Origins and clonal evolution of childhood leukemia

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A B S T R A C T

Childhood leukemia is the most common cancer of children and provides a paradigm for understanding the design principles of cancer. Evidence from twin studies and analysis of Guthrie spots suggests that childhood acute lymphoblastic leukemia (ALL) often arises *in utero*. This information informs studies of the target cell biology of this disease and the exposures that may initiate ALL-associated genetic lesions. Understanding how the initiating lesions in ALL predispose cells to subsequent transformation is an important but poorly understood area. The mechanisms by which the additional mutations that are necessary for transformation to frank leukemia are generated and selected are also important, and recent studies implicate RAG gene products in this process. Leukemic clones appear to evolve in a branching manner such that, at presentation, the marrow is replete with multiple variegated subtypes proving a diverse substrate for selection in response to therapy. Beyond genetic heterogeneity, leukemic cells exhibit epigenetic heterogeneity in respect of their immunophenotypes and functional properties, including cell-cycle status and niche residence. Thus, both genetic and epigenetic variation must be considered when evaluating the response of leukemic cells to therapy.

Learning goals

At the conclusion of this activity, participants should:

- understand the nature of cell hierarchies in human lymphoid development and how they inform the target cell biology of childhood ALL;
- understand the differences between, and implications for therapy, of linear *versus* branching clonal evolution;
- appreciate the role of epigenetic variation and niche in leukemia-cell biology and therapy resistance.

Introduction

Around 1600 children under 14 years of age are diagnosed with cancer every year in the UK. Worldwide, it is estimated that this number is greater than 175,000 children, and up to 55% do not survive.

In the UK, during the period 1996-2005, the total age-standardized incidence of cancer in children under 15 years of age was 142 cases per million and the cumulative risk of developing cancer within 15 years of birth was 1 in 484. The incidence is highest at 2-3 years of age and falls to a minimum around nine years of age. Age 10-14 years then sees the beginning of the increase in incidence with age, which continues through adulthood.

Age distributions and sex ratios vary markedly between types of childhood cancer. Overall incidence is 20% higher in boys than in girls. Leukemia represents a significant fraction of childhood cancers (Figure 1), and acute lymphoblastic leukemia (ALL) is the most common subtype. With an average incidence of around 500 cases per year in the UK, ALL is the most common childhood cancer, and despite considerable therapeutic advances, mortality is approximately 20%. The treatment is also very toxic, resulting in life-long and devastating side-effects. Infant leukemia has, in general, a very poor prognosis (50%-60% mortality) and, in some cases, for example those with a mixed lineage leukemia (*MLL*) rearrangement, an even worse outcome may be predicted. At the genetic level, childhood ALL (cALL) is heterogeneous and is largely characterized by deletions/copy number alterations and chromosomal translocations (Figure 2).[1] The mechanisms underlying genetic disruption are not clear, but RAG protein is a likely agent for many of the mutational events observed. This is supported by recent whole genome sequencing data,[2] which highlight the presence of RAG switch sequences at many of the disrupted regions in childhood ALL samples. Thus, one may speculate that B cells subject to differentiation arrest at a stage where RAG genes are active may sustain RAG-mediated recombination events at loci which harbor cryptic switch sites not cleaved during normal B-cell differentiation and B-cell receptor recombination. The pattern of genetic abnormalities seen in adult ALL is distinct from ALL in children (Figure 2).[1] The reasons for this are obscure, but suggest differences in the underlying biology and target cells, and may also reflect the fact that different environmental exposures initiate leukemogenesis in adults and children. It is important to remember that, in many cases, childhood ALL arises

in utero.[3] Cellular hierarchies in fetal hematopoiesis are thought to be distinct from their adult counterparts, and evidence in murine systems identifies significant differences in the cartography of hematopoiesis at different developmental stages, as well as functional differences in both growth factor responsiveness and potency of different progenitor cell classes.[4,5] With respect to exposures, it is widely assumed that, should they initiate childhood leukemia, then most likely their action is transplacental. The human placenta changes in structure and permeability during gestation, and recent evidence suggests that it contains significant numbers of hematopoietic stem cells.[6,7]

Many of the somatic genetic lesions in childhood ALL occur during fetal development,[3] and TEL-AML1 is a key example. The TEL-AML1 fusion gene consists of the transcription factors TEL (ETV 6) (residues 1-336) and AML1B (RUNX-1) (residues 21-480).[8] It is created by the t(12;21) translocation, which is the most common structural chromosomal alteration in pediatric cancer, occurring in approximately 25% of pediatric common B-cell precursor acute lymphoblastic leukemia (pre-B ALL) cases.[9,10] The TEL-AML1 fusion gene arises predominantly *in utero*.[11] producing a persistent but clinically covert pre-leukemic clone, which represents only a minor component of the bone marrow (BM). Experimental modeling using both mouse and human cells supports a first hit function for TEL-AML1 in pre-leukemia initiation, but also indicates that it is insufficient for development of overt leukemia.[12-17] This is consistent with the long latency of the disease (up to a decade after birth), the low concordance rate seen in twins, and the observation that TEL-AML1-containing B-lineage clones are present in 1% of healthy births; this rate is 100 times higher than the frequency of leukemia.[11,18] This implies the requirement for additional genetic events for progression to frank leukemia, and indeed, this is associated with hallmark secondary changes, including deletions of the remaining TEL allele and dysregulation of other genes such as Pax5.[19-22] It has been widely held that mutations in leukemia are acquired in a linear fashion (Figure 3, left). An alternative view is that evolution of the leukemic clone proceeds along Darwinian lines in a branching manner (Figure 3, right). A crucial difference between these ideas is that the linear acquisition model produces leukemic cells all containing the same spectrum of mutations, while the branching model predicts that a leukemic clone may at any given time be composed of cells containing a spectrum of different mutations. Evidence supporting this latter view has been reported by both the Greaves and Dick groups.[23,24] The presence of genetically variegated leukemic cells has significant implications for selection during therapy and for disease relapse. Comparison of matched diagnostic and relapse material shows that relapse may be initiated from major or minor clones present at diagnosis.[23]

Significant interest now focuses on the genetic variegation present in childhood leukemia, and more broadly in cancer in general. It is important to consider the possible roles of epigenetic variation in both the evolution and biology of childhood leukemia, as well as therapy resistance in this setting. Epigenetics in this context is used in the spirit of Waddington's epigenetic landscape: the notion that cells with the same genetic complement can exist in different functional states, be that related to cell type, differentiation stage, or functional state, e.g. cycle status. Many of

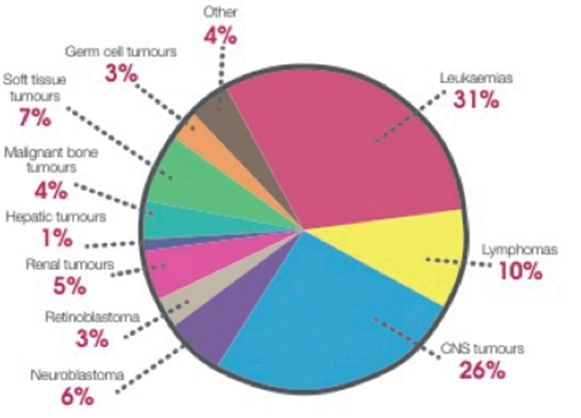

Figure 1. UK Childhood Cancer diagnoses based on data for 2001-2010. Provided by the national registry for childhood tumors.

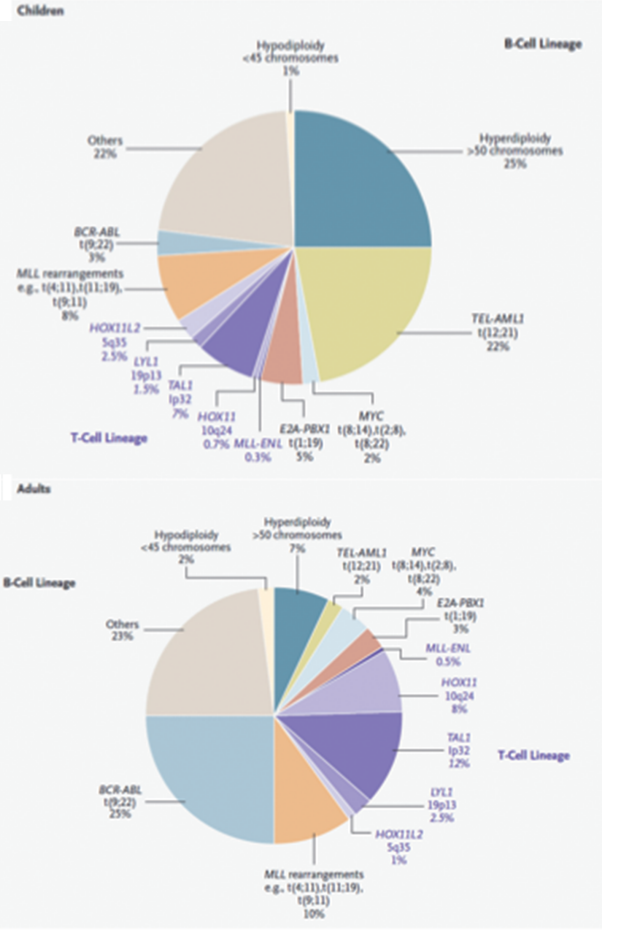

Figure 2. Genetic abnormalities in adult and childhood lymphoid cell malignancies (after Pui *et al.*[2]). Note the different spectrum of underlying mutations in the two settings. In particular, TEL-AML1 accounts for a substantial fraction of cases in children but is rarely seen in adults.

these properties may be intrinsically controlled, but equally may be modified or driven by interactions with extrinsic regulators, including most potently the niche. One of the key epigenetic concepts in leukemia has been that of cancer stem cells (CSCs). The CSC hypothesis postulates that tumor growth is maintained by a subpopulation of cancer cells, which retain self-renewal and differentiation capacity.[25] These cells sit at the apex of the cellular hierarchy and it has been proposed that they are responsible not only for disease initiation and maintenance, but also for relapse. Functional assessment of the ability of immunophenotypically defined subpopulations of blasts to initiate and maintain leukemia in xenograft assays has been key to understanding the extent of functional hierarchies in human leukemic clones. Using this approach, CSCs were generally thought to be relatively rare cells, but ongoing technical refinements in the mouse models suggest that, in many cases, the frequency of these cells may have been significantly under-estimated.[26,27] At the least, there is wide variation in CSC frequency between different tumor types, and perhaps also during disease progression. The CSC hypothesis is currently a subject of controversy[28] and few studies so far have directly examined its clinical significance.[29-32]

Most leukemic cells in pre-B-ALL exhibit features of B-progenitor/precursor cells with co-expression of CD19 and CD10 accompanied by clonal rearrangement of IgH indicative of a pre-B-cell identity. The study of tumor-propagating cells (TPC) in childhood ALL has been both confusing[14,27,33,34] and compounded by the fact that general conclusions about the nature of pre-B-ALL-propagating cells are hard to draw without reference to either disease subtypes, mutational load or progression stage. Nevertheless, it now seems clear that there is significant plasticity in the immunophenotype of cancer-initiating cells in childhood ALL,[23] which both confounds the attribution of the cancer stem cell concept and prevents the firm definition of tumor-propagating cells in this disease. Thus while the value of epigenetics of differentiation state as defined by immunophenotype in childhood ALL seems less than clear, it may be useful to consider heterogeneity of functional properties of leukemic cells from a different perspective. To this end, we have reported that the cycling state of leukemic cells may be important for their response to therapy in childhood leukemia.[35] By following children undergoing chemotherapy, we were able to show that quiescent cells were preferentially resistant to treatment at early stages (Figure 4). How quiescence is regulated in these cells is unclear, as is whether it is determined by

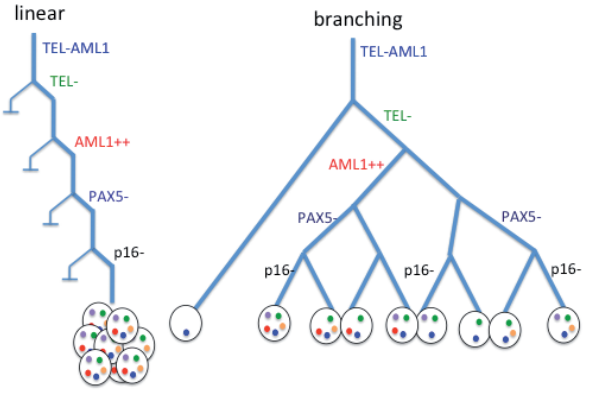

Figure 3. Idealized linear and branching modes of the acquisition of mutations during leukemogenesis of childhood acute lymphoblastic leukemia. Note that in the linear scenario all cells have a common pattern of mutations whereas in the branching mode a variegated pattern is obtained.

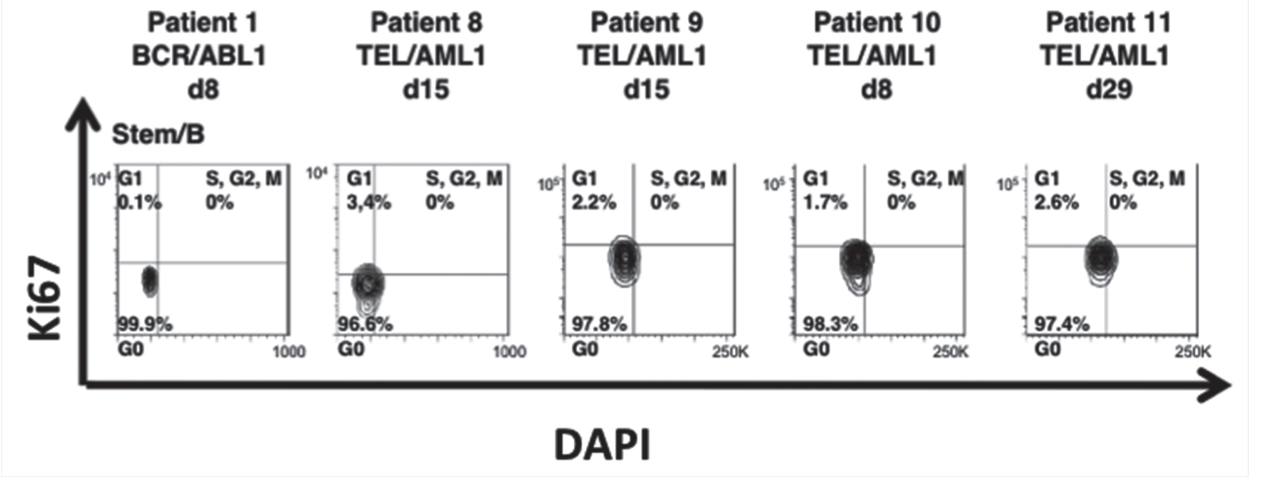

Figure 4. Analysis of cycle status of residual leukemic cells in children undergoing induction chemotherapy for B-cell precursor acute lymphoblastic leukemia. The characteristic genetic abnormality and day of induction chemotherapy at which the samples were taken is shown. Cycle status was determined using DAPI and Ki67staining. Note the enrichment of cells in the G0 phase of the cell cycle. (Modified from Lutz *et al.*[35]).

intrinsic or extrinsic cues. The interaction of cancer cells with host niches has attracted recent attention in many cancers, including ALL.[36] Residence of cancer-propagating cells (CPCs) within preferential microenvironmental niches may play a major part in evading therapy, but the nature of the niches involved and the mechanisms protecting CPCs remain largely unknown. We have explored how ALL cells interact with the niche and how these interactions are modified in response to therapy.[37] In xenograft models of childhood ALL, leukemic cells significantly damage and remodel the BM niche. Post-treatment, small foci of ALL cells are retained, surrounded by sheaths of supporting cells that provide a novel post-therapy protective niche, including Nestin-positive mesenchymal cells (Figure 5).[37] Considerable cytokine crosstalk is involved both in the establishment of this niche and in the interactions between niche and leukemic cells that help them evade therapy. We investigated patients' BM biopsies and found evidence of a similar process in patients receiving induction therapy. The interplay between genetic variants and the epigenetic variation seen in the cycle of niche residency is not understood, but promises to be a fruitful area of investigation.

Genetic variegation poses significant challenges for elimination of leukemic clones. This notwithstanding, it is interesting to note that the majority of leukemic cells in TEL-AML1 ALL retain TEL-AML1 (the initiating mutation) despite extensive variegation in other mutations.[23] Since TEL-AMl1 is a first event, this may reflect a genetic founder effect of limited functional significance. Alternatively, cells may remain dependent on or 'addicted' to its functions. In support of the latter hypothesis, knockdown of TEL-AML1 using interfering hairpin RNAs directed against the junction of the fusion gene significantly impact leukemic-cell function.[38,39] This result places emphasis on understanding the function and target gene biology of the TEL-AML1 fusion. The molecular mechanisms of TEL-AML1 action are not fully understood. TEL-AML1 contains the DNA-binding domain of AML1 (RUNX1) and it is likely that at least some of its targets are normal targets of AML1. In this context it has been suggested that TEL-AML1 may function as a negative regulator, recruiting co-repressors via the TEL-portion of the fusion in a manner that produces heritable epigenetic changes at the level of chromatin structure.[40-43] TEL-AML1 may thus impact the transcriptional network at more than one level, both superimposing its own transcriptional activities and subverting the normal functions of AML1. This may be carried out through runt homeodomain (RHD)-mediated interactions with SMADs leading to alterations in TGFβ responsiveness, or possibly with Pax5, which has been shown to interact with AML1 as well as the AML1-ETO fusion.[13,44-46] Thus understanding TEL-AML1 function requires delineation of the transcriptional networks that it nucleates, their relationship to those associated with AML1 or other Runx activities within the cells of interest, and an appreciation of how these change in the different cellular contexts within the differentiation hierarchies and in response to genetic changes associated with progression.

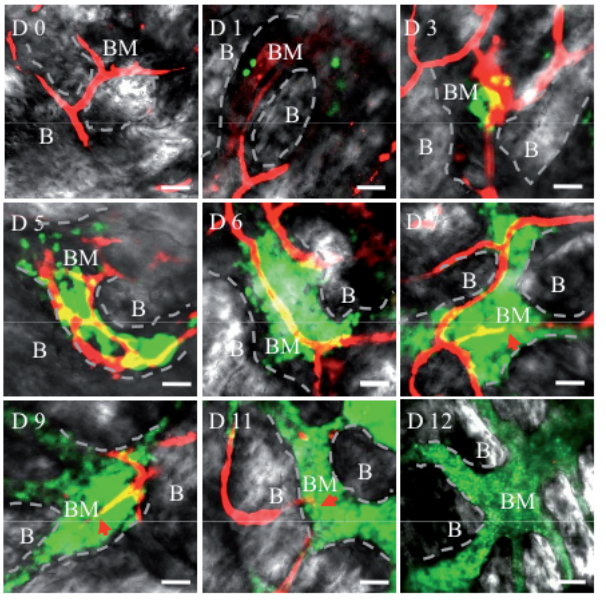

Figure 5. Leukemic cells remodel their bone marrow niches in xenograft models of B-cell precursor acute lymphoblastic leukemia. Leukemic cells (green: transduced with a green fluorescent protein containing vector) are imaged through the calvarium at the indicated time points (days post engraftment in immuno-deficient mice). The endosteal surface where normal stem cells are thought to reside is indicated with a dotted line and the vascular components are shown in red (after Duan *et al.*[37]).

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