Staining with Highly Sensitive Coomassie Brilliant Blue SeePico™ Stain after Flamingo™ Fluorescent Gel Stain Is Useful for Cancer Proteomic Analysis by Means of Two-dimensional Gel Electrophoresis

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Abstract. *Highly sensitive Coomassie brilliant blue SeePico™ Stain was applied for proteomic analysis using two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). After staining with Flamingo™ Fluorescent Gel Stain, the images of the protein spots were analyzed, and 424 protein spots were detected. After washing with Milli-Q water three times, the gels were re-stained with SeePico™ Stain and the images of the protein spots were analyzed; 272 spots were detected. To assess whether SeePico™ Stain alters MS analysis, a spot was picked up and was analyzed by LC-MS/MS. The MS analysis showed good protein identification. These results show a possible role for SeePico™ Stain in cancer proteomics using 2-DE and MS.*

Proteomics is an established molecular profiling technology that may significantly accelerate human cancer research. Development of high-throughput proteomic analysis provides a new tool to study biomarkers and the pathogenesis of cancer. Proteomic differential display analysis has been used to characterize the molecular events occurring in cancer (1). Two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) are important techniques in proteomics to identify comparatively protein expression profiles which may be associated with cancer. 2-DE simultaneously separates thousands of proteins from complex biological samples according to the isoelectric point in the first dimension and molecular weight in the second dimension (2). For the discovery of biomarker and target proteins, it is most important to be able to display extremely small amount of proteins. For this purpose, nowadays, applications of very sensitive fluorescent dyes for 2-DE have been developed (3). After gel image analysis, particular protein spots should be picked up for MS analysis. This gel picking procedure is a very important step, because if the wrong spots are picked up, incorrect MS analysis results are obtained. Commercial gel spot picker machines are very popular for gel spot picking, and automatically select and cut the spots (4). However, since these machines are very expensive, many investigators still use Coomassie brilliant blue (CBB) staining or silver staining after fluorescent staining. CBB staining is not sensitive and the gel image pattern obtained by silver staining is not completely the same as that obtained using fluorescent staining. In the present study we re-stained spots with highly sensitive CBB SeePico™ Stain for the 2-DE gels which had been firstly stained with Flamingo Gel Stain, and selected the protein spots with the aim of the identification of the protein by MS analysis.

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Materials and Methods

Sample preparation. QR-32 was kindly provided by the Laboratory of Pathology, Cancer Institute, Hokkaido University School of Medicine. QR-32 is a transplantable murine fibrosarcoma cell line which has been described previously (5). The nuclear proteins from QR-32 were extracted by means of NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction.

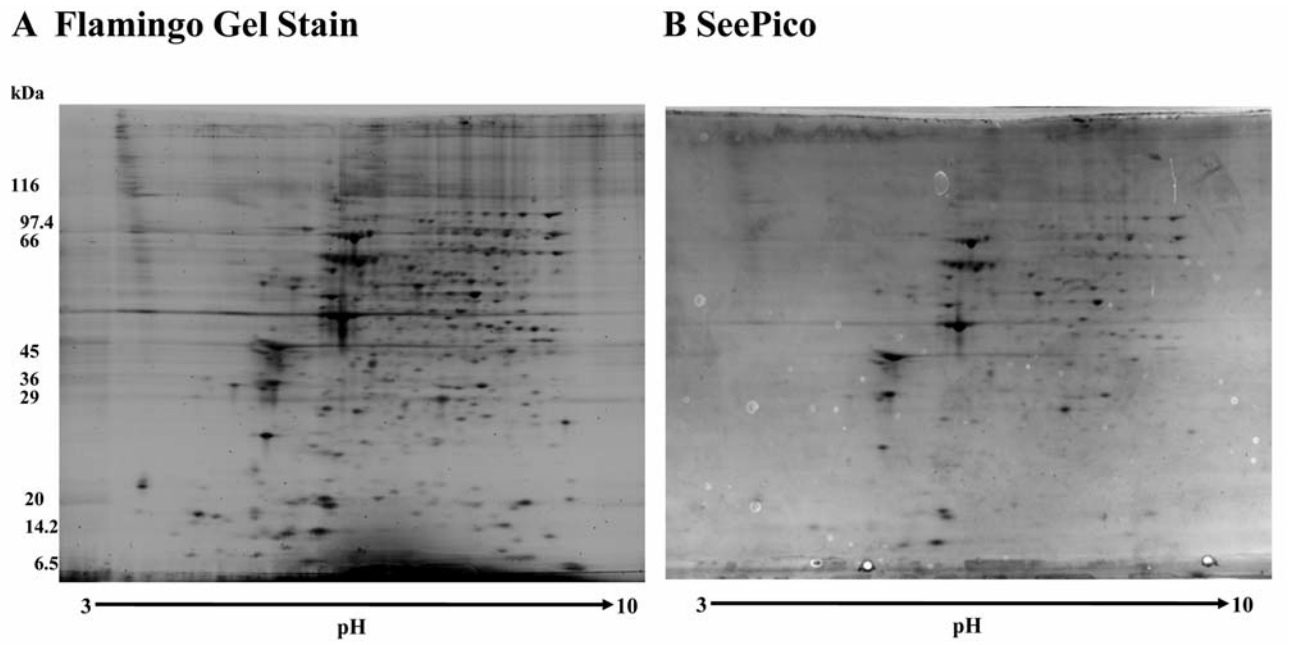

Figure 1. *2-DE patterns of QR32 cell nuclear fraction stained with Flamingo Gel Stain™ and SeePico Stain™. Panel A shows the 2-DE patterns of the nuclear fraction of QR-32 stained with Flamingo Gel Stain™. Panel B shows the 2-DE patterns of the same nuclear fraction of QR-32 stained with SeePico Gel Stain™. Proteins (80 μg) were separated by 2-DE using a pH 3-10 gradient in the first dimension, followed by second-dimensional separation on a precast polyacrylamide gel with a linear concentration gradient of 5-20%.*

2-DE. Eighty μg of protein were used for each 2-DE. For the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip. Samples of QR-32 nuclear proteins were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed using the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. In the second dimension, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (6).

Fluorescent gel staining. After 2-DE, the gels were subjected to fixing with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight. Stained gels were washed with Milli-Q water three times. Agitation was carried out at all stages (7).

Highly sensitive CBB gel staining. After recording images, the gels were washed with Milli-Q water three times, then stained with a highly sensitive CBB gel stain, SeePico™ (Benebiosis Co., Ltd, Seoul, Korea) overnight. Stained gels were washed with Milli-Q water three times. Agitation was carried out at all stages (8).

Image analysis and spot picking. The positions of the protein spots on the gels were recorded by using a ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the proteins were quantified by analyzing the intensity of each spot with Progenesis SameSpot software (Nonlinear Dynamics Ltd. Newcastle Upon Tyne, UK) (8). The differences between expression of spots on the gel stained with Flamingo Gel Stain and on the gel stained with SeePico™ were analyzed. The selected spot whose stained intensity with SeePico™ was moderate, was cut and removed from the gels stained with SeePico™ for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

In-gel digestion. The SeePico™ dye was removed from the gel piece by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM dithiothreitol (DTT) for 15 min. The sample in the gel piece was reduced twice in 50% acetonitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30˚C. The samples were lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were dissolved in 0.1% formic acid (9).

LC-MS/MS. Protein samples dissolved in 0.1% formic acid were centrifuged at 21,500 ×*g* for 5 min and the supernatant was stored at –80˚C until use. An Agilent 1100 LC/MSD Trap XCT (Agilent

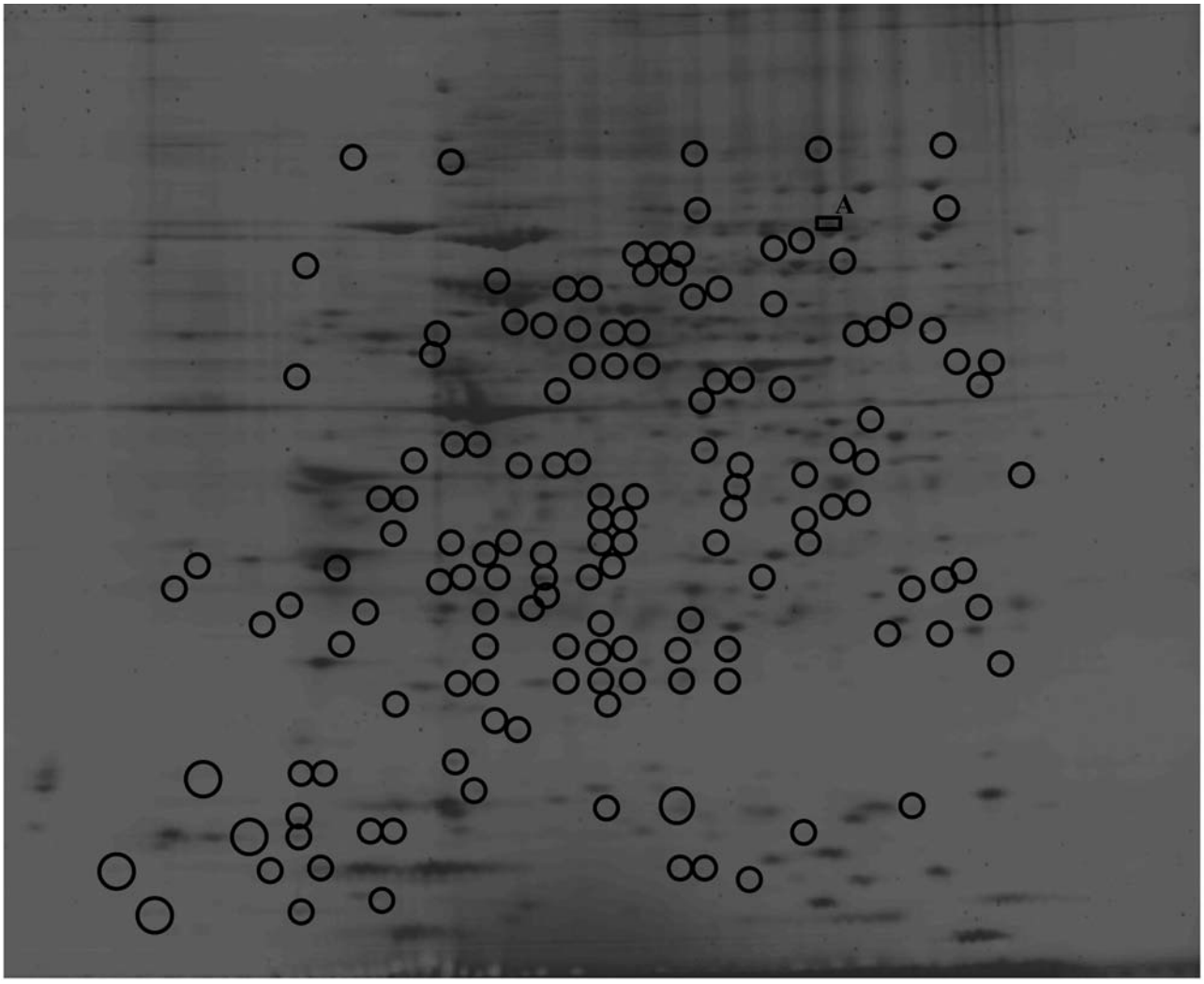

Figure 2. *2-DE pattern of QR32 cell nuclear fraction stained with Flamingo Gel Stain™ . This figure shows the 2-DE patterns of the nuclear fraction of QR-32 stained with Flamingo Gel Stain™ . The encircled spots were not stained with SeePico™ . Spot A was picked up and identified as lamin-A/C by LC-MS/MS.*

Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Twenty-five microliters of each sample were applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm; Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: solvent A: 0.1% formic acid, solvent B: ACN in 0.1% formic acid; column flow: 0.3 μl/min, primary flow: 300 μl/min; gradient: 0-5 min 2% solvent B, 60 min 60% solvent B; stop time: 60 min. Protein identification was performed in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS Ions search engine (http://www.matrixscience.com/search_form_select.html). The criteria for the positive identification of protein was set as follows: filter by protein score >10.0, and filter peptide by score >8, % scored peak intensity. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (10, 11).

Results

Detection of protein spots in 2-DE gels stained with Flamingo™ Fluorescent Gel Stain and SeePico™ . Protein expression was assessed in three samples of QR-32 nuclear fraction. Figure 1A shows the 2-DE gel stained with Flamingo™ Fluorescent Gel Stain, and Figure 1B shows the 2-DE gel stained with SeePico™ . The gel stained with Flamingo™ Fluorescent Gel Stain showed 424 spots; on the other hand, the gel stained with SeePico™ showed 272 spots. Figure 2 shows the spots which were recognized in only the gel stained with Flamingo™ Fluorescent Gel Stain. The encircled spots were not stained with SeePico™ .

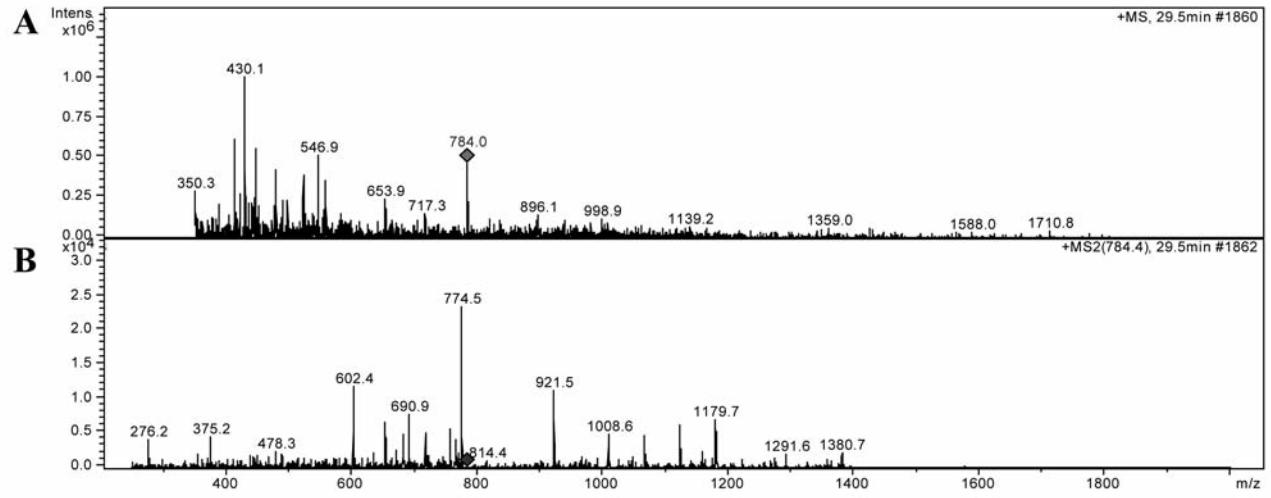

Figure 3. *MS and MS/MS spectra of trypsin-digested spot A. A: LC-MS spectra of trypsin-digested spot A. B: LC-MS/MS spectrum of a precursor ion with m/z 784.0 marked by a diamond in (A).*

Identification of the spot stained with SeePico™ . To evaluate the use of SeePico™ for proteomics, we picked up spot A (Figure 2) which was re-stained with SeePico™ after staining with Flamingo™ Fluorescent Gel Stain, and analyzed the spot by means of LC-MS/MS. The spot was clearly identified as lamin-A/C by using Agilent 1100 LC/MSD Trap XCT. Figure 3 shows MS (A) and MS/MS spectra (B) of the trypsin-digested spot A. The MS/MS spectrum was identified as the partial tryptic peptide NSNLVGAAHEELQQSR, SVGGSGGGSFGDNLVTR, VAVEEVDEEGKFVR, and YALINSTGEEVAmRK from lamin-A/C processed with a spectrum MILL workbench.

Discussion

Proteomic differential display analysis is a very useful and important method for analyzing cancer cells and tissues comparing them with control normal cells and tissues. In this method, cancer-specific proteins are identified by 2-DE and MS techniques. The 2-DE resolves a huge number of proteins by both isoelectric point and molecular weight, and examines changes in protein abundance. Since a large amount of protein sample is necessary for detection of the spots of less abundantly existing proteins, very sensitive fluorescence staining methods are used. An image of protein spots stained with fluorescence is generated by scanning the gel at suitable excitation and emission wavelengths, and this scanning compares the expression levels of the spots quantitatively. To identify the protein spots by MS, it is essential that they are accurately picked up. For this procedure, many investigators use automatic gel picking machines. However, this kind of machine is incredibly expensive. It is also possible to pick spots up from the gel on an LED transilluminator. However, it is visually very challenging for investigators. In the present study, we re-stained spots with highly sensitive CBB SeePico™ Stain for the 2DE gels which were stained with Flamingo Gel Stain first, and tried to pick the protein spots. The protein spot was analyzed by LC-MS/MS, and identified as being lamin-A/C. Since LC-MS/MS showed very clear and good spectra, SeePico™ staining is thought to be suitable for MS analysis.

On the other hand, SeePico™ staining gels showed only 272 spots, while the Flamingo™ Fluorescent Gel Stain showed 424 spots. For cancer proteomics, it is very important to identify cancer-specific, less abundantly existing proteins, because these kinds of proteins are expected to be biomarkers. The development of a more sensitive CBB stain is still awaited.

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