Characterization of panel antibodies for classification of cancer type using novel antibody-based phosphoproteomics

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SUMMARY

Cancer types can be classified according to novel antibody-based proteomics using anti-phosphoprotein monoclonal antibodies (PPmAbs) and multiple discriminant analysis. The antibody-based phosphoproteomics using an antibody panel from over 150 uncharacterized PPmAbs combined with multiple discriminant analysis makes it possible to classify cancer cells. To improve the system, new antibody panels need to be developed using characterized PPmAbs for clinical diagnosis. The uncharacterized 154 PPmAbs were tested for reactivity based on immunohistostaining of several cancer tissues. We focused on AKPS288 PPmAb, and the PPmAb-related antigen was localized in the cytoplasm of tumor cells of the colon and stomach but did not react with non-tumor cells in both tissues. Moreover, the AKPS288 PPmAb showed positive staining in the cytoplasm of normal prostate tissue but not cancer tissue. Based on the mass spectrometry (MS), the PPmAb-related antigen was identified as TATA-element modulatory factor 1 (TMF/ARA160), a tumor-associated antigen (TAA). These results indicate that the use of a novel antibody panel consisting of anti-TAA mAbs could have considerably greater utility for cancer classification than the PPmAb panel with unknown specificity identified in our previous study.

Key words: phosphoproteomics, multiple discriminant analysis, western blotting, mass spectrometry, tumor-associated antigen

INTRODUCTION

After translation, proteins undergo further biochemical modifications under various physiological conditions, thereby altering their properties¹,². When a protein is phosphorylated or dephosphorylated, the local polarity of amino acid residues changes, resulting in a change in the higher-order structure and function of the protein. Protein phosphorylation and dephosphorylation play central roles in intercellular signaling, and the cell cycle and, therefore, abnormalities in and the breakdown of these reactions are thought to be involved in numerous chronic diseases and carcinogenesis³,⁴. Phosphoprotein expression depends on tumor type, and examining phosphoprotein expression profiles may aid cancer diagnosis.

Hundreds of tumor-associated antigens (TAAs) have been reported since their initial discovery in the 1960s by Baldwin⁵, and many studies have examined their application as biomarkers. TAAs are expressed at low levels in some normal tissues but are over-expressed in malignant cells or body fluids, including blood⁶. Many studies have demonstrated that cancer serum contains autoantibodies that react with TAAs⁷. One of the most extensively studied TAAs is the tumor suppressor protein p53. Autoantibodies against p53 were first reported in 1982⁸. Diverse cellular proteins induce autoantibody responses; such proteins include those that protect mRNA from natural physiological degradation such as p62⁹ and CRD-BP¹⁰, oncogene products, such as HER-2/neu¹¹, and differentiation antigens, such as tyrosinase and cancer/testis antigen¹². Tani et al.¹³ detected an
anti-TMF/ARA160 autoantibody in the sera of patients with renal cell carcinoma, indicating that TMF/ARA160 is a TAA. In addition, these reports show that the TAAs can be important molecules of antibody-based cancer proteomics.

We previously demonstrated that it is possible to classify cancer types using antibody-based proteomics with anti-phosphoprotein monoclonal antibodies (PPmAbs) and multiple discriminant analysis\(^{14}\). Specifically, antibody panels from over 150 uncharacterized PPmAbs allow classification of cancer cells using multiple discriminant analysis. Although most cancer proteomics techniques using antibodies target relatively abundant TAAs, our phosphoproteomics technique using the PPmAbs and multiple discriminant analysis targets unidentified proteins. To improve our phosphoproteomics system for clinical diagnosis, it is important to develop new antibody panels using the characterized PPmAbs. However, it is uneconomical and difficult to identify the specificity of over 150 different PPmAbs. Certain procedures might solve these problems, such as evaluating the reactivity of these PPmAbs with tumor tissues and non-tumor tissues using immunohistochemistry, and then focusing on a PPmAb(s), the PPmAb-related antigen(s) identified using MS. In this study, we evaluated the suitability of these procedures for determining the characteristics of a new antibody panel in the phosphoproteomics technique using multiple discriminant analysis.

**MATERIALS AND METHODS**

**Ethics statement**

This study was approved by the ethics committee of the Tokyo Medical and Dental University (Registration No. M2017-151). The study used clinically-obtained and archived formalin-fixed paraffin-embedded (FFPE) tissue specimens; therefore, the ethics committee waived the requirement for specific informed consent in accordance with Ethical Guidelines for Clinical Studies by the Ministry of Health, Labour, and Welfare of Japan. The clinicopathological data of the FFPE tissue samples are shown in Table 1.

**Recombinant proteins**

To examine the reactivity of PPmAbs, four commercial HEK293T cell lysates with overexpressed recombinant proteins, TATA-element modulatory factor 1 (TMF/ARA160), amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL), dermcidin (DCD), and transcription elongation factor SPT5 (hSPT5), were purchased from Novus Biologicals (Littleton, CO, USA).

**Cell lines and cell culture**

Human colorectal cancer cell lines (CoLo-TC, DLD-1, HCT-15 and LoVo)\(^{15–18}\), human stomach cancer cell lines (Ocum-1, Kato-III, MKN-7 and Okajima)\(^{19–21}\), human liver cancer cell lines (HepG2, Huh-7 and Li-7)\(^{22, 23}\), human pancreatic cancer cell lines (MIA PaCa-2, PK-8, PK-9 and PANC-1)\(^{24–27}\), human lung cancer cell lines (A549 and LNCap-FGC)\(^{28, 29}\), human prostate cancer cell lines (DU-145 and LNCap-FGC)\(^{30, 31}\), and human T cell leukemia cell lines (Molt-4)\(^{32}\) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Tokyo, Japan) at 37°C in a humidified atmosphere of 6% CO\(_2\).

**Immunization of mice, generation of hybridomas, and specificity of PPmAbs**

In this study, 154 PPmAbs secreted by hybridomas previously established by Motofuji\(^{14}\) were used. A phosphoprotein enrichment kit for isolating the phosphoprotein mixture as an immunogen from Molt-4 human leukemia cell lysate was purchased from QIAGEN (Hilden, Germany); the phosphoprotein mixture was stored at −80°C as an immunogen for generation of hybridomas and specificity of PPmAbs.

For immunization and generation of hybridomas, BALB/C mice were intraperitoneally (i.p.) injected with 100 \(\mu\)g of the immunogen emulsified with Freund’s complete adjuvant. Mice were subsequently injected i.p. on four additional occasions at 2-week intervals with 100 \(\mu\)g of immunogens emulsified with Freund’s incomplete adjuvant. Immune splenocytes were fused with SP2/0-Ag14 murine myeloma cells using polyethylene Glycol 2,000, and hybridoma cells were generated in hypoxanthine/aminopterin/thymidine culture medium. The hybridoma cells were cloned by limiting dilution with BM Condimed H1 Cloning Supplement (Roche Diagnostics, Mannheim, Germany) twice.

**Indirect immunofluorescence assay (IFA)**

Tumor cell lines were smeared on a glass slide, dried for

| Cancer tissues | Gender (F/M) | Age (years) | Clinical diagnosis | Pathological diagnosis |
|----------------|-------------|-------------|--------------------|-----------------------|
| Stomach        | M           | 66          | Stomach carcinoma  | Double carcinoma of the stomach |
| Colon          | F           | 68          | Colon adenocarcinoma | Well differentiated adenocarcinoma with adenomatous component of the sigmoid |
| Liver          | F           | 76          | Hepatocellular carcinoma | Hepatocellular carcinoma (moderately differentiated) |
| Kidney         | F           | 47          | Renal cell carcinoma | Renal cell carcinoma; clear cell carcinoma of the right kidney. |
| Prostate       | M           | 73          | Prostate adenocarcinoma | Adenocarcinoma of the prostate |

F: Female, M: Male
30 min at 23°C and then fixed with ice-cold methanol for 10 min at −20°C. The cell smears were treated with PPmAb for 30 min at 22°C. After washing with phosphate-buffered saline (PBS), cell smears were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG F(ab’)/2 or IgM F(ab’)/2 (MP Biomedicals, Santa Ana, CA, USA) for 30 min. Staining patterns of cells were examined under a fluorescence microscope (×400 magnification, Eclipse E400, HB-10103AF; Nikon, Tokyo, Japan).

**Immunohistochemistry (IHC)**

Formalin-fixed tissues and Molt-4 cells were analyzed using the standard labeled streptavidin-biotin-peroxidase complex method. Briefly, after routine deparaffinization and rehydration, 3-μm-thick sections were treated with antigen retrieval buffer [10 mM sodium citrate, pH 6.0] in a microwave oven. Then, the sections were treated with 0.3% hydrogen peroxide (H₂O₂) in methanol for 10 min and incubated with each PPmAbs overnight in a moist chamber at 4°C. The nuclei were counterstained with Mayer’s hematoxylin solution.

**Western blot assay (WB)**

Whole cell lysate was obtained from 2×10⁷ cultured Molt-4 cells in 1 mL RIPA buffer [50 mm Tris, 150 mm sodium chloride (NaCl), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, complete mini protease inhibitor cocktail (Roche Diagnostics), pH 8.0] for 10 min and mixed on a rotary shaker. After centrifugation at 14,000×g for 10 min at 4°C to remove cell debris, the lysate was stored at −80°C before use in subsequent experiments. The lysate was fractionated by SDS-PAGE on a 7.5% or 10% polyacrylamide gel, and fractioned proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane using a semi-dry blotting method. The PVDF membrane was treated with 1% bovine serum albumin at 4°C overnight and then with each of the PPmAbs at 22°C for 45 min. After washing with 0.1% (v/v) Tween 20-PBS, the membrane was treated with POD-conjugated anti-mouse IgG + A + M (H & L) or anti-mouse IgM (H & L; American Qualex, San Clemente, CA, USA). Then, the membrane was washed, and the PPmAb reaction was exposed to X-ray films using the ECL-PLUS Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

**Immunoprecipitation (IP) and MS**

Protein L-agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were equilibrated with RIPA buffer, and then 20 μL of the beads (25% wet v/v) was incubated with 1 mL AKPS288 PPmAb for 2 h at 4°C and mixed using a rotary shaker. These beads were incubated with 200 μL of the Molt-4 cell lysate overnight at 4°C and mixed using a rotary shaker. After washing with RIPA buffer, the complex was fractionated using SDS-PAGE with a gel with a high separation ability, the Perfect NT Gel 7.5–15% (DRC, Tokyo, Japan), and the gel was stained with Coomassie Brilliant Blue (CBB). The target area in the gel was cut into pieces, destained with 50% (v/v) acetonitrile, dehydrated with 100% (v/v) acetonitrile, and vacuum-dried. The gel pieces were digested with tryptic digestion solution containing 0.5 μg/μL mass spectrometry-grade trypsin and 50 mM ammonium bicarbonate (NH₄HCO₃) buffer (pH 8.0) for 16 h at 37°C. After incubation, protein fragments were eluted from the gel subfragments with 70% (v/v) acetonitrile and 50 mM Tris-HCl buffer. Digested fragments were loaded into the LC-MS/MS system assembled with the Nanospace SI-2 (Shiseido Fine Chemicals, Tokyo, Japan) and LTQ-Orbitrap Discovery (Thermo Fisher Scientific). Peptide fragments were identified using Mascot (Matrix Science, Boston, MA, USA) and the SwissProt database.

**RESULTS AND DISCUSSION**

**Generation of hybridomas and reactivity of PPmAbs with Molt-4 cells and intra-abdominal tissues**

After immune splenocytes were fused to myeloma cells, the resulting hybridoma cells were cloned. The hybridoma supernatants were screened for anti-phosphoprotein mixture activity using IFA with methanol-fixed Molt-4 cells as antigens. The reactivity of 154 PPmAb hybridoma clones to formalin-fixed Molt-4 cells was evaluated using the colloid bag method and IHC; 89 clones showed strong reactivity. Further, these clones were examined using IHC in formalin-fixed colon, stomach, liver, kidney, and prostate tissues; four and 36 PPmAbs reacted with colon and stomach tissues, respectively; 19, 31, and 23 clones reacted with liver, kidney, and prostate tissues, respectively; and 35 of the 89 clones did not react with any tissues (Table 2). The antigens in these tissues were recognized by 54 clones, including 27, 19, and 8 that reacted with the cytoplasm, nucleus, and both components, respectively. The number of PPmAb clones with both nuclear and cytoplasmic staining was very low, and no PPmAb positively stained the colon. Both tumor and non-tumor regions of the stomach had a high positive staining rate in the nucleus and cytoplasm, indicating that the antigen reacting with PPmAbs is highly expressed in the stomach. Similarly, markedly high antigen expression was observed in the kidney.

For PPmAbs with characteristic reactivity, IHC images of both stomach and prostate cancer tissues are shown in Fig. 1A. The AKPS174 PPmAb exhibited positive staining in the cytoplasm of both tissues and a dot-shaped staining pattern in stomach cancer tissues (Fig. 1A, upper row). The AKPS214 PPmAb showed positive staining in the nuclei of both tissues (Fig. 1A, middle row). In addition, the stainability of AKPS528 PPmAb differed between the tissues; although the nuclei were stained in stomach cancer tissues, the cytoplasm and nuclei were both stained in prostate cancer tissues (Fig. 1A, lower row).
| Tissue | Cytoplasm | Nucleus | Cytoplasm | Nucleus | Cytoplasm and Nucleus |
|--------|-----------|---------|-----------|---------|-----------------------|
|        | T/NT     | T/NT    | T/NT     | T/NT    | T/NT                 |
| Colon  | T/NT     | T/NT    | T/NT     | T/NT    | T/NT                 |
| Stomach| T/NT     | T/NT    | T/NT     | T/NT    | T/NT                 |
| Liver  | T/NT     | T/NT    | T/NT     | T/NT    | T/NT                 |
| Kidney | T/NT     | T/NT    | T/NT     | T/NT    | T/NT                 |
| Prostate| T/NT   | T/NT    | T/NT     | T/NT    | T/NT                 |

| PPmAbs (AKPS series) | Cytoplasm | Nucleus | Cytoplasm and Nucleus |
|----------------------|-----------|---------|-----------------------|
|                      | 1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 | 0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 | 1  2  3  4  5  6  7  8  |
|                      | 1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 | 0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 | 1  2  3  4  5  6  7  8  |
|                      | 1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 | 0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 | 1  2  3  4  5  6  7  8  |
|                      | 1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 | 0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 | 1  2  3  4  5  6  7  8  |

T: tumor part, NT: non-tumor part, −: negative reaction, +: positive reaction, w: weak positive reaction.
Gray squares indicate that different reactivity between cytoplasm and nuclei in tumor or non-tumor tissues.
Fig. 1. Immunohistostaining of anti-phosphoprotein monoclonal antibodies (PPmAbs) in human tumor and non-tumor tissues.

(A) Two tumor tissues, stomach (a1, a3, and a5) and prostate tissues (a2, a4, a6, and a7), were stained with three PPmAbs, i.e., AKPS174 (a1 and a2), AKPS214 (a3 and a4), and AKPS528 (a5, a6, and a7), as described in the Materials and Methods. The AKPS174 PPmAb showed positive staining in the cytoplasm of tumor cells. AKPS214 PPmAb showed positive staining in the nuclei of tumor cells. AKPS528 PPmAb showed positive staining in the nuclei of tumor cells of the stomach, and in the nuclei and cytoplasm of tumor cells of the prostate. (B) Tumor and non-tumor tissues in the colon (b1 and b2), stomach (b3 and b4), and prostate (b5 and b6) were stained with AKPS288 PPmAb, as described in the Materials and Methods. AKPS288 PPmAb showed positive staining in the cytoplasm of tumor parts in the colon and stomach, and in the cytoplasm of non-tumor tissues of the prostate. Scale bars = 50 μm.
Identification of AKPS288 PPmAb-related antigen using IP and MS

We focused on 23 clones (highlighted in gray in Table 2) that showed differential reactivities between tumor and non-tumor tissues. In addition to AKPS288 PPmAb, many other useful PPmAbs (AKPS 378, 277, 281, 421, 431, 422, 158, and 241) were detected. However, most PPmAbs (AKPS 378, 277, 281, 421, and 422) other than AKPS288 PPmAb, either did not react or reacted weakly with the Molt-4 cell lysate as an antigen in WB (data not shown). Although the remaining clones (AKPS 431, 158, and 241) showed positive results in the WB (data not shown), they did not react with multiple FFPE tissue samples in IHC. Conversely, AKPS288 PPmAb showed differential reactivities with colon, stomach, and prostate tissues in the IHC (Fig. 1B and Table 2). For identification of mAb-related antigens using IP and MS, it is necessary to use an mAb with strong binding ability. IgM-kappa APKS288 PPmAb with stronger antigen-binding ability than those of other IgG PPmAbs is suitable for the identifying mAb-related antigens. Based on these characteristics of AKPS288 PPmAb, we focused on AKPS288 PPmAb in this study.

To identify the AKPS288 PPmAb-related antigen, we first evaluated the binding of the AKPS288 PPmAb to Protein L-agarose beads. Although Protein L binds to the kappa light chain of mouse Ig, without interfering with the antigen-binding site of the antibody, it does not bind to the kappa light chain of bovine Ig. The Protein L-agarose beads were incubated with the diluted culture supernatant of AKPS288 PPmAb (1:1, 1:10, and 1:100) and treated with the Molt-4 whole cell lysate. The complexed proteins were separated using SDS-PAGE and analyzed using WB with POD-conjugated anti-mouse IgM (Fig. 2A). An AKPS288 PPmAb-related antigen of 160 kDa was detected using the non-diluted culture supernatant. Therefore, we used the non-diluted culture supernatant of AKPS288 PPmAb for MS. Both 80 kDa and 40 kDa proteins were detected using WB. The proteins were detected using increasing concentrations of AKPS288 PPmAb, and were not detected without the addition of AKPS288 PPmAb (Fig. 2A Lane NC); therefore, we presume that these proteins were associated with the IgM-related molecule of the AKPS288 PPmAb.

The same protocol was performed without WB; instead, after the SDS-PAGE, the gel was stained with CBB (Fig. 2B). Two faint bands (band-1 and -2) containing the target protein were observed at approximately 160 kDa. Both bands were cut into pieces for protein digestion and analyzed using LC-MS/MS after peptide extraction. The peptide fragments were identified using Mascot and the SwissProt database. Four candidate proteins with a sequence coverage greater than 5% were identified, i.e., TATA-element modulatory factor 1 (TMF/ARA160), amyllo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL), dermcidin (DCD) and transcription elongation factor SPT5 (hSPT5). The reactivity of four commercially available cell lysates containing the recombinant proteins was tested using WB using the AKPS288 PPmAb, and only the recombinant TMF/ARA160 protein showed positive results. Accordingly, “AKPS288 PPmAb-related antigen” was identified as TMF/ARA160 (Fig. 2C). The typical sequence coverage for “confident” protein identification is 30%; the TMF/ARA160 sequence coverages were 41.99% and 45.75%, considered highly reliable. The mobility of the recombinant TMF/ARA160 protein on an SDS gel was slightly greater than that of the Molt-4 cell lysate as a positive control; this high molecular mass might be explained by recombination or posttranslational modification.

Reactivity of AKPS288 PPmAb with cell lines using IFA

The expression of the AKPS288 PPmAb-related antigen in 20 methanol-fixed cell lines in addition to that in the Molt-4 cell line (colon, stomach, other intra-abdominal, and prostate cancers) was investigated using IFA (Fig. 3 and Table 3). In positive cells, the cytoplasm showed bright green fluorescence with a characteristic dot pattern in the colon, liver, and prostate cancer cell lines. However, a homogeneous speckle pattern was observed in stomach cancer cell lines. These results indicate that the AKPS288 PPmAb-related antigens spread throughout the cytoplasm. The positive rate of over 70% in fluorescence-positive cell lines (colon, liver, and prostate cancers and T cell leukemia) indicates that the AKPS288 PPmAb-related antigens are constitutively expressed in these tumor cells. Interestingly, a prostate cancer cell line (LNCap-FGC) also showed a positive rate of 75%; however, the prostate cancer tumor tissue did not exhibit positive staining results with IHC (Fig. 1B, lower row). These results indicate that the AKPS288 PPmAb-related antigen treated with formalin may be more fragile than that treated with cold methanol. Yamane et al. (33) first revealed the unique localization of TMF/ARA160 surrounding the Golgi cisternae by immunofluorescence imaging and demonstrated its concentration at the budding structures at the tips of the cisternae by immunoelectron microscopy. Their data support our IHC and IFA results, strongly indicating that the AKPS288 PPmAb recognized TMF/ARA160 molecules in the cytoplasmic Golgi apparatus.

CONCLUSION

In this study, we showed the candidate specificity of a new antibody panel using IHC and MS. We previously found that it is possible to classify cancer using antibody-based proteomics with PPmAbs and multiple discriminant analysis (14). Specifically, the antibody panel of uncharacterized PPmAbs makes it possible to classify cancer cells using multiple discriminant analysis. To improve our system for clinical diagnosis, the new antibody panel needs to be developed using characterized PPmAbs. However, it is uneconomical
Fig. 2. Identification of the AKPS288 anti-phosphoprotein monoclonal antibodies (PPmAb)-related antigen using immunoprecipitation (IP) and mass spectrometry (MS).

Protein L-agarose beads were incubated with the AKPS288 PPmAb and Molt-4 whole cell lysate. After washing the beads, the complex proteins were separated using SDS-PAGE and blotted onto a PVDF membrane as described in the Materials and Methods. (A) The PVDF membrane was incubated with the diluted AKPS288 PPmAb and POD-conjugated anti-mouse IgM antibody. As a positive control, the Molt-4 cell lysate separated electrophoretically was treated with both antibodies (Lane PC). As a negative control for the non-specific binding of the AKPS288 PPmAb-related antigen to Protein L-agarose beads, Protein L-agarose beads were incubated with the Molt-4 whole cell lysate with culture medium containing 10% FCS, instead of AKPS288 PPmAb (Lane NC). (B) After the IP, the SDS gel was stained with CBB, and two faint bands (band-1 and -2) at approximately 160 kDa were cut into pieces for MS, as described in the Materials and Methods. Four proteins (TMF/ARA160, AGL, DCD and hSPT5) with coverage rates greater than 5% were candidate AKPS288 PPmAb-related antigens. (C) The reactivities of the four candidate recombinant proteins and Molt-4 cell lysate (PC) with AKPS288 PPmAb were tested using western blotting (WB). As a negative control for the non-specific binding of POD-conjugated anti-mouse IgM antibody as the secondary antibody (NC), the HEK293T cell lysate containing an empty vector for recombination was treated with the POD-conjugated anti-mouse IgM antibody without a primary antibody. The AKPS288 PPmAb-related antigen is indicated by arrows.
More than 50 anti-TAA antibodies/mAbs were chosen for generation of the antibody panels: (i) overexpressed antigen such as Her2/neu34), (ii) cancer testis antigen such as MAGE family35), (iii) mutant antigen such as p53 protein36), (iv) differentiation antigen such as gp10037), (v) carcinogenesis virus antigen such as human papillomavirus (HPV) E638). The reactivity between the anti-TAA antibody panel and some cancer cells was investigated using IFA and then analyzed using multiple discriminant analysis, and the properties of the new antibody panel will be evaluated from the results.

In the future, patient blood samples and tissue extracts using the antibody microarray analysis combined with the new antibody panel may be used in research and clinical examinations for cancer diagnosis. In addition, to investigate the profile of TAA expression in tumor tissue, it may be more and difficult to identify the specificity of all PPmAbs. To solve this problem, we focused on the reactivity of the AKPS288 PPmAb with cancer tissues in IHC; the AKPS288 PPmAb stained the antigen localized in the cytoplasm of tumor cells of the colon and stomach but did not react with non-tumor cells in both tissues. Moreover, the AKPS288 PPmAb-related antigen was identified using MS as TMF/ARA160, a TAA. These results highlight the significance of PPmAbs with TAA specificity, showing that a new antibody panel composed of anti-TAA antibodies has a much greater potential of cancer classification by antibody-based proteomics compared to that of PPmAbs with previously unknown specificity.

Based on this research, we are planning to prepare antibody panels consisting of various anti-TAA antibodies/mAbs. More than 50 anti-TAA antibodies/mAbs were chosen for generation of the antibody panels: (i) overexpressed antigen such as Her2/neu34), (ii) cancer testis antigen such as MAGE family35), (iii) mutant antigen such as p53 protein36), (iv) differentiation antigen such as gp10037), (v) carcinogenesis virus antigen such as human papillomavirus (HPV) E638). The reactivity between the anti-TAA antibody panel and some cancer cells was investigated using IFA and then analyzed using multiple discriminant analysis, and the properties of the new antibody panel will be evaluated from the results. In the future, patient blood samples and tissue extracts using the antibody microarray analysis combined with the new antibody panel may be used in research and clinical examinations for cancer diagnosis. In addition, to investigate the profile of TAA expression in tumor tissue, it may be more difficult to identify the specificity of all PPmAbs. To solve this problem, we focused on the reactivity of the AKPS288 PPmAb with cancer tissues in IHC; the AKPS288 PPmAb stained the antigen localized in the cytoplasm of tumor cells of the colon and stomach but did not react with non-tumor cells in both tissues. Moreover, the AKPS288 PPmAb-related antigen was identified using MS as TMF/ARA160, a TAA. These results highlight the significance of PPmAbs with TAA specificity, showing that a new antibody panel composed of anti-TAA antibodies has a much greater potential of cancer classification by antibody-based proteomics compared to that of PPmAbs with previously unknown specificity.

Table 3. The frequency of positive reactions for the AKPS288 PPmAb-related antigen in cancer cell lines by IFA.

| Derived from | Cell line | (%) positivity | Derived from | Cell line | (%) positivity |
|--------------|-----------|----------------|--------------|-----------|----------------|
| Colon        | HCT-15    | 88.7           | Pancreas     | PK-9      | 49.8           |
|              | DLD-1     | 88.3           |              | PANC-1    | 24.7           |
|              | CoLo-TC   | 77.3           |              | MIA PaCa-2| 13.3           |
|              | LoVo      | 24.0           |              | PK-8      | 12.0           |
| Stomach      | Ocum-1    | 29.0           | Lung         | A549      | 35.5           |
|              | Kato-III  | 19.0           |              | EBC-1     | 7.3            |
|              | MKN-7     | 5.3            |              | N231      | 1.0            |
|              | Okajima    | 0.0            | Prostate     | LNCap-FGC | 75.0           |
| Liver        | Li-7      | 70.0           |              | DU-145    | 23.0           |
|              | Huh-7     | 38.7           | T cell       | Molt-4    | 98.3           |
|              | HepG2     | 30.7           |              |           |                |

Three hundred cells per cell line were examined at a magnification of ×400 and the positive frequency for the AKPS288 PPmAb-related antigen was calculated from fluorescent-positive cells.

Fig. 3. Reactivity of AKPS288 PPmAb with tumor cell lines using indirect immunofluorescence assay (IFA).

Seven kinds of human tumor cell lines were smeared on a glass slide and treated with the AKPS288 PPmAb, followed by FITC-conjugated anti-mouse IgM antibody. Cells were examined under a fluorescence microscope (×400). Scale bars=25 μm.
advantageous to examine mRNA expression of TAAs in tumor tissues.

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ABBREVIATIONS

AGL; amylo-1, 6-glucosidase, 4-alpha-glucanotransferase, CBB; Coomassie brilliant blue, DCD; dermcidin, FFPE; formalin-fixed paraffin-embedded, FITC; fluorescein isothiocyanate, hSPT5; transcription elongation factor SPT5, HPV; human papillomavirus, IFA; indirect immunofluorescence assay, Ig; Immunoglobulin, IHC; immunohistochemistry, IP; immunoprecipitation, MS; mass spectrometry, PBS; phosphate-buffered saline, POD; peroxidase, PPmAb; anti-phosphoprotein monoclonal antibody, PVDF; polyvinylidene difluoride, RIPA; radio immunoprecipitation assay, SDS; sodium dodecyl sulfate, SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis, TAA; tumor-associated antigen, TMF/ARA160; TATA-element modulatory factor 1, WB; western blot assay

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