Proteomic Analysis of Human Acute Leukemia Cells: Insight into Their Classification

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ABSTRACT

Purpose: **French-American-British (FAB) classification of acute leukemia with genetic heterogeneity is important for treatment and prognosis. However, the distinct protein profiles that contribute to the subtypes and facilitate molecular definition of acute leukemia classification are still unclear.**

Experimental Design: **The proteins of leukemic cells from 61 cases of acute leukemia characterized by FAB classification were separated by two-dimensional electrophoresis, and the differentially expressed protein spots were identified by both matrix-assisted laser desorption/ionization–time-of-flight–mass spectrometry (MALDI-TOF-MS) and tandem electrospray ionization MS (ESI-MS/MS).**

Results: **The distinct protein profiles of acute leukemia FAB types or subtypes were successfully explored, including acute myeloid leukemia (AML), its subtypes (M2, M3, and M5) and acute lymphoid leukemia (ALL), which were homogeneous within substantial samples of the respective subgroups but clearly differed from all other subgroups. We found a group of proteins that were highly expressed in M2 and M3, rather than other subtypes. Among them, myeloid-related proteins 8 and 14 were first reported to mark AML differentiation and to differentiate AML from ALL. Heat shock 27 kDa protein 1 and other proteins that are highly expressed in ALL may play important roles in clinically distinguishing AML from ALL. Another set of proteins up-regulated was restricted to granulocytic lineage leukemia. High-level expression of NM23-H1 was found in all but the M3a subtype, with favorable prognosis.**

Conclusions: **These data have implications in delineating the pathways of aberrant gene expression underlying the pathogenesis of acute leukemia and could facilitate molecular definition of FAB classification. The extension of the present analysis to currently less well-defined acute leukemias will identify additional subgroups.**

INTRODUCTION

Acute leukemia is a heterogeneous disease with distinct biological and prognostic groupings, and the accurate classification is critical for treatment and prognosis. Classification of acute leukemia began with the observation of variability in clinical outcome and subtle differences in nuclear morphology. With the introduction of French-American-British (FAB) classification in 1976 (1), diagnosis and classification have been based on cytomorphology and cytochemistry, which provide the basis for the classification of acute leukemia into those arising from lymphoid precursors [acute lymphoid leukemia (ALL)] or from myeloid precursors [acute myeloid leukemia (AML)]. FAB classification was further solidified by the techniques of immunophenotyping, cytogenetics, and molecular genetics (2) and has been improved over the past decades. The new WHO classifications of acute leukemia (3) are presently used more often in clinical settings, which combine the analysis of morphology, immunophenotyping, cytogenetics, and molecular genetics of acute leukemia cells. Still, the present leukemia classification remains imperfect, and errors do occur. Moreover, no single test is currently sufficient for establishing the diagnosis, and current clinical practice involves experienced hematopathologist's interpretation of the tumor's morphology, histochemistry, immunophenotyping, and cytogenetic analysis. The types of acute leukemia with the unknown cytogenetic abnormalilties in the new WHO classification system are still diagnosed according to FAB classification, and the molecular characteristics that contribute to the subtypes of FAB classification are still unclear.

The molecular definition of leukemia classification could facilitate development of a more systematic approach to leukemia classification, which could be achieved by the proteomic analysis of distinct protein profiles (DPPs) of leukemia. Recent technological advances in proteomics allow us for the first time to examine the expression profiles at the protein level on genome-wide scale, because the proteomic approach is being actively applied to the molecular analysis of various human cancers such as bladder (4), colorectal (5), breast (6), stomach (7), and liver cancers (8) and leukemia (9). Another approach of DNA microarray analyses also shows the feasibility of the molecular analysis, but it should be stressed that mRNA levels

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do not necessarily correlate with protein levels (10), and disease-relevant information may also be hidden behind the changes in protein modification. In this study, we made a collection of 61 bone marrow samples obtained from *de novo* acute leukemia patients at the time of diagnosis, and attempted to create a set of "class predictor" by obtaining their type-distinct proteome. We showed the correlation of the type-specific DPPs with their corresponding FAB classification. Furthermore, our data have major implications with regard to delineating aberrant gene expression pathways underlying the pathogenesis of acute leukemia. In addition, these analyses may promote the identification of new targets for specific treatment approaches.

MATERIALS AND METHODS

Sources of Cells for Analysis. The leukemic cells were derived from bone marrow aspirates of a group of 61 patients ages 18–64 years (median, 44 years) with acute leukemia at the time of initial diagnosis before chemotherapy, and consent was received from all of the patients. The diagnosis was made according to the FAB classification system on the basis of morphologic features and histochemical staining of cells with peroxidase, esterase, and type-specific fusion genes (such as *PML/RARa* and *bcr/abl*) was detected by reverse transcription-PCR in some cases. Of the 61 cases, there were 10 cases with M1, 10 cases M2, 11 cases M3 [7 cases M3a (big granules), 4 cases M3b (thin granules)] (11), 10 cases M4, 10 cases M5, and 10 cases ALL. Leukemic cells were obtained by Ficoll-Hypaque gradient centrifugation of heparinized bone marrow. Normal white blood cells (NWBCs) including normal lymphoctyes and granulocytes (mainly neutrophils) were isolated from peripheral blood of healthy volunteers ($n = 8$) with gradient Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. The two gradient bands (lymphocytes and monocytes primarily in the top band and granulocytes in the middle band) were washed in PBS. The monocytes in the top band were removed by plastic adherence, as described by Gemmell and Anderson (12), and the nonadherent cells (lymphocytes) were collected as the normal lymphocyte sample and were washed with chilled PBS. The acute leukemia cells and NWBCs were pelleted in microcentrifuge tubes and were frozen at −80°C. Only samples with purity greater than 90% were submitted to subsequent two-dimensional electrophoresis analysis. The purity of the samples was determined by morphologic analysis under microscope.

Two-Dimensional Electrophoresis. Frozen cell samples were dissolved in lysis buffer (100 μL per 10^7cells), containing 40 mmol/L Tris, 7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1 -propanesulfonate (CHAPS), 1% dithiothereitol (DTT), 1 mmol/L EDTA, 0.1 mg/mL RNase A, 0.1 mg/mL DNase, and 1× protease inhibitor cocktail (Roche Diagnostic), additionally applying ultrasound for better solubilization. After centrifugation at 12,000 × *g* (Eppendorf, Hamburg, Germany) for 30 min at 4°C, the supernatant was used as the two-dimensional electrophoresisl sample, and the protein concentration was determined by the Bradford method (13) with a commercial Bradford reagent (Bio-Rad Laboratories, Hercules, CA). The protein samples were stored in aliquots and were frozen at −80°C until used.

Two-dimensional electrophoresis was performed as described by GÖrg *et al.* (14) with some modification. Briefly, 125 μg (about 10^6 cells) of proteins for analytical gels or 0.6 to 1 mg (about $6\sim10 \times 10^6$ cells) of proteins for micropreparative gels was diluted to 350 μL with rehydration solution [8 mol/L urea, 4% CHAPS, 0.5% immobilized pH gradient (IPG) buffer (Amersham Pharmacia Biotech)] and applied onto 18 cm (pH 3–10) linear immobilized pH gradient Drystrip (Amersham Pharmacia Biotech). The strips were rehydrated for 12 hours at 20°C. The proteins were then focused on the IPGphor system (Amersham Pharmacia Biotech) according to the manufacturer's protocol (15). The strips were equilibrated for 15 minutes in a solution containing 65 mmol/L DTT, 6 mol/L urea, 30% w/v glycerol, 2% w/v SDS, and 50 mmol/L Tris-HCl (pH 8.8). A second equilibration step was also carried out for 15 min in the same solution except for DTT, which was replaced by 259 mmol/L iodoacetamide. Separation in the second-dimensional electrophoresis was carried out in the PROTEAN II Cell (Bio-Rad company) with a 13% SDS-polyacrylamide gel without a stacking gel at a constant current of 20 mA/gel for the initial 40 min and 30 mA/gel thereafter until the bromphenol blue dye marker reached the bottom of the gel. The samples from the same patient were run for at least two times, to determine the variability.

Protein Spots Visualization and Two-Dimensional Images Analysis. Silver nitrate staining according to the protocol of Pasquai *et al.* (16) and Coomassie Brilliant Blue R-250 (0.25% Brilliant Blue, 10% v/v acetic acid, 50% v/v methanol, destained in 20% v/v EtOH, 10% v/v acetic acid) was used for the analytical and micropreparative gels, respectively. The two-dimensional electrophoresisl images were captured with a ImageScanner (Amersham Pharmacia Biotech). Spot detection, quantification and alignment were performed with the ImageMaster 2D Elite 3.10 software (Amersham Pharmacia Biotech). A total of 50 spots were chosen as ubiquitously expressed reference spots to be used as landmarks for the alignment. Spot intensity volumes were normalized for every gel [(spot volume)/Σ(spot volumes) × 10^4] (9) to correct for subtle variation in protein loading and gel staining between the gels to be compared.

In-Gel Digestion and Extraction of Peptides. After matching the micropreparative gel image with the analytical image, in-gel digestion was performed with a previously published protocol (17).

Peptide Mass Fingerprinting by Matrix-Assisted Laser Desorption Ionization–Time-of-Flight–Mass Spectrometry. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitirile and 0.1% trifluoroacetic acid was used for matrix. One μL of the matrix solution and sample solution with a 1-to-1 ratio were mixed and applied onto the target plate. All mass spectra of matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) were obtained on 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 100 ppm and one partial cleavage. Oxidation of methionine and

carbamidomethyl modification of cysteine were considered. The obtained peptide mass fingerprint were used to search through the SWISS-PROT and NCBInr database by the Mascot search engine.[3] Protein identification was repeated at least once with spots from different gels.

Peptide Sequencing by Tandem Electrospray Ionization Mass Spectrometry. Tandem electrospray ionization mass spectrometry (ESI-MS/MS) experiments were performed on a quadrupole (Q)-time of flight 2 (Q-TOF2) hybrid quadrupole/TOF mass spectrometry (Micromass, United Kingdom) with a nanoflow Z-spray source. The mass spectrometer was operated in the positive ion mode with a source temperature at 80°C and a potential of 800-1000 V applied to the Nanospray probe. The database search was finished with the Mascot search engine[3] with Mascot MS/MS ion search. In addition, the amino acid sequences of the peptides were deduced with the peptide sequencing program MasSeq. To confirm whether the differentially expressed protein spot among different subtypes of acute leukemia was the same protein, the protein spot in different gels of the same subtype and different subtypes was identified, respectively.

The highly or specifically expressed proteins (the differentially expressed proteins) in myeloid, granulocytic, lymphoid lineage of acute leukemia, and M2, M3, or M5 subtypes of AML, as compared with other subtypes, were designated with the letters M, G, L, M_2, M_3, and M_5, respectively. The highly or specifically expressed proteins in both M2 and M3 compared with other subtypes were designated with the letter M_{23}. Differentially expressed proteins between acute leukemia and NWBCs (including normal granulocytic cells and normal lymphocytes) were designed with the letter N. The protein spots were numbered in the order in which they were discovered in our work.

Statistical Analysis. The statistical method of the Newman–Keuls test aimed at the identification of statistically significant differences in spot intensities among different samples. The spots of which the intensity was significantly up-regulated by statistical analysis of the Newman–Keuls test or that were specifically expressed in one subtype (or acute leukemia cells) as compared with other subtypes of acute leukemia (or normal white blood cells) were regarded as differential expression. The Fisher exact test was performed for each of the differentially expressed proteins to compare the proportion of the proteins that showed each feature in the same subtype (or acute leukemia group) with the proportion that showed the feature in other subtypes (or normal control group). Differences were considered significant when the P value was < 0.05.

RESULTS

Purity of the Samples and Matching Rate of the Two-Dimensional Maps. For the individual samples of acute leukemia included in the study, the percentage of leukemic cells separated by gradient centrifugation was more than 90%, and the percentage of normal lymphocytes or granulocytes was in the range of 90 to 95%, according to the morphologic analysis. About 1,200 spots could be detected on each two-dimensional electrophoresis gel by the auto-detect spots menu of ImageMaster 2-D Elite 3.0 software and manual clear-up. Approximately 95% of all spots were matched on duplicate gels (the gels of the same sample were produced, assyed, and processed in parallel.), and the intensity of the same spot from different gels showed no significant change. All maps of the different acute leukemia samples identified as the same subtype showed considerable similarity in their protein expression patterns, in which the matching rate ranged between 85 and 95%. Matching rate of the spots in two-dimensional electrophoresis gels among different types or subtypes of gels was about 60 to 70%. The pattern of protein profiling of M4 resembled that of M2 or M5 subtype of acute leukemia, corresponding to the composition of the cells of M4 subtype with granulocytic and monocytic blasts.

Protein Identification by Mass Spectrometry. One hundred twenty protein spots corresponding to 112 different proteins exhibited quantitative and qualitative variances that were significant ($P < 0.05$; the details not listed) and reproducible in different subtypes of acute leukemia and NWBCs, and were identified successfully by both MALDI-TOF-MS and ESI-MS/MS. We have identified the protein spots from different gels, and results showed that the identified protein spots were the same.

Some of the proteins, of which increased expressions were closely correlated with certain acute leukemia types or subtypes, are summarized in Tables 1 to 3. The protein identification was reconfirmed by the ESI-MS/MS approach in addition to MALDI-TOF-MS. Fig. 1 shows the peptide mass fingerprint analyzed by MALDI-TOF-MS and the sequences analyzed by ESI-MS/MS on the protein spot N-11 (NM23-H1).

Differentially Expressed Proteins between Aute Leukemia and Normal White Blood Cells. The differentially expressed proteins in acute leukemia and NWBCs did not include those expressed differentially in certain subtypes of acute leukemia. We identified 41 spots corresponding to 31 proteins in acute leukemia cells, the expressions of which were altered significantly compared with NWBCs (some of them are shown in Table 1). Some of them are shown in Fig. 2*A* and *B*. Some proteins were down-regulated in acute leukemia cells compared with NWBCs. For example, the expression levels of regulatory myosin light chain-2 (MLC-2; spot N20) and regulatory myosin light chain-3 (MLC-3; spot N-19) were detected in normal lymphocytes, but not in ALL cells. Myeloid-related protein 8 (Mrp8; spot N-29) and Mrp 14 (spot N-27) showed about a 9-fold to a 14-fold decrease in AML cells compared with the normal neutrophils.

Differentially Expressed Proteins between Acute Myeloid Leukemia and Acute Lymphoid Leukemia. We identified a group of proteins the expressions of which exhibited lineage specificity with regard to certain types of acute leukemia. Twenty-nine spots identified as 27 proteins were differentially expressed between ALL and AML. Some of them are summarized in Table 2. Among them, myeloperoxidase (MPO; spot M_{23}-5) was already known to be specifically expressed in AML instead of ALL (11). We further found that the amount of MPO was abundant in M2 and M3 as compared with M1, and

[3] Internet address for the Mascot search engine: http: www.matrixscience.co.uk.

Table 1 The differentially expressed proteins between acute leukemia and NWBCs identified by MALDI-TOF-MS

Spot	Protein name	NCBInr ID no.	M_r		pI		Peptides (MALDI/MS)		Sequence coverage (%)	Score	Protein expression
			Theor	Observ	Theor	Observ	Match	Total			
N-1*	Proliferating cell nuclear antigen (PCNA)	gi 4505641	28,750	29,465	4.57	4.49	10	36	45	68	+
N-2	PTK9L protein tyrosine kinase 9-like (A6-related protein);	gi 6005846	39,523	35,674	6.37	6.89	5	11	22	63	+
N-3	Pyridoxine 5′-phosphate oxidase	gi 8922498	29,969	22,325	6.62	6.76	17	34	52	161	+
N-4*	Uracil DNA glycosylase	gi 35053	35,470	38,666	8.22	6.67	6	14	24	64	+
N-5*	Stathmin; leukemia-associated phosphoprotein p18, (Op18)	gi 5031851	17,292	19,231	5.76	5.55	14	57	60	99	+
N-6	Guanine nucleotide binding protein (G protein)	gi 5174447	35,055	35,103	7.60	7.87	12	27	55	137	+
N-7	Rho GDP dissociation inhibitor (GDI) α	gi 4757768	23,193	23,367	5.02	5.00	11	61	62	88	+
N-8	TNF inhibitory protein (TIP)	gi 13775198	10,431	10,531	4.82	4.78	14	57	60	99	+
N-9	Voltage-dependent anion channel 2	gi 15277577	30,393	30,034	6.81	6.72	9	39	42	88	+
N-10	Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor	gi 12707570	31,351	28,564	8.34	6.01	19	40	62	151	+
N-11	NM23-H1	gi 29468184	19,641	21,045	5.83	5.52	11	45	80	97	+
N-12	Apoptosis inhibitor homolog	gi 2134780	56,617	29,976	6.22	5.87	8	19	21	75	+
N-13	Similar to proteasome (prosome, macropain) subunit, α type, 2	gi 12804095	25,138	24,175	7.92	7.68	7	21	45	83	+
N-14	Peroxisomal enoyl-CoA hydratase 1	gi 4503447	35,971	38,456	6.61	6.87	8	22	31	71	+
N-15*	High mobility group box 1 (HMGB 1)	gi 4504425	24,878	22,987	5.62	6.00	13	35	47	73	+
N-16*	Glutathione *S*-transferase–like; glutathione *S*-transferase omega (GST)	gi 4758638	25,019	25,132	6.00	6.21	8	39	47	82	−
N-17*	Chain A, manganese superoxide dismutase (MnSOD)	gi 2780818	22,176	23,218	6.86	7.01	11	61	62	88	−
N-18	Similar to *homo sapiens* mRNA for *KIAA0120* gene with GenBank accession no. D21261.1	gi 9956026	24,438	21,465	8.41	5.23	20	53	74	176	−
N-19	Myosin regulatory light chain 3 (MLC3)	gi 5453740	19,781	20,974	4.67	4.40	10	27	62	97	−
N-20	Myosin regulatory light chain (MLC2)	gi 15809016	19,767	20,196	4.67	4.61	6	23	45	72	−
N-21	ras suppressor protein 1	gi 6912638	31,521	30,567	8.57	7.98	15	50	45	88	−
N-22	Chain A, dimeric form of the haemopexin domain of Mmp9	gi 23200146	22,407	20,869	8.76	8.56	8	19	22	74	−
N-23	Chain B, crystal structure of A Rac-Rho gdi complex	gi 9955206	20,464	15,687	6.16	5.64	11	41	55	104	−
N-24	Lactoferrin	gi 386855	30,563	23,695	8.14	8.01	9	25	35	76	−
N-25	S100 calcium-binding protein A12	gi 5032059	10,569	11,536	5.83	5.42	5	12	39	82	−
N-26	Chain A, carboxylic ester hydrolase, P 1 21 1 space group	gi 23200327	13,622	12,542	5.10	5.13	4	6	56	66	−
N-27	Chain A, crystal structure of the Mrp14 complexed with Chaps	gi 20150229	13,102	14,231	5.71	5.45	9	38	76	97	−
N-28	CFAg (AA 1–94)	gi 29888	10,931	13,895	9.19	5.92	7	52	71	78	−
N-29	S100 calcium-binding protein A8 (Mrp8)	gi 21614544	10,828	10,732	6.51	7.06	7	29	59	89	−

NOTE. The calculation of experimental isoelectric point (pI) and molecular weight (M_r) was based on migration of the protein spot on two-dimensional gels. Differentially expressed proteins between acute leukemia and NWBCs were designed with the letter N. "+" indicates the up-regulated proteins in AL. "−" indicates the down-regulated proteins in AL. A score more than 63 is significant ($P < 0.05$). The protein spots were numbered in the order in which they were discovered in our work.

Abbreviations: ID, identification; Theor, theoretical; Observ, observed; TNF, tumor necrosis factor.

* The proteins that had been reported to be involved in leukemogenesis.

was less abundant in monocytic lineage (M5 subtype of acute leukemia) compared with granulocytic lineage (M1, M2, M3; Fig. 3*A*). The expression levels of Mrp14 (spot M-6) and Mrp8 (spot M-7) were increased about 8-fold in AML cells compared with ALL cells. But they were much higher in normal granulocytes compared with those in AML cells, as described in the former section. Furthermore, the expression levels of them were 5-fold higher in M2 and M3 than in the M1 subtype. Some of the highly expressed proteins in ALL, compared with both AML and NWBCs, are shown in Fig. 3*C*.

Differentially Expressed Proteins between Granulocytic Lineage and Monocytic Lineage in Acute Myeloid Leukemia. Twenty-four protein spots identified as 23 proteins were found to express differentially between granulolcytic lineage (M1, M2, M3) and monocytic lineage (M5; some of them are shown in Table 2).

Table 2 The highly or specifically expressed proteins in myeloid, granulocytic, monocytic, and lymphoid lineage of acute leukemia identified by MALDI-TOF-MS

Spot	Protein name	NCBInr ID no.	M_r		pI		Peptides (MALDI/MS)		Sequence coverage (%)	Score
			Theor	Observ	Theor	Observ	Match	Total		
M-1	Chain A, structure of up1-telomeric DNA complex	gi 5542530	20,831	22,645	6.79	6.89	12	39	49	82
M-2	Unnamed protein product	gi 16553016	20,611	21,435	9.60	5.01	6	14	32	64
M-3	Coactosin-like 1	gi 21624607	15,935	16,887	5.54	5.39	7	18	52	82
M-4	Cytochrome *c* oxidase subunit VIb	gi 4502985	10,186	12,201	6.30	6.54	5	13	65	77
M-5	F-actin capping protein β subunit; Cap Z	gi 4826659	30,609	34,076	5.69	5.52	14	32	53	137
M-6	Chain A, crystal structure of the Mrp14 complexed with CHAPS	gi 20150229	13,102	14,821	5.71	5.46	15	53	82	106
M-7	S100 calcium-binding protein A8 (Mrp8)	gi 21614544	10,828	12,950	6.51	7.27	6	12	60	103
M-8	Nit protein 2	gi 9910460	30,589	35,159	6.82	7.12	16	19	73	207
M-9	Similar to ATP synthase, H^+ transporting, mitochondrial F1 complex,	gi 13111901	40,260	33,420	8.94	7.11	9	18	28	74
M-10*	Glyoxalase I	gi 2135301	20,764	27,988	5.12	4.93	9	25	53	77
M-11*	Phosphoglycerate kinase 1	gi 4505763	44,586	38,698	8.30	6.89	10	15	40	97
M-12	UP1, the two RNA-recognition motif domain of HnrnpA1	gi 2554653	20,784	20,346	7.26	7.46	8	23	63	136
M-13	Proteasome β subunit	gi 631345	25,893	28,912	5.70	5.78	10	17	46	84
M-14	Adenylate kinase, mitochondrial; ATP-AMP transphosphorylase	gi 7524346	25,598	33,193	7.71	8.21	6	11	26	65
M-15	Aldolase A	gi 229674	39,264	31,726	8.39	7.43	12	28	46	137
M-16†	Chain A, myloperoxidase	gi 494394	12,311	15,363	5.77	6.47	14	30	96	185
M-17	Triosephosphate isomerase	gi 999892	26,221	26,899	6.51	7.10	11	35	50	149
M-18	Ubiquinol-cytochrome *c* reductase	gi 5174743	29,633	26,324	8.55	7.12	10	51	33	79
M-19	Cathepsin D	gi 494296	26,229	30,958	5.31	5.80	10	26	25	25
G-1	Similar to ribosomal protein S12; 40S ribosomal protein S12	gi 27485488	14,589	14,390	6.43	6.40	8	23	50	90
G-2	CDC42 GAP-related protein	gi 2326171	25,209	32,661	9.38	7.60	7	20	35	70
G-3	Fatty acid binding protein 5 (psoriasis-associated); E-FABP	gi 4557581	15,155	16,480	6.60	6.41	10	28	56	80
G-4	Actin γ	gi 71625	41,635	40,193	5.31	5.49	22	53	66	126
G-5	α enolase	gi 2661039	36,286	37,635	6.53	6.68	19	42	63	176
G-6	γ-actin	gi 178045	25,862	30,616	5.65	5.41	11	49	39	71
G-7	β5-tubulin	gi 18088719	49,640	40,822	4.75	5.78	20	55	68	126
G-8†	Chain A, myloperoxidase	gi 494394	12,311	15,363	5.77	6.47	14	30	96	185
G-9*	Similar to VENT-like homeobox 2; haemopoietic progenitor homeobox	gi 30153626	36,677	36,889	8.98	7.58	5	8	17	64
G-10	3-Hydroxyacyl-CoA dehydrogenase, isoform 2	gi 2078329	42,097	36,967	9.34	7.65	6	10	26	75
G-11*	Peroxiredoxin 2; thiol-specific antioxidant protein (TSA)	gi 2507169	21,878	25,676	5.66	5.23	7	19	56	93
G-12	P47	gi 5531827	40,547	37,710	5.03	5.32	13	47	40	125
M_5-1	Adenine phosphoribosyltransferase; AMP pyrophosphorylase	gi 4502171	19,595	18,372	5.78	5.24	5	9	41	
M_5-2	Annexin A2; annexin II (lipocortin II)	gi 16306978	38,594	37,731	7.57	7.02	8	11	28	65
M_5-3	HSPC124	gi 6841470	36,519	36,689	5.60	6.06	7	13	29	93
M_5-4	Sulfotransferase family, cytosolic, 1A	gi 10835035	34,174	36,689	5.68	5.65	10	50	41	63
M_5-5	Unnamed protein product	gi 21758578	32,530	32,790	6.71	6.70	17	40	63	71
M_5-6	Inorganic pyrophosphatase 2 isoform 2	gi 29171694	31,556	36,689	7.04	6.21	6	11	29	150
M_5-7	Hypothetical protein	gi 12052834	36,541	36,689	5.96	6.54	8	14	39	82
M_5-8	Unknown (protein for MGC:27221)	gi 16924265	35,735	34,986	8.47	6.70	15	39	56	103
M_5-9	Transgelin 2	gi 12803567	22,392	22,875	8.41	8.45	15	32	53	127
L-1	Immunoglobulin heavy chain	gi 806832	13,704	14,214	8.57	7.05	4	9	65	74
L-2*	Stathmin; leukemia-associated phosphoprotein p18 (Op18)	gi 5031851	17,292	19,699	5.76	4.98	9	22	46	73
L-3*	Heat shock 27kDa protein 1	gi 4504517	22,768	25,534	5.98	6.01	17	51	77	168
L-4	Aldehyde reductase	gi 1633300	36,419	35,256	6.34	6.64	17	38	55	160
L-5	Endoplasmic reticulum protein 29 precursor	gi 5803013	28,975	23,864	6.77	6.76	13	32	55	126
L-6	Cofilin 1 (non-muscle)	gi 5031635	18,491	20,321	8.22	6.41	11	45	56	75

NOTE. The calculation of experimental isoelectric point (pI) and M_r was based on migration of the protein spot on two-dimensional gels. The highly or specifically expressed proteins in myeloid, granulocytic, monocytic, and lymphoid lineage of acute leukemia, as compared with other subtypes, were designated with the letter M, G, M_5, and L, respectively. A score of more than 63 is significant ($P < 0.05$).

Abbreviations: ID, identification; Theor, theoretical; Observ, observed.

* The proteins that had been reported to be involved in leukemogenesis.

† The proteins that had been reported to be associated with acute leukemia classification.

Table 3 Proteins highly or specifically expressed in M2 and/or M3 identified by MALDI-TOF-MS

Spot	Protein name	NCBInr ID no.	M_r		pI		Peptides (MALDI/MS)		Sequence coverage (%)	Score
			Theor	Observ	Theor	Observ	Match	Total		
M_2-1*	Elongation factor 2	gi 181969	39,750	38,978	5.81	5.73	5	8	19	65
M_2-2*	Small GTP binding protein RhoG	gi 20379122	21,261	21,301	8.41	8.12	6	15	50	78
M_3-1	S100 calcium binding protein A 11 (calgizzarin)	gi 503207	11,733	12,437	6.56	6.12	11	33	86	77
M_3-2	Unnamed protein product	gi 21757045	52,406	30,155	4.99	4.64	18	22	48	173
M_3-3	RNA-binding protein regulatory subunit; oncogene *DJ1*	gi 31543380	19,878	19,987	6.33	6.04	5	9	37	65
M_3-4†	Chain A, human cathepsin G	gi 2392230	25,423	14,568	11.51	9.98	4	5	11	67
M_3-5	Human heparin binding protein	gi 2981936	24,261	24,765	9.53	9.07	7	10	31	66
M_3-6	Atomic resolution structure of Hbp	gi 4699717	23,804	24,302	9.53	9.07	5	14	35	66
M_3-7	Azurocidin	gi 28977	26,637	25,578	9.75	9.07	7	10	28	64
M_3-8	Similar to high mobility group protein 2 (HMG-2)	gi 20951578	18,147	17,100	5.17	4.96	8	32	29	80
M_3-9	Similar to retinoic acid, EGF, and NGF up-regulated; REN	gi 27485516	29,624	25,875	6.09	5.48	4	9	26	64
M_3-10	Human protein disulfide isomerase	gi 2098329	13,249	21,054	5.94	4.87	5	26	39	65
M_{23}-1	Chain A, cyclophilin B	gi 1310882	19,648	29,715	9.18	6.47	6	21	35	68
M_{23}-2	Unnamed protein product	gi 21758578	32,530	32,450	6.71	6.80	7	12	35	88
M_{23}-3	Annexin I; lipocortin I	gi 4502101	38,690	34,692	6.57	5.65	14	33	49	138
M_{23}-4†	Chain A, protease 3, myeloblastin	gi 1633225	24,245	30,613	7.79	8.65	8	34	45	68
M_{23}-5†	Chain c, cryogenic crystal structure of human myeloperoxidase isoform c (MPO)	gi 7766942	53,130	32,231	9.48	8.21	16	46	34	101

NOTE. The calculation of experimental isoelectric point (pI) and M_r was based on migration of the protein spot on two-dimensional gels. The highly or specifically expressed proteins in M2, M3, or both M2 and M3 compared with other subtypes were designated with the letter M_2, M_3, or M_{23}, respectively. A score more than 63 is significant ($P < 0.05$).

Abbreviations: ID, identification; Theor, theoretical; Observ, observed.

* The proteins that had been reported to be involved in leukemogenesis.

† The proteins that had been reported to be associated with acute leukemia classification.

Proteins Highly or Specifically Expressed in M2 and/or M3 Cases. Nine protein spots, corresponding to 7 proteins, were found to highly express in M2 and M3 rather than that in other acute leukemia subtypes (Table 3). For example, proteinase 3 (spot M_{23}-4, Fig. 3*A)* was not detected in M1 cases, but predominantly expressed in M2 and M3 subtypes. It was also found in 4 of 10 cases of M4 and 2 of 10 cases of M5. It was negative in all cases of ALL. Azurocidin (17) and cationic antimicrobial peptides (ref. 18; Fig. 3*B*) were observed in M2 and M3 subtypes of AML and in normal neutrophils, but not in M1 subtype. The up-regulated proteins in M2 subtype, compared with other subtypes, are shown in Table 3. Furthermore, 15 protein spots corresponding to 15 proteins, were found to be closely correlated to M3 subtype (Table 3). For example, cathepsin G (M_3-4) was observed in M3, but not in any other subtypes included in this study. NM23-H1 (N-11) was expressed at a lower level in M3 subtype than that in any other subtypes. We further showed that much lower expression of NM23-H1 in M3a (6 of 7 cases), almost as low as in NWBCs, was obviously different from that in M3b and other subtypes (The expression level of them showed an 8-fold to 10-fold increase compared with NWBCs.).

DISCUSSION

The proteomic approach is being actively applied to the molecular analysis of various human cancers such as bladder (4), colorectal (5), breast (6), stomach (7) cancers, and so forth. However, few have been reported on the type-specific proteomic analysis of acute leukemia. In this study, we have used proteomic profiling of human acute leukemia cells by two-dimensional electrophoresis and MS to explore the DPPs for different types of acute leukemia. Because the classification-specific DPPs contribute to the heterogeneity of acute leukemia with distinct biological and prognostic groupings, it could, therefore, facilitate the efforts to establish the molecular definition of acute leukemia classification, and to develop a more systematic approach to acute leukemia classification based on the DPPs. Furthermore, the distinct biological behaviors of different types of leukemia might be attributed to their altered proteome. Thus, the altered proteome implicates the mechanisms of type-specific biological behavior of leukemia.

Our results have shown the differentially expressed proteins in acute leukemia cells, as compared with the NWBCs, and the type-specific DPPs, which belong to myelocytic lineage of acute leukemia, granulocytic lineage of acute leukemia, ALL, and AML subtype (M2, M3, and M5), respectively. Among the highly expressed proteins in acute leukemia cells, as compared with NWBCs, some are already known to be involved in the process of malignant transformation [such as Op18 (19), and NM23-H1 (20)] and abnormal commitment to acute leukemia cell proliferation [such as proliferating cell nuclear antigen (20), uracil-DNA glycosylase (21), and proteasomes (22)], or were associated with antiapoptosis, such as high mobility group box 1 (HGMB 1), apoptosis inhibitor homologue (23). TIP, the expression of which was elevated in acute leukemia, is another apoptosis-inhibitory factor (24). When we considered that ras suppressor 1 is a negative regulator of G protein (25), our data suggested that the up-regulation of G protein was correlated

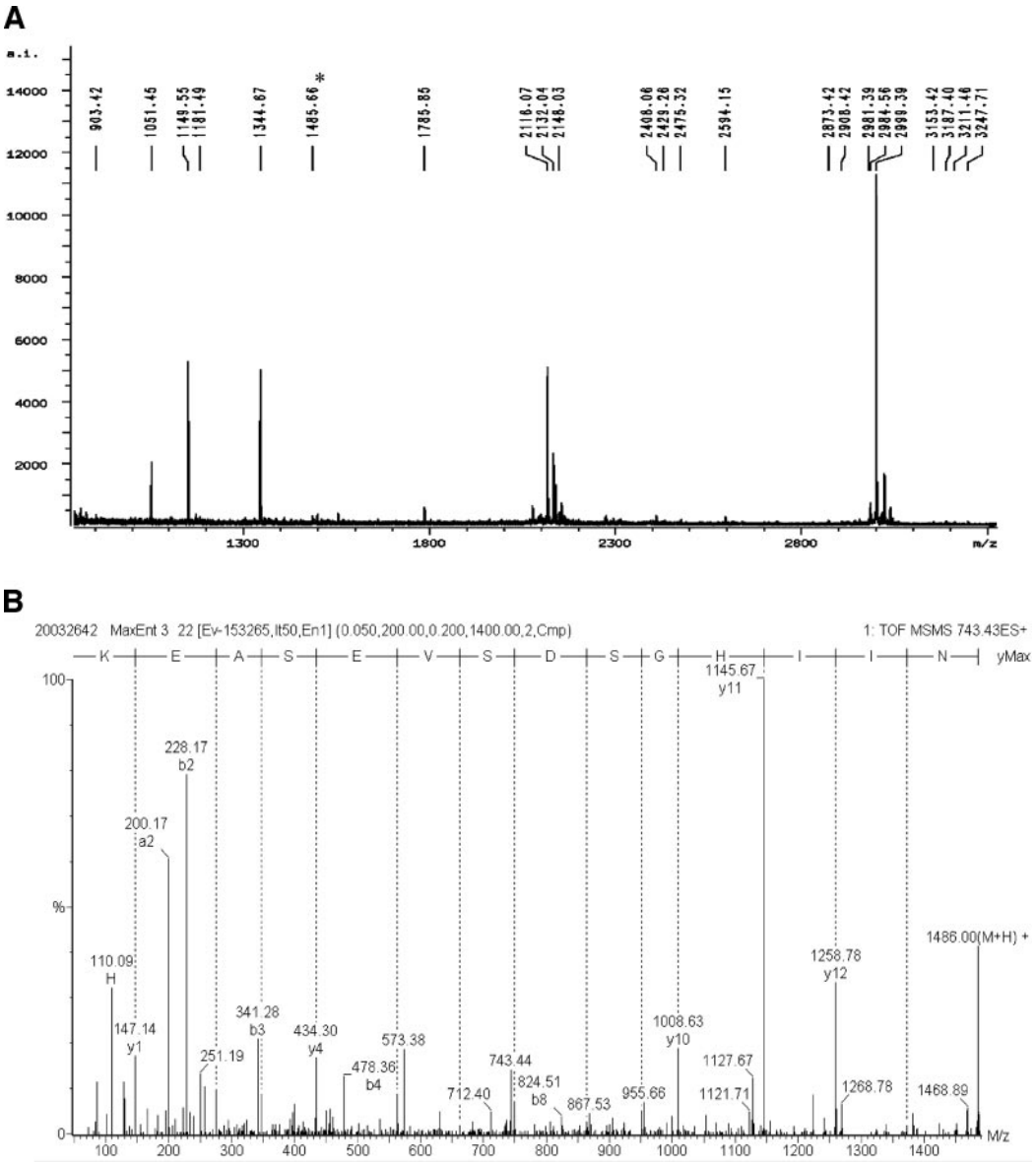

Fig. 1 Identification of spot N-11 by MALDI-TOF-MS and ESI-MS/MS. *A*, peptide mass fingerprint of spot N-11 by MALDI-TOF-MS. After database searching, the identified protein is NM23-H1. Protein score (97) is significant ($P < 0.05$) and sequence coverage reached 80%. *B*, the peptide of 1485.56 chosen from the peptide mass fingerprint for spot N-11 was sequenced by nano-ESI-MS/MS. *, the peptides selected for sequencing by nano-ESI-MS/MS.

with down-regulation of ras suppressor 1, because both were observed in acute leukemia cells in this study. Some proteins involved in differentiation and physiologic functions of NWBCs were down-regulated in acute leukemia in this study, including MRP8, MRP14 (26), which are involved in the maturing process and inflammatory responses of granulocytes, and MLC 2 (27), and of MLC 3 associated with the functions of lymphocyte migration.

Our study discovered two sets of proteins that could be used to discriminate between ALL and AML (Table 2). These identified lineage markers of ALL or AML were known to have roles in different cellular functions, including tumorgenesis, signal transduction, transcription, and so forth. The currently known biology does not provide an imminent explanation for their specific connections with AML or ALL, with the exception of MPO. MPO is considered as the specific marker for AML, as already pointed out by Davey *et al.* (28), and provides inherent resistance of myeloblasts to vincristine (VCR) by degrading VCR in the presence of hydrogen peroxide (29). The other proteins identified to be expressed highly in AML compared with ALL, were known to have roles in drug-resistance [glyoxalase I (30) and phosphoglycerate kinase 1 (31)]. UP1 (32), up-regulated in AML, as compared with NWBCs and ALL, is known to promote telomere elongation and stabilization of

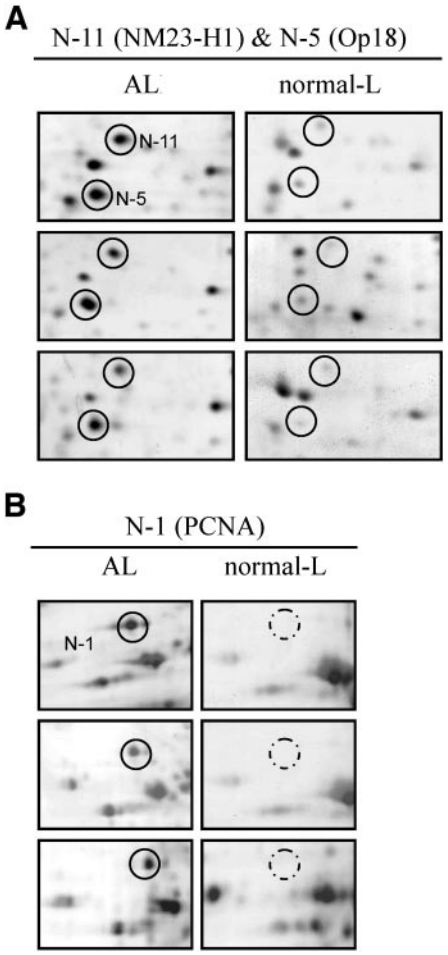

Fig. 2 Close-up sections of up-regulated protein spots in two-dimensional electrophoresis maps of acute leukemia cells compared with NWBCs. *A* and *B*, showing the up-regulation of Op18 (*N-5*), NM23-H1 (*N-11*) and proliferating cell nuclear antigen (*PCNA*; *N-1*) in three cases of acute leukemia (*AL*) as compared with NWBCs (*normal-L*).

telomere length, and to correlate with the immortalization of somatic cells, which implies that its high expression might contribute to the overgrowth of AML cells. The highly expressed proteins in ALL, such as Op18 and HSP 27, may be useful markers for ALL. Among them, the higher expression of Op18 in ALL compared with AML was also in agreement with other reports (33).

We further performed proteomic analyses on the subtypes of AML: M1, M2, M3, M4, and M5, which are defined by their distinct morphologic features according to the FAB classification. We did not find the specific protein in M1 by our methods, which may be the limitation of the techniques we adopted. For example, the abundance of the specific protein in M1 is too low to be observed on the gel; or the specific proteins in M1 may be some membrane proteins that cannot be obtained by our method. We also found that a group of proteins were restricted to M1, M2, and M3 subtypes of the FAB category, and another set of proteins found to be absent in M1, were expressed specifically and highly in M2 and M3. Among them, the different expression levels of proteinase 3 in different acute leukemia subtypes agrees with the published reports (34)

We identified the up-regulation of cathepsin G in M3 subtype as compared with other subtypes, which is consistent with the experiments that a significant decrease in cathepsin G expression occurred in M3 subtype cells undergoing terminal differentiation by the stimulation of all-*trans* retinoic acid (35). NM23-H1 is a differentiation-inhibitory factor and was found up-regulated in all but M3a subtypes of acute leukemia compared with NWBCs. The M3 subtype was divided into M3a and M3b with clinical relevance according to the size of granules in cells (11). M3a has a favorable prognosis as compared with other subtypes of AML. Therefore, NM23-H1 could be an important prognostic factor (36).

Some identified proteins showed isoelectric point and M_r shift from their theoretical values. Changes in isoelectric point and M_r might be attributed to posttranslational modification of the proteins, such as protease digestion, glycosylation, and phos-

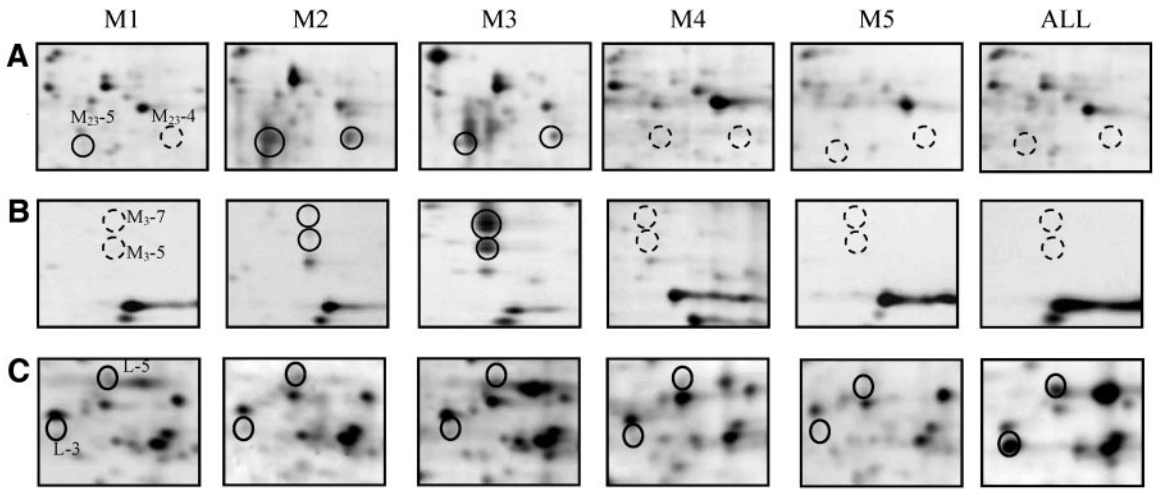

Fig. 3 Close-up sections of differentially expressed protein spots as indicated in two-dimensional electrophoresis maps of different subtypes of acute leukemia cells. *A*, M_{23}*-5*, myeloperoxidase; M_{23}*-4*, proteinase 3. *B*, M_3*-5*, human heparin-binding protein; M_3*-7*, azurocidin. *C*, *L-3*, heat shock 27 kDa protein 1 (HSP27); *L-5*, endoplasmic reticulum protein 29 precursor.

phorylation. Such posttranslational modifications are usually required for proteins to fully perform their biological activities *in vivo*. Because of differential protein processing and posttranslational modifications, multiple protein isoforms of individual protein can be identified on two-dimensional gels. For example, the level of Op18 isoform (spot L-2) was highly elevated in ALL, but was reduced in other types of acute leukemia or absent in NWBCs. These findings suggest that the different protein isoforms may be correlated with specific features or functions of acute leukemia. Thus, they may provide useful diagnostic information and further indicate the need to identify the specific modifications underlying these specific protein isoforms.

The DPPs of different types of acute leukemia could reflect the origin and differentiation stage of the leukemic cells. Such proteins could provide diagnostic confirmation or clarify unusual cases. This point was illustrated by a recent clinical experience. The diagnosis for a patient was M3, according to the morphology with atypical granules in the blast cells of the bone marrow sample. However, the patient didn't respond to the differentiation-inducing therapy with all-*trans* retinoic acid or As_2O_3. We took the opportunity to apply the proteomic analysis method to the bone marrow sample from the patient. The two-dimensional electrophoresis map of the sample showed features of M1, and the proteins specific to the M3 subtype of AML were not found on the two-dimensional electrophoresis map. The *PML/RAR*α fusion gene, expressed in ~95% of the patients with M3, was also shown negative by reverse transcription-PCR. The patient's diagnosis was then revised accordingly, and the treatment was changed from differentiation-inducing therapy to chemotherapy (daunorubicin combined with aracytidine). As a result, the patient gained complete remission within 3 weeks. This experience showed acute leukemia diagnosis could benefit from the analysis of the proteome of acute leukemia cells.

Alizadeh *et al.* (37) have subdivided an entity previously considered homogeneous by various pathologic methods into two, not only new but also prognostically relevant, subgroups by the microarray analyses. Nevertheless, it should be stressed that mRNA levels do not necessarily correlate with protein levels (10). Moreover, disease-relevant information may also be hidden behind changes in protein modification. In addition, our data may have major implications with regard to delineating aberrant gene expression pathways underlying the pathogenesis of acute leukemia. In conclusion, our proteomic analyses show for the first time the type-specific DPPs of acute leukemia based on the FAB classification system. We could expect that the extension of the present analyses to currently less-well-defined acute leukemia would possibly identify additional subgroups of acute leukemia with clinical relevance based on their protein expression profiles. Our analyses also enable us to define the deregulated genes important for the initiation and the progression of AML. Finally, these analyses may promote the identification of new targets for specific treatment approaches.

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