

Establishment of two-dimensional gel electrophoresis profiles of the human acute promyelocytic leukemia cell line NB4

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Received March 30, 2012; Accepted June 11, 2012

DOI: 10.3892/mmr.2012.963

Abstract. To explore optimum conditions for establishing a two-dimensional gel electrophoresis (2-DE) map of the human acute promyelocytic leukemia (APL) cell line NB4 and to analyze its protein profiles, we extracted total proteins from NB4 cells using cell disruption, liquid nitrogen freeze-thawing and fracturing by ultrasound, and quantified the extracted protein samples using Bradford's method. 2-DE was applied to separate the proteins, which were silver-stained in the gel. Well-separated protein spots were selected from the gel using the ImageMaster™ 2D Platinum analysis system. Moreover, the effects of various protein sample sizes (140, 160 and 180 μ g) on the 2-DE maps of the NB4 cells were determined and compared. Matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS), peptide mass fingerprinting (PMF) and database searching were used to identify the proteins. When the quantity of loading proteins was 160 μ g, clear, well-resolved, reproducible 2-DE proteomic profiles of the NB4 cells were obtained. The average number of protein spots in 3 gels was 1160 ± 51 with an average matching rate of 81%. A total of 10 proteins were identified by mass spectrometry and database queries, certain proteins were products of oncogenes and others were involved in cell cycle regulation and signal transduction. In summary, 2-DE profiles of the proteome of NB4 cells were established and certain proteins were identified by MALDI-TOF-MS and PMF which lay the foundation of further proteomic research of NB4 cells. These data should be useful for establishing a human APL proteome database.

Introduction

Acute promyelocytic leukemia (APL) is identified as the M3 subtype of acute myeloid leukemia (AML) and it appears to be the most malignant form of AML, characterized by a severe bleeding tendency and a fatal course of only weeks. Cytogenetically, a specific chromosome translocation, t(15;17) (q22;q21), occurs in more than 95% of APL patients which results in the rearrangement of the promyelocytic leukemia (PML) and retinoic acid receptor α (RAR α) genes and the expression of PML-RAR α chimeric protein (1,2). The front-line treatment for APL is chemotherapy, including the use of anthracycline and cytosine arabinoside, with a complete remission (CR) rate of 75 to 80% in newly diagnosed patients. The use of arsenic trioxide (ATO) from the early 1990s has further improved the clinical outcome of refractory or relapsed APL, as well as newly diagnosed APL (3,4). NB4, a maturation inducible cell line with a t(15;17) marker, is the cell model most commonly used in APL research (5).

Following genomics and transcriptomics, proteomics is considered to be the next step in the study of biological systems (6). Proteomics is the large-scale study of proteins, particularly their structures and functions (7). One of the most promising developments to come from the study of human proteins has been the identification of potential new drugs for the treatment of disease (8). This relies on proteomic information to identify proteins associated with a disease, which computer software may then use as targets in the design of new drugs (8). A proteome is the entire set of proteins expressed by a genome, cell, tissue or organism. More specifically, it is the set of expressed proteins in a given type of cell or organism at a given time under defined conditions (9-11). Proteomics, the study of the proteome, has largely been practiced through the separation of proteins by two-dimensional gel electrophoresis (2-DE) (12,13). In the first dimension, the proteins are separated by isoelectric focusing (IEF), which resolves proteins on the basis of charge. In the second dimension, proteins are separated by molecular weight using SDS-PAGE. The gel is dyed with Coomassie Brilliant Blue or silver to visualize the proteins. Spots on the gel are proteins that have migrated to specific locations. The mass spectrometer has augmented proteomics (14,15). Peptide mass fingerprinting (PMF) involves identifying a protein by cleaving

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Key words: acute promyelocytic leukemia, NB4 cells, two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionization time of flight-mass spectrometry, proteome

it into short peptides and then deducing the protein's identity by matching the observed peptide masses against a sequence database (16,17). Tandem mass spectrometry, however, is able to obtain sequence information from individual peptides by isolating them, colliding them with a non-reactive gas and then cataloging the fragments produced (18,19).

However, due to the high variability of protein expression, certain conditions, including the lysis solution formula, protein preparation method and volume of protein sample, may be suitable for one sample but not be the best choice for another. Therefore, establishing and optimizing the 2-DE technology to suit our particular research objectives became the key challenge of our proteomics study.

Materials and methods

Cell culture. The NB4 cell line was received as a gift from the Shanghai Institute of Hematology, Ruijin Hospital. The cells (1×10^6 /ml) were inoculated in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% heated-fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) in a humidified incubator containing 5% CO₂ and 95% air at 37°C.

Preparation of protein samples. Frozen cells were removed from liquid nitrogen and equilibrated for 10 min at room temperature, then dissolved in lysis buffer (100 μ l per 10^7 cells) containing 40 mmol/l Tris, 7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 1 mmol/l EDTA, 0.1 mg/ml RNase A, 0.1 mg/ml DNase and 1X protease inhibitor cocktail. The cell precipitate was resuspended and oscillated by vortex for ~1-2 min, then frozen and thawed 3 times, with the additional use of ultrasound for improved solubilization. Following centrifugation at 14,000 rpm for 30 min at 4°C, the supernatant was used as the 2-DE sample and the protein concentration was determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA). The protein samples were stored in aliquots and frozen at -80°C until use.

2-DE. Analysis using 2-DE was performed as described by Görg *et al.* (20). IEF was carried out using commercial immobilized pH gradient (IPG) dry strips (18 cm, pH 3.0-10.0 nonlinear; Amersham Pharmacia Biotech) which were rehydrated for 12 h at 20°C in the presence of 140, 160 and 180 μ g protein lysate, respectively. The proteins were then focused using the IPGphor system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Following IEF, the strips were equilibrated twice for 15 min in equilibration buffer containing 6 mol/l urea, 30% glycerol and 2% SDS in 50 mmol/l Tris-HCl buffer (pH 8.8) supplemented with 65 mmol/l DTT for the first treatment and 259 mmol/l iodoacetamide for the second treatment. Second-dimension SDS-PAGE was carried out using a Protean II cell (Bio-Rad) with a 13% SDS-polyacrylamide gel at a constant current of 20 mA/gel for the first 40 min and 30 mA/gel thereafter until the bromophenol blue dye marker reached the bottom of the gel. Each sample was run 3 times. Silver nitrate staining, according to the method of Pasquali *et al.* (21), and Coomassie Brilliant Blue R-250 (0.25% Brilliant Blue) staining was used for the analytical and micropreparative gels, respectively. For differential analysis, the gels were scanned using an ImageScanner

and analyzed using ImageMaster™ 4.01 software (both from Amersham Pharmacia). Only variations above 2 were considered as lower variations were not reproducible.

In-gel digestion and extraction of peptides. Firstly, the proteomics of the NB4 cells at various times were established by silver nitrate-staining. The differentially expressed protein spots were screened using image analysis software and artificially compared. Protein samples (1.2 mg) were then obtained for 2-DE electrophoresis and dyeing by Coomassie Brilliant Blue. The corresponding differential protein spots were identified, cut, decolorized and in-gel digested and the peptides were extracted according to the Thermo Finnigan operation process.

PMF by matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS). Following the matching of the micropreparative gel images and in-gel digestion, 1 μ l sample solution and an equal volume of the saturated matrix solution were mixed and applied to the target plate. All mass spectra were obtained using a Bruker Reflex III MALDI-TOF-MS (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV. Monoisotopic peptide masses were used to search the database, allowing a peptide mass accuracy of 0.3 Da and one partial cleavage. Oxidation of methionine and carbamidomethyl modification of cysteine were considered. The obtained peptide mass fingerprints were used to search through the SWISS-PROT and NCBIInr databases using the Mascot search engine. Protein identification was repeated at least once with spots from separate gels.

Further confirmation by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Certain spots, which we thought significant or for which we could not obtain confirmed results through searching of the SWISS-PROT or NCBIInr databases, were further investigated by ESI-MS/MS using a quadrupole-time of flight 2 (Q-TOF2) hybrid quadrupole/TOF mass spectrometer (Micromass, Manchester, UK) with a nanoflow Z-spray source. The mass spectrometer was operated in the positive ion mode with a source temperature of 80°C and a potential of 800-1,000 V applied to the Nanospray probe. The database search was carried out using the Mascot search engine with a Mascot MS/MS ion search. In addition, the amino acid sequences of the peptides were deduced using the peptide sequencing program MasSeq.

Statistical analysis. The results are the mean \pm standard deviations (SD) of 3 experiments performed in duplicate. Statistical analysis was carried out by the Student's test or one-way analysis of variance (ANOVA) using SPSS software 17.0. The Newman-Keuls test was used for the identification of statistically significant differences in spot vol% among samples. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Protein quantitation. The Bradford assay data were used to draw a standard curve for protein quantitation (Fig. 1). The linear equation was calculated to be: $y = 0.0288x + 0.0726$. The protein concentration of our sample was 11.83 mg/ml.

Table I. Identification of proteins: MALDI-TOF-MS, MALDI-TOF/TOF and UPLC-MS/MS analysis.

Spot	Protein	NCBI nr ID no.	Mw (Da)		pI		Peptides (MALDI/MS)		Sequence coverage (%)	Score
			Theor.	Observ.	Theor.	Observ.	Match	Total		
M160-1	CRP55	gil4757900	48,245	41,610	4.33	3.73	8	16	23	95
M160-2	CRT	gil1905911	48,112	19,441	5.26	6.49	9	22	51	91
M160-2	HSP70	gil52783267	70,854	19,823	5.37	7.07	11	18	19	81
M160-2	HMGB1	gil25090900	24,878	14,841	5.62	5.00	7	20	33	70
M160-2	RanGAP1	gil62911375	23,439	27,647	5.21	5.25	6	17	30	76
M160-2	PGM 1	gil10177166	28,786	26,711	6.64	5.93	11	29	51	119
M160-2	EF-1- β	gil18203449	24,748	32,071	4.50	4.38	6	13	37	70
M160-2	cofilin 1	gil37496526	18,491	19,179	8.22	8.51	10	23	53	101
M160-2	TM	gil66808019	28,938	33,947	4.79	4.70	12	25	28	137
M160-2	P4H	gil46095107	57,081	59,783	4.76	4.67	14	19	37	176

pI, experimental isoelectric point; Mw, molecular weight; CRP55, calreticulin precursor; CRT, calreticulin; HSP70, heat shock 70 kDa protein; HMGB1, high mobility group protein B1; RanGAP1, Ran-specific GTPase-activating protein; PGM 1, phosphoglycerate mutase 1; EF-1- β , elongation factor 1- β ; TM, tropomyosin isoform; P4H, prolyl 4-hydroxylase, β subunit; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight-mass spectrometry; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry.

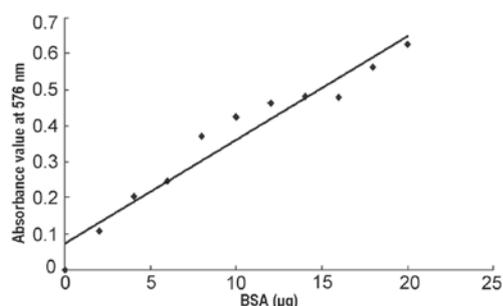


Figure 1. Protein quantitation standard curve.

Proteome expression maps of NB4 cells. Proteome expression maps of the NB4 cells were generated by 2-DE. We detected 1160 \pm 51 protein spots on the silver-stained gel using ImageMaster™ 2D Platinum software and manual clear-up. Approximately 96.3% of all spots were matched and had no significant change in intensity on duplicate gels. All maps demonstrated considerable similarity in their protein expression patterns; the 2-DE fingerprint matching rate of the same cell samples in separate batches was 81%. The spots were distributed in the greatest density at isoelectric points of 4-9 and relative molecular masses of 14-66 kDa (Fig. 2).

Effect of variations in protein sample size on the 2-DE mapping of NB4 cells. In order to confirm the optimum quantity of protein for use in the 2-DE mapping of NB4 cells, we compared and analyzed the effects of various protein sample sizes (140, 160 and 180 μ g) on the 2-DE maps. When 140 μ g protein was used, some of the protein spots appeared unclear or lost, and when the sample size was 180 μ g, some of the spots appeared agglutinated or sedimented. The protein spots were clearest when a 160 μ g sample size was used (Fig. 3).

Identification of proteins: MALDI-TOF-MS, MALDI-TOF/TOF and UPLC-MS/MS analysis. We identified 10 proteins by mass spectrometry and database queries. Some of these were products of oncogenes and others were involved in cell cycle regulation and signal transduction (Table I).

Discussion

Proteomics is a new research field of the post-genomic era, and its aim is the study of the expression and functions of all proteins in cells, tissues and organisms. Proteomic studies enable us to understand vital processes (22,23). There are three pivotal technologies involved in proteomics: 2-DE, mass spectrometric analysis and bioinformatic analysis (24). Therefore, the analytical procedure is usually divided into three steps: firstly, the separation of proteins from samples by 2-DE; secondly, the identification of the isolated proteins by mass spectrometry, and finally the storing, handling and comparison of data relating to proteins by bioinformatic analysis. Proteomics is rapidly developing and has become a research hotspot in the field of life science. therefore, how to get 2-DE maps with high resolution, high throughput and high reproducibility is the main bottleneck of proteomic technology development. Although the invention of IPG adhesive strips has greatly improved the reproducibility of the separation of proteins by 2-DE, there are numerous persistently complicating factors that had to be mastered as we established a 2-DE technology that was suitable for our particular research objectives (25). Therefore, in the current study we optimized each technical link of the proteomics technology in order to explore the optimum conditions for establishing a 2-DE map of NB4 cells.

Protein sample preparation is the first step in the 2-DE process; it is also the promoter of the technical system. Therefore, the quality of the protein preparation is likely to

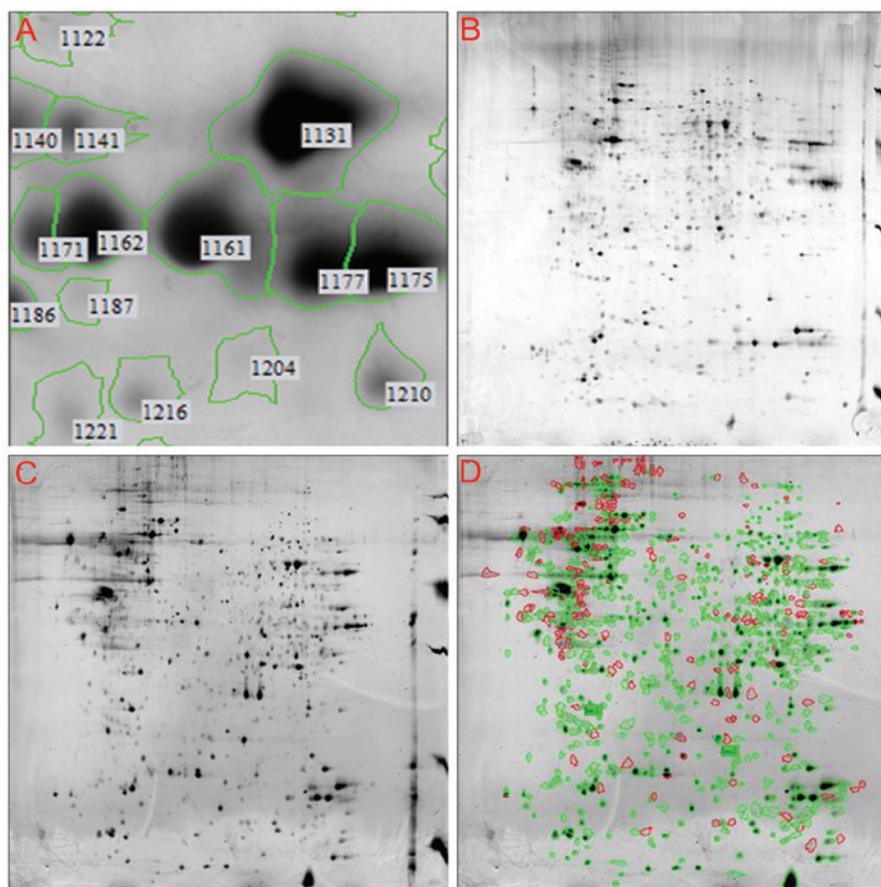


Figure 2. Resolution and matching rate of 2-DE maps of the human acute promyelocytic leukemia cell line NB4. (A) Detection of protein spots by 2-DE in NB4 cells; (B) 2-DE map of NB4 cells (Reference gel); (C) 2-DE map of NB4 cells (from various batches); (D) stacking chart of (B and C); green markers represent the matching protein spots and red represents unmatched proteins. 2-DE, two-dimensional electrophoresis.

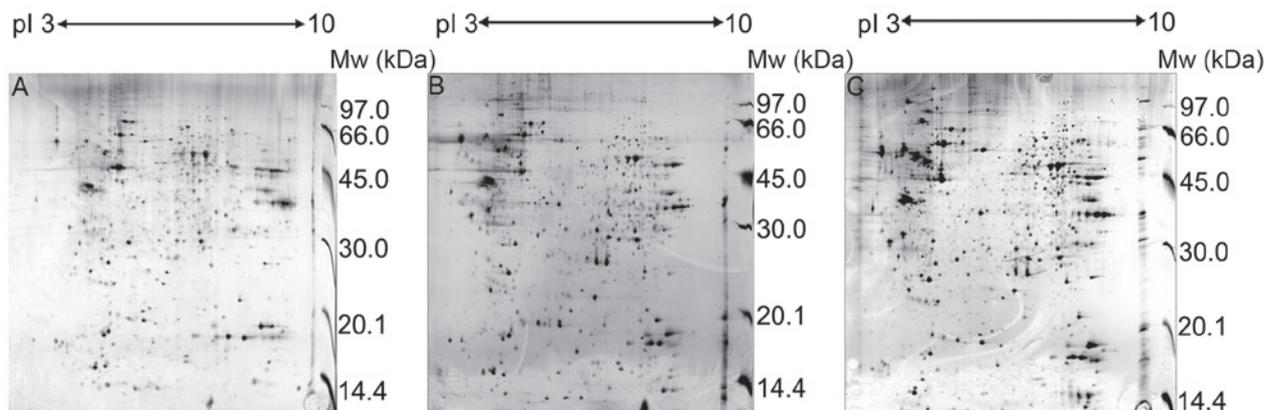


Figure 3. Effect of various protein sample sizes on the 2-DE mapping of NB4 cells. 2-DE maps prepared from (A) 140 μg , (B) 160 μg and (C) 180 μg protein samples. 2-DE, two-dimensional electrophoresis.

directly affect the effectiveness of 2-DE and determine the accuracy and comprehensiveness of the final results. NB4 is a suspension cell line. We followed the basic principles of protein sample preparation and referred to the related literature (26) for filtered cells and extracted proteins. We added the protease inhibitor to a lysis solution and used repeated liquid nitrogen freeze-thawing to improve protein solubility prior to total protein extraction. Ultrasound and nucleic acid enzymes were additionally applied to remove the influence of nucleic

acid contaminants. Following centrifugation at 14,000 rpm for 30 min at 4°C, the impact of lipids and polysaccharides were minimized as well.

Protein sample size in IEF directly affects the 2-DE spectrum resolution and reproducibility (27,28). When the sample size was too small, some of the protein spots appeared unclear or lost, while when the sample size was too large, some of the spots appeared agglutinated or sedimented. In our study, when a 160 μg protein sample was used, the protein spots appeared

comprehensive and clear. Moreover, the parameter settings of the IPGphor IEF and the SDS-PAGE vertical electrophoresis apparatus (29), the distribution of the acrylamide gel, the closeness of the contact between the IPG strip and the SDS-PAGE gel during electrophoretic transfer, dyeing methods and other factors also impact 2-DE mapping results (30).

Using these protein sample preparation, 2-DE, gel staining and scanning and image analysis methods and a series of other standardized operations, we obtained high resolution, reproducible 2-DE maps of the NB4 cells and successfully identified 10 differentially expressed protein spots, setting up a study platform for the NB4 cell proteome. Furthermore, this experiment not only provides useful information for further analysis and for building a related protein expression database, but also lays a foundation in the search for valuable tumor markers and a therapeutic molecular target for APL.

Acknowledgements

This study was supported by the Natural Science Foundation of China (no. 30701133) and the Shaanxi Province Science and Technology Development Fund of China (2010K01-135). We express our gratitude to Dr Hongli Wang for access to the Proteome Laboratory of the Institute of Basic Medical Sciences, National Center of Biomedical Analysis (Beijing, China).

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