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LEADING ARTICLE

Proteomic analysis of childhood leukemia

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Childhood acute lymphoblastic and myeloid leukemias are stratified into molecular and cytogenetic subgroups important for prognosis and therapy. Studies have shown that gene expression profiles can discriminate between leukemia subtypes. Thus, proteome analysis similarly holds the potential for characterizing different subtypes of childhood leukemia. We used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to analyze cell lysates from childhood leukemia cell lines as well as pretreatment leukemic bone marrow derived from childhood leukemia cases. Comparison of the acute myeloid leukemia (AML) cell line, Kasumi, and the biphenotypic myelomonocytic cell line, MV4;11, with the acute lymphoblastic leukemia (ALL) cell lines, 697 and REH, revealed many differentially expressed proteins. In particular, one 8.3 kDa protein has been identified as a C-terminal truncated ubiquitin. Analysis of childhood leukemia bone marrow showed differentially expressed proteins between AML and ALL, including a similar peak at 8.3 kDa, as well as several proteins that differentiate between the ALL t(12;21) and hyperdiploid subtypes. These results demonstrate the potential for proteome analysis to distinguish between various forms of childhood leukemia. Future analyses are warranted to validate these findings and to investigate the role of the C-terminal truncated ubiquitin in the etiology of ALL.

Introduction

Leukemia is the most common form of cancer in children, representing approximately 25–35% of childhood cancers.[1] In the US, 2400 new cases of acute lymphoblastic leukemia (ALL) are diagnosed each year.[2] Leukemias are classified into different subtypes on the basis of their immunophenotypic and cytogenetic characteristics, and have variable prognoses and most likely differing etiologies. For example, ALL is more common in children than acute myeloid leukemia (AML) and mixed lineage leukemia (MLL).[3] MLL usually occurs very early in life and is characterized by translocations and other rearrangements involving chromosome band 11q23 where the *MLL* gene resides.[4] Typical translocations include t(4;11), t(6;11), and t(9;11). ALL can be divided into several subtypes by molecular and cytogenetic techniques with high hyperdiploid (greater than 50 chromosomes) being the most common, followed by cases harboring t(12;21) (*TEL-AML1*) and t(1;19) (*E2A-PBX*). AML

often contains translocations or inversions such as t(8;21) and inv(16).[5] While classification provides important information to the clinician, the process is expensive, time consuming, and requires trained cytogeneticists.

Recently, various groups have begun to apply cDNA microarrays in an attempt to subclassify childhood leukemias with some success. In particular, distinct patterns of gene expression have been shown to differentiate between ALL and AML subtypes.[6–9] Proteome analysis of childhood leukemia subtypes may yield similar results and further aide the diagnostic process,[10–12] as well as increase the precision of epidemiologic studies to detect associations between subtypes of leukemia with environmental exposures. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has successfully been used to analyze differentially expressed proteins in multiple studies of malignancies such as breast cancer[13–18] and to distinguish between rat prostate cancer cell lines of variable metastatic potential.[19]

Here, we have applied SELDI-TOF MS proteomic technology based on ProteinChip® Arrays from Ciphergen Biosystems to analyze the proteomes of four childhood leukemia cell lines (two ALL cell lines harboring t(12;21) and t(1;19), a MLL cell line harboring t(4;11), and an AML line harboring t(8;21)), as well as 20 childhood leukemia bone marrow samples of different subtypes. We hypothesized that array-based proteomics could detect differentially expressed proteins among different forms of childhood leukemia. Differences in protein expression are reported between the ALL and AML cell lines and bone marrow, as well as between the ALL t(12;21) and high hyperdiploid subtypes. These results suggest that proteome differences can further the understanding of the etiologies of leukemias and aid the diagnostic process.

Materials and methods

Cell culture

All cells were maintained at a density of 0.5×10^6 cells/ml at 37°C and 5% CO_2. Kasumi, 697 (German Collection of Microorganisms and Cell Cultures, Germany), and REH (American Type Culture Collection, Manassas, VA, USA) cells were cultured in 90% RPMI 1640 and 10% FBS while MV4;11 (American Type Culture Collection, Manassas, VA, USA) were cultured in 90% Isocove's modified Dulbecco's medium with 4 mM L-glutamine and 10% FBS. Cell lines were monitored for growth and maintained at logarithmic growth phase. Cells were clear of contamination and media was kept separate with cell lines handled individually to prevent cross contamination. Cells were harvested by centrifugation at 12 000 r.p.m. for 10 min and cell pellets were kept at −80°C until extraction.

Childhood leukemia bone marrow

Twenty pretreatment childhood leukemia bone marrow samples were chosen from the Northern California Childhood Leukemia Study (NCCLS) based on sample availability and diversity by leukemia subtype as follows: five ALL t(12;21), six ALL with high hyperdiploidy, two ALL and five AML with normal karyotypes, one AML inv(16), and one AML with a complex karyotype (47, XY, −9, +12, +Marker). The NCCLS is an ongoing population-based case–control study of childhood leukemia that investigates environmental and genetic risk factors. In collaboration with nine pediatric hospitals in Northern and Central California, the NCCLS collects pretreatment bone marrow specimens at the time of the diagnosis in about 78% of participating children (under age 15 years) diagnosed with leukemia.[20] Bone marrow was washed in PBS and bone marrow mononuclear cells were isolated with Ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA). A solution of 80% fetal bovine sera and 20% DMSO was used for storage in liquid nitrogen.

Preparation of whole cell extracts

Cells were thawed on ice, pelleted, and washed twice in $1 \times$ PBS. Pellets were then resuspended to $100 \mu l/10^6$ cells in lysis buffer containing 25 mM HEPES-KOH pH 7.4, 50 mM KCl, 4 mM $MgCl_2$, 1% Triton, and 1 mM phenylmethylsulphonyl fluoride. Cells were vortexed vigorously, followed by three freeze/thaw cycles. Samples were placed on ice for 30 min and spun at high speed ($\sim 16\,000\,g$) for 20 min at 4°C. Supernatants were aliquotted and stored at -80°C. Protein concentrations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA). Following sample titration, $4 \mu g$ was determined to be the optimal protein quantity for analysis.

ProteinChip Array preparation

Samples were analyzed in duplicate on ProteinChip Arrays: IMAC3-Cu (immobilized metal affinity capture), Q10 and SAX2 (strong anion exchange with and without hydrophobic barrier, respectively), and CM10 (weak cation exchange) (Ciphergen Biosystems, Fremont, CA, USA). Internal controls were included on each array and a coefficient of variance (CV) was calculated for internal controls as well as sample duplicates to ensure both interarray and intra-array reproducibility. A CV less than 30% was accepted.

IMAC3-Cu protocol

IMAC3-Cu arrays were charged with 100 mM copper sulfate ($CuSO_4$), rinsed with deionized (DI) H_2O, neutralized with 50 mM sodium acetate (NaOAc) (pH 4), and washed again with DI H_2O. Arrays were pre-equilibrated with PBS (pH 4) and $10 \mu l$ of sample previously diluted to $400 \mu g/ml$ in PBS (pH 4) was applied. Following incubation for 30 min, spots were washed three times with PBS and two times with DI H_2O. Sinapinic acid (SPA) prepared as a saturated solution in 50% acetonitrile (MeCN) and 0.5% trifluoroacetic acid (TFA) was added to each spot. Arrays were air dried and analyzed by the ProteinChip Reader (Series PBS II, Ciphergen Biosystems).

CM10, Q10, and SAX2 protocols

Barring the $CuSO_4$ application, the CM10, Q10, and SAX2 arrays were prepared using the same protocol with appropriate binding buffers. 100 mM ammonium acetate (pH 4) was used for CM10 arrays and 100 mM NaOAc (pH 8) was used for the Q10 and SAX2 arrays.

SELDI-TOF MS analysis

For each array type, the optimal data acquisition protocol was established and all arrays of the same chemistry were analyzed using the same protocol. The baseline was subtracted and spectra were normalized by total ion current. Peaks were manually labeled in the mass-to-charge (m/z) range of 1500–20 000. Raw data consisting of m/z and corresponding peak intensities were collected. Clusters of proteins with similar molecular weights were generated (cluster mass window of 0.3%) and the median intensity of clusters was compared using the Biomarker Wizard software (Ciphergen Biosystems). Statistical analysis of clusters was performed with this software using the Mann–Whitney and Kruskal–Wallis tests.

Protein purification

REH protein extract was fractionated using a Microcon YM30 membrane (Millipore, Billerica, MA, USA) and eluted with increasing concentrations of MeCN in 0.1% TFA. Fractions were further enriched using a Vivapure C18 microspin column (Vivascience, Germany) and eluted with 80% MeCN. The sample was run on a 12% SDS-PAGE gel and stained with Colloidal Blue (Invitrogen, Carlsbad, CA, USA). The bands containing the protein of interest were removed and one gel piece was passively eluted for molecular weight and purity confirmation. Five μl of solvent (50% formic acid, 25% MeCN, 15% isopropanol, 10% H_2O) were added to the gel piece, incubated for 2 h at room temperature, and 2 μl were applied to a NP20 array (Ciphergen Biosystems) followed by addition of $1 \mu l$ CHCA and analysis with a ProteinChip Reader.

Trypsin digestion

Coomassie stain and SDS were removed from the remaining gel pieces with methanol/acetic acid and acetonitrile/pH 8 ammonium bicarbonate solutions. The gel pieces were dehydrated using 100% MeCN and dried at 70°C in a SpeedVac. The dried gel pieces containing the 8.3 kDa marker were rehydrated with 15 μl of modified trypsin (Roche Applied Science, Indianapolis, IN, USA), resuspended at 2 ng/μl in 50 mM ammonium bicarbonate (pH 8), and incubated for 16 h at 37°C.

Protein identification

Single MS and MS/MS spectra of peptides resulting from trypsin digestion were acquired on a Q-Star tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Ciphergen PCI-1000 ProteinChip Interface. Of each protease digest $1 \mu l$ was spotted on a NP20 ProteinChip Array followed by $1 \mu l$ of saturated CHCA in 50% MeCN and 0.5% TFA. Spectra were collected from 1 to 3 kDa in single MS mode. Specific ions were selected and introduced into the collision cell for collision-induced dissociation (CID) fragmentation. The CID spectral data

was submitted to the database-mining tool Mascot (Matrix Sciences) for protein identification.

Results

SELDI-TOF MS analysis of leukemic cell lines

Comparison of spectra from the ALL, AML, and MLL cell lines revealed many differentially expressed proteins. Comparison of ALL with the AML and MLL cell lines revealed 17 proteins that were significantly differentially expressed ($P < 0.005$) using the SAX2 array, three of which exhibited at least a 20-fold greater expression in the ALL cell lines than in AML and MLL. One particular 8.3 kDa protein was highly expressed in REH and 697, with minimal expression observed in MV4;11 or Kasumi ($P < 0.005$) (Figure 1). The low expression of this protein peak in AML and MLL in addition to the low *P*-value and high peak intensity, indicating a high relative abundance of the protein, led us to choose it for identification. Analysis of the REH and 697 spectra showed three protein peaks that were differentially expressed, however, these peaks were of low intensities and were not ideally suited for identification.

Similar results were seen with the IMAC3 and CM10 arrays. On the IMAC3 arrays, six proteins were significantly different between REH and 697 ($P < 0.01$) and comparison of REH and 697 with Kasumi and MV4;11 showed significant differences in 18 protein peaks. There was a 10-fold difference in intensity of three of these peaks and one showed more than a 50-fold difference. While none of the peaks detected in the CM10 spectra differed significantly between REH and 697, 10 peaks showed significant differences upon comparison of AML and MLL with the ALL spectra ($P < 0.001$), four of which had greater than a five-fold difference.

SELDI-TOF MS bone marrow results

Q10 array analysis of the 20 childhood leukemia bone marrow samples revealed 40 significantly different protein peaks between the ALL and AML cases ($P < 0.01$). Interestingly, an 8.3 kDa protein peak with a two-fold greater expression in the ALL than AML samples was among these differences ($P < 0.005$) (Figure 2). Restricting the analysis to comparison of the ALL t(12;21) and high hyperdiploid cases with the normal AML cases resulted in 41 significantly different peaks ($P < 0.01$). Here we
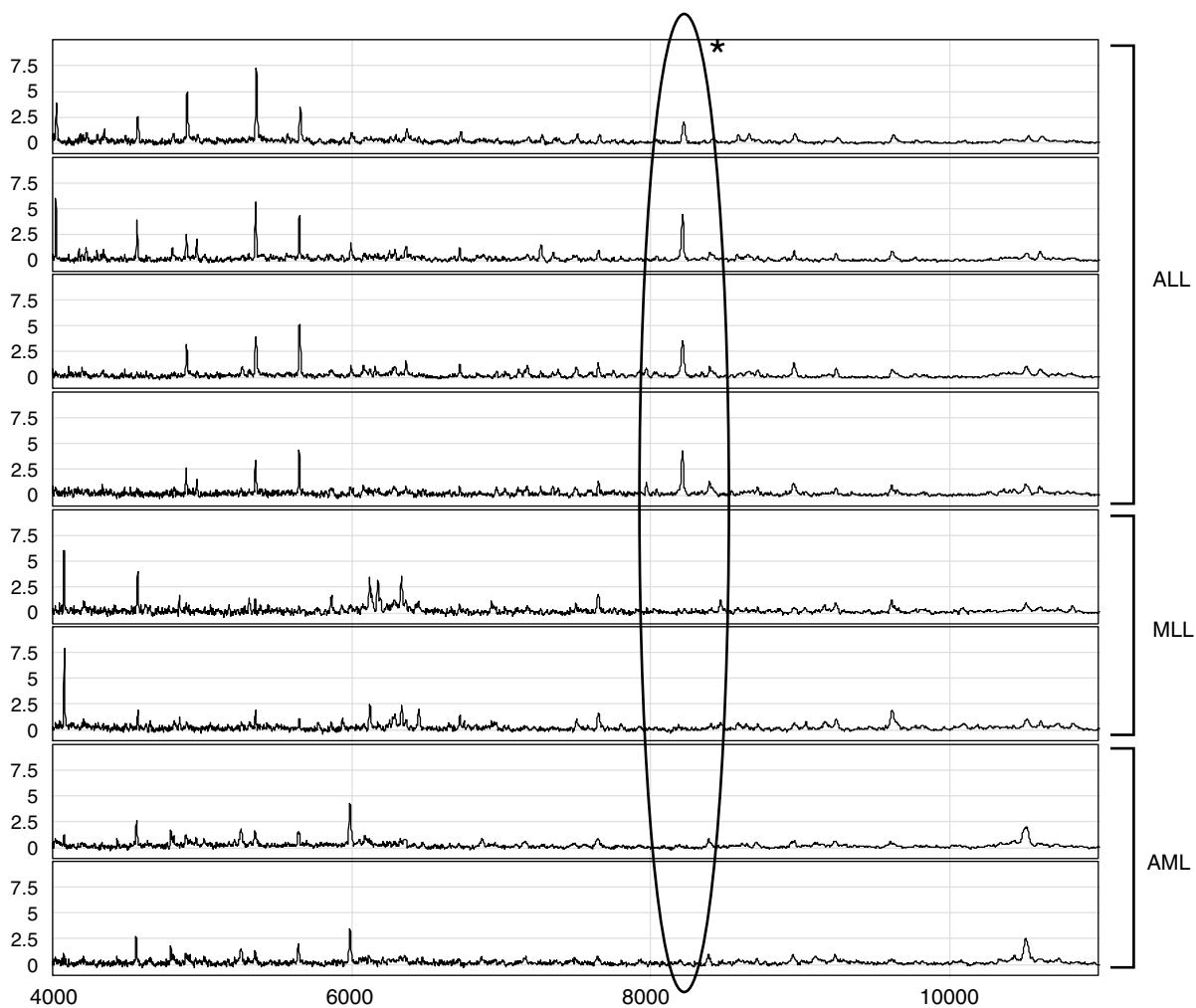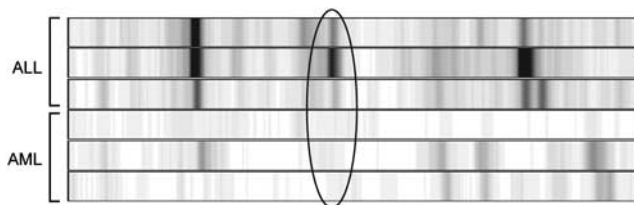

Figure 1   Spectra from SELDI-TOF MS analysis of REH, 697, MV4;11, and Kasumi cell lines. Protein (4 μg) from each cell type was analyzed on SAX2 ProteinChip® Arrays. ALL cell lines shown are REH and 697, the MLL cell line is MV4;11, and the AML cell line is Kasumi. The asterisk indicates the differentially expressed protein at 8.3 kDa.

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found a 3.5-fold increase in expression of the 8.3 kDa protein among the ALL compared to the AML cases ($P=0.0001$). Comparison of the ALL t(12;21) and high hyperdiploid cases revealed a number of significantly different peaks ($P<0.01$), some of which were present only in one of the subtypes (Supplementary Figure 1). Of these 12 peaks, a 9.75 kDa protein exhibited a three-fold higher expression in cases with high hyperdiploidy than t(12;21) ($P<0.005$) (Figure 3).

Protein identification of the 8.3 kDa protein

As mentioned previously, the 8.3 kDa protein in the cell lines on the SAX2 array was chosen for identification primarily because of its high expression in the ALL but not in the AML or MLL cell lines. Fractionation of the REH cell lysate using a Microcon YM30 membrane showed the highest concentration of the 8.3 kDa protein in the flow-through and 10% MeCN fractions (data not shown). Combination of these two fractions and application to the C18 column led to further enrichment of the 8.3 kDa protein as determined by ProteinChip® Array analysis (data not shown). One-dimensional SDS-PAGE analysis then revealed a single, clean band, but with an apparent molecular
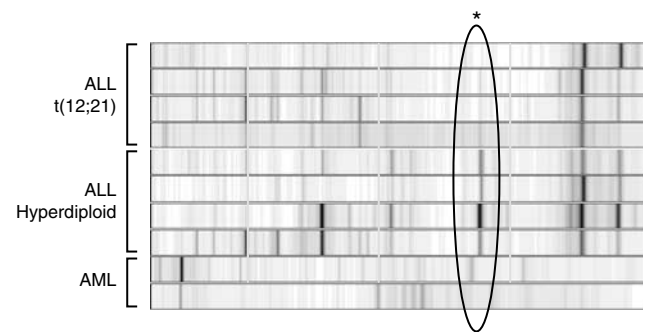
weight of 6 kDa, which is smaller than the expected weight of 8.3 kDa (Figure 4a). However, NP20 ProteinChip Array analysis of the band confirmed the purity and expected molecular weight of 8.3 kDa (Figure 4b). The appearance of the band at 6 kDa was most likely due to inherent properties of the protein that caused it to run faster than expected on the gel. Following trypsin digestion of the protein, three peptides with *m/z* values of 1039.5, 1067.6, and 1787.9 were detected by single MS in addition to trypsin autolysis peptides (Figure 5). Amino-acid sequencing of the three peptide fragments by MS/MS confirmed the same protein identity for all three peptides to be ubiquitin (Supplementary Figure 2).

Discussion

In the present study, we have identified in childhood ALL, AML, and MLL cell lines the differential expression of a C-terminal

Figure 2 Childhood leukemia bone marrow expression of an 8.3 kDa protein. Protein (4 µg) from childhood leukemia bone marrow was analyzed on a Q10 array. The asterisk indicates the differentially expressed protein at 8.3 kDa.

Figure 3 Differential protein expression of childhood ALL subtypes. Q10 analysis of bone marrow demonstrates differential expression of a 9.75 kDa protein between ALL t(12;21) and high hyperdiploidy subtypes. The asterisk indicates the 9.75 kDa protein.
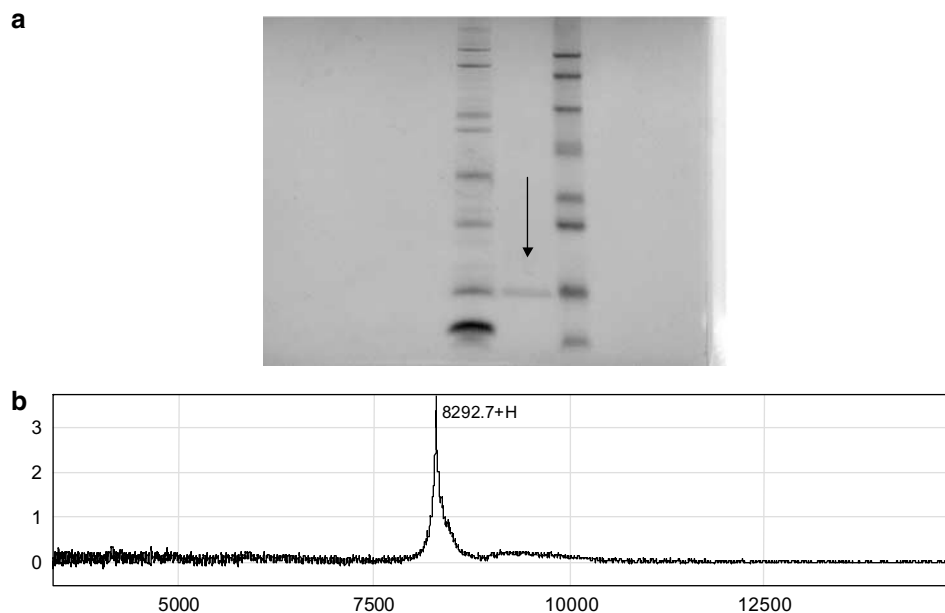
Figure 4 (a) Purification of the 8.3 kDa protein. The C18 fraction of the REH sample containing the 8.3 kDa protein was run on a 12% gel for final purification. The arrow indicates the band of interest at 6 kDa between two marker lanes. (b) SELDI-TOF MS confirmation of the 8.3 kDa protein. Following excision of the band of interest shown on the gel in (a) and passive elution as described in the Materials and methods, 2 µl of sample was analyzed by SELDI-TOF MS on a NP20 array to confirm the presence and molecular weight of the 8.3 kDa protein.
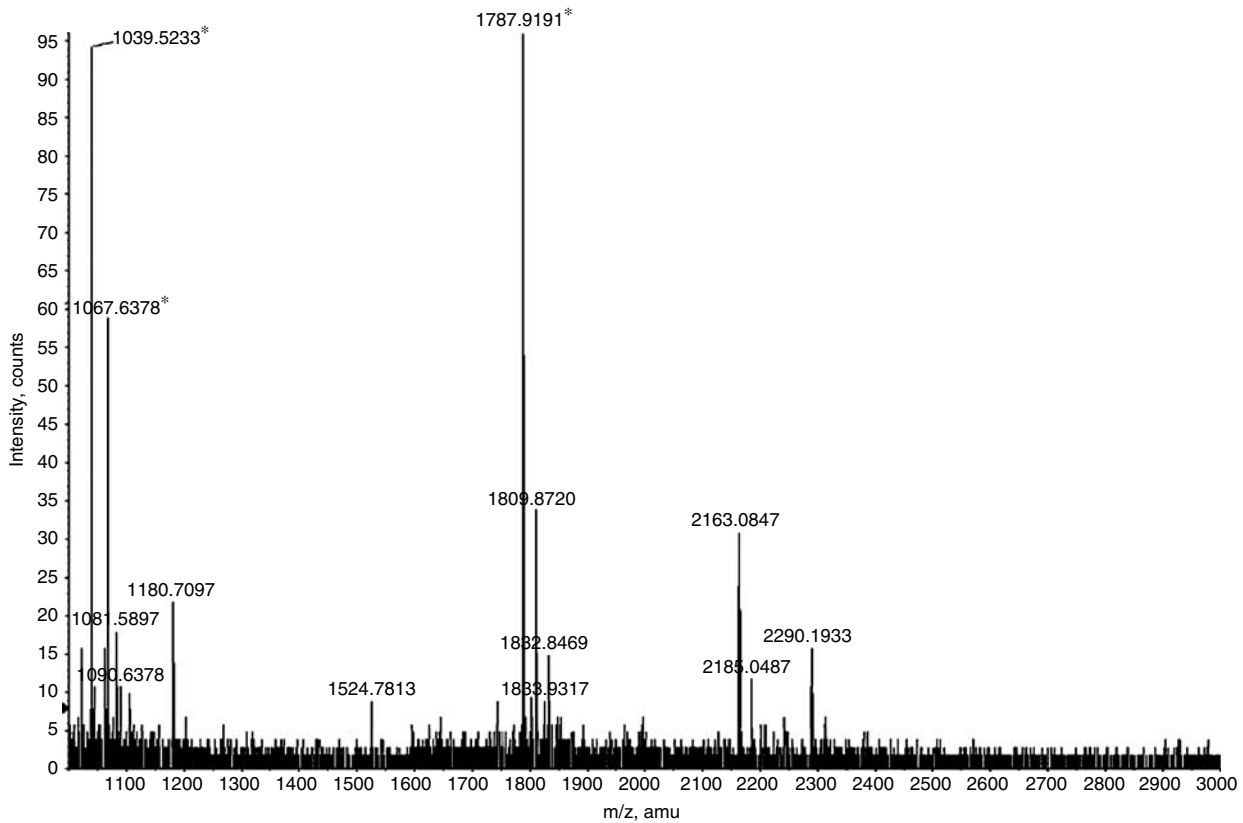
Figure 5 Trypsin digest of the 8.3 kDa protein. The purified 8.3 kDa protein was digested with trypsin yielding three peptides in addition to autolysis products of trypsin. The three peptides from the 8.3 kDa protein are indicated by asterisks at *m/z* values of 1039.5, 1067.6 and 1787.9.

truncated version of ubiquitin, a universal protein that is crucial to cellular processes. Consistent with these findings, greater expression of an 8.3 kDa protein also was observed in ALL bone marrow when compared with AML. This finding was even more pronounced when the analysis was restricted to the two most common childhood ALL subtypes, t(12;21) and high hyperdiploidy, compared with karyotypically normal AML. Although limited sample material prevented the identification of the differentially expressed protein in the bone marrow, the difference in expression is consistent with that reported in the cell lines and suggests that this finding may not be specific to cell lines.

Previous studies have demonstrated abnormally high levels of proteasome complexes in malignant hematopoietic cell lines of both lymphoid and myeloid origin, and also leukemic cells from bone marrow of ALL and AML patients.[21,22] While it has been established that ubiquitin gene expression is altered upon cell proliferation and differentiation, this is the first time differential expression of a truncated form of ubiquitin has been reported. Ubiquitin has a molecular weight of 8565 Da but the differentially expressed protein reported here is 8.3 kDa. Molecular weight calculation indicates that the best agreement of the calculated to observed value would be a three amino-acid truncation at the C-terminus (Supplementary Figure 3). Trypsin cleavage occurs at the C-terminal end of arginine or lysine, which would preclude it from generating the 73 amino-acid truncated ubiquitin protein observed, thus suggesting that the truncated ubiquitin is generated through biological processes. Covalent binding of the C-terminal glycine (Gly76) to a lysine residue on a substrate protein targets that protein for degradation by the UPP.[23,24] This process of protein degradation is crucial to

regulation of various cellular processes including cell cycle progression, cellular growth and proliferation, and DNA repair.[23,25] The importance of the C-terminus in conjugation of ubiquitin to target proteins implies that a truncated version may alter conjugation and possibly the UPP degradation of targeted proteins. This may have a variety of cellular consequences due to the diverse role of the UPP in regulation of cellular processes. While the exact biological implications of this observed difference in the C-terminal truncated version of ubiquitin and the role it plays in the etiology of leukemia are unknown, functional studies are currently underway.

This study also demonstrates the differences in protein expression among various childhood leukemias. While few differences were seen between spectra of the ALL cell lines, REH and 697, comparison of the ALL cells with the AML and MLL cell lines revealed several significantly different protein peaks on the SAX2, IMAC3, and CM10 arrays. The bone marrow results supported these findings with many differentially expressed proteins found between the ALL and AML cases. More importantly, analysis of the ALL subtypes, t(12;21) and high hyperdiploidy, indicates that there are differences in protein expression between these leukemias. Although heterogeneity in protein expression exists among individuals, both similarities and differences are apparent between the two ALL subtypes (Figure 3, Supplementary Figure 1). Specifically, proteins present exclusively in one subtype are evident (Supplementary Figure 1). Similarities among the ALL cases are expected due to a common lineage but differentially expressed proteins among the two ALL subtypes, such as the 9.75 kDa protein reported here, are crucial to understanding the etiologies of these leukemias. Such differences clearly indicate that characterization of childhood

leukemia proteomes can help distinguish between these two clinically diverse subtypes as well as lead to the identification of differentially expressed proteins useful for diagnostic, mechanistic, and etiologic studies aimed at improving therapy and understanding etiology. Although previously reported drawbacks of SELDI-TOF MS analysis include instrument drift, mass spectrometer calibration, and sample processing,[26,27] these obstacles were addressed in this study by analyzing all samples on the same instrument, daily instrument calibration, and standardized sample processing. An additional downside to SELDI-TOF MS is the inability to directly identify proteins such as the 9.75 kDa protein reported in this study. Protein identification requires additional sample material and techniques such as MS/MS. This may not be a problem in a study with ample biological material such as serum. However, as in the case of this study, limited sample material such as bone marrow may prove to be difficult. This obstacle may be overcome in future identification of the proteins reported here through identification of pooled samples.

In conclusion, the present study demonstrates the differential expression of a C-terminal truncated version of ubiquitin between childhood ALL, MLL, and AML cell lines as well as characterization of protein expression of these leukemias in both cell lines and bone marrow. These results demonstrate the potential of proteome differences to distinguish between various leukemias and lead to discovery of potential biomarkers and/or proteins involved in the development of leukemias. Further investigation is needed on the role of the C-terminal truncated ubiquitin in the etiology of leukemia and future analyses of additional childhood leukemia cases are needed to validate these findings.

Acknowledgements

Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu).

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