Amino Acids (2011) 40:943–951
DOI 10.1007/s00726-010-0718-9

ORIGINAL ARTICLE

Proteomic analysis of childhood de novo acute myeloid leukemia and myelodysplastic syndrome/AML: correlation to molecular and cytogenetic analyses

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Received: 27 May 2010 / Accepted: 3 August 2010 / Published online: 14 August 2010

Abstract The aim of this study was to investigate the progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML) and to provide additional data regarding the proteomic analysis of AML. The protein profiles obtained were correlated to cytogenetic and molecular analyses. Bone marrow (BM) and peripheral blood (PB) samples were obtained during MDS diagnosis, at MDS transformation to AML, at de novo AML diagnosis and 3 months following treatment. As controls, non-leukemic pediatric patients were studied. Cytogenetic and molecular analyses were carried out by G banding and polymerase chain reaction followed by sequencing, respectively. Differential proteomic analysis was performed by two-dimensional gel electrophoresis and protein identification by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. No significant correlations were noted between protein patterns and cytogenetic or molecular analyses. Certain suppressor genes, metabolic enzymes, immunoglobulins and actin-binding proteins were differentially expressed by BM or PB plasma and cell lysates compared to controls. The obtained data showed that vitamin D and gelsolin played contradicting roles in contributing and restraining leukemogenesis, while MOES, EZRI and AIFM1 could be considered as biomarkers for AML.

Keywords Childhood AML · MDS · Proteomics · Mass spectrometry · Two-dimensional gel electrophoresis

Introduction

Myelodysplastic syndromes (MDS) are a rare group of disorders, accounting for about 4% of childhood malignancies (Niemeyer 2009). They are characterized by clonal proliferation of hematopoietic cells, morphologic dysplasia, progressive cytopenias and increased risk of developing acute myeloid leukemia (AML) (Cazzola and Malcovati 2009; Kar et al. 2009). Childhood AML is a hematological malignancy, accounting for 15–20% of childhood leukemia that shows diverse genetic abnormalities and a high degree of heterogeneity in response to therapy.

Extended cytogenetic and molecular analyses have been used to stratify both MDS and AML. Monosomy 7 is considered as the most common cytogenetic abnormality in childhood MDS, seen in approximately 30% of cases and is associated with good prognosis (Niemeyer 2009). In AML, reciprocal chromosomal abnormalities such as t(15;17) or the inv(16) have been associated with frequently favorable clinical behavior (Thiede et al. 2006), whereas other

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0718-9) contains supplementary material, which is available to authorized users.

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cytogenetic aberrations indicate leukemia with intermediate or high risk of relapse. However, in a large proportion of both cases the genetic lesion is yet undefined.

Molecular analyses have yielded novel molecular markers, which are important for pertinent diagnostics of MDS and AML. For instance, in AML, internal tandem duplications (ITDs) of the fms-related tyrosine kinase 3 gene (*FLT3*) are considered predictive for increased risk of treatment failure after conventional chemotherapy and reduced overall survival. *FLT3* mutations in newly diagnosed MDS occur in about 3–5% of patients and yet have not been related to disease progression (Small, 2006). Conversely, *RAS* mutations are detected in MDS at a low frequency (Niemeyer 2009); however, they have been associated with a poor outcome in both MDS and AML (Bowen et al. 2005). Remarkably, MDS with *RAS* gene mutations tends to transform into leukemia (Sheng et al. 1997). A more encouraging outcome has been established in cytogenetically normal AML cases with mutations in the nucleophosmin gene (*NPM1*), whereas in MDS, *NPM1* mutations have been sporadically detected (Rau and Brown 2009).

Although progress has been made regarding the cytogenetic and molecular characterization of both myeloid malignancies, the mechanism of MDS transformation to AML (MDS/AML) as well as the heterogeneous nature of AML in response to therapy and subsequent risk of relapse remains to be elucidated. It seems likely that a favorable approach to screen for de novo AML and MDS/AML will be to determine the protein profile. Protein patterns could provide valuable information regarding leukemogenesis, effective diagnosis, prognosis and novel therapeutic targets, which will enable the development of targeted AML therapy.

To gain insight into those constraints, we attempted to detect changes in protein expression levels in the bone marrow (BM) and peripheral blood (PB) plasma and cell lysates of pediatric patients with MDS, MDS/AML or de novo AML and from samples 3 months following treatment. In a clinical practice, plasma and cell lysates are particularly promising for proteomic research to benefit prognosis and response to therapy. The purpose of this study was to verify and validate protein candidates relevant to the prediction of the mechanism of MDS progression to AML and to further characterize differentially expressed proteins between de novo AML and MDS/AML, to provide additional data regarding the identification of novel biomarkers related to the disease diagnosis, prognosis and personalized therapeutic treatment. Eventually, the obtained protein patterns were correlated to clinical and laboratory patients' characteristics, and cytogenetic and molecular analyses.

Materials and methods

Patients

Bone marrow and PB samples were analyzed from five pediatric patients (1–15 years) with AML diagnosed according to French–American–British (FAB) Cooperative Group criteria and immunophenotype. Of those, 3/5 were diagnosed with de novo AML and 2/5 with MDS/AML. The study population included two patients with M1, one patient with M2, one patient with M4 and one patient with M5b FAB subtypes. This study was approved by the Medical School of the University of Athens Ethics Board in Greece.

Sample collection and processing

Bone marrow and PB samples were collected from three de novo AML patients at diagnosis and from two patients with MDS before AML transformation, at MDS/AML diagnosis and 3 months following treatment. As controls, BM and PB samples from three non-leukemic pediatric patients were studied. The collected BM and PB samples were centrifuged at $1{,}800\times g$ for 10 min, to isolate BM and PB plasma, respectively. The BM and PB cell lysates were isolated by Ficoll–Hypaque density gradient centrifugation at $1{,}800\times g$ for 15 min. Proteins were extracted from cells using TRI Reagent (Ambion Ltd., London, UK) following the manufacturer's recommendations. Protein concentrations were determined by Bradford Reagent (Bio-Rad, Hercules, CA, USA). All samples were stored at −80°C until use.

Cytogenetic analysis

Cytogenetic investigations were performed by G-banding analysis in all patients at diagnosis. Additionally, interphase fluorescence in situ hybridization (iFISH) (Katsibardi et al. 2010) was used to monitor *TEL/AML1* fusion gene level t(12;21) (p12q22), *BCR/ABL* fusion gene t(9;22) (p34q11), *AML1/ETO* fusion gene t(8;21) (q22q22) and *MLL* gene rearrangement t(4;11) (q21q23).

Molecular analyses of *NPM1*, *FLT3* and *RAS* mutations

Genomic DNA was extracted from BM samples at AML diagnosis according to the standard phenol–chloroform protocol. Exon 12 of the *NPM1* gene was amplified by polymerase chain reaction (PCR). The primers and the procedure were adapted from Döhner et al. (2005). Mutational analyses of the *FLT3*/AL (activation loop) at positions D835/I836, *FLT3*/ITD and *RAS* genes (*NRAS*, *HRAS*

and *KRAS*) were performed as previously described (Braoudaki et al. 2008).

DNA sequencing

Direct sequencing of both strands of each PCR product was carried out on an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

Protein pre-fractionation

Two different commercially available protein partitioning products were used for analysis of low abundant proteins in BM and PB plasma samples derived from all five AML cases; however, depletion of high abundant proteins was not performed on BM and PB cell lysates. The ProteoMiner protein enrichment kit (Biorad, Hercules, CA, USA) was used to uncover low abundance proteins, whereas the Vivapure Anti-HSA kit was used for effective human albumin depletion (Sartorius Stedium Biotech, Gottingen, Germany), both according to the manufacturer's instructions. Protein concentrations were determined by Bradford Reagent (Bio-Rad, Hercules, CA, USA).

Two-dimensional electrophoresis (2DE)

In brief, 1 mg of total protein was loaded on immobilized pH gradient strips for 2DE. The first-dimensional separation was performed on an IPGphor isoelectric focusing system, whereas the second-dimension electrophoresis was carried out in 12% SDS–polyacrylamide gels using PROTEAN apparatus (Bio-Rad). The gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) and scanned in a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) using the PD-Quest v8.0 2DE analysis software. To increase sensitivity, all samples were run on several gels each with different pH range starting from 3 to 10 NL and following with smaller pH ranges, such as 4–7 L. Gel reproducibility was assessed by running duplicate gels of each protein extract for each pH range IPG strips. The procedure is described by Tsangaris et al. (2006).

Peptide mass fingerprinting by MALDI-TOF MS

All spots automatically detected by Melanie 4.02 software were excised by the Proteiner SPII (Bruker Daltonics, Bremen, Germany) and dried in a speed vacuum concentrator (MaxiDry Plus, Heto, Denmark). MS analyses were performed on a MALDI-MS MS in a time of flight mass spectrometer (Ultraflex II, Bruker Daltonics, Germany). The detailed procedure is described by Kolialexi et al. (2008).

Western blot analysis

Western blot analysis was performed as described by Anagnostopoulos et al. (2010). In brief, immunoblots were blocked in 5% w/v nonfat dry milk in Tris-buffered saline Tween 20 (TBST) solution for 1 h at room temperature. Next, the membranes were incubated overnight at 4°C with the appropriate dilution of the following primary antibodies (Santa-Cruz Biotechnology Inc. CA, USA) against moesin (sc58806). Following washing, the membranes were incubated with the corresponding anti-mouse HRP-conjugated secondary antibody (Santa-Cruz Biotechnology Inc. CA, USA). The membranes were washed with ECL West Pico (Pierce Biotechnology Inc., Rockford, USA) Detection System to visualize the protein bands. Western blots were scanned with a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and images were analyzed by Quantity One image processing software (Bio-Rad, Hercules, CA, USA). All experiments were carried out in triplicate.

Statistical data analysis

Mean densitometry values of all individual protein spots obtained from each group of samples (MDS, de novo AML, MDS/AML, 3 months following treatment, non-leukemic patients) were first checked for normal distribution by application of the Kolmogorov–Smirnov for test with the use of the StatPlus 2007 software (AnalystSoft, Vancouver, Canada). A two-pair t test assuming unequal variances was employed to analyze the protein data with normally distributed densitometric values, whereas the protein data without normally distributed densitometric values were compared for statistical significance by Mann–Whitney non-parametric test using the GraphPad Instat 3 software (GraphPad software Inc. La Jolla, CA). Statistical significance was defined as $p < 0.05$. For the Western blot experiments, the optical density means of the bands for each protein detected between the different sample leukemic groups and the controls were compared with two sample t tests assuming unequal variances. Statistical significance was defined as $p < 0.01$.

Results

Protein analyses

From each de novo AML patients ($n = 3$), 2 BM and PB plasma and 2 BM and PB cell lysates were electrophoresed. From the MDS/AML patients ($n = 2$), the following samples were analyzed: BM and PB plasma during MDS before AML transformation, BM and PB plasma and cell lysates from MDS/AML at diagnosis, and BM and PB

plasma samples obtained 3 months following treatment. All samples were run on gels with 3–10NL and 4–7L pH ranges. Figure 1 shows representative images of BM samples derived from MDS at diagnosis, MDS/AML and 3 months following treatment. In addition, BM and PB plasma samples obtained from de novo AML and MDS/AML patients at diagnosis were depleted of high abundant proteins using independently ProteoMiner and Vivapure Technologies (Fig. 2). Their low abundant protein content was analyzed by 2DE. All pre-fractionated protein samples were run on gels with 3–10 NL pH range. Thus, according to the described approach, the overall number of gels evaluated in the relevant groups was 80. Accordingly, a total of 36 samples were analyzed in the three non-leukemic patients (12 samples/patient), who served as controls.

Protein identification in BM plasma samples

A mean of 342 ± 17 spots per gel were compared between the BM plasma samples and the controls and, in total, 27 proteins were found to be differentially expressed. The expression level of those proteins was altered significantly. Among these, four proteins were down-regulated in BM samples derived from the MDS cases before transformation to AML as compared to the control. Furthermore, differential expression of 17 proteins was found from the BM samples at de novo AML and MDS/AML cases and 6 proteins were found up-regulated in the BM samples obtained 3 months following treatment.

The vast majority of proteins that were found differentially expressed in BM plasma samples compared to the BM controls were important acute phase proteins such as

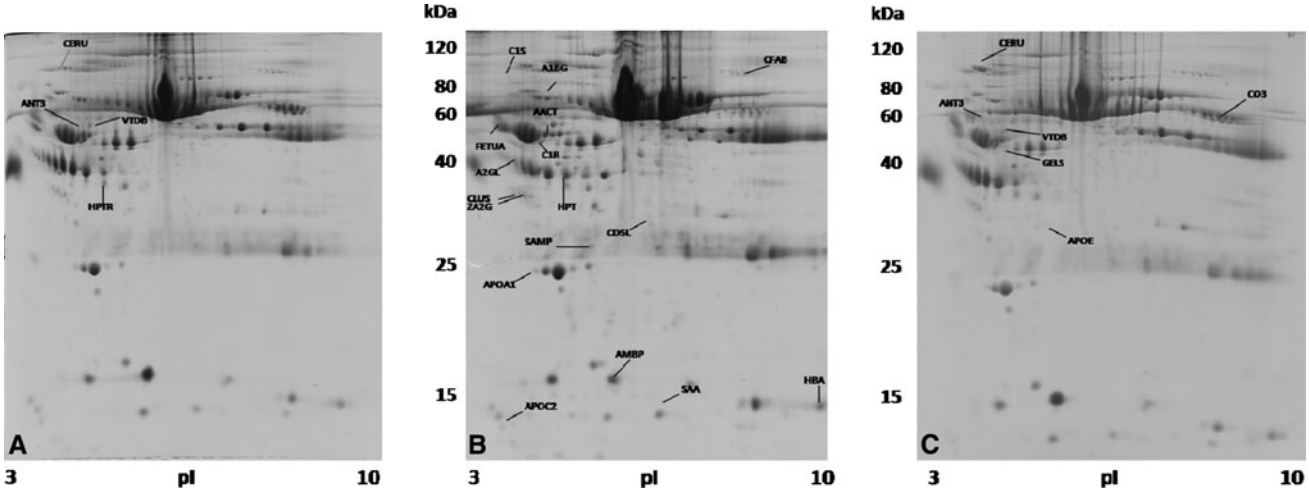

Fig. 1 Representative gel images of BM plasma derived from patients at MDS (**a**), at MDS/AML diagnosis (**b**) and from plasma samples 3 months following treatment (**c**). The differentially expressed spots are annotated and indicated by *arrows*

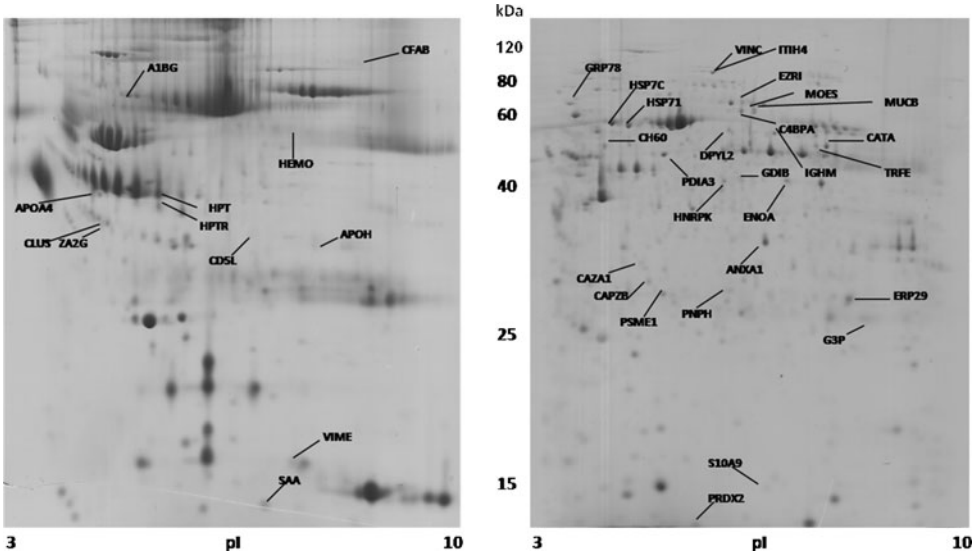

Fig. 2 Representative gel images of PB plasma samples derived from a patient with de novo AML at diagnosis, showing: **a** PB plasma sample before depletion and **b** PB plasma sample following depletion. The differentially expressed spots are annotated and indicated by *arrows*

ceruloplasmin (CERU), serum amyloid P component (SAMP), serum amyloid A precursor (SAA), complement C3 precursor (CO3), leucine-rich alpha-2-glycoprotein precursor (A2GL) and clusterin (CLUS). It is well known that acute phase protein levels fluctuate in response to inflammation.

Protein identification in PB plasma samples

Regarding the proteins extracted from PB plasma samples, 22 were found differentially expressed, from the 374 ± 26 spots per gel analyzed, between the PB samples as compared to the control. Among these, 4 proteins were significantly down-regulated in the PB plasma samples derived from the MDS cases before transformation to AML as compared to the control; 12 proteins were found to be differentially expressed from the PB samples for all AML cases; and 6 proteins were up-regulated from the PB plasma samples obtained 3 months following treatment. In general, functional analysis showed that the majority of the detected proteins in the PB samples were metabolic enzymes, structural proteins, signal transduction mediators and immunoglobulins.

Of note, vitamin D-binding protein precursor (VTDB), CERU and antithrombin-III (ANT3) were found to be significantly down-regulated in the BM and PB plasma samples derived from the MDS cases, whereas in the BM plasma samples obtained 3 months following treatment, all three were significantly up-regulated ($p < 0.05$). In addition, although a lack of gelsolin (GELS) was observed in all samples from de novo AML and MDS/AML cases at diagnoses, increased expression was detected in almost all BM and PB plasma samples obtained 3 months following treatment.

Protein identification in BM and PB cell lysates

Nineteen proteins were found to be differentially expressed from 965 ± 56 spots per gel analyzed, between BM cell lysates and controls, while 14 proteins from 950 ± 68 spots per gel tested were up-regulated in the PB cell lysates when compared with the controls. Interestingly, apoptosis inducing factor 1 (AIFM1) was up-regulated in both BM and PB cell lysates derived from 3/5 AML patients (2 de novo AML and 1 MDS/AML). All three patients were associated with a favorable prognosis. The majority of the remaining proteins identified were metabolic enzymes and heat shock proteins (HSPs).

Protein identification following depletion in BM and PB plasma samples

The pre-fractionated BM plasma protein samples from all AML patients ($n = 5$) at diagnosis revealed *N*-acetyl D glucosamine kinase (NAGK) and complement factor H-related protein 1 (FHR1) from the 418 ± 42 spots analyzed per gel, whereas from the pre-fractionated PB plasma samples of the same patients, 27 proteins were identified among the 436 ± 45 spots per gel analyzed. Among them were four HSPs: heat shock 70 kDa protein 1 (HSP70), heat shock cognate 71 kDa protein 1 (HSP71), 60 kDa heat shock protein (HSP60) and 78 kDa glucose regulated protein (HSP78). HSPs expression was significantly higher ($p < 0.05$) in the AML cases when compared to control groups. Increased levels of suppressor genes (annexin 10), metabolic enzymes (alpha-enolase, CATA, protein disulfide isomerase A3 and peroxiredoxin-2), immunoglobulins (Ig mu chain C region and Ig mu heavy chain disease protein) and actin-binding proteins (ezrin and moesin) were observed in all PB plasma pre-fractionated samples from AML cases at diagnosis. The elevated expression of moesin compared to control samples was confirmed by Western blot analysis (Fig. 3). Of note, the immunoblot signal intensities were in a linear range.

Protein identification and patient characteristics

The laboratory and clinical parameters of all patients were examined in relation to the protein profiles obtained. There was no correlation between the individual protein expression levels and laboratory or clinical characteristics such as white blood cell count (WBC), platelet count, blast percentage, age and sex. Among the five AML patients, two succumbed following viral infections ($n = 2$, one de novo and one MDS/AML), while the other three remained in complete remission.

Protein identification and cytogenetic analysis

In this study, chromosomal aberrations were observed in 3/5 cases. In 2/5 patients, the *AML1/ETO* fusion gene t(8;21)(q21;q22) was detected, which was principally found in the AML M2 and M1 FAB subtypes. *MLL* gene rearrangements with chromosome 11q23 abnormality were detected in 2/5 cases: one AML M4 and one M5b. There was no association between protein expression patterns in patients bearing cytogenetic abnormalities with those who did not.

Protein identification and molecular analysis

NPM1 gene mutations were detected in 2/5 patients with AML (both patients were de novo AML: one M1 AML and one M2 AML). No common *RAS* mutations were observed. Overall, *FLT3*/ITD mutations were found in 2/5 AML patients. Of these, one patient also had an *NPM1* mutation. All BM samples isolated from patients carrying *NPM1* and/or *FLT3*/ITD mutations had a similar expression pattern

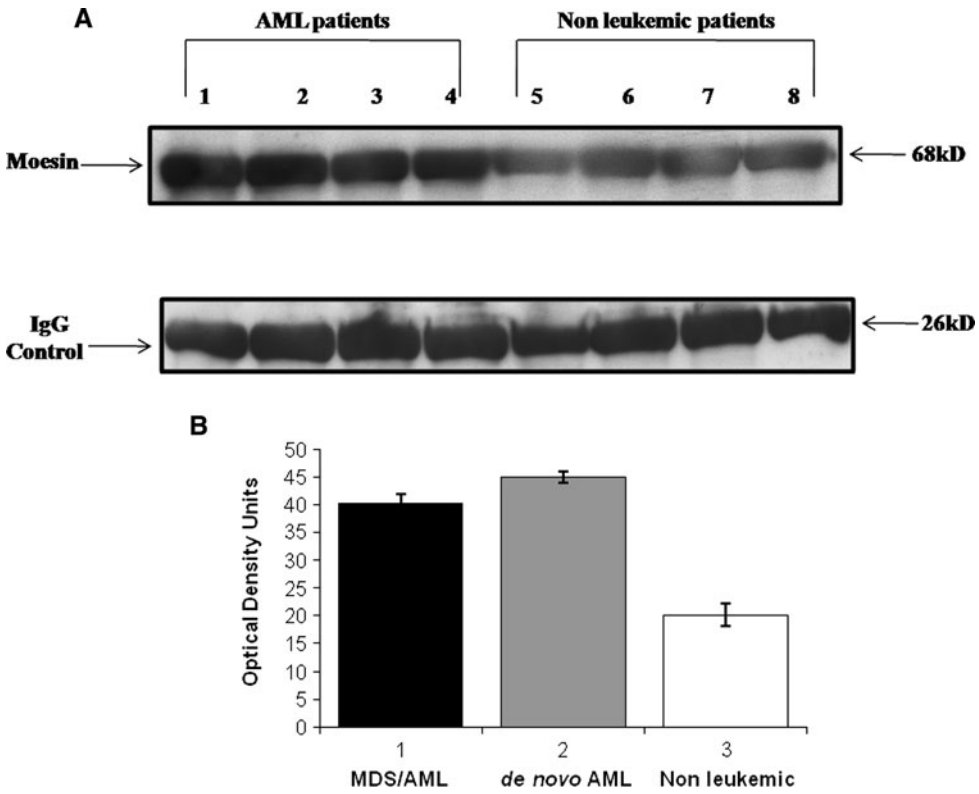

Fig. 3 **a** Western blot analysis of moesin expression in de novo AML patients (*Lanes 1* and *2*), MDS/AML patients (*Lanes 3* and *4*) and non-leukemic patients (*Lanes 5–8*). **b** Quantification of moesin content using scanning densitometry. Each *bar* represents the mean optical density ± SD of three independent experiments. Differences were significant at the level of $p < 0.01$

with the wild-type cases. No correlation was observed between the levels of protein expression in patients harboring any gene mutations with those who did not.

Discussion

In this setting, we aimed to characterize the proteomic mechanism underlying MDS progression to AML and provide additional data regarding the identification of potential protein biomarkers suitable for early AML diagnosis, prognosis and individualized therapy. At present, there have been comparatively several reports focusing on identifying protein biomarkers related to AML diagnostics and therapeutics (Emura et al. 2000; Smith et al. 2005; López-Pedrera et al. 2006; Sjøholt et al. 2006; Walters et al. 2006; Pekova et al. 2009) However, to our knowledge, this is the first attempt aiding to provide insights into the biology of MDS transformation to AML and the intermittent advancement to AML therapy.

In an attempt to ascertain a possible linkage between cytogenetic analysis and overt levels of protein expression, we examined whether there was a positive correlation between protein patterns in patients presenting with recurrent cytogenetic abnormalities and those bearing no cytogenetic aberrations. Our findings revealed a negative correlation. Regarding the *AML1/ETO* positive cases, the presence of AML1 and ETO proteins was not detected since both were considered to be low abundant proteins in cell lysates.

We endeavored to correlate protein signatures with the most common genetic abnormalities (*FLT3*, *NRAS*, *HRAS*, *KRAS* and *NPM1* mutations) associated with childhood AML. *RAS* gene mutations were not detected in any of the cases studied and thus a possible correlation with protein expression was not feasible. In contrast, *NPM1* and *FLT3* gene mutations were observed in 2/5 of the AML cases studied. The *NPM1* mutations were detected in cases with de novo AML. Few studies have reported the presence of *NPM1* mutations in patients with MDS (Zhang et al. 2007), while others showed that *NPM1* exon 12 mutations are absent in MDS (Shiseki et al. 2007). In BM from patients harboring *NPM1* mutations, the levels of protein expression were comparable to those in BM isolated from patients without *NPM1* gene mutations. A similar tendency was observed in patients bearing an activating *FLT3/*ITD mutation. More specifically, no discrete expression patterns in terms of *FLT3*/ITD gene mutations were detected. On the contrary, in studies conducted by Kornblau et al. (2009), distinct protein expression patterns for *FLT3*/ITD were identified.

Several proteins were found to be differentially expressed between BM plasma and controls. The majority of proteins was acute phase proteins derived from de novo AML and MDS/AML patients and from samples obtained 3 months following treatment. The most imperative proteins with

significantly higher levels of expression in BM samples from de novo AML and MDS/AML cases included clusterin, which has been implicated in various cell functions such as carcinogenesis and tumor progression, serum amyloid P protein, which plays an important role in the clearance of the apoptotic cells (Rithidech et al. 2007), and zinc-alpha-2 glycoprotein, which is considered to participate in tumor proliferation and has also been designated as a potential biomarker of different types of carcinomas (Hassan et al. 2008). The absence of all these proteins in MDS cases needs to be elucidated regarding the potential role of these proteins in AML transformation.

The protein patterns obtained from the analyses of AML plasma samples did not exhibit significant disparities between BM and PB specimens for most proteins with regard to the level of expression, molecular weight (MW) and isoelectric points (pI). More specifically, with regard to the molecular weight and/or isoelectric points, 14 proteins exerted differences. These included CERU, VTDB, ANT3, haptoglobin-related protein (HPTR), CLUS, apolipoprotein A1 (APOA1), alpha-1B glycoprotein (A1BG), alpha-2-HS glycoprotein (FETUA), AMBP protein (AMBP), GELS, complement C1r subcomponent (C1R), hemoglobin subunit alpha (HBA), complement C1s subcomponent (CO3) and vimentin (VIME).

The majority of proteins present in both BM and PB samples from MDS samples were found to be down-regulated. There was a significant decrease in levels of expression of three particular proteins including CERU, ANT3 and VTDB. Previous reports suggested that MDS was characterized by anemia associated with copper deficiency or other cytopenia refractory to treatment (Kumar et al. 2005; Fong et al. 2007). The mechanism of anemia in copper deficiency may be related to the decreased levels of CERU obtained in MDS cases in this study.

ANT3 is considered as the major inhibitor of blood coagulation. The occurrence of various coagulation abnormalities in patients with AML is well established (Pabinger-Fasching 1991; Dixit et al. 2006). In the current study, low levels of ANT3 were detected in MDS cases, whereas elevated expression was associated with samples obtained from patients 3 months following treatment. Dixit et al. (2006) has previously demonstrated low levels of ANT3 in AML patients at diagnosis, which arose to normal levels at the end of induction therapy. No reports were found regarding the role of ANT3 in MDS.

VTDB deficiency has been known to be associated with cancer progression. In the current study, VTDB deficiency was found in BM and PB plasma samples derived from MDS patients, whereas elevated levels of VTDB were detected in the BM plasma derived from the samples obtained 3 months following treatment from the same patients. This trend could prove crucial, since it obviously supports the contributing role of VTDB deficiency in leukemogenesis. Previous reports (Trump et al. 2010) also proposed a possible link between VTDB and pathogenesis, progression and cancer therapy.

GELS is a calcium-regulated actin-binding protein often associated with several pathological conditions including tumorigenesis. It has been also considered as a marker for poor outcome in some tumor types (Verrills et al. 2006). Though GELS was not detected in any of the de novo AML or MDS/AML samples, elevated levels of expression were observed in almost all samples obtained 3 months following treatment, suggesting that GELS might act as an AML suppressor protein.

The protein patterns obtained during the screening of cell lysates revealed significant consistencies between BM and PB specimens for most proteins. Notably, heat shock cognate 71 kDa protein (HSP7C) with MW 71 kDa was identified with MW 80 kDa in both BM and PB cell lysates, whereas catalase (CATA) with MW 59 and pI 7 was observed with MW 65 kDa and pI 5 in PB plasma cell lysates possibly due to post-translation modifications. Regarding the levels of expression, significantly elevated levels of AIFM1 protein were detected in both BM and PB cell lysates. All these cases remain in complete remission and involve both de novo AML and MDS/AML patients. This protein has not been found to be differentially expressed in any of the BM or PB plasma specimens we screened so far, possibly due to its selective expression in blast cells. In the present study, the elevated expression of AIFM1 protein was associated with favorable patient prognosis and thus it is likely that this protein could be employed as an AML-targeted therapeutic biomarker.

The human plasma is composed of approximately 50% of albumin and 20–25% of immunoglobulins. We used two different pre-fractionation tools to remove these high abundant proteins and subsequently increase the diversity of proteins present in plasma. Following protein pre-fractionation, various additional proteins were identified in the BM and PB plasma of the de novo AML and MDS/AML samples studied. More specifically, among all AML patients as compared to the control group, we identified six proteins with differences regarding their MW and pI, including 60 kDa heat shock protein (CH60), heat shock 70 kDa protein 1A (HSP71), F-actin-capping protein subunit beta (CAPZB), Ig mu chain C region (IGHM), Ig mu heavy chain disease protein (MUCB) and peroxiredoxin 2 (PRDX), as well as several differentially expressed proteins. We found that certain suppressor genes, metabolic enzymes, immunoglobulins and actin-binding proteins were significantly and consistently up-regulated. In particular, one suppressor gene, annexin A10, was found to be up-regulated in all AML samples. According to López-Pedrera et al. (2006), the function of annexin A10 remains

unclear; however, its down-regulation has been related to high-grade and high-stage tumors and early tumor recurrence.

Four metabolic enzymes were found to be up-regulated in the majority of the BM plasma de novo AML and MDS/AML samples analyzed following protein depletion. These included the alpha-enolase enzyme, which has been identified as a product of several types of tumors, the protein disulfide isomerase 3 and the antioxidant enzymes catalase, known to be involved in carcinogenesis and tumor progression and PRDX, which is not only involved in cellular proliferation, differentiation and apoptosis but also functions as a molecular chaperon during oxidative stress and as a tumor suppressor molecule (Petrak et al. 2008). Along with the metabolic enzymes, two major actin-binding proteins were also differentially expressed: moesin (MOES) and ezrin (EZRI). More specifically, compared to the control group, both proteins were up-regulated in the PB plasma AML diagnostic samples. These findings were also supported by observations revealed by using Western blot analysis. MOES and EZRI are members of the ezrin–radixin–moesin (ERM) family of proteins involved in the structural integrity of microvilli and in signal transduction to the actin cytoskeleton. The cytoskeleton of mammalian cells participates in several vital cellular activities, involving determining and altering shape, movement, cell division, cell-to-cell communication, cell anchorage and organization of the intracellular milieu (Trofatter et al. 1993). Therefore, any defect in the expression levels of MOES or EZRI could affect any of the above-described processes and have a subsequent effect on growth control. These data evidently suggest MOES and/or EZRI as possible markers for AML diagnosis.

In the present report, all four heat shock proteins (HSPs) (HSP60, HSP70, HSP71 and HSP78) that were up-regulated in the PB plasma protein-depleted samples as compared to control groups were consistent among the AMLs. HSP70 plays an important role in anti-tumor immune response, while its levels may reflect the severity of disease progression (Yeh et al. 2009). Elevated expression levels of HSP70 have been previously detected in AML and MDS cases (Thomas et al. 2005; Duval et al. 2006) directly linking its up-regulation to leukemogenesis (Ota et al. 2003). Additional studies have documented over-expression of HSP27, HSP60, HSP70, HSP90 and HSP110 in patients with poor MDS prognosis. Studying the expression of HSPs in blasts could provide key information in understanding MDS progression, since they are considered to play a prevailing role in apoptosis (Duval et al. 2006).

In summary, the current study was shown to provide significant insights into the growing role of several proteins in MDS progression to AML and to offer additional data for developing novel therapeutic targets and valuable biomarkers for stratifying patients with AML. Generally, there was good evidence that VTDB and GELS played contradicting roles in contributing and restraining leukemogenesis, respectively, and that MOES, EZRI and AIFM1 afford potentially useful biomarkers related to AML. Additional analyses including studies at the mRNA level are warranted to validate and uncover the role of our findings.

Acknowledgments The authors wish to thank Dr. Alexandra L. Perry for assistance with editing.

Conflict of interest None.

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