Chapter 14
Leukemic Stem Cells in Acute Lymphoblastic Leukemia

Ugo Testa

Abstract Acute lymphoblastic leukemia (ALL) is observed in both children and adults, with >60 % of cases occurring at age <20 years (peak incidence at ages between 2 and 5 years). The survival of pediatric ALL, particularly of children ALLs is around 90 %, while the prognosis of adults and infant ALLs is poor. According to their differentiation features, ALLs are distinguished in B-ALLs and T-ALLs. T-ALLs are less frequent than B-ALLs, accounting for 25 % of adult T-ALLs and 10–15 % of pediatric ALLs. The nature and the frequencies of the stem cells or leukemia-initiating cells in ALLs were intensively investigated during last years. The ensemble of these studies carried out on the characterization of leukemic stem cells in B-ALLs indicate that the putative stem cells responsible for initiating and maintaining B-ALLs are not a fixed cellular identity (i.e., cells with $CD34^+CD19^+$ or $CD34^+CD19^-$ or $CD34^-$ have been shown to possess leukemia-initiating capacity), but themselves evolve both in their genotype and phenotype. This conclusion was strongly supported by studies carried out in twins: basically, these studies have shown that the ALL-specific fusion events occur in utero during embryonic/fetal development, generating a preleukemic clone, clinically silent; the preleukemic clone may progress to full leukemia development through the acquisition of new genetic abnormalities, such as point mutations, deletions and/or duplications. In addition to these findings, another very important contribution derived from the study of B-ALL leukemia-initiating cells is that these cells are not only phenotypically heterogeneous, but also genotypically heterogeneous: their heterogeneity reflect the heterogeneity of the bulk tumor cells. Furthermore, relapsing B-ALLs are issued from leukemic stem cell populations, representing a major or minor clone at presentation.

Keywords Hematopoiesis • Hematopoietic stem cells • Leukemic stem cells • Acute lymphoblastic leukemias

U. Testa (✉)
Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy
e-mail: ugo.testa@iss.it

S. Babashah (ed.), *Cancer Stem Cells: Emerging Concepts and Future Perspectives in Translational Oncology*, DOI 10.1007/978-3-319-21030-8_14

1 Normal Hematopoiesis

Hematopoiesis represents a complex multistep differentiation system initiated by undifferentiated stem cells (hematopoietic stem cells, HSCs) undergoing a process of self-maintenance and of cell differentiation through the generation of a series of hematopoietic progenitor cells (HPCs), multipotent and unipotent. The homeostasis of blood production is carefully controlled through regulation of self-renewal, lineage specification, differentiation and maturation. Therefore, the two essential processes regulating hematopoiesis are mainly dependent on the capacity of HSCs to both self-renew and differentiate. The studies carried out on both murine and human HSCs and HPCs during the l ast decades have led to elucidate in part the mechanisms and the cellular steps through which HSCs progressively differentiate.

To assay human hematopoietic stem and progenitor cells, immunodeficient animals, such as nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, are currently used. Cells endowed with the capacity of repopulating at long-term the hematopoietic system of these immunodeficient mice, as well as of secondary animal recipients, are thought to be HSCs. The NOD/SCID repopulating assay provided evidence that human HSCs are present in the $CD34^+CD38^-$ fraction of human hematopoietic cells. Using NOD/SCID mice strains with enhanced immunosuppression as recipients, it was shown that also the $CD34^+/CD38^+$ cell fraction possesses some repopulating activity. However, $CD34^+/CD38^+$ cells possess only a short-term SCID-repopulating activity, while the long-term repopulating activity is limited to the $CD34^+/CD38^-$ cell population (Hogan et al. 2002). It is important to mention that several studies have characterized a rare SCID-repopulating population observed at the level of $CD34^-Lin^-$ cells: these cells, like $CD34^+/CD38^-$ cells possess a long-term repopulating capacity. Importantly, $CD34^-$ HSCs are able to generate in vivo $CD34^+$ HSCs (Kimura et al. 2010).

According to their capacity of repopulating hematopoiesis, the hematopoietic stem cell pool can be subdivided into three groups: short-term HSCs, capable of generating clones of differentiating cells for only 4–6 weeks; intermediate-term HSCs, capable of sustaining a differentiating cell progeny for 6–8 months before becoming extinct; long-term HSCs, capable of maintaining hematopoiesis indefinitely (Benviste et al. 2010).

The classical model, the *clonal succession model*, of hematopoietic hierarchy implies that all mature cells of the peripheral blood are the progeny of a single long-term hematopoietic stem cell. An alternative model, the *clonal diversity model*, proposes that distinct hematopoietic stem cell types are capable to contribute to the formation of all lineages, but are programmed to do so in a highly biased fashion, in part related to microenvironmental stimulations. Recent studies provide support to the second model. These observations are compatible with the view that the hematopoietic system is maintained by a continuum of hematopoietic stem cell subtypes, rather than a functional uniform stem cell pool.

All blood elements are generated at the level of the sites where hematopoietic stem cells reside (i.e., in the fetal liver during fetal life and in the bone marrow

during postnatal life), with the exception of T-cells that are generated at the level of the thymus from lymphoid progenitors derived from HSCs and migrating in this site. The first-cellular elements generated by the differentiation of HSCs consist of progenitors of more restricted differentiation capacity, until to generate unipotent progenitors. Collectively, all this process is known as lineage commitment or cell fate decision. Following a model proposed for many decades, the hematopoietic stem cell first originates a common myeloid progenitor (CMP), able to generate all myeloid lineages (granulocytes, monocytes, dendritic cells, erythrocytes and megakaryocytes) and a common lymphoid progenitor (CLP), able to generate all lymphoid elements, B, T and NK lymphocytes. Alternatively to this view, more recently it was proposed an alternative myeloid-based model, postulating that HSCs first diverge into a CMP and a common lymphoid-myeloid progenitor (CLMP). The CMPL in turn generates T and B-cell progenitors through a bipotential myeloid-T progenitor and myeloid-B progenitor stage, respectively (Kawamoto et al. 2010).

This last model received support from recent studies based on the characterization of early human hemato-lymphopoietic progenitors. Thus, according to the actual view during normal human hematopoiesis two types of multipotent progenitors are generated: a CMP, isolated from bone marrow as a $CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-$ cell, unable to generate every type of lymphoid cells, and capable of generating all myeloid elements through an intermediate differentiative pathway involving the generation of bipotent G/Mo and Mk/E progenitors; a CLMP which can be identified as a $CD34^+CD38^-CD45RA^+Thy\text{-}1^{neg\text{-}low}$ cell, capable of generating in vitro and in vivo all lymphoid elements (T, B and NK lymphocytes) and some myeloid elements such as monocytes/macrophages and dendritic cells, but not erythroid, megakaryocytic or granulocytic elements (Doulatov et al. 2010).

More recently, the fractionation of human cord blood and bone marrow $CD34^+$ cells into $CD133^+$ and $CD133^-$ subfractions allowed to propose a revised dichotomy model, where the HSCs are able to generate two types of multipotent progenitors, a CMP, here defined as a common erythro-myeloid progenitor (EMP) capable of generating erythroid cells, megakaryocytes, basophilic and eosinophilic granulocytes, and a LMPP, capable of generating lymphoid elements, dendritic cells and granulocytes (Gorgens et al. 2013).

The large majority of HSCs are $CD34^+$. Many studies have shown that in various hematopoietic tissues (bone marrow, cord blood, peripheral blood, and mobilized peripheral blood), the $CD34^+CD38^-$ cell fraction is enriched in early multipotent progenitors, while the $CD34^+CD38^+$ fraction is enriched in committed progenitors. Other membrane markers expressed on $CD34^+CD38^-$ cells further enrich for HSCs, such as CD90. Furthermore, it was shown that CD45RA was expressed on HPCs, but not on HSCs. Therefore, the selection of $CD34^+CD38^-CD90^+CD45RA^-$ allowed to considerably enrich in HSCs. CD49f was shown to be a more reliable marker for human HSCs than CD90 in that virtually all HSCs were shown to be $CD49f^+$ (Notta et al. 2011a). The combination of some membrane markers allows also to enrich for selected types of HPCs: thus, $CD34^+CD38^-CD90^{low/-}CD45RA^+CD135^+$ (Doulatov et al. 2010) or $CD34^+CD38^-CD45RA^+CD10^-$ (Goardon et al. 2011) allows to identify and to enrich for progenitors with LMPP features. Gorgens et al. (2013) have

identified different subsets of HPCs according to the membrane antigen phenotype: (1) the majority of erythro-megakaryocytic progenitors are observed among $CD34^+CD133^-$ cells; furthermore, the eosinophilic and basophilic granulocytic progenitors are also observed among $CD34^+CD133^-$ cells (therefore, the large majority of MEPs are observed among $CD34^+CD133^-$ cells); (2) MPPs, LMPPs, MPLs and GMPs reside at the level of the $CD34^+CD133^+CD138^{-/low}CD45RA^-$; LMPPs as $CD34^+CD133^+CD38^{-/low}CD45RA^+CD10^-$; GMPs as $CD34^+CD133^+CD38^+CD45RA^+CD10^-CD7^-$. These progresses at the level of the identification of various subtypes of hematopoietic stem/progenitor cells were of fundamental importance for the study of the microRNAs at the level of the various types of HPCs.

In addition to $CD34^+$ HSCs, the human HSC hierarchy contains a rare $CD34^-$ population, able to repopulate hematopoiesis into immunodeficient mice. These cells were characterized as $CD34^-CD38^-CD93^+$ cells, have characteristics of HSCs and can be placed in the HSC hierarchy above $CD34^+$ HSCs. A remarkable property of these cells is that consists in an active NOTCH signaling (Anjos-Afonso et al. 2013).

HSCs exhibit several biological properties different from other hematopoietic cells, including HPCs. Thus, given their consistent longevity, HSCs are exposed during their long life-time to various stress stimuli, including reactive oxygen species, nutrient fluctuation and DNA damage and then they possess particular mechanisms that govern their integrity through the unfolded protein response pathway (van Galen et al. 2014). Another remarkable property of HSCs is their requirement for a highly controlled rate of protein synthesis, lower than that observed in other hematopoietic cells (Signer et al. 2014). HSCs are located at the level of peculiar tissual areas known as stem cell niches, essential for maintaining and regulating these cells: these niches are perivascular and are composed by mesenchymal stromal cells and endothelial cells and are usually located near trabecular bone (Morrison and Scadden 2014). HSC populations are heterogeneous, as shown by the intrinsically determined heterogeneity in differentiation potentiality of long-term HSCs and neutrophil-restricted human HSC with rapid, but transient repopulating activities (Miller et al. 2013). A striking example of HSC heterogeneity was obtained in a recent study showing that the expression of the glycoprotein von Willebrand Factor (vWF) identifies a HSC subset that is primed for megakaryocytic production in response to thrombopoietin; vWF^+ $HSCs^+$ are able through their self-renewal to generate both vWF^+ and vWF^- daughter HSCs (Sanjan-Pla et al. 2013).

Among the various factors, low oxygen tension (hypoxia) plays a key role in maintaining undifferentiated states of hematopoietic stem cells: gradients of oxygen between 1 % in hypoxic niche and 6 % in the sinusoidal cavity exist in the human bone marrow. Therefore, HSCs and primitive HPCs exhibit a hypoxic profile and harbor metabolic properties of hypoxic cells, such as enhanced anaerobic glycolysis and reduced flux through the Krebs cycle. Therefore, the hypoxic microenvironment plays a major role in metabolic reprogramming of HSCs and in regulation of their function. Cellular responses to hypoxia are mediated by hypoxia-inducible factors (HIFs), regulating gene expression in a way that permits to the cells an adaption to the hypoxic condition. Using a HIF-1-mediated modification of the gene

expression program, hematopoietic stem cells adapt to the hypoxic microenvironment within stem cell niches by utilizing glycolysis instead of mitochondrial phosphorylation (Simsek et al. 2010); HIF-1α deficiency at the level of the hematopoietic stem cell compartment causes an increased cell cycling rate and progressive loss of long-term repopulating activity (Takubo et al. 2010). HIF-2α is less expressed than HIF-1α in HSCs, but both HIF-1α and HIF-2α transcripts are more abundantly expressed in HSCs than in HPCs. In contrast to the findings observed in studies of HIF-1α gene knockout, acute (inducible) or constitutive conditional deletion of HIF-2α specifically at the level of the hematopoietic system, had no impact on HSC survival and self-renewal. Therefore, these observations suggest that HIF-1α and HIF-2α may have different functions in mouse HSCs (Guitart et al. 2013). In contrast to the findings observed in murine HSCs, HIF-2α knockdown in human cord blood HSCs and early HPCs showed an inhibitory effect on their proliferation, a reduced ability to form erythroid colonies in vitro and an impaired ability to reconstitute hematopoiesis in vivo, due to enhanced production of ROS and increased endoplasmic reticulum stress (Rouault-Pierre et al. 2013).

Lymphocyte development implies the regulated production of B- and T-lymphocytes from HSCs through a series of progenitors exhibiting a progressively restricted differentiation capacity. The first of these differentiative steps involves the generation of multipotential progenitors, termed multipotent progenitors (MPPs), which have still the capacity to generate all hematopoietic lineages, but have lost the self-renewal property. The up-regulation of the membrane receptor on these cells characterizes the lymphoid commitment; these cells have been described as LMPPs. The subsequent step of differentiation is characterized by the loss of the myeloid potential and by the up-regulation of the IL-7R; these progenitors are known as Common Lymphoid Progenitors (CLPs). CLPs have the capacity to generate all types of lymphoid elements, including B-, T- and NK-lymphocytes, as well as lymphoid dendritic cells. The commitment to the B-cell or T-cell lineages following migration to the thymus implies the loss of the potential for other lineages. A number of transcription factors important for early B-lymphoid commitment, including PU.1, Ikaros, Pax5 Hhex and E2A, promote B-cell differentiation. On the other hand, following migration to the thymus, a distinct group of transcription factors, including E2A, Lyl1, TCF1, PU.1, Myb, GATA-3, C/EBPalpha and Mef2c, promotes the early steps of T-cell differentiation and the expansion of early thymocyte progenitors (ETPs).

The first early stages of human T-cell differentiation have been in part delineated, providing a model of T-cell differentiation which implies: (i) first the migration of lymphoid stem/progenitor cells; (ii) the initial differentiation of these cells reaching a differentiation stage (DN1) at whose level are still multipotent (i.e., they can generate T and B-lymphocytes, macrophages and dendritic cells); (iii) during the subsequent stage of differentiation (DN2) the thymic progenitor cells become located in thymic niches where, under the effect of Notch1 and IL-7, undergo the first steps of T-cell commitment; (iv) under the effect of thymic microenvironment, the expression of the Bcl11b transcription factor is induced in thymic progenitors and this determines a full T-cell commitment with activation of T-cell receptor expression

and repression of alternative differentiation cell fates and blocking of stem/progenitor cell properties (Di Santo 2010). The analysis of early T-cell development showed some peculiar findings. The thymus lacks HSCs or other cell types with self-renewing properties, but is continuously seeded by progenitors deriving from bone marrow. The fraction of most immature mouse thymic progenitors displays in vitro and in vivo capacity to generate both T-lymphocytes and myeloid cells (Bell and Bhandoola 2008; Wada et al. 2008). According to these findings it was suggested that the loss of myeloid potential is a relatively late event during T-cell differentiation in the thymus. Luc et al. (2012) have provided evidence at the single cell level that the earliest progenitors in the mouse neonatal thymus possess combined B and T lymphocyte, granulocyte and monocyte, but not erythro-megakaryocytic potential. However, in conflict with these studies, Schlenner et al. (2010) reported that, while the large majority of ETPs originated from IL7RA$^+$ progenitors, only 20 % of thymic neutrophils originated from these progenitors, thus questioning the physiologic relevance of this differentiation pathway.

Studies carried out on human CB progenitors have led to identify CLMPs with a membrane phenotype corresponding to CD34$^+$Thy1$^{low/-}$CD45RA$^+$CD10$^+$CD7$^-$, with a multipotential lymphoid potential (B-, T- and NK lymphocytes), with a myeloid (granulocytes and monocytes) potential and lacking staminal, repopulating activity (Doulatov et al. 2010). The multipotential potential, combined with a myeloid potential of CLMPs, was further confirmed by Goardon et al. (2011) showing that CD34$^+$CD38$^-$Thy1$^-$CD45RA$^+$CD10$^+$CD7$^-$ cells isolated from normal bone marrow have the potential to generate B, T, NK cells, as well as myeloid cells in vivo in NSG mice. Gorgens et al. (2013) identified a cell fraction highly enriched in LMPPs (Lympho-Myeloid Progenitors) with a membrane phenotype CD34$^+$CD133$^+$CD38$^{low/-}$CD45RA$^+$: subdivision of these cells according to CD10 positivity leads to identify a CD10$^+$ fraction lacking granulocyte potential, but retaining lymphoid potential, corresponding to the MLP described by Doulatov, and a CD10$^-$ fraction corresponding to LMPPs. The presence of an early lymphoid-primed human progenitor was obtained by Kohn et al. showing that CD10$^-$CD62L^{high} progenitors isolated from bone marrow have lymphoid and monocytic potential; these cells can be placed at an intermediate stage of differentiation between HSCs and CD34$^+$CD10$^+$ lymphoid progenitors (Kohn et al. 2012).

Some studies have challenged the view that LMMPs possess only lympho-myeloid potential, showing that these cells possess erythro-megakaryocytic potential in vivo (Boyer et al. 2011). However, Boiers et al. (2013) have provided evidence about the existence in mice of lympho-myeloid progenitors emerging during development prior to definitive HSCs and playing a physiologic relevant role in the generation of a lymphoid and myeloid cell progeny. This progenitor (characterized as a cell with an IL7Ralpha$^+$Kit$^+$Flt3$^+$Lin$^-$ membrane phenotype) first appears in yolk sac and contributes physiologically to the establishment of lymphoid and some myeloid components of the immune system. Additional evidence about a physiologic role of early ETPs in promoting granulocytic differentiation at the level of the thymus derives from another recent study (De Obaldia et al. 2013). In fact, these authors have shown that the analysis of various animal models in which ETPs are

absent either for abrogation of the thymic settling or for the inhibition of early thymic development by IL7Ralpha or HES1 genetic knockdown, showed a marked reduction of the thymic granulocytes (De Obaldia et al. 2013).

Signaling via NOTCH receptors is essential for the generation of ETPs in the thymus. NOTCH signaling acts through two different mechanisms, both up-regulating T-cell lineage-specific gene expression and antagonizing alternative differentiation cell fates, as progenitor cell commit to the T-cell lineage. Particularly, the NOTCH-induced transcription factor HES1 acts as a repressor of C/EBPalpha and of the myeloid differentiation program of ETPs (De Obaldia et al. 2014).

Given the complexity of the process of T-cell differentiation from HSCs, it is not surprising that HSC transplantation is followed by a period of immune deficiency due to the paucity of T-cell reconstitution. This problem has stimulated the research on the identification of T lymphocyte progenitors capable of thymus engrafting capacity and of T-cell reconstitution into immunodeficient mice. In this context, both pro-T1 ($CD34^+CD7^+CD5^-$) and pro-T2 ($CD34^+CD7^+CD5^+$) cells were capable of improving and of accelerating HSC-mediated reconstitution of T lymphopoiesis into immunodeficient NOD/SCID mice (Awong et al. 2013).

A recent study has provided evidence about a peculiar mechanism involved in thymic lymphopoiesis and required to maintain a normal, non-neoplastic T-lymphopoiesis. This mechanism is based on the continuous supply of bone marrow-derived progenitors to the thymus: these progenitors replace thymus-resident progenitors. This continuous replacement is based on a progenitor strategy of competition between the bone marrow-derived progenitors and the thymus-resident progenitors for the T-cell growth factor IL-7: under normal conditions and then in the presence of bone marrow-derived progenitors, the availability of IL-7 for thymus-resident progenitors is low and these cells undergo apoptosis. In the absence of incoming bone marrow progenitors, the thymus-resident progenitors proliferate and differentiate, generating T lymphoid cells. However, surprisingly in the absence of a progenitor competition mechanism the endogenous progenitors undergo transformation, generating tumors similar to T-ALLs and with genetic abnormalities, such as NOTCH1 mutations, typical of these leukemias (Martins et al. 2014). Therefore, the physiologic competition between bone marrow-derived progenitors and thymus-resident progenitors, greatly reduces the chances that these last progenitors can undergo malignant transformation.

A key event occurring during B lymphopoiesis is represented by immunoglobulin (Ig) gene rearrangements promoted by the activation of recombination enzymes (RAG-1 and RAG-2 and terminal deoxynucleotidyl transferase promote the D-to-J and V-to-DJ rearrangements at the level Ig heavy chain locus during the differentiation from the CLP to the pre-B stage). At the pre-B-cell differentiation stage, signaling through the pre-B-cell antigen receptor determines the induction of VJ_L rearrangements and allelic exclusion at the Ig heavy chain locus, thus determining the formation of a functional B-cell antigen receptor on the surface of immature B-cells. This rearrangement machinery is timely orchestrated by a number of transcription factors, such as PU.1, PAX5, E2A and EBP, playing a key role in the control of B-lymphopoiesis.

On the other hand, studies on B-lymphopoiesis starting from CLMPs have shown a sequence of events that determines starting from $CD34^{+}CD38^{-}CD45RA^{+}CD10^{-}$ cells the progressive generation of progenitors (pre-pro-B-cells and multilineage CLP/early-B-cells) characterized first by the acquisition of CD19 expression and then CD10 expression; the progenitors thus generated act in turn as precursors for distinct pro-B, pre-B-cells (Sanz et al. 2010). During the early stages of human B lymphopoiesis the level of CD10 expression is an important marker of B-cell commitment and differentiation. In fact, $CD34^{+}CD10^{high}$ cells express CD19 and lymphocyte transcription factors and correspond to loss of myeloid differentiation potential; in contrast, $CD34^{+}CD10^{low}$ cells showed a multiple differentiation potential, being capable of generating lymphocytes, plasmocytoid and conventional dendritic cells and myeloid cells (Ichii et al. 2010).

2 B-cell Acute Lymphoblastic Leukemias; Molecular Abnormalities

The large majority of B-ALLs display chromosomal abnormalities detectable by conventional cytogenetic studies. According to these abnormalities, B-ALLs can be subdivided in: high hyperploidy, consisting in the gain of at least five chromosomes and representing the most frequent pediatric B-ALL subtype; a rare hypodiploid B-ALL subtype with <44 chromosomes; a spectrum of chromosomal translocations, including: t(12;21)(p13;q22) encoding the fusion protein ETV6-RUNX1 (TEL-AML1) (20–25 % of pediatric B-ALLs); t(9;22)(q34;q11.2) encoding BCR-ABL1 (representing about 2 % of pediatric B-ALLs); t(1;19)(q23;p13.3) encoding TCF3-PBX1 (E2A-PBX1) (about 4 % of pediatric B-ALLs); rearrangement of MLL at 11q23 with different partner genes, the most common being AF4 (about 6 % of pediatric B-ALLs); rearrangement of CRLF2 with P2RY8 or with the immunoglobulin heavy chain locus (occurring in B-progenitor cell B-ALLs); rearrangement of IGH locus with a wide range of partner genes, including IL3, CEBPE, BCL2, EPOR, ID4 (Table 14.1).

2.1 ETV6-RUNX1 B-ALLs

Approximately 25 % of B-ALLs are characterized by a balanced t(12;21) chromosomal translocation that generates the ETV6-RUNX1 fusion gene. This B-ALL subtype, associated with a favorable prognosis, was the object of intensive studies showing that the ETV6-RUNX1 translocation is the initiating key event of this B-ALL and occurs prenatally in a committed B-cell progenitor (Greaves and Wiemels 2003). However, the fusion ETV6-RUNX1 gene was not sufficient by itself to induce the full development of overt leukemia and a number of studies have

Table 14.1 Genetic abnormalities found in B-ALLs and their prognosis

Main genetic abnormality	Genes specifically involved	Additional genetic abnormalities and biological features	Prognosis
Hyperdiploidy (>50 and <66 chromosomes). About 30 % of pediatric B-ALL; about 10–13 % of adult B-ALL	Chromosomes X, 4, 6, 10, 14, 17, 18 triploid Chromosome 21 tetraploid	CAN of several genes, including IKZF1, PAX5, ETV6, CDKN2A, RB1, TCF3 Mutations in MAPK signaling pathway (HRAS, NRAS, FLT3, PTPN11) BCR-ABL in adult patients (14 %)	Good
Hypodiploidy (<46 chromosomes) High hypodiploidy (40–45 chromosomes) Low hypodiploidy (33–39 chromosomes) Very low hypodiploidy (30–32 chromosomes) Near Haploidy (23–29 chromosomes)	Chromosomes 3, 7, 17, 15, 16 13, 9, 4, 12 and 20 are the most frequently lost	In low-hypodiploid ALLs: TP53, IKZF2, RB1 Near-haploid ALLs: RTK, Ras pathway, IKZF3	Adverse clinical outcome, low and near haploid karyotype, particularly
t(12;21)(p13;q22) About 25 % of pediatric B-ALL	ETV6-RUNX1 (TEL-AML1)	Low number of somatic mutations (7/leukemia) and none of them was recurrent CNAs: ETV6 (54 %), CDKN2A (22 %), PAX5 (22 %), BTG1 (6 %), TBL1XR1, RAG1, RAG2	Good
t(9;22)(q34;q11) 25–30 % of adult B-ALL; 3–5 % of pediatric B-ALL	BCR/ABL1 (Philadelphia chromosome, $Ph^{+)}$	Frequent (>60 %) deletion of the transcription factor Ikaros (IKZF1). Frequent (48 %) deletion of CDKN2A. CRLF2 genomic rearrangement. Ras signaling pathway mutations. TCF3 and PAX5 (45 %) deletions	Poor in adult Intermediate in children
t(1;19)(q23;p13) balanced and unbalanced translocations. About 3 % of pediatric B-ALL	TCF3-PBX1	1q gain; CAN of CDKN2A/B (23 %) and PAX5 (18 %); alterations of B-cell pathway genes	Intermediate

(continued)

Table 14.1 (continued)

Main genetic abnormality	Genes specifically involved	Additional genetic abnormalities and biological features	Prognosis
BCR-ABL1 like 10–15 % of pediatric B-ALL		Gene expression profile similar to BCR-ABL1-positive B-ALLs. About 50 % of these B-ALLs have CRLF2 rearrangements with JAK1/2 mutations. Mutations of ABL1, ABL2, EPOR, JAK2 and PDGFRB in cases without CRLF2 rearrangements. Frequent BTG1 deletion (31 %)	Poor in adult Intermediate in children
MLL rearrangements (1q23) 6 % of pediatric ALL, particularly frequent among infants t(4;11)(q21;23) t(9;11)(p22q23) t(11;19)(q23;p13.3) t(10;11)(p13-14;q14-21)	MLL-AFF(AF4) MLL-MLLT3(AF9) MLL-ENL MLL-MLLT10(AF10)	Additional genetic mutations are infrequent in these B-ALLs. CNAs are very rare, with the exception of CDKN2A (31 %). KRAS or NRAS mutations in 16 % of patients. FLT3 mutations in a part of patients	Poor
B-ALL with ERG deletion About 3 % of pediatric B-ALLs	ERG	Frequent IKZF1 deletion (38 % of patients), CDKN2A/B (20 %), PAX5 (12 %), ETV6 (3 %), BTG1 (6 %)	Good
B-ALL with CRLF2 rearrangement (Cytokine Receptor Like Factor 2) 7 % of B-ALLs; 53 % of Down patients with ALL	CRLF2, CRLF2-P2RY8, CRLF2-IGH	JAK1 and JAK2 mutations; deletion or mutation of IKZF1	Poor/intermediate
iAMP2 3 % of pediatric B-ALL	Amplification and deletion of parts of chromosome 21	Frequent RB1 (39 %), PAR1 (30 %), ETV6 (21 %) and IKZF1 (25 %) deletions. P2RY8-CRLF2 translocation (38 %)	Poor/intermediate

shown that additional mutations are required for the development of this B-ALL. Genome profiling studies have shown that the additional genetic events occurring in ETV6-RUNX1 B-ALLs are mainly represented by copy number aberrations (CNAs), mainly deletions, affecting genes involved in the control of B-lymphocyte proliferation, development and differentiation, such as CDKN2A, PAX5, BTG1, TBL1XR1, RAG1, RAG2 and the WT copy of ETV6 (Mullighan et al. 2007). These CNAs are related to a mechanism of aberrant RAG endonuclease targeting the promoters, enhancers and first exons of genes that normally regulate B-cell differentiation and represent the largely more frequent secondary events occurring in ETV6-RUNX1 B-ALL, while point mutations are much more rare events (Papamannuil et al. 2014).

2.2 Hyperdiploid B-ALLs

Hyperdiploid B-ALLs are the commonest subtype of B-ALLs in childhood, accounting for about 30 % of all pediatric B-ALLs, include leukemias with >50 and <66 chromosomes and usually have a good prognosis, due to a good response to standard therapy. These leukemias do not display a random pattern of chromosome gain, usually involving gains of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 and all these gains are triploid, with the exception of chromosome 21 gain that is tetraploid. In some cases, the chromosome gains are associated with the classical translocations observed in B-ALLs and these forms are associated with a less good prognosis. Genomic studies have shown some recurrent abnormalities in these B-ALLs, including copy number alterations at the level of some genes, including CDKN2A and mutations in MAPK signaling pathway (KRAS, PTPN11 and FLT3) and in histone-modifying CREB-binding protein gene (particularly in relapsing cases) (Inthal et al. 2012; Paulsson et al. 2008, 2010). Case et al. (2008) showed that 58 % of hyperdiploid B-ALLs exhibited mutations of genes affecting the RAS pathway, the more common being mutations at the level of KRAS or NRAS.

High hyperploid B-ALL is less frequent among adolescent and adult B-ALL patients, its frequency being estimated around 10 %. The presence of two primary genetic aberrations within the same clone is B-ALL is rare, but the contemporaneous presence of hyperploidy and the BCR/ABL translocation is a notable exception. The frequency of these "double-hit" B-ALL in pediatric patients is very low, due to the low frequency of BCR-ABL-positive in children, but is clearly higher in adult B-ALL where the incidence of BCR-ABL-positive B-ALLs is markedly more pronounced (14 % of adult BCR-ABL$^+$ B-ALLs are hyperdiploid and 13 % of adult BCR-ABL$^-$ B-ALLs are hyperdiploid). The comparison of the pattern of chromosome gains in the two groups of hyperdiploid B-ALLs was comparable, with the exception of trisomy of chromosome 2 which was much more frequent among Ph$^+$ hyperdiploid B-ALLs, than Ph$^-$ hyperdiploid B-ALLs (Chilton et al. 2014).

Subclonal analysis provided evidence that the numerical chromosome aberrations are the primary events and arose before structural events, suggesting a stepwise evolution of the leukemic clone (Paulsson et al. 2010).

2.3 Hypodiploid B-ALLs

Hypodiploidy is observed in 5–8 % of ALLs and can be subdivided into: (a) high hypodiploidy (40–45 chromosomes), (b) low hypodiploidy (33–39 chromosomes); very low hypodiploidy (30–32 chromosomes) and near haploidy (23–29 chromosomes), associated with distinct genetic and clinical features. The majority of hypodiploid patients has 45 chromosomes; low hypodiploidy and near haploidy B-ALLs are rare and are associated with a very negative prognosis.

A recent study provided fundamental information about the genomic landscape of hypodiploid B-ALLs, showing that these ALLs form a peculiar subtype of B-ALLs, distinct from other B-ALL subtypes. In fact, this study showed that low hypodiploid B-ALLs have a very frequent mutation of TP53 and frequent inactivating mutations (53 %) of the IKAROS family gene IKZF2 (HELIOS) and of the retinoblastoma gene (RB1, 41 %); near-haploid ALLs with 24–31 chromosomes harbor genetic abnormalities at the level of the Ras signaling pathway (71 %) and of the IKAROS family gene IKZF3 (AIOLOS). Very interestingly, the TP53 mutation found in low-hypodiploid B-ALL cells was also observed in matched non-tumor cells, suggesting germline inheritance; according to this observation it was suggested that low hypodiploid B-ALL could represent a manifestation of Li-Fraumeni syndrome (Holmfeldt et al. 2013). It is of interest to note that in the majority of these patients with TP53 mutated, both TP53 alleles are mutated or one is mutated and the other one is deleted (Stengel et al. 2014). Other studies have confirmed the very frequent (93 %) occurrence of TP53 mutation in low hyperdiploid B-ALLs; importantly, in these B-ALLs the normal TP53 allele was lost due to monosomy 17 (Muhlbacher et al. 2014).

2.4 BCR-ABL1 ALLs

This subgroup of B-ALLs is characterized by the formation of the BCR-ABL1 fusion transcript due to der(22) of the t(9;22)(q34;q11) translocation, or Philadelphia (Ph) chromosome. BCR-ABL1 represents about 25–30 % of adult B-ALLs and 3–5 % of pediatric B-ALLs. About 30 % of B-ALL patients display the p210 BCR-ABL fusion protein (formed by the breakpoint in the middle of BCR), while the remaining 70 % display the p190 BCR-ABL fusion protein (resulting from breakpoints in the BCR minor cluster region within the BCR intron 1). Both fusion proteins have a transforming potential of hematopoietic cells and induce a syndrome similar to CML in mice. Genome-wide analysis of B-ALLs showed that 83 % of these

leukemias display deletion of transcription factor Ikaros (IKZF1), a master regulator of B-cell differentiation. The IKZF1 deletions resulted in haploinsufficiency, expression of a dominant negative form of Ikaros or the complete loss of Ikaros expression (Mullighan et al. 2008b). The presence of Ikaros deletions represent an important prognostic factor of BCR-ABL B-ALLs both in pediatric and adult patients because they are associated with a poorer outcome and resistance to treatment with the BCR-ABL TKIImatinib.

2.5 BCR-ABL1-Like ALLs

IKZF1 alterations are observed also in a group of B-ALL patients, not displaying BCR-ABL1 translocation; these B-ALLs correspond to about 15 % of all pediatric B-ALLs and 30 % of adult B-ALLs, have a poor outcome and usually exhibit a gene expression profile similar to BCR-ABL1-positive ALLs and these cases are referred as Ph-like ALLs. Approximately 50 % of Ph-like ALL patients have rearrangements of CRLF2, with concomitant JAK 1 or 2 mutations. Transcriptome and wide-genome sequencing studies have shown that Ph-like B-ALLs without CRLF2 rearrangements frequently display genetic abnormalities activating cytokine receptors and tyrosine kinases, such as ABL1, ABL2, EPOR, JAK2 and PDGFRB. The deregulation of these kinases derives from fusion events involving these genes and resulting in a deregulated tyrosine kinase activity. About 20 % of Ph-like B-ALLs lack a chimeric fusion, but possess activating mutations of either IL7R, FLT3 or focal deletions of SH2B3 encoding LNK (Roberts et al. 2012).

Recently, Roberts et al. (2014a) have published the results of the genomic profiling of 154 patients with Ph-like B-ALLs. This large genomic screening allowed to establish that 91 % of patients with Ph-like ALLs display kinase-activating lesions consisting either in rearrangements involving ABL1, ABL2, CRLF2, CSF1R, EPOR, JAK2, NTRK3, PDGFRB, PTK2B, TSLP or TRYK2 or sequence mutations involving FLT3, IL7R or SH2B3. Alterations of ABL1, ABL2, CSF1R, JAK2 and PDGFRB resulted in Stat5 activation and cytokine-independent proliferation. Importantly, some of these abnormalities are clearly sensitive to available kinase inhibitors. Given these observations, it seemed logical to perform a clinical approach of individually treating Ph-like ALLs according to their mutational status at the level of cytokine receptors or tyrosine kinase. This approach considerably improved the outcome of this type of B-ALL patients (Roberts et al. 2014b).

2.6 B-ALLs with ERG Deletion

Recently, a new subtype of B-ALL, characterized by deletion of the ETS-family transcription factor ERG, was identified. In this B-ALL subtype, associated with a peculiar gene expression profile, ERG deletions involve an internal set of exons,

resulting in loss of the central inhibitory domain and expression of truncated ERG isoform that acts as competitive inhibitor of WT-ERG (Harvey et al. 2010). A more recent study based on the analysis of a large cohort of patients showed that the frequency of B-ALLs with ERG deletions correspond to 3.2 %. ERG deletion was mutually exclusive with other genetic lesions and was characterized by aberrant CD2 expression and frequent IKZF1 deletions (Clappier et al. 2014). In spite of the presence in this B-ALL subtype of frequent IKZF1 deletions, the prognosis was good.

2.7 MLL-Rearranged B-ALLs

MLL-rearranged B-ALLs represent about 6 % of pediatric ALLs, are characterized by a poor prognosis and are particularly frequent in infants, where they occur in about two third of infants with ALLs. Many partner genes of MLL rearrangements have been identified, but the more frequent is AF4, observed in about 50 % of cases. MLL-rearranged B-ALLs are characterized by a peculiar pattern of gene expression characterized by high expression of class I HOX genes, cooperating together with MLL fusions in inducing leukemia and in maintaining a stem cell-like state of differentiation (Faber et al. 2009). It is important to point out that MLL translocations arise in utero and rapidly lead to the development of overt leukemia, at birth or shortly after.

At variance with other B-ALL subtypes, additional genetic mutations are infrequent in MLL-rearranged B-ALLs. Particularly, copy number alterations are very rare in MLL-rearranged B-ALLs at diagnosis (Bardini et al. 2010). Ras mutations are observed in a minority of B-ALL patients with MLL rearrangements (about 16 % of these patients display either NRAS or KRAS mutated) (Driessen et al. 2013). Furthermore, FLT3 kinase domain mutations are also reported in a variable fraction of infants with MLL-AF4 ALLs. This conclusion was directly supported by whole genome sequencing of MLL-AF4 pro-B ALLs, showing the absence of CNAs in these ALLs and the occurrence of very few somatic mutations (a mean of 5 mutations) (Dobbins et al. 2013).

MLL-rearranged B-ALLs have a peculiar epigenetic profile, with signatures of cytosine, microRNA and H3K79 methylation differing from either types of B-ALLs. The increased H3K79 methylation derives from the enhanced activity of the histone methyltransferase DOT1L. Importantly, suppression of DOT1L expression into human and murine MLL-AF4 leukemic cells determines an inhibition of the MLL-induced expression program, differentiation and/or apoptosis of leukemic cells and blockade of leukemogenesis (Krivstov et al. 2008; Jo et al. 2011).

Recent studies showed that MLL fusion proteins are regulated in leukemia cells via proteolysis by the proteasome; furthermore, at variance with other oncoproteins, MLL-fusion proteins are expressed in leukemic cells at low levels. The addition of Bortezomib, a proteasome inhibitor, induced a clear increase of MLL-AF4 protein levels and apoptosis of leukemic cells through activation of the extrinsic apoptotic

pathway. Specific gene silencing experiments provided evidence that the high sensitivity of this ALL subtype is specifically dependent on the presence of the MLL-AF4 fusion protein (Liu et al. 2014b).

2.8 B-ALL with CRLF2 Rearrangement

Some B-ALLs were characterized by rearrangements involving the Cytokine Receptor-Like Factor 2 (CRLF2), also known as Thymic Stromal-Derived Lymphopoietin (TSLP) Receptor. Together with the IL-7Ralpha chain, CRLF2 forms a heterodimeric receptor for TSLP. A first type of CRLF2 abnormality was identified by Mullighan and coworkers in 2008, showing a recurrent interstitial deletion of pseudoautosomal region 1 of chromosome X and Y in B-ALL that juxtaposes the coding region of CRLF2 with noncoding exon of P2RY8 (purinergic receptor gene): the CRLF2-P2RY8 fusion was observed in 7 % of patients with B-ALL and in 53 % of Down patients with ALL (Mullighan et al. 2008a). Subsequent gene expression profiling studies have shown that 14 % of high-risk B-ALLs display hyperexpression of CRLF2; all these cases harbored a rearrangement of the CRLF2 gene: 32 % had the CRLF2-P2RY8 fusion and 62 % had a translocation of the immunoglobulin heavy chain gene IgH on 14q32 to CRLF2 (Harvey et al. 2010). CRLF2 rearrangements were associated with activating mutations of JAK1 or JAK2, deletion or mutation of IKZF1, and a peculiar Hispanic/Latin ethnicity (Harvey et al. 2010).

Less frequently, CRLF2 harbors a Phe232Cys gain-of-function mutation that promotes constitutive dimerization and cytokine-independent growth (Shochat et al. 2014). It is important to underline that CRLF2 rearrangements are frequently (up to 50 %) observed in BCR-ABL1-like ALLs (Harvey et al. 2010). B-ALL with rearranged CRLF2 displays a transcriptional signature that greatly overlaps with a BCR/ABL signature and is enriched for genes involved in cytokine receptor and JAK-STAT signaling; furthermore, these ALLs are associated with a poor outcome (Yoda et al. 2010). As above mentioned, about 50 % of CRLF2-rearranged B-ALLs display activating mutations of JAK1 and JAK2; in non-Down syndrome ALLs, CDLF2 alterations and JAK2 mutations are associated with IKZF1 deletion/mutation.

Recently, it was reported the full-exome sequencing of Down syndrome B-ALLs, showing the frequent occurrence of some driver mutations including RAS mutations (36 % of cases), JAK2 mutations (29 % of cases) or CRLF2-P2RY8 fusions (34 %); RAS mutations were shown to be mutually exclusive with JAK2 mutations. Clonal architecture analysis suggested that CRLF2 rearrangement represents the initial oncogenic event, followed by JAK2 or RAS mutations as secondary events driving subclonal expansions (Nikolaev et al. 2014).

2.9 B-ALLs with MYC Translocations

Chromosomal rearrangements involving the MYC gene, located on band 8q24, are a typical characteristic cytogenetic abnormality of Burkitt lymphoma and several subsets of other mature B-cell neoplasms. The MYC rearrangement determines a major dysregulation of the MYC oncogene and plays a key role in genesis of these diseases by juxtaposing the MYC gene to immunoglobulin genes. The major cytogenetic abnormality observed in Burkitt lymphoma is the MYC-immunoglobulin heavy chain gene (IGH) rearrangement t(8;14)(q34;q32), followed by MYC-rearrangement t(8;22)(q24;q11). Although MYC rearrangements are mainly found in mature B-cell lymphoid neoplasias, cases of B-ALL carrying the MYC rearrangement are observed. Around 3 % of adult B-ALLs show the chromosomal translocation t(8;14)(q34;q32) and display a mature B-ALL or Burkitt-type ALL immunophenotype. In these B-ALLs, the location of the chromosomal breaks in the IGH locus occur at the level of the joining and the eight different switch regions of this gene (Burmeister et al. 2013). Immunophenotypic features of these B-ALLs are compatible with Burkitt type ALL/Burkitt lymphoma (i.e., $CD34^+$, $CD19^+$, $CD22^+$, $HLA\text{-}DR^+$, $CD10^+$, sIg^+, TdT^-). The majority (>60 %) of adult B-ALLs with MYC rearranged display TP53 mutations and have a poor outcome (Stengel et al. 2014). Rare cases display the combined translocations of both MYC and MLL translocations (Meeker et al. 2011). The majority of B-ALL with Burkitt-type MYC rearrangements have a mature B-cell phenotype; however, some cases display a FAB L3 morphology and a B-precursor immunophenotype and lack to express surface Igs (Navid et al. 1999).

In some patients a Burkitt-type ALL was observed in association with the translocation t(14;18)(q32;q21), typical of follicular lymphomas: in these rare B-ALL patients this translocation was found in association with various types of MYC translocations, such as the classical Burkitt t(8;14)(q34;q32) or der(14)t(14;19) or the Burkitt variant t(8;22)(q34;q11) or the non-Burkitt MYC rearrangement t(8;9)(q24;p13); these B-ALLs are associated with a very negative prognosis (Dunphy et al. 2003; D'Achille et al. 2006).

2.10 iAMP21 B-ALL

About 2 % of B-ALLs show an intrachromosomal amplification of one copy of chromosome 21, iAMAP2, which defines a distinct B-ALL subgroup with prognostic and therapeutic implications. Initial studies have shown the complex nature of chromosome 21 structure in these patients with a common 6.6 mb-common region of amplification on chromosome 21 containing RUNX1 and c common region of deletion at the telomere. Gene profiling studies have failed to detect in these patients consistent abnormalities of relevant genes present on chromosome 21. More recently, studies have greatly contributed to understand the molecular pathogenesis

of these B-ALLs showing numerous copy number alterations mostly targeting chromosome 21 and involving deletion of IKZF1 (22 %), CDKN2A/B (17 %), PAX 5 (8 %), ETV6 (19 %) and RB1 (37 %) (Rand et al. 2011). Furthermore, the P2RY8-CRLF2 fusion was observed in 38 % of iAMP21 patients (Russell et al. 2009). Analysis of the clonal architecture of these B-ALLs showed that the various abnormalities are secondary to chromosome 21 rearrangements (Rand et al. 2011). Initial clinical studies, where the iAMP2 B-ALLs were treated with standard protocols showed a poor outcome; the outcome of these leukemias, however, clearly improved when intensified treatments were introduced for high-risk B-ALLs (Harrison et al. 2014).

Interestingly, a recent study showed that 3 % of iAMP21 B-ALLs display a constitutional Robertsonian translocation between chromosomes 15 and 21, rob (15;21) (q10;q10). Individuals born with this rare constitutional translocation have about 2,700 fold increased risk of developing iAMP21 B-ALL compared to the general population. In these cases, amplification is initiated by a chromothripsis event involving both sister chromatids of the Robertsonian chromosome; subsequently, duplication of the entire chromosome 21 occurs. In sporadic iAMP21 cases, breakage-fusion-bridge cycles are typically the initiating event, frequently followed by chromothripsis (Li et al. 2014).

2.11 Clinical-Molecular Classification of B-ALLs

Current risk classification of B-ALLs included several pretreatment clinical features including white blood cell count, age and the presence or the absence of recurrent cytogenetic abnormalities and analysis of minimal residual disease at the end of induction therapy and classifies these leukemias into four different groups: low, standard/intermediate, high and very high (Schultz et al. 2007). Very high-risk B-ALLs corresponded to about 4–5 % of pediatric B-ALLs and included leukemias with BCR-ABL translocation or hypodiploidy, failure to achieve a complete remission at the end of induction therapy (with >25 % of leukemic blasts). Low-risk B-ALLs represented about 27–30 % of all B-ALLs and include leukemias with the t(12;21)(TEL/AML1) or simultaneous trisomies of chromosomes 4, 10 and 17 (hyperdiploid B-ALLs).

Recently, a new simplified risk stratification of pediatric B-ALLs was proposed, based on the integration of cytogenetic and genomic data (the genomic data were related to the major CNAs observed in B-ALLs, concerning eight genes, IKZF1, CDKN2A/B, PAR1, BTG1, EBF1, PAX5, ETV6 and RB1). This classification identified two groups: a god-risk group included patients with ETV6-RUNX1, high hyperploidy, normal copy-number status for all eight genes, isolated deletions affecting ETV6/PAX5/BTG1, and ETV6 deletions with a single additional deletion of BTG1/PAX5/CDKN2A/B; a poor risk group including all the other genetic features. The clinical data observed on >1,500 B-ALL patients supported a significant

difference between the two groups of patients at the level of event-free survival (94 % vs 79 %) and relapse rate (4 % vs 17 %) (Moorman et al. 2014).

Harvey et al. (2010) have performed a gene expression profiling study to attempt a better characterization and classification of high-risk pediatric B-ALLs. Unsupervised clustering of gene expression profiling showed 8 unique cluster groups with these high-risk B-ALLs. Only clusters 1 and 2, corresponding each to about 10–11 % of total high-risk B-ALLs, were associated with known chromosomal translocations: cluster 1 with MLL rearrangements and cluster 2 with t(1;19) (TCF3-PBX1). Clusters 3 and 4, corresponding each at about 5–6 % of total B-ALLs, are characterized by the presence of a very high frequency of CDKN2A deletions (80–90 % of cases) and by the frequent (cluster 3, 25 %) or very frequent (cluster 4, 85 %) PAX5 deletions; cluster 3 displays a relapse-free survival (RFS) at 4 years in the average, while cluster 4 displays a RFS lower than then average of the whole high-risk group. Cluster 5 is a small group corresponding at about 5 % of these patients and shows frequent ETV6 (40 %) and IKZF1 (30 %) deletions. Cluster 6 corresponds to 10 % of high-risk B-ALLs and is characterized by frequent (40 %) ERG deletions and is the group displaying the best prognosis, with a RFS at 4 years of 94 % of patients. The cluster 7 represents the largest group (about 35 %) of high-risk B-ALLs and is characterized by the presence of multiple CNAa, involving CDKN2A, IKZF1, PAX5 and also ETV6 and IL-3RA; about 10 % of these B-ALLs display CRLF2 rearrangements; these patients display a RFS moderately lower than the average of the whole high-risk B-ALL group (Harvey et al. 2010). The group 8 corresponds at about 11 % of high-risk B-ALLs and is characterized by the very frequent (75 %) CRLF2 rearrangements and by the presence of multiple CNAs, particularly frequent IKZF1 (>90 %) and CDKN2A/B (about 60 %) deletions, and by frequent (50 %) JAK1/JAK2 mutations; this group is associated at a poor prognosis with a RFS markedly lower (23 % at 4 years) than the average (66 % at 4 years) of the whole high-risk B-ALL group.

2.12 Relapsed B-ALLs

Despite intensive chemotherapy, about 20 % of pediatric patients and >50 % of adult patients with B-ALL do not achieve a complete remission or relapse after chemotherapy. As above mentioned, several chromosomal alterations, such as BCR-ABL1 and MLL rearrangements are associated with high rates of relapse; however, all B-ALL subtypes may relapse, including B-ALL subtypes with favorable prognosis. Since the prognosis of relapsed B-ALL patients is usually poor, there is a consistent interest to characterize at molecular and clonal/subclonal level relapsed B-ALL. Thus, several studies have performed microarray profiling studies comparing matched leukemic samples at diagnosis and relapse to identify new mutations occurring only at relapse and to determine the genetic heterogeneity at clonal level of B-ALLs and to understand how this heterogeneity may affect B-ALL relapse. The initial studies involving the matched analysis of B-ALL patients

showed that the majority of B-ALLs display significant changes in the spectrum of genetic alterations from the diagnosis to the relapse and that many of these alterations relapse-acquired, such as those occurring at the level of IKZF1 and CDKN2A/B, are in fact present at low level at diagnosis, confined to rare tumor subclone (Yang et al. 2008). Mullighan et al. (2011) have sequenced 300 relevant genes in matched diagnosis and relapse B-ALL samples. Using this approach they identified 52 somatic mutations in 32 genes, many of which seem to be acquired at relapse, and particularly at the level of the transcriptional co-activators CREBBP and NCOR1, come transcription factors (ERG, SPI1, TCF4 and TCF7L2) and many components of the Ras signaling pathway. Particularly, they showed that 18 % of released B-ALL displayed sequence or deletion mutations of CREBBP and that these alterations were present only in a part of these patients at diagnosis. Inthal et al. (2012) observed a very high incidence (63 % of cases) of CREBBP mutations among relapsing high hyperploid B-ALL, while these mutations were observed on only 19 % of these relapsing patients at diagnosis. Interestingly, CREBBP mutations at diagnosis were not observed in long-term survivor patients with high hyperploid B-ALLs.

A recent study by Meyer and coworkers provided evidence that about 10 % of relapsed B-ALL pediatric patients display relapse-specific mutations at the level of the 5′-nuceotidase NT5C2, an enzyme that is responsible for the inactivation of nucleoside-analog chemotherapy drugs. These mutations conferred increased enzymatic activity and resistance to treatment with nucleoside analog therapies (Meyer et al. 2013). Irving and coworkers observed a high prevalence (37 % of cases) of somatic mutations activating the Ras pathway (KRAS, NRAS, FLT3 and PTPN11) at the level of a large population of relapsing pediatric B-ALL patients (Irving et al. 2014). Using sensitive allelic specific assays it was possible to demonstrate the existence of low-level mutated subpopulations in the majority of these patients at diagnosis (Irving et al. 2014). Mar et al. (2014) have analyzed a group of relapsing pediatric B-ALL patients and through analysis with matched diagnosis samples they showed that the somatic mutations in epigenetic regulators, such as CREBBP, KDM6A, MLL2, SETD2 and MSH6 are enriched at relapse. They interpreted these findings suggesting that therapy may have applied a selective pressure to acquire or select for rare subclones possessing these mutations.

3 T-cell Acute Lymphoblastic Leukemias; Molecular Abnormalities

T-cell acute lymphoblastic leukemias (T-ALLs) are leukemic processes involving the uncontrolled proliferation of T-cell progenitors/precursors. T-ALLs account for about 25 % of adult ALLs and 10–15 % of pediatric leukemias. At the clinical level, patients with T-ALLs show diffuse BM infiltration by immature T lymphoblasts, mediastinal masses associated to pleural effusions, high white blood cell counts. The prognosis of T-ALLs has improved in the last years due to the development of specific chemotherapy-based treatments; however, the current curative treatment

rate does bypass 75 % in children and 50 % in adults. Gene expression profiling studies have shown the existence of three main types of T-ALL subtypes, indicating three different stages of differentiation at which T-ALL blasts are blocked: (a) early immature T-ALLs indicating an early block in T-cell differentiation at a very early stage; (b) early cortical T-ALLs, characterized by positivity for CD1a, CD4 and CD8 and usually associated with activation of homeobox genes, such as TLX1 and TLX3, NKX2.1 and NKX2.2; (c) late cortical thymocytes expressing CD4, CD8 and CD3 and usually showing activation of the TAL1 transcription factor (Ferrando et al. 2002).

Early immature T-LL is characterized by the specific immunophenotype $CD1a^{-}$, $CD8^{-}$ and $CD5^{weak/-}$, with stem cell or myeloid marker expression. The early immature T-ALL subgroup represents a peculiar subtype of T-ALL, with a unique genetic basis, as supported by various lines of evidence: (i) expression of LYL1 oncogene and co-expression of LMO2; (ii) high prevalence of 5q,13q and 11q chromosomal deletions and absence of deletions of the short arm of chromosome 9; (iii) expression of the stem/progenitor marker CD34 and of the myeloid markers CD33 and CD13 (Ferrando et al. 2002). These observations were confirmed in more recent studies: Homminga et al. (2011) in a large microarray analysis of T-ALLs identified an immature cluster, largely corresponding to the early immature T-ALL subgroup; Coustan-Smith et al. (2009) defined a subgroup of T-ALLs, characterized by the absent expression of CD4, CD13, CD33, CD11b and named these leukemias subgroup as ETP T-ALLs. Recent studies have provided a molecular characterization of the fine genetic defects occurring in early immature T-ALLs. First, Homminga et al. (2011) have reported in these T-ALLs the recurrent rearrangements, resulting in overexpression of the MEGFC2 gene, encoding a key transcriptional regulator of lymphoid development, highly expressed only in immature thymocytes. Second, Zhang et al. (2012) have carried out a fundamental study reporting whole-sequence analysis of 52 ETP T-ALLs and have described the main genetic alterations occurring in this T-ALL subgroup: (i) high frequency (67 %) of activating mutations of cytokine receptor pathways and RAS signaling pathways, including IL7R, FLT3, JAK1, JAK3, SH2B3, NRAS, KRAS and BRAF; (ii) inactivating mutations (58 %) at the level of transcription factors, acting as regulators of hematopoietic differentiation, such as ETV6, RUNX1, IKAROS (IKZF1), GATA3 and EP300; (iii) inactivating mutations (48 %) at the level of genes encoding histone modifiers, such as SETD2, SUZ12, E2H2, EED. Furthermore, some of these T-ALLs displayed multiple genome rearrangements, suggesting the occurrence of genomic instability, in association with alterations of genes related to DNA mismatch repair. It is of interest to note that the mutational spectrum of ETP T-ALLs is similar to myeloid tumors and the global transcriptional profile is that of normal HSCs and myeloid LSCs.

In line with these results, other studies showed that about 50 % of adult immature T-ALLs display mutations of myeloid-specific oncogenes and tumor suppressor genes, including IDH1, IDH2, FLT3, NRAS and DNMT3A; in this study it was also noted that mutations of the ETV6 tumor suppressor gene are particularly frequent (25 %), resulting in the expression of a ETV6 truncated form with dominant negative activity (Van Vlierberghe et al. 2011). A recent study reported a whole exome

sequencing of adult ETP T-ALLs providing several novel and interesting findings: DNMT3A is mutated in 16 % of cases, while this gene was found not mutated in pediatric ETP T-ALLs; FLT3 was found to be mutated in 35 % of these patients; mutations of other epigenetic regulator genes, such as MLL2 (10 %), EZH2 (6 %), SH2B3 (6 %) and SUZ12 (1 %) were also frequent; novel recurrent mutations in the genes FAT1 (25 %), FAT3 (20 %) and DNM2 (35 %) have been identified; finally, PRC2 mutations, frequent in pediatric ETP T-ALLs are rare in adult ETP T-ALLs (Neumann et al. 2013).

Recent studies characterized pediatric T-ALLs in whom induction therapy failed to induce disease remission. A subgroup of these chemotherapy-resistant patients was characterized at molecular level by the absence of biallelic TCRγ locus deletion (ABD), a characteristic of early thymocyte precursors before V(D)J recombination, and aT-cellular level by a T early precursor cell phenotype (Gutierrez et al. 2010). Zuurbier et al. (2014) have recently characterized these ABD T-ALLs at molecular level showing frequent NOTCH1/FBW7 mutations (57 %), but absent WT1, PHF6 and PTEN/AKT mutations.

Studies carried out in the last years have shown that T-ALL development results from a multistep oncogenic process involving the acquisition of multiple somatic genetic abnormalities at the level of the NOTCH signaling pathway, transcription factors, signaling oncogenes and tumor suppressors (reviewed in Van Vlierberghe and Ferrando 2012). The most frequent genetic alterations occurring in T-ALL is represented by the deletion of the CDKN2A locus, present on chromosome 9p22 and occurring in about 70 % of cases: this locus englobes two different tumor suppressor genes, p14/INK4A and p16/ARF, both involved in the control of cell cycle. The activation of the NOTCH signaling pathway is frequently observed in T-ALLs: in fact, 60 % of T-ALLs, display activating mutations of the NOTCH1 (either at the level of the HD domain of this receptor, or of the PEST domain), while 20 % of T-ALL cases exhibit activation of NOTCH1 mediated by mutations of the FBXW7 gene (encoding a protein involved in the control of stability of NOTCH1 and other relevant oncoproteins such as MYC, MCL1, CyclinE) (reviewed in Tosello and Ferrando 2013). Both the physiologic and oncogenic effects of NOTCH1 require translocation of the intracellular portion of the NOTCH1 receptor to the nucleus, where it activates a specific program of gene expression. In this context, NOTCH1 is a key regulator of the proliferation of T-ALL blasts by controlling various genes involved in the control of cell growth. Among these genes a key role is played by c-myc, whose expression is transcriptionally controlled through the binding to an enhancer present at the level of the proximal c-myc promoter (Herranz et al. 2014).

In about 40 % of T-ALLs, chromosomal translocations juxtaposing transcription factors playing a key role in the control of T-cell differentiation and regulatory elements located in proximity of the T-cell receptor genes are observed (reviewed in Van Viberghe and Ferrando 2012). These T-ALL-specific transcription factors acting as oncogenes for the development of this leukemia are: some members of the HLH family of transcription factors, such as LYL1, TAL1; some members of the LIM-only domain (LMO) family, such as LMO1 and LMO2; some members of the homeobox gene family, including some HOXA genes, TLX1/HOXD11 and TLX3/

HOX11L2; some key oncogenes, such as MYB and MYC; TAN1, a truncated, constitutively active form of the NOTCH1 membrane receptor. In other cases, these transcription factors are activated by genetic abnormalities, different from those involving TCR-associated chromosomal abnormalities, such as duplications of the MYB oncogene, small activating deletions of LMO2 and TAL1 and the translocation determining the activation of TLX3/HOX11L2 gene through its juxtaposition near to the BL11B gene locus (reviewed in Van Vlierberghe and Ferrando 2012).

Homminga et al. (2011) have performed an integrated transcriptomic and genomic analysis of T-ALLs, identifying two T-ALL potential subgroups lacking known oncogenic rearrangements and representing about 20 % of pediatric T-ALLs. One of these two subtypes is associated with cortical thymocyte differentiation block and by very frequent overexpression of NKX2-1/NKX2-2, for which genes frequent rearrangements have been observed (in about 60 % of cases). The second subtype was associated with immature cell development, high expression of MEFC2 transcription factor and rearrangements of MEFC2 or of transcription factors directly targeting MEF2C (in about 50 % of cases). Ectopic expression of NKX2-1 of MEFC2 induces oncogenic effects and interferes with T-cell differentiation. A subsequent study clearly showed that MEFC-dysregulated T-ALLs represent a subgroup of ETP T-ALLs and are characterized by an early T-ALL gene signature and have non-rearranged T-cell receptors, in line with their early T-cell differentiation block (Zuurbier et al. 2014).

Additional mutations, occurring at the level of various transcription factor tumor suppressor genes, such as BCL11B, ETV6, LEF1, PHF6, RUNX1 and WT1, have been reported in T-ALLs. Furthermore, genetic alterations at the level of various signaling pathways have been described in T-ALLs, such as: activating mutations of the cytokine receptors IL7R and FLT3; activating mutations of the transducing proteins JAK1 and JAK3; deletions of the PTPN2 gene; activating mutations of the RAS signaling pathway; deletions and mutations of the PTEN gene (reviewed in Van Vlierberghe and Ferrando 2012).

Recent studies have reported mutations of some genes involved in the epigenetic control of gene expression, such as EZH2, EED, SETD2 and SUZ12, thus highlighting a possible role of altered epigenetic regulation in T-cell oncogenesis (Ntziachristos et al. 2012). Finally, a recent study identified three new oncogenic driver genes in T-ALLs. Thus, CNOT3 was identified as a tumor repressor gene mutated in about 8 % of adult T-ALLs; mutations affecting the ribosomal proteins RPL5 and RPL10 have been detected in about 10 % of pediatric T-ALLs (De Keersmaecker et al. 2013). The mechanism through which mutations of ribosomal RPL proteins affect leukemia development is largely unknown; however, a recent study, based on the expression of the mutant Rpl10-R981 in yeast, suggested that T-cellular adaptation to the presence of this mutant implies changes in gene expression that in long-term undermine cellular homeostasis (Sulima et al. 2014).

All these observations have led to propose a molecular classification of T-ALLs which identifies T-ALL subtypes, each characterized by a type of mutation specific and considered a driver event. Thus, according to this molecular classification the mutations occurring in T-ALLs are classified as type A mutations (Driving

oncogenes that characterize and define different genetic T-ALL subgroups) and type B mutations (common genetic abnormalities that can be found in all T-ALL genetic subgroups). According to this classification, six T-ALL genetic subgroups have been identified, TAL/LMO, TLX1, TLX3, HOXA, MYB, ETP (or LYL1), whose main features are reported in Table 14.2.

The prognostic significance of the various type B gene mutations and abnormalities occurring in T-ALLs was recently evaluated in adult T-ALL patients. Homozygous deletion of CDKN2A, mostly present in cortical/mature T-ALLs, was associated with favorable outcome, compared to the rest of T-ALLs not displaying this abnormality. TP53 heterozygous deletion, observed in about 10 % of T-ALL patients, was associated with worse clinical outcome. NOTCH1 and FBW7 mutants, very frequent among cortical/mature T-ALLs, were associated to a better outcome than non-mutant T-ALLs. Similarly, favorable outcome was observed in adult T-ALL patients with heterozygous inactivating mutations or deletions in the BCL11B tumor suppressor gene. In contrast, somatic mutations in genes targeting the epigenetic regulators DNMT3A and IDH1/2, uniquely present in the early immature adult T-ALL group, are associated with negative prognosis (Van Vlierberghe et al. 2013).

Trinquand et al. (2013) have proposed a simplified classification of adult T-ALL, based on the presence or not in these leukemias of NOTCH1/FBXW7 mutations: the absence of these mutations was found to be associated with a poor prognosis. The group of NOTCH1/FBXW7 mutations was associated with a good prognosis at the condition that in these leukemias are absent KRAS, NRAS and PTEN mutations. According to this classification, about 50 % of adult T-ALLs are predicted to have a "good" outcome and about 50 % a poor outcome. Grossmann et al. (2013) have recently shown the negative impact of RUNX1 and DNMT3A mutations at the level of the early T-ALLs: the presence of these mutations was associated with a short survival.

3.1 Relapsed T-ALLs

The problem of relapsed T-ALLs is a major problem because these ALLs are usually more resistant to treatment than B-ALLs and exhibit a greater tendency to relapse. Relapsed T-ALLs have a poor prognosis. The large majority of T-ALL patients either relapse on-therapy or immediately after or within 2 years after the end of treatment; only about 10 % of these patients relapse 2.5 years after the end of treatment. In an initial study, Szczepanski et al. (2003) have used clonal T-cell receptor gene rearrangements to study the clonal derivation of relapsed T-ALLs. In the majority of cases clonal TCR rearrangements in paired T-ALL specimens were similar at diagnosis and at relapse, thus suggesting that the relapsing clone evolved from the same leukemic clone present at diagnosis. However, in two late-relapsing patients it was noted that the TCR rearrangements observed at diagnosis and at relapse are completely different, thus suggesting that the relapsing clone evolved

Table 14.2 T-ALL genetic subgroups with the main features

Subgroup (frequency, %)	Rearrangement	Gene involved	Additional genetic abnormalities	T-cell differentiation block
TAL/LMO (30–35 %)	t(1;14)(p32;q11)/1p32 deletion t(1;7)(p32;q34)/1p32 deletion t(7;9)(q34;p32) t(11;14)(p15;q11) t(7;11)(q34;p15) t(11;14)(p13;q11)/11p13 deletion t(7;11)(p34;p13)/11p13 deletion	TAL1 (SIL/TAL1) TAL1 (SIL/TAL1) TAL2 LMO1 LMO1 LMO2 LMO2	NOTCH pathway mutation (50 % of cases) Frequent PTEN mutation/deletion (30–40 % of cases) Frequent MYC translocations (17 % of cases) Absent IL-7R mutations CDKN2A deletion (53 %) CASP8 deletion	Pre-αβ/TCR-αβ (Early T/Cortical) Immature/Pre-αβ/TCR-αβ
TLX1 (8 %)	t(10;14)(q24;q11) t(7;10)(q34;q24)	TLX1/HOX11	PTPN2 deletion (about 50 % of cases) Absent IL-7R. Frequent PHF6 mutation (43 %) Frequent BCL11B mutation (50 %) CDKN2A deletion (89 %)	Immature/Pre-αβ The capacity to block T-cell differentiation is due to interaction with Ets1 and block of TCR gene expression
TLX3 (24 %)	t(5;14)(q35;q32)	TLX3/HOX11L2	Frequent WT1 gene mutations (35 %) Absent PTEN mutation/deletion Frequent IL-7R mutations (16 %) Frequent PHF6 mutations (43 %) Frequent BCL11B mutation (25 %) CDKN2A deletion (78 %) RB1 deletion	Immature/Pre-αβ The capacity to block T-cell differentiation is due to interaction with Ets1 and block of TCR gene expression

HOXA (15 %)	inv(7)(p15q34) t(7;7)(p15;q34) t(10;11)(p13;q14) t(11;19)(q23;p13) 9q34 deletion	HOXA HOXA CAML-AF10 MLL-ENL SET-NUP214	Frequent IL-7R mutation (38 %) Frequent (50 %) PHF6 mutations in SET-NUP214 Frequent RAS mutations in MLL-ENL CDKN2A deletion (67 %)	TCR αβ or TCR γδ Immature/ϒδ lineage Immature/ϒδ lineage Immature/ϒδ lineage
NKX2-1 and NKX2-2 (5 %)	inv(14)(q13q32.33) inv(14)(q11.2q13) t(7;14)(q34;q13) t(14;20)(q11;p11)	NKX2-1 (IGH) NKX2-1 (TRA) NKX2-2 (TRB) NKX2-2 (TRB)		Cortical thymocyte stage
MEF2 C (2 %)	del(5)(q14) der(5)t(4;5)(q26;q14)	MEF2C MEF2C (4q26)	NOTCH1/FBXW7 abnormalities (60 %) No mutations of PHF6 and WT1 PTEN/AKT mutations (13 %)	Early thymocyte progenitor

independently from the initial leukemic clone observed at diagnosis. In a second later study the same authors have explored a group of late-relapsing T-ALLs showing that there was evidence of: (a) a common clonal origin between diagnosis and relapse in 64 % of cases; (b) a different clonal origin between diagnosis and relapse in the remaining 36 % of cases (Szczepanski et al. 2011).

Mullighan et al. (2008a) studied copy number alterations in 14 matched T-ALL samples at diagnosis and at relapse, showing that the mean frequency of CNAs remained unchanged, but many of these alterations changed from diagnosis to relapse. A clonal relationship between the diagnosis and relapse T-ALL was observed in about 71 % of T-ALL cases; according to these findings it was suggested that the relapse CNAs either were present at diagnosis at low/very low levels and positively select at relapse or were acquired as new genetic alterations after the initial induction therapy. Tzoneva et al. (2013) have studied the mutational profile by using whole-exome sequencing of 5 T-ALL patients at diagnosis and at relapse: they identified a total of 60 mutations of whom 17 were present at diagnosis and at relapse, 24 were selectively present only at relapse and 19 were present only at diagnosis. About 80 % of these leukemias displayed at least one mutation at relapse, observed also at diagnosis; 20 % mutations all different at relapse, compared to those observed at diagnosis. The most relevant finding of whole-exome sequencing of relapsed T-ALL was the identification of mutations of the cytosolic 5′-nucleotidase II gene (NT5C2), encoding a 5′-nucleotidase enzyme responsible for the inactivation of nucleoside-analog chemotherapy drugs; NT5C2 mutant proteins display increased nucleotidase activity in vitro and confer resistance to 6-mercaptopurine and 6-thioguanine when expressed in T-lymphoblasts.

4 Leukemic Stem Cells in Acute Lymphoblastic Leukemias

4.1 B-ALLs

The nature and the frequencies of the stem cells or leukemia initiating cells in ALLs have been a contentious issue. In an initial study, Cobaleda et al. (2000) have shown that, regardless of the heterogeneity in maturation characteristics of the leukemic cells, only primitive blasts with a $CD34^{+}CD38^{-}$ immunophenotype were able to transfer BCR/ABL-positive ALLs into immunodeficient NOD/SCID mice. According to this finding, these cells were defined as SCID leukemia-initiating cells (SL-IC). Subsequently, it was proposed that an aberrant $CD19^{+}CD34^{+}$ lymphoid cell that lacks CD38 (the normal counterpart of this cell does not exist) expression could represent a candidate leukemic stem cell population in ALLs (Castor et al. 2005). This conclusion was based on the observation that clinically and genetically different subtypes of B-ALLs originate from different stages of hematopoietic differentiation: ETV6-RUNX1 (TEL-AML1) fusions-positive ALLs originated from committed B-cell progenitors, while major breakpoint BCR-ABL1 fusions (encoding P210 BCR-ABL1) originated at the level of HSCs (Castor et al. 2005). In

contrast, minor breakpoint BCR-ABL1 fusions (encoding P190 BCR-ABL1) had an origin at the level of cells with a B-cell progenitor phenotype (Castor et al. 2005). According to these findings it was proposed that P190 and P210 BCR-ABL1 were distinct tumor biological and clinical entities (Castor et al. 2005). In line with this observation, using samples of patients with ETV6-RUNX1 (TEL-AML) fusion, $CD34^{+}CD38^{low}CD19^{+}$ leukemic blasts were shown to be able to re-initiate and sustain leukemic growth in immunodeficient NOD/SCID mice. Particularly, using three samples from three patients with TEL/AML1-positive ALLs, only $CD34^{+}CD38^{low}CD19^{+}$ cells were able to engraft primary and secondary SCID mice, while transplantation of $CD34^{+}CD38^{-}CD19^{+}$ cells from only one of the three patients led to a low level of engraftment in primary, but not in secondary mice. Importantly, lentiviral transduction of normal cord blood progenitor cells with the ETV6-RUNX1 fusion gene led to the formation of cells with the aberrant $CD34^{+}CD38^{low}CD19^{+}$ immunophenotype (Hong et al. 2008). More recently, Cox et al. (2009) have provided evidence that in pediatric ALLs there is a minority (i.e., <1 %) of $CD133^{+}CD19^{-}$ cells that are capable of initiating and maintaining in vitro long-term cultures of B-ALL cells and of engrafting serial NOD/SCID recipient mice, with development of a B-ALL process; in contrast, there was no detectable engraftment with $CD133^{+}/CD19^{+}$ cells (Cox et al. 2009). At the level of the $CD133^{+}$ cell population, only $CD133^{+}/CD38^{-}$ cells were able to engraft immunodeficient mice.

The Philadelphia chromosome t(9;22) leading to the BCR/ABL fusion oncogene and the translocation t(4;11) with formation of the MLL/AF4 fusion oncogene have been associated with a particular poor outcome. Hotfilder et al. (2005) have analyzed 8 leukemic samples with ALL/t(9;22) and 12 with ALL/t(4;11) and have isolated immature $CD34^{+}CD19^{-}$ leukemic cells from these samples, showing by in situ hybridization that about 60 % of these cells carry the leukemic translocation. Through in vitro colony assays it was shown that myelo-erythroid colonies generated by $CD34^{+}CD19^{-}$ cells do not originate from a progenitor that carries the leukemic translocation. According to these findings it was concluded that childhood high-risk ALL/t(9;22) and t(4;11) originate in a primitive $CD34^{+}CD19^{-}$ progenitor/stem cell, without a myelo-erythroid developmental potential.

Subsequent studies have raised some doubts that ALL LSCs can be simply identified as $CD34^{+}CD38^{low}CD19^{+}$ cells. In fact, several studies have identified candidate LSCs in both rare, immature populations as well as conversely, across several immunophenotypically distinct groups of more mature cells (Cox et al. 2004; Kong et al. 2008; Le Viseur et al. 2008; Vormoor 2009). Particularly, it was shown that sorted $CD34^{+}CD19^{-}$, $CD34^{+}CD19^{+}$ and $CD34^{-}CD19^{+}$ cell populations all contain leukemia-initiating cells, although with different frequency. Importantly, each of these populations re-establish the complete immunophenotype of the original leukemia and is able so self-renew: this observation demonstrates the ability of B-ALL blasts to move back and forth between the different populations. It is important to note that in these studies the intrafemoral injection of sorted leukemic cells allowed a reproducible and efficient leukemic engraftment into the immunodeficient mice. Therefore, the intrafemoral injection of leukemic cells seems to be a robust transplantation assay to evaluate cell populations that are able to maintain ALLs in vivo.

It is also of interest to note that, in spite the efficient leukemic transplantation procedure developed in this study, not all ALL samples were able to engraft into NOD/SCID mice: particularly, while six out of seven high-risk ALLs were capable to grow into NOD/SCID mice, only two out six standard-risk patients engrafted (Le Viseur et al. 2008). Morisot et al. (2010) confirmed the preferential tendency of high-risk ALLs to grow in xenograft assay into highly immunodeficient mice: particularly, they observed that mice transplanted with primary samples from ALL patients at relapse developed leukemias in mice at 1–3 months post-transplantation, while those transplanted with primary samples from ALL patients at diagnosis developed leukemias more slowly, at 2–7 months post-transplant. Importantly, LSC frequency in precursor-B ALL was high, being evaluated in a range comprised between 1 % and 24 %. Other studies have shown that in primary childhood B-cell precursors both $CD34^+CD38^+CD19^+$ and $CD34^+CD38^-CD19^+$ cells exhibit an in vivo leukemogenic potential when grafted to a NOD/SCID mouse; in contrast, $CD34^+CD38^-CD19^-CD10^-$ cells do not generate a leukemic progeny in NOD/SCID mice, but a normal multilineage hematopoietic cell progeny (Kong et al. 2008). In a more recent study, Kong et al. (2014) have shown that $CD34^+CD38^-CD58^-$ cells are the leukemia-initiating cell population of Ph^+ ALLs. These studies were prompted by the observation that Ph^+ B-ALL patients with a predominant $CD34^+CD38^-CD58^-$ phenotype have a poorer prognosis than those with predominant $CD34^+CD38^+$ and/or $CD34^+CD58^+$ phenotypes. Importantly, only $CD34^+CD38^-CD58^-$, but not $CD34^+CD38^-CD58^+$ or $CD34^+CD38^+CD58^-$ or $CD34^+CD38^+CD58^+$ cells were able to engraft immunodeficient mice. The heterogeneity of immunophenotypic features of LICs in B-ALL was confirmed by Diamanti et al. (2012). In fact, these authors have shown that $CD34^+CD19^-$, $CD34^+CD19^+$ and $CD34^-$ cells isolated from B-cell precursor ALLs, all contain LICs.

An important property of the NOD/SCID model of ALL is its capacity to retain the genotypic and phenotypic properties of the original patient samples which provides a relatively accurate representation of the human disease. However, the NOD/SCID mice model possesses also some important intrinsic limitations and its capacity to be permissive for leukemic growth is certainly limited (Kennedy and Barabé 2008). These limitations are seemingly related to the lack of a supportive microenvironment or to the residual host's immune system preventing the engraftment of leukemic cells, blocking their capacity to reach tissual niches suitable for their survival and proliferation. In some recent studies new attempts have been made to develop new NOD/SCID transplantation assays based on the inoculation of leukemic cells in the spleen or in the liver. Thus, Wang et al. (2012) have reported the successful engraftment of ALL cells in NOD/SCID mice via intrasplenic inoculations: this assay implies the pre-treatment of mice with anti-CD122 mAb. This assay allowed the engraftment of ALL cells in 5 out 11 cases, with serial transplantation of the engrafted ALLs. Cheung et al. (2010) have reported the successful engraftment by ALL cells after direct intrahepatic injection into unconditioned newborn NOD/SCID mice. Five out 13 ALL samples engrafted into NOD/SCID mice using the intrahepatic route of leukemia cell injection.

A recent study provided interesting findings related to the frequency of LICs among different B-ALL subtypes, and in relationship with various immunophenotypic leukemic subpopulations (Rehe et al. 2013). It is important to point out that in this study were included pediatric B-ALLs pertaining to various B-ALL subtypes. Using these leukemic samples, it was investigated a possible relationship between membrane differentiation markers (CD34 expressed at the level of pro-B and pre-B1 lymphoid cells, CD10 expressed from pre-B1 to immature B-cells and CD20 expressed at low levels in preB-cells and at high levels in immature and mature B-cells) and LICs. The frequency of leukemia-initiating cells, as well as their kinetics of engraftment into immunodeficient mice, was comparable in leukemic blasts sorted according to the low/absent or high expression of either CD34, CD10 or CD20, thus indicating the absence of a link between leukemic cell differentiation status (as evaluated through the study of membrane markers) and LIC properties. Interestingly, the transcriptomic analysis of sorted $CD34^{+}$ and $CD34^{low/-}$ B-ALL cells showed a remarkable difference in their transcriptomic profile, with $CD34^{+}$ cells resembling normal B progenitors; however, in spite these differences in gene expression pattern, both these cell populations display a similar leukemia-initiating capacity (Rehe et al. 2013). A recent study directly addressed the problem of defining the stem cell program of purified populations of leukemic Stem/progenitor cells isolated from B-ALLs, compared to their normal counterpart. This type of analysis provided important data to define the stemness program of leukemic cells. To perform this analysis five populations of normal early lymphoid cells have been evaluated: HSC ($CD34^{+}CD38^{-}CD19^{-}$), Early Lymphoid Progenitor Cells (ELPC, $CD34^{+}CD38^{+}CD19^{-}$), Pro-B ($CD34^{+}CD38^{+}CD19^{+}$), Pre-B ($CD34^{-}CD19^{+}IgM^{-}$) and Immature/Mature B ($CD34^{-}CD19^{+}IgM^{+}$); these cell populations, at the HSC stage express "self-renewal" genes, including HOXB4, BMI1, TEL, AML1, PTEN, IKZF1, MLL and GFI1 and progressively acquire the expression of genes essential for B-cell development, such as, TCF3, EBF1, SPI1 and IKZF1 first, then DNTT, PAX5, VPREB1, RAG ½, LEF1 and IGLL1. On the other hand, four populations of leukemic cells have been isolated from TEL-AML1 B-ALLs: $CD34^{+}CD38^{-}CD19^{+}$, a leukemic-specific, early stem/progenitor cell population; $CD34^{+}CD38^{+}CD19^{+}$ operatively defined as ALL-Pro-B for its immunophenotypical similarity to normal Pro-B; $CD34^{-}CD38^{+}CD19^{+}$ defined as ALL Pre-B and, finally, $CD34^{-}CD38^{-}CD19^{+}$, defined as ALL-IM/M-B. All these four leukemic cell populations displayed a similar transcriptomic profile, independently on their phenotypic features and resembling all normal HSCs or ELPCs (Fan et al. 2014). This observation strongly supports the functional studies on isolated leukemic subpopulations showing that different immunophenotypical fractions of leukemic lymphoblasts contain LSCs.

The pattern of B-ALL growth into immunodeficient mice may reflect the leukemia prognosis. Thus, Meyer et al. (2011) have investigated the engraftment properties and impact on patient outcome of 50 pediatric B-ALL samples transplanted into NOD/SCID mice. Time to development of leukemia (TTL) into immunodeficient mice was determined for each patient sample engrafted as weeks from transplant to overt leukemia: accordingly, patients with a TTL <10 weeks were classified

as TTL^{short}, while those with prolonged time of NOD/SCID engraftment were classified as TTL^{long}. Importantly, patients whose leukemia samples exhibited TTL^{short} exhibited a clearly shorter survival compared to those with late leukemia onset. B-ALLs growing into NOD/SCID mice with a TTL^{short} pattern are associated with a gene expression signature characterized by high expression of signaling pathways involved in cell growth and apoptosis. These findings were confirmed and extended by the same authors in a subsequent study showing that an intact apoptosome function was associated with a TTL^{long} phenotype, good treatment response and better patient survival, while deficient apoptosome function was associated with rapid engraftment (TTL^{short} phenotype) and early relapse (Queudeville et al. 2012).

The differences observed between the different studies on the identification of leukemia-initiating cells in ALLs may be in part related to the intrinsic biologic heterogeneity of the leukemic stem cells observed in different B-ALL specimens, but are related also to technical differences in the methodology used to test in vivo the leukemia-initiating capacity of leukemic cell subpopulations. The assay-related variables are the following: (i) the NOD/SCID model (the NOD/SCID Gamma mice or NOD/SCID mice pre-treated with anti-NK lymphocytes lytic antibodies seem to be better recipients than the classical NOD/SCID mice); (ii) the site of leukemic cell injection into mice (the intrafemoral injection of candidate cells leads to a markedly more sensitive stem cell assay, compared to intravenous injection); (iii) the conditioning or not of recipient mice with irradiation. In line with this conclusion, a recent study provided important observations to optimize the experimental conditions for xenotransplant assays of human B-ALL samples. In fact, Patel et al. (2014) have explored a possible role of total body irradiation (TBI) pre-conditioning on the engraftment of human pediatric B-ALL cells into NSG mice. They observed that TBI preconditioning was associated with a markedly higher proportion of engrafting samples observed that TBI preconditioning was associated with a markedly higher proportion of engrafting samples (11/12), compared with no TBI (7/13). The analysis of B-ALL subtypes growing in the immunodeficient mice showed that while t(4;11) B-ALLs were able to grow efficiently also in unconditioned NSG recipient, the other B-ALL subtypes required TBI preconditioning for efficient engraftment into NSG mice. The superiority of the TBI preconditioning was apparent, not only when leukemic cells were injected IV, but also when leukemic cells were inoculated into bone marrow. The requirement for TBI preconditioning was related to the capacity of TBI to induce SDF-1 alpha release by bone marrow stromal cells and acting as a strong chemoattractant and homing factor for B-ALL progenitors.

Considering the ensemble of these studies one must conclude that the putative stem cells responsible for initiating and maintaining B-ALLs are not a fixed cell identity, but themselves evolve both in genotype and phenotype. This conclusion is supported by twin studies. These studies were based on the analysis of leukemic and pre-leukemic stem cell populations in the pair of identical twin discordant for ETV6-RUNX-positive ALL (Greaves and Wiemels 2003). As mentioned above, a subpopulation of $CD34^{+}CD38^{-}CD19^{+}$ cells was shown to be able to transfer the leukemia into NOD/SCID mice (Hong et al. 2008). These putative leukemic stem cells are present in both the healthy twin with pre-leukemia and in her co-twin with

ETV6-RUNX1-positive ALL: however, in the former one these cells are much less frequent than in the latter one. Importantly, pre-leukemic stem cells present in the healthy twin are genotypically and phenotypically distinct from leukemic stem cells observed in the twin with ETV6-RUNX1-positive ALL (Hong et al. 2008).

There is compelling evidence that several of the common translocations (i.e., MLL-AF4, TEL-AML1, BCR-ABL) that are seen in pediatric B-ALLs often originate prenatally in utero during embryonic/fetal development (Greaves and Wiemels 2003). The first evidence about the in utero origin of childhood B-ALLs is issued from studies in twins. In fact, leukemic cells isolated from identical twins with B-ALL share unique, specific, clonal chromosome rearrangements, a finding highly compatible with the hypothesis that these specific leukemogenic abnormalities derive from spontaneous mutagenic events occurring in utero (Ford et al. 1993; Wiemels et al. 1999). A second line of evidence indicates that during in utero development these leukemic fusion genes may arise in a population of mesodermal stem cells capable of differentiate during development in a variety of mesoderm-derived tissues, including Hematopoietic Stem Cells and Mesenchymal Stem Cells. This hypothesis was tested by investigating whether bone marrow-mesenchymal stem cells from childhood leukemia harbor leukemia-specific fusion genes. Mesenchymal Stem Cells of childhood B-ALLs carrying TEL-AML1 and BCR-ABL do not express the fusion transcripts; however, MLL-AF4 was detected and expressed in bone marrow-Mesenchymal Stem Cells from all cases of MLL-AF4-positive B-ALLs (Menendez et al. 2009). These observations indicate that MLL-AF4 arises in a population of mesodermal stem cells generating both hematopoietic and mesenchymal cells. Third, a prenatal origin of childhood ALLs was further supported by the detection of clonotypic immunoglobulin gene rearrangements on neonatal blot spots of children with various subtypes of ALLs (Greaves et al. 2003). Fourth, Teuffel et al. reported the results of a study carried out in 5-year-old monozygotic twins with concordant B-all displaying translocation of ETV6 and RUNX1 genes (ET6-RUNX1 fusion). Separate leukemic clones were identified in the diagnostic samples since distinct IGH and IGK gene rearrangements could be detected; importantly, both the identical ETV6-RUNX1 fusion sequence and the distinct immunoglobulin gene rearrangements were identified in the neonatal spots, thus unambiguously indicating that the separate leukemic clones evolved before birth (Teuffel et al. 2004). The study of twins with ALL was also of fundamental importance to determine the timing of mutation acquisition required for leukemia development. These studies were triggered by the observation that leukemic fusions are detectable in cord blood from healthy newborn infants at rates about 100 fold higher than the incidence of ALLs, thus suggesting a strict need for additional mutations in leukemia development (Mori et al. 2002).

According to these findings it was proposed a model suggesting that the ALL-specific fusion events occur in utero during embryonic/fetal development, generating a preleukemic clone, clinically silent; the preleukemic clone may progress to full leukemic transformation through the acquisition of new genetic abnormalities, such as point mutations, deletions and/or duplications. Thus, according to this model it is expected that TEL-AML1 fusion should occur in about 1 % of newborns,

taking into account the cumulative incidence of TEL-AML1$^+$ B-ALL in children of about 0.01 %. This expectation of incidence of TEL-AML1 fusion in newborns has been met by the study of Mori and coworkers reporting an incidence of about 1 % of TEL-AML1 among British-Italian newborns (Mori et al. 2002). These findings were confirmed by other investigators and, particularly, by Eguchi-Ishimal et al. (2002) showing that 1.5 % of tested cord bloods are positive for TEL-AML1 fusion. A Danish group has recently challenged this view, showing that the proportion of newborns with detectable TEL-AML1 fusion was lower (about 0.01 %), implying that a high proportion of infants born with detectable TEL-AML1 fusion develop TEL-AML1$^+$ B-ALL (Lausten-Thomsen et al. 2011). However, this was an isolated finding since the majority of other studies have shown high frequencies of TEL-AML1 fusions among newborns. Particularly, in the study led by Skorvaga et al. (2014), it was reported a frequency of 4 % of newborns exhibiting TEL-AML1 fusions. It is very important to point out that the TEL-AML1 transcripts are expressed in cord blood cells at very low levels, estimated as low as about one to five copies per 10^5 cells.

ETV6-RUNX1-positive ALLs, in addition to the fusion ETV6-RUNX1 gene, also have multiple copy number alterations (CNA), as revealed by genome-wide single-nucleotide polymorphism arrays: recurrent CNAs are seemingly driver events. The analysis of CNAs in five pairs of monozygotic twins with concordant ETV6-RUNX1-positive ALL showed that all the driver CNAs were discordant within each of the five twin pairs, thus suggesting that they are secondary to the prenatal gene fusion event (Bateman et al. 2010). In other studies the whole genomes of leukemic cells from some twin pairs with ALL have been sequenced, showing that few (5–10) shared prenatal coding-region single nucleotide variants were limited to the putative initiating lesions, while a relatively more abundant (15–20) non-synonymous single-nucleotide variants were distinct between tumors and, therefore, secondary and postnatal. These variants do not seem to affect genes relevant for the leukemogenic process, in agreement with the view that the leukemic development of ETV6-RUNX1-positive ALLs may be triggered by the initial fusion event and few CAN driver events (Ma et al. 2013).

The study of twins was also of fundamental importance to determine the timing and developmental sequence of molecular events in BCR-ABL1$^+$ ALL, usually associated with deletion of the IKAROS (IKZF1) gene. Through the analysis of the status of BCR-ABL1 and IKZF1 genes in some pairs of monozygotic twins concordant or discordant for Ph$^+$ ALL, it was reached the conclusion that the BCR-ABL1 is an initiation event occurring in utero, while the IKZF1 is a secondary and probably post-natal mutation. In the absence of the IKZF1 mutation, the leukemic clone remains clinically silent (Cazzaniga et al. 2011).

Similarly, there is evidence that MLL gene rearrangement with one of its fusion partner (AF4, ENL or AF9 genes) is an initiation, prenatal event. The penetrance of this genetic abnormality is very high and the concordance of ALLs in monozygotic twins bearing MLL rearrangements is near to 100 %. However, the study of rare cases of discordance of MLL-rearranged ALLs in monozygotic twins allowed to support the prenatal origin of MLL gene fusion event (Chuk et al. 2009).

Recent studies support the existence of genetic heterogeneity of leukemia initiating cells in ALLs. Using a multi-color, multi-plexed FISH method allowing the detection of the most recurrent or common genetic events occurring in a TEL-AML1 ALL (TEL-AML1 fusion, duplication of the fusion, extra copies of chromosome 21, deletion of unrearranged AML1 allele, mono- or bi-allelic deletions of PAX5 and CDKN2A/p16), it was possible to analyze individual stem cell clones in 30 patient's TEL-AML1 ALLs. This study provided evidence that leukemic stem cells in each patient are highly heterogeneous for their genetic alterations (Greaves 2009, 2010). These observations were definitely supported by a study carried out in ETV6-RUNX1-positive ALLs by multiplexing fluorescence in situ hybridization using the probes characterizing all known driver mutations occurring in this ALL subtype (TEL-AML1 fusion gene and few driver copy number alterations). This analysis allowed to define a composite picture of subclonal architecture, showing the existence of ALL subclones displaying a variegated genetics and complex evolutionary histories (Anderson et al. 2011). Leukemia-initiating cells are equally heterogeneous in the genetical abnormalities, showing a level of subclonal complexity highly similar to that observed in the bulk tumor cells (Anderson et al. 2011). Interestingly, the analysis of relapsing cases of ETV6-RUNX1-positive ALLs provided evidence that, irrespective of the time of relapse, the relapsing clone was derived from either a major or minor clone at presentation. Genetic events frequently observed in relapsing ETV6-RUNX1 ALLs are deletions of CDKN2A/B and gain of chromosome 16 (van Delft et al. 2011).

Ph^+ ALLs, rare in children (<5 % of pediatric ALLs) but frequent in adults (~35 % of adult ALLs), resemble CML lymphoid blast crisis and have a poor prognosis. The genetic lesions that cooperate with BCR-ABL to induce ALL have been in part characterized, including the frequent (>80 %) deletion of IK2F1 encoding the transcription factor Ikaros (Mullighan et al. 2008b), of PAX5 transcription factor (about 50 %) and of the inhibitors of cyclin D-dependent kinases CDKN2a/B (about 55 %) (Mullighan et al. 2008c). Taking advantage on the presence of frequent genetic abnormalities in Ph^+ ALLs attempts have been made to understand how the variability in these genetic abnormalities may reflect a genetic heterogeneity at the level of the leukemic stem cell compartment. Using various strains of NOD/SCID mice Notta et al. (2011b) have defined two subtypes of Ph^+ ALLs: one causing an aggressive disease in immunodeficient mice, the other inducing a non-aggressive leukemia in mice. The analysis of genetic lesions in these two subgroups showed: (a) similar frequencies of IKF1 deletions in the two groups; (b) marked differences in the frequencies of CDKN2A/B and PAX5 in the two groups (for CDKN2A/B 90 % in the aggressive group vs 0 % in the non-aggressive group; for PAX5 60 % in the aggressive group vs 10 % in the non-aggressive group) (Notta et al. 2011b). The analysis of clinical outcome showed a trend towards poorer outcome of aggressive patients with early relapse. By combining the xenografting and the DNA copy number alteration profiling it was provided evidence that genetic diversity occurs in functionally defined leukemia initiating cell subclones and that many patient samples contain multiple genetically distinct subclones. Reconstructing the subclonal evolution of leukemia-initiating cells of several ALL samples by copy number alteration

profiling allowed to support a branching multi-clonal evolution model of ALL leukemogenesis: for some patients, the predominant clone repopulated xenografts, whereas in other ones the predominant clone was competed by minor subclones. Reconstitution of xenografts with the predominant clone observed in the cells of the patients was associated with an aggressive growth in immunodeficient animals, a poorer patient outcome and the presence of additional mutations, particularly deletion of CDKN2A/B (Notta et al. 2011b).

The clonal architecture of MLL-AF4 infant B-ALLs was recently explored. As above mentioned, the MLL translocation with the AF4 partner gene is believed to be the initiating event occurring in utero. At variance with other B-ALLs, the disease development of MLL^+ ALLs does not seem to need additional, cooperating genetic abnormalities. However, although the copy number alterations are rare in MLL-AF4 patients at diagnosis, their number is more numerous at relapse, thus indicating genetic evolution of persisting MLL^+ leukemic clones (Bardini et al. 2010). Through the analysis of Ig/TCR rearrangement of MLL-AF4 at diagnosis and of xenograft leukemias derived the ALL samples, Bardini et al. (2014) have shown that MLL-AF4 ALLs are composed by a branching clonal and subclonal leukemia architecture, already at diagnosis; furthermore, investigation of paired leukemia samples at diagnosis and at relapse, indicated that relapse frequently occurs from clones pre-existing at diagnosis. Importantly, all the identified leukemic subclones are reflected at the level of leukemia-initiating cells, thus indicating that the cellular leukemic clonal/subclonal heterogeneity is dictated by a corresponding heterogeneity at the level of LSCs. Additional evidence in favor of clonally-related, but distinct subsets of leukemia-initiating cells was issued from the study of xenografts of high-risk precursor B-cell ALLs (Schmitz et al. 2011).

As above mentioned, TEL-AML1 confers a self-renewal advantage to stem cells. Some information are available about the mechanisms through which TEL-AML1 sustains stem cell growth and induces a growth advantage. Thus, it was shown that expression of TEL-AML1 in human cord blood progenitor cells led to expansion of a candidate preleukemia stem cell population with an early B phenotype ($CD34^+CD38^-CD19^+$) and a pronounced growth advantage in the presence of TGF-β (markedly reduced growth inhibition by TGF-β).

At the end of this section, it is important to mention new exciting therapeutic development obtained in the treatment of B-ALL with relapsing, refractory disease through CD19 targeting. As above shown, one of the antigens most frequently reported as expressed in B-ALL leukemia-initiating cells is CD19. This antigen is expressed during all stages of B-cell differentiation and this is expressed on the large majority of B-leukemic cells. The new therapeutic protocol consisted in the infusion of autologous T-lymphocytes transduced with CD19-directed chimeric antigen receptor lentiviral vector: through this procedure T-lymphocytes were redirected to address their cytotoxic activity to cells expressing CD19 (Mude et al. 2014; Lee et al. 2015). The advantage of this genetically engineered immunotherapy is double being mediated by cytotoxic T-lymphocytes highly efficient against $CD19^+$ cells and capable of long half-life in vivo and of tissutal trafficking. Recently, clinical data were made available about first B-ALL therapy treated with this new

approach. 90 % of the treated patients initially achieved a complete response. Of the patients who had a complete response 70 % remained in remission with an event-free survival rate of 67 % and overall survival rate of 78 % at 2 years (Mude et al. 2014). Taking into account the data on the CD19 expression of B-ALL leukemic stem cells, one could expect these findings and could speculate that only in a part of these patients displaying persistent complete remission the treatment could be curative (i.e., in those patients expressing CD19 at the level of all subpopulations of leukemia-initiating cells). It is important to note that these results are considerably better than those achieved using the best chemotherapy protocols for relapsed ALLs, allowing complete remission rates <25 % and median response duration <10 weeks.

4.2 T-ALLs

T-ALLs are about 15 % of all pediatric ALL cases. Difficulties in maintaining primary cultures of T-ALL cells and in developing in vivo models of T-ALL growth have limited for long time investigations into the biology of this malignancy. Studies carried out in these last years have in part elucidated the nature of the leukemia cells initiating T-ALLs. In this context, Cox et al. (2007) have sorted T-ALL cells for expression of CD34, CD4 and CD7: cells capable of in vitro and in vivo leukemic long-term growth were found among $CD34^+/CD7^-$, but not $CD34^+/CD4^+$ and $CD34^+/CD7^+$ (Cox et al. 2007). Importantly, in these experiments, 5×10^5 to 1×10^7 unsorted leukemia cells were required for engraftment, thus indicating that leukemia-initiating cells are rare in human T-ALLs.

In a subsequent study, Armstrong et al. (2009) have shown that the intrabone infusion of T-ALL blasts resulted in the constant engraftment of leukemic cells, with equally very high levels of engraftment into secondary and tertiary mice. The frequency of LICs into various T-ALL samples was variable and 10,000 leukemic cells were required to obtain engraftment into 100 % of immunodeficient animals. Importantly, in this study experimental conditions suitable for the maintaining of T-ALL LICs were determined, showing that co-culture of primary human T-ALL with a stromal line (MS5) expressing the NOTCH ligand delta-like-1 (DL1) reproducibly allowed to maintain T-ALL LICs and long-term growth of T-ALL cells. The sustained activation of the NOTCH signaling pathway into these cultures was strictly required for the survival and proliferation of leukemic cells: in fact, inhibition of the NOTCH pathway into primary cell cultures abolished in vitro cell growth of leukemic cells and in vivo T-LIC capacity.

Chiu et al. (2010) have used a stromal co-culture assay and NOD/SCID/IL-2Rγ^{null} (NSG) xenograft model using intrafemoral injection to characterize LICs from primary T-ALLs. Using this approach it was shown that $CD7^+CD1a^-$ cells isolated from primary T-ALL samples are responsive in vitro to proliferative signals mediated through NOTCH activation and are able to initiate leukemia into immunodeficient mice (Chiu et al. 2010). Expansion and clonal selection of leukemic cells generated by $CD7^+CD1a^-$ cells leads to the generation of a heterogeneous leukemic

cell population, with $CD7^+CD1a^+$ cells acquiring the property of LICs. Importantly, $CD7^+CD1a^-$ cells were shown to be resistant to glucocorticoid treatment and could be responsible for the development of drug-resistant T-ALLs.

Gerby et al. (2011) have fractionated T-ALL cells from primary leukemias into three cell fractions according to CD34 and CD7 positivity: the $CD34^+CD7^-$ fraction contained normal HSCs and HPCs; the $CD34^+CD7^+$ cell population was enriched in leukemia-initiating cells and proliferated in response to NOTCH activation and was inhibited by NOTCH inhibitors; $CD34^-CD7^+$ cell population contained more differentiated leukemic cells.

It is of interest to note that an optimal detection of leukemic stem cells in T-ALL samples requires fresh cells since the standard cryopreservation techniques determine a clear decrease of the frequency of these cells (Greystoke et al. 2013).

Interestingly, in some rare AML subtypes it was identified the existence of peculiar T-lymphocytic leukemia-initiating cells. These AMLs pertain to the group of AML samples unable to engraft into NOD/SCID mice (corresponding to about 40 % of total AMLs): about 30 % of these AMLs unable to grow into NOD/SCID mice are, however, capable of engrafting NOD/SCID/IL-$2R\gamma^{null}$ mice, but generated into these animals a monoclonal T-cell lymphoproliferative disorder similar to T-ALL. These grafts displayed self-renewal capacity as demonstrated by in vivo serial passages and their leukemia-propagating activity was restricted to $CD34^+$ cells. Molecular studies showed that these AML patient-derived LICs constantly expressed the MLL-AFX1 fusion product (Risueno et al. 2011).

Studies on the phenotype of a peculiar form of T-ALL, early T-cell precursor leukemia, suggest a peculiar origin of this T-ALL type. This type of T-ALL is characterized by an early T-cell precursor gene-expression signature and is associated with distinctive immunophenotypic features [$CD1a^-$, $CD8^-$, $CD5^{weak}$ with stem cell (CD34 and CD117) and myeloid (CD11b and CD13) markers] (Coustan-Smith et al. 2009). According to these findings it was suggested that this T-ALL is issued from the malignant transformation of early precursor T-cell, a subset of highly undifferentiated thymocytes representing immigrant T-cells from the bone marrow to the thymus, capable of multilineage differentiation (Bell and Bhandoola 2008; Wada et al. 2008).

As above mentioned, studies carried out in B-ALLs have shown a clonal heterogeneity at the level of both bulk leukemic cells and LICs. Similar evidence start to be obtained also for T-ALLs. In fact, Blackburn and coworkers, using a zebrafish transgenic model of T-ALL have obtained evidence about functional variation at the level of individual clones, with a minority of clones acquiring the capacity to activate AKT pathway and to increase their number of leukemia-propagating cells (Blackburn et al. 2014). These clones exhibited increased c-myc levels and are resistant to dexamethasone. According to these observations, it was suggested that T-ALL clones spontaneously and continuously evolve to leukemia progression through cellular mechanisms involving an increased frequency of LICs (Blackburn et al. 2014). The problem of tumor cell heterogeneity as a consequence of clonal LSC heterogeneity was specifically addressed by Clappier and coworkers. These authors have comparatively analyzed genetic lesions in T-cell ALL samples and in

xenograft derived from these samples: compared with paired diagnosis samples, the xenograft leukemias often contained additional genomic lesions occurring at the level of oncogenes and/or tumor suppressor genes and derive from minoritary subclones present in the patients at diagnosis. Furthermore, comparison of paired diagnosis and relapse samples showed that xenograft leukemias for their genetic abnormalities resembled more relapse samples than bulk diagnosis samples. Therefore, the establishment of T-ALL in immunodeficient mice is dependent on tumor cell heterogeneity existing in leukemic samples, selects and expands a more aggressive malignancy, recapitulating the leukemic progression and relapse of patients (Clappier et al. 2011).

5 Animal Models of Lymphoblastic Leukemias

5.1 Animal Models of T-ALL

5.1.1 NOTCH1

As above mentioned activating gain of function mutations in NOTCH1 have been observed in 50–70 % of patients with T-ALL. Initial studies carried out in animal models have shown that gain-of function NOTCH alleles that constitutively activate strong downstream signals are efficient inducers of leukemia in mice, while gain-of function NOTCH1 mutations commonly found in individuals with T-ALLs act as only weak tumor initiators (Chiang et al. 2008). However, these low, non-leukemogenic NOTCH1 mutants are able to complement other leukemogenic events, such as KRas activation (Chiang et al. 2008). Furthermore, NOTCH1 mutations have been identified in transgenic mouse T-ALL models driven by $KRas^{G12D}$ (Kindler et al. 2008). A NOTCH1 mutant, consisting of the transmembrane and intracellular domain of NOTCH1 (ICN1 mutant) was able to induce T-ALL in BM cells after transplant in mice (D'Altri et al. 2011).

As above indicated, PI3K-AKT pathway activation occurs in >85 % of T-ALL cases through various molecular mechanisms; activation of PI3K-AKT has been shown to collaborate with NOTCH1 in inducing leukemia development (Medyouf et al. 2010). The membrane IGF1R is an important target of NOTCH1 and its overexpression, frequently observed in T-ALLs, seems to represent one of the mechanisms through which Notch activation stimulate LICs in T-ALLs (Medyouf et al. 2012).

Calcineurin is a key determinant of Notch-mediated T leukemogenesis: in fact, calcineurin activation was found to be critical for leukemia initiating/propagating cell activity in T-ALL induced in mice by ICN1 NOTCH1 mutant (Gachet et al. 2013). Using a zebrafish T-ALL model, Blackburn et al. (2012) have reached the important conclusion that the primary role of NOTCH signaling in T-ALL development consists in the expansion of a population of pre-malignant early thymocytes and the acquisition of additional mutations by these cells is required for the full

transformation to leukemic progenitor cells. NOTCH1 cooperates also with ZMIZ1, a transcriptional coactivator of the protein inhibitor of activated STAT-like family, to induce T-ALL in mice. ZMIZ1 functionally interacts with NOTCH1 to promote c-MYC transcription and activity. ZMIZ1 inhibition slowed the growth and increased the sensitivity of tumor cells to NOTCH inhibitors (Rakowski et al. 2013).

The analysis of mouse models of NOTCH-induced T-ALL showed a differential effect of supraphysiological NOTCH signaling at the level of the leukemic and normal stem cell compartment: in fact, the enhanced NOTCH signaling promoted LSC activity in T-cell progenitors, but progressively extinguishes self-renewal of normal HSCs (Chiang et al. 2013). Other recent studies clearly showed that NOTCH1 expression at the level of human HSCs triggers T-cell differentiation as supported by studies based on the xenograft of human HSCs transduced with a constitutively active form of NOTCH1 (Haji et al. 2014).

The frequent occurrence of activating NOTCH1 mutations in T-ALLs and the key oncogenic role played by these mutations in leukemia development, have led to clinical trials evaluating the therapeutic effect of gamma-secretase inhibitors (GSI) that prevent NOTCH1 activation. However, the clinical responses to these drugs have been consistently limited in the time for the development of drug resistance. A recent study provided evidence that the resistance to GSI was due to the presence among naïve T-ALL cells of rare persister cells that through an epigenetic mechanism activate distinct signaling and transcriptional programs leading to drug resistance. The drug resistance of persister cells seems to be due to the expression of the transcription factor BRD4, essential for the viability of these cells and for the induction of c-myc and BCL2 expression in these cells. The essential role of BRD4 in mediating GSI-resistance of persister cells is supported by experiments carried out with the BRD4 inhibitor TQ1: this molecule induces growth arrest and apoptosis of persister cells (Knoechel et al. 2014).

5.1.2 FBXW7

FBXW7 is a constituent of the SCF (Sp1-Cul1-Fbox) ubiquitin ligase complex that controls the degradation and half-life of key proteins controlling fundamental cell pathways, such as Myc, NOTCH1, CyclinE and Mcl1. This gene is mutated in about 20 % of T-ALL patients: these mutations are usually heterozygous and cluster at the level of the substrate-binding domain. Monoallelic deletion of FBXW7 at the level of the hematopoietic system fail to induce leukemia; in contrast, complete FBXW7 deletion can lead to T-ALL development, but with low penetrance (Matsuoka et al. 2008). The development of a new generation of mice carrying Cre-inducible Fbxw7 heterozygous mutants, allowed to demonstrate that Fbxw7 deficiency does not affect HSC function and differentiation, but increases the number of leukemia-initiating cells; furthermore, Fbxw7 mutations cooperate with NOTCH1 mutations to induce T-ALL development in mice (King et al. 2013). The leukemia-promoting activity of Fbxw7 mutants correlated with their capacity to induce c-myc accumulation (King et al. 2013).

5.1.3 TAL1

TAL1, a transcription factor acting as a master regulator of hematopoiesis is mutated or translocated in about 25 % of childhood T-ALLs. TAL1 transgenic mice develop lymphomas with a mixed T and B-cell phenotype (Condorelli et al. 1996). Importantly, other studies have shown that transgenic mice expressing TAL 1 DNA binding mutants still develop T-cell leukemias/lymphomas (O'Neil et al. 2001). In line with these findings recent studies have shown that the oncogenetic role of TAL1 is played through its regulatory partners (including E2A, RUNX1, GATA3 and LMO1/2) through activation of MYB (Sanda et al. 2012) and microRNA-222 activation with consequent FBXW7 protein down-regulation (Mansour et al. 2013).

5.1.4 PTEN

One model was based on PTEN deletion in mouse hematopoietic cells that leads to a myeloproliferative disease, followed by T-ALL. In this model, PTEN inactivation in hematopoietic stem cells serves as a first hit to activate the PI3K-AKT pathway, conferring survival and proliferative advantages, and to promote genetic instability, leading to additional alterations: among them, the activation of beta-catenin may contribute to the acquisition of self-renewal capacity of leukemic stem cells, while t(14;15) chromosomal translocation results in T-lineage-specific overexpression of c-myc which may lead to T-ALL development (Guo et al. 2008). Therefore, the PTEN null model, with functionally defined populations of leukemic cells, one endowed with leukemia-initiating capacity ($CD3^{+}c\text{-}kit^{mid}$) and the other with blast properties ($CD3^{+}c\text{-}kit^{-}$), provides a unique opportunity to evaluate the effect of small molecule inhibitors on T-ALL development and, particularly, their capacity to target leukemic stem cells. Thus Schubbert et al. (2014) using this mouse model of T-ALL have shown that leukemia-initiating cells are targetable using combination therapy directed against the deregulated PI3K pathway and Myc. In both these models, an expansion of $c\text{-}kit^{+}CD3^{+}Lin^{-}$ cells is observed. Subsequent studies have shown that tumorigenesis in the context of a deficiency of PTEN in T-cell progenitors appears to be critically dependent on PI3Kγ and PI3Kδ isoforms (Subramanian et al. 2012).

5.1.5 IL-7R

The transduction of a mutant activating IL-7R into early thymocytes allowed to develop a model of human ETP-ALLs. In fact, in a recent study Treanor et al. (2014) showed that the transplantation of mouse early thymocytes $p19^{Arf-/-}$ transduced with a mutant IL-7R into recipient mice generated in vivo the formation of ETP-ALLs blocked at an early stage of differentiation, at which myeloid lineage and T-lymphoid differentiation programs co-exist.

5.1.6 LMO2

The cellular origin of T-ALL was investigated in a leukemia model of LMO2 oncogene activation. Particularly, to investigate the cellular origin of T-ALL, a cell mapping strategy was applied to a mouse T-ALL model to determine when the leukemia-initiating cell is established within the thymus. LMO2 transgenic mice express LMO2 in the thymus and develop T-cell leukemia similar to human T-ALL, after a latency of about 10 months. Analysis of the thymus cell populations during this time showed a preleukemic phenotype characterized by the accumulation of immature $CD4^{-}CD8^{-}$ thymocytes. These observations indicate that LMO2 promotes self-renewal of preleukemic thymocytes, providing a mechanism through which committed T-cells can accumulate additional genetic mutations required for leukemic transformation (McCormack et al. 2010). These findings were reinforced by an additional study showing that mice transgenic for TAL1 and LMO1 show an expanded population of primitive thymocyte progenitors inhibited in their terminal differentiation; these oncogenes provide a favorable context for the acquisition of activating NOTCH1 mutations and the emergence of self-renewing leukemia initiating cells (Tremblay et al. 2010). The model of T-ALL development induced by LMO2 in cooperation with TAL1 was further explored showing that T-ALL cells generated in these mice are heterogeneous and only 1 out 10,000 leukemic cells was able to generate a leukemic process after transplantation. The leukemia-initiating-capacity of these leukemias requires NOTCH1 signaling since it was inhibited by γ-secretase inhibitors (Tatarek et al. 2011). In addition to NOTCH1 also LYL1 was essential for mediating the leukemogenic activity of LMO2. LYL1, as well as TAL1, is required for the binding to DNA of LMO2. While TAL1 expression in thymocytes is dispensable for LMO2, LYL1 expression in thymocytes is strictly required for LMO2 leukemogenic activity, particularly for that concerns induction of self-renewal of thymocytes and of stem cell-like gene signature (McCormack et al. 2013).

The mouse models of LMO2-induced T-ALL have been explored to determine its effect at the level of thymocyte progenitors. These studies have shown that the most remarkable effect of LMO2 consist in blocking the differentiation of T-cell progenitor cells and in inducing a stem cell signature into these progenitors (Cleveland et al. 2013). Using transgenic mice with enforced expression of LMO2 in T-cells by the CD2 promoter/enhancer, it was provided evidence that LMO2 induces T-cell leukemia by two pathways: in one pathway there was coordinated activation of LYL1, HHEX and MYCN, while in the other pathway NOTCH1 target genes are activated. It is of interest to note that the gene activation pathway involving LYL1, HHEX and MYCN is commonly observed in early T-cell precursor ALLs. Conditional inactivation of HHEX in CD2-LMO2 transgenic mice clearly attenuated T-ALL development (Smith et al. 2014).

The capacity of LMO2 to induce selectively T-cell leukemias is impressively demonstrated by the gene therapy studies performed by gamma-retroviral gene transfer for severe combined immunodeficiency-X1 and showing the development of T-ALL in 4 out 20 patients treated due to integration of the retroviral vector 5′ of the LMO2 gene (Hacein-Bey-Abina et al. 2008).

5.1.7 ERG-Induced T-ALLs

The Ets-related gene (ERG) is an Ets-transcription factor required for hematopoietic stem cell development and maintenance-ERG is well expressed in HSCs and HPCs and its expression is lost during hematopoietic differentiation. ERG expression is down-regulated during early T-lymphopoiesis, being absent in T lymphopoiesis, being absent in T-lymphocytes; however, ERG expression is maintained in T-ALLs. In about 50 % of T-ALLs ERG is overexpressed and its overexpression is associated with a negative outcome of these leukemias (Baldus et al. 2006). Given these findings, the effects of ERG overexpression in hematopoietic cells have been explored. Thus, using either a vav promoter-driven ERG transgenic overexpression (Tsuzuki et al. 2011), or retroviral-mediated ERG overexpression in bone marrow transplant mice (Thoms et al. 2011), two studies have reported the development of ERG-induced T-cell leukemias. In one of these two studies it was shown also that ERG expression in T-ALL cells is mediated by the binding of TAL1, LMO2, LYL1 to an enhancer element present in the promoter of ERG gene (Thoms et al. 2011).

5.2 Animal Models of B-ALL

5.2.1 ETV6-RUNX1

Numerous attempts have been made to develop a mouse model of ETV6-RUNX1 B-ALL. Thus, various investigators have attempted to induce leukemia formation through retroviral transduction of bone marrow cells or fetal liver with ETV6-RUNX1 vectors: no incidence of leukemia was observed and only an increase of immature B-lymphoid cells was detected. Bernardin et al. (2002) reported a low frequency (2 out 9) of leukemia induction following enforced expression of ETV6-RUNX1 in mouse bone marrow cells; the efficiency of leukemia induction markedly increased (6 out 8) when ETV6-RunX1 was expressed into p16/p19-negative mouse bone marrow cells. Similarly, studies based on the transgenic mice model showed that the expression of ETV6-RUNX1 under control of the heavy chain immunoglobulin promoter failed to induce leukemia formation. For leukemia development a secondary genetic event is required, such as co-expression of other mutant genes or mutations induced by carcinogens or irradiation (van der Weyden et al. 2011; Li et al. 2013). Interestingly, it was shown that ETV6-RUNX1 renders prone to leukemia development (after mutagenesis) only when expressed at the level of HSCs, but not of lymphoid progenitor cells; in line with these findings, ETV6-RUNX1 increases the number of HSCs and maintain these cells in a quiescent state (Schindler et al. 2009).

In line with findings observed using murine bone marrow cells, also using human cord blood fractions enriched in HSC/HPCs or in B-cell progenitor cells and transduced with TEL-AML1 expression vectors, it was reached the conclusion that ETV6-RUNX1 was not competent to confer self-renewal ability on progenitor cells

and to initiate leukemogenesis (Fan et al. 2014). $Lin^{-}CD34^{+}CD38^{-}CD49f^{+}$ CB-cells, transduced with TEL-AML1 were able to induce preleukemia when injected into NOD/SCID mice (Fan et al. 2014).

5.2.2 E2A-HLF

The oncogenic fusion protein E2A-HLF is a chimeric transcription factor that arises from the t(17;19) translocation in children B-ALLs and is associated with a very poor outcome. Various animal models have been reported. Two initial transgenic models based on the expression of the fusion gene E2A-HLF under the control of the Eμ enhancer with the SV40 promoter provided evidence about a significant transforming activity with a variable proportion (20–60 %) of animals displaying the formation of lymphomas, mostly of the T-cell lineage.

A more recent study showed that the transduction of a murine stem cell retrovirus to induce E2A-HLF expression failed to induce leukemia development; however, when BM cells were transduced to express E2A-HLF together with Bcl-2 and transplanted, induce the formation of B-ALLs, resembling human B-ALLs (Smith et al. 2002).

Yamasaki et al. (2010) have used an inducible knock-in approach to induce E2A-HLF expression in hematopoietic cells; however, using this approach no leukemia formation was observed. Through insertional mutagenesis secondary events required for leukemia development have been identified: particularly, the Zfp521/ZNF521 gene was identified as a cooperative gene for E2A-HLF to develop B-ALL.

Other studies were focused to determine the essential role of LMO2 in mediating the oncogenic activity of the E2A-HLF fusion gene: in fact, it was shown that E2A-HLF induces LMO2 expression in primary B-ALL cells and this expression is essential for leukemic survival (Hirose et al. 2010); E2A-HLF was able to immortalize primary lymphoid progenitors and this effect is mimicked by induced expression in these cells of LMO2 and Bcl-2 (De Boer et al. 2011).

5.2.3 E2A-PBX1

The mechanisms of E2A-PBX1-mediated pre-B-cell transformation and the molecular nature of direct E2A-PBX1 target genes and pathways remain largely unknown. Initial attempts at modeling E2a-PBX1-driven human B-ALLs have been unsuccessful because caused myeloid leukemias, but not B-ALLs. A first model able to replicate in mice E2A-PBX1 B-ALL was based on the development of transgenic mice in which the expression of the fusion gene was under the control of lymphoid-specific Lck upstream sequence, Eμ enhancer and TCR Vβ promoter: mice developing leukemia at late times, died for a B-ALL or mixed lineage ALL. The long latency required for leukemia development was reduced by co-expression experiments with Hox gene overexpression by viral insertional mutagenesis (Bijl et al. 2005).

5.2.4 BCR-ABL

Various experimental approaches have been explored to try to develop a suitable model of BCR-ABL$^+$ B-ALL. Using a retroviral bone marrow transduction/transplantation model, both p190 and p210 induce a fatal myeloproliferative disease in recipients of transduced marrow. When donors are pre-treated with 5-FU, recipients of p190 or p210-transduced bone marrow develop a mixture of CML and B-lymphoid leukemia. P190 is more potent than p210 for induction of B-lymphoid leukemia (McLaughlin et al. 1989). BCR-ABL-induced B-cell leukemia requires 4–12 weeks for its development and involves only the B-lymphoid lineage: these findings imply that it originates at the level of a B-cell-restricted progenitor and requires the acquisition of additional genetic events for leukemia development (Li et al. 1999). Using this mouse ALL model it was possible to demonstrate the essential role of the PI3K pathway, and particularly of mTOR kinase, in the BCR-ABL1-mediated transformation of B lymphoid progenitors (Janes et al. 2010).

In order to establish an efficient model of BCR-ABL B-ALL, Williams et al. (2006) have first developed a strategy to obtain the immortalization and growth factor independence of these cells through $p19^{Arf}$ knockout: importantly, these cells do not undergo apoptosis when transduced with BCR-ABL expression vectors. Introduction of Bcr-Abkl into $p19^{Arf}$-deficient bone marrow progenitors induces rapid ex vivo outgrowth of pre-B lymphoid cells and induces a highly aggressive form of B-ALL when inoculated into syngeneic mice. Virtually, all the pre-B-cells obtained through this procedure have leukemic potential, as supported by the observation that as low as 20 such cells when infused into healthy syngeneic mice induce a rapidly fatal, transplantable B-ALL.

A recent study identified in the mice the type of B-cell progenitor that seems to be more prone to leukemic transformation by BCR-ABL. Particularly, these studies have provided evidence that B-1 progenitors (i.e., those more responsive to IL-7 and the only ones responsive to TYSLP) when transduced with BCR-ABL initiate the leukemic process more rapidly than do BCR-ABL expressing B-2 progenitors (Montecino-Rodriguez et al. 2014).

5.2.5 BCR-ABL-Like ALLs

Ph-like ALLs represent a subgroup of high-risk B-ALLs characterized by a gene expression profile similar to Ph$^+$ ALLs, poor prognosis and recurrent CRLF2 rearrangements, JAK1/2 point mutations, JAK2 fusion genes and tyrosine kinase mutations. No animal genetic models of this ALL subtype have been reported. However, Maude et al. (2012) have reported the successful xenotransplantation of Ph-like ALL blasts into NSG mice (with 18/21 leukemia engraftment) and have used these xenografts to demonstrate the sensitivity of leukemic cells to targeting with JAK or mTOR inhibitors.

5.2.6 MLL-Rearranged ALLs

The development of animal models of MLL-rearranged ALLs has represented the object of intensive studies during these last years. Metzler et al. (2006) have used the invertor conditional technology to create a mouse model of MLL-AF4. Transgenic mice expressing this fusion gene invariably developed B-cell neoplasias, but of more mature phenotype than usually observed in pediatric B-ALLs.

Chen et al. (2006) have produced MLL-AF4 knock-in mice by homologous recombination in embryonic stem cells: these mice have an increased number of lymphoid and myeloid cells in hematopoietic tissues and after a prolonged latency developed hematologic malignancies, most frequently consisting in B-cell lymphomas. These observations have suggested that MLL-AF4 per se is not sufficient to induce the development of an overt malignancy and additional secondary mutations are required. Using a slightly different transgenic approach, Krivstov et al. (2008) provided evidence that the expression of a MLL-AF4 allele resulted in the development of AML, and less frequently of pre-B ALL.

In subsequent studies it was explored the in vivo transforming potential of both MLL-AF4 and of its fusion reciprocal AF4-MLL. Transplantation of purified preparations of progenitor/stem hematopoietic cells transduced with MLL-AF4 failed to induce leukemia development, while the transplantation of the cells transduced with AF4-MLL elicited the formation of proB-ALL, B/T biphenotypic leukemias or mixed lineage leukemia. According to these findings it was proposed that the t(4;11) leukemia is based on two oncoproteins, MLL-AF4 and its reciprocal AF4-MLL (Bursen et al. 2010).

In other studies attempts have been made to induce oncogenic transformation of either human $CD34^+$ cells (Montes et al. 2011) or human embryonic stem cells (Bueno et al. 2012) by transducing MLL-AF4 in these cells. In human $CD34^+$ cells MLL-AF4 expression enhanced the hematopoietic repopulating cell function and clonogenic potential, but failed to induce leukemia development (Montes et al. 2011). In embryonic stem cells, MLL-AF4 expression enhanced the hemogenic specification, but impaired further hematopoietic commitment in favor of an endothelial cell fate (Bueno et al. 2012).

Since FLT3 is highly expressed in MLL-$AF4^+$ pro-B ALLs, it seemed of particular interest to investigate a possible cooperation between MLL-AF4 and FLT3 in the transformation of human $CD34^+$ cells. However, the results of these studies showed that FLT3 activation was not sufficient to immortalize or transform MLL-AF4-expressing human $CD34^+$ stem/progenitor cells, thus suggesting the existence of alternative genetic and/or epigenetic cooperating oncogenic lesions (Montes et al. 2014). Similar experiments have been carried out in human ESCs showing that FLT3 activation cooperates with MLL-AF4 fusion protein to abrogate the hematopoietic specification of ESCs, but was unable to immortalize/transform ESC-derived hematopoietic cells, again suggesting the need for alternative genetic cooperating hits (Bueno et al. 2013).

5.2.7 Stat5b/Pax5

As above mentioned, somatic alterations of Pax5, a transcription factor acting downstream with respect to the transcription factors TCF3 and EBF1 to commit lymphoid progenitors to a B-cell fate, are frequent (up to 50 %) in the high-risk BCR-ABL1-positive and BCR-ABL1-like B-ALLs. Given the essential role of Pax5 in B-cell development, Pax5-deficient mice are arrested at the pro-B-cell stage in the bone marrow. Studies carried out in transgenic model of B-ALL driven by the expression of Stat5 constitutively active (Stat5-CA) in hematopoietic cell suggested a tumor suppressor role for Pax5: in fact, Stat5-CA mice usually develop B-ALLs with a long latency and low penetrance; this tumorigenic process is markedly accelerated by Pax5 heterozygosity (Heltemes-Harris et al. 2011). Interestingly, tumors arising in Stat5-CA; $Pax5^{+/-}$ mice invariably retain the WT Pax5 allele (Heltemes-Harris et al. 2011). More recently, this model was re-explored using transgenic RNAi to reversibly suppress endogenous Pax5 expression in the hematopoietic compartment of mice: restoring endogenous Pax5 expression in established B-ALLs triggers B-cell differentiation inducing durable disease remission. It is important to note that even brief Pax5 restoration in B-ALL cells was sufficient to cause rapid cell cycle exit and inhibition of their leukemia-initiating-capacity (Liu et al. 2014a).

Recent studies have reported the frequent (about 9 % of cases) occurrence of activating Stat5b mutations in T-ALL patients. These Stat5b-mutated T-ALLs are characterized by Bcl-X_L overexpression and by apparently absent chromosomic abnormalities (Kontro et al. 2014). Another recent study confirmed the frequent Stat5b mutations (6.3 % of cases) in T-ALL patients. In this study it was shown that Stat5b mutations occur in the phosphotyrosine binding pocket of Stat5b (N642H). Interestingly, in two patients studied at diagnosis and relapse it was shown that in one patient the Stat5b mutation was present only at diagnosis, while in the other patient the Stat5 mutation was at the heterozygous state at diagnosis and at the homozygous state at relapse. Stat5b-mutated T-ALLs exhibited a higher tendency at relapse than the Stat5b-WT T-ALLs (Baudapalli et al. 2014). At the biochemical level, the mutant Stat5b resulted in constitutive Stat5b phosphorylation, activation of Stat5 target genes and growth factor independent proliferation.

6 Conclusion

Tremendous progresses have been made in the understanding of the molecular abnormalities observed in ALLs. These information have been essential for a molecular classification of these diseases in subgroups, and for the identification of new therapeutic targets. Importantly, the identification of these various molecular abnormalities have provided precious molecular markers for the identification of tumor cell subpopulations and for the understanding of cellular and molecular dynamic during tumor development and progression.

The parallel development of studies on the identification and characterization of leukemic stem cells into these tumors and their integration with molecular studies has provided the basis for a consistent initial understanding of the early stages of ALL development, with identification of putative leukemia-initiating cells and for definition of their heterogeneity and changes during tumor development/progression. It is largely expected that these studies will contribute to an improvement in the efficacy of the therapy of ALLs, particularly through targeting of membrane antigens selectively or particularly expressed on leukemic stem cells.

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