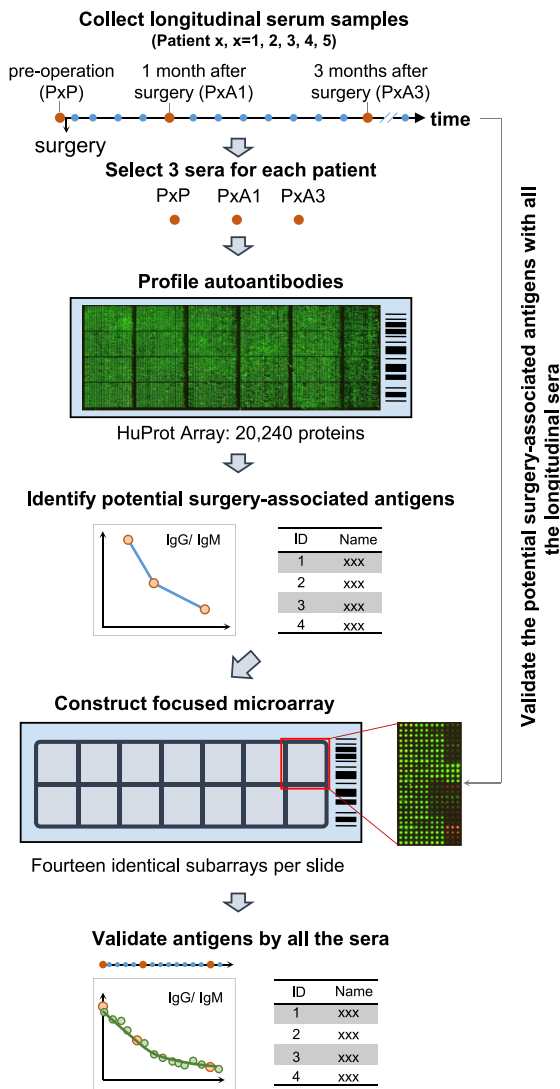


Fig. 1. The schematic diagram and work flow. Serum samples were collected longitudinally from the same patient at different time points before and after surgery. Representative samples were probed on the HuProt proteome microarray to identify candidates of surgery-associated antigens. A focused protein microarray with 14 identical subarrays was constructed by including the candidates of surgery-associated antigens. All the serum samples collected from the same patient were incubated on the focused microarray, some of the surgery-associated antigens were confirmed. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

changed. Since the cost of the HuProt human proteome microarray is high, to analyze and validate the candidates in a more economic and efficient way, a list of candidate antigens was selected to generate a focused microarray. All the serial sera were screened by the focused microarray to validate and characterize the surgery-associated autoantibodies.

3.2. The autoantibody repertoires are extremely steady for at least 3 months after surgery but with high variation among individuals

Three longitudinal samples for each patient were selected to profile autoantibody repertoires using the HuProt human proteome microarray, *i.e.*, the day before surgery, ~1 month and ~3 months after surgery (Fig. 1). IgG and IgM were simultaneously detected for each sample displaying green and red on the microarray image, respectively. Through the overall microarray image, it is clear that the autoantibody (both IgG and IgM) profiles of the same patient are almost

the same among PxP, PxA1 and PxA3. While the autoantibody (both IgG and IgM) profiles of different patients are obviously different (Fig. 2a).

To quantitatively demonstrate the difference between the samples, we calculated Pearson correlation coefficients between any two samples using the normalized microarray data. The Pearson correlation coefficients between sera derived from the same patient are very high both for IgG (0.96 ± 0.032) and IgM (0.942 ± 0.019), similar to that between replicated experiments (Fig. S2). Surprisingly, the correlation coefficients among the pre-operative sera from different patients are 0.425 ± 0.091 and 0.61 ± 0.033 for IgG and IgM, respectively (Figs. 2b–d and S2), which are surprisingly low. Furthermore, the IgM autoantibodies have higher correlations among individuals than IgG autoantibodies, demonstrating higher similarity. The correlation coefficients of IgG between the twins' sera (P4 and P5) are slightly higher than that of others (0.552 ± 0.014 versus 0.411 ± 0.085 , $p < 0.01$, Fig. S2e), whereas it is equivalent for IgM (0.604 ± 0.020 versus 0.612 ± 0.034 , $p = 0.53$, Fig. S2f), suggesting the autoantibody repertoires may be predominantly influenced by individual experiences and also related to inherited background.

To investigate the autoantibody similarity among the sera, we next calculated the shared antigen amounts. A threshold for each protein was determined based on both the signal intensity and the protein concentration (more details in Methods and Materials). The total positive IgG and IgM autoantibodies for all sera were $2,845 \pm 205$ and $3,224 \pm 222$, respectively. The shared autoantibody portion among three sera from the same patient is high for both IgG and IgM (Figs. 2e and S2g–j), whereas it is low for different patients (Fig. 2f). We defined the shared autoantibody as an autoantibody showed positive signal in all the tested samples, and defined the total autoantibodies as a non-redundant collection of autoantibodies showed positive in any of the tested samples. For each of the three groups, *i.e.*, PxP, PxA1 and PxA3, among the 5 samples, we counted the number of the shared autoantibodies, as well as the number of the total autoantibodies. We then calculated the ratios (the number of the shared autoantibodies/ the number of the total autoantibodies), which indicates the shared positive autoantibody portion among individuals. Interestingly, the ratios of shared to total positive autoantibody numbers are significantly higher for IgM (~25%) than that for IgG (~14%) in all the three groups (Fig. 2f). These results indicated IgM autoantibodies are generally more common in population. This is in consistency with the fact that natural IgM autoantibodies are broadly and consistently exist in healthy population [37,38].

3.3. Decreases in autoantibody levels are commonly observed in all patients

To identify the antigens to which the autoantibody levels vary in response to surgery, we compared the autoantibodies levels before and after surgery for each patient. We can test more samples on the HuProt microarray to identify these antigens, however, the cost of this microarray is too high to be applied in the analysis of a large amount of samples. Thus, we decided to construct a focused microarray that contains 14 identical subarrays on a single microarray with only some selected candidates, then perform the validate on this microarray with an independent cohort of samples. To cover the true surgery-associated antigens that may have subtle change in antibody level as many as possible, we first set a lower criterion, *i.e.*, the signal intensity ratios of PxP/PxA1 and PxA1/PxA3 are over 1.25 or PxP/PxA3 is over 2 either for IgG or IgM. In addition, the microarray images were further manually inspected to exclude proteins with dirty or missed spots or the spots with abnormally high background. A total of 151 proteins (Tables 2 and S2) with decreased antibody level were identified, 57 proteins with higher signal intensity in pre-operative sera and larger range of variation were selected. In addition, five proteins, to which the antibody levels are sharply increased

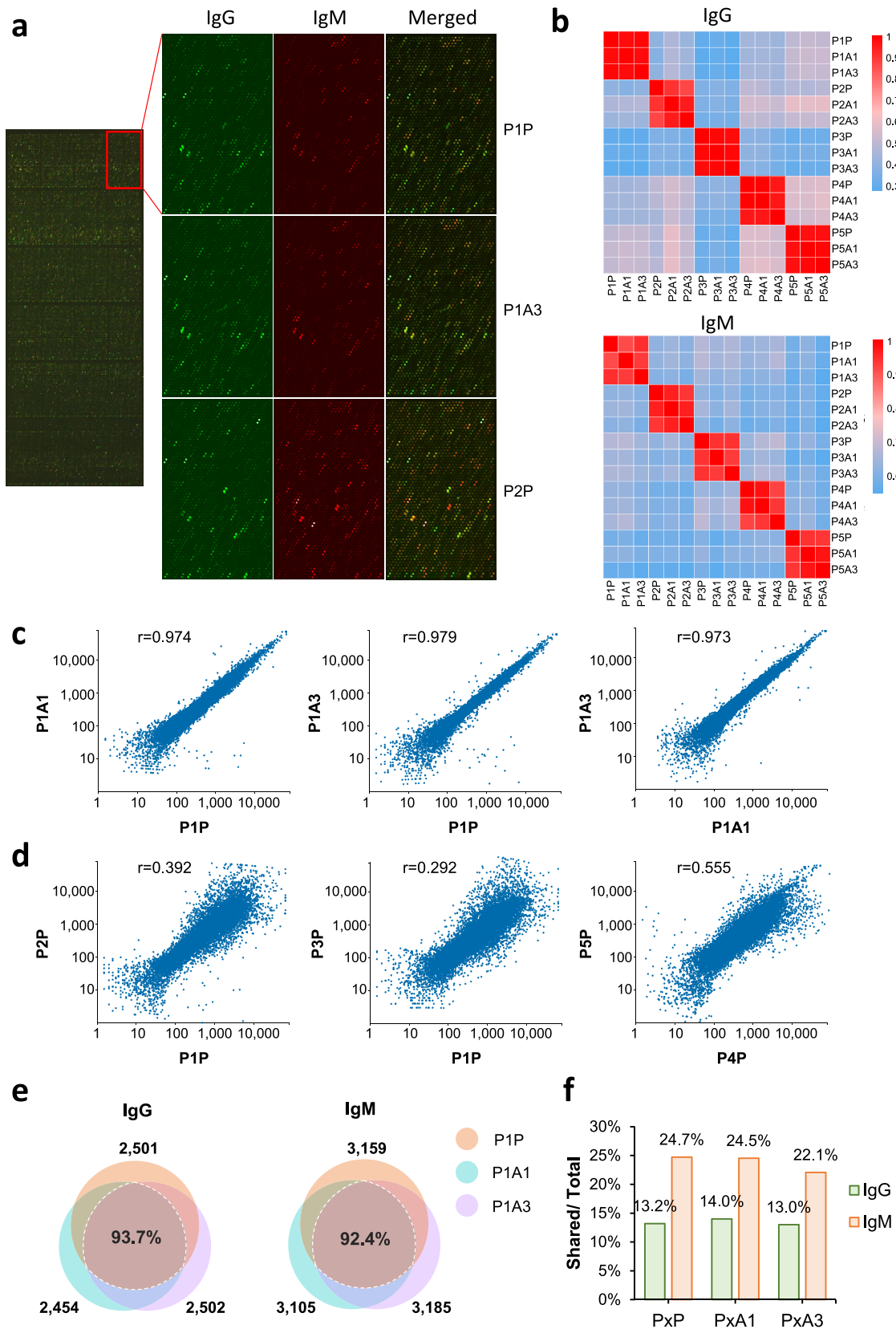


Fig. 2. The serum autoantibody repertoires of the lung adenocarcinoma patients. (a) Representative proteome microarray results. (b) Pearson correlation coefficient matrix of IgG (upper) and IgM (lower). Each square represents the correlation coefficient of auto-antibody repertoires of two sera. (c) The correlations of the overall IgG signal intensities among samples from the same patient. (d) The correlations of the overall IgG signal intensities among samples from different patients. (e) The amounts of the positive autoantibodies (IgG and IgM) and the shared portion (the ratio of shared to total) of the three longitudinal sera from P1. (f) The ratios of shared/total positive autoantibodies of 5 sera for IgG and IgM in three groups, which are pre-operative (PxP), 1 month after surgery (PxA1) and 3 months after surgery (PxA3), respectively (x is the number of the samples, i.e., 1, 2, 3, 4 or 5). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 2
Summary of surgery-associated candidate autoantigens.

	Hits of proteome microarray screening			Selected for further evaluation		
	IgG	IgM	Total	IgG	IgM	Total
P1	25	5	30	7	3	10
P2	33	15	48	13	5	18
P3	24	9	33	13	8	21
P4	7	19	26	3	7	10
P5	16	25	41	2	10	12
Overlapped	-14	-13	-27	-8	-6	-14
Total	91	60	151	30	27	57

after surgery in some patients and a set of 12 additional proteins, including proteins with no change of antibody levels and known TAAs, are also included on the focused microarray as controls.

All the sera collected from the five patients, 68 in total (Table S1), were tested on the focused microarray. For the potential surgery-associated autoantibodies, their increase or decrease could be confirmed through the analysis of samples collected at more time points from the same patient. Six autoantigens, *i.e.*, LSP1, RGS20, and SNRPA were validated for the first patient (P1), LSP1 for P2, CDH12 for P3, PVALB for P4 and SPP1 for P5 (Figs. 3 and S3). For example, the results of HuProt microarray showed that the IgG autoantibody signal against LSP1 is gradually decreasing from P1P to P1A3, while there was no change for the surrounding spots (Fig. 3a). Obvious decrease was observed for anti-LSP1 IgG started immediately after surgery and the trend of decrease continued for a long period of time, whereas there was no change for anti-RHOD IgG and anti-HRAS IgG, these results indicated that the change of autoantibody levels is antigen specific (Fig. 3b). It is worth noting that the level of anti-LSP1 IgG in P2 also continuously decreased after surgery, but not in other patients (Fig. 3c). For P1, the IgM autoantibodies against RGS20 and SNRPA also significantly decreased after surgery (Fig. 3d and e). But anti-p53 IgG that was positive in sera of P1 before surgery had no significant change within three months after surgery (Fig. S3a), suggesting the surgery-associated autoantibodies may be independent to each other and had different mechanisms. For P5, anti-SPP1 IgG rapidly decreased after surgery and remained at a low level for at least 443 days (Fig. 3f). For P5, anti-PVALB IgM slowly decreased and 443 days later, this signal is very low and comparable as in the other patients (Fig. 3h). For P3, anti-CDH12 IgM gradually decreased to a low level about 100 days later and kept constant. About 14 months after surgery (422 days), bone metastasis was found by CT scan and then confirmed by biopsy, followed by chemotherapy, however there is no significant rebound of the autoantibody level. While Anti-CDH12 antibody response was not detectable in other patients (Fig. S3b). For all the autoantibodies mentioned above, the other autoantibody isotype (IgM or IgG) of the same patients remained unchanged during the process for most cases, suggesting that the change in response to surgery is antibody isotype specific (Fig. S3). These results demonstrate that the existence of surgery-associated autoantibodies/autoantigens is ubiquitous in lung adenocarcinoma patients and individual specific.

3.4. The biomarkers are of low prevalence but complementary in lung adenocarcinoma patients

The surgery-associated autoantibodies consistently tend to have a higher level in pre-operative sera from the corresponding patients than from other patients, except for anti-LSP1 IgG in P2 (Fig. 3), suggesting that the initial signal intensity in pre-operative sera could be used as an indicator to predict whether it could be a surgery-associated marker for a specific patient. Based on this observation, we roughly evaluated the prevalence of the autoantibodies as surgery-associated biomarkers, through detecting the levels of the antibodies of pre-operative sera from a cohort of 45 lung adenocarcinoma

patients (Table S3). Not surprisingly, the levels of autoantibodies varied in cohort 2 (Fig. 4). To calculate the prevalence of a specific surgery-associated autoantibody, a strict threshold was set based on both the signal intensity distributions of cohort 2 and the signal intensity of corresponding patient(s). For each autoantibody, z scores were calculated. In most cases, potential surgery-associated autoantibody for a given patient of cohort 2 was called out when Z score >2 (Fig. 4). The prevalence of each autoantigen in lung adenocarcinoma patients is low (2%–14%), but they are independent to each other. To sum up, a total of ~35% (16/45) lung adenocarcinoma patients have one of these 6 autoantibodies as surgery associated markers (Fig. 4g). These results suggest a combined panel of surgery-associated autoantigens may serve as a general marker for lung adenocarcinoma.

To investigate whether the identified autoantibodies are tumor-specific, we tested the antibody responses in 45 patients with non-cancer lung lesion and 46 healthy controls. The benign group consists of 21 patients with fibrous hyperplasia, 6 with hamartoma, 4 with pulmonary bulla and 11 with other lung diseases. The patients from both groups have similar age ranges (56.3 ± 13.7 and 56.9 ± 11.2) and gender ratios (female to male ratio of 26:19 and 23:23) with the tumor group. In most cases, the signal intensities of the antibodies are generally higher in tumor group than benign and healthy control groups (Fig. S4). Under the cutoff values set same as in Fig. 4, these autoantibodies also have a higher positive rate in tumor group than healthy controls while for anti-LSP1 (Fig. S4a) and anti-PVALB (Fig. S4c), some control sera have high signal intensities. These data suggest that the autoantibodies are associated to tumor to a certain extent.

4. Discussion

In the present study, through longitudinal serum autoantibody profiling by HuProt human proteome microarray, we found that the serum autoantibody repertoires are extremely stable, even after the surgery of lung adenocarcinoma. We also confirmed the high variation of autoantibody repertoires among individuals. We further investigated the proteins with decreased antibody responses after tumor excision or surgery which we named surgery-associated biomarkers. Through the proteomic screening by longitudinal sera from 5 lung adenocarcinoma patients, we preliminarily identified a total of 151 candidates, 57 of which were selected for further validation. Under a stringent criterion, 6 proteins were then verified to have dramatically decreased antibody responses and each patient had 1–3 such autoantibodies correspondingly. In addition, by a larger cohort that consists of lung adenocarcinoma patients, benign and healthy controls, we observed that these 6 autoantibodies had a low prevalence. Based on the observations and preliminary data, we found that the surgery-associated autoantibodies widely exist in lung adenocarcinoma patients, but the specific antigens are highly variable among individuals.

Protein microarray is a powerful tool for serum autoantibody profiling, one marked advantage of which is its capability of parallel analysis and compare a set of samples in a short period [39,40]. The HuProt microarray is a commercial product, and the current version

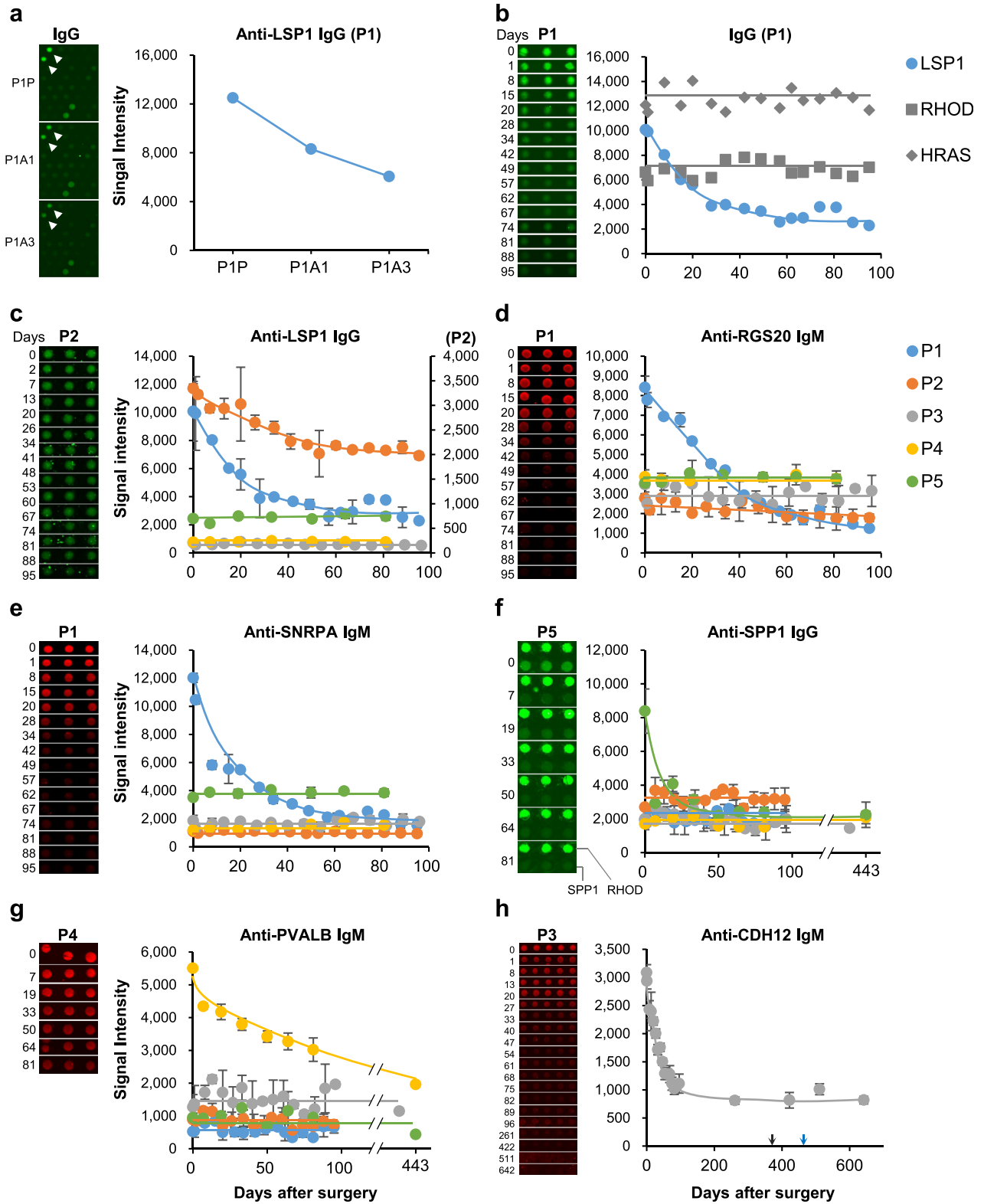


Fig. 3. Several serum autoantibodies with level decreased in response to surgery were validated. (a) The serum levels of anti-LSP1 IgG in samples of P1 according to the results of HuProt microarray. The gray arrows indicate the spots of LSP1 in the microarray, and the corresponding quantitative signal intensities are shown in the chart on the right. (b) The microarray segments of anti-LSP1 IgG for all the 14 sera from P1 detected by the focused microarray, the autoantibody levels of two unrelated proteins, *i.e.*, RHOD and HRAS, were included as controls. (c–h) Other examples, *e.g.*, Anti-LSP1 IgG, anti-RGS20 IgM, anti-SNRPA IgM, anti-SPP1 IgG, anti-PVALB IgM and Anti-CDH12 IgM. “Days” mean days after surgery. All experiments were performed at least twice, the mean and standard deviations were calculated.

contains 20,240 recombinant proteins and providing an ideal platform for studying the autoantibody repertoire at proteome level. This microarray has been widely applied in protein- molecule interaction

identification [41–43], monoclonal antibody specificity evaluation [44] and serum autoantibody profiling [33,34,45]. Herein, we applied this tool for serum autoantibody profile to investigate the extent of

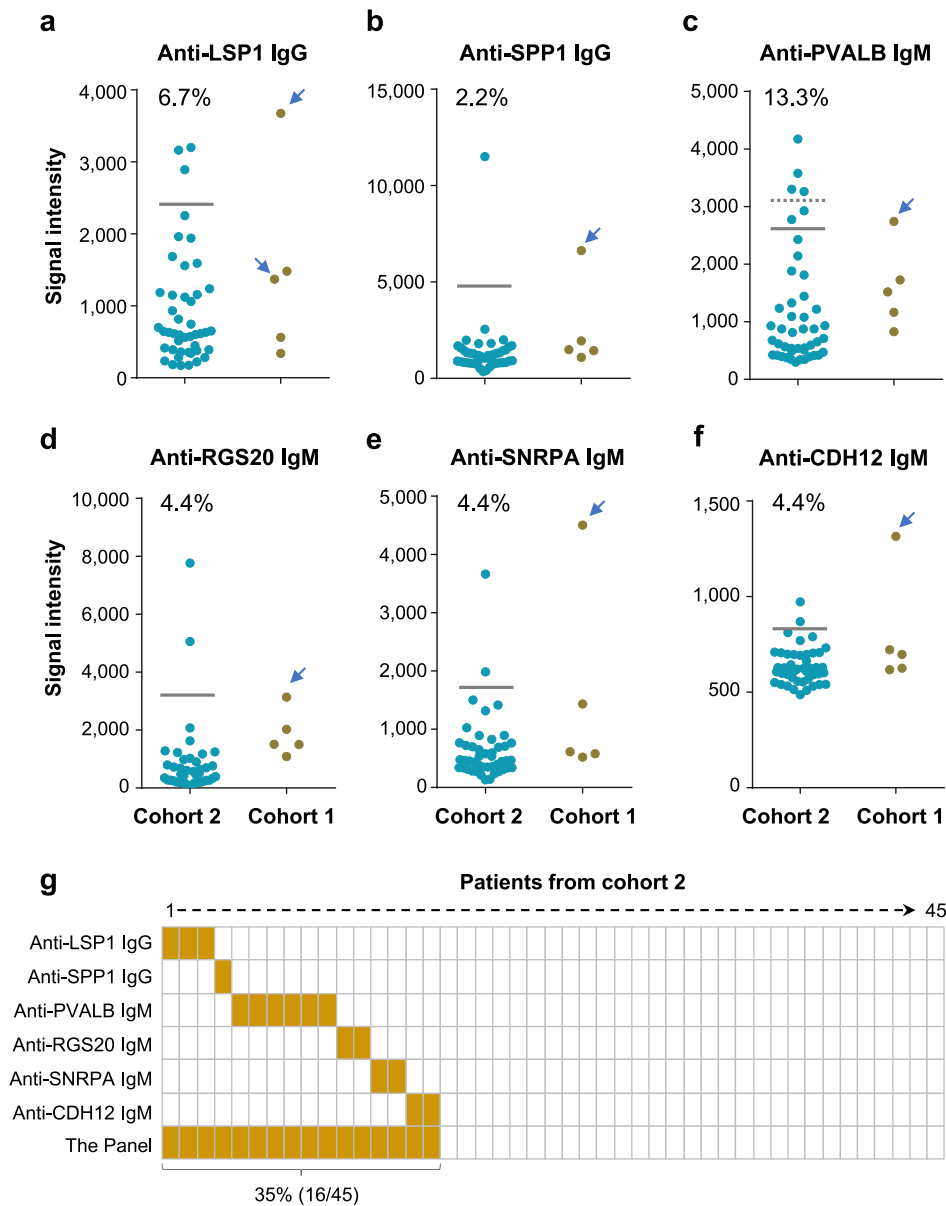


Fig. 4. Prevalence of the surgery associated autoantibodies in lung adenocarcinoma patients. Prevalence of the surgery-associated autoantibodies of anti-LSP1 IgG (a), anti-SPP1 IgG (b), anti-PVALB IgM (c), anti-RGS20 IgM (d), anti-SNRPA IgM (e) and anti-CDH12 IgM (f). Each spot represents one pre-operative serum (probed on the focused microarray at a dilution of 1:80) either from Cohort 1 (P1, P2, P3, P4 and P5) or Cohort 2 (45 patients). The blue arrows indicate the patients whose autoantibody levels decreased after surgery, the numbers indicate the positive rates, and the gray bars indicate the threshold values. $Z > 1.6$ for anti-PVALB IgM (c) and $Z > 2$ for others. The dashed line indicates signal of $Z = 2$ in (c). (g) The distributions of the 6 antigens in cohort 2 and the coverage as a panel. Each rectangular box represents one sera with positive signal indicated in brown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alteration of autoantibody repertoire upon surgery, to identify surgery-associated autoantibodies and to reveal the differences among individuals.

It is known that antibody repertoires are stable in sera of healthy populations, which may result from a long half-life of 21–30 days of immunoglobulins in sera, and also due to the existence of long-lived plasma cells [46,47]. However, to our knowledge, globally, the change of autoantibody profile in response to cancer treatment has not been reported. We find that the autoantibody repertoires are extremely stable after surgery, suggesting a widely existing physiological background of autoantibodies. In addition, the autoantibody signature is of high variation between individuals, as is in consistency with the previous studies that the autoantibody repertoires are variable in healthy people [46,48]. Compared to IgG, IgM autoantibodies are shared more frequently across individuals, which is in consistency with the knowledge that IgM is the predominant isotype of natural

antibodies recognizing a variety of antigens with lower affinities [37,38,47,49]. Based on these previous studies and our findings, we hypothesize that the structure of the autoantibody repertoire (IgG and IgM) consists of three parts. The first part is the natural autoantibodies that arise in an antigen-independent way, majorly IgM. The second part is disease related antibodies, such as TAAs, induced by disease or other associated factors. The third part is physiological autoantibodies targeting specific self-antigens. The possible functions of the autoantibodies include the maintenance of homeostasis [50] and the protection against inner pathological factors [27]. For instance, Carmi and coworkers found that natural IgGs targeting tumor surface proteins exist in the serum of healthy people and account for the effect of allogeneic tumor rejection [27]. The high variabilities among individuals may result from the different physiological experiences and the variable clonal expansion process. Furthermore, the high background and high individual difference of

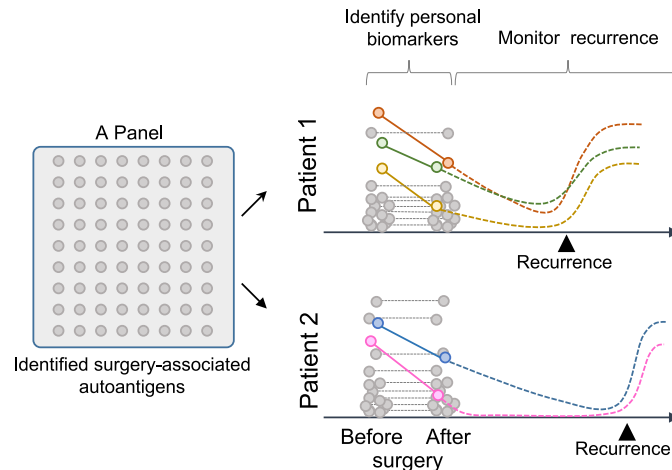


Fig. 5. A model of potential application of surgery-associated autoantibodies for personalized tumor recurrence monitoring. A panel of pre-identified surgery-associated antigens that derived from a larger cohort study may cover most, if not all, lung cancer patients. For a specific patient, antibodies against all the antigens of the panel in the sera of both before and after surgery are detected and compared to define the patient specific biomarkers. These serum biomarkers could be continuously monitored so as to alarm the tumor recurrence when they tend to increase. Each spot represents a surgery-associated antigen previously identified by a larger cohort and the colored spots indicated the effective ones for the specific patients.

serum autoantibodies confirms the difficulty to identify and verify sole TAA as biomarker for the diagnosis of specific type of cancer, such as lung cancer. Interestingly, the similarity of the IgG autoantibody repertoires between the identical twins (P4 and P5) are slightly higher than that between the others, suggesting autoantibody repertoires are partially influenced by inherited background. This study may facilitate the understanding of the effect of inherited background on B cell repertoire dynamics, in response to vaccination [51,52] or under some particular autoimmune diseases [53].

Serum autoantibodies have the potential to indicate tumor burden, thus, could be applied to monitor treatment response and recurrence. Several studies reported decreased antigen-specific autoantibody titers in response to cancer treatment, and there are a set of identified antigens, e.g., p53 [11,12,14], NY-ESO-1 [17], MAGE-B2 [18] and so on. Theoretically, the reason of the autoantibody decrease is thought to be the remove or reduction of the antigen together with the tumor to prevent the continuing stimulation of the immune system [24]. One possible contributor is tumor infiltrating B lymphocytes (TIBs) which could be activated by the tumor antigens and secreted antibodies. Removal of tumor tissue together with TIBs eradicates the resource of the autoantibodies that could be transferred to blood [18,21]. However, for the recognition of TAAs, it is not always in consistence between the serum and supernatant of cultured TIBs [22]. In addition, no IgMs but IgGs could be detected in the serum of SICD mice engulfed with human tumor tissue that contains TIBs, [22], however, tumor-associated IgM autoantibodies are not rare [3,54]. We found in this study, that 4 of 6 autoantibodies are IgMs. These observations suggest there are other resources of the antibodies.

Extensive identification of treatment-associated autoantibodies is barely studied. It is still unclear whether such types of biomarkers are occasional or ubiquitous, irrespective of specific antigens. We found it is fairly common in lung adenocarcinoma patients upon surgery, but the specific antigens largely differ among patients, which is actually in consistence with the low positive rate of a single specific TAA. However, it is possible to identify a single or a set of individually specific autoantigens, which could serve as biomarker for personalized monitoring. In addition, the prevalence of the antigens as surgery-associated biomarkers is generally low while they are independent to each other. It is possible that the combination of these antigens with those from previous studies and some new antigens identified by more patients could form a panel with a high coverage. Thus it is possible that autoantibodies could be widely applied

for monitoring the treatment response and recurrence of cancer patients that were subjected to resection.

We proposed a model to address how to apply the surgery-associated autoantibodies to precision medicine in terms of monitoring tumor recurrence (Fig. 5). More surgery-associated antigens could be identified by profiling sera from more patients using our strategy and constitute a panel to cover most, if not all, lung cancer patients. For a patient, the binding of serum autoantibodies to this panel of antigens could be assessed by appropriate means, for example, protein microarray. We can compare the binding patterns of sera before and after surgery, thus identify potential biomarker/s which is patient specific. These serum biomarkers could be continuously monitored so as to alarm the tumor recurrence when they tend to increase. Certainly, further studies are needed to answer the remaining questions, for example, why there is no expected increase for some of the surgery/treatment-associated autoantibodies at the time of tumor recurrence [12,16]. In our case, it is not clear why the level of anti-CDH12 IgM decreasing after surgery in P3 did not arise when bone metastasis occurred. Our model, for the first time, describes the strategy of autoantibody biomarkers based precision medicine, whereas the concept would be useful for other types of cancer biomarkers both for diagnosis and therapy.

Five proteins to which the autoantibody levels sharply increased after surgery were also selected for further validation, two of which were verified, i.e., anti-VSIG2 IgM in P3 and anti-TSTA3 IgG in P5 (Fig. S5). The level of autoantibodies increased after cancer treatment are barely reported except for the antigens related to autoimmune diseases for immunotherapy [5,20] or tumor associated antigens for chemotherapy [17,19]. It may partially result from that the intracellular proteins are exposed to the immune system because of injured cells during surgery.

In the present study, by analyzing longitudinal sera collected at different time points, remarkable changes of the levels of the identified autoantibodies were observed, providing not only what extent but also what pace and the beginning time of the antibody level changes are. It is anticipated that the half-times of these antibodies could be readily characterized.

There are some limitations. Firstly, the sample volume is small. In one aspect, the identified biomarkers are not fully demonstrated to bring importance for direct application, and further studies are needed. However, we believe the limit number of samples can still successfully elucidate that the existence of surgery-associated autoantibodies, which is not occasional in some cases but prevalent in

cancer patients. In another aspect, since the prevalence of an individual antigen is low, we may fail to identify some surgery-associated autoantibodies in the present study. Further study using more diverse samples are needed to identify more biomarkers and confirm our findings in a broader range. Secondly, the period of follow-ups is limited, so we did not experimentally prove the identified antigens could be applied for personalized monitoring of tumor recurrence. We are keep collecting sera from the five patients, and the samples will be analyzed in future study. In addition, only pre-operative sera were used to roughly assess the prevalence of the identified autoantibodies as surgery-associated biomarkers, so it may could not precisely indicate the exact prevalence, to strengthen this, more longitudinal samples are needed in the future. Last, the antibodies haven't been fully demonstrated to be tumor-related. Although the antibodies tend to have higher signal intensities in lung adenocarcinoma patients than control groups, they are also positive in some control individuals, such as anti-LSP1 and anti-PVALB (Fig. S4a and c), indicating there are additional factors that may responsible for the generation of these antibodies. The observation that the level of the antibodies dramatically decreases in response to tumor resection provided a supporting evidence that the autoantibodies are related to tumor. This emphasized the necessary of comparison the autoantibody level between pre and after surgery to identify the personalized biomarkers in practical application. In addition, for the 5 patients, no bronchial obstruction and infection was observed through CT image or blood test performed before surgery, suggesting the autoantibodies were not elicited by other biological processes in the tumor micro-environment.

Taken together, this study provides an effective strategy to identify personalized surgery-associated autoantibody biomarkers. By analyzing the longitudinal serum samples, we found that the autoantibody repertoires from the same patient are very stable ever after resection of tumor, however, there are dramatic differences between different patients. The antigens we identified in this way are specific to different patients. After further study, these antigens may serve as biomarker that could alarm the recurrence of lung cancer, individually or in combination, thus to some extent, enable personalized medicine. Importantly, since tumor-associated autoantibodies are able to indicate tumor burden, they may have prognosis value. Whether the existence of tumor-associated autoantibodies is beneficial or not is antigen dependent, in another word, is still controversial [55], our study suggests that the antigens with changed autoantibody levels may provide better targets for prognosis and the heterogeneity of the autoantibodies should be fully considered.

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Data availability

The HuProt microarray data are deposited on Protein Microarray Database (<http://www.proteinmicroarray.cn>) under the accession number PMDE239. Additional data related to this paper may be requested from the authors.

Declaration of Competing Interest

Two relevant patents which have been applied in China (application number: 202010053544.X and 202010054978.1) are filed by Shanghai Jiao Tong University and Ruijin Hospital. S.-C. T., Y. L., H.-C. L. and C.-Q. L. are relevant to these patents. Other authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2020.102674](https://doi.org/10.1016/j.ebiom.2020.102674).

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