Review Article

Emerging technologies in paediatric leukaemia

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Abstract: Genetic changes, in particular chromosomal aberrations, are a hallmark of acute lymphoblastic lymphoma (ALL) and accurate detection of them is important in ensuring assignment to the appropriate drug protocol. Our ability to detect these genetic changes has been somewhat limited in the past due to the necessity to analyse mitotically active cells by conventional G-banded metaphase analysis and by mutational analysis of individual genes. Advances in technology include high resolution, microarray-based techniques that permit examination of the whole genome. Here we will review the current available methodology and discuss how the technology is being integrated into the diagnostic setting.

Keywords: Karyotyping; DNA microarrays; high throughput DNA sequencing

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Introduction

Acute lymphoblastic leukaemia (ALL) is a malignant disease of the bone marrow characterized by the accumulation of immature lymphoid cells. It is the most common form of childhood malignancy in children from 0-14 years, accounting for almost 25% of paediatric cancer (1). Risk stratification of childhood ALL patients and adaptation of therapy based on this has been central to the improvement to the five-year survival rates of children diagnosed with ALL, with the majority of children diagnosed with ALL now expected to be five-year survivors (2,3). Notwithstanding this, 20% of patients still relapse and of these only half will survive. A considerable proportion of these patients who relapse lack the high risk-stratifying genetic changes that are included in current ALL treatment protocols.

Currently therapy is adjusted by three parameters: clinical risk factors, genetic subtypes and early response to therapy (4). The clinical risk factors are protocol dependent but may include age, gender, immunophenotype and white cell count at diagnosis. Our ability to detect genetic change is pivotal in ensuring that appropriate treatment is administered, which in turn has an impact on response to therapy. This approach was developed following the realization that paediatric ALL is a heterogeneous disease consisting of various subtypes that differ markedly in their response to chemotherapy (5,6).

The first correlation between prognosis and karyotype at diagnosis in ALL was made by Secker-Walker *et al*. in 1978 (7). However, early attempts to elucidate the genetic changes associated with ALL were hampered by technical issues surrounding the culturing of leukaemic cells. Notoriously poor morphology also made conventional G-banded metaphase analysis difficult. The first large series of newly diagnosed ALL cases analyzed for cytogenetic and prognostic correlations was by the Third International Workshop on Chromosomes in Leukaemia (8). Clonal chromosomal aberrations were detected in ~66% of patients reviewed by the workshop and these non-random changes could be used to identify both high-risk and low-risk ALL (9).

Conventional cytogenetic analysis is now an essential component of the multidisciplinary approach to the diagnosis, classification and risk-stratification of patients with acute leukaemia (10). The introduction of molecular-based cytogenetic techniques such as fluorescent in situ hybridisation (FISH) as an adjunct to conventional cytogenetics has improved the abnormality detection rate with ~90% of ALL cases having detectable chromosomal aberrations at presentation (11-13). The use of FISH led to the discovery of clinically relevant abnormalities that were

previously unidentified (13-15) and in addition permitted the detection of chromosomal abnormalities in samples that had previously failed cytogenetic analysis or where, due to limitations of conventional cytogenetic analysis including poor morphology, no abnormal clone had been detected.

Genetic classification of paediatric ALL

The increased knowledge of the genomic aberrations associated with ALL have enabled the disease to be subdivided into subgroups with different prognostic and clinical features based on the presence of specific acquired genetic aberrations namely aneuploidies and chromosomal translocations.

Traditionally, ALL has been classified according to phenotype into precursor T-cell, precursor B-cell (BCP) and mature B-cell (Burkitt) ALL, which are then further classified according to recurrent cytogenetic abnormalities.

Ploidy status remains the most well-documented and easily characterized genetic change in ALL and is divided into the following entities based on chromosomal number: near-haploidy (25-29 chromosomes), low hypodiploidy (31-39 chromosomes), high hypodiploidy/hypodiploidy (40-44/<45 chromosomes), diploidy/pseudodiploidy (46 chromosomes), low hyperdipoidy (47-50 chromosomes), high hyperdiploidy (51-67 chromosomes), near-triploidy (66-79 chromosomes), near-tetraploidy (84-100 chromosomes). Some numerical changes may be single abnormalities such as trisomy 5 and trisomy 21, while others are secondary changes associated with specific structural abnormalities (16-18).

Chromosome rearrangements include t(12;21) *ETV6-RUNX1*, t(1;19) *TCF3-PBX1*, t(9;22) *BCR-ABL1* and *MLL* rearrangement in B-progenitor ALL and rearrangement of *TLX1*, *TLX3*, *LYL1*, *TAL1* and *KMT2A* genes in T-lineage ALL (19-24).

Cytogenetics still remains the "gold standard" for the genetic classification of ALL, although more recent advances in technology such as gene expression profiling and microarray technology have been proposed as alternative methods. Adoption of some of these methods into routine clinical practice will undoubtedly improve our ability to risk stratify patients and ultimately lead to improved survival.

Identification of genomic abnormalities in ALL

The completion of the Human Genome Mapping Project (25) enabled the development of probes for any known DNA sequence and in turn led to the improvement of existing techniques such as FISH. This increased knowledge and the ability to spot a multitude of DNA sequences onto a slide have enabled array-based genome-wide studies to be undertaken. These genome-wide microarray studies examining gene expression, copy number abnormalities (CNAs) and loss of heterozygosity (LOH) have further enhanced our knowledge providing insights into the biology of ALL (26-28). There are other methods such as DNA methylation profiling and microRNA expression which continue to provide additional information on the pathology of ALL, but these will not be discussed in this review.

Gene expression profiling

Gene expression profiling was initially used to distinguish between acute myeloid leukaemia (AML) and ALL samples (29). Gene expression profiles (GEPs) for 6,817 genes were analysed in bone marrow samples of 27 ALL and 11 AML patients resulting in a set of 50 genes that could discriminate between ALL and AML. Another study showed that they were able to distinguish between AML cases and ALL cases with and without *KMT2A* rearrangements on the basis of GEP alone (30). In the same year Yeoh *et al.* analysed GEPs of the leukaemic blast cells from 360 paediatric ALL patients (31). Unsupervised hierarchical clustering identified six major leukaemia subtypes that corresponded to T-ALL, hyperdiploid with >50 chromosomes, *BCR-ABL1*, *E2A-PBX1*, *ETV6-RUNX1* and *KMT2A* gene rearrangement. A subgroup of 14 cases was also identified that had normal, pseudodiploid or hyperdiploid karyotypes and lacked any consistent cytogenetic abnormality. They were able to show that by using a computer-assisted supervised learning algorithm a diagnostic accuracy of 96% could be achieved using a 271 gene classifier (31). A number of other GEP studies have since been published with accuracies ranging from 95-100% (32-36). Although gene expression patterns can be defined by the characteristic translocations they do not correlate, in general, with the submicroscopic genetic changes that are now being observed (37,38).

The MILE (Microarray Innovations in Leukaemia) study, sponsored by Roche, specifically examined the diagnostic utility of GEP on 3,334 adult and paediatric patients, including 382 diagnostic specimens from children with ALL (36). The purpose of this study was to assess the clinical utility of GEP as a single test to subtype

leukaemias into the conventional categories of myeloid and lymphoid malignancies. It was a two phased study with a retrospective biomarker discovery phase using a commercially available whole-genome microarray performed on 2,096 patients with leukaemias and MDS, followed by an independent validation phase using a customised chip on a cohort of 1,191 patients (36). The first phase of this study demonstrated an accuracy of 92.2% for the 18 diagnostic classes, with 7 of the 18 classes showing a ≥94.6% concordance. Lower sensitivities were seen with entities with known biologic heterogeneity such as ALL with a hyperdiploid karyotype (75.8%). Overall all 18 classes could be predicted with a median sensitivity of 92.1% and a median specificity of 99.7% (36). For their validation cohort of 1,152 patients, which was an independent and blinded set for the classification algorithm developed in stage 1, there was an overall observed accuracy of the classifier prediction of 88.1%. This was increased to 91.5% when restricted to the 14 distinct classes of leukaemia. When these classes were refined further to eight of the 14 represented acute leukaemia classes, GEP diagnoses were concordant with the gold standard cytogenetic diagnoses in ≥95.0% analyses. Discrepancies were again observed in cases of heterogeneous disease, but in 29/51 discrepant results, re-examination of the specimen led to confirmation of the microarray result.

GEP has also highlighted a novel subgroup of BCP ALL with deregulated expression of *CRLF2*. This group comprises ~6% of paediatric BCP ALL and has been associated with an inferior outcome, although this association is not borne out by all studies (39-42). It is likely that other novel subgroups could be identified using this approach.

Although the diagnostic accuracy of GEP appears to be similar to existing methodologies such as conventional cytogenetics, it has failed to become part of the routine diagnostic work-up of ALL patients. Its shortfall has been an inability for it to accurately categorize in particular the hyperdiploid group of ALL patients. The ability to diagnose numerical chromosomal abnormalities is important for those laboratories who are unable to perform routine cytogenetic analysis and who rely on chromosomal copy number in order to stratify their patients (3). From a more practical perspective, gene expression profiling is not routinely performed in the clinical diagnostic laboratory and the expertise in interpretation of this data may not be readily available. Furthermore, its inability to outperform existing techniques brings with it a reluctance to adopt GEP in clinical practice.

Microarray techniques

There are a number of other microarray platforms that can be used for genome-wide analyses of ALL samples that could rival cytogenetics as the gold standard. The basis of these arrays is that a labelled sample of test DNA is hybridized to a chip spotted with thousands to millions of probes, each which is specific to a different region of the genome. Unlike conventional cytogenetics, these platforms are not reliant on the availability of mitotically dividing cells and samples do not need to be cultured. These techniques however are unable to detect balanced translocations since they are not accompanied by copy number changes, however unbalanced translocations associated with copy number changes are detectable. Interestingly, it appears that there are small focal deletions associated with some "balanced" translocations that although do not categorically show the balanced translocation do indicate that it may be present (43).

Early array studies in ALL used bacterial artificial chromosome (BAC) comparative genomic hybridization (CGH) arrays, in which test and reference DNA were hybridized simultaneously using probes derived from BACs, each of which contains a large fragment of DNA up to several hundred kilobases in size (27,44-49). Due to the large probe size however the resolution of these arrays was unable to accurately define small chromosomal aberrations. Although able to detect the majority of CNAs, they may miss small focal CNAs which have been shown to be characteristic of ALL (45). Arrays are more sensitive than conventional karyotyping when referring to the detection of small chromosomal aberrations, but they do have difficulty in distinguishing between multiple large clones. It is generally considered that the array will be able to detect clones that are present in 30% of cells, therefore the technology may struggle to detect smaller subclones that would have been seen using conventional G-banded metaphase analysis.

The use of oligonucleotide arrays, where the probe size generally ranges between 100 and 200 nucleotides, has improved the detection of smaller genetic lesions. The short probe size allows the genome to be interrogated at a very high resolution and arrays can be customized so that specific regions can be intensely examined (50-52). CGH and single nucleotide polymorphism (SNP) arrays or a combination of the two, have been valuable in the discovery of novel chromosomal changes that had previously been unable to be seen due to the limitations of conventional cytogenetic techniques.

SNP arrays in particular have been crucial in broadening our knowledge of the biological changes associated with ALL. Unlike CGH arrays, SNP arrays have oligonucleotide probes that flank known SNPs, originally used for genome-wide marker and linkage association studies for inherited and acquired disease, their ability to detect CNAs as well as to genotype has been exploited in their use in the study of cancer. From paired SNP profiles of a tumour sample and a reference sample, ideally a matched normal sample from the same patient, it is possible to detect LOH in a cancer (53). LOH can arise from a deletion or from a deletion with a reduplication of the non-deleted region. Reduplication of the non-deleted region is termed copy-neutral LOH or acquired uniparental disomy and is unable to be detected by molecular cytogenetics or by CGH array alone. The detection of copy-neutral LOH is especially important in cancer as it may indicate duplication of a mutated gene or a silenced tumour suppressor gene (54).

The first published SNP study in ALL examined ten paediatric cases using the Affymetrix 10K SNP array that examined approximately 11,000 markers (55). However in comparison to the SNP arrays now available this array is considered low resolution given the average distance of 100-200 kb between markers. Nevertheless eight cases of LOH were detected, including a region of chromosome 9p where the *CDKN2A/CDKN2B* tumour suppressor gene resides. Mullighan *et al.* published a much larger study two years later where they examined 242 paediatric ALL samples using three different Affymetrix SNP arrays that together examined over 350,000 markers with an intermarker resolution of less than 5 kb (56). They were also able to run paired constitutional samples that enabled them to determine whether any areas of LOH were inherited or somatic (56). They also showed that ALL was a fairly genetically stable disease with a relatively low number (mean of 6.46 lesions per case) of CNAs identified (56). There was however significant variation in the number of CNAs across the leukaemic subtypes. In particular, gains of DNA were uncommon with the exception of high hyperdiploid ALL, and included amplification of *MYB* in T-ALL and focal internal amplifications of *PAX5* in BCP ALL. Those patients with a *KMT2A* gene rearrangement typically presented in early infancy and had few additional genetic lesions, in contrast to those with *ETV6-RUNX1* and *BCR-ABL1* ALL who presented later in childhood and had more than six lesions per case. These findings, in addition to providing evidence that SNP arrays could be used to enhance the information obtained by conventional cytogenetics, also provided further support to the concept that initiating translocations develop early in childhood and that subsequent changes are required for leukaemogenesis (57).

It is evident that there are many alterations in the genome that are now detectable using microarray technology, but that not all of these changes have an impact on prognosis. *PAX5* is a known target of genetic alteration in B-ALL and has been shown to be affected by a number of mutations including deletions, intragenic amplifications, multiple translocations and sequence mutations (58-60). Despite multiple studies being undertaken, there is still no established link between *PAX5* alterations and outcome. Whilst these changes are somehow involved in the biology of the disease they have little impact on current drug regimes and therefore on prognosis. In contrast, *IKZF1* deletions occur in 80% of Philadelphia-positive (*BCR-ABL1*) BCP-ALL and are associated with a markedly inferior prognosis (61). The deletions occur most commonly in the middle of one allele, creating a dominant negative isoform (62). *IKZF1* deletions and sequence mutations can also occur in Philadelphia-negative BCP-ALL and are also associated with a poor prognosis. A total of 15-30% of BCP-ALL patients have deletions of *IKZF1*, whilst a smaller number have deleterious sequence mutations (63). Either change results in an increased risk of treatment failure, with IKZF1 status shown to be an independent risk factor (63).

There is more than one way that *IKZF1* can be altered, all of which will affect prognosis and response to treatment. These alterations include large deletions of the gene resulting in haploinsufficiency, focal intragenic deletions that result in expression of aberrant dominant-negative IKAROS isoforms (most commonly deletions of coding exons 3-6) and sequence mutations. There is however no single microarray that is able to accurately detect all of these changes. Unless there are sufficient probes with a high enough density along the gene, small, very focal alterations of *IKZF1* may be missed. A customized array approach may be necessary with a higher density along areas of the genome implicated in disease. Customized arrays, although ensuring that the areas of known importance are covered with a sufficient density of probes, inhibit the ability to detect novel changes in other areas of the genome. Novel changes that could potentially be regions for drug-targeted therapy or involved in the pathogenesis of the disease could be overlooked. Next-generation sequencing could overcome this problem.

There also remains the problem of selecting an appropriate reference DNA against which the results are normalized. The best reference DNA is of the patient's

normal genome. This allows the determination of which variants are inherited and which are true somatic mutations specific to the tumour genome. Acquisition of the normal genome can be problematic in the context of leukaemia, given that the individual does not usually have a sample taken for DNA extraction prior to a diagnosis of ALL. Remission samples are usually used as the reference at relapse and can also be used for retrospective screening with a paired diagnostic sample. Alternative references can also be used such as pooled unrelated reference DNA or databases where the results are filtered against known inherited changes. Some authors have recommended the use of a standard collection of normal reference samples that have been extensively characterized such as the HapMap (64). Neither of these alternatives however is ideal as interpretation can be difficult and changes can be misinterpreted in instances where the individual has a rare copy number polymorphism not apparent in the pooled reference sample or when the data from the database is extracted from a less dense probe set (65). The appropriate reference is particularly important in the context of CN-LOH to distinguish it between inherited homozygosity and thereby reduce the number of false calls (66). Correct normalization, taking into consideration the possibility of gross chromosomal abnormalities given that aneuploidy is a common feature of cancer, is also important to prevent misinterpretation of the genome.

The use of arrays in the diagnostic setting is slowly gaining momentum. Conventional G-banded metaphase analysis however still remains the gold standard. A number of laboratories are now using customized arrays in association with FISH for haematological malignancies, however the full implementation of arrays into the diagnostic setting is hampered a little by existing clinical trials. Ongoing clinical trials that commenced with conventional cytogenetics as the methodology of choice must continue using it to enable accurate comparison of data. Attempts are being made to examine existing datasets by array in an attempt to integrate the cytogenetic findings with the CNAs detected by the new technologies. It is envisaged that this will continue and in turn refine the current classification system and therefore improve patient outcome (67).

Next-generation sequencing

Next-generation sequencing has the ability to detect even more novel changes and will undoubtedly play a role in the diagnosis of ALL in the future. Initial studies have shown the power of this technology lies in its ability to discover in an unbiased way tumour-specific somatic mutations that other platforms have not (68). Unlike the existing alternatives such as GEP, CGH or SNP-arrays, sequencing can simultaneously detect copy number, SNP genotypes and sequence mutations (69).

A study of high-risk B-ALL cases by transcriptomic resequencing identified novel gene rearrangements including *STRN3-JAK2* and *NUP214-ABL1* that had previously only been reported in T-lineage ALL. As both of these gene rearrangements result in constitutive kinase activation, their discovery enables the potential of treatment with a tyrosine-kinase inhibitor (70). Undoubtedly other novel genomic alterations will be discovered providing new targets for drug therapy.

As with array technology, the detection abilityof sequencing has also improved. Ley *et al.* published a paper describing the genetic changes detected by whole genome sequencing in an adult AML patient with a normal karyotype (68). Their study showed focal insertions and deletions in two genes and non-synonymous somatic mutations occurring in eight genes. Two years later the same sample was resequenced which resulted in the detection of a previously unidentified frameshift deletion in *DNMT3A* (71). Following this discovery a large number of samples were screened confirming its presence in 22-30% of AML patients. Its discovery has led to a potentially drug-targetable mutation in AML.

There are a number of different types of sequencing that can be utilized, dependent on the information required, these include whole-genome sequencing (WGS), whole-exome sequencing (WES) and messenger RNA sequencing (mRNA Seq). Each sequencing type has its own strengths and weaknesses. WGS requires the preparation of DNA libraries, the amount of starting DNA required being dependent on how the DNA library is constructed—paired-end or mate-pair. It does however permit the detection of variation across the entire genome. This type of sequencing will gives the most complete coverage of the genome, but it does mean that genomic changes with, currently, unknown significance are discovered which are of little use in the diagnostic setting without a direct implication to prognosis or treatment direction. More focused WGS is being used in some of the larger research institutions where areas of known genetic instability are sequenced more deeply than other regions. Whilst this may detect novel changes in the genome, it may overlook other areas that are also implicated in the disease. The difficulty from a diagnostic perspective

is finding the ideal platform or methodology that lies somewhere between the two.

WES is used for studying what is contained within the exome and untranslated regions. As the exome only accounts for ~1.4% of the genome, it enables multiple samples to be pooled and sequenced together (72). Its strength lies in deep coverage of the exome, however it will not detect variation in the non-coding regions.

mRNA Seq will detect mutations as well as rare transcripts and permits the precise quantitation of expressed transcripts. In comparison to GEP, the dynamic range of expression can be more accurately quantified, as well as permitting the detection of rare transcripts (73).

Despite what appears to be an explosion of publications utilizing next-generation sequencing to detect various types of mutations, there are only a small number of studies that have looked at paediatric ALL using whole genome/exome/transcriptome sequencing and that has been restricted to BCP-ALL. These studies however have all focused on specific cytogenetic subgroups or on relapsed samples rather on the group as a whole (74-77). Nevertheless a number of recurrent somatic mutations have been discovered relating to genes encoding for transcription factors, as well as proteins and kinases and have contributed to a better understanding of the leukaemic process.

A key issue with all types of sequencing, for the diagnostic laboratory, is the generation of the data and how to interpret them. As with microarray data there are a number of algorithms employed to normalize the data and if not used appropriately, novel changes may easily be overlooked.

Appropriately paired sample analysis is also important to the understanding of the pathogenesis of disease. They enable novel genetic changes to be observed throughout the disease course and to ascertain whether the CNA is biologically important or merely a passenger in the disease course.

The cost of performing whole genome sequencing has dropped significantly over the last few years, and will continue to do so, making it a viable option for most laboratories in the future. The introduction of next-generation sequencing to the diagnostic setting is only hampered by the fact that there is still so much to learn. Whilst the ability to detect the mutations is clearly important in our understanding of the pathophysiology of the disease, what is not clear is the effect that these novel changes have on the disease course and how they respond to treatment. Functional studies must be diligently undertaken in order for the patient to reap the rewards of the available technology.

Conclusions

The concept of personalized medicine has never been more achievable than with the ability to sequence entire genomes. As the price of the technology continues to fall, it is not unrealistic to suggest that within the next decade children presenting with ALL will receive personalized treatment tailor-made to the sequenced profile of their disease. However in order for this to occur, large cohorts of samples must be sequenced in depth and the resulting novel genomic changes studied closely. In the meantime, due to the additional information that can be obtained with regards to CNAs and LOH, SNP and CGH + SNP arrays will become more utilized in the diagnostic setting most likely at the expense of the more labour-intensive conventional G-banded metaphase analysis. The identification of novel changes will hopefully expand our knowledge of the pathogenesis of ALL, leading to the optimization of therapeutic targets. This in turn will result in the genetic profile being used to detect early markers of disease, to risk stratify and most importantly to direct therapeutic management in ALL so that all children can expect to be five year survivors.

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Footnote

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