Recognition of Recurrent Protein Expression Patterns in Pediatric Acute Myeloid Leukemia Identified New Therapeutic Targets

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Abstract

Heterogeneity in the genetic landscape of pediatric acute myeloid leukemia (AML) makes personalized medicine challenging. As genetic events are mediated by the expression and function of proteins, recognition of recurrent protein patterns could enable classification of pediatric AML patients and could reveal crucial protein dependencies. This could help to rationally select combinations of therapeutic targets. To determine whether protein expression levels could be clustered into functionally relevant groups, custom reverse-phase protein arrays were performed on pediatric AML (n = 95) and CD34+ normal bone marrow (n = 10) clinical specimens using 194 validated antibodies. To analyze proteins in the context of other proteins, all proteins were assembled into 31 protein functional groups (PFG). For each PFG, an optimal number of protein clusters was defined that represented distinct transition states. Block clustering analysis revealed strong correlations between various protein clusters and identified the existence of 12 protein constellations stratifying patients into 8 protein signatures. Signatures were correlated with therapeutic outcome, as well as certain laboratory and demographic characteristics. Comparison of acute lymphoblastic leukemia specimens from the same array and AML pediatric patient specimens demonstrated disease-specific signatures, but also identified the existence of shared constellations, suggesting joint protein deregulation between the diseases.

Implication: Recognition of altered proteins in particular signatures suggests rational combinations of targets that could facilitate stratified targeted therapy. Mol Cancer Res; 16(8):1275–86. ©2018 AACR.

See related article by Hoff et al., p. 1263

Introduction

Pediatric acute myeloid leukemia (AML) is a heterogeneous disease and accounts for approximately 15% of childhood acute leukemia. Despite significant improvements in therapy over the past decades, survival rates remain at 65% to 70% (1–3). Moreover, 30% of the pediatric patients will relapse and prognosis after relapse is dismal. As further therapy intensification leads to increased toxicities, other treatment options are needed for those not responding to, or relapsing after, standard chemotherapy regimens. A better understanding of the molecular underpinnings will yield insights into the involvement of crucial protein regulatory pathways, providing opportunities to aid in the development of more targeted therapies. This strategy could allow the development of specific targeted therapies in response to changes in protein-regulatory pathways, furthering the goal of precision medicine.

Many genes have been identified that contribute to the pathogenesis of leukemia, but each is present in only a fraction of patients, and most patients have more than one driver mutation (4, 5). This leads to a complex interplay between genetic events in an infinite number of combinations, making application to individual patients extremely difficult. Notwithstanding, a recent study in 1,540 adult AML patients identified 5,234 driver mutations across 76 genes or genomic regions and enabled recognition of 11 subgroups based on patterns of genes that were frequently comutated (4). Inopportunistically, most genetic events are not targetable, which makes it challenging to use genomic classifications clinically. Hence, it is important to know what characterizes those combinations of recurrent genetic events and their effect on the leukemic cells, to enable correct therapeutic targeting.

Because upstream genetic and epigenetic events are predominately mediated by the expression and activation of proteins, we hypothesize that, despite molecular heterogeneity within leukemia, pediatric AML would coalesce into a limited number...
of recurrent protein expression patterns that could be used to identify therapeutic targets. Gene expression profiling (GEP) has already revealed recurrent patterns of mRNA expression that confer some prognostic information, but this has not been leveraged to direct individualized therapy. Measuring protein expression levels has two significant advantages over GEP: (i) it provides accurate knowledge of expression levels, while mRNA infrequently correlates with protein levels (6–12); and (ii) it could reveal knowledge of posttranslational modifications (PTM) and activation status, which GEP cannot. We hypothesized that analyzing proteins in a network-based fashion would identify important protein expression patterns that could characterize subpopulations of pediatric AML and identify targetable protein pathways.

Materials and Methods

Patient population

Bone marrow (n = 39) and peripheral blood (n = 53) samples were acquired on the basis of availability from 87 newly diagnosed, 7 relapsed, and 1 primary refractory pediatric AML patients that were evaluated at Texas Children’s Hospital (TXCH; Houston, TX; n = 56), University Medical Center Groningen (UMCG; Groningen, the Netherlands; n = 31), and the MD Anderson Cancer Center (MDACC; Houston, TX; n = 8) between 1990 and 2016. Samples were collected prior to therapy in accordance with protocols approved by the Institutional Review Board (IRB) of the participating institutions. Informed consent was obtained in accordance with the Declaration of Helsinki. Associated demographics are described in Table 1. Samples were analyzed according to the MDACC lab protocol Lab06-0654, approved by the MDACC IRB. Patients were treated under a variety of protocols as listed in Supplementary Table S1 and evaluated for outcome. Seventy-eight (82%) achieved complete remission (CR) and 33 (42%) subsequently relapsed. Thirty-one patients underwent stem cell transplantation from either a matched related donor (n = 9), matched unrelated donor (n = 14), mismatched unrelated donor (n = 1), a haploidentical donor (n = 3), double cord blood transplant (n = 2), or unknown (n = 2). Forty-six (48%) were still alive at the end of follow-up (82–716 weeks). Mutation analysis was restricted to routine diagnostics.

RPPA methodology

Proteomic profiling was performed by using the reverse-phase protein array (RPPA) methodology on 95 samples from pediatric AML patients, together with 73 samples from pediatric acute lymphoblastic leukemia (ALL) patients (57 pre-B ALL and 16 T-ALL). Patient demographics are presented in Supplementary Table S2. 10 cryopreserved normal bone marrow CD34+ (All-Cells), and 95 leukemic cell line samples. Cell lines were obtained from the ATCC and different laboratories and were tested for mycoplasma using the Mycoplasma PCR Detection Kit (Applied Biological Materials Inc. catalog no. G238). The methods and validation of the technique, as well as the processes used for antibody normalization are fully described in previous publications (13–15). Briefly, whole-cell lysate protein preparations were prepared from cryopreserved (MDACC; n = 3, TXCH; n = 22, UMCG; n = 31) or fresh (MDACC; n = 5, TXCH; n = 34) mononuclear cell fractions of ficolled bone marrow or peripheral blood samples. Samples with <80% blasts underwent CD3/19 depletion using magnetic bead separation (Miltenyi Biotec). Samples were normalized to a concentration of 1 × 10^6 cells/μL and printed in five serial (1:2) dilutions onto slides along with normalization and expression controls. Slides were probed with 194 primary antibodies and a secondary antibody to amplify the signal, and finally a stable dye to precipitate protein signal (16). All antibodies were strictly validated by performing Western blot analysis against a panel of standard cell lines. Antibodies were then tested on a RPPA with over a hundred cell lines, and a Pearson correlation coefficient between Western blot analysis and RPPA was calculated (14, 17). The panel of antibodies used in this study comprised antibodies against 149 different proteins in addition to 45 antibodies recognizing PTMs including 36 antibodies targeting phosphorylation sites, 6 targeting cleaved forms of caspases, NOTCH1 or PARP1, and 3 targeting Histone 3’ methylation sites. A “Rosetta Stone” table of manufacturer, antibody name, Pearson correlation coefficient for validation, and primary and secondary antibody dilution can be found in Supplementary Table S3. Stained slides were quantitated using Microvige software (Version 3.4, Vigne Tech).

Protein and antibody naming nomenclature

Because neither the HUGO (18), HUPO (19), or MiMi (20) naming systems account for PTM, we used the nomenclature in which the HUGO name is followed by a period, then the type of PTM, "p" for phosphorylated, "cl" for cleaved, or "Me" for methylation, followed by the letter code for the affected amino acid and its sequence position. For example, AKT1.pT308 is AKT1 phosphorylated on Threonine at position 308.

Table 1. Patient characteristics

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</tr>
<tr>
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</tr>
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<tr>
<td>Alive</td>
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<td>48</td>
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</table>

NOTE: Cytogenetic aberrations were classified as favorable, intermediate, and unfavorable cytogenetics. Favorable: inv(16), t(8;21), t(15;17) PML/RARA, hyperdiploid, mutated CEBPA; unfavorable: monosomy 7 and t(9;22) BCR/ABL1 translocation. Patients that were not classified as favorable or unfavorable were defined as having intermediate cytogenetics.
after the protein name enables alphabetical sorting and inclusion of the affected site.

Data processing and normalization

SuperCurve algorithms generated a single value of protein concentration from the five serial dilutions (21). Loading control (22) and topographical normalization (23) procedures were performed to account for protein concentration and background staining variations. Because all samples had replicates, the average expression level of the replicates was used as a single expression level. Row and column median subtraction were performed on all samples. All protein expression levels were normalized relative to the median of the normal CD34⁺ bone marrow samples.

Computational analysis

Overall computational analysis was done using the "meta-Galaxy" analysis as described by Hu and colleagues (unpublished data), which identified protein expression patterns in the context of related functional groups. As we thought that we would lose too much information by taking all protein information together and analyze all at once, we hypothesized that data analysis would be better informed if the proteins were first analyzed in the context of smaller functionally related groups, which we called a "Protein Functional Group" (PFG). To form those PFGs, the 194 antibodies were allocated into 31 PFGs based on their known functionality or pathway membership as described in the existing scientific literature and based on strong associations between a protein and protein members from a PFG. Because proteins have multiple functions and interact with many other proteins in the cell, proteins could belong to more than one PFG. The core protein members of each PFG are shown in Supplementary Table S4.

Next, we wanted to see whether we could identify patterns in the protein expression data of each PFG that allowed clustering of patients. Therefore, progeny clustering (a bootstrapping and stability based method for selecting the cluster number; ref. 24) in combination with k-means (for generating cluster memberships; ref. 25) was applied to each PFG and identified an optimal number of subsets of patients that expressed similar (correlated) expression of core protein members. Each subset of patients within a PFG was then defined as a "protein cluster." The measure of stability was based on a co-occurrence probability matrix that captured true and false classifications. For some PFGs, an alternative number of clusters was chosen or small clusters were merged into the closed group to make more biologically relevant clusters. Linear discriminant analysis (26) was applied to determine which of the identified protein clusters within each PFG was most similar to the protein expression levels of the normal CD34⁺ samples. This protein cluster was then set as protein cluster 1, meaning that protein cluster 1 was by convention the closest to the normal CD34⁺ cells. Principal component analysis (PCA; ref. 27) was used to visualize the distribution of the different clusters relative to the normal CD34⁺ bone marrow samples.

Figure 1.
The optimal number of protein clusters that was identified for each protein functional group. A, The optimal number of protein clusters defined for each 31 PFG is shown. Functional patterns were designated as "normal-like" (checkered pattern) or "leukemia-specific" (solid fill) based on colocalization, or geographical distinction in PCA plot compared with the normal bone marrow CD34⁺ control cells. B, Comparison of protein patterns found in the pediatric AML patient samples and the leukemic cell lines. Green ticks indicate that the protein cluster was mimicked by at least one of the cell lines. Red crosses show that none of the cell lines did express a similar protein pattern.

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Associations between protein clusters and clinical variables were assessed using the Fisher exact test for categorical variables and the Kruskal–Wallis test by ranks for continuous variables. Survival curves were generated using the Kaplan–Meier estimator.

Protein networks integrated protein associations obtained from the STRING (28) database (combined score > 0.9) and computationally reconstructed edges from RPPA data using graphical lasso (29) and StARS (for model selection based on stability; ref. 30).

To then build the bigger model in which we included all of the protein data together, protein clusters from each PFG were assembled into a large binary dataset that indicated the protein cluster membership for each patient: 1 if a patient was a member of that particular protein cluster, 0 if a patient was not. Block clustering (31) analysis was applied to the matrix to search for recurrent patterns between protein clusters from various PFGs. Protein clusters that tended to co-correlate strongly with each other were defined as a "protein constellation." Next, a group of patients with similar patterns of protein constellations was defined as a "protein signature." The optimal number of protein constellations that formed expression signatures was obtained by calculating the largest sum of the squared difference between the expected and observed values, divided by the expected value of each box (coordinate between a signature and a constellation). The expected value was defined as the sum of cluster membership within a constellation, divided by the proportion of patients in a given signature. Survival curves and clinical associations were generated similarly as for the PFG. Lists of proteins that were expressed at significantly higher or lower values relative to the normal CD34+ cells were generated for each constellation and for each signature using the Wilcoxon signed-rank test with an FDR-adjusted P value (P < 0.01). The 30 proteins that provided the most information to discriminate between the signatures were selected using random Forest (32). All the statistical tests and plots were generated in R (Version 0.99.484 – 2009–2015 RStudio, Inc.). Protein networks were generated in Cytoscape (Version 3.3.0; ref. 33).

Results

PFGs express "normal-like" and "leukemia-specific" protein patterns

To assess protein expression in relation to other proteins that are functionally related or interact with each other in similar signaling pathways, proteins were analyzed in the context of their PFG. First, progeny clustering was applied to each PFG to cluster patients into an optimal number of protein clusters based on similar (correlated) protein expression patterns of the core PFG components. The optimal number of protein clusters ranged from 3 to 6 clusters (Fig. 1A). The measure of cluster stability was based on a co-occurrence probability matrix that captured true and false classifications (Supplementary Fig. S1). Overall, clusters showed high stability and reproducibility with scores of 0.6 to 0.8. Using both bone marrow and peripheral blood samples combined did not influence the clustering analysis (see annotation bar above heatmap; Fig. 2A; Supplementary Fig. S2). PCA was performed to visualize the distribution of the relative protein expression for patients in their assigned protein cluster relative to that of normal CD34+ samples. All but three PFGs (cell cycle, protein kinase C, and transcription) had at least one protein cluster with similarities to the normal CD34+ samples (checkerboard pattern; Fig. 1A), defined as a protein expression pattern that overlapped with the median relative protein expression level was calculated for each protein cluster and overlaid onto the networks. Comparison of the networks between protein clusters recognized transition states that represented variation in relative expression and activation along with deactivation of different protein components. For instance, for the proteins involved in the "Hypoxia" PFG, we found an optimal number of 4 protein clusters (C1, C2, C3, and C4; Fig. 2A). By convention, protein cluster C1 was statistically determined as closest to normal and had most of the proteins expressed within the range of the normal CD34+ cells (relatively normal expression was shown in the range of cyan–green–yellow). This cluster showed overlap with most of the CD34+ samples in the PCA plot (Fig. 2C). As result, protein clusters C2, C4, and C3 were all defined as having "on"-states (expression shown in red), respectively showing higher cumulative expression for "VASP," "KDR," "VHL," and "HIF1A." Another example shows the PFG "CREB," in which we could identify 5...
Table 2. Demographics and laboratory features for the protein expression signatures

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<th>6</th>
<th>7</th>
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<td>13</td>
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<td></td>
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<td>50</td>
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**NOTE:** Significant patient characteristics and laboratory features are shown for the overall patient cohort as well as for each protein expression signature. Other nonsignificant variables that were evaluated, but which lacked association with any of the protein expression signatures included age at diagnosis, gender, cytogenetic prognostic risk groups, transplantation status, absolute blast count in the peripheral blood, number of monocytes in the bone marrow and peripheral blood, lactate dehydrogenase, albumin, creatinine, fibrinogen, and human leukocyte antigen-antigen D related.

distinct protein clusters (C1, C2, C3, C4, and C5; figures are published online, http://qutublab.rice.edu/pediatric-aml/creb/). PCA clearly demonstrated overlap between CD34+ samples and C1. Cluster C2, C3 showed a pattern consistent with lower expression of "BECN1" in C2 and CREB1.pS133 in C3. C5 showed lower expression of CREB1, CREB1.pS133, and BECN1, and higher expression of ATF3, whereas C4 expressed opposite levels of C4: high CREB1, CREB1.pS133, and BECN1, and lower expression of ATF3.

Protein constellations reveal recurrent pathway expression patterns

To test if we could identify protein clusters that co-occurred recurrently with each other, with the thought to find a more structured way to classify pediatric AML without losing valuable information by taking all the protein together weighted equally, as done by the unsupervised hierarchical clustering approach (Supplementary Fig. S3), the "meta-Galaxy" analysis was used. Therefore, the 136 protein clusters, which is the sum of all the identified protein clusters in the 31 PFG, were combined into a single binary matrix, which we called a "meta-Galaxy". As patients were always represented by one of the protein clusters in each PFG, in the end each patient was a member of 31 out of the 136 protein clusters.

Block clustering was performed to identify correlation within a group of protein clusters, which we defined as a "protein constellation" (horizontally in Fig. 3A). A group of patients that showed a similar pattern of protein constellation membership was defined as a "protein expression signature" (vertically in Fig. 3A). An optimization calculation was applied and determined the optimal number of 12 protein constellations and 8 protein signatures. This was determined by selecting the matrix where the sum of the squared difference between the expected and observed occurrence was maximal. The same constellation, signature 3 had an observed presence of 37% (284/760). Signature 4 showed an observed occurrence above expected (78%, 125/160), whereas signature 7 was below expected (9%, 13/152) \((P < 0.001)\). A list of the protein clusters in each constellation is shown in Supplementary Table S5.

Protein expression signatures correlate with clinical features and outcome

For this population of patients treated with chemotherapy regimens based on ADE (Cytarabine, Daunomycin, and Etoposide), survival rates ranged from 27% to 75% (Fig. 3B). Signatures showed no significant differences in survival when simultaneously compared as individual signatures. However, when signatures were combined into favorable (signature 3, and 6) intermediate (signature 2, 5, and 7) and unfavorable (signature 1, 4 and 8) groups, similar to what is done with cytogenetics, protein signatures were significantly prognostic \((P = 0.018)\). Disease free survival (DFS) was defined as the time post induction therapy that led to CR, without the occurrence of an event (relapse or death) and varied from 20% to 75% with an overall event rate of 64% \((N = 58/911)\) (Fig. 3C). High disease-free survival was observed in signature 3 \((N = 6/6, median NR)\) compared to signature 1 \((N = 3/8, median 39 weeks)\) \((P = 0.040)\).

In order to assess which other variables in our data set had influence on the estimated survival probability, and to demonstrate the impact of the protein information in addition to the conventional prognostic factors like cytogenetics, we performed a multivariate Cox Proportional Hazard analysis (Supplementary Table S6). This analysis resulted in a significant contribution for unfavorable protein signatures \((HR=1.89, P = 0.033)\) and for favorable cytogenetics \((HR=0.32, P = 0.0154)\), indicating a strong relationship between unfavorable protein signatures and a decrease in the survival probability.

Signatures were also correlated with patient features and laboratory variables (Table 2). Hispanic ethnicity \((overall N = 28/95, 29%)\) was high in signature 7 and 8 \((53%, 67%)\) and low in signature 4 and 5 \((5%, 8%)\) \((P = 0.002)\) (Fig. 3A, line 3 of annotations). 11q23 rearrangements \((overall N = 16/95, 17%)\) were overrepresented in signature 2 and 7 \((44%, 26%)\) and underrepresented in signature 4 and 6 \((5%, 0%)\) \((P = 0.027)\), and the complex karyotype was prominent in signature 8 \((N = 4/6, 67%)\) compared to the overall population \((N = 16/95, 17%)\). Moreover, signatures were associated with FAB classification \((P = 0.025)\) with M5 cases highly present in signature 5 and 8, and M7 present in signature 6, and with several laboratory features including white blood cell count, the percentage of bone marrow and peripheral blood blasts, hemoglobin and platelet count.
Figure 3.
"Meta-Galaxy" analysis identifies the existence of 12 protein constellations and 8 protein expression signatures. A, Block clustering applied on a binary matrix that indicates the protein cluster membership for each patient identified an optimal number of 12 protein constellations (horizontally); a group of protein clusters from various PFG. Patients that express similar combinations of protein constellations form 8 protein expression signatures (vertically). Each column represents a single patient, and each row represents a single protein cluster. Annotations denote patient characteristics, including: gender, age, ethnicity, and cytogenetic risk groups. Kaplan–Meier curves for overall survival (B) and disease-free survival (C) based on the 8 protein signatures are shown. Line colors for each signature match with the colored annotation bar on top of the "meta-Galaxy".
Because we observed similarities between some of the protein clusters and the normal CD34+ samples, we searched for constellations that were more or less frequently associated with the "normal-like" protein clusters compared with overall. Constellation 12 included 7 protein clusters (n = 7/10, 70.0%) that had sufficient overlap with the CD34+ samples and constellation 4 had 3 normal-like protein clusters (n = 3/7, 42.9%). Oppositely, constellation 8 (n = 1/12, 8.3%), 9 (n = 0/4, 0%), and 11 (n = 1/8, 12.5%), all had a much lower frequency of normal-like protein clusters compared with overall (n = 40/136, 29.4%).

Protein expression signatures and constellations are specific to ALL and AML

ALL and AML are thought to arise from different cells of origin and have different underlying pathophysiology, disease outcome, and treatment strategies. However, as both are acute hematologic malignancies involving deregulated growth, proliferation, and differentiation, we hypothesized that some overlap would exist in protein expression patterns. Although patient-specific protein patterns would help identify novel targets for a specific patient, similarities between the two diseases might identify crucial protein pathways that play a key role in the development or progression. As we had pediatric AML and ALL on the same RPPA array, this enabled direct comparison between their protein expression levels. Details on the 73 pediatric ALL samples can be found in the accompanying pediatric ALL manuscript and in Supplementary Table S2.

Pathway analysis on the combined AML and ALL samples was done similarly as done for the analysis restricted to the pediatric AML samples and resulted in a total of 142 protein clusters for the 31 PFG. Block clustering along with the optimization calculations suggested an optimal number of 12 protein constellations and 12 protein expression signatures (Fig. 4A). A list of the protein clusters represented in each constellation is showed in Supplementary Table S7. Most constellations were associated with a protein expression signature (Fig. 4A). A list of the protein clusters assigned to each constellation is showed in Supplementary Table S7. Most constellations were associated with a single disease, with constellation 1–4 only found in ALL and constellation 7–10 and 12 only found in AML. However, constellation 5, 6, and 11 showed overlap between ALL and AML. Clear separation was observed for T-ALL exclusive signatures 1 and 2, B-ALL–specific signatures 3–5, and AML-specific signatures 7–12. Only signature 6 contained a mixture of B-ALL and AML, as it was formed by 4 pediatric ALL patients and 4 pediatric AML patients. This signature was characterized by nearly unanimous adoption of the constellation 11 pattern, which was characterized by proteins from PFG “mTOR signaling,” “SMAD,” “ribosome,” and “HIPPO” (Fig. 4B). Patients that comprised this mixed signature 6 also fell within one particular signature when considered separately; all AML cases were found as part of signature 7 in the ALL “meta-Galaxy” (accompanying manuscript), and all of the AML cases fell within signature 3 of the AML “meta-Galaxy.” However, none of the clinical features or patient demographics was similar between the AML and ALL cases, emphasizing again that most protein patterns are not driven by genetic events.

Use of protein expression levels to select therapy

To use the “meta-Galaxy” approach in the process of finding combinational therapy that could be applied to a selected subgroup of patients that share similar protein expression patterns, proteins that were greatly up or down regulated in a given signature compared with the 10 healthy CD34+ samples were identified as potential drug targets (Figures available online, http://gutublab.nice.edu/pediatric-aml/global/). We propose that higher expressed proteins could act as targets for inhibition or deactivation, whereas lower expressed proteins could function as targets for replacement. However, this hypothesis first requires validation by in vivo experiments. Figure 5A shows an example of the proteins that were significantly changed in signature 4, one of the signatures with an unfavorable survival. Colors show the intensity of the protein expression. From here, combinations of proteins could rationally be selected and could then be targeted together, either alone, or in addition to standard therapy, in distinct classes of patients.

Next, we defined 19 "universals," proteins that were changed in the same direction in at least 6 of the 8 signatures (Fig. 5B). Those proteins were particularly involved in the PFG “Apoptosis Occurring,” “Cell Cycle,” “Differentiation,” “Transcription,” and “STAT.” Interestingly, GATA1 and STAT1 were universally lower expressed in both pediatric AML and adult AML patients (unpublished data), and RB1,pS807_R811, a phosphorylation event that deactivates the RB1 protein, showed universally opposite expression in children and adults, being predominantly unphosphorylated (active) in pediatric patients and highly phosphorylated (inactive) in adults (unpublished data). For pediatric AML and ALL samples, comparable expressions were seen for the higher expressed universals CASP7,c1198 and CDKN1B,pS10, and the lower expressed JUN,pS73 and GATA1 (accompanying manuscript).

Discriminative proteins could classify patients

Aiming to assign patients into one of the defined protein expression signatures using only a limited selection of proteins, random Forest selected the 30 most discriminative proteins (Supplementary Fig. S4). These proteins classified patients with an error rate of 23% (correct classifications n = 73/95); signature 1 (n = 9/11, 82%), signature 2 (n = 13/16, 81%), signature 4 (n = 18/20, 90%), and signature 7 (n = 16/19, 84%) enabled high prediction accuracy, whereas patients in signature 8 (n = 2/6, 33%) were much harder to predict. The 4 classified patients were assigned to signature 2 following this classification, which had nearly similar protein expression patterns.

Cell lines only partially mimic protein clusters

As cell lines are one of the most extensively used resources to perform validation experiments and to reveal the underlying pathophysiology of leukemia, we thought that it would be useful to examine protein expression levels in commonly used leukemic cell lines, and to compare those with the patterns that were found in our cohort of pediatric AML patients. Therefore, a separate RPPA was performed on 95 leukemic cell lines probed with 235 antibodies of which 163 antibodies overlapped with the pediatric array (n = 163/194, 84.0%). Cell lines were collected on the basis of their availability and represented a selection of the most frequently used cell lines (e.g., Kasumi-1, HL-60, Molm13/14, OCIAML3, Jurkat, and REH). To allow comparison between the cell line array and the pediatric acute leukemia array, the healthy CD34+ samples that were printed on both arrays were aligned.

Overall, unsupervised hierarchical clustering and PCA showed clear skewing in protein levels measured in the cell lines and pediatric samples (Supplementary Fig. S5). Only one patient mixed with the cell lines; the rest of the cell line samples strongly clustered among each other. When comparing cell lines with patient samples on the level of protein clusters, for only 80 of...
Figure 4. “Meta-Galaxy” analysis enables clear separation between the pediatric ALL and pediatric AML patient samples. A, Block clustering applied to the binary matrix that indicate protein cluster membership for the pediatric ALL and pediatric AML patient samples combined, identified the existence of 12 protein constellations (horizontally) and 12 protein expression signatures (vertically). Annotations suggest clear separation in protein patterns for T-ALL (magenta; signature 1 and 2), B-ALL (yellow; signature 3, 4, and 5), and AML (blue; signature 7, 8, 9, 10, 11, and 12) and show patients characteristics, including: gender, age, ethnicity, and cytogenetic risk groups. B, Summary expression plot of constellation 11 that characterizes patients that fall into the mixed signature 6 (light green). Significantly upregulated proteins compared with the normal CD34+ samples are shown in red and significantly downregulated proteins compared with the normal CD34+ samples are shown in blue.
that were universally changed in the same direction in 6 of the 8 signatures were determined as "universal." Colors show the relative median expression of each protein within that signature and ranged from the highest (maroon) to the lowest (dark blue) expression. Nonsignificantly different proteins compared with normal CD34+ samples are shown in white (blank).

The 136 proteins cluster (58.8%), a minimum of one cell line analogous was found that had a comparable expression pattern. None of the protein constellations or signatures was found. An overview of protein clusters that are expressed in the cell lines is shown in Fig. 1B and Supplementary Table S8.

Pediatric leukemia web portal

In addition to the figures that are shown for the "Hypoxia" PFG in this article, results from each PFG analysis could be assessed online at http://qutublab.rice.edu/pediatric-aml.

Discussion

The extreme degree of genetic heterogeneity within pediatric AML makes therapy tailored to small subgroups of patients difficult, and most of the mutations do not currently have a drug that specifically targets the mutated form. We hypothesized that pediatric AML could be classified on the basis of recurrent patterns of protein expression and activation, and that recognition of key components within crucial protein pathways could help in targeted therapy guidance.

In this study, we used the two-layer "meta-Galaxy" approach that defined signatures by first looking at protein expression patterns within a PFG and from there built higher order structures by performing hierarchical clustering. In contrast to the traditional unsupervised clustering approaches that directly cluster patients and proteins by weighting all proteins equally, this approach takes known relationships into account, and from there clusters proteins and patients that were using the importance that the progeny clustering gave them.

Because most potential drugs target proteins and current classification systems based on recurrent genetic events have not yet led to an improvement in outcome for pediatric AML, we further hypothesized that a protein classification system could aid in providing therapeutic guidance by recognition of crucial protein pathways that could be targeted by known therapeutics. Thereby, we propose that combinations of higher expressed proteins could be inhibited with small-molecule inhibitors, and that lower expressed proteins could be reactivated or replaced to restore their expression. Recently, we demonstrated in adult AML that subgroups of patients with high levels of MDM2, in association with low MDM4 and low TP53 total and phosphorylation levels, were the most sensitive to MDM2 inhibitions, such as Nutlin (34). A combination to test in our patient population could be to combine inhibition of MTOR and CDKN1B, a protein that in its phosphorylated form promotes cell-cycle progression (35). Those two proteins were overexpressed together in signature 4. Another potential target to replace or to activate would be GATA1, a transcription factor important for the development and maturation of the hematopoietic stem cell (36). This protein was universally repressed in all patient signatures and was also universally low in pediatric ALL. However, as most cell lines do not recapitulate most of the protein clusters seen in patients, the use of cell lines in the validation process should be guarded.

If targeting patients based on specific changes in their protein expression levels does predict targets, then rapid determination of protein expression of patients, preferentially prior to the initiation of consolidation therapy would be required to use this in the selection of therapy. To make this possible, we have defined a minimal set of 30 proteins that could potentially classify patients into signatures with an accuracy of 76.8%. Development of an ELISA or forward-phase protein array of this limited set, once confirmed for a larger dataset, could then provide results within one day and thereby serve as an important step in therapy selection.

Simultaneous analysis of pediatric ALL and AML showed signatures that were almost exclusively either ALL or AML, confirming that ALL and AML are indeed the result of different underlying biology, not only on a genetic basis, but also on the level of protein expression and activation. However, overlapping utilization of some constellations was observed, suggesting that some protein constellations are shared between the diseases. These constellations had protein expression patterns that...
suggested increases in mTOR signaling and SMAD proteins that can mediate increases in growth and proliferation, while simultaneously, induction of components of apoptosis suggested that these cells were under increased apoptotic stress. This suggests metabolic pathways that are deregulated in common between ALL and AML and suggests that some therapeutics targeting these proteins could be effective in these otherwise disparate diseases.

A limitation of the study was the restricted number of patient samples and antibodies used, limiting the number and depth of pathway analysis. Although AML in children is much less common than pediatric ALL and AML in adults, the use of only 95 patient samples restricted the number of observed signatures and therefore reduced the precision of classification and limited the ability to find robust correlations with disease outcome. Moreover, as AML is thought to arise from leukemic stem-like cells (LSC; CD34+/CD38−) that are known to express proteins differentially compared with bulk leukemic cells (37), an even more representable classification should be reasonable if we would use LSCs instead of bulk (CD3−CD19+) leukemia cells. We have already collected AML samples from a large cooperative clinical trial to validate our findings in a larger cohort and to compare proteomics in different cell populations. These prospectively collected AML samples collected from a Children’s Oncology Group cooperative group trial have available genetic mutational pro
time in pediatric AML that could distinguish subgroups of patients by using similar characteristics based on their proteomics. Recognition of potential proteins targets within signatures now needs to be validated. In the end, this approach should be applied to different AML age subsets and to different cell populations to provide superior insights in leukemic drivers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Recognition of Recurrent Protein Expression Patterns in Pediatric Acute Myeloid Leukemia Identified New Therapeutic Targets

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