Original Article

Protein Profiling as a Useful Diagnostic Tool to Classify Patients with Acute Myeloid Leukemia of Different Cytogenetic Abnormalities

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Background: Several nonrandom chromosomal abnormalities have been identified in acute myeloid leukemia [AML] and are strong determinants of prognostic outcome and therapeutic response. Because clinical outcomes of AML patients with cytogenetic aberrations differ considerably, we hypothesized that their proteomes may also differ, particularly in their expression patterns and protein interaction pathways.

Objective: To study the protein profiling that is related to different karyotypes of AML patients.

Materials and Methods: We performed proteomic analysis using 20 AML samples with various cytogenetic abnormalities including t(8;21) (n = 4), t(15;17) (n = 3), inv(16) (n = 4), trisomy 8 (n = 3), trisomy 11 (n = 3) and trisomy 21 (n = 3). Proteins from bone marrow cells were separated by two-dimensional gel electrophoresis and the protein profiles were compared among the samples.

Results: Favorable karyotypes, such as t(8;21), t(15;17) and inv(16), showed similar protein profiles within their own groups but differed from all other subgroups, whereas the trisomy group had similar protein profiles only within the same French-American-British morphological classification. As previously reported, some identified proteins by LC/MS/MS spectrometer, including transgelin-2, were also expressed in leukemic cells from patients or leukemia cell lines.

Conclusion: Unique proteomic patterns were identified in some AML subgroups. AML patients may be further sub-classified using protein profiles generated by this approach in combination with the current standard diagnostic methods.

Keywords: proteomics, leukemia, acute myeloid leukemia, classification, diagnostic methods

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Various nonrandom chromosomal abnormalities have been consistently identified in acute myeloid leukemia [AML]. The aberrations can include chromosome translocations, such as t (8; 21), t (15; 17) or inv (16), and abnormal chromosome numbers such as monosomy and Trisomy[1,2]. These cytogenetic abnormalities strongly affect therapeutic response. Whereas t (8; 21), t (15; 17) and inv (16) are associated with favorable prognosis, −5/−7 portend poor response to chemotherapy[2], and trisomy of several chromosomes in AML patients are associated with

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heterogeneous responses to treatment and to intermediate disease risk. However, although various cytogenetic aberrations are linked to outcomes in AML, the distinct protein profiles that contribute to these subtypes are still unclear.

Molecular analysis could help classify AML into various subtypes using microarray-based techniques[3,4]. Previous studies have shown results of DNA microarray analysis that provided a basis for molecular classification of AML. However, as RNA levels do not necessarily determine protein levels, in this study we analyzed 20 bone marrow samples to create protein profiles by obtaining their cytogenetics and FAB classifications. This study may also help to diagnosis and classify AML subtypes and improve understanding of aberrant gene product pathways, possibly leading to new targeted treatments.

Materials and Methods

Leukemia samples

We used archived samples from 20 adult Thai patients with AML patients who had been newly diagnosed for mononuclear cells [MNC] collection (Table 1). This study was approved by the Ethical Committee for Human Research, Faculty of Medicine Siriraj Hospital (No. 543/2551 (EC1)). Conventional chromosome banding studies were performed using standard techniques and chromosomal abnormalities were described according to the International System for Cytogenetic Nomenclature[5,6]. Briefly, bone marrow cells were cultured in RPMI 1640 medium supplemented with 20% fetal calf serum at 37°C for 24 hours. Unstimulated and stimulated cultures were created as previously described. We made standard cytogenetic preparations; at least 15 metaphases were analyzed. Mononuclear cells were isolated using Ficoll-hypaque density gradient centrifugation and were stored at -80°C[7,8].

Two-dimensional gel electrophoresis [2-DE]

We performed 2-DE using the immobiline/polyacrylamide system. After dissolving samples by lysis buffer, samples were subjected overnight to in-gel rehydration of 70 mm (analytical runs) and 180 mm (preparative runs), using nonlinear pH 3 to 10, IPG gel strips (GE Healthcare, Sweden). The first dimension [IEF] was performed at 6500 V, using a Pharmacia LKB Multiphor II system. The IPG strips were equilibrated in two steps of equilibration buffer. The first step employed 50 mM Tris-HCl buffer, pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 1% DTT; 2.5% iodoacetamide replaced DTT in the second step. The IPG strips were then subjected to 2-D 14% T SDS polyacrylamide gels. Electrophoresis of the minigel was performed in a Hoefer system at 20 mA, room temperature for 2 hours. After electrophoresis, proteins were visualized by CBR-250 staining or SYPRO Ruby staining.

Gel scanning and image analysis

Gels were scanned using a GS-700 Imaging Densitometer (BioRad, USA). Melanie II system (BioRad, USA) was used for computer analysis.

Trypsin in-gel digestion

Protein spots were excised and transferred to 0.5 mL microcentrifuge tubes. Fifty microliters of 0.1 M NH_4HCO_3 in 50% ACN was added. The gel was incubated three times for 20 minutes at 37°C. The solvent was discarded and gel particles were dried completely by SpeedVac. Reduction and alkylation was performed by swelling the gel pieces in 50 mL buffer solution (0.1M NH_4HCO_3, 10 mM DTT, and 1 mM EDTA) and incubated at 67°C for 45 minutes. After cooling, the excess liquid was removed and quickly replaced by the same volume of freshly prepared 100 mM iodoacetamide in 0.1 M NH_4HCO_3 solution. The mixture was incubated at room temperature in the dark for 30 minutes. The iodoacetamide solution was removed and the gel pieces were washed with 50% ACN in water, three times for 10 minutes each time, and the gel pieces were completely dried. Aliquots (1 mg trypsin/10 mL of 1% acetic acid) of trypsin (Promega Corporation, USA) were prepared and stored at 22°C. Fifty microliters of digestion buffer (0.05 M Tris-HCl, 10% ACN, 1 mM $CaCl_2$, pH 8.5) and 1 mL of trypsin were added to the gel pieces. After incubating the reaction mixture at 37°C overnight, the digestion buffer was removed and saved. The gel pieces were then extracted by adding 60 mL of 2% freshly prepared TFA and incubating for 30 minutes at 60°C. The extract and saved digestion buffer were finally pooled and dried.

Protein identification by Liquid chromatography-Mass spectrometer [LC/MS/MS]

We carried out LC/MS/MS analyses using a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, UK) equipped with a Z-spray ion source working in the nano-electrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 mm id 6150 mm C18 PepMap

Table 1. Cytogenetic and FAB classification of 20 AML patients used in the proteomic analysis

Sample ID	FAB	Karyotypes
TA1	M2	46, XX, t (8; 21) (q22; q22)
TA2	M2	46, XX, t (8; 21) (q22; q22) [8]/45, X, t (8; 21) (q22; q22) [9]
TA3	M2	45, X, t (8; 21) (q22; q22)
TA4	M2	46, XY, t (8; 21) (q22; q22)
TP1	M3	46, XX, t (15; 17) (q22; q21)
TP2	M3	46, XX, t (15; 17) (q22; q21)
TP3	M3	46, XX, t (15; 17) (q22; q21)
IN1	M4	46, XY, inv (16) (p13 q22)
IN2	M4	46, XY, inv (16) (p13 q22)
IN3	M4	46, XX, inv (16) (p13 q22)
IN4	M1	48, XX, +9, +22, inv (16) (p13 q22)
TR81	M4	47, XX, +8
TR82	M4	47, XY, +8
TR83	M2	47, XY, +8
TR111	M1	47, XX, +11
TR112	M0	47, XY, +11
TR113	M2	47, XY, +11
TR211	M5	47, XX, +21
TR212	M2	46, X, -Y, +21
TR213	M2	47, XY, +21

column (LC Packings, The Netherlands). Eluents A and B were 0.1% formic acid in 97% water/3% ACN, and 0.1% formic acid in 97% ACN, respectively. Six microliters of sample were injected into the nano-LC system, and separation was performed using the following gradient: 0 minute 7% B, 35 minutes 50% B, 45 minutes 80% B, 49 minutes 80% B, 50 minutes 7% B, and 60 minutes 7% B. Database searches were performed with ProteinLynx screening Swiss-Prot and NCBI. For some proteins that were difficult to find, we used NCB Inr, the MASCOT search tool available on the Matrix Science site.

Results

Approximately more than 280 protein spots were observed in each sample. The protein profiles from favorable karyotype, including t (8; 21), t (15; 17) and inv (16), showed similar expression profiles within their own groups but differed from all other subgroups. The protein pattern of trisomy cytogenetics subgroup showed the similar results within the same FAB classification (Figure 1). This study identified the protein spots with expressed in all chromosome abnormalities samples as shown in Table 2 and Figure 2. Interestingly, we also identified the unique spots from different subgroups. Heat shock protein 90 AB1 was expressed only in the t (8; 21) group; cyclophilin and eosinophil lysophosplipase were shown in the inv (16) group; and transgelin-2 was identified as specific target of trisomy 8 and trisomy 11 (Table 3).

Discussion

Until now, few proteomic data for specific AML karyotypes have been reported[10,11], although we have some drugs that target aberrant proteins in other blood cancers (such as imatinib for BCR-ABL protein in CML or all-trans-retinoic acid for PML-RAR protein in APL)[9]. In the present study, we analyzed protein profiles of AML cellsusing 2-DE and LC/MS/MS to identify proteins that are differently expressed in each subgroup.

This study identified several proteins in AML patients, similar to previous studies of chaperonin, protein disulfide isomerase, thioredoxin and prolyl 4-hydroxylase etc[10,12,13]. We also showed the identified proteins as specific targets in each cytogenetic group; for example, Hsp90AB1 was a specific target in t (8; 21) samples, cyclophilin and eosinophil lysophospholipase in inv (16) samples and transgelin-2 in trisomy 8 and 11 samples. Our findings were dissimilar to the German study[10]. Jie-Wei Cui et al reported that their distinct protein profiles [DPPs] can be used to classify acute leukemia samples into different FAB subgroups[7]. Similarly, each group of FAB subtype in our samples

Table 2. The protein spots that were identified in all karyotypes

Spot ID	Protein name	PI	MW (kDa)
1	Prolyl 4-hydroxylase beta subunit	4.7	57
2	ATP synthase beta subunit	5.1	56
3	Chaperonin	5.5	61
4	Heat shock protein 70	5.7	73
5	Protein disulfide isomerase	5.9	56
6	Human rab GDI	5.9	51
7	Human erythrocyte catalase	6.7	56
8	Enolase 1	7.0	47
9	ATP synthase H transporting mitochondria	5.0	56
10	GAPD17	8.6	36
11	Manganese superoxide dismustase (MuSOD)	8.4	25
12	Heterogeneous nuclear ribonucleoprotein A1	7.8	22
13	Thioredoxin	4.8	11
14	Cofilin 1 non muscle	8.2	18
15	Carbonic anhydrase 1	6.6	29

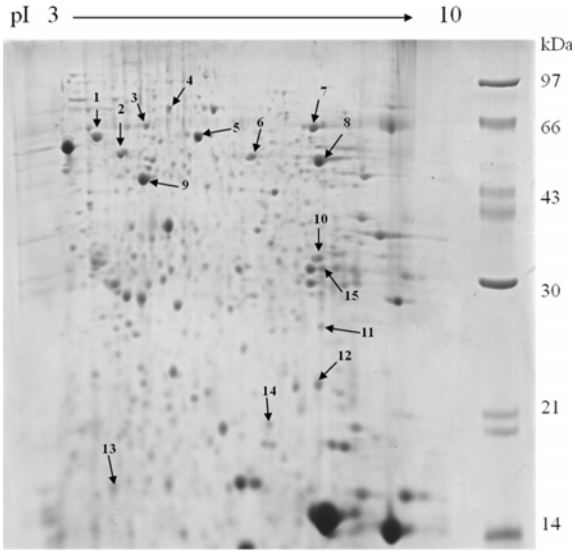

Figure 1. 2-D PAGE results show protein spots that were identified in all karyotypes.

had a similar protein profile in its own group, especially in the trisomy 8 and trisomy 11 karyo types. In addition, transgelin-2 is reportedly expressed in leukemic cells from patients and from leukemia cell lines[14,15]. Some studies also report proteomic analyses of AML that focus on molecular genetic abnormalities[16-18].

According to the result that the favorable groups showed similar protein profiles within its own group whereas the trisomy subgroups had similar protein profiles within the same FAB classification, suggested that chromosomal abnormality in favorable subtypes could be the first event of the disease, whereas trisomy may be a secondary aberrant event in the process of leukemogenesis.

Schochet et al performed microarray analyses for gene expression;they established 36 gene patterns in three distinct AML karyotypes: t (8; 21), t (15; 17) and inv (16)[3]. We compared our protein data to this result and did not find a strong correspondence. However, mRNA expressions do not necessarily correlate with protein levels. Bulkhi et al previously generated data of protein interaction networks specific to different cytogenetic subgroups. The major regulatory network and signaling pathways affected by these networks appear to be MAPK8, MYC for complex aberrant karyotypes, JUN and MYC for inv (16), and TP53 for t (8; 21)[10]. The present study also analyzed our protein interaction data, but found no significant relationship.

Conclusion

Our data suggests that classifying AML patients may be possible using the proteomic profiles generated by this approach in combination with the current standard diagnostic methods. Characterization of related signaling pathways of identified proteins is ongoing. This research should enhance our understanding of AML pathogenesis and potentially leads to the better design of novel protein-targeted therapy.

Table 3. Protein identified as a specific targets of cytogenetic groups

Protein name	PI	MW (kDa)
Proteins identified as specific targets of t (8; 21)		
Heat shock protein 90 AB1	4.7	40
Proteins identified as specific targets of inv (16)		
Cyclophilin	9.7	7.2
Eosinophil lysophospholipase	22	16
Proteins identified as specific targets of trisomy 8 and trisomy 11		
Transgelin-2	8.4	22

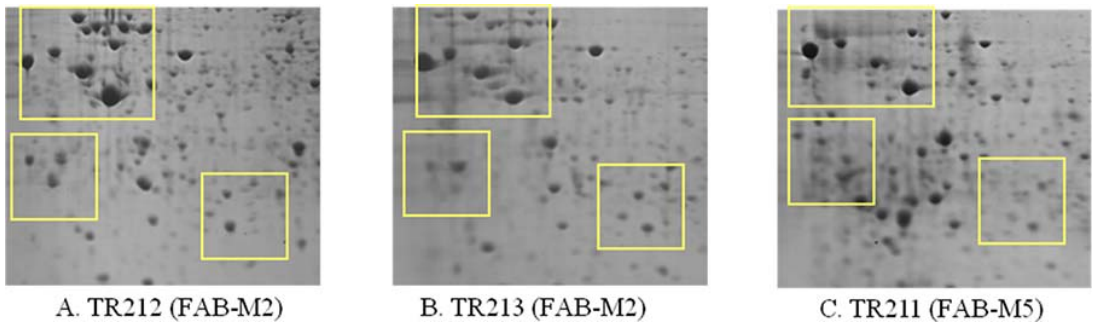

Figure 2. Close-up sections of similar (A and B) and different (C) protein expression patterns among trisomy 21 samples.

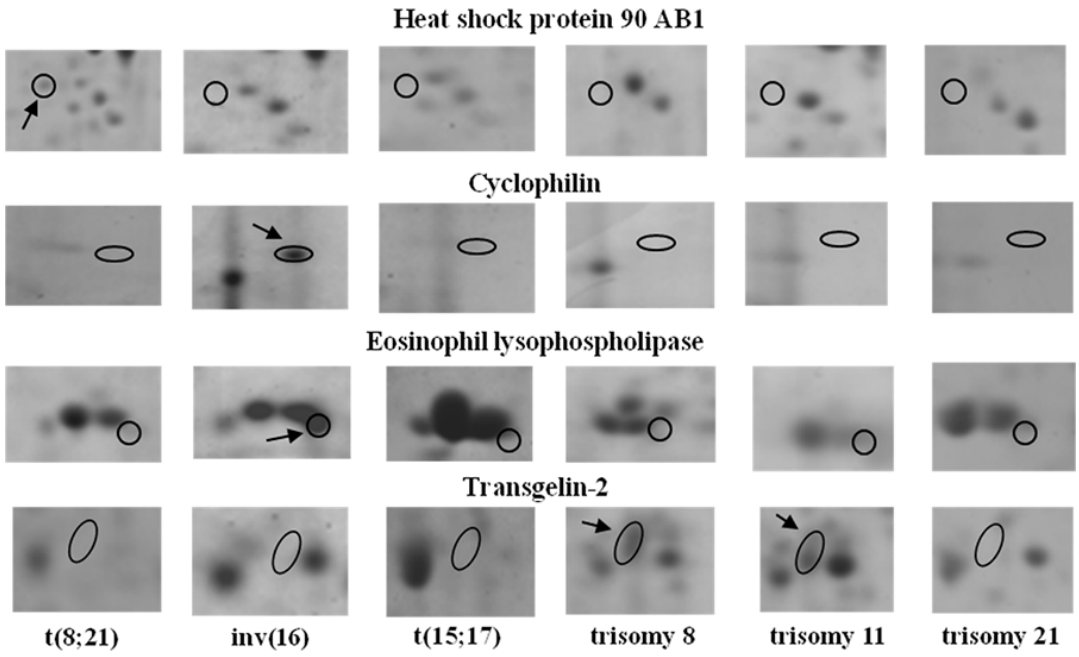

Figure 3. Close-up sections of differentially expressed protein spots as indicated in 2-DE maps of different AML karyotypes.

What is already known on this topic?

Previous studies using DNA microarray analysis provided a basis for molecular classification of AML. One study also reported distinct protein profiles [DPPs] that can used to classify acute leukemia sample into different FAB subgroups.

What this study adds?

The present study identified protein profiles that were specific to each FAB subtype and also showed specific proteins expressed in each cytogenetic group. These proteins are not similar to those found in previous studies.

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Potential conflicts of interest

The authors declare no conflict of interest.

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