SHORT COMMUNICATION

Proteomic analysis of acute myeloid leukemia: Identification of potential early biomarkers and therapeutic targets

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The main goal of this study was to analyze, using proteomic techniques, changes in protein expression of acute myeloid leukemia (AML) cells that could give insights into a better early prognosis for tumor pathophysiology. Proteomic analysis of different subtypes of AML cells was carried out using 2-DE and MALDI-TOF PMF analysis. Proteins identified as more significantly altered between the different AMLs belonged to the group of suppressor genes, metabolic enzymes, antioxidants, structural proteins and signal transduction mediators. Among them, seven identified proteins were found significantly altered in almost all the AML blast cells analyzed in relation to normal mononuclear blood cells: alpha-enolase, RhoGDI2, annexin A10, catalase, peroxiredoxin 2, tromomyosin 3, and lipocortin 1 (annexin 1). These differentially expressed proteins are known to play important roles in cellular functions such as glycolysis, tumor suppression, apoptosis, angiogenesis and metastasis, and they might contribute to the adverse evolution of the disease. Proteomic analysis has identified for the first time novel proteins that may either help to form a differential prognosis or be used as markers for disease outcome, thus providing potential new targets for rational pathogenesis-based therapies of AML.

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Leukemogenesis in humans appears to be a multistep process. Many different causes could alter normal hematopoiesis, including overexpression of certain oncogenes, altered intracellular pathways, aberrant behavior of oncoproteins and expression of chromosomal abnormalities, among others [1]. Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and over-proliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts [2]. Any myeloid lineage can be affected and the extent of maturation of the leukemia blasts can also vary. In different cases of the disease, there may be considerable variation of clinical behavior and of prognosis.

Many subsets of disease that comprise AML are best defined by the specific molecular genetic derangement that is the root cause of the clonal hemopathy [3–5]. However, AML patients with no detectable cytogenetic abnormalities make up the largest subgroup, and represent a variety of cytogenetically silent molecular genetic abnormalities. Thus, while the molecular pathogenesis is increasingly well understood for the patients with balanced reciprocal translocations, for the remaining AML patients, the candidate oncogenes and molecular pathogenesis await discovery.

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Abbreviations: AML, acute myeloid leukemia; **AL**, acute leukemia; **ANX A10**, annexin A 10; **MBC**, mononuclear blood cell

In contrast to chronic myelogenous leukemia, there is abundant evidence that mutations in two or more genes are necessary to cause AML. More than 200 different chromosome translocations and other mutational events have been described in AML cells [6]. Mutated tyrosine kinases such as FLT3 or kit, activated alleles of N-RAS or K-RAS, and constitutive expression of transcription factors, such as NF-κB, have been found in a significant percentage of AML patients, and associated with a significantly greater risk of relapse and reduced survival [1, 7–9].

The mechanism by which cells receive and transmit mitogenic signals is complex and involves the coordinated action of many different proteins, commonly represented by so-called signal transduction cascades [2]. As such cascades might be overactive in leukemia cells compared to normal hematopoietic cells, a therapeutic opportunity is provided. Moreover, an activating mutation in one of the proteins responsible for transmitting proliferative signals in AML defines a potential target.

In recent years, a considerable research effort has been directed towards the identification of markers to guide effective consideration of AML treatment. DNA microarray analysis has been applied to identify molecular markers of human hematological malignancies [10]. However, the relatively low correlation between the abundance of a given mRNA and that of the encoded protein makes it important to characterize the protein profile directly, or the 'proteome' of malignant cells in addition to the 'transcriptome'. Discrepancies between gene transcription and protein expression are the result of post-transcriptional regulation, including altered mRNA translation, and protein stability. In neoplastic cells, additional mechanisms include proteasome-mediated degradation of specific proteins induced by leukemogenic oncogenes [6]. For these reasons, analysis of protein expression is both complementary and additive to the data obtained by transcriptomic analysis.

In the present work we have studied the pattern of differential protein expression of blasts from AML patients in comparison to matched healthy individuals. We show differential expression of proteins between AML blast cells and normal mononuclear cells, identifying potential marker proteins associated with some characteristics of the pathology, thereby revealing the potential value in this approach for a better characterization of the pathophysiology of AML.

A total of 13 patients with AML, diagnosed according to the classification of the World Health Organization (WHO) committee [11], as well as 10 healthy controls were entered into this study after informed consent. Blast cells were obtained from bone marrow aspirates at the time of diagnosis, and isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of 10×10^6–20×10^6 cells in RPMI 1640 supplemented with 10% DMSO and 10% FBS, employing a method of controlled freezing and storage in liquid nitrogen. Cells were thawed at 37°C by progressively adding RPMI 1640 containing 20% FBS and 0.16 kU/mL deoxyribonuclease I (Sigma, St Louis, MO, USA). Contamination by other leukocytic cell types was assessed by dual color FACS analysis (FACScan, BD Biosciences, San Jose, CA, USA) of each sample using mAbs against the appropriate cell marker (antibodies against CD14, CD2, CD19 and CD15 were used to assess contamination with monocytes, T lymphocytes, B lymphocytes and neutrophils, respectively; Caltag Laboratories, San Francisco, CA, USA). Peripheral blood samples from 10 healthy volunteers were collected in sterile pre-cooled tubes containing 0.129 M sodium citrate (1:9) (Becton Dickinson Vacutainer System Europe, Meylan, France) as the anticoagulant and centrifuged immediately at $500 \times g$ for 10 min at 4°C to remove platelets. Platelet-free pellets were suspended in PBS and centrifuged over a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient. Mononuclear blood cells (MBCs) were collected from the interface of plasma with Ficoll and suspended in PBS. Cells were then washed with chilled PBS, pelleted. and frozen at −80°C. The details of patients examined in the study are indicated in Table 1.

After thawing, cell pellets were resuspended with lysis buffer (200 μL/10^7 cells) containing 7.0 M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholytes and 1% DTT. Extracted proteins were separated by centrifugation at $12\,000 \times g$ for 15 min and the supernatant was used as the 2-DE sample. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of the whole cell lysates were stored at −80°C until used. IPG strips (7 cm, pH range 3–10, Bio-Rad) were passively rehydrated with 150 μg protein lysate in 150 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% Triton X-100, 0.5% pharmalyte 3–10 and 0.001% bromophenol blue) for 12 h. IEF was carried out at 20°C, using a PROTEAN IEF system (Bio-Rad). Focusing was started with a conditioning step of 250 V for 20 min, followed by a voltage ramping step to 4000 V for 2 h, and a final focusing step of 10 000 Vh. Thereafter, the strips were soaked in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.001% bromophenol blue) containing 20 mg/mL DTT for 10 min and then in equilibration solution containing 25 mg/mL iodoacetamide for an additional period of 10 min. Second dimension was carried out in 12.5% polyacrylamide gels at 35 mA/gel Mini-Protean 3 Cell, Bio-Rad).

Gels used for peptide identification and analysis were stained with colloidal CBB stain (BioSafe, Bio-Rad). Gel images were obtained using a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed with the PDQuest 7.1.0 2-D analysis software (Bio-Rad). To accurately compare spot quantities between gels, image spot quantities were normalized dividing the raw quantity of each spot in a gel by the total quantity of all the valid spots in that gel. Samples from ten control subjects were used to generate the control maps. Fluctuations in the protein expression levels among the control maps were monitored in densitometric analysis, and a CV was generated for the mean value of each spot to better define changes in protein expression between normal MBCs and leukemic blast cells.

Table 1. Clinical details of AML patients

No.	Sex	Age (years)	WHO classification[a)]	Blasts (%)	WBC ($\times 10^9$/liter)[b)]
1	F	75	Acute Leukemia without maturation	87	81.8
2	F	3	Acute Leukemia without maturation	84	N/A
3	F	72	Acute Leukemia with maturation	54	114.5
4	F	68	Acute Leukemia with maturation	25	N/A
5	M	61	Acute promyelocytic leukemia	93	11.1
6	M	32	Acute promyelocytic leukemia	84	3.8
7	F	36	Acute promyelocytic leukemia	85	0.9
8	M	49	Acute promyelocytic leukemia	79	0.7
9	F	17	Acute promyelocytic leukemia	51	30.1
10	F	84	Acute myelomonocytic leukemia	65	78.8
11	M	65	Acute monocytic leukemia	76	93.0
12	F	72	Acute monocytic leukemia	85	156.0
13	M	51	Acute megakaryocytic leukemia	85	16.6

a) AML subtype according to the WHO classification of neoplastic diseases of hematopoietic tissues, which combines the analysis of morphology, immunophenotyping, cytogenetics, and molecular genetics of acute leukemia cells.

b) WBC, white blood cell count; N/A, not available.

Protein spots of interest were manually excised from preparative gels, transferred to Eppendorf tubes and subjected to MS analysis. Peptide mass fingerprints of spots were analyzed in the mass range of 800–3500 Da at the Proteomics Service of National Biotechnology Center (CSIC, Madrid, Spain) by MALDI-TOF/MS (Reflex IV; Bruker Daltonics, Bremen, Germany). Proteins were identified by searches in Swiss-Prot and TrEMBL databases using the peptide mass fingerprints generated by MALDI-TOF MS by means of MASCOT software (Matrix Science, London, UK).

At least two separate experiments were performed for all samples, and similar protein spot patterns were obtained. Approximately 500 protein spots were detected on the CBB-stained gels from the donors' material. Scanned images were employed for analysis using the PDQuest software. Near 85% of all spots were matched on duplicate gels (the gels of the sample were produced, assayed, 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 (AL) samples identified as the same subtype showed considerable similarity in their protein expression patterns, in which the matching rate ranged between 85 and 95%. The pattern of protein profiling of AL with maturation resembled that of acute promyelocytic or monocytic subtypes of AML, being similar to the composition of the acute monocytic leukemia subtype with granulocytic and mainly monocytic blasts.

Representative gels of the highly expressed proteins in control MBCs and myeloid subtypes are shown in Fig. 1. These gels are annotated to show the location of spots excised and identified by PMF for purposes of in-gel calibration for p*I* and molecular mass. The proteins identified are listed in Table 2. The predicted molecular mass and p*I* of protein spots extrapolated from in-gel calibration spots were used to corroborate protein identifications. We identified 48 spots in AML cells from which 28 corresponded to known proteins. The expression of these proteins was altered significantly compared with normal MBCs ($p < 0.05$). Variability of the differentially expressed protein spots among the ten controls used was minimal, as demonstrated by CV analysis. Some of these identified spots are shown in Fig. 1A and B and are listed in Table 3.

As a general view, altered expression of signal transduction proteins, such as disulfide isomerase, zinc finger proteins or lipoocortin 1, and metabolic enzymes such as alpha-enolase, triosephosphate isomerase, fumarase hydratase or ATP synthase (which may reflect the overall changes in cellular metabolism and growth rate that occur during malignancy [12]) were observed in almost all AML subtypes.

The proteomic analysis of leukemic blast samples allowed the identification for the first time of several proteins differentially expressed in AML that are known to play important roles in cellular functions such as glycolysis, tumor suppression, apoptosis, angiogenesis and metastasis. As already demonstrated for other types of cancer cells [13–18], these proteins might also contribute to the adverse evolution of AML. Among them, the more significantly altered proteins between the AML samples and the control group, as subdivided by biological functions, were the following: (i) suppressor genes: Rho GDP dissociation inhibitor beta, which was significantly inhibited in all AML subtypes *vs.* control cells, and annexin A 10 (ANX A10), which was significantly reduced in all AML subtypes except in acute monocytic leukemia; (ii) glycolytic enzyme: alpha-enolase, significantly increased in all AML subtypes except for AL without maturation *vs.* control cells; (iii) antioxidant enzymes:

Table 2. The highly expressed proteins in AML cells and normal MBCs identified by MALDI-TOF/MS peptide mass fingerprinting

Spot no.	NCBI accession no.	Protein name	Sequence coverage	Functional classification	Mol. mass (kDa)	p*I*
1	gi/4502101	Lipocortin 1 (annexin 1)	56%	Signal	38.9	6.6
2	gi/10835002	Rho-GDI beta	29%	transduction	23.0	5.1
3	gi/42718005	Dystrobrevin alpha	34%		65.8	8.7
4	gi/5689750	Annexin A10	26%		37.7	5.2
5	gi/48375108	Annexin A10 short isoform	36%		23.1	5.8
6	gi/55665380	Zinc finger, MYND domain	15%		42.3	5.8
7	gi/52694665	Zinc finger protein BTB	24%		73.4	6.03
8	gi/401774	Ribosomal protein S6 kinase 3	19%		29.0	6.0
9	gi/6435686	Transforming protein RhoA	22%		21.4	5.8
10	gi/55958481	Ras association domain family 4	21%		45.8	6.7
11	gi/1208427	Disulfide isomerase ER-60	32%		56.7	6.0
12	gi/107834	T-cell receptor delta chain	27%		12.2	5.04
13	gi/4503571	Enolase 1	44%	Metabolism	47.5	7.0
14	gi/37499465	Apolipoprotein A-I	14%		30.7	7.1
15	gi/40889561	Triosephosphate isomerase	43%		26.5	6.5
16	gi/442753	Hemoglobin, chain D	46%		15.9	7.9
17	gi/178779	Apolipoprotein A-IV precursor	11%		34.4	5.7
18	gi/28279801	NIT1 protein	17%		26.9	5.8
19	gi/4557525	Dihydrolipoamide dehydrogenase precursor	8%		54.2	7.6
20	gi/32880021	Fumarase hydratase, cytoplasmic isoform	14%		50.1	6.9
21	gi/1922287	Enoyl CoA hydratase	18%		31.8	8.3
22	gi/57209199	Voltage-dependent anion channel 2	22%		30.8	8.0
23	gi/4378804	Hemoglobin beta (fragment)	81%		4.5	9.4
24	gi/50345982	ATP synthase, H+ transporting	25%		54.5	8.2
25	gi/4557014	Catalase	57%	Cellular defense	59.9	6.9
26	gi/33188452	Peroxiredoxin 2	34%		21.9	5.6
27	gi/55665778	Tropomyosin 3	43%	Structural	26.5	4.8
28	gi/15277503	ACTB protein	16%		40.5	5.5

Table 3. Differentially expressed proteins between AMLs and normal MBCs

Protein name	Changes in protein expression *vs.* normal mononuclear blood cells (%)					
	Acute leukemia without maturation (patient 2)	Acute leukemia with maturation (patient 4)	Acute promyelocytic leukemia (patient 8)	Acute monocytic leukemia (patient 12)	Acute megakaryocytic leukemia (patient 13)	CV in MBCs
Enolase 1	74.6	2344.9	346.0	1730.9	2686.2	17.5%
Rho-GDI beta	67.3	25.7	31.5	33.2	82.4	3.9%
Annexin A10 short isoform	67.5	69.4	40.2	1008.1	37.7	10.3%
Catalase	308.0	52.6	225.4	288.2	173.8	22.0%
Peroxiredoxin 2	4557.1	101.95	379.97	261.26	272.52	7.7%
Tropomyosin 3	15.4	31.0	15.3	25.9	12.8	5.7%
Lipocortin 1	1096.4	102.8	564.3	184.9	376.5	11.6%

catalase, significantly increased in AL without maturation, promyelocytic and monocytic leukemia subtypes, as well as peroxirredoxin-2, which was significantly increased in all AML subtypes except for AL with maturation; (iv) structural proteins: tropomyosin 3, significantly reduced in all AML subtypes; and (v) signal transduction mediators: lipocortin 1, significantly increased in all AML subtypes except for AL with maturation *vs.* control cells. The approach we adopted,

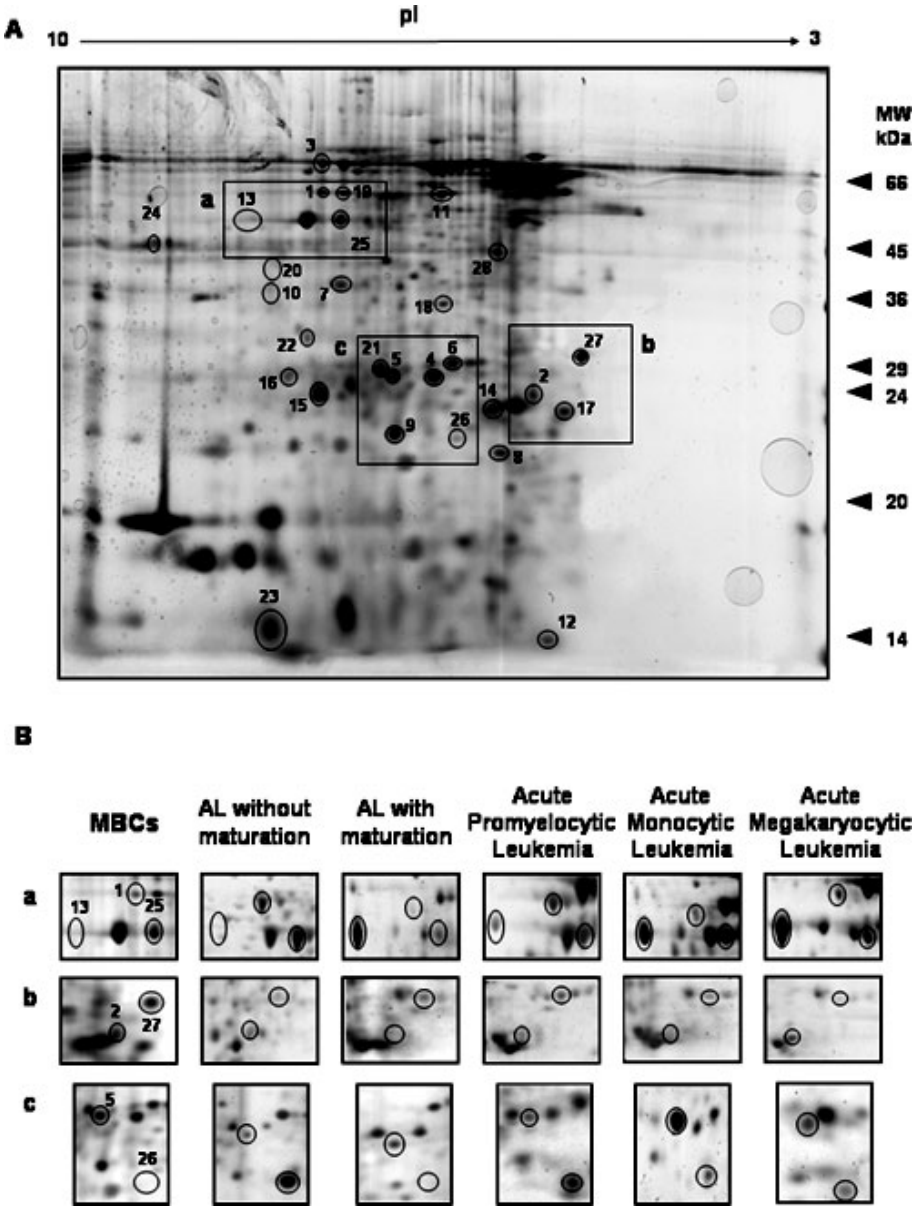

Figure 1. A representative 2-D gel image showing the highly expressed proteins in AML subtypes and normal MBCs. (A) The scanned image of the CBB-stained gel was used to detect and compare protein spots. The positions of proteins expressed differentially among AML patients and healthy donors are enclosed in circles, indexed as numbered, and the positions of molecular mass standards (in kDa) are shown on the right. The complete list of proteins is given in Table 2. (B) Close-up sections of differentially expressed protein spots as indicated in 2-DE maps of different AML subtypes compared with normal MBCs. (a) 1, lipocortin 1; 13, alpha-enolase; (b) 25, catalase; 2, Rho-GDI 2; 27, tropomyosin 3; (c) 5, Annexin A10, short isoform; 26, peroxiredoxin 2.

using p*I* 3–10 2-D gels and CBB staining, was designed to detect prominent changes in expression of major proteins. Therefore, in the following, only those proteins will be discussed that seem to have some relevance for AML as based on literature data.

Alpha-enolase was found to be expressed at higher levels in most AML subtypes. This enzyme is involved in glycolysis and catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which in turns is dephosphorylated into pyruvate to yield ATP. Enolase has been previously identified as a product of several types of tumors, and enolase activity has been detected in the sera of some cancer patients [13, 19]. Moreover, the differential expression of this enzyme has been demonstrated to imply a difference of maturation and differentiation or activation among neoplastic cells [20].

Two suppressor genes were found to be down-regulated in leukemia samples: Rho GDI2 and ANX A10. Rho GTPases are critical elements of signal transduction pathways implicated in promoting a variety of cellular processes, such as changes in morphology, motility, and adhesion [21]. Misregulation of Rho GTPases has been proven to contribute to cancer cell invasion and metastasis. Accordingly, the inhibitor Rho GDI2 has been demonstrated to be a metastatic suppressor gene, and its loss is a marker of aggressive human cancer [14]. Thus, the down-regulation of this protein observed in all AML subtypes is likely to contribute to the adverse development of this malignancy.

The second potential suppressor gene found to be down-regulated in AML blasts was ANX A10, which is a novel member of the ANX family whose function remains unclear. However, recent studies have demonstrated that ANX A10 down-regulation correlated with high-grade and high-stage tumors and early tumor recurrence. Moreover, its down-regulation has been also associated with malignant phenotype in liver cells, and correlates with vascular invasion and progression of hepatocellular carcinoma [15].

On the other hand, two antioxidant enzymes were found significantly increased in almost all leukemia subtypes analyzed: catalase and peroxiredoxin 2. Oxidants are known to modulate cell proliferation and apoptosis, and induce synthesis of growth factors that play an important role in tumor growth and invasion. Antioxidant enzymes and thiol proteins regulating cellular redox state constitute the major cellular protection against oxidants. Consequently, they are also associated with both carcinogenesis and tumor progression [16]. This has consequences not only for tumor behavior but also for resistance of tumor cells to cytotoxic drugs and radiation.

Densitometric analysis also indicated a significant reduction in the expression of tropomyosin 3 between the AML cells and the normal MBCs. Tropomyosin are a family of cytoskeletal proteins that bind to and stabilize actin in microfilaments. The loss of tropomyosin expression in tumor cells may prevent proper assembly of microfilaments, and, consequently, contribute to the invasive and metastatic properties of cancer cells [17]. In addition, reduced tropomyosin levels have also been correlated with metastatic potential in lung carcinoma and melanoma cells. Moreover, down-regulation of tropomyosin in various human cancer cells has been shown to be dependent on Ras/MEK/ERK pathway activation and correlated with growth transformation [22]. Furthermore, we have found that the down-regulation of tropomyosin in AML cells was accompanied by constitutive activation of the MEK/ERK pathway (unpublished data).

In this regard, the increased levels of lipocortin 1 (annexin 1) observed in AML cells may also be interesting. Lipocortin 1 is a calcium- and phospholipid-binding protein, either cytosolic or associated with the membrane or the cytoskeleton in a calcium-dependent manner [23]. Lipocortin 1 is a substrate for protein kinase C and protein-tyrosine kinases. This fact, coupled with multiple phosphorylation sites and its calcium- and phospholipid-binding properties, may be indicative of a role for lipocortin 1 in signal transduction as a means to affecting its pleiotropic physiological roles. In agreement with our results, a recent study has shown that increasing the expression of lipocortin 1 leads to constitutive activation of ERK1/2 in RAW macrophages [18].

Various annexins have been implicated in cellular processes, including modulation of phospholipase A2 activity and inflammation, immune response, proliferation, blood coagulation, differentiation, membrane skeletal linkage and intracellular signal transduction. Moreover, the multifactorial downstream effects of the action of lipocortin 1, including antiproliferative, anticoagulant and antiendotoxic effects, might be translocated by the ERK signaling pathway, which serves to couple a wide range of stimuli to the regulation of a variety of cellular functions [18].

Taken together, our data suggest that pathological changes in acute leukemia may be reflected in proteomic patterns in cells, and thus identification of these altered protein patterns represents a way of discovering new potential tumor markers. Emerging studies, using multidimensional chromatography combined with SELDI-TOF technique followed by MALDI-TOF/TOF analysis, have identified the changes in the proteome associated with the inducible AML1-ETO expression in AML cells [24]. Targets of transcription factors involved in myeloid stem cell development and leukemia, or new protein expressed and/or activated in response to anticancer reagents have also been identified by 2-DE and MS [25], and amino acid-coded mass tagging coupled with ESI-MS [26].

Proteins identified by the proteomic approach employed in this study were those with relatively high abundance; we suggest, therefore, that the proteins differentially expressed may have value in the diagnostic of AML. Reliable prognostic indicators are still required to predict and further characterize the clinically variable course of AML. The further extension of the present analyses to currently less-well-defined AL, through the study of a wider spectrum of different AML samples, would possibly identify additional protein expression profiles, providing a complete map of regulatory molecules to be used in prognostic and therapy response evaluation at the single case level. Disease-relevant information will also be hidden behind changes in protein modification or subcellular localization. Such changes are detectable only at protein level. Ultimately, data from the protein and the transcript level will be required in combination with in-depth knowledge of the involved genetic circuits to distinguish between disease-relevant and secondary effectors. Understanding these complex changes taking place at the molecular level – the molecular anatomy of the disease – should lead to the identification of precise prognostic markers and novel drug intervention sites and, ultimately, to a specific tailored therapy, individually designed for a patient based on his or her particular molecular anatomy.

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