Perspectives of gene expression profiling for diagnosis and therapy in haematological malignancies

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Abstract
Considering the heterogeneity of leukaemias and the widening spectrum of therapeutic strategies, novel diagnostic methods are urgently needed for haematological malignancies. For a decade, gene expression profiling (GEP) has been applied in leukaemia research. Thus, various studies demonstrated worldwide that the majority of genetically defined leukaemia subtypes are accurately predictable by GEP, for example, with respect to reciprocal rearrangements in acute myeloid leukaemia (AML). Moreover, novel prognostically relevant gene classifiers were developed as, for example, in normal karyotype AML. Considering the lymphatic malignancies, GEP studies defined novel clinically relevant subtypes in diffuse large B cell lymphoma (DLBCL), and improved the discrimination of Burkitt lymphoma and DLBCL cases, overcoming considerable overlaps of these entities that exist from morphological and genetic perspectives. Treatment-specific sensitivity assays are being developed for targeted drugs such as farnesyl transferase inhibitors in AML or imatinib in BCR-ABL1 positive acute lymphoblastic leukaemia (ALL). Irrespectively of these proceedings, an introduction of the microarray technology in haematological practice requires diagnostic algorithms and strategies for interaction with currently established diagnostic techniques. Large multicentre studies such as the MILE Study (Microarray Innovations in LEukemia) aim at translating this methodology into clinical routine workflows and to catalyze this process.

Keywords: gene expression profiling (GEP); acute myeloid leukaemia (AML); acute lymphoblastic leukaemia (ALL); lymphatic malignancies; MILE study

INTRODUCTION
Diagnosis and therapy of haematological malignancies are based on a variety of genetically defined subentities [1]. For classification of the individual leukaemia subtypes, multiple diagnostic techniques have to be integrated: in acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL), morphology is complemented by chromosomal banding analysis. Together with fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) based techniques a specific composition is able to define chromosomal or molecular genetic alterations in ≥90% of cases. Immunophenotyping by multiparameter flow cytometry is applied to determine the involved hematopoietic lineage [2]. In the lymphatic neoplasms, morphology requires
the contribution of immunophenotyping, immunohistochemistry or FISH to define the individual lymphoma subtype.

Today, the detection of many subtypes of leukaemia or lymphoma are highly relevant for therapeutic decisions: for example, in acute promyelocytic leukaemia (APL) with t(15;17)/PML-RARA, disease-free survival is achieved in >80% of cases by a combination of all-trans-retinoid acid (ATRA) with cytotoxic chemotherapy, whereas in complex aberrant karyotype AML (≥3 clonal cytogenetic aberrations) long-term overall survival is <20%, irrespective of intensive therapeutic approaches including stem cell transplantation (SCT) [3–7]. In B-lineage ALL, paediatric patients with the t(12;21)/RUNX1-ETV6 (AML1-ETV6) are cured in most cases by standard chemotherapy, whilst prognosis remains much poorer in Philadelphia positive ALL with the t(9;22)(q34;q11)/BCR-ABL1, even when the specific tyrosine kinase inhibitor, imatinib, is combined with chemotherapy [8].

Thus, obtaining the exact diagnosis in haematological malignancies is obligatory. However, comprehensive diagnostic algorithms including morphology, cytogenetics and molecular genetics, and flow cytometry are time-consuming and labour-intensive as well as rely heavily on expert knowledge [2]. First, variances in the clinical courses occur even within distinct genetically based subtypes. This is only in part explained by the coincidence of known genetic alterations, for example, of a prognostically favourable NPM1 mutation and a prognostically adverse FLT3-ITD mutation in AML [9]. Refractoriness to induction therapy is seen in 10–20% of patients, but the individual response is currently not exactly predictable at the time of diagnosis.

In lymphomas, difficulties arise from overlaps of diverse lymphoma subentities, while a correct subclassification is essential for the definition of the intensity of treatment. On the other hand, the indication to regimens such as high-dose methotrexate (MTX) for patients with Burkitt’s lymphoma (BL) has to be performed with caution due to the toxicity profile of the therapeutic regimen [10, 11]. Considering these shortcomings of current diagnostic approaches, the search for novel diagnostic techniques in haematology continues [12].

After pioneering experiments from Golub et al. [13], who demonstrated the prediction of various leukaemia subclasses by gene expression profiling (GEP) algorithms, this novel technology has been explored in great detail for its potential application in diagnostics of haematological malignancies [14]. One main focus was the reproduction of known subentities based on specific gene expression patterns (‘class prediction’) [13, 15–17]. Aiming to go beyond the borders of current diagnostic methods, several study groups revealed novel prognostically relevant subgroups of patients (‘class discovery’); for example, in normal karyotype AML [18–20]. With respect to lymphomas, GEP analyses identified new prognostically relevant subtypes in diffuse large B-cell lymphoma (DLBCL) [21, 22] and were able to improve the difficult discrimination of BL and DLBCL cases [10, 23]. Irrespective of these proceedings, the future role of GEP in the diagnosis and therapy of haematological malignancies is still under discussion.

TECHNICAL ASPECTS

Gene expression analyses require high amounts of malignant cells, a precondition which is fulfilled in practically all bone marrow samples of an acute leukaemia biopsy at diagnosis [24]. A typical leukaemia sample shipment can be performed as whole bone marrow at ambient temperature. Variable storage time of samples and different sample handling procedures had been demonstrated to have no influence on a diagnostic signature further proving the robustness of the assay [25].

For microarray studies, diverse platforms are available. Spotted glass slide arrays allow the application of a variety of immobilized probes—cDNA, oligonucleotides or genomic fragments. The highest specificity is achieved with DNA–oligonucleotide microarrays of 40–60-mer length with a lower risk of cross-hybridization [26]. Affymetrix microarrays consist of 25-mer in situ synthesized probes [27].

Microarray data analysis starts with data preprocessing and quality control checks to identify array artefacts and to evaluate the homogeneity of experimental groups [24]. Often, patients are grouped according to predefined characteristics with supervised analyses [28, 29], while unsupervised analyses test the hypothesis whether specific characteristics have a correlate at the level of distinct gene expression signatures. For the interpretation of data, hierarchical clustering organizes data into patterns with similar signatures and allows the visualization of data for interpretation of biological relationships.
and to discover novel subtypes [30]. With principal component analysis (PCA), the dimensionality of array data is reduced, and visualization and interpretation of large data sets are facilitated [31].

**GENE EXPRESSION ANALYSES IN AML**

Most aberrations as categorized in the WHO 2008 classification as ‘AML with recurrent genetic abnormalities’ [1] are accurately predictable with gene expression analyses: This applies first to the favourable reciprocal translocations t(15;17)/PML-RARA in APL, t(8;21)/RUNXI-RUNX1 and inv(16)/CBFB-MYH11. Distinct gene expression patterns were described for these alterations with discrimination accuracies up to 100% [32–35]. Also, the prognostically adverse 11q23/MLL rearrangements, which are frequently associated with previous exposition to topoisomerase-II inhibitors, can be separated from other AML subtypes. 11q23/MLL rearranged cases differed in AML and ALL according to different transcriptional programs with involvement of regulators of B cell development such as PAX5 and EBF in the B-lineage cases [36]. Specific gene expression patterns were also determined for some rare translocations, for example, AML with t(8;16)(p11:p13) which results in a fusion of the AML with t(8;16)(p11;p13) which results in a mined for some rare translocations, for example, Specific gene expression patterns were also determined for these alterations with discrimination accuracies up to 100% [32–35]. Also, the prognostically adverse 11q23/MLL rearrangements, which are frequently associated with previous exposition to topoisomerase-II inhibitors, can be separated from other AML subtypes. 11q23/MLL rearranged cases differed in AML and ALL according to different transcriptional programs with involvement of regulators of B cell development such as PAX5 and EBF in the B-lineage cases [36]. Specific gene expression patterns were also determined for some rare translocations, for example, AML with t(8;16)(p11:p13) which results in a fusion of the MYST3 and CREBBP genes [37]. Vicinity of this subtype to 11q23/MLL rearranged AML was suggested due to the common association to previous exposition to topoisomerase inhibitors and due to overlapping gene signatures with HOX gene dysregulation [38, 39].

At the same time, identification of gene expression patterns was performed for the more heterogeneous subgroup of AML patients with complex aberrant karyotypes, being characterized by non-random combinations of numerical or structural gains and losses. The specific gene expression patterns were characterized by upregulation of genes with a DNA repair function such as RAD21 [7, 40–42].

Considering molecular markers, the prognostically favourable nucleophosmin (NPM1) mutations with their high frequency especially in normal karyotype AML [9, 43] were observed to result in highly specific gene expression signatures by various study groups [44]. In both adult and paediatric leukaemias, the respective expression patterns were characterized by activation of distinct HOXA and HOXB cluster genes, a homeodomain-containing family of transcription factors [45, 46], and by involvement of genes with a function in signalling and apoptosis [47].

Regarding the FLT3 mutations—another frequent molecular marker in normal karyotype AML with negative prognostic impact—reproduction of a characteristic signature for the fms-related tyrosine kinase 3 based on GEP is still under debate. While some studies demonstrated a clear separation of FLT3-ITD (internal tandem duplications) and FLT3-TKD (tyrosine kinase domain mutations) with high accuracy [48, 49], others reported lower accuracy in the prediction of the FLT3-ITD event [35] or found significant overlaps for FLT3-ITD and FLT3-TKD mutated cases [50]. These difficulties in precisely determining the FLT3 mutation status by a gene expression signature might be due to other variables or genetic alterations which influence the FLT3 pathway. Bullinger et al. [51] were not able to define a specific gene expression signature being exactly correlated to the FLT3 mutation status as defined by PCR assays, but found a novel signature which showed overlaps to FLT3 mutated AML and correlations to FLT3 pathway activation. This signature had even stronger correlations with the clinical outcome when compared to the ‘classical’ FLT3 mutations.

Importantly, GEP studies further identified new prognostic biomarkers, especially in the heterogeneous group of normal karyotype AML. Bullinger et al. [18] were able to subdivide normal karyotype cases into two clusters which were either characterized by overexpression of several transcriptional regulators such as GATA2 or by involvement of genes being responsible for leukocyte differentiation and immune response. The prognostic power of this gene signature was independently reproduced by Radmacher et al. [19]. However, more examples of such independent validation of these novel classifiers will be needed before an introduction of prognostic biomarker gene sets in clinical practice seems justified.

Aiming to clarify the mechanisms of resistance and therapy response in AML, Heuser et al. [52] compared the gene expression profiles of hematopoietic stem cells of responders and non-responders to induction therapy. While responders showed clustering with the hematopoietic stem cells of healthy individuals, non-responders were observed to harbour overexpression of genes being highly expressed
in normal hematopoietic progenitor cells. Thus, a specific transcriptional program in the hematopoietic progenitor cells might modify resistance. High expression of the prognostically adverse brain and acute leukaemia, cytoplasmic (BAALC) gene was associated with overexpression of genes being involved in drug resistance (MDRI) and stem cell markers (CD34 or KIT) in a study of Langer et al. [53]. Aiming to predict response to targeted therapy approaches, Raponi et al. [54] developed a distinct gene expression ratio algorithm of the RASGRF1 (leading to RAS activation) and aprataxin (APTX) genes which was able to predict the response to the farnesyltransferase inhibitor tipifarnib. Such approaches might be highly useful to perform selection of patients for distinct therapeutic strategies [12].

**GENE EXPRESSION ANALYSES**

**IN ALL**

In ALL, class prediction can be performed according to the involved lymphatic lineage [16, 55–57] to different maturation stages as defined by the immunophenotypes [58], or according to genetically defined subtypes. In paediatric ALL, the favourable t(12;21)/ETV6–RUNX1 (TEL-AML1) is clearly separated from other subtypes, for example, by overexpression of the telomeric repeat binding factor 2 (TERF2) gene, which has a coding function for a telomere specific protein [16, 57, 59]. 11q23/MLL rearranged ALL—which has an adverse prognosis—and is characterized by dysregulation of certain members of the HOX gene family [16, 36, 60, 61].

In contrast, gene expression patterns of the t(9;22)/BCR-ABL1 in Philadelphia positive ALL are similar to ALL cases without known genetic rearrangements and do not display a unique pattern in GEP studies [17, 58, 62]. Considering the therapeutic option of imatinib for BCR-ABL1 positive ALL and the adverse prognostic profile of this subtype, other methods will be needed to close this gap in the cascade of markers in ALL being accurately recognized by underlying gene signatures.

Above all, GEP deepened our understanding of leukaemogenic pathways in ALL. It is interesting to briefly mention that the general technical platform of microarrays also allows other genomics assays to be performed. In children with ALL without known genetic abnormalities, single nucleotide polymorphism (SNP) microarray studies had demonstrated that alterations of genes being involved in B-cell development such as IKZF1 (coding for the transcription factor IKAROS) or PAX5 were linked to an inferior prognosis. An analysis of the gene expression signatures showed similarity of the respective cases to the profile of BCR-ABL1 positive ALL [63, 64].

Yet another application of microarrays was demonstrated by designing experiments that interrogate the expression status of microRNAs (miRNAs). A tumour suppressor miRNA (miR–203), which is inactivated in human tumours by both genetic and epigenetic mechanisms, controls the protein expression levels of the chimeric BCR-ABL1 produced by the Philadelphia chromosome in BCR-ABL1 positive ALL and chronic myeloid leukaemia (CML). Re-expression of miR–203 by exogenous transfection or epigenetic drugs was demonstrated to inhibit the proliferation of tumour cells in an ABL1-dependent manner. Thus, modulation of certain miRNAs might be explored for future therapeutic strategies in BCR-ABL1 expressing malignancies [65, 66].

Similar to approaches in AML, new gene expression-based classifiers are being developed in ALL to improve prognostic predictions. In paediatric ALL, long-term prognosis was predictable with GEP with 78% accuracy and the achievement of complete molecular remission following induction therapy with 100% accuracy [56, 67]. Another study combining GEP and quantitative PCR identified a three-gene classifier based on the glutamine synthetase (GLUL), ornithine decarboxylase antizyme inhibitor (AZIN) and immunoglobulin J chain (IGJ) genes to predict prognosis in paediatric pre–B-ALL [68]. For Philadelphia chromosome positive ALL, a classifier was described to have predictive power for detecting sensitivity to the tyrosine kinase inhibitor, imatinib, and for the development of secondary resistance [69]. Chiaretti et al. [70] were able to classify patients according to the response to induction therapy and the duration of remission in adult T-ALL.

**GENE EXPRESSION ANALYSES**

**IN LYMPHOMA**

With current diagnostic procedures, classification of immature B-cell lymphomas is facing several problems: First, DLBCL demonstrates a variable clinical course, which correlates to morphological, cytogenetic and immunological diversity as observed in this entity. With GEP analyses, three new prognostically
relevant subclasses were described: germinal centre B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL and primary mediastinal B-cell lymphoma (PMBL). Two common genetic events in DLBCL, namely, the bcl-2 translocation and c-rel amplification were detected in the GCB subgroup which only showed the highest 5-year survival rate. At the same time, a 17-gene predictor was developed to predict overall survival in DLBCL patients after chemotherapy [21, 22].

With GEP studies, these different DLBCL subtypes could be linked to diverse molecular pathways: in the ABC subtype, Lenz et al. [71] described frequent occurrence of a distinct amplicon on chromosome 19 which was characterized by upregulation of the SPIB gene encoding an ETS family transcription factor. As suggested by RNA-mediated interference experiments, the respective gene had an oncogene function in the DLBCL subtype only. Another study performed a subdivision of DLBCL cases according to a three-gene classifier: the favourable ‘stromal-1’ group was characterized by extracellular matrix deposition and histiocytic infiltration, whereas the prognostically adverse ‘stromal-2’ signature revealed high tumour blood-vessel density. Thus, outcome in DLBCL seemed to be influenced by differences in immune cells, fibrosis and angiogenesis in the microenvironment of the lymphoma [23].

Another clinically relevant problem is the morphological discrimination of BL and DLBCL cases [72], as some cases of aggressive lymphatic malignancies display morphological, immunophenotypic and genetic features of classical BL. Historically, such cases were classified as ‘atypical BL’ or ‘Burkitt-like lymphoma’. MYC-rearrangements are characteristic for classical BL, but as they are detectable as well in ≤10% of DLBCL cases, the respective mutation status does not qualify to perform a clear discrimination of both entities [11]. However, a clear separation of DLBCL and BL is highly relevant for therapeutic decisions as more intensive treatment approaches are necessary for patients with BL.

Hummel et al. [73] and Dave et al. [10] both presented novel gene expression classifiers to identify all pathologically verified BL cases. BL was characterized by a high level of c-MYC target gene expression and the expression of a subgroup of germinal-centre B-cell genes. Further, this study demonstrated that, in some instances, patient samples which previously had been classified as DLBCL showed gene expression signatures corresponding to BL. Interestingly, overall survival was better when these patients had received intensive chemotherapy protocols instead of lower-dose regimens. In children with lymphatic malignancies, Klapper et al. [74] performed the reclassification of 31% of morphologically defined DLBCL cases as molecular BL. Thus, molecular BL might be more frequent in children than previously known and GEP may be included to define not only the correct entity but also to guide treatment.

Finally, microarray analyses revealed new diagnostic markers in lymphatic malignancies. Wellmann et al. [75] observed an exclusive occurrence of the clusterin gene (CLU) in anaplastic large-cell lymphoma (ALCL) cell lines, whereas the respective gene was not detected in various other lymphoma cell lines. Clusterin represents a highly conserved glycoprotein which is involved in various processes such as intercellular and cell matrix interactions or apoptosis, but the exact functional role in lymphoma still has to be determined.

**TRANSLATING GEP INTO CLINICAL ROUTINE WORKFLOWS**

Recently, the MILE (Microarray Innovations in LEukemia) study research program was performed in eleven laboratories across three continents: seven from the European Leukemia Network (ELN; www.leukemia-net.org), three from the US and one in Singapore. The MILE study aims to investigate whether the standardized method of GEP with low technical failure rate and simplified standard operating procedures may improve current diagnostic ‘gold standards’ as an adjunct to conventional algorithms and potentially offers a reliable diagnostic and prognostic tool for many patients who do not have access to a state-of-the-art ‘gold standard’ workup.

The first stage was designed as biomarker discovery phase. Whole-genome gene expression profiles (GEP) from 16 distinct categories of clinically relevant leukaemias and myelodysplastic syndromes (MDS), as well as non-leukemic and healthy bone marrow samples as controls, were generated and were compared to diagnostic routine assays (‘gold standard’). Data from the completed MILE stage I included 2143 adult and pediatric samples, tested with HG-U133 Plus 2.0 microarrays [76]. In order to assess the clinical utility of microarray-based diagnostics, a stage II was subsequently performed,
further prospectively collecting 1191 patient samples and using a customized microarray and sample preparation workflow.

The MILE study further has demonstrated that gene expression microarrays can be specifically designed to focus on specific assays, such as subclassifying acute leukaemias. A recent study including 160 patient samples representing eleven subtypes of acute and chronic leukaemias, MDS and non-leukaemia specimens as a control group confirmed the robustness of such an assay [77]. In this study, all samples had been centrally collected and diagnosed as part of the daily routine in the Munich Leukemia Laboratory and the custom leukaemia microarray demonstrated a high technical precision and reproducibility of quadruplicate mononuclear cell lysates in four different laboratories in Germany (D), Austria (A) and Switzerland (CH) (the DACH study).

Also for lymphomas, class-specific arrays have demonstrated utility, such as a custom oligonucleotide microarray, with 2524 unique genes that are expressed differentially among the various forms of non-Hodgkin’s lymphoma. As reported by Dave and colleagues, GEP could reliably distinguish BL from DLBCL [10].

DISCUSSION
Within the last decade since the introduction of GEP in leukaemia research [13] most genetically and immunologically defined subgroups of AML and ALL were precisely characterized by specific gene expression patterns and a large number of these signatures was validated by independent study groups [78]. While most studies focused on the reproduction of reciprocal rearrangements [16, 17, 33, 35], other cytogenetic subgroups such as complex aberrant karyotype cases were also linked to characteristic gene expression signatures [7, 40–42]. Thus, with some exceptions, for example, signatures defining FLT3-ITD mutated AML [35, 51] or BCR-ABL1-positive ALL [58, 62], a large spectrum of genetic markers can be detected in a single approach using microarray technology which strongly underlines the potential for applying this technique in daily clinical routine. Thus, screening for a large panel of genetic parameters would be facilitated and could be done with a single diagnostic approach. This might be combined with already established techniques such as PCR [79]. In addition, GEP might allow to select parameters for minimal residual disease (MRD) diagnostics during follow-up of the disease, for example, by quantitative PCR [80, 81].

Secondly, GEP analyses were able to define new prognostic parameters, for example, in normal karyotype AML. As some study groups presented an independent reproduction of these classifiers [18, 19, 82], these new prognostic markers might even contribute to the selection of patients for allogeneic stem cell transplantation in the future [83]. Facing the increasing number of targeted therapy studies, the development of compound specific sensitivity assays with GEP [54, 69, 84, 85] is another promising field [12]. In AML, it was possible to predict the response to the farnesyltransferase inhibitor tipifarnib based on the ratio of the expression of the RASGRF1 and APTX genes [54, 84]. In patients with BCR-ABL1 positive ALL, Hofmann et al. [69] were able to predict the sensitivity to the tyrosine kinase inhibition with imatinib based on the expression of 95 genes, and a panel of 56 highly differentially expressed genes was associated with the development of secondary resistance to imatinib. A similar approach was undertaken by Holleman et al. [86] who developed a combined gene expression score indicating individual resistance patterns for steroids, vincristine, asparaginase and daunorubicin in childhood ALL. Considering lymphoma entities, GEP studies have provided a new prognostically relevant subclassification of DLBCL cases [21, 22] and improved the discrimination of BL and DLBCL based on novel molecular insights [10, 73].

Thus, summarizing the wealth of GEP data obtained in haematological malignancies it becomes clear that microarray technology has proven its potential to improve disease subclassification and to lead the way for individualized prognostic predictions. The availability of this standardized and objective methodology in an increasing number of laboratories worldwide, the stability of gene expression signatures irrespective of preanalytic conditions, and the high interlaboratory reproducibility further support the inclusion of this platform into routine workflows [25, 77, 87].

Several requirements have to be addressed before an introduction of GEP in clinical practice can be realized [28]: Validated diagnostic algorithms are needed to prepare the interaction of the array technology with currently established methods [79]. It remains to be defined which gene expression patterns require additional confirmation by established methods such as PCR and in which entities
a molecular diagnosis by microarray analysis solely would be sufficient. The utility of microarray analysis to lead to biomarkers for follow-up diagnostics with quantitative real-time PCR should be further explored [81]. Moreover, new prognostic gene classifiers will have to be continuously validated by independent study groups [18, 19], and their utility has to be compared with classic risk factors [29]. Also, assays to predict the response to treatment specific compounds warrant further development [54]. Finally, one may think of a strategy to combine GEP assays with newer array applications such as genome-wide SNPs analyses or with next-generation high-throughput sequencing [10, 88–90]. In the mean time, international multicentre efforts such as the MILE study have already demonstrated that classification of various leukaemia subtypes based on GEP can be standardized between different laboratories and aim to catalyze the translation of this technology into daily routine diagnostics [76]. However, costs of high-throughput genomics analyses have to be carefully assessed in comparison to current diagnostic approaches and can’t just be added to existing algorithms. Yet, such health economic calculations may as well be performed factoring in parameters such as labour-intensity, requirement of expert knowledge, robustness and turn-around time of results for the multitude of today’s standard genetic and molecular techniques to diagnose and subclassify haematological malignancies.

**Key points**

- GEP using high-density microarrays is a widely used research method for characterizing leukaemia and lymphomas.
- Robust biomarker signatures have been identified to allow a precise classification of many leukaemia subtypes using differential gene expression patterns.
- Expression microarray analyses also led to a better molecular understanding of lymphoma subtypes.
- Given the high technical precision of the microarray platform, its utility in routine diagnostic workflows and guiding treatment decisions is currently being evaluated.

**References**

Gene expression profiling in haematological malignancies


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