The Potential of Protein Expression Profiles in Categorization Risks for Acute Myeloid Leukemia: A Pilot Study

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ABSTRACT

Currently, there are no markers to predict response to acute myeloid leukemia (AML) therapy and patients have to wait for a period of 3-6 months to see treatment response. The study aimed to analyze changes in protein expression in AML cells between different categorization risk groups using proteomics techniques. Six peripheral blood (PB) and six bone marrow (BM) samples at diagnosis and remission times were collected from AML patients. Another Six PB samples were collected from different categories of AML. All samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The levels of proteins in patients with AML were compared at different categorization risk, individual response to treatment and clinical characteristics. Twenty-one and 145 differentially expressed proteins were identified with disease progression and risk categories of AML, respectively. Three (3) proteins were noticeably highly expressed out of the range of others proteins by at least 3- fold difference between diagnosis and remission. Two other of proteins were up regulated by more than 10 folds between risk categories of AML. Furthermore, 4 proteins were found to be expressed in one risk category, but were not detectable in other two risk categories. The study showed that a panel of differentially expressed protein profiles might serve as more objective biomarkers for accurate stratification of different risk categories of AML.

Keywords: Acute Myeloid Leukemia, Proteomics, Risk category, Biomarker discovery

ÖZET

Akut Miyeloid Lösemide Protein Ekspresyon Profillerinin Potansiyeli: Ön Çalışma

Günümüzde, akut miyeloid lösemide (AML) tedavi yanıtını belirleyen marker yoktur. Hastalar, tedavi yanıtını görebilmek için 3-6 ay kadar beklemeleri gerekmektedir. Bu çalışmada farklı risk gruplarında AML hücrelerinin protein ekspresyon değişiklikleri, proteomiks tekniği ile çalışılmıştır. Altı AML hastasının tanı anında ve remisyonda alınan periferik kan (PK) ve kemik iliği (Kİ) örnekleri analiz edilmiştir. Diğer risk kategorisinden 6 AML hastasının PK analiz edildi. Örneklerin hepsi liquid chromatography tandem mass spectrometry (LC-MS/MS)'de analiz edildi. Hastaların protein ekspresyonları, farklı risk kategorisine, tedavi yanıtına ve klinik özelliklerine göre analiz edildi. Hastalık tedavi yanıtlarına göre 21, risk gruplarına göre bakıldığında ise 145 farklı protein ekspresyonun olduğu saptandı. Üç proteinin tanı anında ve remsiyonda normal aralıkların çok üzerinde; en az üç kat yüksek olarak eksprese olduğu saptandı. Farklı iki proteinin ise farklı risk gruplarında en az 10 kat faarklı olarak eksprese olduğu görüldü. Ayrıca, 4 protein bir risk grubunda eksprese olur ikeni, diğer iki risk grubunda ekspresyonu saptanacak düzeylerde değildi. Bu çalışma farklı protein ekspresyon panlellerinin, farklı risk sınıflamalarında daha objektif biyomarker olarak kullanılabileceğini göstermiştir.

Anahtar Kelimeler: Akut Miyeloid Lösemi, Protemiks, Risk kategorisi, Biyomarker keşfi

INTRODUCTION

Leukemia is a malignant disorder of hematopoietic stem cells (HSCs) that occurs as a result of uncontrolled growth and differentiation as well as self-renewal capacity. In acute myeloid leukemia (AML), HSCs persist in an immature state because their development or differentiation is blocked at the level of progenitor cells. Consequently, leukemic cells (blasts) reproduce and accumulate rapidly.[1] One of the main goals of proteomics studies in cancer research is to characterize proteins expressed in abnormal cells. Basically, proteomics has the capacity to identify abnormally expressed proteins in malignant cells and the expression of proteins of the metabolic and signaling pathways will help to elucidate the mechanisms underlying the initiation and development of neoplasia.[2] Advancement technologies in proteomics studies have increased the opportunities for discovering diseases biomarkers, particularly cancers. The detection of sensitive biomarkers present at low concentrations in human plasma of AML patients has a higher value for development of new methods of determining the prognosis of AML. Such biomarkers will complement the more classical diagnostic techniques such as cytogenetic, hematology, and other clinical tests conducted on a routine basis. More importantly, this innovative approach would minimize the use of invasive procedures such as bone marrow biopsies, thereby avoiding associated complications.[3,4]

Survival of AML patients is dependent on strategic therapy. Mortality in these patients is often related to failure in the initiation of chemotherapy and early relapse.[5] Thus, risk stratification based on prognostic factors is essential to adapt a therapeutic regime for positive clinical outcome. This may be achieved through profiling of differentially expressed plasma peptides and mapping the peptide spectrum specific for the AML subtype. Proteomic profiling is also useful for the clarification of pathogenesis, drug-resistance and prognosis.[6] Consequently, this study was design to analyze changes in protein expression of AML cells between different categorization risk groups using proteomics techniques that could potentially shed insights into leukemic pathophysiology and help in predicting early responses to treatment.

MATERIALS AND METHODS

Study Subjects

Written informed consent was obtained from all participants. The Research Advisory Council and the Office of Research Affairs, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia approved this study. The disease data were compiled and compared against the protein fingerprints derived from the patients' peripheral blood and bone marrow samples.

Sample Collection and Preparation

Peripheral blood (5 mL) and bone marrow (2 mL) were collected from patients with AML in ethylenediamine tetraacetic acid (EDTA) tubes at diagnosis and remission time. Blood samples were subjected to Ficoll-Paque density gradient centrifugation to obtain plasma and mononuclear cells as previously described.[5] Briefly, fresh balanced salt solution (BSS) was prepared by adding 1 mM phenylmethylsulfonyl fluoride (PMSF) to phosphate-buffered saline (PBS) to a final concentration of 20 μM and benzamidine at a final concentration of 830 μM. The PMSF was dissolved in ethanol while benzamidine was dissolved in distilled water. Equal volumes of EDTA anti-coagulated blood and freshly prepared BSS were added to a 15-mL tube, mixed thoroughly, and gently vortexed. A 22G syringe needle was inserted through the septum of a bottle containing well-mixed Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA, USA), the bottle inverted, and required volume of Ficoll-Paque PLUS withdrawn. A 22G needle and syringe was inserted vertically into the 15-mL test tube containing anti-coagulated blood until it reached the bottom. The Ficoll-Paque PLUS was gently and slowly released to form a layer under the blood sample, while avoiding mixing. The sample then was centrifuged for 30 min at 318 × g (Eppendorf, Hamburg, Germany) at room temperature. The upper layer was withdrawn using a clean pasture pipette, without disturbing the peripheral mononuclear cell layer at the interface, transferred to a sterile 1.5-mL centrifuge tube, labeled and stored at –80^{0}C and used later for these experiments.

Depletion of Acute Myeloid Leukemia Samples

Crude plasma samples were depleted using Pierce® Albumin/IgG Removal Kit (according to manufacturer's instructions) prior to proteomics analysis in order to remove the most abundance proteins especially albumin and IgG and determination of total protein concentration was performed by Bradford technique.[7]

Disease Progression

In this experiment, 6 samples were analyzed in two arms. The first arm compared BM plasma and PB plasma at diagnosis to evaluate the differences in the number of proteins identified in each sample type and to observe the changes in expression between these two types of bodily fluid compartments specimens as baseline protein patterns. The second arm of the analysis was to evaluate changes in the expression of proteins in PB plasma at the time of diagnosis compared to protein changes at remission period.

Risk Categories

The comparison was made between different categorization of 6 AML leukemia samples including low-, intermediate- and high-risks in order to follow the differentiation of proteins expressions within these risks using PBP samples at time of diagnosis.

Sample Preparation and Protein In-solution Digestion

All samples were normalized for protein concentration. From each sample group, equal amount (100 μg) of complex protein mixtures derived from depleted plasma was taken from disease progression and risk category and exchanged twice with 500 μL of 0.1% RapiGest (Waters, Manchester, UK) using a 3-kDa ultra filtration device (Millipore), previously described.[8,9] Protein concentrations of 0.5 to 1 μg/μL were achieved at the end of in-solution tryptic digestion. Briefly, the proteins were denatured in 0.1% RapiGest SF at 80^{0}C for 15 min, reduced in 10 mM DTT at 60^{0}C for 30 min, centrifuged briefly at 13,000 RPM for 10 seconds to bring together the condensation under the tube cap, allowed to cool to room temperature, and alkylated in 10 mM iodoacetamide (IAA) for 40 min at room temperature in the dark. The samples were then trypsin-digested at an enzyme: protein ratio (w/w; 1 μg/μL trypsin concentration) of 1:50 overnight at 37^{0}C with gentle shaking. The digestion/ RapiGest was quenched by incubation with 4 μL of 12 M HCl at 37^{0}C for 15 min, followed by centrifugation at 13,000 RPM for 10 min. The samples were then diluted to 5 pmol/μL or 5- to 10-fold with aqueous 0.1% formic acid to achieve a load of approximately 3 μg for the analytical column of the mass spectrometer. All samples were spiked with yeast alcohol dehydrogenase (ADH; P00330) as an internal standard at a concentration of 200 fmol per injection to facilitate absolute quantitation as previously described.[10,11]

Protein Identification by Synapt G2 Mass Spectrometry

A one-dimensional, (1-D) Nano Acquity liquid chromatography coupled with a Synapt G2 high definition tandem mass spectrometry (Waters, Manchester, UK) was used to generate expression proteomics data for three subtypes of AML samples. The optimization of the instrument was as previously described [10]. Briefly, the detector set up was done using 2-ng/μL-leucine enkephalin (556.277 Da). Mass/charge (m/z) calibration was achieved with a separate infusion of 500 fmol [Glu] 1-Fibrinopeptide B (GluFib, 785.843 Da), on a Trizaic Infusion tile using the automation of the Mass Lynx IntelliStart. Other parameters that were preset were capillary voltage at 3.6 Kv, sample cone at 50 V, extraction cone of 5 V, source temperature at 100^{0}C, cone gas flow rate of 10 L/h, Nano-flow gas pressure at 0.6 bars, and a purge gas flow rate of 600 L/h. All analyses were performed on a Trizaic Nano source (Waters, Manchester, UK), using the positive ion mobility mode nanoESI at slow flow rate.

A 3 μl sample containing approximately 3 μg of the digested protein was loaded onto the column, and samples were infused using the Acquity Sample Manager with a mobile phase consisting of A1 (99% water +1% acetonitrile + 0.1% formic acid) and B1 (100% acetonitrile + 0.1% formic acid, with a sample flow rate of 0.450 μl/min). Data-in-

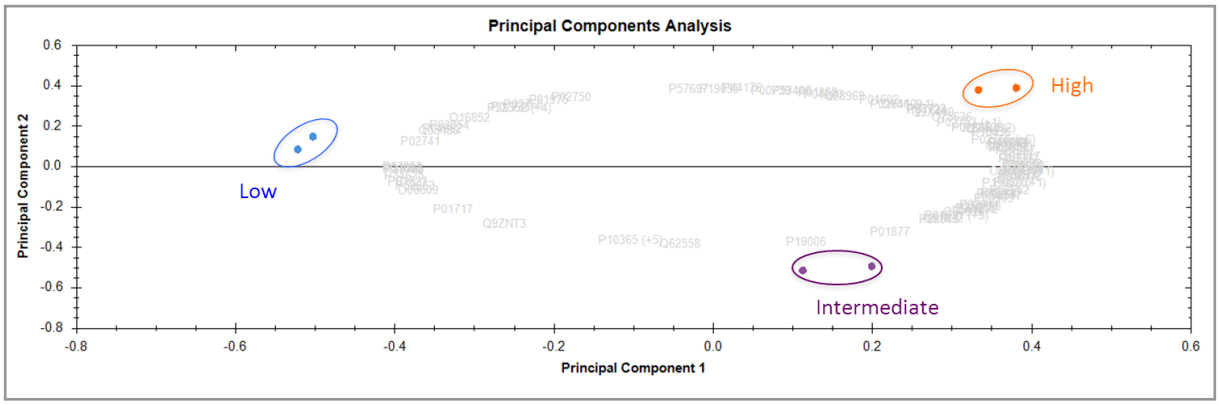

Figure 1. Unsupervised Principal Component Analysis (PCA) plot using the 145 proteins that were significantly differentially expressed between low- (purple), intermediate- (blue), and high-risk (orange) plasma samples form AML patients. The numbers in grey scale are the accession numbers of each of the proteins. The PCA plot was generated from Progenesis QI for proteomics using the normalized quantitative dataset of protein expression changes across the three sample sub-groups.

dependent acquisition/iron mobility separation experiments (MSEs) were performed, and data were acquired over an m/z range of 50 - 2000 Da, scan time of 1 sec, ramped transfer collision energy of 20 to 50 V, and total acquisition time of 120 min. All samples were run in triplicate and repeated in 2 different experiments to ensure reproducibility of results. The data were accessed via the Mass Lynx program (Version. 4.1, SCN833, Waters, Manchester, UK) using the resolution and positive-polarity modes. The data were background subtracted, smoothed, and de-isotoped at a medium threshold. Protein Lynx Global Server (PLGS) 2.2 (Waters, Manchester, UK) and Progenesis LC/MS (QI) were used for all automated data processing and database searches.

The generated peptide masses were compared against the SwissProt protein sequence database and cross-validated on Uniprot human proteome database using PLGS 2.5/Progenesis QI (Waters, UK, Nonlinear, UK) for protein identification and differential analysis as previously described.10, 11

Data Analysis and Informatics

Progenesis QI (Nonlinear Dynamics, Newcastle, UK) software was used for data processing and search. The search criteria were; 1 missed cleavage, max protein mass= 1000 kDa, trypsin, and Carbamidomethyl C fixed and oxidation M variable modifications. The software generated normalized label-free relative quantification analyses and significantly differentially polypeptides were subjected to unsupervised principal component analyses (PCA) for all sample groups.

Multivariate data analysis Variance (ANOVA) at $p \leq 0.05$ was used to identify significant alterations in regulated proteins and in addition, the expression level of at least > 1.5-fold change between paired of samples being compared.

RESULTS

Differentially Expressed Proteins in Acute Myeloid Leukemia Patients at Diagnosis and Remission

Among the limitations facing prospective sample collection for expression analysis is logistics in prospective serial collection of samples at different stages of disease progression. Hence it was possible to have either BMP or PBP and where possible both from the same individual patients at diagnosis. While in majority of cases, only PBP was available as at time of disease remission. Marked differentially expressed proteins were observed when all the three sample types including BMP and PBP at diagnosis as well as PBP at remission were subjected to non-gel-based and label-free in-solution expression proteomics analyses. In total, 39 identified proteins were differentially expressed across all the three sample types. However, only 21 of the 39 proteins showed significant ($p < 0.05$) expression changes and at least ≥ 1.5-fold difference

Table 1. List of identified differentially expressed with their description and their levels of expression changes between peripheral blood samples at diagnosis and at remission. (In bold are some of the proteins with expression levels of > 3-folds)

Accession#	Max Fold Change	Highest Mean Condition	Lowest Mean Condition	Protein Description
O83974	1.06	Diagnosis	Remission	Nucleoside diphosphate kinase (EC 2.7.4.6) (NDK).
P02048	2.60	Diagnosis	Remission	Hemoglobin beta chain.
P02774	2.26	Remission	Diagnosis	Vitamin D-binding protein precursor (DBP) (Group-specific component).
P01617	2.02	Diagnosis	Remission	Ig kappa chain V-II region TEW.
P01623	1.35	Remission	Diagnosis	Ig kappa chain V-III region WOL.
P02735	**3.70**	**Diagnosis**	**Remission**	**Serum amyloid A protein precursor (SAA) [Contains:** Amyloid protein A SAA1,SAA2].
P04217	2.16	Remission	Diagnosis	Alpha-1B-glycoprotein.
P80748	1.82	Remission	Diagnosis	Ig lambda chain V-III region LOI.
Q60397	**5.81**	**Diagnosis**	**Remission**	**Guanine nucleotide-binding protein G(k), alpha subunit.**
P01766	1.73	Diagnosis	Remission	Ig heavy chain V-III region BRO.
P01922	**4.62**	**Diagnosis**	**Remission**	**Hemoglobin alpha chain.**
P00738	1.86	Diagnosis	Remission	Haptoglobin-2 precursor.
P01009	1.53	Diagnosis	Remission	Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor).
P01876	1.68	Diagnosis	Remission	Ig alpha-1 chain C region.
Q28522	1.03	Diagnosis	Remission	Serum albumin precursor (Fragment).
P02790	1.63	Remission	Diagnosis	Hemopexin precursor (Beta-1B-glycoprotein).
P01871	2.49	Diagnosis	Remission	Ig MU chain C region.
P02769	1.88	Remission	Diagnosis	Serum albumin precursor (Allergen Bos d 6).
P01860	1.32	Diagnosis	Remission	Ig gamma-3 chain C region (Heavy chain disease protein).

between PB plasma samples obtained at diagnosis and at remission from the same individual patients. Three of the 21 proteins with expression difference of greater than 3-fold were listed in (Table 1).

Differentially Expressed Plasma Proteins Between Different Risk Categories of Acute Myeloid Leukemia

Approximately 225-250 unique proteins species were identified from each of triplicate runs from all sample groups including low-, intermediate-, and high-risk patients. Among these proteins, 145 were significantly differentially expressed with at least ≥1.5-fold change ($p < 0.05$). The dataset of 145 differentially expressed proteins was subjected to unsupervised Principal Component Analysis (PCA) and samples were distinctively clustered into three separate groups (Figure 1). The majority of the differentially expressed proteins were up-regulated among the high AML-risk category, followed in decreasing order of expression of the proteins in the intermediate- and low risk categories respectively as listed in Table 2.

DISCUSSION

Despite advanced improvement in the diagnosis and management of hematological diseases including AML, accurate prognostication of disease often remain elusive using currently available clinical and cytogenetic parameters. The heterogeneity of AML is detectable via morphological and immunological evaluation as well as through their cytogenetic and molecular characteristics. These cellular characteristics are defined by the molecular entities, which could be targeted for the development of diagnostic and prognostic profiles, and for the identification of disease subtypes.[12] Despite the currently available therapeutic methods, one of which is stem cell transplantation, the rate of recovery for patients with AML is still rather low. While long-term survival is high in older adults with AML, the majority of patients are incapable of tolerating chemotherapy. Even though the patholo-

Table 2. List of peripheral blood identified differentially expressed proteins from peripheral blood samples between different risk categories of AML patients

Accession	Peptide count	Peptides used for Quant	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
Q29443	18	3	1.87E-05	10.0016	High	Low	Serotransferrin precursor (Siderophilin) (Beta-1-metal-binding globulin).
P02753	4	3	3.60E-05	3.012	High	Low	Plasma retinol-binding protein precursor (PRBP) (RBP).
O74109	2	2	4.55E-05	3.851	High	Intermediate	Acidic ribosomal protein P0 homolog (L10E).
Q9WZ72	1	1	5.65E-05	6.410	Intermediate	Low	30S ribosomal protein S6.
P44176	1	1	0.000114372	3.166	High	Intermediate	Protein trpH.
P01880	6	6	0.000119906	26.529	Low	High	Ig delta chain C region.
P02741	8	8	0.000189273	2.834	Low	Intermediate	C-reactive protein precursor.
P19006	**13**	**1**	**0.000204301**	**Infinity**	**Intermediate**	**Low**	**Haptoglobin alpha and beta chains.**
P02911	**3**	**3**	**0.014019105**	**1854.053**	**Intermediate**	**Low**	**Lysine-arginine-ornithine-binding periplasmic protein.**
P15514	**1**	**1**	**0.033543696**	**112.135**	**Low**	**Intermediate**	**Amphiregulin precursor (AR) (Colorectum cell-derived).**
P36421	**1**	**1**	**0.04183228**	**Infinity**	**Intermediate**	**High**	**Tyrosyl-tRNA synthetase, cytoplasmic (EC 6.1.1.1).**
P23722	3	2	0.000249133	2.332	High	Low	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.13).
P02760	10	10	0.000252698	2.109	Low	Intermediate	AMBP protein precursor [Contains: Alpha-1-microglobulin].
P01602	2	2	0.000262566	7.169	High	Intermediate	Ig kappa chain V-I region HK102 precursor.
P02652	7	7	0.000294418	3.199	Intermediate	Low	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II).
P04262	1	1	0.000314701	2.313	Low	Intermediate	Keratin, type II cytoskeletal 68 kDa, component IB.
P21667	5	2	0.000325455	39.825	High	Low	Hemoglobin beta chain.
P01871	23	9	0.000384945	1.963	High	Intermediate	Ig MU chain C region.
P02765	15	14	0.000386585	2.132	High	Low	Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Alph-2-HS-glycoprotein).
O08609	2	1	0.00040561	6.050	Low	High	Transcription factor-like protein 4 (Max-like bHLHZip protein).
O16852	2	2	0.000491965	2.854	Low	Intermediate	Proliferating cell nuclear antigen (PCNA) (Cyclin).
P01860	12	5	0.000513446	5.569	High	Intermediate	Ig gamma-3 chain C region (Heavy chain disease protein).
P53853	1	1	0.000531913	8.174	Low	High	Hypothetical 30.6 kDa protein in RPA49-SUI1 integrity.
P52263	1	1	0.000546844	2.622	Low	High	Ovomucoid (Fragment).
P02054	3	1	0.000560865	2.725	Low	Intermediate	Hemoglobin beta chain.
P54398	3	2	0.00062032	2.758	High	Low	Fat body protein P6.
P02647	28	21	0.000644801	1.892	Intermediate	Low	Apolipoprotein A-I precursor (Apo-AI).
O75636	7	7	0.000655277	2.602	High	Low	Ficolin 3 precursor (Collagen/fibrinogen domain-containing protein 3).
P02655	2	2	0.00094464	7.785	High	Low	Apolipoprotein C-II precursor (Apo-CII).
P016319	7	3	0.000985358	8.544	Intermediate	Low	Ig kappa chain V-II region 26-10.
P05090	7	7	0.000985493	2.620	High	Low	Apolipoprotein D precursor (ApoD).
P34625	1	1	0.00113035	27.706	Low	High	Hypothetical 10.6 kDa protein ZK353.2 in chromosome III.
P04208	4	1	0.001164622	1.842	Intermediate	High	Ig lambda chain V-I region WAH.
P01877	20	3	0.001298204	2.070	Intermediate	Low	Ig alpha-2 chain C region.
P02768	77	32	0.001319554	1.949	High	Intermediate	Serum albumin precursor.
P19652	10	9	0.001334186	1.680	High	Intermediate	Alpha-1-acid glycoprotein 2 precursor (AGP 2).
P29621	4	2	0.001350336	1.846	Intermediate	Low	Kallikrein-binding protein precursor (KBP).
P02750	15	15	0.001458032	2.490	Low	Intermediate	Leucine-rich alpha-2-glycoprotein (LRG).
P25311	15	15	0.001486352	1.796	High	Intermediate	Zinc-alpha-2-glycoprotein precursor (Zn-alpha-2-glycoprotein).
P02735	5	5	0.001487066	3.804	Low	Intermediate	Serum amyloid A protein precursor (SAA) [Contains: Amyloid protein A].
P02656	4	2	0.001548529	4.508	High	Intermediate	Apolipoprotein C-III precursor (Apo-CIII).
P01975	4	2	0.00166841	2.165	Low	Intermediate	Hemoglobin alpha chain.
P50512	2	2	0.001838578	4.443	High	Low	RNA polymerase sigma-32 factor.
Q58998	1	1	0.002023546	6.297	Intermediate	Low	Probable ribose 5-phosphate isomerase (EC 5.3.1.6)
Q00137	2	2	0.002121304	1.919	Low	Intermediate	Hypothetical gene 8 membrane protein.
P58517	1	1	0.002168685	6.652	Intermediate	Low	Serine protease inhibitor 4 (PI-4) (Fragment).
P01717	2	1	0.002612881	12.074	Low	High	Ig lambda chain V-IV region Hil.

Table 2. List of peripheral blood identified differentially expressed proteins from peripheral blood samples between different risk categories of AML patients *(Continiu)*

Accession	Peptide count	Peptides used for Quant	Anova (p) change	Max fold mean condition	Highest mean condition	Lowest	Description
P98049	1	1	0.002986535	5.594	Intermediate	Low	Cytochrome c oxidase polypeptide II (EC 1.9.3.1).
P53466	3	1	0.003026519	2.524	High	Intermediate	Actin, cytoskeletal 2 (LPC2).
P01630	1	1	0.003039135	3.532	High	Low	Ig kappa chain V-II region 7S34.1.
P07490	2	1	0.003137362	2.866	High	Low	Progonadoliberin I precursor [Contains: Gonadoliberin II].
P01621	2	1	0.003272698	5.787	Low	High	Ig kappa chain V-III region NG9 precursor (Fragment).
P01857	15	5	0.003351987	1.907	High	Low	Ig gamma-1 chain C region.
P01011	17	15	0.003405063	1.651	Low	Intermediate	Alpha-1-antichymotrypsin precursor (ACT).
P31227	3	3	0.003424816	1.500	Low	High	S-modulin (Sensitivity-modulating protein).
P09871	10	10	0.003514922	1.547	High	Low	Complement C1s component precursor (EC 3.4.21.42)
P49064	15	4	0.003568207	2.425	High	Low	Serum albumin precursor (Allergen Fel d 2).
P01862	5	2	0.003616366	1.771	Low	Intermediate	Ig gamma-2 chain C region.
Q9X480	2	2	0.003655911	2.118	High	Low	Putative signal peptide peptidase sppA (EC 3.4.21).
P29610	3	3	0.003661547	1.550	High	Low	Cytochrome c1, heme protein, mitochondrial precursor.
Q28969	1	1	0.00375721	4.033	High	Intermediate	Nitric-oxide synthase, endothelial (EC 1.14.13.39).
P06727	23	23	0.003832552	1.856	High	Low	Apolipoprotein A-IV precursor (Apo-AIV).
Q09163	4	3	0.004158717	1.827	Low	High	Delta-like protein precursor (DLK) (Preadipocyte factor 1).
Q00835	1	1	0.004316318	8.127	Intermediate	Low	Trichodiene synthase (EC 4.2.3.6) (Sesquiterpene cyclase).
Q28522	35	3	0.004421518	7.094	High	Low	Serum albumin precursor (Fragment).
P27425	13	2	0.004543771	4.626	High	Low	Serotransferrin precursor (Siderophilin) (Beta-1-metal binding globulin).
P18436	2	1	0.004599351	2.107	High	Low	Hemoglobin gamma chain.
P04090	2	2	0.005033086	1.677	Low	High	Prorelaxin H2 precursor.
P00738	42	16	0.005072056	1.560	Low	High	Haptoglobin-2 precursor.
O60493	2	2	0.00508749	7.187	High	Low	Sorting nexin 3 (SDP3 protein).
P56410	11	2	0.005121536	2.622	Intermediate	Low	Ovotransferrin.
P50450	5	1	0.005482226	1.834	Low	High	Thyroxine-binding globulin precursor (T4-binding globulin).
P00969	2	2	0.005512066	8.731	Intermediate	Low	DNA ligase (EC 6.5.1.1) (Polydeoxyribonucleotide synthase).
Q05186	1	1	0.005570395	4.633	Low	Intermediate	Reticulocalbin 1 precursor.
P07724	10	1	0.005644092	2.050	High	Low	Serum albumin precursor.
P33213	5	5	0.005692555	2.075	Intermediate	Low	Nodulation protein nolX.
P18902	2	1	0.005852563	3.176	High	Low	Plasma retinol-binding protein (PRBP) (RBP).
P01884	2	1	0.005950654	1.825	Low	Intermediate	Beta-2-microglobulin precursor.
P39187	1	1	0.006075748	1.736	Low	High	Protein ytfJ precursor.
P02790	21	19	0.006116569	1.854	High	Intermediate	Hemopexin precursor (Beta-1B-glycoprotein).
Q62558	17	4	0.006132337	2.236	Intermediate	High	Haptoglobin precursor.
P19002	7	1	0.006135569	2.669	High	Low	Hemoglobin alpha-1, alpha-2, and alpha-3 chains.
P06123	1	1	0.006432199	7.061	High	Low	Ferredoxin-like protein in vnf region.
P57697	1	1	0.00649747	5.219	High	Intermediate	Proteasome alpha subunit (EC 3.4.25.1) (Multicatal).
Q51832	2	2	0.007133136	2.543	Intermediate	Low	Ribonuclease HII (EC 3.1.26.4) (RNase HII) (Fragment).
P33165	2	1	0.007464601	3.651	Low	High	Elongation factor Tu (EF-Tu).
Q61147	16	4	0.007730386	3.483	High	Low	Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase).
P04170	2	2	0.007834218	1.703	Intermediate	High	Rubredoxin (Rd).
P19626	1	1	0.008320912	2.348	Intermediate	Low	Myosin regulatory light chain 2.
P19035	3	2	0.008619178	4.930	High	Intermediate	Apolipoprotein C-III (Apo-CIII) (Fragment).
Q9PRL9	4	2	0.008649686	1.624	High	Low	Hemoglobin alpha-I chain.
Q51929	1	1	0.009014932	1.860	Low	Intermediate	Major cold-shock protein (Fragment).
Q9ZE57	1	1	0.009220375	2.281	High	Low	Hypothetical protein RP090.
P01763	3	2	0.009823443	1.885	Low	High	Ig heavy chain V-III region WEA.
P01781	4	2	0.010223347	1.511	Low	Intermediate	Ig heavy chain V-III region GAL.
P51884	8	6	0.010322692	1.755	High	Low	Lumican precursor (LUM) (Keratan sulfate proteoglycan lumican).
P09904	4	1	0.011026794	1.678	High	Low	Hemoglobin alpha chain.
P01743	2	2	0.011393916	4.619	High	Low	Ig heavy chain V-I region HG3 precursor.

Table 2. List of peripheral blood identified differentially expressed proteins from peripheral blood samples between different risk categories of AML patients (*Continiu*)

Accession	Peptide count	Peptides used for Quant	Anova (p)	Max fold change	Highest mean condition	Lowest mean condition	Description
P10365	3	3	0.011657067	3.012	Intermediate	High	Actin.
P04442	4	2	0.011928285	1.581	Intermediate	Low	Hemoglobin alpha-D chain.
Q9P4C2	7	1	0.012101487	1.763	Intermediate	Low	Alcohol dehydrogenase II (EC 1.1.1.1).
P01617	2	2	0.012223837	2.319	High	Low	Ig kappa chain V-II region TEW.
Q9ZNT3	1	1	0.012614214	87.409	Low	High	Actin-depolymerizing factor 5 (ADF-5) (AtADF5).
P06267	1	1	0.012872182	2.268	High	Low	Light-independent protochlorophyllide reductase iron-sulfur-ATP-binding protein.
P20284	2	1	0.012912974	7.010	Intermediate	Low	30S ribosomal protein S19P (HmaS19) (HS18).
P00739	32	8	0.013284412	2.228	High	Intermediate	Haptoglobin-related protein precursor.
P27169	9	9	0.013389271	1.736	High	Intermediate	Serum paraoxonase/arylesterase 1 (EC 3.1.1.2) (EC 3.1.8.1).
P22352	4	4	0.014523229	2.117	Low	Intermediate	Plasma glutathione peroxidase precursor (EC 1.11.1.9)
P04220	15	1	0.015439708	3.003	High	Intermediate	Ig MU heavy chain disease protein (BOT).
P46755	4	3	0.017212861	1.522	Low	Intermediate	Mitochondrial ribosomal protein S4.
P01876	22	2	0.017265181	1.786	Intermediate	Low	Ig alpha-1 chain C region.
P41263	3	3	0.017476193	1.683	High	Low	Plasma retinol-binding protein precursor (PRBP).
P73836	2	2	0.017971459	1.709	Intermediate	Low	Hypothetical 10.7 kDa protein ssr3402 precursor.
Q03044	2	1	0.018080499	2.041	Low	Intermediate	Alpha-1-antiproteinase precursor (Alpha-1-antitrypsin)
P02788	8	2	0.020697749	2.304	Intermediate	Low	Lactotransferrin precursor (Lactoferrin) [Contains; Lactoferroxin A, B and C].
P07461	1	1	0.021366279	3.514	Intermediate	Low	Myosin regulatory light chain, smooth muscle.
P08835	9	1	0.021598078	6.494	Low	High	Serum albumin precursor (Fragment).
P05543	11	6	0.022442445	1.814	Low	Intermediate	Thyroxine-binding globulin precursor (T4-binding globulin).
P00915	2	1	0.023901829	4.762	High	Low	Carbonic anhydrase I (EC 4.2.1.1) (Carbonate dehydrogenase).
P43067	3	1	0.024431418	7.063	High	Low	Alcohol dehydrogenase 1 (EC 1.1.1.1) (40 kDa allergen).
P01610	2	1	0.024732175	8.178	Intermediate	Low	Ig kappa chain V-I region WEA.
Q04791	3	3	0.025244992	2.486	Intermediate	Low	Fatty acyl-CoA hydrolase precursor, medium chain (EC; 3.1.2.-).
P38792	2	1	0.026964975	1.808	Low	Intermediate	Exosome complex exonuclease RRP4 (EC 3.1.13.-) (Ri).
P28030	1	1	0.029020189	2.701	Low	High	Thermostable direct hemolysin precursor.
P50828	3	2	0.029509667	6.195	High	Low	Hemopexin precursor (Hyaluronidase) (EC 3.2.1.35).
P02023	22	2	0.029551727	1.848	High	Low	Hemoglobin beta chain.
P21380	8	1	0.03028008	1.989	Intermediate	Low	Hemoglobin beta chain.
P00488	9	9	0.030287679	1.597	High	Low	Coagulation factor XIII A chain precursor (EC 2.3.2.13).
P00331	6	2	0.030341899	2.771	High	Low	Alcohol dehydrogenase II (EC 1.1.1.1).
P02746	3	2	0.030684442	3.279	Low	Intermediate	Complement C1q subcomponent, B chain precursor.
P35217	2	1	0.032376447	21.464	High	Low	Carbonic anhydrase I (EC 4.2.1.1) (Carbonate dehydrogenase).
P01600	2	1	0.032645748	1.943	High	Intermediate	Ig kappa chain V-I region Hau.
P01594	2	1	0.03295854	4.125	High	Intermediate	Ig kappa chain V-I region AU.
P01024	40	38	0.033275096	1.552	High	Intermediate	Complement C3 precursor [Contains: C3a anaphylatoxin].
P09235	1	1	0.033829635	1.553	Low	Intermediate	Interferon alpha-9 precursor.
P00761	6	5	0.03447314	1.775	Low	Intermediate	Trypsin precursor (EC 3.4.21.4).
P01966	8	1	0.037099364	16.199	Intermediate	Low	Hemoglobin alpha chain.
P36957	1	1	0.037248952	3.939	Intermediate	High	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex.
P05611	1	1	0.03741116	2.102	High	Low	Ovomucoid (Fragment).
Q28932	7	1	0.038642909	2.839	High	Low	Hemoglobin beta chain.
P81398	4	4	0.039208009	1.737	High	Low	Rhodocetin beta subunit.
Q09886	1	1	0.039832596	2.635	High	Low	Hypothetical 13.7 kDa protein C584.12 in chromosome III.
O74258	4	2	0.040479378	1.844	Intermediate	High	Actin.
P07414	6	1	0.043441192	3.278	High	Low	Hemoglobin alpha chain.
P42538	4	2	0.044487833	1.810	Intermediate	Low	Hypothetical 37.2 kDa protein (ORF3).

gy and the potential targets of AML have been well studied thus far, the disease sometimes cannot be effectively treated using standard pharmacological treatment techniques.[13]

This current study was has attempted to identify novel proteins that could be used as biomarkers for diagnosis, prognosis and mode of treatment of AML. The discovery of potential biomarkers for AML was explored via proteomic profiling of samples from AML patients at diagnosis and at disease remission and with special focus on different categorization of AML including low, intermediate, and high-risk groups. This type of analysis might offer a more objective way to stratified AML patients into different risk categories based on their unique protein fingerprints. Our evaluation of protein patterns between BM and PB were very similar implying that molecular changes in BM of AML patients could be well studied using peripheral blood samples that offers less degree of invasiveness compared with BM biopsy and or aspiration.

Marked quantitative changes were observed when PB plasma at diagnosis and at remission from the same individual subjects was compared. Among the 21 identified differentially expressed proteins, three of the 21 proteins with expression difference of greater than 3-fold were further evaluated for their role in the progression and response to achieving remission of the malignant cell clones. One of them is Guanine nucleotide-binding protein G (k), α-subunit, which belongs to the family of G proteins. Some of the members of G proteins are known to function as intracellular molecular switches as well as their involvement in different signal transduction across the cell membrane.[14]

Of interest to the study is our observation of more than 5-fold difference in the expression of the Guanine nucleotide-binding protein G (k), α-subunit between samples at diagnosis and remission.

Another protein with significantly up-regulated between samples at diagnosis and remission period is Serum amyloid A protein precursor (SAA; containing both amyloid precursor proteins SAA1 and SAA2). Increase plasma concentrations of these proteins are associated with changes during the acute phase response. While the proteins have been described to produce in the liver by the hepatocytes, however, extra hepatic productions have also be reported.[15] Differential expression of serum amyloid A protein precursor has also been described also been reported in pediatric AML plasma samples.[16]

The fact that some of the identified proteins in this present study consisting of mainly samples from Adult AML patients might be indicative of similarity in the disease progression between pediatrics and adult AML patients.

Putting all these together signifies the potential roles of these molecules for prognostication of remission status of AML patients at diagnosis thus leading to the so-called personalized medicine.

In addition, it might be considered as a form of validation of potential usefulness of some of these proteins that shares some similarity in some of the differentially express proteins between adult and pediatrics AML subjects.

Different risk categories in AML including low-, intermediate-, and high-risk groups are being associated with probability of their success or failure to respond to therapy. We have identified some proteins that are significantly differentially expressed. In addition to quantitative changes in the levels of expression of some proteins, we have observed unique qualitative changes in four proteins that are either present in one of the three and absent in the other two, or in contrast, differentially expressed in two categories and absent in the third category.

Amphiregulin is a member of the epidermal growth factor family and with ability to promote the growth of normal epithelial cells as well its ability to restrains the growth of some malignant cells.[17] Recently, Amphiregulin has been implicated with association human ovarian cancer progression.[18,19] In this present study, we found Amphiregulin precursor (colorectum cell-derived growth factor) to be expressed in low risk AML samples and absent in both intermediate and high risk groups. While Amphiregulin role has not been extensively studied among hematological malignancy, its observed expression in this study is similar to changes in the plasma and bone marrow analyzed samples among low and high-risk pediatrics AML patients supporting its potential usefulness in risk stratification of both pediatrics as well as adult AML patients and

they may contribute to better our understanding of the adverse heterogeneity of the disease at protein level.

Conclusion

This study has demonstrated application of expression proteomics towards discovery of novel biomarkers for objective stratification of AML risk categories as well as potential new targets for signature-based therapies of AML and for monitoring disease outcome.

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