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Serum autoantibody profiling using a natural glycoprotein microarray for the prognosis of early melanoma

Yashu Liu¹, Jintang He¹, Xiaolei Xie¹, Gang Su¹, Seagal-Teitz Tennenbaum¹, Michael S. Sabel¹, and David M. Lubman^{1,*}

¹Department of Surgery, University of Michigan Medical Center

Abstract

The poor prognosis of melanoma and the high-cost of lymph node biopsy for melanoma patients have led to an urgent need for the discovery of convenient and accurate prognostic indicators. Here, we have developed a natural glycoprotein microarray to discover serum autoantibodies to distinguish between patients with node negative melanoma and node positive melanoma. Dual-lectin affinity chromatography was used to extract glycoproteins from a melanoma cell line. Liquid-based reverse phase separation and microarray platforms were then applied to separate and spot these natural proteins on nitrocellulose slides. The serum autoantibodies were investigated by exposing these proteins to sera from 43 patients which have already been diagnosed to have different stages of early melanoma. The combination of 9 fractions provides a 55% sensitivity with 100% specificity for the detection of node positive against node negative and a 62% sensitivity with 100% specificity for the detection of node negative against node positive. Recombinant proteins were used to confirm the results using a sample set with 79 patients with diagnosed melanoma. The response of sera against recombinant 94 kD glucose-regulated protein (GRP94), acid ceramidase (ASAH1), cathepsin D (CTSD), and lactate dehydrogenase B (LDHB) shared a similar pattern to the fractions where they were identified. The glycoarray platform provides a convenient and highly-reproducible method to profile autoantibodies which could be used as serum biomarkers for prognosis of melanoma.

Keywords

Natural glycoprotein microarray; melanoma prognosis; autoantibody profiling

Introduction

Melanoma is a cancer that begins in the melanocytes and causes the majority (75%) of skin cancer-related death¹. In 2009, there were 68,720 new cases of invasive melanoma and 8,650 melanoma-related deaths.² Survival rates are excellent, over 90%, with thin melanoma (<1mm in Breslow thickness), but rapidly decrease with increasing thickness and nodal involvement. Once melanoma metastasizes to the regional lymph nodes, the survival drops to less than 60%.

To select clinically node negative melanoma patients, sentinel lymph node (SLN) biopsy is recommended to determine the presence of microscopic regional nodal metastases. In this case, the patients we studied have already been confirmed to be melanoma patients. The aim of our study is to discover accurate and convenient serum biomarkers to separate between

*To whom correspondence should be addressed. David M. Lubman, Department of Surgery, The University of Michigan Medical Center, 1150 West Medical Center Drive, Building MSRB1 Rm A510B, Ann Arbor, MI 48109-0656. dmlubman@umich.edu.

melanoma patients with node negative and those with node positive disease. The detection and eradication of nodal micrometastases is not only associated with improved outcome, but also helps select patients for adjuvant systemic therapy.³ SLN biopsy is recommended for patients with melanomas 1.0mm or thicker, or for patients with melanoma <1.0mm but with other adverse features such as ulceration, high mitotic rate or angiolymphatic invasion.⁴ Using this criteria, regional micrometastases are identified in approximately 20 to 25% of patients, exposing a large number of patients to the cost and morbidity of the procedure to benefit a small fraction. As with SLN biopsy, adjuvant therapy is offered to a large number of patients even though benefit is realized in only a few. Novel biomarkers that may better stratify early-stage patients by risk of regional and distant recurrence may allow for a more selective approach to surgical and adjuvant therapy.

For many solid tumors, genomic analysis has been investigated as a method of stratifying risk, but for melanoma, where there is rarely primary tumor available for analysis after the initial diagnostic biopsy, this is not clinically feasible. An alternate approach is the use of serum biomarkers. The response of the immune system in patients with different types of cancer has been a recent focus of biomarker research⁵. Tumor cells produce many aberrant proteins and isoforms of proteins with abnormal post-translational modifications (PTMs), all of which are termed as “tumor-specific antigens,” and these antigens may induce an anti-tumor immune response.^{6–8} This may control the growth of the tumor, and escape from immune recognition may be associated with an increased propensity to metastasize. Monitoring the presence or absence of antibodies in the serum that recognize specific tumor antigens can provide insights into the propensity of melanoma to metastasize, serving as biomarkers of melanoma biology and perhaps identifying ideal targets for therapeutic intervention.

Since most of the key molecules involved in the innate and adaptive immune response are glycoproteins⁹, we have focused our targets on glycoproteins and their interacting proteins in this study using dual-lectin affinity chromatography to extract these proteins from a melanoma cell line. Liquid-based reverse phase separation and natural protein microarray were then applied to separate the enriched proteins and spot the separated fractions on nitrocellulose slides. The specific serum autoantibodies which could be used for the prognosis of melanoma were discovered by exposing the slides to sera from patients with different stages of melanoma and control subjects. Mass spectrometry was applied to identify the proteins in these fractions of interest. This method provides an effective means to obtain a panel of auto-antibodies for melanoma prognosis with high reproducibility. Based on the results of protein identification, we performed a confirmation study against a sample set of melanoma patients using full length recombinant proteins.

Materials and methods

Sera

Serum samples were obtained from 79 patients with melanoma (see Table 1). The study was approved by the University of Michigan Institutional Review Board. The collection of clinical data and blood samples was performed only after patients gave informed consent. Blood was obtained from patients between the ages of 18 and 75 with documented melanoma 2 to 3 weeks prior to undergoing wide excision and lymph node surgery (either SLN biopsy if node negative or lymph node dissection if clinically node positive). Samples were also collected from healthy volunteers and patients with either basal cell carcinoma or squamous cell carcinoma (non-melanoma skin cancer) due to surgical excision. Blood was allowed to clot at room temperature, after which the tubes were centrifuged at 600 g for 10 minutes. The serum phase was then harvested and frozen in 1-mL aliquots.

Cell lysis

The cells used in this work were from a melanoma cell line generated from a resected intra-abdominal metastasis that occurred 4 years after the resection of a thin melanoma. Cells were harvested when they reached ~90% confluence and washed once with PBS and then suspended in lysis buffer (1% octyl- β -D-glucopyranoside (OG), 20 mM Tris-HCl, pH7.4, 150 mM NaCl and 1% protease inhibitor mixture (sigma). The cells were homogenized with 30 strokes in a tight-fitting Dounce glass homogenizer. The cell lysate was centrifuged at 40,000g for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined by the Bradford method.¹⁰

Lectin Extraction

A Pierce centrifuge column was packed with 3 mL of agarose-bound Concanavalin (ConA) and 3 mL of agarose-bound wheat germ agglutinin (WGA). The column was first washed with 3 volume of binding buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 1mM MgCl₂, 1mM CaCl₂ and 1mM MnCl₂). Cell lysate containing 20 mg of proteins was diluted four times with binding buffer, and passed through the column twice. The column was washed with 4 volumes of binding buffer to remove the nonspecific binding proteins. Finally, the captured glycoproteins were released with 18 mL of elution buffer (300 mM N-acetylglucosamine and 300 mM methyl- α -D-mannopyranoside in binding buffer, pH 7.4). The eluted fractions were concentrated using Microcon YM-10 protocol to 600 μ l. The protein concentration was then measured to evaluate the yield of glycoproteins. The lectin affinity chromatography was repeated using a cell lysate containing 3 mg of total proteins to evaluate the reproducibility of cell lysis.

Reverse Phase HPLC Separation

An ODSIII-E (8 $\times$ 33 mm) column (Eprogen, Inc.) was used to separate the glycoproteins extracted from the melanoma cell line. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The gradient was run from 5% to 15% B in 1 min, 15% to 25% in 2 min, 25% to 31% in 2 min, 31% to 45% in 10 min, 41% to 47% in 6 min, 47% to 67% in 4 min, 67% to 100% B in 3 min, and reduced to 5% B in 1 min after maintaining 100% B for 1 min. The flow rate was 1 ml/min and the column temperature 60 °C. UV absorption was monitored at 214 nm. The fractions were collected using an automated fraction collector (model SC100; Beckman Coulter) in 1 mL 96-well plates (Greiner Bio-One). Approximately 50% of each protein sample was transferred to a 200 μ L 96-well clear printing plate (Bio-rad) and was completely dried using a speedvac concentrator and stored at -80 °C for the printing of the microarray. The remaining portion of each sample was transferred to a 1.5 mL tube and stored at -80 °C for protein identification.

Microarray printing

The fractions in the 96-well printing plate were dissolved in 50 μ L printing buffer which was composed of 62.5 mM Tris-HCl (pH 6.8), 1% w/v sodium dodecyl sulfate (SDS), 5% w/v dithiothreitol (DTT) and 1% glycerol in 1 $\times$ PBS. The fractions from the printing plate were spotted onto nitrocellulose (Whatman, USA) slides using a non-contact piezoelectric printer (nanoplotter 2 GeSiM). The total volume of each spot is 2.5nL, and the distance between spots was 600 μ m. Printed slides were dried on the printer deck overnight and stored in a refrigerator desiccated at 4 °C if the slides were not used immediately. Each fraction was printed in each block and 14 identical blocks were printed on each slide.

Autoantibody detection

The slides were washed three times with 0.1% Tween-20 in PBS buffer (PBST) and then blocked with 1% bovine serum albumin (Roche) in PBST for one hour. The blocked slides

were dried by centrifugation and inserted into a SIMplex (GenTel Bioscience) multi-array device which divides each slide by 16 wells. The wells separate the neighboring blocks and prevent cross contamination. Each serum sample was diluted 1:200 in probe buffer which consisted of 1% BSA, 0.05% Triton X-100, 0.1% brij-30 (Sigma-aldrich, USA) in $1 \times$ PBS. One hundred microliters of each diluted serum sample were applied to the protein array and the hybridization was performed in a humidified chamber for one hour. The slides were hybridized in diluted serum for 2 hrs using a mini-rotator at 4 °C. After hybridization, slides were washed five times using probe buffer for 5min each time, and then re-hybridized with goat-anti-human IgG (H+L) conjugated with Alexafluor 647 (1 μ g/mL, Invitrogen, Calsbad, CA) for 1hr at 4 °C. The slides were washed five times again with probe buffer for 5 min each and dried. All slides were scanned using an Axon 4000B microarray scanner (Axon Instruments, Foster City, USA). Sera from 27 patients with node negative and 16 patients with node positive were used for the autoantibody detection.

Microarray Data Acquisition and Analysis

Genepix Pro 6.0 was used to extract the numerical data from each spot on the slides. The background subtracted median intensity of each spot was taken as a single data point into analysis. The non-parametric Wilcoxin rank-sum test was applied to assess the difference of the humoral response between sera from different stages of melanoma. A *p* value threshold of 0.05 was used to determine differential fractions for further study. All the statistical analyses were performed using SPSS 13.0.

Protein identification by LC-MS/MS

The proteins of selected fractions were digested with trypsin at 37 °C overnight. The resulting peptides were analyzed by LC-MS/MS using an LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was performed on a Paradigm MG4 micropump system (Michrom Biosciences Inc., Auburn, CA) equipped with a C18 separation column (0.1 mm $\times$ 150 mm, C18 AQ particles, 5 μ m, 120 Å, Michrom Biosciences Inc., Auburn, CA). Peptides were separated with a linear gradient of acetonitrile/water containing 0.1% formic acid at a flow rate of 300 nL/min. A 90 min linear gradient from 5 to 40% acetonitrile was used. The MS instrument was operated in positive ion mode. The ESI spray voltage was set at 2.5 KV, and the capillary voltage at 30 V. The ion activation was achieved by utilizing helium at a normalized collision energy of 35%. The data were acquired in data-dependent mode using the Xcaliber software. For each cycle of one full mass scan (range of *m/z* 400–2000), the three most intense ions in the spectrum were selected for tandem MS analysis, unless they appeared in the dynamic or mass exclusion lists.

All MS/MS spectra were searched against the IPI database (IPI.human.v3.39). The search was performed using SEQUEST algorithm version 27 incorporated in Bioworks software version 3.1 SR1 (Thermo Finnigan). The search parameters were as follows: (1) Fixed modification, Carbamidomethyl of C; (2) variable modification, oxidation of M; (3) allowing two missed cleavages; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 0.0 Da; (6) peptide charges +1, +2, and +3. The identified peptides were processed by the Trans-Proteomic Pipeline (TPP) 11. This software includes both the PeptideProphet and ProteinProphet programs. The database search results were first validated using the PeptideProphet software, and then the peptides were assigned for protein identification using the ProteinProphet software. In this study, both the PeptideProphet probability score and the ProteinProphet probability score were set to be higher than 0.9. This resulted in an overall false positive rate below 1% 12.

Confirmation using recombinant proteins

Full-length recombinant proteins 75 kD glucose-regulated protein (GRP75), 94 kD glucose-regulated protein (GRP94), acid ceramidase (ASAH1), cathepsin D (CTSD), and lactate dehydrogenase B (LDHB) were purchased from Abnova Corporation (Taiwan), and Abcam (USA). The concentration of each recombinant protein was 100 µg/mL. Each recombinant protein was printed in triplicate and 14 identical blocks were printed on each slide. Samples from different groups were perfectly balanced on the 14 blocks of all slides to eliminate bias from block-to-block variation and slide-to-slide variation (Supplemental Fig. S1). The procedure of hybridization of the recombinant protein array was the same as the protein array of these fractions. A sample set containing 79 patients (48 patients with node negative melanoma, 31 patients with node positive melanoma) was used in the confirmation study.

Results

We used the native proteins extracted by a dual-lectin column from the melanoma cell line as baits to detect the presence of autoantibodies in the sera from different stages of melanoma patients. Figure 1 illustrates schematically the methodology employed in this work.

Lectin extraction and HPLC separation

The dual-lectin (ConA & WGA) affinity chromatography was applied to extract both glycoproteins and proteins interacting with these glycoproteins from the cell lysates. The procedure was repeated using independent cell lysates containing 3 mg and 20 mg of proteins. The yields of lectin extracted proteins were 131 µg (4.27%) and 854 µg (4.38%), respectively. We further separated these proteins based on their hydrophobicity through a non-porous RP-HPLC column. Forty-seven fractions were subsequently printed onto nitrocellulose slides for microarray-based autoantibody discovery. The reproducibility of glycoprotein extraction and RP-HPLC separation is shown in Supplemental Fig. S2. The ConA lectin was used to test the existence of glycoproteins in each fraction (Supplemental Fig. S3).

Autoantibody discovery

The 47 fractions separated by lectin extraction and RP-HPLC were used for the discovery of autoantibodies from 27 diagnosed patients with node negative melanoma and 16 patients with node positive melanoma (see Supplemental Table 1).

Here we applied two statistical approaches including Outlier-Sum Statistics and non-parametric Wilcoxon Rank-Sum Test to determine which fractions can be used to distinguish between patients with node negative and node positive melanoma. All the intensities were normalized by median absolute deviation (MAD) and the normalized data were then applied to further analysis. The Outlier-sum test was performed to select the fractions that react with only a subset of the samples in the node negative or node positive group using the background-subtracted median spot intensities. The non-parametric Wilcoxon Rank-Sum Test was performed for each fraction on the array. A list of fractions which exhibited significantly different response to the serum between the node negative and node positive group ($p < 0.05$) was generated through the test. Finally, we found 9 fractions that exhibited different reactivity between the node negative and node positive group (Fig. 2). Patients with node negative melanoma showed a higher humoral response to 3 fractions while patients with node positive melanoma showed a higher humoral response to the other 6 fractions.

The reactivity of these fractions to node negative and node positive can be transformed to combined receiver operating characteristic (ROC) curves using Canonical Discriminant Analysis, which maximizes the separation of the existing groups. The combination of 3 fractions with higher reactivity to node positive melanoma gives an AUROC of 0.833 (a 55% of sensitivity with 100% of specificity, as shown in Fig. 3a), and the combination of the other 6 fractions with higher response to node negative melanoma gives an AUROC of 0.822 (a 62% of sensitivity with 100% specificity, as shown in Fig. 3b).

Protein identification

LC-MS/MS was used to identify the proteins in these 9 fractions and their adjacent fractions. Only proteins with greater than 10% coverage were considered in the analysis and a minimum of three high-scoring peptides were required for positive identification. As expected, multiple proteins were identified in each of the fractions. The identified proteins were screened based on an assumption that their reactivity profile should be consistent with their appearance in the neighboring fractions. If the fraction of interest was unique and did not lie between adjacent reactive fractions then only the highest scoring protein that was not found in adjacent fractions in the separation profile was considered a true hit, since the adjacent fractions did not elicit a humoral response. If the fraction of interest was part of a group of fractions eliciting a positive response within a separation profile, the common protein identified in all these fractions was considered a true hit. Furthermore, MALDI-QIT-TOF (Shimadzu) was applied to reconfirm that the highest peaks of the MS spectrum acquired from each fraction belong to the peptides from the chosen protein. In this manner, we identified 5 proteins including 75 kD glucose-regulated protein (GRP75), 94 kD glucose-regulated protein (GRP94), acid ceramidase (ASAH1), cathepsin D (CTSD) (Fig. 4), and lactate dehydrogenase B (LDHB) from the 9 selected fractions.

Confirmation using recombinant proteins

To confirm our results with a different platform and larger set, we used full length commercial recombinant proteins of GRP75, GRP94, ASAH1, CTSD and LDHB to discover the levels of their antibodies in sera of 48 patients with node negative melanoma and 31 patients with node positive melanoma (Fig. 5 & Table 1). Serum samples of melanoma patients in the discovery set (27 node negative and 16 node positive) were also used in the confirmation set. Sera from 8 patients with non-melanoma skin cancer and 9 healthy subjects were also used together as the non-melanoma group in the experiment (Supplemental Fig.S4). However, we only focused on the difference of serum autoantibodies between patients with node negative and node positive where the purpose of this study is to profile serum autoantibodies to facilitate the prognosis of melanoma.

A saturation test was performed to make sure that the amount of each available auto-antibody in sera is lower than the binding capacity of the specific recombinant protein. The saturation test showed that 1:200 dilution of serum provides the optimal performance for autoantibody response with unsaturated signals and high signal/background ratio.

GRP94, ASAH1, CTSD and LDHB showed a similar pattern of humoral response to the fractions from which they were identified. GRP94 showed higher reactivity to some of the node positive group while the other 3 proteins (ASAH1, CTSD and LDHB) had higher reactivity to some of the node negative group. The antibody level of GRP94 was elevated in the serum from 29% patients with node positive (9 of 31) and 0% patients with node negative (0 of 48). The combination of ASAH1, CTSD and LDHB provides a 40% sensitivity (19 of 48) for the detection of node negative with 100% specificity. The performance of these recombinant proteins on distinguishing between the patients with node

negative and node positive melanoma is shown in a scatter plot of the overall response (Fig. 6a), and the combined ROC curve has an AUROC of 0.746 (Fig. 6b).

Discussion

The profiling of auto-antibodies in the sera of melanoma patients is an attractive concept for the prognosis of melanoma. Antibody responses to melanoma-associated antigens are considered to be important in controlling melanoma progression. Monitoring the level of these serum antibodies may provide a useful clue to estimate the metastatic potential of melanoma. Herein, we investigated the alteration of serum autoantibodies during melanoma progression, using the sera from patients with different stages of melanoma (node negative and node positive). Note that the goal of the present study is to find useful and convenient biomarkers for melanoma prognosis, where the patients have already been diagnosed with melanoma. We therefore only focused on the change of serum autoantibodies between patients with node negative and patients with node positive.

Protein microarrays have been shown to be well suited for the study of autoantibody responses. In a previous study¹³, a two-dimensional liquid-based separation and natural protein microarray was used to screen serum auto-antibodies for the early detection of pancreatic cancer. This method allows these proteins to be printed with their PTMs as expressed in the disease state and to be easily identified by mass spectrometry. Usually over 1,000 fractions were collected and printed, which is time-consuming and introduces variations in reproducibility, more importantly, only a small proportion of these fractions have protein with PTMs in them. It has been reported that immunoglobulins in the serum can recognize both proteins and many of their attached oligosaccharides.¹⁴ Here, we report a dual-lectin affinity chromatography based natural glycoprotein microarray to select a subproteome of target glycoproteins and their interacting proteins for autoantibody discovery in patients with melanoma. This strategy allows efficient and convenient separation of glycoproteins, which are usually present at low cellular levels under native conditions.

We chose ConA and WGA to perform the lectin-affinity chromatography to extract a large set of N-glycosylated proteins. ConA has a preference for mannoses structure and WGA has a preference for GlcNAc, both of which are the core structures of N-linked oligosaccharides. Although several studies suggest that the binding specificity of ConA and WGA can not cover all types of N-linked glycoproteins, to our knowledge, ConA and WGA are the most commonly used lectins for top-down N-linked glycoprotein profiling.^{15–16} Also, our results from lectin-blot suggest that the N-linked glycoproteins containing oligosaccharides preferred by other lectins are also captured by dual-lectin affinity chromatography in this study as shown in Supplemental Fig.S5. Both AAL and SNA showed significantly stronger response against the glycoproteins enriched by ConA and WGA than the proteins from the whole cell lysate.

Although auto-antibodies and the progression of tumors are found to be strongly correlated, it is difficult to use a single autoantibody for the surveillance for cancer. In most cases, auto-antibodies to specific proteins occur only in 10–20% of patients' sera. This is evident in our study, where only a subset of node negative or node positive patients showed increased reactivity against one of the 9 fractions, while the signals from the other samples remain the same. Combining the 9 fractions as a panel provides a 55% sensitivity with 100% specificity for the presence of nodal metastases and a 62% sensitivity with 100% specificity for the absence of nodal disease in patients with melanoma.

In order to confirm the results acquired from the natural protein microarray, five full-length recombinant proteins were selected based on the protein identification from the selected fractions and the commercial availability. GRP94 showed higher reactivity to some of the node positive patients while the other 3 proteins (ASAH1, CTSD and LDHB) had higher reactivity to some of the node negative patients. GRP94 belongs to the heat shock proteins (HSP) family, which plays a key role in the generation of humoral immune response in patients with different tumors.^{17–18} Studies have shown that GRP94 purified from human melanoma lines can represent melanoma antigens and stimulate T cells recognition of these antigens.¹⁹ The expression of GRP94 was found to be higher in metastatic melanoma than primary specimens.²⁰ In our study, we found the serum autoantibody against GRP94 from 29% of patients with node positive melanoma, which suggests that the serum anti-GRP94 can also be used as a useful indicator of melanoma progression.

Hyper-expression of GRP94 has also been found to be a marker for aggressive behavior in many tumors and other diseases.^{21–22} Therefore, the elevation of anti-GRP94 may also be induced by other factors besides malignant tumors. Here, we found that some healthy subjects and patients with non-melanoma skin cancers (control subjects) also showed high anti-GRP94 level in their sera (Supplemental Fig.S4). However, since the aim of our study is to find accurate biomarkers for melanoma prognosis instead of diagnosis, the increase of anti-GRP94 in some of the control subjects does not affect its performance for distinguishing between the node positive and node negative melanoma. On the contrary, the results suggest that the high level of serum anti-GRP94 may be used as a risk factor for many other diseases after further investigation.

Increased expression of ASAH1, CTSD and LDHB has been described in melanoma, and serum levels of LDH are associated with prognosis in stage IV melanoma.^{23–26} In this study, an autoantibody to one of these proteins was found in 40% of node negative patients (19 of 48) but none of the node positive patients (0 of 31). It has been shown by numerous observations that tumor cells can induce antigen-specific CD4⁺ and CD8⁺ T cell tolerance.^{27–29} In this way, tumor variants can escape from the immune selection process and become metastatic by developing in immunocompetent hosts.^{30–31} Consistent with these studies, our results suggest that the up-regulation of anti-ASAH1, anti-CTSD and anti-LDHB antibodies may play an important role in preventing melanoma metastasis, and the loss of these antibodies may represent an important step in melanoma progression. The combined AUC of serum antibodies against GRP94, ASAH1, CTSD and LDHB was 0.746 (Fig. 6b), which shows that serum antibodies against these recombinant proteins can be applied as biomarker candidates to monitor the progression of melanoma. Our findings provide valuable clues about the immune system response during melanoma progression and further discovery of prognostic and potential therapeutic targets.

The recombinant proteins shared a similar pattern of response to the fractions from which they were identified, but the natural proteins exhibited a better performance than the recombinant proteins on distinguishing between node negative and node positive patients. In addition, recombinant GRP75 did not show different reactivity between ENN and ENP. One possible reason for this could be that recombinant proteins synthesized in *E. coli* did not have key post-translational modifications responsible for the antigenicity of the endogenous proteins. This is a common problem when using the recombinant proteins without the PTM to confirm the results from the natural protein microarray³². However, due to the high reproducibility and convenience of our strategy in the present study (Supplemental Fig.S2), we are able to use these fractions as an alternative means to distinguish between patients with node negative and node positive melanoma. Overall, these auto-antibodies discovered in our work provide a potential means to aid the prognosis of melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Humoral response experimental overview

Proteins were first extracted from melanoma cell lines then dual-lectin (ConA&WGA) affinity chromatography was applied to enrich the N-linked glycoproteins and proteins interacting with these glycoproteins. These proteins were separated by a reverse phase HPLC then the separated fractions were spotted on nitrocellulose slides. Antibody-antigen response of each spot was detected using anti-human IgG conjugated to a fluorophore after probing with sera from patients with early node negative melanoma (node negative) and patients with early node positive melanoma (node positive). Following statistical analysis, fractions which exhibited a different humoral response between the node negative and node positive were selected, and then proteins from these fractions were identified by ESI-MS/MS and MALDI-QIT. We further investigated the performance of these proteins on the prognosis of different stages of melanoma, using recombinant proteins and a confirmation set of melanoma patients.

Figure 2. Heat map with dendrograms of hierarchical clustering for the natural glycoprotein microarray

The heat map was based on the intensities of response from 9 fractions against sera from node negative and node positive patients. Each column indicates a fraction which showed significantly different response between node negative and node positive ($p < 0.05$), and each row indicates a serum from patient with node negative or node positive. The intensity of response increases from red color to blue color. The 9 fractions allow us to distinguish between most of the patients with ENN (red rectangle) and the patients with ENP (blue rectangle). The heat map was generated with R language.

Figure 3. Performance of Selected fractions and protein identification

Forty-seven fractions collected from RP-HPLC fractionation were used as bait to explore the different levels of serum autoantibodies between patients with early node negative melanoma (node negative) and early node positive melanoma (node positive). Serum from patients with node negative showed higher humoral response to 3 fractions while serum from patients with node positive showed higher humoral response to the other 6 fractions. (a) The combined Receiver Operating Characteristic (ROC) Curve for the 3 fractions which showed higher reactivity to serum of node positive. (b) The combined Receiver Operating Characteristic (ROC) Curve for the 6 fractions which showed higher reactivity to serum of node negative.

Figure 4. The reactivity of selected fractions against serum and protein identification
 (a) The scatter plot of the reactivity of fraction 43, 44 and 45 to the serum from different melanoma patients. Each spot represents the normalized intensity of serum autoantibodies from one patient against one of the selected fractions. Patients with node negative showed significantly higher response to fraction 43, 44 and 45, compared to the node positive group (* $p < 0.05$; ** $p < 0.01$). (b) MS/MS sequencing data of 4 peptides from cathepsin D (CTSD) identified from the fractions 43, 44 and 45.

Figure 5. The scatter plot of the reactivity of recombinant GRP94, ASAH1, CTSD and LDHB to serum from patients with node negative and patients with node positive

Each spot represents the normalized intensity of serum autoantibodies from one patient against one of the recombinant proteins. These recombinant proteins showed the similar pattern of humoral response to the fractions from which they were identified.

Figure 6. Performance of recombinant GRP94, ASAHI, CTSD and LDHB

(a) Performance of the 4 recombinant proteins on distinguishing between patients with node negative and patients with node positive. (b) The combined ROC Curve for the 4 recombinant proteins.

Table 1

Melanoma patients utilized in study

Characteristic	Total	Node Negative	Node Positive
Number	79	48	31
Age			
Mean (SD)	51.9 (12.6)	51.8 (13.2)	52.0 (12.0)
Gender			
Males: N (%)	38 (48.1)	23 (47.9)	15 (48.4)
Females: N (%)	41 (51.9)	25 (52.1)	16 (51.6)
Breslow Thickness			
Mean (SD)	2.39 (1.65)	1.94 (1.15)	3.11 (2.04)
Mitotic Rate			
Mean (SD)	5.51 (5.72)	4.45 (4.93)	7.04 (6.50)
Ulceration			
Absent: N (%)	24 (30.4)	35 (74.5)	16 (50)
Present: N (%)	51 (64.6)	11 (23.4)	13 (40.6)
Unknown: N (%)	4 (5.0)	2 (4.1)	2 (6.4)