

Hematological Malignancy

Acute Leukemia Subclassification: A Marker Protein Expression Perspective

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Improved leukemia classification and tailoring of therapy have greatly improved patient outcome particularly for children with acute leukemia (AL). Using immunophenotyping, molecular genetics and cytogenetics the low hanging fruits of biomedical research have been successfully incorporated in routine diagnosis of leukemia subclasses. Future improvements in the classification and understanding of leukemia biology will very likely be more slow and laborious.

Recently, gene expression profiling has provided a framework for the global molecular analysis of hematological cancers, and high throughput proteomic analysis of leukemia samples is on the way. Here we consider classification of acute leukemia samples by flow cytometry using the marker proteins of immunophenotyping as a component of the proteome. Marker protein expressions are converted into quantitative expression values and subjected to computational analysis. Quantitative multivariate analysis from panels of marker proteins has demonstrated that marker protein expression profiles can distinguish MLLre from non-MLLre ALL cases and also allow to specifically distinguish MLL/AF4 cases. Potentially, these quantitative expression analyses can be used in clinical diagnosis. Immunophenotypic data collection using flow cytometry is a fast and relatively easily accessible technology that has already been implemented in most centers for leukemia diagnosis and the translation into quantitative expression data sets is a matter of flow cytometer settings and output calibration. However, before application in clinical diagnostics can occur it is crucial that quantitative immunophenotypic data set analysis is validated in independent experiments and in large data sets.

Keywords: ALL; Quantitative flow cytometry; Marker proteins; Immunophenotyping

INTRODUCTION

The outcome for children with acute leukemia (AL) has improved remarkably over the past three decades. Current therapies achieve an event free survival of more than 75% for paediatric acute lymphoblastic leukemia (ALL) patients. But a significant challenge remains and it is believed that refined subclassification of leukemia and the identification of prognostic determinants will guide precisely tailored therapy and improve patients' outcome.

At present the diagnosis of patients with AL includes a series of clinical features and a combination of laboratory methods to classify the bone marrow and peripheral blood (BM/PB) sample of patients. The biological classification of AL samples has merged from a morphology-based system towards a molecular diagnostic system that involves the recognition of the cell lineage of origin and the presence of somatically acquired genetic abnormalities. The recognition of the cell lineage of origin is aided by immunologic surface and cytoplasmic markers and is found to be an important application using flow cytometry. Today immunophenotyping using flow cytometry is one of the fastest and most widely applied diagnostic methods in leukemia diagnosis and minimal residual disease evaluation. Much progress in the immunophenotyping of AL was made, thanks to the production of a palette of distinct fluorochromes and monoclonal antibodies (mAbs) to

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a variety of antigens expressed by normal and neoplastic hematopoietic cells and instrumentation that allows the simultaneous measurements of several cellular antigen expressions, cell size and cytoplasmic complexity (see for a recent review Schabath *et al.* [1] and several reviews herein). Nationwide and international cross laboratory validation studies [2–6] have arrived to the general consensus that multiparameter flow cytometry is a powerful diagnostic tool for the characterization of ALs and that it can be used to define subclasses that correlate with prognosis. Classifications include immunophenotypic subgroups of precursor B-, T-cell ALL, and immature acute myeloid leukemia (AML) [2,4–6]. Expression of empirically discovered single antigens or composite immunophenotypic patterns are used for this classification and have resulted in diagnostic screening of AL patient samples with panels of antibodies. More recently several efforts have been made to identify immunophenotypic characteristics of the major genotypic leukemia categories (see for a complete review Hrusak and Porwit-MacDonald [7]). In fact, recurrent genetic aberrations in AL samples are recognized to have important prognostic value (reviewed by Carroll *et al.* [8]).

HIGH THROUGHPUT LEUKEMIA PROFILING

The complete sequencing of the human genome and advancement in technologies, specifically the introduction in basic and applied research of microarrays, has engaged a number of investigators in applying these technologies to clinical leukemia samples. With the aim to improve diagnosis and risk classification and to understand the biology of leukemic cells, gene expression patterns (transcriptome) of patient samples are explored for the identification of leukemia subtype-specific expression signatures.

In a pivotal study Golub *et al.* [9] demonstrated the feasibility of using microarrays to distinguish AML and ALL samples on the basis of differential gene expression signatures and showed that using a set of genes as class predictors, unselected AL samples can be classified. In two large-scale microarray studies [10,11] it was shown that, using supervised learning algorithms, AL cases cluster according to recurrent cytogenetic aberrations associated with pediatric ALL: T-ALL, E2A-PBX1, BCR-ABL, TEL/AML1, MLL rearranged and hyperdiploid subtypes findings demonstrate that these cytogenetic aberrations define specific biological groups of ALL. But when unsupervised learning approaches were applied, a novel ALL subtype was discovered that lacks any of the above or other genetic aberrations but had a common expression profile [10]. Indeed one of the important perspectives of the application of high throughput technologies is the potency of class

discovery and identification of intrinsic biological clusters of AL [8].

To analyze large data sets of gene expression profiles two main approaches are employed. An unsupervised learning method uses pattern recognition algorithms and aggregates samples into groups based on an overall similarity of gene expression profiles without prior knowledge of specific relationship. The identified groups can then be verified for clinical relevance or concordance with known subclasses. In contrast, supervised learning technique group patient samples in classification categories based on known differences such as common translocations, or clinical categories and compare expression profiles of the defined groups.

Armstrong *et al.* [12] used expression microarrays for class discovery and proposed that mixed-lineage leukemia (MLL) including patients with leukemias involving MLL rearrangements form a distinct clinical entity from ALL and AML. Recently, Tsutsumi *et al.* [13] analyzed in a retrospective study that a large cohort of MLL patients using unsupervised learning algorithm could distinguish two groups of patients with distinct gene expression profiles. Importantly, the two subgroups occurred to correlate exclusively with prognosis and not with any other clinico-pathological feature.

PROTEOMICS

Transcriptome analysis, that uses microarray techniques to screen large numbers of genes for mRNA expression levels, can be considered an indirect approach to analyze the functional status of a cell or tissue. Actually, the functional effectors of cellular pathways and processes are proteins that are encoded by approximately 30,000 genes while the number of proteins exceeds 300,000. This higher complexity of the proteome (the entire protein complement of a tissue or cell) is due to multiple alternative splicing variants and post-translational modifications. Proteomic technology has advanced to the level where proteomic patterns can be analyzed as fingerprints in much the same way as microarray data are analyzed. Proteomic pattern analysis of large cohorts of data using both supervised and unsupervised learning methods will in the near future uncover novel biomarkers and distinguish prognostically relevant subclasses of hematolymphoid neoplasms [14].

Proteomic pattern analysis is especially attractive for exploring the presence of novel disease entities since it is an open system; it does not require identification or isolation of each protein composing the overall pattern. This is in contrast to microarrays, where only “known” target sequences can be printed on the array and interrogated for expression levels.

At present high throughput proteomic analysis of leukemias has not yet been performed. Presently investigations have focussed on a segment of the proteome of leukemic blast cells, the cluster differentiation (CD) antigens that were successfully explored for AL subclass identification [15].

FLOW CYTOMETRIC IMMUNOPHENOTYPING OF AL

Flow cytometric immunophenotyping developed independently of other diagnostic approaches in AL and involves the characterization of expression of a growing number of marker proteins among which the CD antigens are the most prominent. The first markers extensively studied showed phenotypic similarities between leukemic and healthy cells that allowed the establishment of lineage of origin and maturation stage of the pathologic cells. Later, leukemia-associated phenotypes were identified and a number of reports have shown that in most precursor B-ALL cases blast cells display aberrant phenotypes [16–20]. More recently studies have attempted to identify immunophenotypic characteristics of the major genotypic leukemia categories (see for a complete review Hrusak and Porwit-MacDonald [7]). The expression of a single molecule (NG2 in the case of MLL translocations; CBFbeta/SMMHC the product of *inv(16)*, or the hybrid PML/RARalpha protein) can rarely predict a molecular genetic subtype and scoring strategies have been constructed which simultaneously take into account the expression levels of several molecules to describe a phenotypic entity that corresponds to a genotypic subtype of leukemia [21,22].

Classical immunophenotyping by flow cytometry generates several parameters to describe the expression of a given marker protein: the percentage of positive cells, the mean (or the median) fluorescence intensity and the coefficient of variation (CV). A common cut-off value for positivity is 20% of positive cells, which results in a rather arbitrary distinction between positive and negative cells. Mean fluorescence intensity (MFI) is a principal measure of the antigen expression that depends on the number of antibodies bound to each gated cell. The CV describes how much the expression of a certain molecule is homogeneous or heterogeneous in a certain population. Potentially, MFI and CV values of marker expression should allow objective profiling of leukemia samples.

QUANTITATIVE MULTIPARAMETRIC IMMUNOPHENOTYPING

Flow cytometry is a powerful technique that allows the analysis of the expression of a wide range

of molecules and multiple color labeling further allows the analysis of expression in subsets of cells. Usually flow cytometry results are simplified and expressed in a qualitative or semiquantitative manner. But accurate phenotyping of genotypic leukemia classes cannot be obtained by either positive or negative antigen definition and requires a more accurate phenotypic description. To characterize the phenotype of ETV6/AML1 ALLs objectively, De Zen *et al.* [21] translated absolute levels of antigen expression from arbitrary units (fluorescence channels) into molecules equivalent of soluble fluorochrome (MESF) and the CV was used as a measure of the degree of antigen expression in the population of leukemic blast cells. Using a scoring system with Boolean logic operators the combined expression of six antigens could be correlated with the specific ETV6/AML1 genotype.

In an immunophenotypic study of myelodysplastic syndromes (MDS) Maynadie *et al.* [23] chose to express fluorescence labeling in a simplified numerical form, corresponding to the ratio of the fluorescence of the whole cell subset gated for each marker and the mean of autofluorescence. Applying unsupervised learning algorithms on the data set obtained for 207 MDS patients and 68 controls using 14 markers, they obtained a clustering of patients that at least in part correlated with the French-American-British (FAB) classification supporting the view that quantitative immunophenotyping by flow cytometry may be a future tool for the characterization of MDS.

ALL CLASS DISCRIMINATING GENES ARE MARKER PROTEINS

In pediatric ALL the number of class discriminating genes identified by microarray analysis varies markedly from group to group [8]. Importantly, Carroll *et al.* [8] concluded that the number of genes required to diagnose all of the known ALL subtypes in parallel can be as few as 20. Similarly, Kohlmann *et al.* [24] arrived at a similar observation: only a small set of differentially expressed genes is necessary to discriminate eight different AL subtypes. Interestingly, among class discriminating genes there are a number of known surface markers including CD14, CD19, CD79a, CD24, CD43 and CD44 that distinguish ALL from MLL samples [12]. Yeoh *et al.* [10] report that CD19, CD22, CD10 characterize B-ALL and T-ALL cases are characterized by over-expression of genes coding for CD2, CD3 and CD8. This has two important implications: (i) few genes contain a substantial amount of diagnostic information and (ii) diagnostic classification using these and other genes that will be discovered in the future may well

be accomplished using methods like multiparameter flow cytometry.

CONCORDANCE BETWEEN MARKER PROTEIN AND GENE EXPRESSION

In order to verify concordance between protein (flow cytometry data) and gene expression microarray data in ALs, Kern *et al.* [25] studied the protein expression and mRNA expression in terms of presence or absence for 39 relevant genes in 113 patients of AML, B- and T-ALL. In microarray expression analysis of BM samples all nucleated cells, obtained after a Ficoll-Hypaque gradient centrifugation, are present in the preparation and thus included in the analysis, therefore the same cell population was also included in the flow cytometry analysis. Protein expression of the relevant marker genes was analyzed on all cells gated in a forward versus side scatter plot, hence including lymphocytes, leukemic blast cells, monocytes, and granulocytes. The presence of all nucleated cells in the analysis has major influence on the results obtained. Depending on the relative infiltration of leukemic blast cells in the sample the contribution of the other cell populations will vary.

However, in spite of variability in sample composition, Kern *et al.* [25] found that overall 69.1% congruent results were obtained: congruency both in positivity or negativity with respect to the expression of a certain marker at RNA and protein level. Some markers stand out for high congruency, notably those markers that are lineage characteristic: CD22, CD79a, CD19, CD10 and TdT for B-lineage leukemia samples, MPO, CD13 and CD33 for AML lineage and CD3 and TdT for T-lineage leukemia samples. Discordant results between microarray and flow cytometry were ascribed to genes with low mRNA and low protein expression levels. Congruency of microarray and flow cytometry results for lineage-specific markers was also noted in other studies [10,12]. The comparison between flow cytometry and microarray data are generally hampered by a lack of quantitative values. For comparison purposes Kern *et al.* [25] used arbitrary cut-off levels to distinguish between the presence or absence which, especially in the case of weakly expressed markers/genes, may have a major impact on interpretation of congruency.

COMPUTATIONAL ANALYSIS OF FLOW-CYTOMETRY ANTIGEN EXPRESSION PROFILES IN PREB-ALL

Recently, we used the marker protein segment of the proteome of ALL blast cells to investigate

samples of pediatric preB-ALL patients [15]. Expression data of 16 marker proteins included in the AIEOP panel for routine clinical analysis of B-ALL were converted into molecules of equivalent soluble fluorophore (MESF); MESF data together with CV data were compiled in a marker protein expression data base of 145 patients. Using this database we first tested the potential of univariate and multivariate computational procedures to discriminate between samples positive for mixed lineage leukemia translocations (MLL+) and samples negative for these translocations (MLL-). Our analysis confirmed the results of Armstrong *et al.* [12] who, using microarray expression analysis distinguished MLL+ samples from ALL and AML samples. For 145 samples tested, 21 variables discriminated between MLL+ and MLL- phenotypes. However, when MLL+ and MLL- populations were explored through an unsupervised method using all 32 variables in a principal component analysis (MESF and CV values for each marker protein) it appeared that the MLL+ samples were split into two distinct MLL+ clusters. One cluster was more similar to, and mixed with, the MLL- cluster and another was further separated more distinct from MLL-. Retrospective analysis revealed that the more separate MLL+ cluster comprises exclusively MLL/AF4 samples, while the other group represented MLL with various translocation partners. To our knowledge this is the first time that phenotypic heterogeneity has been described. Whether MLL/AF4 distinction from other MLL samples has prognostic relevance, MLL itself being a prognostic entity with poor prognosis, awaits further prospective analysis (Fig. 1).

CONCLUSIONS AND FUTURE PROSPECTS

Next to global analyses of transcript and protein expression patterns, a segmental analysis of the proteome using phenotypic marker protein expression will no doubt refine predictive AL classification systems.

Potentially, these analyses can be used in clinical diagnosis. Immunophenotypic data acquisition using flow cytometry is a fast and relatively easily accessible technology that has already been implemented in most centers for leukemia diagnosis. The translation into quantitative data sets is a matter of cross laboratory output calibration. However, before application in clinical diagnostics can occur, it is crucial that quantitative immunophenotypic data set analysis is validated in independent experiments and in large data sets that mirror the normal distribution of leukemia subtypes seen in the clinical setting.

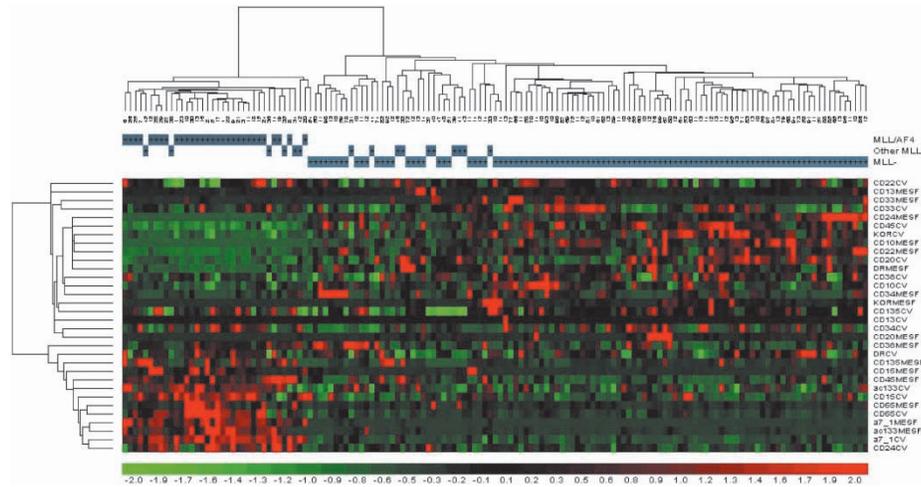


FIGURE 1 Hierarchical cluster analysis of flow cytometry profiles of 145 patient samples illustrates the distinction between MLL/AF4, MLL positive samples non AF4, and MLL- samples. Expression data of 16 marker proteins (MESF and CV values) were grouped using hierarchical agglomerative clustering. Hierarchical clustering and expression maps of clustered markers were obtained using dChip software on standardized data (available at <http://www.dchip.org>). The clustering algorithm is based on Pearson's correlation coefficient and centroid linkage. In the expression maps, each cell represents the normalized expression level of a single marker in a single sample; red and green, values above and below, respectively, the mean value for that marker across all of the samples. Color saturation is proportional to the magnitude of the difference from the average expression level.

Recently, in our laboratory a set of immunophenotypic expression data of a cohort of AML patients has been analyzed using this methodology (in preparation). Supervised analysis of marker protein analysis does allow to some extent the distinction of FAB groups. However, class distinction largely improved using unsupervised hierarchical clustering and discriminating antigens identified four clusters that correspond to the four major classes of chromosomal aberrations among AML patients: t(8;21), inv(16), t(15;17) and samples negative for these aberration. The classification was highly specific (96%) but had a low sensitivity for negative samples. The sensitivity for t(8;21) and t(15;17) samples was high, but in this case specificity was low. The prognostic value of the four classes described by immunophenotypic expression data awaits further analysis.

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