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Cytogenetics and Molecular Genetics of Acute Lymphoblastic Leukemia

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a neoplastic disease characterized by clonal expansion of leukemic cells in the bone marrow (BM), lymph nodes, thymus, or spleen. ALL is a genetic disease because essentially most patients harbor acquired genetic alterations (somatic mutations) that contribute to the increased proliferation, prolonged survival, and/or impaired differentiation of the lymphoid hematopoietic progenitors. In the majority, albeit not all, patients diagnosed with ALL, one or more of these genetic alterations are in the form of nonrandom numerical or structural chromosome aberrations that can be detected microscopically [1,2]. The application of contemporary genome-wide molecular analyses continues to reveal many additional genetic rearrangements that are not detectable cytogenetically [3].

Several of the ALL-specific chromosome aberrations and their molecular counterparts have been included in the 2008 World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues (Table 1), and together with morphology, cytochemistry, immunophenotype and clinical characteristics are being used to define individual disease entities within B-lineage ALL (B-ALL) [4]. Although several specific recurrent chromosome aberrations and gene mutations also occur in T-lineage ALL (T-ALL), at present they are not used to delineate separate entities within T-ALL [5].

Notably, cytogenetic and, increasingly, molecular genetic findings at diagnosis constitute important, independent prognostic factors in both childhood and adult ALL [6–17].

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Accordingly, cytogenetic and molecular analyses are considered obligatory for analyzing outcome of many clinical trials, and detection of specific chromosome aberrations and/or their molecular equivalents, such as t(9;22)(q34;q11.2) and *BCR-ABL1*, is used to assign ALL patients to specific targeted therapy [18]. In the first part of this article, we will briefly describe the cytogenetic methodology, followed by a summary of major cytogenetic findings and their clinical relevance in ALL. In the second part, we will review modern molecular techniques and their application in the research on genetics and epigenetics of ALL.

CYTOGENETICS

Standard cytogenetic analysis

This technique reveals all microscopically detectable chromosome aberrations occurring simultaneously in leukemic cells, regardless of whether these aberrations are numerical or structural, or, in the case of the latter, balanced or unbalanced. To obtain analyzable metaphase cells, pretreatment samples are subjected to unstimulated short-term (24- or 48-hour) cultures *in vitro*, at the end of which the cells are treated with a compound that arrests dividing cells in metaphase (e.g., colcemide), hypotonic solution and repeated changes of fixative solution. Thereafter, microscope slides containing metaphase spreads are made, appropriately aged, stained using banding techniques (most often G-banding), and analyzed under microscope. Twenty or more metaphase cells are usually analyzed. While in cases with an abnormal karyotype analysis of <20 cells can be acceptable, for a case to be reliably determined as cytogenetically normal, an analysis of at least 20 karyotypes from a BM sample is required [19]. To be considered relevant, the identified chromosome aberrations must be clonal, i.e., present in a minimum of two metaphase cells in the case of an identical structural aberration and gain of the same, structurally intact chromosome (trisomy), or in at least three cells in the case of a missing chromosome (monosomy) [20].

Fluorescence *in situ* hybridization (FISH)

Many chromosome aberrations recurrent in ALL can also be detected using molecular-cytogenetic techniques, such as FISH. This method employs cDNA or genomic DNA fragments (probes) complementary to specific sequences in the human genome that are labeled with fluorochromes and hybridized to fixed metaphase chromosomes and/or interphase nuclei. The locations of the fluorescent signals are then visualized using a fluorescence microscope. Several types of probes are available, including 1) centromeric probes consisting of chromosome-specific DNA repeats (satellite DNA), which are useful for detection of numerical aberrations such as trisomies and monosomies; 2) whole chromosome painting (WCP) probes containing numerous unique DNA sequences capable of binding to the entire length of specific chromosomes, which allow the identification of individual chromosomes or their parts participating in structural aberrations in metaphase cells; and 3) locus-specific probes hybridizing to particular sequences within individual genes - they are used to detect recurrent structural abnormalities such as translocations, inversions, or deletions [21].

FISH, as well as molecular genetic techniques, such as reverse transcriptase polymerase chain reaction (RT-PCR), are especially valuable in patients for whom standard cytogenetic analysis yields no analyzable metaphase cells or only a few cells of poor quality. They are also essential for identification of cytogenetically cryptic abnormalities, a prime example of which is t(12;21)(p13;q22), a translocation that involves the juxtaposition of similarly banded regions and thus cannot be discerned reliably in G-banded preparations [22]. Additionally, both FISH and RT-PCR are invaluable for detection or confirmation of suspected variants of recurrent aberrations, such as cryptic insertions between seemingly

intact chromosomes 9 and 22 leading to the *BCR-ABL1* gene fusion [23], which in most ALL patients is generated by the typical t(9;22)(q34;q11.2).

Multicolor FISH

In a portion of ALL patients, cytogenetic preparations are of suboptimal quality, with poor chromosome morphology and indistinct banding, making the interpretation of karyotypes difficult. In these and some good quality cases, the karyotype may contain aberrations unrecognizable using G-banding, e.g., marker or certain ring chromosomes, or only partially recognizable, e.g., an unidentified chromosome segment attached to a known chromosome, usually designated “add”. Analysis of such cases can be greatly aided by application of multicolor FISH techniques. Both spectral karyotyping (SKY) and multiplex FISH (M-FISH) utilize WCP probes specific for each of the 22 pairs of autosomal chromosomes as well as the sex chromosomes X and Y, and allow simultaneous display of all chromosome pairs in different colors. Studies of pediatric ALL that applied SKY or M-FISH, complemented in some instances by FISH with locus-specific probes, identified cryptic translocations, and revealed the origin and chromosomal composition of marker and derivative chromosomes [24–27]. In ALL patients with high hyperdiploidy with poor chromosome morphology, SKY allowed accurate characterization of all numerical aberrations and of the chromosomal origin of segments involved in interchromosomal structural rearrangements [28]. In patients with t(12;21), SKY revealed nonrandom unbalanced translocations of chromosome 6 [29]. Nordgren et al. [25] concluded that although in most instances the combination of standard cytogenetic analysis and interphase FISH was sufficient for the detection of prognostically relevant chromosome aberrations, SKY (and M-FISH) can greatly improve the accuracy of karyotype interpretation, especially in patients with very complex chromosome rearrangements.

Success rates of cytogenetic analyses and rates of aberration detection in ALL

Meaningful results of standard cytogenetic analysis can be obtained in most patients with ALL. In large studies of adult ALL, between 70% and 75% of samples analyzed cytogenetically were deemed successful [13–15,19]. In one-third of cases with results described as unsuccessful, the sample processed cytogenetically yielded no or only a few analyzable mitotic cells, whereas in the remaining cases, the karyotypes were obtained but were considered to be of too poor quality to allow unambiguous interpretation [15,19]. Higher success rates, 83% and 91%, were reported by two large studies of childhood ALL [9,30]. Importantly, bone marrow samples are preferable to blood ones since the latter had a significantly higher rate of unacceptable cytogenetic results compared with BM [11,14]. As shown in a very large cooperative UKCCG study of over 2,300 pediatric ALL patients, the success rate can be increased by the use of interphase FISH with probes capable of detecting prognostically relevant chromosome abnormalities from 83% when only G-banding was applied to 91% when interphase FISH was carried out on the fixed cell suspensions used for cytogenetic analysis [30].

Among successfully analyzed patients, one or more clonal aberration has been detected in 60% to 79% of adults [13–15,19], and 57% to 82% of children [9,30,31] with ALL. In general, the rates of aberration detection were higher in more recent series compared with the earliest ones [14,19], and, as mentioned before, they can be further increased by the use of FISH and SKY.

Prognostic relevance of cytogenetics in ALL

The first major study demonstrating the independent prognostic significance of cytogenetic findings at diagnosis in ALL was the Third International Workshop on Chromosomes in Leukemia [6,7,10]. Subsequent studies confirmed the Workshop’s results and refined them

by providing data on clinical relevance of further recurrent aberrations as well as elucidating the molecular basis and biological consequences of many of these aberrations. Table 2 summarizes the incidence and prognostic impact of the major cytogenetic findings in childhood and adult ALL.

High hyperdiploidy, i.e., karyotypes containing modal chromosome number of 51 to 67 chromosomes, defines one of the largest cytogenetic subsets of childhood ALL, comprising 25–30% of patients with B-ALL [32]. High hyperdiploidy is less frequent in adult ALL, being seen in 2–10% of patients [8,14], and it is very rare in T-ALL. The distribution of specific chromosome gains is nonrandom, with the most often gained chromosomes 21, X, 14, 6, 18, 4, 17 and 10, each of which is gained in over 50% of hyperdiploid ALL patients, followed by chromosomes 8, 5, 11, and 12, gains of which occur more often in patients with 57 or more chromosomes [33]. The prognosis of children with high hyperdiploidy is excellent, with CR rates approaching 100% in some studies, the 5-year event-free survival (EFS) rates between 71% and 83% [9,34–36] and 5-year overall survival (OS) rates of approximately 90% [35,36]. Outcome of adults with high hyperdiploidy has been improved in some [8,14], but not all [12,13], studies in relation to other cytogenetic groups, but it is not comparable to the excellent outcome of children with high hyperdiploidy. In the latter age group, prognosis may also be influenced by specific cytogenetic features of the hyperdiploid karyotype. Patients with the concurrent presence of +4, +10 and +17 have been reported to have especially favorable prognosis [37], as were those with +4 and +18 in another study [35]. On the other hand, the relatively rare patients with recurrent translocations, such as t(9;22)(q34;q11), t(1;19)(q23;p13) or translocations involving 11q23, have outcomes similar to non-hyperdiploid patients with these aberrations [35,38], and thus are often excluded from the high hyperdiploidy category. Approximately one-half of the high hyperdiploid patients harbor other structural aberrations, such as duplications and gains of 1q, del(6q) or i(17)(q10), but their presence does not seem to influence prognosis [35,39], with a possible exception of prognostically adverse i(17)(q10) [39]. Importantly, since hyperdiploid leukemic cells sometimes fail to proliferate in culture, ALL patients with unsuccessful cytogenetic analysis or those with normal karyotypes should be analyzed using FISH with centromeric probes, flow cytometry to measure DNA index or single nucleotide polymorphism genomic microarray [30,32,40].

Another large cytogenetic subset of pediatric ALL is characterized by the presence of a cryptic t(12;21)(p13;q22)/*ETV6-RUNX1*(*TEL-AML1*). While this translocation, detectable using FISH and/or RT-PCR, occurs in approximately 25% of children with pre-B-ALL [36,41], it is almost non-existent in adult ALL. The prognosis of children with t(12;21) is excellent, both among patients with standard-risk and those with high-risk ALL according to the NCI criteria, with 94% of the patients experiencing rapid early responses to therapy [36,41]. Three-fourth of t(12;21) patients harbor additional genetic changes, most often deletions of 12p with loss of the *ETV6* gene (in 55–70% of patients), +21 (in 15–20%) and an extra der(21)t(12;21) (in 10–15%) [42,43]. Stams et al. [43] reported that disease-free survival of patients with +der(21)t(12;21) and those without secondary aberrations was worse than DFS of patients with del(12p) and +21, whereas in a study of Attarbaschi et al. [42] worse EFS was associated with a secondary deletion of non-translocated *ETV6* allele. Further studies are needed to clarify the prognostic role of secondary aberrations in B-ALL patients with t(12;21).

The most frequent abnormality among adults with ALL is the Philadelphia chromosome (Ph), i.e., t(9;22)(q34;q11.2)/*BCR-ABL1*, which is detected in 11–29% of patients [8,11–15]. In contrast, t(9;22) is relatively rare in children (1–3%) [9,31,44]. With rare exceptions [13,14], Ph+ patients are diagnosed with B-ALL. Prognosis of both adults and children treated with standard chemotherapy is very poor, with less than 5% of adults being cured

[18]. The only potentially curative therapy is allogeneic hematopoietic stem cell transplantation (HSCT), although this procedure is associated with increased treatment related mortality [45]. Administration of imatinib as part of induction therapy resulted in higher rates of complete remission (CR) and improved survival of patients who underwent transplantation [46,47], although patients receiving imatinib may develop resistance to the drug and relapse. Other tyrosine kinase inhibitors (e.g., dasatinib) that may overcome the mechanisms of resistance are currently being tested [18]. Approximately two-thirds of newly diagnosed patients harbor one or more secondary chromosome aberrations in addition to t(9;22), most frequently an extra copy of der(22)t(9;22), -7 or loss of 7p arm, an abnormality of 9p, +21, +8, and +X. High hyperdiploidy is detected in approximately 15% of Ph+ patients [14,48,49]. The presence of secondary aberrations as such, regardless of type, when compared with a sole t(9;22), did not affect prognosis in pediatric [48] or adult [49] studies in the pre-imatinib era, but it was found to be an independent, adverse prognostic factor in adult Japanese patients receiving imatinib as part of their treatment [50]. In that latter study [50], both +der(22)t(9;22) and abnormalities of 9p had a negative impact on disease-free survival. Abnormal 9p portended worse outcome also in other studies [45,48,51], whereas +der(22)t(9;22) conferred poor prognosis in some [49] but better outcome in other [45] studies. Notably, half of the patients with +der(22)t(9;22) analyzed by Fielding et al. [45] also had high hyperdiploidy, which was suggested to confer improved outcome in both adult [51] and pediatric [48] series. The presence of secondary -7 was associated with lower complete remission (CR) rates in the pre-imatinib era [49,51]; it is unclear if this is also the case in Ph+ patients treated with imatinib.

Translocations involving band 11q23/*MLL* are detected in two-thirds of infants with ALL [52]. Their incidence in older children and adults is much lower, 1–2% and 4–9%, respectively [8,9,11–14,38]. By far the most common among 11q23 rearrangements is t(4;11)(q21;q23)/*MLL-AFF1(AF4)*, detected in over 50% of patients, followed by t(11;19)(q23;p13.3)/*MLL-MLLT1(ENL)* and, less commonly, t(9;11)(p22;q23)/*MLL-MLLT3(AF9)*, t(10;11)(p13–15;q14–21)/*MLL-MLLT10(AF10)* and others [52,53]. The t(4;11) predicts a poor prognosis in both children and adults, with particularly dismal outcome in patients with a poor early response to prednisone, infants aged <3 months and older adults [8,11–14,54,55]. The outcome of patients with t(11;19) is also generally poor, especially in infants under the age of 1 year. In older children with t(11;19), those with T-ALL had a better outcome than patients with B-ALL [54]. Secondary aberrations are detected in one third of patients with t(4;11), the most frequent of which are +X, abnormalities of 7p [including i(7)(q10)] and 9p and +8 [53,55]. Similarly, secondary chromosomal abnormalities are detected in one-half of patients with t(11;19) who most often carry secondary +X, +8 and del(6q) [53]. A large, multi-institutional study has conclusively shown that secondary aberrations do not affect prognosis of infants or older children with ALL and t(4;11), t(11;19)(q23;p13.3), or other 11q23 translocations, which were analyzed as a one group [53].

Translocation (1;19)(q23;p13.3)/*TCF3(E2A)-PBX1* occurs in 1–3% of adult [8,11,13,14,56] and 1–6% pediatric ALL [9,38,57,58], and can be in either balanced or unbalanced form, as der(19)t(1;19) with 2 normal chromosomes 1. Most patients have pseudodiploid karyotypes, and almost all are diagnosed with pre-B ALL. Outcome of patients with t(1;19) is controversial. In adult studies it was reported as poor [8,13], relatively favorable [11] or not different from the outcome of Ph-negative patients without t(1;19) [14]. In pediatric series, the initially unfavorable prognosis, especially for the patients with balanced t(1;19) [57], has been improved by the use of more effective therapies [44,58]. However, t(1;19)/*TCF3-PBX1* was found to be an independent risk factor for isolated CNS relapse in children [58]. A recent study of adult ALL has suggested that prognosis of patients with t(1;19) can be markedly improved by the hyper-CVAD regimen [56].

Recent studies identified an intrachromosomal amplification of chromosome 21 (iAMP21) as a novel recurrent abnormality in B-ALL [59–61]. Approximately 2% of children and <0.5% of adults display iAMP21. In contrast to acute myeloid leukemia (AML), where similar iAMP21 does not involve the amplification of the *RUNX1* gene [62,63], virtually all ALL patients with iAMP21 show multiple extra copies of *RUNX1* in structurally rearranged abnormal chromosomes, which are composed of chromosome 21 material only [59–61]. The outcome is poor, with ALL patients with iAMP21 having a 3-fold increased risk of relapse and twice the risk of death compared with those without this abnormality [61].

Complex karyotype, defined as 3 or 5 chromosome aberrations, is a well-established adverse prognostic factor in AML [64]. Only few studies investigated prognostic significance of a complex karyotype in ALL. While Wetzler et al. [49] did not detect any impact of karyotype complexity (with 3 or 5 aberrations) on cumulative incidence of relapse or OS of Ph-positive patients, Moorman et al. [14] reported that ALL patients with a complex karyotype with 5 aberrations who did not harbor an established translocation (4% of adults with ALL) had a significantly inferior EFS and OS, and that complex karyotype was an independent adverse prognostic factor within the Ph-negative patient cohort. This observation requires confirmation.

T-ALL occurs in 16–25% of adult [4,8,11,12–14] and 8–15% of childhood ALL [4,9,38]. At diagnosis, the proportion of cytogenetically normal cases is higher in T-ALL than B-ALL, with approximately 50% of T-ALL patients having a normal karyotype. Roughly one-third of T-ALL patients have a translocation involving one of the T-cell receptor genes (*TCR*), with a breakpoint at 14q11 (*TCRA/TCRD*) or 7q34 (*TCRB*). The most common of these translocations in adults is t(10;14)(q24;q11.2), which results in overexpression of the *TLX1* (*HOX11*) gene, and is associated with a favorable outcome [8,12]. Detected in 3–6% of childhood T-ALL, t(1;14)(p32;q11.2) juxtaposes *TCRD* and *TAL1* (*SCL*) resulting in overexpression of *TAL1*. *TAL1* overexpression is also brought about by a cryptic deletion of chromosome 1, which causes a *TAL1-STIL* fusion. In pediatric T-ALL, *TAL1-STIL* fusion occurs in 16–26% of the cases, making the combination of the two rearrangements involving *TAL1* the most frequent abnormality in childhood T-ALL [65,66]. In contrast, the frequency of these rearrangements in adult T-ALL is very low [66]. Similarly, a cryptic t(5;14)(q35;q32), juxtaposing *TLX3* to *BCL11B*, occurs in 20–30% of pediatric T-ALL; but is less common in adults [2,67].

A novel genetic phenomenon in T-cell ALL, namely cryptic extrachromosomal amplification of a segment from chromosome 9 containing a fusion between *ABL1* and *NUP214* (nucleoporin) was recently described [68–70]. The amplified *NUP214-ABL1* sequences are located on submicroscopic circular extrachromosomal DNA molecules called episomes. *NUP214-ABL1* is a constitutively activated tyrosine kinase activating similar pathways as *BCR-ABL1*, and is sensitive to inhibition with tyrosine kinase inhibitors, especially nilotinib and dasatinib [71]. Notably, essentially all patients harbor rearrangements of *TLX1* or *TLX3* or their ectopic expression, and most also carry hemi- or homozygous deletions of *CDKN2A* and *CDKN2B*, and chromosome aberrations, including +8, t(7;10)(q35;q24) or t(10;14)(q24;q11). In a study of Graux et al. [70], the estimated 5-year OS rate was 49%±11%.

MOLECULAR GENETICS

Expression microarray analysis

Array-based gene-expression profiling (GEP) combines synthesis of cDNA from the entire mRNA transcriptome with DNA array technology to evaluate the entire “transcriptome” of samples. Rather than evaluating changes in copy number or sequence of the nuclear DNA,

GEP is used to determine the level at which genes are expressed in samples compared with controls. Sample cDNA is generated from mRNA, labeled with fluorochromes, and subsequently hybridized to chips spotted with probes corresponding to known transcripts. Fluorescent signals are captured and analyzed. The intensity of the signal at each spot corresponds to the amount of cDNA and thus mRNA or expression of the gene targeted by the spot on the probe.

Retrospective studies have demonstrated that expression profiling can be effective in classifying lymphoblastic leukemias into recognized, prognostically important subtypes including *BCR-ABL1*, *TCF3-PBX1*, hyperdiploid, 11q23/*MLL* rearranged, *ETV6-RUNX1*, and T-ALL [16,72,73]. Within T-ALL overexpression of the *Hox11* ophan homeobox gene in both pediatric and adult cases was associated with excellent prognosis when treated with modern combination chemotherapy, while cases at high risk of early relapse failure are included largely in the TAL1- and LYL-positive groups [Ferrando, 2003 #762]. GEP has also identified a new subtype of ALL without *BCR-ABL1* fusion but with a similar expression profile to *BCR-ABL1* leukemia [73]. In addition, GEP has shown that lymphoblastic leukemias with *MLL* rearrangements have a unique gene expression profile separate from other ALL and AML [74]. GEP has been retrospectively used to identify gene expression signatures at diagnosis that were predictive of early response and long-term outcome in NCI defined high-risk childhood ALL. These profiles were generated from patient samples treated on COG 1961 and then validated in three independent cohorts from POG trials, the German Cooperative Study Group for Childhood ALL (COALL) and the Dutch Childhood Oncology Group (DCOG) protocols. Although the expression profiling correlated with outcome in univariable analysis, they lost significance when known predictors of outcome like age, WBC, and genotype were accounted for, suggesting that the profiling does not predict outcome beyond previously established prognostic factors in high-risk pediatric precursor B-ALL [75]. However, retrospective GEP in conjunction with flow cytometry, and SNP array analysis has recently been used to identify a subtype of T-ALL (early T-cell precursor, ETP-ALL), which has a poor prognosis with standard intensive chemotherapy [76]. It remains to be seen, whether augmented therapy, like HSCT in first remission will benefit patients prospectively identified with ETP-ALL by GEP.

Although expression arrays analyze gene expression, and thus could potentially identify highly differentially expressed genes as target entry points for biological studies, for the most part expression profiling has been used for classification of leukemias, rather than the identification of genes whose aberrant expression may be important in leukemic transformation. Exceptions include *HOXA9* [77], *MEIS1* [78], and *FLT3* [79] in *MLL* leukemias, and the erythropoietin receptor in *ETV6-RUNX1* leukemias [73].

Expression of specific genes identified as aberrantly expressed in other malignancies has shown that low *ERG* (v-ets erythroblastosis virus E26 oncogene homolog) and *BAALC* (brain and acute leukemia, cytoplasm) identify adult T-ALL patients with a distinctly favorable long-term outcome [Baldus, 2007 #685].

Array based comparative genomic hybridization

Array based comparative genomic hybridization (a-CGH) is a recently developed technology that combines the ability to screen the entire genome like conventional comparative genomic hybridization (CGH) with the ability to detect small variations in DNA copy number. This broad-spectrum, high-resolution technique uses cloned DNA fragments of relatively large size (100–200 kb), or, more recently, long (60–75 nucleotides) oligonucleotides spotted onto a solid matrix. The resolution of this technique is determined by the size of and distance between the clones used to construct the array. Test sample and control DNA are labeled with different fluorochromes and then hybridized to the DNA on

the array. The fluorescent signals are captured from each spot on the array and analyzed. The fluorescent signal ratio is used to determine gain or loss of test DNA compared with the control DNA at each spot on the array. For example, if test DNA was labeled with a green fluorochrome, and control DNA with a red fluorochrome, a predominantly green signal at a spot would indicate gain of test DNA at that region. A yellow signal would indicate relatively equal amount of test and control DNA, and a red signal, loss of test DNA at the corresponding region [80]. A-CGH augments traditional karyotyping in the detection of deletions or amplifications smaller than those that can be found by banding techniques, and allows analysis of samples for which metaphase chromosomes are not available. For example, using a tiling path 33K BAC array, Kuchinskaya et al. [81] detected copy number alterations, several of which were below the resolution of standard G-banding analysis, in ~80% of pediatric ALL patients with either a normal karyotype or an unsuccessful cytogenetic analysis. Two other studies reported patterns of genomic imbalances in Down syndrome children with ALL [82,83].

Single Nucleotide Polymorphism (SNP) array analysis

Single nucleotide polymorphism (SNP) arrays are oligonucleotide arrays with probes specific for regions flanking SNPs. When labeled genomic DNA from an individual is hybridized to the array, the DNA will bind with greater frequency to the probes that correspond to that individual's SNPs, and those regions of the chip will fluoresce with greater intensity. These chips were designed for genome wide screens to identify SNP linked to inherited or acquired disease [3]. However, recently, using these types of chips, researchers have been able to identify new leukemia associated genes by identifying small regions of acquired deletions, amplifications, and uniparental disomy in acute leukemias [40,84,85] and associated some of these genetic aberrations with high-risk leukemias [17,86].

Some important general themes are revealed by these studies in addition to the specific genes identified. First, high resolution analysis of SNP-arrays confirms pre-established cytogenetic anomalies and identifies submicroscopic anomalies, including unbalanced chromosome translocations. Thus, some leukemias with unidentified lesions on routine cytogenetic analysis can be shown to have common lesions such as a t(12;21) when evaluated at a higher resolution [85]. In addition, SNP-chip analysis permits more accurate detection of hyperdiploid ALL than the DNA index method [40]. Second, multiple independent copy number aberrations are present in the leukemic samples, suggesting that multiple complementary genetic lesions are required for leukemic transformation. Third, although there seems to be multiple aberrations in each leukemia, the overall numbers of aberrations are not very high. For example, one study identified a mean of 6.46 aberrations per leukemia [84], while another study identified a mean of 4.2 for B-lineage ALL and 2.6 for T-ALL [85]. These findings suggest that although multiple mutations are associated with leukemic transformation, marked global genomic instability is not an underlying mechanism leading to leukemic transformation. Interestingly, multiple studies also showed significant differences in the frequency of aberrations among specific ALL subtypes [17,84] with average frequencies ranging from 1 in 11q23/*MLL* rearranged leukemias to 11 in hyperdiploid ALL with >50 chromosomes.

Finally, the study of sub-microscopic aberrations has identified aberrations in a number of genes involved with B-cell differentiation and cell cycle regulation. Two SNP-array studies of pediatric ALL reported frequent deletions or other inactivating mutations in regulators of B-cell development, with the paired box gene *PAX5*, mapped to 9p13.2, being the most frequent target in B precursor leukemias [84,85]. The larger study, which also included resequencing of *PAX5* in the entire cohort, found this gene to be altered in 32% of precursor B-ALLs. Mutations in order of frequency included focal deletions of all or part of *PAX5*,

deletions of chromosome 9 or 9p, broad deletions involving *PAX5* and flanking genes, and large 9p deletions involving the 3' region of *PAX5*. Although various mechanisms were involved, including lack of expression of the altered allele, altered proteins (including 3 different fusion proteins) that lack either the DNA-binding domain or transcriptional regulatory domain, or promoter mutations, all of the mutations resulted in loss of function of *PAX5* [84]. Additional regulators of B-cell development found mutated in both studies include *EBF1* and *IKZF1* (Ikaros). The larger study found frequent mutations in additional transcription factors associated with B-cell development including *AIOLOS*, *LEF1*, *RAG1*, and *RAG2*. A third, and larger still, study of pediatric ALL also found *PAX5* frequently mutated but the other genes were not found to be mutated at high frequencies [40].

In addition to B-cell differentiation mutations, SNP arrays confirmed previously known involvement of cell cycle regulation gene mutations in leukemogenesis. *CDKN2A* located at 9p21.3, encodes *p16^{INK4a}* and *p14^{ARF}*. This region was frequently mutated in SNP-array studies in leukemia [40,84,85]. *P16* deletion or inactivation was associated with both B-ALL and T-ALL, although it was more common in T-ALL [40,85]. *P16* deletion was due to monosomy 9, 9p deletions, submicroscopic deletions, or uni-parental disomy of 9p in one study [40]. This study also found a high frequency of 12p/*ETV6* deletions, two-thirds of which were in leukemias with *ETV6/RUNX1*.

More recently, *IKZF1* inactivating mutations (complete deletions, or partial deletions, some of which result in a dominant negative $\Delta 3-6$ isoform) have been found by SNP-arrays in 84% of ALLs with *BCR-ABL1*, but not in CML. Additionally, *IKZF1* mutations were found to be acquired at conversion from a chronic phase CML to blast crisis in a small sample where DNA was available pre and post conversion [86]. Subsequently, these investigators also found that *IKZF1* inactivating mutations are associated with poor outcomes in pediatric ALL without *BCR-ABL1*. In two large independent cohorts, one of high-risk ALL (including patients with CNS or testicular disease, 11q23/*MLL* rearrangements, age >10 years, or high WBC), and the second consisting of both standard and high-risk patients, *IKZF1* inactivating deletions were associated with minimal residual disease, hematologic relapse, and any relapse. Interestingly, leukemia with *IKZF1* mutations without *BCR-ABL1* has gene expression signatures similar to *BCR-ABL1*-positive ALL [17].

Resequencing of candidate genes

Resequencing of candidate genes has identified a large number of genes that are frequently mutated in specific subgroups of ALL (Table 3). Resequencing requires *a priori* knowledge of a candidate gene that may be mutated. Once a target gene is identified, primers specific to the gene or suspected region of the gene are used to amplify the gene of interest, which is then sequenced and compared to the known sequence of the gene. Several genes, first identified as targets by SNP arrays, have been shown to also harbor small mutations in ALL samples. Some of these, including *PAX5* [84], *IKZF1* [84], and *CDKN2A* [87], have been further studied by resequencing, with identification of additional mutations in each case. These mutations are typically missense or nonsense mutations, or microdeletions that most commonly result in loss of function of the gene product.

Mutations that result in gain of function or constitutive activation of proteins can also be found by resequencing. Mutations in *FLT3* and multiple genes involved in the RAS/MAPK cascade have been shown to have acquired activating mutations in ALL. *FLT3* activating mutations are present in up to 15% of ALLs with *MLL* rearrangements [79]. These mutations result in constitutively active FLT3, a membrane receptor tyrosine kinase important for early hematopoietic development. Additionally, *FLT3* mutations have been found in 3–10% of unselected cases of ALL, and seem to be associated with the high hyperdiploid subgroup [88–90]. *PTPN11*, *KRAS*, and *NRAS* also have activating point

mutations in a subset of ALL patients, and are frequently associated with high hyperdiploid leukemia, being present in approximately 7%, 16%, and 17% of patients, respectively [88–91]. These mutations are usually mutually exclusive, suggesting that a single mutation resulting in constitutive activation of the receptor tyrosine kinase – RAS pathway is sufficient to dysregulate this pathway in leukemogenesis. Together these mutations are present in approximately 30% of high hyperdiploid pediatric ALLs [89].

NOTCH1, a gene demonstrated to be important in lymphocyte lineage specification, was first identified as a potential oncogene in T-ALL as a translocation partner in the rare t(7;9) (q34;q34.4) found in <1% of T-ALL [92]. Resequencing of functional domains of *NOTCH1* revealed that activating *NOTCH1* mutations are present in the majority of primary pediatric and adolescent T-ALL, with mutations occurring in all of the molecular subtypes of T-ALL [93]. These mutations were found to involve either the heterodimer domain (HD) or the PEST domain of the NOTCH1 protein. Mutations involving the HD, which is responsible for stable noncovalent association between the extracellular and transmembrane components of inactivated NOTCH1, produce ligand independent activation of NOTCH1. Frameshift mutations result in complete or partial loss of the C terminal negative regulatory PEST domain [93], and increased levels of intracellular NOTCH1 due to impaired degradation of the activated receptor. More recently, a third type of activating *NOTCH1* mutation, internal tandem duplication of the juxtamembrane portion, has been identified in 7 of 210 primary T-ALL samples from pediatric and young adult T-ALL patients. Like HD mutations, these result in constitutive ligand independent activation of NOTCH1 [94]. NOTCH1 seems to control cell proliferation by regulating several signaling pathways such as NFκB [Osipo, 2008 #763] and PI3K-AKT-mTOR [Chan, 2007 #764]. Interestingly, mutational loss of PTEN can bypass the requirement for NOTCH1 signaling [Palomero, 2007 #765]. These data suggest that a comprehensive phenotyping is needed to better understand the leukemogenic events and further design of future therapeutic strategies.

Epigenetic changes associated with ALL

DNA methylation, a type of reversible epigenetic regulation that typically results in downregulation of genes is prevalent in cancer. Analysis of single gene and genome wide analysis have revealed that aberrant DNA methylation is common in both adult and pediatric ALL. Methylation of multiple gene promoters lead to downregulation of tumor suppressor genes or pathways, one of the best described is the methylation and loss of expression of *CDKN2A* and *CDKN2B* [95].

Timing of mutations associated with ALL

Although little is known about proximate causes of small point mutations associated with ALL, the timing of some gross chromosomal rearrangements can be identified in certain cases of childhood ALL. Studies of monozygotic twins with leukemia have used clone-specific gene rearrangements, such as unique breakpoint regions in *MLL* or *ETV6-RUNX1* fusions, or unique immunoglobulin heavy chain or T-cell receptor gene rearrangements, to demonstrate that leukemias in each twin developed from a single, identical clone. In all likelihood, the leukemia initially developed in one twin *in utero*, and subsequently “metastasized” via the shared placental circulation to the second twin [96]. Guthrie cards (absorbent cards onto which neonatal blood spots are collected for metabolic and/or genetic screening) have been retrospectively evaluated in pediatric leukemia patients. These studies revealed that all studied ALLs with *MLL-AFF1* fusions, most with *ETV6-RUNX1* fusions, some hyperdiploid, and some with *TCF3-PBX1* fusions develop prenatally [97].

Perspectives for the future

Recently, the entire genome of leukemic cells from a cytogenetically normal AML sample was sequenced and compared to the patient's constitutional genome established from a skin biopsy. Although the entire genome was sequenced, the initial report focused on analysis of known gene coding sequences. Ten single nucleotide variants were found in the leukemic cells compared to the skin cells, and eight of these were in genes not previously associated with AML [98]. Although in its infancy, rapid advances in high throughput "next generation sequencing", suggest that whole genome sequencing of ALL samples may become rapidly available and contribute to risk stratification in the not too distant future.

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Table 1

World Health Organization classification of acute lymphoblastic leukemia

Category in WHO Classification
PRECURSOR LYMPHOID NEOPLASMS
B lymphoblastic leukemia/lymphoma
B lymphoblastic leukemia/lymphoma, NOS
B lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
B lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2); <i>BCR-ABL1</i>
B lymphoblastic leukemia/lymphoma with t(v;11q23); <i>MLL</i> rearranged
B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); <i>TEL-AML1 (ETV6-RUNX1)</i>
B lymphoblastic leukemia/lymphoma with hyperdiploidy
B lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)
B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); <i>IL3-IGH</i>
B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); <i>E2A-PBX1 (TCF3-PBX1)</i>
T lymphoblastic leukemia/lymphoma

Data from Swerdlow SH, Campo E, Harris NL, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Lyon, France, IARC, 2008.

Table 2

Major chromosome aberrations in pediatric and adult acute lymphoblastic leukemia: frequency and prognostic significance

Chromosome aberration/ genes involved	Children		Adults	
	Frequency [reference no.]	Clinical outcome [reference no.]	Frequency [reference no.]	Clinical outcome [reference no.]
High hyperdiploidy	23–30% [9,34,32,36,42]	Favorable [9,34–36,38]	7–8% [8,11,13,14]	Favorable [8,11,14] Intermediate [13]
Hypodiploidy	6% [9]	Intermediate for patients with 45 chromosomes [99] Adverse for patients with <45 chromosomes [99] Intermediate for patients with <46 chromosomes [9]	7–8% [8,11,15]	Adverse [8,11]
Near-haploidy	0.4–0.7% [9]	Adverse [9,99]	Rare	Not determined
t(12;21)(p13;q22)/ <i>ETV6-RUNX1 (TEL-AML1)</i>	22–26% [36,41,100]	Favorable [36,41,100]	0–4% [101,102]	Not determined
t(9;22)(q34;q11.2)/ <i>BCR-ABL1</i>	1–3% [9,44]	Adverse [9,36,38,48]	11–29% [8,11–15]	Adverse [8,11–15]
t(4;11)(q21;q23)/ <i>MLL-AFF1 (AF4)</i>	1–2% [9,38] 55% of infants [52]	Adverse [9,38,52]	4–9% [8,11–14]	Adverse [8,11–14]
t(1;19)(q23;p13.3)/ der(19)t(1;19)(q23;p13.3)/ <i>PBX1-TCF3 (E2A)</i>	1–6% [9,38,57,58]	Favorable [9] Intermediate [38,57]	1–3% [8,11,13,14,56]	Favorable [56] Relatively favorable [11] Intermediate [14] Adverse [8,13]
t(10;14)(q24;q11)/ <i>TCRA/TCRD-TLX1 (HOX11)</i>	Rare	Not determined	0.6–3% [8,12,13]	Favorable [8,11] Intermediate [14]
del(6q)	6%–9% [9,103]	Not prognostic [9,103]	3–7% [8,11–14]	Intermediate [8,13,14]
Abnormal 9p	7–11% [9,104]	Not prognostic [9] Adverse [104]	5–15% [8,11,12,14]	Favorable [14] Relatively favorable [12,13] Intermediate [8,11]
Abnormal 12p	3%–9% [9,38,105]	Not prognostic [9,38, 105]	4–5% [8,11,12,14]	Favorable [11,12]
Normal karyotype (no aberration detected)	31–42% [9,38,99]	Relatively favorable [38,99]	15–34% [8,11–14]	Relatively favorable [8,12–14] Intermediate [8,14]

Table 3

Genes commonly mutated in acute lymphoblastic leukemia

Disease*	Gene	Function	Approximate Frequency % [†]	References	Comments
B-ALL and T-ALL	<i>CDKN2A/2B</i>	Cell cycle modulator	30 and 70	Mullighan and Downing [3]	deletions
B-ALL and T-ALL	<i>PAX5</i>	B-cell differentiation	30 and 10	Mullighan and Downing [3]	deletions, deletions in T-ALL are large and typically include <i>CDKN2A</i>
B-ALL and T-ALL	<i>EBF</i>	B-cell differentiation	4 and 6	Mullighan and Downing [3]	deletions
B-ALL and T-ALL	<i>RBI</i>	Cell cycle modulator	4 and 12	Mullighan and Downing [3]	deletions
T-ALL	<i>NOTCH1</i>	T-cell differentiation	56	Weng et al. [93] Sulis et al. [94]	Activating HD and PEST domain mutations
B-ALL	<i>ETV6</i>	Transcription factor	26	Mullighan and Downing [3]	deletions, also frequently involved on translocations
B-ALL	<i>NRAS</i>	Ras pathway	17	Case et al. [88]	activating point mutation codons 12, 13, 61
B-ALL	<i>KRAS</i>	Ras pathway	16	Case et al. [88]	activating point mutation codons 12, 13, 61
B-ALL	<i>IKZF1</i>	B-cell differentiation	8	Mullighan and Downing [3]	84% of <i>BCR-ABL1</i> ALL; complete or partial deletions
B-ALL	<i>PTPN11</i>	Ras pathway	7	Tartaglia et al. [91]	activating point mutations Exons 3 and 13
B-ALL	<i>E2-2</i>	B-cell differentiation	6	Kuiper et al. [85]	deletions
B-ALL	<i>FLT3</i>	Ras pathway	3	Case et al. [88]; Armstrong et al. [85]	activating ITD or point mutations, 15% of <i>MLL</i> ALL rearranged

* B-ALL includes precursor B-ALL

[†]Frequencies only approximate due to variation in sample size and sample selection in different studies