

Characterization of new serum biomarkers in breast cancer using lipid microarrays

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Abstract Breast cancer is the most common form of cancer among women. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including sensitivity, stability, and easy detection. Anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune diseases, but their potential for cancer diagnosis has not been explored. Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which could be sensed by the immune system and cause the production of autoantibodies. Discovery of anti-lipid antibodies could be used as biomarkers for early breast cancer diagnosis. We describe here a more sensitive and accurate method for lipid microarray detection using dual fluorescent labeling, and used it to examine global anti-lipid profiles in the MMTV-Neu transgenic breast cancer model. We conclude that, at the current technology, lipid microarray is not a preferred method for anti-lipid antibody detection in breast cancer animal models. Our result will help the future application of lipid microarrays in identifying anti-lipid autoantibodies in breast cancer and other human diseases.

Keywords Breast cancer · Lipid · Lipid microarray · Autoantibody · Transgenic mouse model

Introduction

Breast cancer is the most common form of cancer among women. However, despite many advances in technology, imaging, and genomic information, few reliable biomarkers for early breast cancer diagnosis are currently available. Improved prognosis could be achieved with better early screening tests, which would lead to earlier diagnosis. The ideal cancer screening test is one that is minimally invasive, inexpensive, and highly sensitive and specific. Much effort has been expended to identify new markers for breast cancer, including gene expression profiling using oligo or cDNA microarrays [1] or protein expression alteration analysis using mass spectrometry [2]. Another new and promising approach for early detection is to look for the immune response to cancer, instead of cancer itself. Multiple studies have shown that cancer patients produce detectable autoantibodies to tumor-associated antigens that are overexpressed by neoplastic cells [3]. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including increased sensitivity, better stability, and easy detection. Several potential autoantibodies have already been identified in breast cancer, although the ones found thus far are not universally present [3, 4].

The use of autoantibodies as cancer diagnostic biomarkers has been limited thus far to protein antigens. In contrast, anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune disease, but their potential for cancer diagnosis has not yet been explored. Metabolism of lipids immediately follows cellular stimulation, resulting in various lipid metabolites (e.g. diacylglycerol, lysophospholipid, and ceramide). In fact, several lipid metabolic/signaling pathways have been found to associate directly with cancer. For example, fatty acid α -methylacyl-CoA

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racemase, which is involved in peroxisomal β -oxidation of dietary branched-chain fatty acids, is one of the most overexpressed genes in prostate cancer and is directly associated with prostate cancer risk [5–7]. Fatty acid synthase, which synthesizes fatty acids from acetyl-CoA and malonyl-CoA, is highly expressed in aggressive localized and metastatic prostate cancer [8, 9]. Moreover, one of the genes frequently inactivated in prostate cancer is phosphatase and tensin homolog, which controls the production of phosphatidylinositol-3,4,5-triphosphate production and antagonizes the PI3K/AKT pathway [7]. Finally, using magnetic resonance imaging, magnetic resonance spectroscopy, and mass spectrometry, specific changes in choline phospholipid metabolism has been found to associate with more aggressive cancer phenotypes [10, 11].

Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which would be sensed by the immune system and cause the production of novel autoantibodies. These anti-lipid antibodies may be very useful markers for cancer diagnosis and prognosis. Due to technical difficulties, there has been no systemic study on production of anti-lipid antibodies during cancer progression. The technology now exists to explore this question at a “lipidomic” level, using the newly developed approach of lipid microarrays, which exhibit great sensitivity and specificity, and have been used successfully to identify autoantibodies against lipids in multiple sclerosis (MS) patients and in an MS animal model [12]. In the current study, we have developed a dual fluorescent labeling method for lipid array detection, and used this method to look for the potential anti-lipid antibodies in a transgenic breast cancer model.

Materials and methods

Chemicals, lipids, and antibodies

Regular chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Casein was from USB (Cleveland, OH, USA). Odyssey blocking buffer was from LI-COR Biosciences (Lincoln, NE, USA). Fatty acid-free bovine serum albumin (BSA) was from Millipore (Billerica, MA, USA). Lipids were obtained from Matreya (Pleasant Gap, PA, USA), Avanti Polar Lipids (Alabaster, AL, USA), EMD Biosciences (Gibbstown, NJ, USA), and Sigma-Aldrich. They were dissolved in the solvents suggested by the manufacturers at a working concentration of 200 μ M (see Supplementary Table 1). Rabbit asialo-GM1 antibody (#1950) was from Matreya. GD3 mouse monoclonal antibody (#MAB2053) was from Millipore. Goat anti-

rabbit and mouse IgG conjugated with IRDye 800 or horseradish peroxidase (HRP) were from Rockland Immunochemicals. Alexa 680-conjugated goat anti-mouse and anti-rabbit IgG were from Invitrogen (Carlsbad, CA, USA).

Preparation of lipid arrays

Of each lipid, 200 pmol (in 20 μ l volume) was spotted on membranes by hand under flowing nitrogen gas using glass micro-pipets. The size of each lipid spots and antibody responsiveness in different experiments are consistent using this spotting method, as demonstrated by two independent experiments in Fig. 1a and b. Printed membranes were stored dry in the refrigerator. Nitrocellulose and regular polyvinylidene fluoride (PVDF) membranes were purchased from BioRad (Hercules, CA, USA). The low fluorescence PVDF membrane (Hybond-LFP) was from GE Healthcare Life Sciences (Piscataway, NJ, USA).

Mice and blood collection

All animal experiments were performed at Stony Brook University animal facility in accordance with AAALAC guidelines and with Stony Brook University Institutional Animal Care and Use Committee approval. FVB/N mice and MMTV-Neu mice on an inbred FVB/N background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The transgenic animals were confirmed by PCR analysis of genomic DNA from tail biopsy. Blood was drawn from the submandibular area of age-matched FVB/N and MMTV-Neu mice using a goldenrod animal lancet from Medipoint (Mineola, NY, USA). After collection, the blood was allowed to clot for 30 min at room temperature in 1.1 ml Z-GEL serum collecting tubes (Sarstedt, Newton, NC, USA). After 1 min spin in a microcentrifuge at top speed, the supernatant was transferred to a new Eppendorf tube and stored in the freezer (-20°C) until use.

Probing lipid arrays

Lipid arrays were blocked overnight at 4°C with a blocking buffer in PBS (5% fatty-acid-free BSA, 1% casein or Odyssey blocking buffer). Each array was incubated with serum sample (1:200 dilution in the blocking buffer with a total volume of 1 ml), and / or the control rabbit anti Asialo-GM1 (1:5,000) or mouse GD3 mouse monoclonal antibody (1:1,000) at room temperature for 3 h. The membranes were washed twice with blocking buffer (10 min each time), and then incubated with secondary antibodies (1:5,000) diluted in the blocking buffer (Alexa 680-conjugated anti-rabbit or anti-mouse IgG, or IRDye

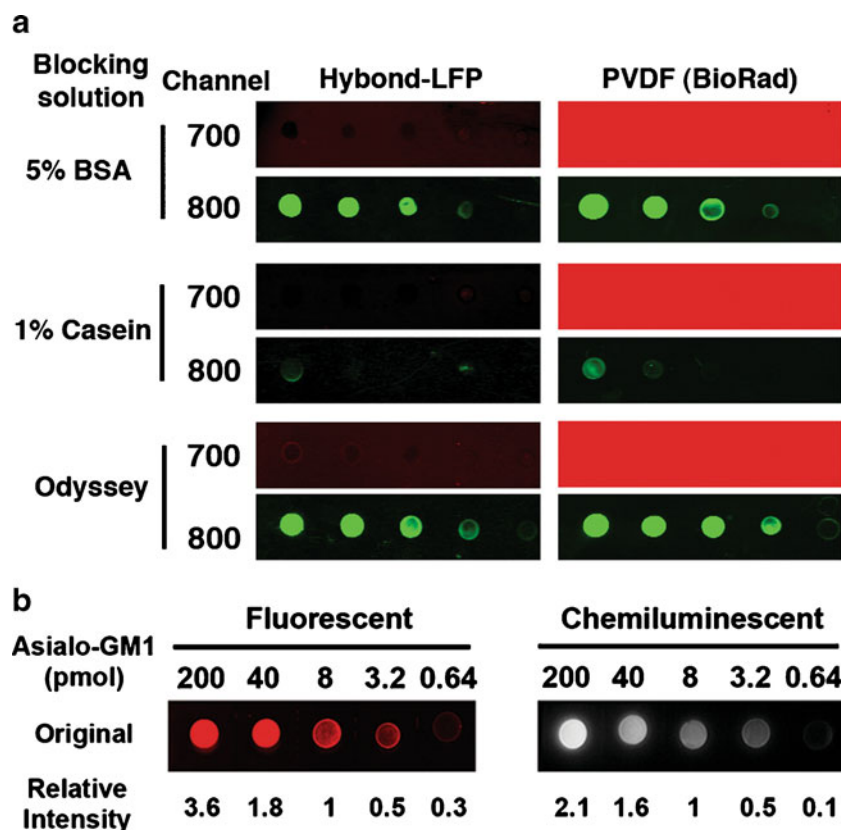


Fig. 1 Improvement of the current lipid microarray methodology. **a** Asialo-GM1 was serially diluted (200, 40, 8, 3.2, and 0.64 pmol) and spotted on either Hybond-LFP or regular PVDF (BioRad). The membranes were blocked in 5% BSA, 1% casein or Odyssey (from LI-COR) blocking buffers, then detected by a polyclonal rabbit asialo-GM1 antibody and followed by IRDye 800 goat anti-rabbit secondary antibody. The membranes were then scanned in both channel 700 (red) and channel 800 (green) using a LI-COR Odyssey instrument. **b** Asialo-GM1 was serially diluted and spotted on Hybond-LFP as above. The membranes were blocked in Odyssey blocking buffer, detected by a polyclonal rabbit asialo-GM1 antibody, and then

followed by a goat anti-rabbit secondary antibody conjugated to Alexa 680 (upper panel) or HRP (lower panel). For the fluorescent detection, the membrane was directly scanned in the channel 700 using a LI-COR Odyssey instrument. For the chemiluminescent detection, the membrane was incubated with the SuperSignal West Pico Chemiluminescent Substrate and the signal was detected by a CCD camera on an Alpha Innotech Gel Imaging System with the FluorChem Q application. The relative intensity values of each spots were compared to that from 8 pmol Asialo-GM1 (assigned an arbitrary unit of 1), based on the intensity values generated by the LI-COR Odyssey or Alpha Innotech Gel Imaging System software

800-conjugated goat anti-rabbit or goat anti-mouse antibodies) for 2 h at room temperature. After two more washes (10 min each time), the arrays were scanned using an Odyssey infrared imaging system from LI-COR Biosciences. For the chemiluminescent detection, the arrays were blocked with Odyssey blocking buffer, and incubated with the rabbit anti Asialo-GM1 and HRP-conjugated goat anti-rabbit secondary antibodies. After two washes, the membranes were incubated with the SuperSignal West Pico Chemiluminescent Substrate from Thermo Fisher Scientific (Rockford, IL, USA) and detected by a charge coupled device (CCD) camera on an Alpha Innotech Gel Imaging System with the FluorChem Q application from Cell Biosciences, Inc. (Santa Clara, CA, USA). The quantification of fluorescent and chemiluminescent signals was performed using software installed on Odyssey and Alpha Innotech Gel Imaging Systems, respectively.

Results and discussion

Improvement of the currently existing lipid microarray methodology

In the lipid array protocol of identifying autoantibodies against lipids in MS patients and in an MS animal model [12], chemiluminescent detection was used to detect antibody reactivity to lipids and glycolipids spotted on PVDF membranes. Chemiluminescent detection relies on an enzymatic reaction to generate light, which is detected by a CCD camera or imaged on film. This enzymatic reaction is dynamic, constantly changing over time. Some samples produce bright light for a short time and others produce comparatively weak light, but for a long period of time. Therefore, images must be collected in a certain time period. This time dependence of the signal compromises quantifica-

tion and accuracy. We decided to improve this technique by using a dual-labeled fluorescent detection, which can be detected by an LI-COR Odyssey infrared imaging instrument.

The first two issues we set up to test were the membrane support and blocking solution. The PVDF membrane was used to spot lipids in the original lipid arrays. However, autofluorescence was consistently high on PVDF membrane when the fluorescently labeled secondary antibodies were used (result not shown). Although the infrared dyes used in the LI-COR Odyssey imaging system is supposed to have low background, the mouse serum generated a very high autofluorescent background. We then decided to test if other membranes could lower the autofluorescent background. Nitrocellulose membrane has an intrinsic low background and is recommended for Western blotting using fluorescently labeled antibodies. However, this type of membrane reacted to some solvents used to dissolve lipids, which generated high background (data not shown). We then chose to use a new type of PVDF from Amersham, Hybond-LFP, which the manufacturer claimed to have low fluorescent background for the Western blotting application. We compared the performance of regular PVDF and Hybond-LFP in three different blocking buffers. Different amounts of asialo-GM1 (200, 40, 8, 3.2, and 0.64 pmol) were spotted on either Hybond-LFP or regular PVDF (BioRad). The membranes were blocked in BSA, casein or Odyssey blocking buffers, detected by a polyclonal rabbit asialo-GM1 antibody, followed by IRDye 800 goat anti-rabbit (green channel) secondary antibody. Although no secondary antibodies were used for channel 700, the background in this channel is extremely high on the regular PVDF membranes in all three blocking conditions (Fig. 1a, right panel). The background in channel 700, however, was minimal on Hybond-LFP (Fig. 1a, left panel). The background in channel 800 was low and was very similar in all blocking solutions on both regular PVDF and Hybond-LFP. Among all blocking solutions, Odyssey blocking buffer was able to detect as little as 0.64 pmol of Asialo-GM1, thus gave the best sensitivity (Fig. 1a, bottom, left panel). We, therefore, decided to use the Hybond-LFP PVDF membrane and Odyssey blocking buffer for our subsequent experiments.

In the previous chemiluminescent detection, 10–100 pmol of lipids were used to prepare the lipid arrays [12] suggesting our fluorescent detection method is more sensitive. To avoid the variations caused by different reagents and blocking solution, we performed experiments to directly compare the chemiluminescent detection using a CCD camera and our fluorescent detection using the Odyssey imaging system. The chemiluminescent signal was detected by one of the most advanced chemiluminescent detection system, Alpha Innotech Gel Imaging System, which can approach the sensitivity of film, while far

exceeding film with respect to linear dynamic range and the ability to obtain quantitative information from the image (Based on Alpha Innotech Application Technique Note 122). Different amounts of asialo-GM1 were spotted in duplicate on Hybond-LFP as described above. After blocking with Odyssey blocking buffer and incubating with the rabbit asialo-GM1 antibody, the membranes were then incubated with goat anti-rabbit labeled with HRP (for chemiluminescent detection), or with Alexa-680 (for LI-COR Odyssey detection), respectively. To compare two different imaging systems, we generated the relative intensity values based on that of 8 pmol asialo-GM1 (Fig. 1b). The signals detected by all antibody-based methods are not linear to the level of their targets. However, both methods were able to reflect the differences of the relative amounts of lipid. Compared to the chemiluminescent detection, one advantage of the fluorescent detection method is the sensitivity. While the chemiluminescent detection barely detected the lowest amount of Asialo-GM1 tested in our experiment (0.64 pmol), the fluorescent detection method generated a stronger or more visible signal from the same amount of lipid. Another advantage of the fluorescent detection is the better dynamic range. In the tested dilution range, the relative intensity value is from 0 (background) to 3.6 for the fluorescent detection, and from 0 (background) to 2.1 for the chemiluminescent detection. In summary, our results suggest that the fluorescent dye-based detection improved the sensitivity of detection as well as provided the better assessment of relative lipid levels.

Array validation

We then validated our lipid arrays using polyclonal and monoclonal antibodies with defined specificities. The polyclonal antibody for Asialo-GM1 bound specifically to GM1, but not to the closely related gangliosides GM2 or GM3 (Fig. 2). The monoclonal antibody against GD3

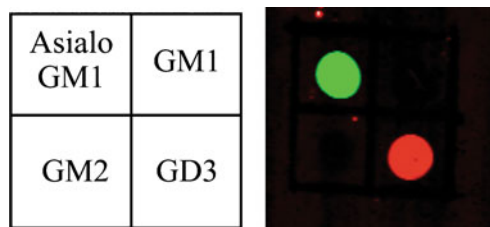


Fig. 2 Dual channel detection of lipids by specific antibodies. Of Asialo-GM1, GM1, GM2 or GD3, 200 pmol were each spotted on PVDF membrane. The membranes were then blocked in Odyssey blocking buffer, incubated with rabbit asialo-GM1 polyclonal and mouse monoclonal GD3 antibodies, and detected by Alexa 680 goat anti-mouse (red channel) and IRDye 800 goat anti-rabbit (green channel) secondary antibodies. Asialo-GM1 and GD3 antibodies have reactivity only to the corresponding lipids. Non-specific signal to irrelevant lipids was not detected

specifically bound to GD3, but not to asialo-GM1, GM1, and GM2 (Fig. 2). Incubation of the secondary antibodies without the primary antibodies did not show reactivity against lipids (data not shown). The specificity of lipid array and dual-color fluorescent labeling thus allows us to label mouse serum samples using one color and the polyclonal rabbit antibody against a lipid (such as asialo-GM1) using another color simultaneously. The reactivity to asialo-GM1 can then be used as an internal control, thus providing a better method to quantitate fluorescent intensity on different membranes.

Profiling lipid-specific antibody responses in sera at different stages of breast cancers

Since breast cancers are very heterogeneous in their causes and outcomes, an experimental system with a defined genetic background will minimize experimental variation. We chose to perform our experiments using the widely-used Neu transgenic model developed by Dr. William Muller [13]. In this mouse model, expression of the rat Her-2/Neu wild-type gene is driven in mouse mammary via an MMTV promoter. Focal mammary tumors first appear at 4 months. Both virgin and breeder mice develop tumors. Tumors arise as foci in hyperplastic, dysplastic mammary glands. Seventy-two percent of tumor-bearing mice that live to 8 months or longer develop metastatic disease to the lung. The experimental procedure is illustrated in Fig. 3 and includes the following steps: collecting sera from transgenic Her-2/neu and wild-type control FVB/N mice at the same age, generating lipid arrays, and probing the anti-lipid reactivity of the sera (Fig. 3).

Thirty-nine individual lipids, such as glycerophospholipids, sphingolipids, sterol lipids or their oxidized forms, were printed in ordered arrays on Hybond-LFP membranes as described in the “Material and methods” section (Fig. 4a). A serial dilution of Asialo-GM1 was used as internal control to normalize the variations in each array (the last row of the array in Fig. 4). To follow the emergence of anti-lipid antibodies, sera were collected before and after visible tumors are observed (4, 6, and 8 months) and assayed at a range of dilutions using the lipid arrays. Sera from wild-type mice at the same ages were used for controls. A rabbit polyclonal antibody against Asialo-GM1 was added to the sera from the experimental and control mice. The lipid arrays were blocked, probed with sera or defined antibodies, and detected using secondary antibodies conjugated to Alexa 680 or IRDye 800. After washing, the arrays were visualized and quantified using an LI-COR Odyssey infrared imaging system. In the red channel, the control rabbit anti-Asialo-GM1/Alexa 680 goat anti-rabbit antibodies detect the asialo-GM1 internal control (the last row), and some other lipids as well (Fig. 4b). The intensities of the internal controls, Asialo-GM1 spots on the lipid arrays, were similar at the same dilutions in both arrays, supporting that there is minimal array variation. Some lipid spots are labeled in the green channel probed with mouse sera/IRDye800 goat anti-mouse antibody, i.e., GM1, GM2, GD3, GD1a, PI(3,5)P2, PI, PGPC, PazePC, POPC, PI(3,4, 5)P3, 18:1 DG, Cardiolipin, LacCer, Cer, GlcCer, and PGE2 (see full names in the supplemental table; Fig. 4b). However, sera from cancer mice at different ages did not show stronger responsiveness to the tested lipids comparing to sera from

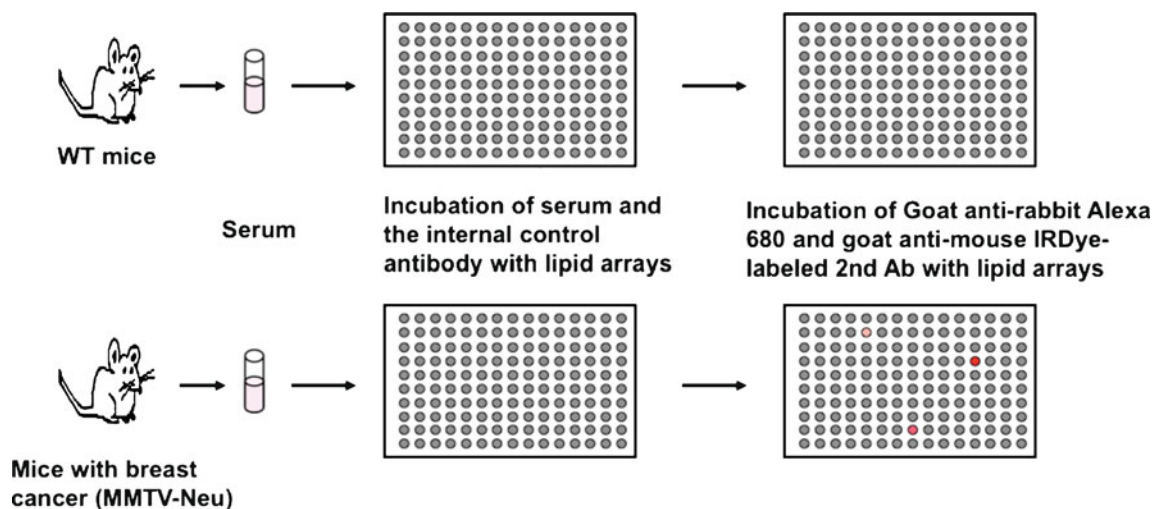
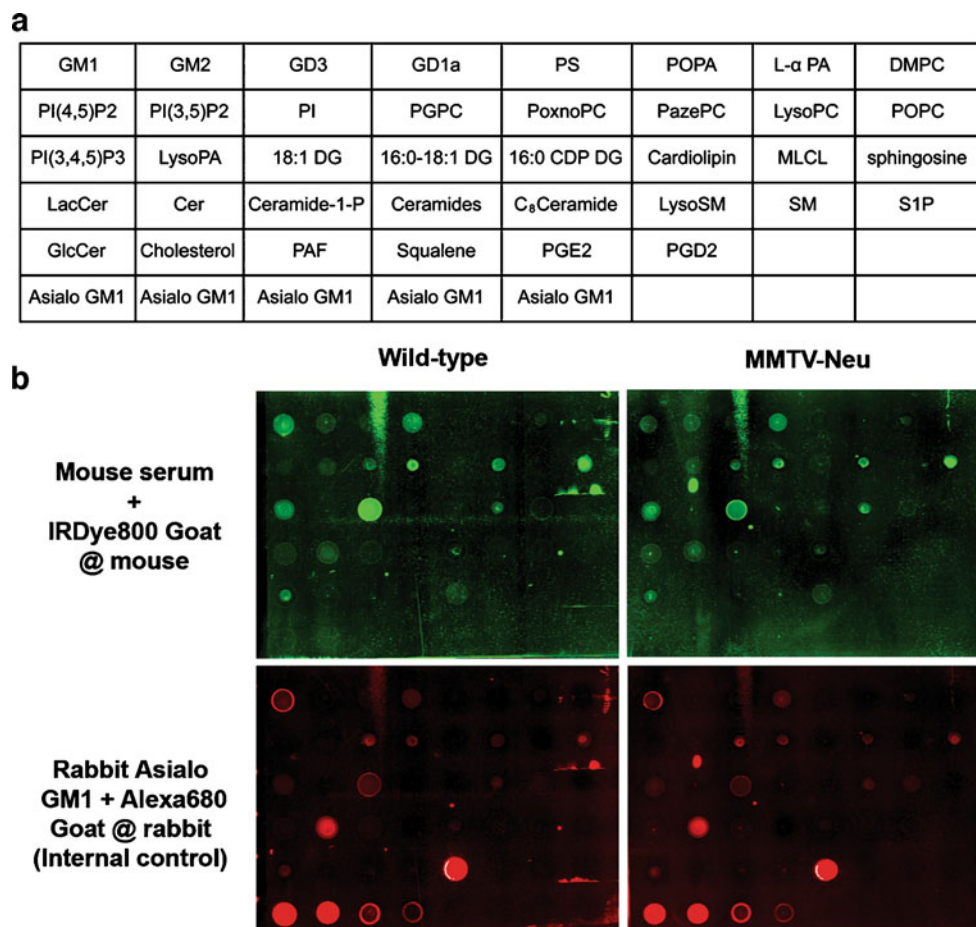


Fig. 3 Steps of detecting specific anti-lipid serum responses in the MMTV-Neu transgenic breast mice. (1) Sera are collected from the age-matched FVB/N wild-type and MMTV-Neu transgenic mice. (2) Membranes spotted with different lipids are incubated with mouse

sera and a rabbit polyclonal Asialo-GM1 antibody (internal control). (3) The membranes were then detected with Alexa 680-conjugated goat anti-rabbit and IRDye 800-conjugated goat anti-mouse antibodies

Fig. 4 Searching for new anti-lipid antibodies in the MMTV-*Neu* breast cancer mouse model using lipid microarrays. **a** Lipids spotted on microarrays. Of each individual lipid, 200 pmol was spotted on the Hybond-LFP membranes. The last row was spotted with a serial dilution of Asialo-GM1, which were used as an internal control for different membranes. **b** No differences in serum responses to lipids between the wild-type and MMTV-*Neu* mice. Membranes spotted with different lipids were incubated with sera collected from the FVB/N wild-type and MMTV-*Neu* transgenic mice and a rabbit polyclonal Asialo-GM1 antibody. The membranes were then detected with Alexa 680-conjugated goat anti-rabbit (*red channel*) and IRDye 800-conjugated goat anti-mouse (*green channel*) antibodies. The anti-Asialo-GM1 intensity was used as an internal control for different membranes



normal mice as expected (Fig. 4b, only the results from 8-month-old mice are shown here). The serum responsiveness to GM1 and 18:1 DG was actually decreased by more than onefold in the cancer mice, suggesting that they might be potential biomarkers for breast cancer, although we cannot explain the decrease of serum responsiveness in cancer mice. The differences of the intensity value of serum responsiveness to the rest of lipids between the normal and cancer mice were less than 1.1-fold. The immunoreactivity of the mouse sera to these lipids may reflect the existence of low level of antibodies against some lipids in mice, or non-specific antibody-lipid binding.

Several possibilities may account for the lack of the serum reactivity in the transgenic breast cancer mice in the current study. First, the number of lipids tested in the current studies is far less than the number of lipids that exists in cells. Lipids are structurally highly diverse owing to the many possible variations of the lipid building blocks and how these blocks are linked. A conserved estimation of the theoretical number of lipids covering major lipid classes is close to 200,000 [14]. Second, not all known cellular lipids have been chemically synthesized or purified from cells or tissues, thus they are not commercially available for preparing lipid arrays. Third, the lipids spotted on the

PVDF membranes may be misoriented and have different conformations comparing to those in cell membranes. The anti-lipid antibodies in the transgenic mice, if there is any, recognize only the lipids in cell membranes, and cannot recognize the “denatured” cognate lipids. Finally, our current detection method has not reached the level of sensitivity to detect the weak immune response that may occur. Based on our current pilot study, we conclude that at present, the lipid microarray technology is not a preferred method for anti-lipid antibody detection in breast cancer diagnosis and prognosis.

Nevertheless, as the major components of cell structure and signaling molecules, deregulation of lipid levels is expected to play important roles in tumorigenesis and trigger immune responses. Indeed, recent reports describe anti-lipid antibody production in cancer patients [15]. In one study, a patient with breast cancer and another with colorectal carcinoma experienced dramatic exacerbation of their pre-existing anti-phospholipid syndrome after surgery [16]. In another study, anti-GD1 IgM was found to be specifically augmented in patients with organ-confined prostate cancer (stage T1/T2), but not in patients with unconfined prostate cancer (T3/T4), benign prostate hyperplasia or healthy people [17]. In principle, lipid microarray

is still the most powerful method to explore potential anti-lipid autoantibodies in breast cancer patients at a “lipidomic” level. Compared to DNA and protein microarrays, the production of lipid arrays, especially the handling of the solvents used to dissolve lipids, is relatively challenging. Development of better lipid array techniques in the future will definitely help to delineate the functions of lipids in cancer and lead to discover some useful lipid or anti-lipid biomarkers. Some recent progress on this topic has shed light on increasing the accessibility of lipid microarray to the research community. Among those, a new method has recently been developed to create micropatterned lipid bilayer arrays using a 3D microfluidic system [18]. The dual fluorescent labeling detection based on the LI-COR infrared imaging system and preliminary results described in our current study would certainly provide some useful information for identifying new anti-lipid autoantibodies in the future.

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