CANCER GENOMICS & PROTEOMICS 7: 17-24 (2010)

Identification of Melanoma Antigens Using a Serological Proteome Approach (SERPA)

AYAKO SUZUKI[1*], AKIRA IIZUKA[1*], MASARU KOMIYAMA[1], MASAKO TAKIKAWA[1], AKIKO KUME[1], SACHIKO TAI[1], CHIE OHSHITA[1], AYUMI KURUSU[1], YOUJI NAKAMURA[1], AKIFUMI YAMAMOTO[4], NAOYA YAMAZAKI[3], SHUSUKE YOSHIKAWA[2], YOSHIO KIYOHARA[2] and YASUTO AKIYAMA[1]

[1]*Immunotherapy Division, Shizuoka Cancer Center Research Institute and*
[2]*Department of Dermatology, Shizuoka Cancer Center Hospital, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan;*
[3]*Department of Dermatology, National Cancer Center Hospital, Chuo-ku, Tokyo 104, Japan;*
[4]*Department of Dermatology, Saitama Medical University, Iruma-gun, Saitama 350-0495, Japan*

Abstract. *Background: Melanoma is an intractable cancer with a poor prognosis and increasing prevalence worldwide. Specific biomarkers for early diagnosis have yet to be found. Materials and Methods: Serum samples from melanoma patients and healthy volunteers were utilized for identifying melanoma marker proteins using a serological proteome approach. Specifically, G361 cell protein spots separated by 2-dimensional gel electrophoresis and transferred to a membrane were incubated with patient sera, and positive spots that reacted with more than 5 serum samples were identified using time of flight mass spectrometry. Results: Only patient sera showed many spots reacted in G361 gels. A total of 13 positive spots were detected and 5 proteins were identified: eukaryotic elongation factor2 (EEF2), enolase1 (ENO1), aldolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heterogeneous nuclear ribonucleoproteins (HNRNP) A2B1. The mRNAs of four proteins (EEF2, ENO1, ALDOA and HNRNPA2B1) were highly expressed in G361 cells compared with melanocytes. EEF2, ENO1 and ALDOA mRNAs were also frequently expressed in other melanoma cell lines. Conclusion: The autoantibody-based proteomic approach was effective for investigating melanoma biomarkers. This study might contribute to the development of a diagnostic device for the early detection of cancer.*

*Both authors contributed equally to this work.

Correspondence to: Yasuto Akiyama, MD, Immunotherapy Division, Shizuoka Cancer Center Research Institute, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan. Tel: +81 559895222 (Ext. 5330), Fax: +81 559896085, e-mail: y.akiyama@scchr.jp

Key Words: Malignant melanoma, autoantibody, melanoma markers, two-dimensional gel electrophoresis (2-DE), serological proteome approach, SERPA.

Melanoma is one of the most malignant tumor types and is frequently seen in Western countries. The incidence of melanoma is steadily increasing worldwide, even in Asia, and the mortality rate is high once it becomes metastatic (5-year survival rate less than 10%). Early-stage primary melanoma can be cured by intensive therapy, however, most melanomas have a very poor prognosis because of metastasis and rapid progression.

Within the last decade, immunotherapeutic approaches using peptides and cell-based vaccines have been tried and found effective in some restricted cases (1, 2). With regard to immunotherapy, it is very important to identify highly immunogenic cancer antigens. The sera derived from cancer patients should contain a lot of autoantibodies against various cancer-related antigens (3-7). It is reasonable to believe that because tumor cells release large quantities of tumor antigens after necrosis or apoptosis, autoantibodies against them could be spontaneously produced. In that sense, serum from cancer patients is a precious source of cancer antigens (8, 9). Interestingly, Chapman *et al.* (7) reported that 76% of 104 lung cancer patients exhibited autoantibodies to at least 1 of 7 cancer antigens and testing plasma might aid the early detection of lung cancer.

It is not entirely clear how intracellular proteins become autoantigens, however, it has been suggested that i) posttranscriptional modifications enhance immunogenicity (3), ii) specific autoantigens are fetal proteins highly expressed in tumor cells (4), and iii) abnormal increases in some kinds of proteins are responsible (5-7). Therefore, serum samples of cancer patients are a highly relevant source of cancer antigens. However, it is difficult to find antigens for translational or clinical applications. A recently

established serum autoantibody-based proteomic approach enables one to identify multiple antigens that a serum antibody can respond (10).

In the present study, we comprehensively investigated melanoma antigens expressed in G361, a representative melanoma cell line, by means of the autoantibody-based proteomic approach. To find novel target antigens, we separated G361 protein lysate by 2-dimensional gel electrophoresis (2-DE) and determined melanoma antigen spots by Western blotting using 10 samples of melanoma patient sera and then identified target proteins by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF-MS).

Materials and Methods

Cell culture. Human melanoma cell lines (G361, C32, RPMI7951, MEWO, A375) were obtained from the American Type Culture Collection (Rockville, MD, USA). NCC-KT, KU-MELTC-1, TDMM1, SEKI, MMG-1, MMG-3 were described previously (11). Normal human melanocytes were purchased from KURABO Industries Ltd., Osaka, Japan. MEL-SCC001 and MEL-SCC018 are primary cell lines established in our laboratory. All melanoma cell lines were cultured in RPMI-1645 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, NY, USA), 2 mM glutamine (Lonza, Walkersville, MD, USA), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Only G361 and normal melanocytes were utilized for 2-DE analysis, and all other cell lines for reverse transcription (RT)-PCR analysis.

Human sera. The present clinical research using sera from 10 melanoma patients (MEL001 to 010) and 3 healthy donors was approved by the Institutional Review Board of the National Cancer Center (NCC) and Shizuoka Cancer Center (SCC), Japan. Ten melanoma cases were randomly selected from metastatic (stage IV) patients previously treated with chemotherapy. All patients gave written informed consent.

Protein extraction. The G361 cell pellets were incubated in a lysis buffer (50 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS), homogenated with a digital sonifier (Branson, Danbury, CT, USA) and then centrifuged at 100,000 rpm for 30 min at 4˚C. The supernatants were dialyzed using a mini dialysis kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) at 15˚C for more than 3 hours.

Two-dimensional gel electrophoresis (2-DE). The amount of protein extracted from G361 and melanocyte cells was determined using the Quick start Bradford dye reagent (Biorad Laboratories, Hercules, CA, USA). Either 150 µg of protein for Western blotting or 500 µg for picking gels were added to the same volume of 2x sample buffer (7 M urea, 2 M thiourea, and 4% CHAPS) and made up to 450 µl with rehydration buffer (GE Healthcare) including 1% Farmalight (GE Healthcare). The sample solution was centrifuged at 2,500 rpm for 10 min at 4˚C. The supernatant was added to a 24 cm pH 3-11 NL immobiline gel strip (IPG•GE Healthcare). The strips were rehydrated with the protein sample for 12 h at 20˚C and 30 V using an IPGphor system (GE Healthcare). Isoelectric focusing was performed at 35 kVh (8,000 V) at 20˚C. After the first dimension of electrophoresis, strips were washed for 15 min twice with equilibration buffer. The strips were then washed with 1x running buffer, loaded onto running gels and electrophoresed with 1% agarose gel. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in Ettan 6 gel tanks (GE Healthcare) at 1 W per gel at 15˚C overnight. After the electrophoresis, proteins in gels were transferred onto PVDF membrane (Millipore corp., Billerica, MA, USA) or visualized by staining with Sypro ruby (Invitrogen, Carlsbad, CA, USA).

Western blotting. Proteins were transferred onto PVDF membranes with CAPS buffer (pH 11, Sigma-Aldrich, Inc. St. Louis, MO, USA), using Hoefer transfer equipment (GE Healthcare) at 180 V for 1 h. Proteins transferred to PVDF membranes were dyed with Cy5 (GE Healthcare) following the manufacturer's directions. Membranes were incubated with TBS buffer containing 0.1% tween 20 (TBS-T) and 3% bovine serum albumin (BSA) for 1 h or overnight. The membranes were incubated for 1 h at room temperature with patient serum at a dilution of 1:100 with TBS-T buffer. After three washes with TBS-T buffer, the membranes were incubated with secondary antibody [horseradish peroxidase (HRP)-conjugated sheep anti-human IgG polyclonal antibody; GE Healthcare] at a dilution of 1:10000 with TBS-T buffer containing 3% ECL blocking agent (GE Healthcare). The reaction was detected with ECL plus (GE Healthcare) according to the manufacturer's instructions. Chemiluminescent signals were scanned with a Typhoon 9410 (GE Healthcare), and specific spots positively stained with both Cy5 and ECL plus were recognized on the membrane image.

Gel staining and imaging analysis. After electrophoresis, picking gels were fixed with 10% acetic acid (Wako), 7% methanol (Wako) for 3 h and stained with Sypro ruby (Invitrogen) for more than 3 hours. After staining, the gels were destained with 10% acetic acid, 7% methanol for 3 hours.

Specific protein spots as candidates were recognized concomitantly by ECL plus and Sypro ruby staining on the G361 gel images using a Typhoon 9410 imager (GE Healthcare). The ECL plus images were scanned with a 488 nm laser and 520BP 40 emission filter. The Cy5 images were scanned with a 633 nm laser and 670BP 30 emission filter. Sypro ruby-stained gels were scanned with a 633 nm laser and 457BP emission filter. The photomultiplier tube (PMT) was set to ensure a maximum intensity of between 50,000 and 80,000 pixels. Images were quantitatively analyzed with the Decyder software package in the differential in-gel analysis (DIA) mode (GE Healthcare). First of all, protein spots with a DIA score of more than 10,000 detected with each patient serum in only the G361 membrane compared with melanocytes were identified as targets. To pick specific protein spots, spots with a DIA score of more than 50,000 detected in more than 5 melanoma patient sera were recognized. All of these spots from Sypro ruby-stained G361 gels were removed, processed and analyzed by MALDI-TOF MS.

In-gel digestion and mass spectrometry (MS). The gels were dehydrated with 50 mM bicarbonic ammonium 50% acetonitrile for 20 min. After 3 rounds of dehydration, the gel pieces were further dehydrated in 100% acetonitrile for 10 min and then dried absolutely at 37˚C. For gel digestion, MS grade trypsin (Promega,

Madison, WI, USA) at 125 ng in 0.1% RapiGest (Waters, Milford, MA, USA)/100 mM NH_4HCO_3 was added to the gel pieces and which were then incubated at 37˚C for 1 h. The trypsin solution was removed and the peptides were extracted with 1% trifluoroacetic acid (TFA)/ 80% acetonitrile for 30 min, and concentrated by vacuum centrifuge. The concentrated peptide solutions were condensed with Zip Tip C18 tips (Millipore) and mixed with α-cyano-4-hydroxy-*trans*-cinnamic acid (Sigma, St. Louis, MO, USA) and spotted onto target plates. The peptides were identified by MALD-TOF-MS using a 4700 Proteomic Analyzer (Applied Biosystems, Foster City, CA, USA). The mass range was set at 700 to 4000 (m/z). Data acquisition was carried out using 4000 Series Explorer Software, V 3.6 (Applied Biosystems). Post-analysis data processing was performed using Mascot software (Matrix Science Inc. Boston, MA, USA).

Real-time PCR analysis of identified proteins. Real-time PCR analysis of identified proteins using a 7500 Real-Time PCR System (Applied Biosystems) was performed. The method of real-time PCR was described previously (11). Briefly, all PCR primers and TaqMan probes were designed and purchased from Applied Biosystems. Total RNA was extracted from cells using the NucleoSpin RNA kit (NIPPON Genetics, Japan) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems). TaqMan quantitative PCRs were carried out in 20 μl volumes containing 1 μl of cDNA and 1 μl of primer/probe mixture in PCR master mix at 50˚C for 2 minutes and 95˚C for 10 minutes followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The level of target mRNA expression was normalized to the expression of the β-actin gene, and mRNA levels were rated using normal melanocytes as the control.

In order to investigate relevant mRNA expression in other melanoma cell lines, regular RT-PCR was performed, which is described elsewhere (12). The primer sequences used were 5'-CTTCCCCCAAGTTATCAAATCCAAG-3' and 5'-GGTCACTCAG AGCCTTGTAGACAGC-3' for aldolase A (ALDOA); 5'-CTGTGC TGATGATGAACAAGATGG-3' and 5'-GTCAAAGTACCTGTCA CCCCA CAG-3' for eukaryotic elongation factor2 (EEF2); 5'-ACTGCTATTGGGAAAGCTGGCTAC-3' and 5'-CACTGTGAGA TCATCCCCCAC TAC-3' for enolase1 (ENO1); and 5'-GAT CTGATGGATATGGCAGTGGAC-3' and 5'-GAAGAAGCTCA GTATCG GCTCCTC-3' for heterogeneous nuclear ribonucleoproteins (HNRNP) A2B1; and 5'-GGCTACAGCTTCACCACCAC-3' and 5'-GTACTT GCGCTCAGGAGGAG-3' for β-actin.

Results

Reactivity of melanoma patient sera with protein extracts from melanocytes or G361. Sera were obtained from 10 melanoma patients and 3 healthy volunteers. The protein concentrations of sera were not significantly different between the two groups (data not shown). First of all, volunteer sera did not react with any significant spots in melanocyte or G361 gels (Figure 1). Melanoma patient sera showed many spots reacted in G361 gels, while fewer spots reacted with patient sera in melanocyte gels (Figure 2). G361-specific spots were selected by subtracting the spots detected in the melanocyte gel from each patient serum-treated G361 gel. Set A shows the number of spots

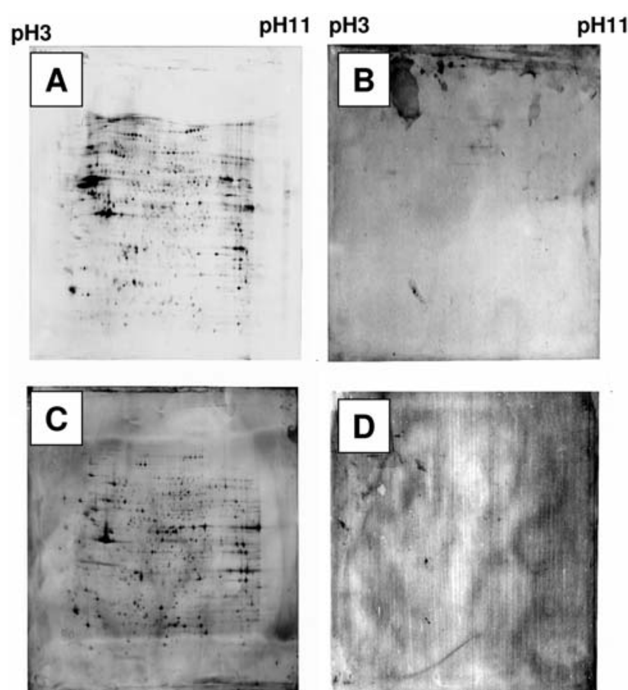

Figure 1. *Melanocyte and G361-derived protein spots on 2-DE gel reacted with healthy volunteer serum. Cy5 images and chemiluminescent signals by ECL plus were scanned with Typhoon 9410, and specific spots positively stained with both Cy5 and ECL plus were recognized on the same membrane. A and B, melanocyte gel; C and D, G361 gel; A and C, Cy5-stained images; B and D, chemiluminescent signals by ECL-plus. The pI range on immobiline gel strip is shown at the top of the panel.*

with a DIA score of more than 10,000 detected in only the G361 gels, and set B indicates the one with a score of more than 50,000 detected in more than 5 patient sera (Table I). Through 10 patient sera, 13 spots in total which met set B condition were finally identified and utilized for TOF-MS analysis.

MALDI-TOF MS/MS analysis of the differential protein spots. Thirteen proteins, all reacting with more than 5 patient sera, were plotted on a picking gel as target proteins (Figure 3). The cut-off condition for spot identification was as follows: i) spots with a DIA score of more than 50,000, and ii) spots detected with more than 5 melanoma patient sera. Table II shows the results of identification. Spots #1-3 and #4-6 corresponded to EEF2 and ENO1, respectively. The pairs of #8-9, #10-11 and #12-13 belonged to ALDOA, GAPDH and HNRNP A2B1, respectively. The process of identification was repeated more than twice for each target protein spot. Table III shows the frequency of identified spots in melanocyte and G361 gels. EEF2 was G361-specific and ENO1 was highly identified in G361, while also seen in melanocytes in a few cases.

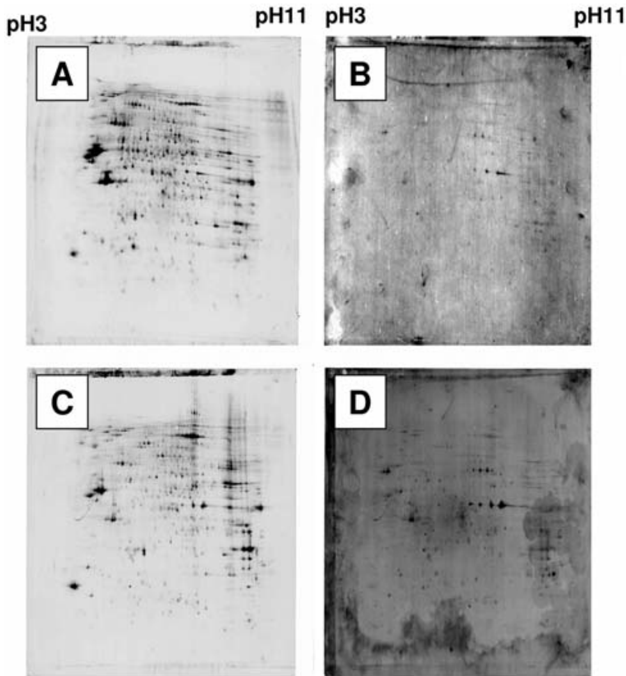

Figure 2. *Melanocyte and G361-derived protein spots on 2-DE gel reacted with melanoma patient serum. Cy5 images and chemiluminescent signals by ECL-plus were scanned with Typhoon 9410, and specific spots positively stained with both Cy5 and ECL plus were recognized on the same membrane. A and B, melanocyte gel; C and D, G361 gel; A and C, Cy5-stained images; B and D, chemiluminescent signals by ECL-plus. The pI range on immobiline gel strip is shown at the top of the panel.*

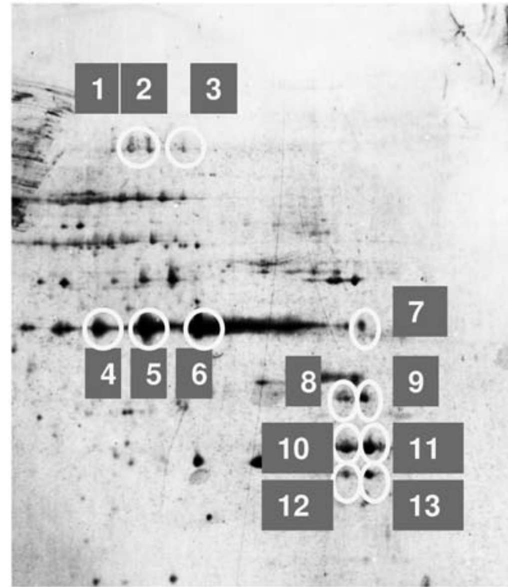

Figure 3. *Identification of 13 target spots on G361 picking 2-DE gel stained by Sypro ruby. Thirteen proteins, all reacting with more than 5 patient sera, were plotted on a picking gel as target proteins. Each spot is marked with a circle.*

Table I. *Evaluation of target spots from G361 gels in stringent conditions.*

Patient serum	Target spot# Set A*	Target spot# Set B**
MEL001	31	6
MEL002	13	6
MEL003	19	5
MEL004	6	2
MEL005	25	8
MEL006	14	7
MEL007	61	6
MEL008	61	3
MEL009	17	7
MEL010	33	5

*Set A: protein spots with a DIA score of more than 10,000 detected in only the G361 gels. **Set B: protein spots with a DIA score of more than 50,000 detected in more than 5 melanoma patient sera.

The expression of target proteins in melanocyte and melanoma cell lines. The mRNA levels of *ENO1, EEF2, ALDOA, GAPDH* and *HNRNPA2/B1* in G361 cells are shown in Figure 4. All levels were several fold higher in G361 cells than in normal melanocyte cells. The expression of *ALDOA* was most prominent (more than 14-fold). Figure 5 shows *ENO1, EEF2, ALDOA* and *HNRNPA2/B1* mRNA levels in 13 melanoma cell lines including G361 cells. All mRNAs were detected in most of the melanoma cell lines and the expression of *HNRNPA2/B1* mRNA was relatively stronger than other genes, while all mRNA expression levels in melanocytes were low.

Discussion

Over the past decade, it has been demonstrated that cancer is immunogenic, and multiple cancer antigens have been identified from clinical samples. The most important evidence suggesting the immunogenicity of tumors is the presence of autoantibodies in cancer patient plasma, which demonstrated that cancer is basically immunogenic under conditions where a humoral immune response could be induced in the individual. Autoantibody induction also indicates the individual exposure of the immune surveillance system to cancer, that is to say, the malignant transformation of normal somatic cells. On the other hand, Shoshan *et al.* (9) indicated that the repertoire of serum autoantibodies differs between healthy people and cancer patients, and in cancer patients the antibody repertoire is expanded with a wide range of reactivity against many cancer-related

Table II. *Identification of target protein spots.*

Spot#	Name	pI	MW	MS score
①	eukaryotic translation elongation factor 2 (EEF2)	6.41	95277	59
②	eukaryotic translation elongation factor 2 (EEF2)	6.41	95277	224
③	eukaryotic translation elongation factor 2 (EEF2)	6.41	95277	98
④	Enolase 1 (ENO1)	7.01	47079	116
⑤	Enolase 1 (ENO1)	7.01	47139	254
⑥	Enolase 1 (ENO1)	7.01	47079	211
⑦	unnamed protein product	9.1	50095	78
⑧	aldolase A	8.34	39307	412
⑨	aldolase A	8.34	39307	362
⑩	glycelaldehyde-3- phosphate dehydrogenase (GAPDH)	8.26	36031	218
⑪	glycelaldehyde-3- phosphate dehydrogenase (GAPDH)	8.26	36031	266
⑫	similar to Heterogenerous nuclear ribonucleoproteins A2/B1 (hnRNP A2hnRNP B1) isoform	8.97	37407	210
⑬	similar to Heterogenerous nuclear ribonucleoproteins A2/B1 (hnRNP A2hnRNP B1) isoform	8.97	37407	514

Table III. *Frequency of identified spots in melanocyte and G361 gels treated with 10 patient sera.*

Spot #	Frequency	
	Melanocyte	G361
①	0/10	7/10
②	0/10	6/10
③	0/10	5/10
④	3/10	7/10
⑤	2/10	10/10
⑥	3/10	10/10
⑧	2/10	6/10
⑨	5/10	8/10
⑩	2/10	8/10
⑪	3/10	7/10

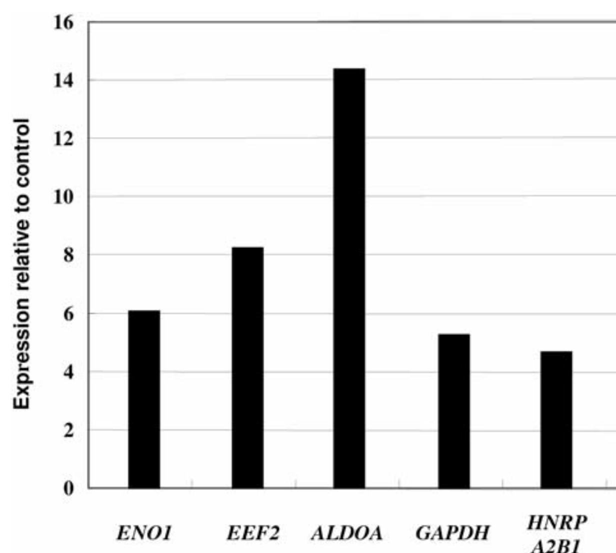

Figure 4. *Levels of mRNA of 5 candidate proteins in G361 cells. The levels of mRNA of 5 candidate proteins in G361 cell line were investigated using a real-time PCR. The mRNA expression is reported relative to that of normal melanocytes as the control. EEF2: eukaryotic elongation factor 2; ENO1: enolase-1; ALDOA: aldolase A; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HNRNPA2B1: heterogeneous nuclear ribonucleoproteins A2B1.*

antigens. This is why autoantibodies reflect the humoral immune response profiling against cancer and might be sensitive biomarkers for detecting early forms of cancer.

Serological proteome analysis (SERPA) has been described as a classical proteomic method based on protein separation by 2-DE and identification by TOF-MS. Cancer cell-derived protein extracts are separated on 2-DE gels and transferred to PVDF membranes, and the blots are incubated with sera from cancer patients or healthy volunteers (13). In last decade, many cancer-associated antigens have been identified using SERPA in cancer cells or tissues including those from kidney (10), lung (14), breast (15), ovary (16) and esophageal cancer (17). These observations demonstrated the great potential of autoantibodies for the identification of novel cancer antigens like the serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX). Furthermore, more importantly, sera from healthy volunteers did not react with cancer protein spots separated on 2-DE gels, whereas cancer patient sera strongly reacted, which might suggest the specificity of SERPA (10, 16). However, there are some questions regarding SERPA based on humoral proteomics. Autoantibody-matched antigens are generally all located intracellularlly, and few membrane-associated proteins are identified using SERPA. The reason why few extracellular proteins are detected is not clear.

In the present study we identified 5 melanoma marker candidate proteins, ENO1, EEF2, ALDOA, GAPDH and HNRPA2B1. ENO1, otherwise known as neuron-specific

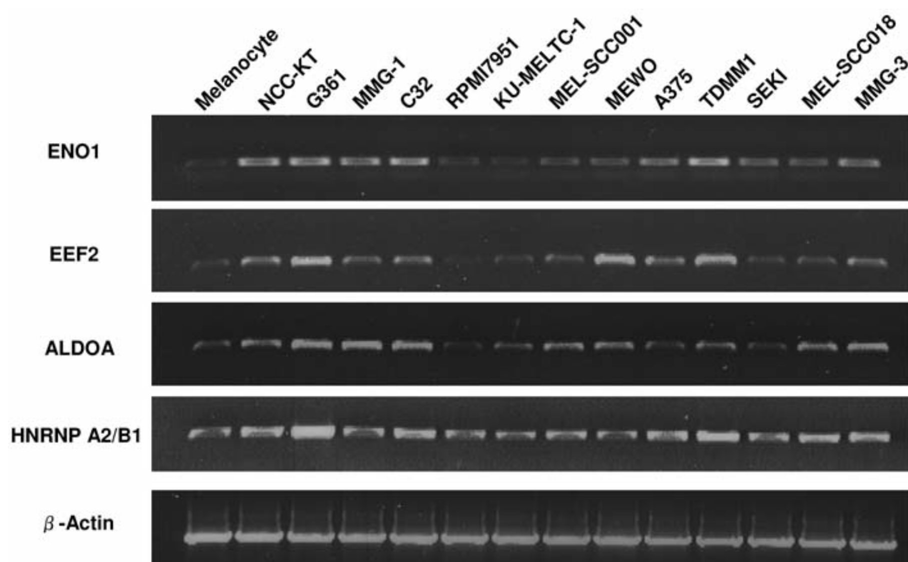

Figure 5. *Expression of 4 candidate mRNAs in melanoma cell lines. ENO1, EEF2, ALDOA and HNRNP A2/B1 mRNA expressions were investigated using a regular RT-PCR in 13 melanoma cell lines including G361 cells.*

enolase (NSE), is a well-known tumor marker for small cell lung cancer and melanoma. Autoantibody against ENO1 was also produced in patients with lung cancer and melanoma associated with autoimmune retinopathy (18). EEF2 was demonstrated to be overexpressed in gastrointestinal and hepatocellular cancer and its knockdown induced G_2/M arrest (19). ALDOA is commonly expressed in tumor tissue such as hepatocellular and pancreatic cancer. More interestingly, Mizuno *et al*. (20) showed that siRNA-mediated silencing of the hypoxia-inducible factor (HIF)1α gene significantly reduced the expression of ALDOA in pancreatic cancer cells, which may suggest that ALDOA is closely linked to the pathway induced by hypoxia. The expression of these three proteins has not previously been reported in melanomas.

Recently, a new technology named cancer immunomics has been developed for the early detection of prostate cancer. Specifically, Bradford *et al*. (21) was able to identify specific peptides that react with autoantibodies present in patient sera using a phage-epitope microarray and autoantibody signature. This approach may represent the next generation of novel humoral proteomics approach using cancer epitope-display on phages instead of conventional 2-DE gels.

Finally, in the current study, we identified melanoma-associated proteins using SERPA. As yet, most tumor markers of melanoma have potent sensitivity and specificity in advanced stage to some extent, however few markers helpful for early stage detection have been reported. These might suggest that SERPA can be a powerful tool to identify novel melanoma biomarkers.

Acknowledgements

This work was supported in part by a grant in cooperation with Innovative Technology and Advanced Research in an Evolutional Area (CITY AREA) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1 Rosenberg SA, Yang JC and Restifo NP: Cancer immunotherapy: moving beyond current vaccines. Nat Med *10*: 909-915, 2004.
2 Nestle FO, Banchereau J and Hart DA: Dendritic cells: on the move from bench to bedside. Nat Med *7*: 761-765, 2001.
3 Von Mensdorff-Pouilly S, Gourevitch MM, Kenemans P, Verstraeten AA, Van Kamp GJ, Kok A, van Uffelen K, Snijdewint FG, Paul MA, Meijer S and Hilgers J: An enzyme-linked immunosorbent assay for the measurement of circulating antibodies to polymorphic epithelial mucin (MUC1). Tumour Biol *19*: 186-195, 1998.
4 Ura Y, Ochi Y, Hamazu M, Ishida M, Nakajima K and Watanabe T: Studies on circulating antibody against carcinoembyonic antigen (CEA) and CEA-like antigen in cancer patients. Cancer Lett *25*: 283-295, 1985.
5 Regidor PA, Regidor M, Callies R and Schindler AE: Detection of p53 auto-antibodies in the sera of breast cancer patients with a new recurrence using an ELISA assay. Does a correction with the recurrence-free interval exist? Eur J Gynaecol Oncol *17*: 192-199, 1996.
6 Lokshin AE, Winans M, Landsittel D, Marrangoni AM, Velikokhatnaya L, Modugno F, Nolen BM and Gorelik E: Circulating IL-8 and anti-IL-8 autoantibody in patients with ovarian cancer. Gynecol Oncol *102*: 244-251, 2006.

7 Chapman CJ, Murray A, McElveen JE, Sahin U, Luxemburger U, Tureci O, Wiewrodt R, Barnes AC and Robertson JF: Autoantibodies in lung cancer: possibilities for early detection and subsequent cure. Thorax *63*: 228-233, 2008.
8 Lu H, Goodell V and Disis ML: Humoral immunity directed against tumor-associated antigens as potential biomarkers for the early diagnosis of cancer. J Proteome Res *7*: 1388-1394, 2008.
9 Shoshan SH and Admon A: Novel technologies for cancer biomarker discovery: humoral proteomics. Cacer Biomark *3*: 141-152, 2007.
10 Klade CS, Voss T, Krystek E, Ahorn H, Zatloukai K, Pummer K and Adolf GR: Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. Proteomics *1*: 890-898, 2001.
11 Takikawa M, Akiyama Y, Ashizawa T, Yamamoto A, Yamazaki N, Kiyohara Y, Oku N and Yamaguchi K: Identification of melanoma-specific serological markers using proteomic analyses. Proteomics-CA *3*: 552-562, 2009.
12 Liu F, Akiyama Y, Tai S, Maruyama K, Kawaguchi Y, Muramatsu K and Yamaguchi K: Changes in the expression of CD106, osteogenic genes, and transcription factors involved in the osteogenic differentiation of human bone marrow mesenchymal stem cells. J Bone Miner Metab *26*: 312-320, 2008.
13 Hardouin J, Jean-Paul Lasserre, Sylvius L, Joubert-Caron R and Caron M: Cancer Immunomics From serological proteome analysis to multiple affinity protein profiling. Ann NY Acad Sci *1107*: 223-230, 2007.
14 Li C, Xiao Z, Chen Z, Zhang X, Li J, Wu X, Li X, Yi H, Li M, Zhu G and Liang S: Proteome analysis of human lung squamous carcinoma. Proteomics *6*: 547-558, 2006.
15 Hamrita B, Chahed K, Kabbage M, Guillier CL, Trimeche M, Chaieb A and Chouchane L: Identification of tumor antigens that elicit a humoral immune response in breast cancer patients' sera by serological proteome analysis (SERPA). Clin Chim Acta *393*: 95-102, 2008.
16 Gragnon A, Kim JH, Schorge JO, Ye B, Liu B, Hasselblatt K, Welch WR, Bandera CA and Mok SC: Use of a combination of approaches to identify and validate relevant tumor-associated antigens and their corresponding antoantibodies in ovarian cancer patients. Clin Cancer Res *14*: 764-771, 2008.
17 Fujita Y, Nakanishi T, Hiramatsu M, Mabuchi H, Miyamoto Y, Miyamoto A, Shimizu A and Tanigawa N: Proteomics-based approach identifying autoantibody against peroxiredoxin VI as a novel serum marker in esophageal squamous cell carcinoma. Clin Cancer Res *12*: 6415-6420, 2006.
18 Ren G and Adamus G: Cellular targets of anti-alpha-enolase autoantibodies of patients with autoimmune retinopathy. J Autoimmun *23*: 161-167, 2004.
19 Nakamura J, Aoyagi S, Nanchi I, Nakatsuka S, Hirata E, Shibata S, Fukuda M, Yamamoto Y, Fukuda I, Tatsumi N, Ueda T, Fujiki F, Nomura M, Nishida S, Shirakata T, Hosen N, Tsuboi A, Oka Y, Nezu R, Mori M, Doki Y, Aozasa K, Sugiyama H and Oji Y: Overexpression of eukaryotic elongation factor EEF2 in gastrointestinal cancers and its involvement in G_2/M progression in the cell cycle. Int J Oncol *34*: 1181-1189, 2009.
20 Mizuno T, Nagao M, Yamada Y, Narikiyo M, Ueno M, Miyagishi M, Taira K and Nakajima Y: Small-interfering RNA expression vector targeting hypoxia-inducible factor 1 alpha inhibits tumor growth in hepatobilliary and pancreatic cancers. Cancer Gene Ther *13*: 131-140, 2006.
21 Bradford TJ, Wang X and Chinnaiyam AM: Cancer immunomics: Using autoantibody signatures in the early detection of prostate cancer. Urol Oncol *24*: 237-242, 2006.

Received October 30, 2009
Revised January 11, 2010
Accepted January 12, 2010