Identification of leukemia restricted surface membrane proteome by biotinylation strategy

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Identification of leukemia restricted surface membrane proteome by biotinylation strategy

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Abstract

Identification of leukemia restricted surface membrane proteome by biotinylation strategy

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Numerous cell surface proteins of leukemia cells such as CD33 and CD52 have been used as diagnostic and therapeutic targets. Thus profiling of the cell surface proteome and proteins restricted to specific types of leukemia can provide a way to identify novel targets for leukemia diagnosis and therapy. However there has been little data pertaining to the comprehensive analysis of surface membrane proteins because there are few effective strategies for profiling surface membrane proteomes. In this study, I report on the application of quantitative proteomic techniques that incorporate affinity-capture and purification on monomeric avidin columns to find all biotinylated cell surface proteins from leukemia cell lines and leukemia patient samples.

Surface proteins were biotinylated and purified. Following solubilization, the proteins were resolved by 2-D PAGE using carrier ampholytes (pI 4 to 8)

in the first dimension, then visualized either by mass spectrometry-compatible silver staining or hybridization with streptavidin-HRP complex. The patterns visualized by silver stain and hybridization appear to be virtually identical from 6 different leukemia cells and patient samples. I have identifiend both well-known surface proteins such as CD antigens, immunoglobulins, receptors and also chaperonic proteins including heat shock proteins(HSPs) and protein disulfide isomerases (PDIs) which were previously considered to be largely ctoplasmic or endoplasmic reticulum proteins.

I also analyzed and compared gel images among six human leukemia cell lines to find those proteins restricted to specific cell lines. 120 of 749 total spots were cell-line specific. All 120 spots were analyzed and 57 spots were identified as 36 different proteins by MALDI-TOF. Thirty-five out of 36 spots had matched spots in different gels or identified in the same gel of different leukemia cells. we found a glycophorin restricted to K562 leukemia cell line that had no matched spot or did not identified in different leukemia cells. This finding suggests our method for gel image analysis and comparison may effective approach for the identification of the restricted proteins on a particular leukemia cells.

Comprehensive profiling of the leukemia cell surface proteome and gel image analysis and comparison provide an effective approach for the identification of commonly occurring proteins as well as proteins with restricted expression patterns to a specific cell line.

Key Words: surface membrane proteins, proteomes, leukemia

Identification of leukemia restricted surface membrane proteome by biotinylation strategy

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I. Introduction

Leukemia is a malignant cancer of the bone marrow and blood, which is characterized by uncontrolled accumulation of blood cells.[1] As in many cancers, protein expressions on the leukemia cell surface have provided many important clues to the diagnosis and treatment of this disease. Recently, numerous cell surface proteins have become targets in leukemia therapy. For example, Alemtuzumab is a humanized anti-CD52 monoclonal antibody that has been approved by the United States Food and Drug Administration for the treatment of fludarabine-refractory chronic lymphocytic leukemia (CLL). This

antibody binds to CD52, an antigen highly expressed on both B and T lymphocytes and on monocytes. The binding of alemtuzumab to CD52 can produce cell death in three ways: antibody dependent cellular cytotoxicity, complement activation, and direct induction of apoptosis.[2,3]

Gemtuzumab ozogamicin (GO) is comprised of a humanized anti CD 33 MoAb that is linked covalently to a semisynthetic derivative of a potent cytotoxic, calicheamicin.[4] The CD33 antigen, a 67-kD glycosylated transmembrane protein of unknown function, is expressed on most hematopoietic stem cells: on both mature and immature myeloid cells, including colony-forming progenitor cells; and on erythroid, megakaryocytic, and multipotent progenitors.[5] CD33 is also found on leukemic blasts from the majority of patients with AML and myelodysplastic syndromes (MDS). Because CD33 has little expression outside the hematopoietic system, it represents an attractive target for MoAb-based therapy in patients with AML. MoAb-mediated cross linking of CD33 inhibits proliferation of normal and myeloid leukemia cells.[6] Radio-labeled anti-CD33 MoAb rapidly saturates peripheral blood (PB) and bone marrow (BM) in in vitro studies and this phenomenon indicate rapid cellular internalization of the MoAb. These properties of CD33 make it a suitable target for MoAb-based therapy.

Cell surface proteins which are only expressed in specific types of

leukemia(s) or which undergo certain modifications only in the presence of leukemia might be used in various forms of immunotherapy, including antibody-based treatments and vaccines. Therefore profiles of the cell surface proteome and of proteins restricted to certain leukemia(s) may lead to the identification of novel targets for leukemia diagnosis and therapy.

Due to the lack of dependable strategies for profiling surface membrane proteomes, little data exists related to the comprehensive analysis of surface membrane proteins.[7-9] Recently, a global surface protein biotinylation strategy, coupled with the use of MS, was applied to the identification of cell surface proteins of *Helicobacter pylori*[10] and a variety of cancer cell types, and in the latter case identified a considerable number of chaperone proteins.[11,12] The high affinity and specific nature of avidin-biotin interactions have been used in diverse applications in immunology, histochemistry, *in situ* hybridization, affinity chromatography and many other areas.[13-17] Biotinylation reagents serve as tags which transform difficult to detect molecules into probes recognizable by a labeled detection reagents. Once tagged with biotin, a molecule of interest, such as an antibody or receptor ligand, can be used to probe cells, tissues or proteins immobilized on blots or arrays. The molecule thus tagged is then detected using a labeled avidin conjugate. While the binding of biotin to native avidin or streptavidin

is basically irreversible, modified avidins can bind biotinylated probes reversibly, making them valuable reagents for isolation and purification of biotinylated molecules from complex mixtures. I report here on the profiling of surface proteins of human leukemia cell lines by 2D image analysis and identification of proteins restricted to specific types of leukemia cell line(s) by comparing 6 human leukemia cell lines.

II. Materials and Methods

1. Samples

The non-adherent human leukemia cell lines, Sup B-15 (acute lymphoblastic leukemia-B cell), CEM/C2 (acute lymphoblastic leukemia-T cell), KG1 (acute myelogenous leukemia), U937 (acute monoblastic leukemia), HL-60 (acute promyelocytic leukemia), and K562 (erythroleukemia) were grown at 37°C in a 6% CO_2-humidified incubator in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Freshly isolated lymphoblastic leukemia cells were obtained by leukapheresis from 2 patients. One is a B-lymphoblastic leukemia patient and the other is a T-lymphoblastic leukemia patient.

2. Biotinylation of membrane proteins

Cultured cells grown in 225 cm^2 tissue culture flasks were harvested by centrifugation (1000 x g, 10 min at 4^oC) and washed three times with RPMI-1640 (without added serum or protein). Cells were suspended at 2.5 x 10^7 cells/ml in RPMI-1640 (without added serum or protein) containing 0.5 mg/ml EZ-Link-Sulfo-NHS-LC-Biotin was added and incubated at 37^oC for 10 min. The biotinylation reaction was terminated by addition of Tris-HCL (pH 7.5) to a final concentration of 50 mM. Following biotinylation, the cells were washed in PBS, pelleted by centrifugation and solubilized in PBS containing 2% NP-40. The cells were further disrupted by brief sonication.

3. Purification of biotinylated membrane proteins

Solubilized biotinylated membrane proteins from the human leukemia cell lines were purified on ImmunoPure Immobilized Monomeric Avidin columns (Pierce, Rockford, IL, USA), with modifications of the protocol supplied by the manufacturer. Briefly, 2.5 ml columns of Immobilized Monomeric Avidin were prepared and extensively washed with PBS. The columns were washed with 2mM D-biotin in PBS in order to block any non-reversible biotin binding sites on the column. The loosely bound biotin was removed from the reversible biotin binding sites by washing with 12 ml of 0.1M glycine (pH

2.8), and the columns were then extensively washed with PBS. The disrupted cells with membrane proteins biotinylation were again subjected to sonication, after which the solubilization solution was clarified by centrifugation (14,000 rpm for 20 min at 4^{o}C). The solubilization solution was passed through the Immobilized Monomeric Avidin columns three times, after which the column was again extensively washed with PBS containing 1% NP-40. The bound biotinylated proteins were eluted from the column with 5mM D-biotin in PBS containing 1% NP-40. Fractions containing eluted protein were concentrated on Centricon YM-3 columns (Millipore Corp, Bedford, MA, USA).

4. 2-D PAGE and western blotting

Biotinylated proteins were analyzed with 2-D PAGE. Proteins were solubilized with lysis buffer containing 8M urea, 2% pH 3.5-10 carrier ampholytes, 2% β-mercaptoethanol, 2% NP-40 and 10 mM PMSF. Isoelectric focusing was carried out using either pH 4-8 carrier ampholyte-based tube gels for 13,200 volt-hours at room temperature. First-dimension gels were loaded onto a cassette containing the second-dimension gel after equilibration in second-dimension sample buffer (125 mM Tris, pH 6.8 containing 10% glycerol, 2% SDS, 1% dithiothreitol and bromophenol blue). Separation in the second dimension was performed by electrophoresis in 7-14% polyacrylamide

gradient SDS gels, and the samples were electrophoresed until the dye front reached the opposite end of the gel. Some gels were silver-stained and digitized for pattern analysis. For some other gels, the resolved proteins were transferred to an Immobilon-P PVDF membrane. Unstained membranes were prepared for hybridization by incubation with blocking buffer (consisting of Tris buffered saline (TBS) containing 1.8% nonfat dry milk and 0.1% Tween 20) for 2 hours, then washed and incubated with a horseradish peroxidase conjugated biotin-streptavidin complex (at a 1:400 dilution) for 40 min at room temp. The membranes were washed five times with TBS containing 0.1% Tween 20, once in TBS, briefly incubated in ECL (Enhanced Chemiluminescence), and exposed to XAR-5 X-ray film. Patterns visualized were directly compared to comparable gel silver-stain patterns.

5. In-gel enzymatic digestion and mass spectrometry

Additional two-dimensional gels containing proteins eluted from avidin columns were silver-stained by successive incubations in 0.02% sodium thiosulfate for 2 min, 0.1% silver nitrate for 40 min, and 0.014% formaldehyde plus 2% sodium carbonate. The proteins of interest were excised from the two-dimensional gels and destained for 5 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate as described

previously.[18] Following three washes with water, the gel pieces were dehydrated in 100% acetonitrile for 5 min and dried for 30 min in a vacuum centrifuge. Digestion was performed by addition of 100 ng of trypsin in 200 mM ammonium bicarbonate. Following enzymatic digestion for 18 h at 37 °C, the peptides were extracted twice with 50 µl of 60% acetonitrile/1% trifluoroacetic acid. After removal of acetonitrile in a vacuum centrifuge, the peptides were concentrated using pipette tips (C18, Millipore, Bedford, MA, USA).

Peptide mixtures were analyzed using either an Amersham Biosciences (Arlington Heights, IL, USA) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Pro Version 2.0 mass spectrometer, or by nanoflow capillary liquid chromatography coupled with electrospray quadrupole time of flight tandem mass spectrometry (ESI Q-TOF MS/MS) in the Q-TOF micro (MicroMass, Manchester, UK). The peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in acetonitrile containing 1% trifluoroacetic acid (0.5 µl of sample:0.5 µl of matrix). Peptides were selected in the mass range of 800-3000 Da. MALDI-TOF MS gave a peptide mass fingerprint for each spot based on the molecular mass of trypsin-digested products. I calibrated the spectra with known trypsin-digested peptides and NCBI databases. ESI MS/MS tandem spectra were recorded in

the automated MS to MS/MS switching mode, with an *m*/*z*-dependent set of collision offset values. Doubly to triply-charged ions were selected and fragmented, with argon used as the collision gas. The acquired spectra were processed and searched against a non-redundant SwissProt protein sequence data base using the ProteinLynx global server.

6. 2-D image analysis and comparison

2-D images of human leukemia cell lines were analyzed and compared using Progenesis Workstation version 2002.01software (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK). Before I analyzed the gel image we selected a set of best gel images of 6 human leukemia cell lines. The automatic analysis protocol included spot detection, warping, background subtraction, average gel creation, matching, and reference gel modification. After manually removing false positive spots, matching was done using a default, fully automated, method. A minor amount of spot editing was performed including spot splitting corrections as well as minor match editing. After minor spot editing, matched spots shared the same numbers through synchronization. The results were exported to Excel for analysis.

III. Results

1. Biotinylation of surface membrane proteins

Figure 1A shows the biotinylated proteins in whole cell lysates of U937 acute monoblastic leukemia cells subsequent to cell surface labeling. The pattern of visualized biotinylated proteins has an abundance of separated proteins which do not show up in silver stained 2-D gels of the same whole cell lysates(Fig. 1B). Many of the resolved biotinylated proteins show trains of spots, as one might anticipate with proteins that go through many post-translational modifications such as glycosylation, phosphorylation and sulfation. While some proteins appear in the same location in both the silver-stained (Fig. 1B) and biotinylated (Fig. 1A) 2-D patterns, most biotinylated proteins fail to appear in the silver stained 2-D pattern of whole cell lysates. This strongly suggests that I obtain a selective and enhanced visualization of low abundance proteins through biotinylation of the surface membrane.

Figure 2 shows the similarity of silver stained 2-D images of whole cell lysates from 6 different leukemia cells. Most proteins of whole cell lysates fail to appear in the biotinylated 2-D patterns.

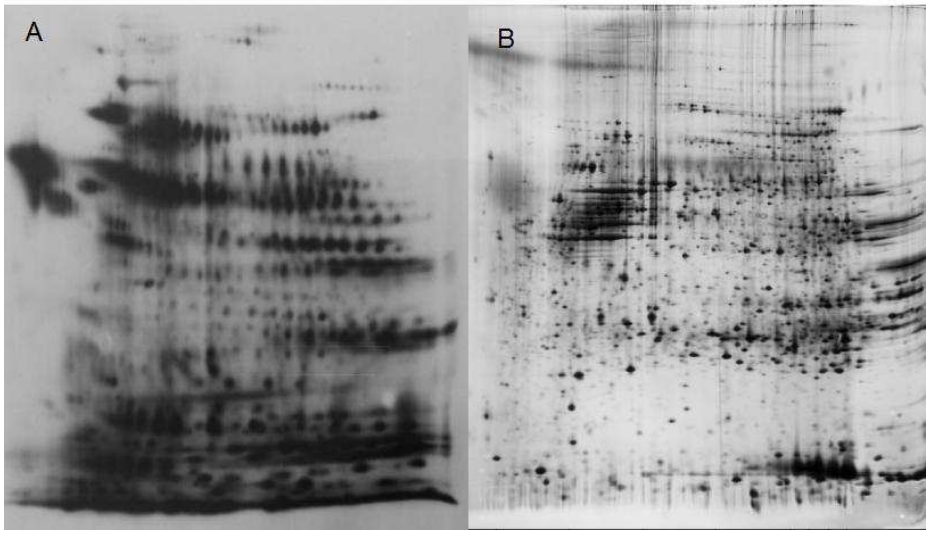

Figure 1. Visualization of biotinylated surface proteins in U937 acute monoblastic leukemia cells.

Detection of biotinylated surface proteins of U937 cells. Surface proteins of intact U937 cells were biotinylated, solubilized, resolved by 2-D PAGE, and then transferred to PDVF membranes(A). They were visualized by hybridization with streptavidin-HRP complex. Interestingly, a lot of proteins were detected, which were not present in the 2-D gels of same whole cell lysates shown in B.

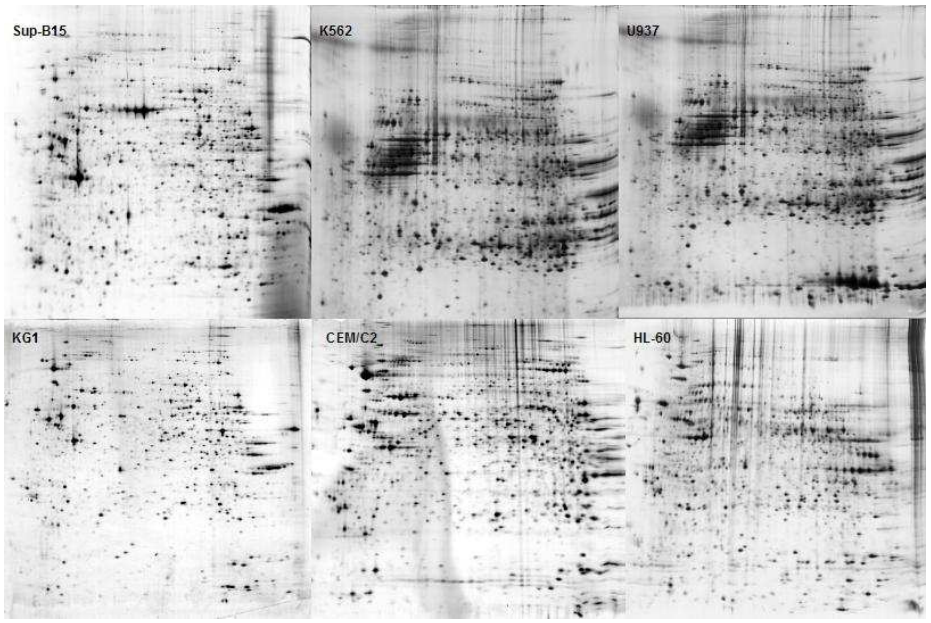

Figure 2. Similarity of silver stained images of whole cell lysates from 6 different leukemia cells.

Expression patterns of whole cell lysates from 6 different leukemia cells were similar each other but most proteins fail to appear in the biotinylated 2-D patterns.

2. Purification of biotinylated surface membrane proteins

Figure 3 shows that the pattern of biotinylated proteins from the U937 cell line visualized by hybridization closely resembles the pattern obtained from silver stained gels of another aliquot of the same preparation that were bound to a monomeric avidin column. The patterns visualized by silver stain and hybridization appear to be virtually identical. Figure 4 shows that the pattern of biotinylated proteins obtained from silver stained gels of 6 different

leukemia cells. The patterns visualized by silver stain and hybridization appear to be identical. The results of comparison of these biotinylated proteins from 6 different leukemia cells have described later.

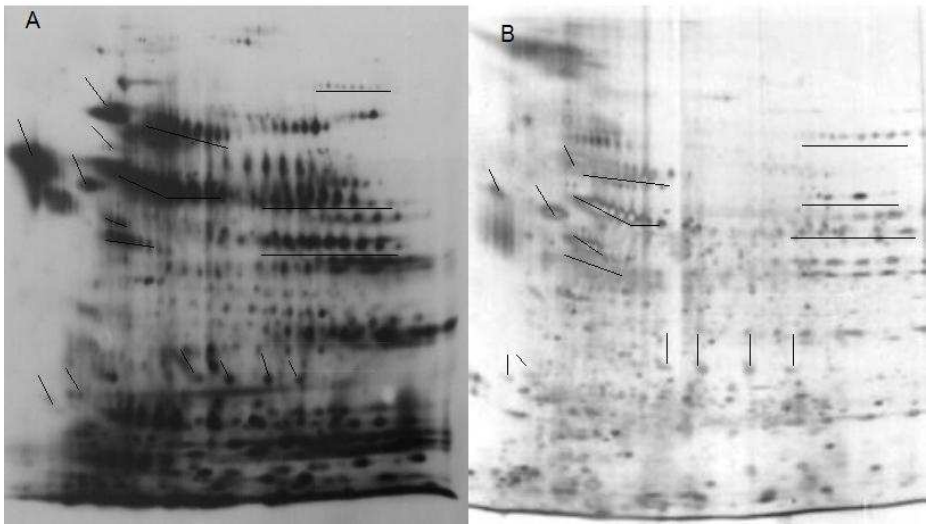

Figure 3. Similarity of U937 cell line biotinylation patterns as visualized by hybridization and silver-stained images of the same monomeric avidin column eluate.

Surface proteins of U937 were biotinylated and purified as described in “Experimental Procedure”. Following solubilization, the proteins were resolved by 2D PAGE using carrier ampholytes (pI 4 to 8) in the first dimension, then visualized either by mass spectrometry-compatible silver staining or hybridization with streptavidin-HRP complex, as described in “Experimental procedures”. Solid lines point to biotinylated proteins that were identified by mass spectrometry. Interestingly, the patterns visualized by silver stain and hybridization appear to be virtually identical.

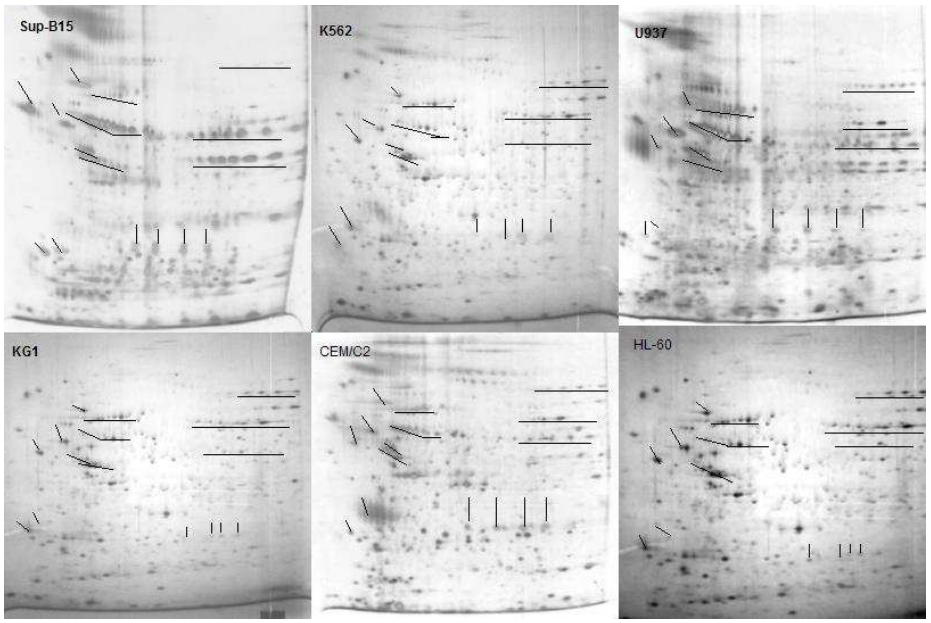

Figure 4. Similarity of biotinylation patterns as visualized by silver-stained images of 6 different leukemia cells.

3. Identification of biotinylated surface membrane proteins

Figure 5 shows that the pattern obtained from the Sup-B15 cell line (3A), B-lymphoblastic leukemia cell line, and the CEM/C2 cell line(3C), T-lymphoblastic leukemia cell line closely resembles the pattern obtained from the B-lymphoblastic leukemia patient sample (3B) and the T-lymphoblastic leukemia patient sample (3D). These silver stained 2-D gels of biotinylated proteins were identical to the pattern of biotinylated proteins visualized by hybridization. Solid lines point to biotinylated proteins obtained form four different cells shared common proteins, while showing other proteins that

were restricted to each cell populaion (Table 1).

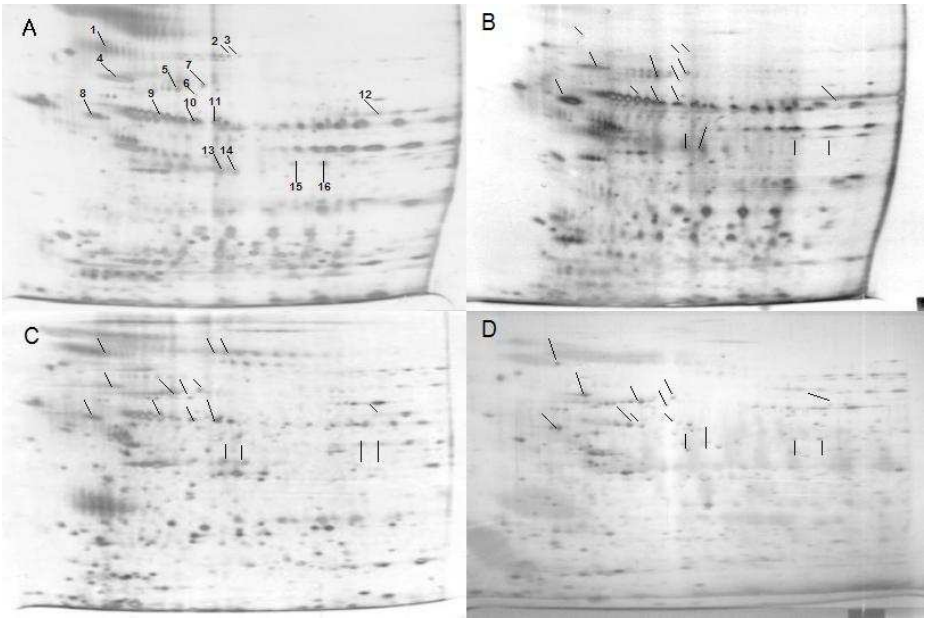

Figure 5. Similarity of biotinylation patterns between the Sup-B15, B-lymphoblastic leukemia cell line (A) and the CEM/C2, T-lymphoblastic leukemia cell line (C) as visualized by silver-stained images and B-lymphoblastic leukemia patient sample (B) and T-lymphoblastic leukemia patient sample (D).

Identified proteins are numerically labeled and indicated by lines. Solid lines point to biotinylated proteins obtained from four different cells which were highly similar to one another.

Table 1. Identified surface proteins from Sup-B15 acute B-lymphoblastic leukemia cell line.

Number	Gene symbol	Protein name
1	ITGB2	Integrin beta chain, beta 2 precursor
2	TFRC	Transferrin receptor
3	TFRC	Transferrin receptor
4	HSPA5	78kDa Glucose regulated protein, BiP
5	HSPA8	HSP 70Kda protein 8
6	HSPA2	Heak shock 70kDa protein 2
7	HSPA9B	GRP75
8	PDI	Protein disulfide isomerase
9	HSPD1	60kDa heat shock protein
10	JC5704	Protein disulfide isomerase
11	JC5704	Protein disulfide isomerase
12	CAT	Catalase
13	P10319	HLA-Bw58
14	P10319	HLA-Bw58
15	CD38	CD38 antigen
16	CD38	CD38 antigen

This proteins numbered in figure 5 were submitted to MALDI-TOF MS analysis and identified

Figure 6 shows profiling of the major chaperonic proteins of the 6 different leukemia cell line. There is relatively large set of proteins with chaperon functions, including heat shock proteins (HSPs), GRP78, HSP70, HSP60 and PDIs which are previously considered to be largely cytoplasmic or endoplasmic reticulum (ER) proteins. I confirmed CD38 expression on the cell surface of CEM/C2, acute T-lymphoblastic leukemia cell line, with monoclonal antibody to CD38 (Fig7).

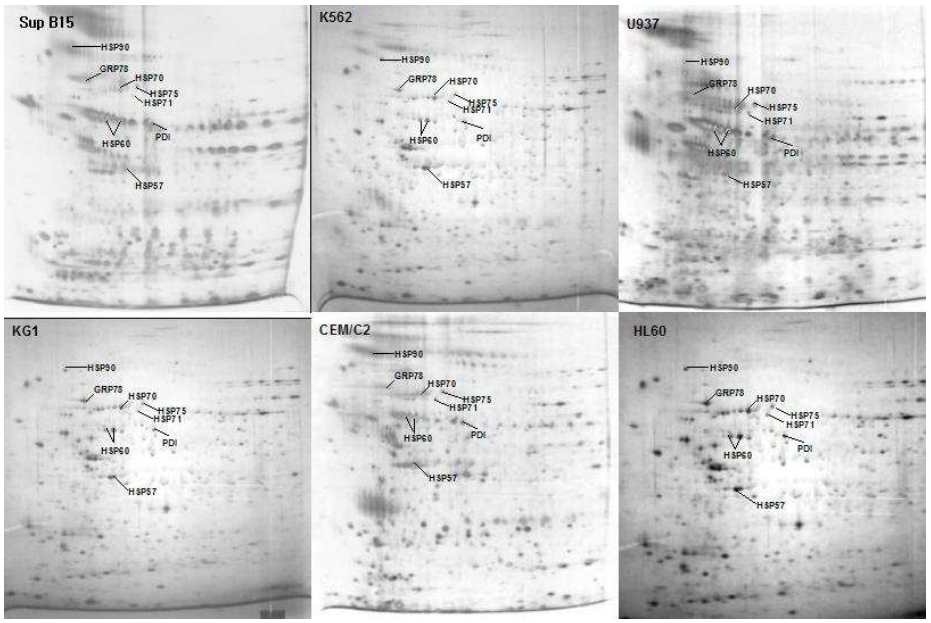

Figure 6. Expression of chaperone proteins from 6 different leukemia cell lines.

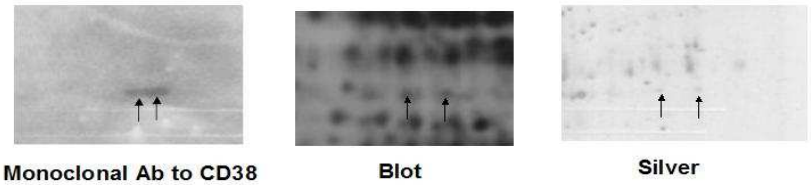

Figure 7. CD 38 expressions on the cell surface of CEM/C2 leukemia cell line with monoclonal antibody to CD38.

4. 2-D gel image analysis and comparison

Gel images from six different cell lines were compared to one another using Progenesis software. After automated processing, matched spots were individually confirmed by manual examination. The synchronization then gave matched spots the same number. In this study, a total of 749 spots were detected and analyzed from 6 leukemia cell lines. 695 spots were biotinylated and 54 spots non-biotinylated. 40 out of 749 (5%) spots were shared among 6 different cell lines. They were HSP90, HSP70, HSP60, PDI, catalase, GRP78, GRP75, CD38, integrin beta chain and transferring receptor (Table 1). 106 spots (14.2%) in 5 cell lines, 142 spots (19.0%) in 4 cell lines, 177 spots (23.6%) in 3 cell lines, 164 spots (21.9%) in 2 cell lines, and 120 spots (16.0%) were restricted to a specific cell line. 37 spots were restricted to the U937 cell line, 15 spots to the HL60 cell line, 24 spots to the Sup B-15 cell line, 30 spots to the KG1 cell line, 18 spots to the CEM/C2 cell line, and 6 spots to the K562 cell line. All 120 spots were analyzed and 57 spots were identified as 36 different proteins by MALDI-TOF. Even though some of them are cytosolic proteins, there are CD antigens and surface membrane proteins such as immunoglobulins and MHC molecules (Table 2). Thirty-five out of 36 proteins were restricted to one leukemia cell in this study but had matched spots in different gels or identified in different spot in the same gel of

different cell line. For example, Integrin alpha-L and integrin alpha-X were restricted in HL-60 leukemia cell line in this study but had matched spots in different gels of K562, KG1, Sub-B15. CD38 were restricted in KG1 but had identified spots in Sup-B15 and U937 and had matched spots in different gels of K562, KG1, and HL-60. Among the 36 identified proteins we found a glycophorin restricted to K562 leukemia cell line and had no matched spots in different gels of other leukemia cell line or did not identify in different cell line in this study. Fig 8 displays the restricted pattern of glycophorin in the K562 cell line based on comparison of the 6 leukemia gels.

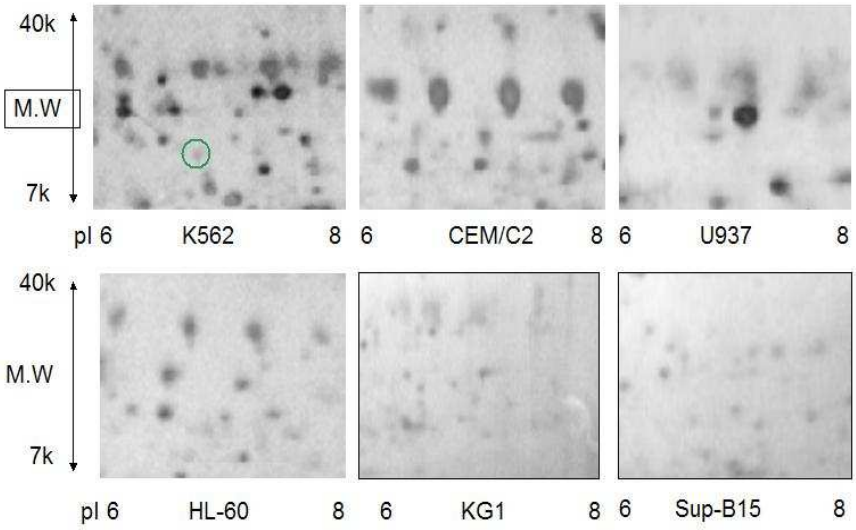

Figure 8. Restricted expression of glycophorin to the K562 cell line.

A circled spot was restricted to K562 cell line. The other spots were located at least 2 leukemia cell lines or restricted to other leukemia cell line.

Table 2. Identified proteins restricted to one leukemia cell.

Ptotein name	K562	CEM/C2	KG1	Sup-B15	U937	HL-60
MHC class I antigen HLA-A	Present	Present	MS	MS	Present	Present
HLA-DRB1	Absent	Present	Present	Present	Absent	Absent
Integrin alpha-L	MS	MS	MS	MS	Absent	Present
Integrin alpha-X	MS	MS	MS	MS	Absent	Present
Integrin beta-1	MS	Present	Present	MS	Present	Present
Integrin beta-2	MS	Present	Present	MS	Present	Present
GRP78	Absent	MS	MS	MS	MS	MS
CD31 antigen	Present	MS	Present	MS	Present	Present
Transferrin receptor protein 1	Present	Present	Present	MS	MS	MS
CD38 antigen (p45)	MS	MS	Present	Present	Present	MS
CD86 antigen	MS	Present	Present	MS	Present	Present
T-cell activation antigen CD26	MS	Present	Present	Present	Present	Present
T cell receptor beta chain	MS	Absent	Present	Absent	Absent	Absent
Heat shock protein HSP 90-beta	Present	MS	MS	Present	MS	MS
Protein disulfide isomerase A3	Present	MS	MS	MS	Present	Present
protein disulfide isomerase (PDI)	Present	Present	Present	MS	MS	MS
60 kDa heat shock protein	Present	MS	MS	MS	MS	MS
Heat shock 71 kDa protein	MS	MS	MS	MS	MS	Present
GRP75	Present	MS	MS	MS	MS	Present
CD45 antigen	Absent	Present	MS	Present	Present	Present
CD147 antigen	MS	Present	Absent	MS	Present	Absent
CD106 antigen	MS	Absent	Present	MS	Present	Absent
CD11b	MS	Present	MS	MS	Present	Present
CD18 antigen	Absent	MS	Absent	Present	Absent	Absent
CD44 antigen	Absent	Present	Absent	Present	MS	Present
Calnexin	Present	Present	Present	Present	Present	Present
Membrane glycoprotein M6-a	Present	MS	MS	MS	MS	MS
Catalase	Absent	Present	Present	Present	MS	MS
CD6	Present	Absent	Present	Present	Absent	Absent
Membrane progesterone receptor	Present	Present	Absent	Absent	Absent	Absent
Activin receptor type IIB	Absent	Present	Absent	Present	Absent	Absent
Flotilin2	MS	MS	Present	Present	Absent	Absent
4F2 antigen Heavy chain	Present	Present	MS	Present	Absent	Absent
Protein-tyrosine kinase	Present	Present	Present	MS	Absent	Absent
P-glycoprotein	Present	Absent	Present	MS	Absent	Absent
Glycophorin	Present	Absent	Absent	Absent	Absent	Absent

IV. Discussion

My approach consists of the biotinylation of surface membrane proteins of surface membrane proteins of leukemia cells, followed by their comprehensive profiling, identification, analysis and comparison. A basic component in a biotin-avidin based application is the moiety to be targeted. In the case of proteins, biotinylation is usually done via the ε-amino group of lysine using an *N*-hydroxysuccinimide (NHS) ester of a biotin analog. To selectively label only those lysine residues that are extracellular in orientation, non-membrane permeable biotin reagents need to be utilized to prevent the entry of biotin into the cell. Sulfo-NHS-LC biotin is water-soluble; thus it is not permeable across hydrophobic lipid bilayers and can be utilized for the selective labeling of surface membrane proteins. Other groups that have utilized sulfo-NHS-biotin for isolation of individual cell surface proteins in intact cells have found selective biotinylation of plasma membrane proteins.[10] My approach for the comprehensive profiling and identification of surface membrane proteins involves the selective biotinylation of the surface proteins of intact cells. As the population of surface membrane proteins that are biotinylated represent low abundance proteins at the whole cell level, it is necessary to utilize an enrichment procedure following biotinylation to allow

identification of the biotinylated proteins. To this end, a column of immobilized monomeric avidin is utilized to bind biotinylated proteins. In this study, column eluates from different cell populations are concentrated 400-fold, and aliquots resolved by 2-D PAGE, followed by either silver staining for some aliquots or transfer to PVDF membranes, the biotinylated proteins are visualized by hybridization with a streptavidin/horseradish peroxidase complex and detected with ECL reagent. As shown in figure 1 and figure 3, I have obtained a selective and enhanced visualization of low abundance proteins through biotinylation of the surface membrane and most proteins captured on the column represented biotinylated proteins.

In this study I have identified both well- known surface proteins such as CD antigens, immunoglobulins, receptors and also chaperonic proteins including heat shock proteins (HSPs) and protein disulfide isomerases (PDIs). Because HSPs and PDIs were previously considered to be largely cytoplasmic or endoplasmic reticulum (ER) proteins, I review current knowledge of cell surface expression of specific chaperone proteins. PDI, like other members of its family, is a soluble protein, not likely to be inserted into the membrane. Some interesting roles have been attributed to cell surface PDI. In general, the protein thioredoxin is seen to be involved in the reducing activity of the cell

exterior,[19] so that macromolecule disulfide bridges coming into contact with or present on the cell surface can be reshuffled or reduced. For example, the cell surface thiol levels in lymphocytes and in fibrosarcoma cells positively correlate with the amount of cell surface PDI.[20,21] In contrast, an inverse correlation has been found in B cell chronic lymphocytic leukemia, in which inhibition of surface-bound PDI with bacitracin or anti-PDI antibodies resulted in a high increment in thiols of the cell membrane proteins.[22] Although the function of these external thiols and their relationships with PDI remain unclear, their importance is suggested by the significant increase in surface thiols in conditions such as lymphocyte activation or leukemia.[20-22]

Recently It has been reported that the surface role of HSP90[23] and HSP70[24]. As shown in figure 6, a large set of chaperone proteins are shared spots especially in acidic portion of gels. But the exact amounts and roles of chaperone proteins on the cell surface are still not clear. Although the functions of some chaperone proteins on the surface membrane of hematopoietic cells or leukemia cells has been demonstrated, a satisfactory explanation of their function remains to be elucidated.

In many instances CD antigens are expressed only at certain stages of

development and the biological functions of many of the identified proteins are still unknown. Unlike the morphological criteria used in classical hematology for the description of specific developmental stages of lymphocytes, the use of monoclonal antibodies allows the objective and precise analysis and standardized typing of mature and immature normal and malignant cells of all lineages. Use of these antibodies also helps to delineate the biologic traits that distinguish normal immuno and hematopoietic cells from their malignant counterparts, which is of fundamental importance in understanding hematological malignancies, especially leukemias. However, these antibody-based methods for the identification of surface membrane proteins such as flow cytometry are expensive and labor-intensive, requiring 5-20μl quantities of fluorescently-labeled antibodies (10-500μg/ml) and allowing concurrent analysis for only a limited number of surface membrane proteins or CD antigens, usually three to four.[25] In this study I identified many surface membrane proteins, including CD antigens, on each 2D gel in human leukemia cell lines at the same time. As shown in figure 5 and table 1, I identified similar surface membrane protein patterns between leukemia patient samples and cell lines. Despite many advances in 2-D technology, there is some limitation. One of them is reproducibility of 2-D gel image especially in the basic portion. Expression of CD38 is in basic portion in 2-D gel image.

This is why I used monoclonal Antibody to CD38 to confirm CD38 expression on the cell surface of leukemia cells in this study.

Two-dimensional electrophoresis is currently the core technology for studying the differences in protein expression levels and their post-translational modifications between various biological samples. The power of the 2-DE technique lies in its capacity to separate simultaneously thousands of proteins for subsequent protein identification and quantitative comparison studies. However despite the significant improvements to the technique such as immobilized pH gradients and its coupling with mass spectrometry analysis it is still difficult to automate and to reproduce the basic portion of 2-D gel images. In this study I have compared 6 different cell lines and only 40 out of 749 (5%) spots were shared among 6 different cell lines. This is because one protein can make various spot numbers on the 2 D-E gels according to posttranslational modifications and sometimes there are streak line instead of line of spots due to lack of reproducibility in the basic portion thus it is very hard to match the spots that identified as same protein. As shown in Table 2, 120 spots were thought to be restricted to one leukemia cell line (16%) were analyzed and 57 spots were identified as 36 different proteins by MALDI-TOF. Thirty-five out of 36 identified spots were restricted to one leukemia

cells in this study but had matched spots in different gel images or present in other position in the same 2-D gel image. As described earlier, some protein makes various numbers of spot on 2-D gel according to post-translational modification. Thus it is very hard to match as the same spots if one protein makes different number of spots in different gels.

Glycophorin is major red cell membraine sialoglycoprotein and has a molecular weight of 36kd. In its native state, glycophorin has little or no direct interaction with the membrane cytoskeleton.[26] The abundance of sialic acid is predominantly responsible for the negative charge of the red cell membrane. Thus, an important function of glycophorin is to minimize cell-cell interactions and prevent red cell aggregation in the circulation.[27] This protein has been found only in the K562 cell line, not in other leukemia cell lines.[26] In this study I identified a glycophorin restricted to K562 leukemia cell line. This finding suggests my method for gel image analysis and comparison can effective approach for the identification of the specific proteins on a particular leukemia cells.

V. Conclusion

The comprehensive profiling of the leukemia cell surface proteome and the gel image analysis and comparison provide an effective approach in identifying commonly occurring proteins as well as proteins with restricted expression patterns in a specific leukemia cell line. It is likely that characterization of the changes that occur at the cell surface during differentiation in response to various stimuli and during leukemogenesis processes will lead to a better understanding of these processes and will further expand the repertoire of diagnostic and therapeutic targets of leukemia.

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin 2003;53:5-26.
2. Dyer MJ, Hale G, Hayhoe FG, Waldmann H. Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. Blood 1989;73:1431-9.
3. Ginaldi L, De Martinis M, Matutes E, Farahat N, Morilla R, Dyer MJ, et al. Levels of expression of CD52 in normal and leukemic B and T cells: correlation with in vivo therapeutic responses to Campath-1H. Leuk Res 1998;22:185-91.
4. Giles FJ. Gemtuzumab ozogamicin: promise and challenge in patients with acute myeloid leukemia. Expert Rev Anticancer Ther 2002;2:630-40.
5. Wellhausen SR, Peiper SC. CD33: biochemical and biological characterization and evaluation of clinical relevance. J Biol Regul Homeost Agents 2002;16:139-43.
6. Bernstein ID. CD33 as a target for selective ablation of acute myeloid leukemia. Clin Lymphoma 2002;2 Suppl 1:S9-11.
7. Morre DJ, Morre DM. Preparation of mammalian plasma membranes by aqueous two-phase partition. Biotechniques 1989;7:946-8, 50-4, 56-8.
8. Jacobson BS, Stolz DB, Schnitzer JE. Identification of endothelial cell-surface proteins as targets for diagnosis and treatment of disease. Nat Med 1996;2:482-4.
9. Harvey S, Zhang Y, Landry F, Miller C, Smith JW. Insights into a plasma membrane signature. Physiol Genomics 2001;5:129-36.
10. Sabarth N, Lamer S, Zimny-Arndt U, Jungblut PR, Meyer TF, Bumann D. Identification of surface proteins of Helicobacter pylori by selective biotinylation, affinity purification, and two-

dimensional gel electrophoresis. J Biol Chem 2002;277:27896-902.

11. Jang JH, Hanash S. Profiling of the cell surface proteome. Proteomics 2003;3:1947-54.
12. Shin BK, Wang H, Yim AM, Le Naour F, Brichory F, Jang JH, et al. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. J Biol Chem 2003;278:7607-16.
13. Wilbur DS, Pathare PM, Hamlin DK, Stayton PS, To R, Klumb LA, et al. Development of new biotin/streptavidin reagents for pretargeting. Biomol Eng 1999;16:113-8.
14. Wilchek M, Bayer EA. Foreword and introduction to the book (strept)avidin-biotin system. Biomol Eng 1999;16:1-4.
15. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem 1991;37:625-36.
16. Bayer EA, Wilchek M. Biotin-binding proteins: overview and prospects. Methods Enzymol 1990;184:49-51.
17. Chapman-Smith A, Cronan JE, Jr. The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. Trends Biochem Sci 1999;24:359-63.
18. Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis 1999;20:601-5.
19. Mandel R, Ryser HJ, Ghani F, Wu M, Peak D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. Proc Natl Acad Sci U S A 1993;90:4112-6.
20. Lawrence DA, Song R, Weber P. Surface thiols of human lymphocytes and their changes after in vitro and in vivo

activation. J Leukoc Biol 1996;60:611-8.

21. Jiang XM, Fitzgerald M, Grant CM, Hogg PJ. Redox control of exofacial protein thiols/disulfides by protein disulfide isomerase. J Biol Chem 1999;274:2416-23.

22. Tager M, Kroning H, Thiel U, Ansorge S. Membrane-bound proteindisulfide isomerase (PDI) is involved in regulation of surface expression of thiols and drug sensitivity of B-CLL cells. Exp Hematol 1997;25:601-7.

23. Tsutsumi S, Neckers L. Extracellular heat shock protein 90: a role for a molecular chaperone in cell motility and cancer metastasis. Cancer Sci 2007;98:1536-9.

24. Mambula SS, Stevenson MA, Ogawa K, Calderwood SK. Mechanisms for Hsp70 secretion: Crossing membranes without a leader. Methods 2007;43:168-75.

25. Jennings CD, Foon KA. Recent advances in flow cytometry: application to the diagnosis of hematologic malignancy. Blood 1997;90:2863-92.

26. Gahmberg CG, Andersson LC. K562--a human leukemia cell line with erythroid features. Semin Hematol 1981;18:72-7.

27. Gahmberg CG, Jokinen M, Karhi KK, Ulmanen I, Kaariainen L, Andersson LC. Biosynthesis of the major human red cell sialoglycoprotein, glycophorin A. A review. Rev Fr Transfus Immunohematol 1981;24:53-73.

Abstract (in Korean)

바이오틴 기법을 이용한 백혈병제한단백질 발굴

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장 준 호

CD33, CD52 와 같은 백혈병세포 특이 단백질은 백혈병의 진단과 치료에 있어 중요한 표적이다. 이러한 백혈병특이단백질 발굴은 백혈병 진단 및 치료제 개발이 매우 중요하다. 하지만 현재까지의 방법으로는 포괄적인 백혈병특이단백질의 발굴이 매우 제한적이다. 이 실험에서 저자는 세포표지단백질의 바이오틴화, 아비딘 칼럼, 2차원 젤, 단백질 발굴 등의 프로테오믹스 기법을 이용하여 백혈병 세포의 포괄적인 표지단백질을 발굴하고자 하였고, 백혈병세포주와 백혈병 환자 세포의 표지단백질 분포를 비교하였으며, 특정 백혈병세포에 국한된 특이단백질을 발굴하고자 6개의 서로 다른 백혈병세포를 비교평가 하였다.

이 실험에서 세포표지단백질을 바이오틴화 하였고 순수분리하였다. Carrier ampholyte를 이용하여 1차원 분리한 후 2차 전기영동을 실시하여 이차원 젤을 얻었다. Mass spectrometry를 위하여 silver 염색을 시행하였고 streptavidin-HRP 복합체를 이용하여 hybridization을 시행하였다. 그 결과 6개의 서로 다른 백혈병 세포

주의 silver 염색 단백질 분포는 hybridization한 바이오틴 분포와 일치하였다.

저자는 이 실험에서 잘 알려진 세포표지단백질인 CD 항원, 면역글로불린, 수용체 등과 함께 과거 세포내 단백질로 알려져 있던 heat shock 단백질과 protein disulfide isomerase 등을 발굴하였다.

또한 6개의 서로 다른 백혈병 세포주를 비교 평가하여 특정 세포주에 국한된 제한 단백질을 찾고자 하였다. 총 749개의 단백질 중 120개가 세포주 제한 단백질로 나타났고, 이를 모두 분석하여 36개의 서로 다른 단백질을 발굴하였다. 36개의 단백질 중 35개는 이 실험에서 특정 백혈병세포제한단백질로 나타났으나 다른 세포주에 일치하는 단백질이 있거나 다른 젤에서 같은 단백질이 발굴되었다. 36개 단백질 중 K562에 국한되어 있는 것으로 알려진 glycophorin을 K562 세포주에서 발굴하였고 이 단백질은 다른 세포주에서 일치하는 단백질이 없었고 다른 젤에서 발굴되지 않았다. 이러한 결과는 바이오틴을 이용한 프로테오믹스 기법이 포괄적인 세포 표지 단백질 발굴 및 특정한 백혈병제한단백질 발굴에 유용함을 나타낸다. 프로테오믹스 기법을 이용한 포괄적인 세포표지 단백질 발굴 및 비교분석은 백혈병 세포에 흔히 존재하는 단백질 및 특정 백혈병제한단백질 발굴에 유용할 것으로 사료된다.

핵심되는 말 : 프로테오믹스, 표지단백질, 백혈병제한단백질