Genomic and Transcriptomic Analyses of Pediatric T-cell Acute Lymphoblastic Leukemia /Lymphoma

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Material and methods

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- Combined interphase fluorescence in situ hybridization (CI-FISH)
- TAF_{I\alpha}/NUP214 fusion transcript
- Notch1 mutation analysis
- Gene expression profiling
  - RNA preparation
  - Gene expression array
  - Data analysis
- MicroRNA profiling

Results

- Copy number and SNP analysis
- Translocations analysis
- Notch1 mutations
- Gene expression analysis
- MicroRNAs expression

Discussion

References
Chapter 4 – Summary conclusion and future perspectives

Acknowledgements
Chapter 1 – Introduction
**T-cell lymphoblastic leukemia/lymphoma**

Acute lymphoblastic leukemia and non-Hodgkins lymphoma are common cancers in children and adolescents, together representing over third of newly diagnosed malignancies in the pediatric age group. T-cell acute lymphoblastic leukemia (T-ALL defines approximately 15% of all newly diagnosed cases of ALL, and lymphoblastic lymphoma (LL) represents approximately 30% of all newly diagnosed cases of non-Hodgkins lymphoma in children. In contrast to ALL, the vast majority of cases of LL are characterized by a T-cell immunophenotype. T-ALL and T-cell lymphoblastic lymphoma (T-LBL) are thought to arise from malignant thymocytes, corresponding to defined stages of intrathymic T-cell differentiation.

Many similarities between T-ALL and T-LBL exist [1]. Both diseases share common characteristics, such as immunophenotypic features, lymphoblast morphology and clinical characteristics, e.g. the median age at diagnosis and the similar outcome after ALL-type chemotherapy.

Because of these overlapping features the two pathologies are often considered the same disease; however, in spite all similarities between T-ALL and T-LBL, there are also obvious differences, first of all the site of presentation of the disease.
Several study groups distinguish leukemia from lymphoma based on the extent of bone marrow (BM) involvement: patients with less than 25% lymphoblasts in the BM and no peripheral blasts are diagnosed as lymphoma; in case of 25% or more BM blasts patients are diagnosed with leukemia. The typical site of relapse differs as well, with predominantly local relapse in lymphoma patients and systemic relapse in T-ALL. The time to relapse tends to be minimally shorter in T-LBL than in T-ALL with a median time to progression or relapse of 10 months in T-LBL compared with 12-13 months in T-ALL patients. These distinct distribution patterns might indicate differences in the homing potentials of T-ALL and T-LBL lymphoblasts and raise the question about whether T-LBL and T-ALL represent one and the same disease, only with two different presentations, or whether T-ALL and T-LBL blasts carry different biological potentials [2].

**Clinical characteristics and outcome in T-ALL and T-LBL**

In general the clinical characteristics show a large overlap between pediatric T-ALL and T-LBL. About one-third of patients are female, and about half of the patients is <10 years of
age at diagnosis (table 1).

<table>
<thead>
<tr>
<th></th>
<th>T-ALL</th>
<th>T-LBL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>26%</td>
<td>31%</td>
</tr>
<tr>
<td>male</td>
<td>74%</td>
<td>69%</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 years</td>
<td>55%</td>
<td>55%</td>
</tr>
<tr>
<td>10–14 years</td>
<td>29%</td>
<td>33%</td>
</tr>
<tr>
<td>14–18 years</td>
<td>16%</td>
<td>11%</td>
</tr>
<tr>
<td>Mediastinal tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>61%</td>
<td>90%</td>
</tr>
<tr>
<td>Bone marrow involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>18%</td>
</tr>
<tr>
<td>Central nervous system involvement</td>
<td>9%</td>
<td>4%</td>
</tr>
<tr>
<td>Lactate dehydrogenase serum level</td>
<td>&lt;500 iu/l</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>≥500 iu/l</td>
<td>79%</td>
</tr>
</tbody>
</table>

Table 1: clinical characteristics of T-ALL and T-LBL in childhood

The best-known difference between T-ALL and T-LBL is the primary site of involvement. T-ALL patients present with BM involvement and lymphoblasts in the peripheral blood (PB), often accompanied by thrombocytopenia. A mediastinal tumor is diagnosed in two-thirds of ALL patients at initial staging. Splenomegaly and adenopathy was reported to be more frequent in T-ALL than in T-LBL. The higher frequency of elevated serum lactate dehydrogenase (LDH) levels in T-ALL might – at least partially – be due to higher tumor burden. Interestingly the frequency of central nervous system (CNS) involvement in T-ALL is more than twice the frequency of CNS involvement in T-LBL, which lends additional support to the hypothesis of differences in the expression of homing factors
between T-ALL and T-LBL lymphoblasts.

In T-LBL patients the primary manifestation of the lymphoma is predominantly the anterior mediastinum, often accompanied by pleural and/or pericardial effusions; less than 20% of T-LBL present with BM involvement at diagnosis. In summary, T-ALL and T-LBL share clinical characteristics but their primary site of the disease and other manifestations differ significantly. However, there is no clinical criterion to clearly distinguish T-ALL from T-LBL besides the blast count in BM at diagnosis [2].

**Minimal disseminated disease (MDD) and Minimal residual disease (MRD) evaluation**

In pediatric ALL but not in LBL MRD diagnostics, based on real time quantitative polymerase chain reaction (RQ-PCR) of immunoglobulin or T-cell receptor (TCR) gene rearrangements or flowcytometry (FC), is regularly used for the stratification of treatment according to the individual kinetic of response. Recently, different works [3-4] reported that FC and RQ-PCR for TCR gene rearrangements identified MDD at diagnosis in a good percentage of T-LBL cases. Both FC and RQ-PCR have been found to be efficient methods for MDD and MRD evaluation in LBL [2]. MRD is defined as the percentage of blasts that persist in the BM after any therapy. The evaluation
of MRD is based on the study of the patient-specific rearrangements of TCR and immunoglobulin (Ig) genes, used as markers of disease, at diagnosis and during three different time points of treatment. On the base of MRD response after the phase Ia (day 33) and before consolidation (day 78) therapy T-ALL patients were stratified in three different risk groups: standard risk (SR), medium risk (MR) and high risk (HR) on the meaning of the low probability to relapse for the standard group and the high probability to relapse for the high risk group. Even many efforts have been done in order to improve the outcome for these patients the prognosis remained still poor, about 70% for T-ALL. By now the study of MRD is the best prognostic tool and is used in many protocols in order to predict the outcome of patients. In the graphic (figure 1) is shown the 5 years event free survival (EFS) of 387 T-ALL patients enrolled in AIEOP-BFM 2000 protocol. As shown in the figure the EFS of the HR group is really worst (40%) than SR and MR groups (96% and 84%); until now no biologic features were provided to justify this difference in the outcome.
Recent trials in T-LBL patients indicate that favorable EFS can be achieved using systemic ALL chemotherapy treatment strategies without local radiotherapy of the mediastinum or other involved sites. However, the main difficulty in the treatment of T-LBL patients is the lack of prognostic parameters. Different from T-ALL with validated impact of parameters, such as prednisone response, BM response and minimal residual disease evaluation, none of these techniques is available for T-LBL patients today. In consequence there is no stratification of treatment intensity according to the individual risk of the T-LBL patient resulting in “intermediated risk T-ALL treatment intensity” for the vast majority of T-LBL patients. Ongoing and future trials on T-LBL patients are necessary to establish prognostic parameters to allow for reduced treatment
intensity in low risk patients and more intense high risk ALL strategies in high-risk T-LBL patients [2].

**Immunophenotype**

The immunophenotype of T-ALL and T-LBL corresponds with defined stages of intrathymic T-cell differentiation. Precursor T-cell most commonly expresses CD7, CD5 and CD2, whereas CD3 is present in the cytoplasm (cyCD3) or on the surface (sCD3). T-ALL and T-LBL are usually TdT positive, a marker that distinguishes them from all other types of lymphoma, except precursor B-cell lymphoblastic lymphoma. In addition to TdT, CD34 and CD99 are also expressed in any combination: alone, neither, or both together. For T-ALL, most study groups use the European Group of the Immunological of Leukaemias (EGIL) classification criteria, while for T-LBL the EGIL classification or the WHO classification are used to group precursor T-cell neoplasm into immunophenotypic subgroups according to their stages of intrathymic differentiation.

In general, T-ALL and T-LBL show overlapping immunophenotype features, but some studies stated that T-LBL have antigen expression profiles consistent with more mature stages of intrathymic T-cell development than T-ALL. However, attempts at separating by immunophenotypic
features have, on the whole, not met with success. Table 2 compares the immunophenotypic characteristics of 186 T-ALL cases and 217 T-LBL cases according to EGIL criteria.

<table>
<thead>
<tr>
<th></th>
<th>T-ALL</th>
<th>T-LBL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of patients (%)</td>
<td>No. of patients (%)</td>
</tr>
<tr>
<td>Pro/pre-T-cell (pro-T: cyCD3⁺, CD7⁺, CD2⁺; pre-T: CD2⁺)</td>
<td>46 (26)</td>
<td>23 (16)</td>
</tr>
<tr>
<td>Intermediate T-cell (CD1a¹)</td>
<td>104 (58)</td>
<td>105 (71)</td>
</tr>
<tr>
<td>Mature T-cell (sCD3⁺, CD1a⁺, CD4 or CD8)</td>
<td>28 (16)</td>
<td>20 (14)</td>
</tr>
<tr>
<td>T-cell, not further classified (CD3⁺, TdT⁺)</td>
<td>8</td>
<td>69</td>
</tr>
</tbody>
</table>

Table 2: Immunophenotypic characteristics according to EGIL classification of pediatric T-ALL and T-LBL series

Due to the limitation in distinguishing cytoplasmatic and surface CD3 expression by immunohystochemical staining, a relevant proportion of T-LBL cases could not be classified into immunophenotypic subgroups. The available data indicate that the immature stages of pro- and pre-T immunophenotypes might indeed be less frequent in T-LBL compared with T-ALL series. In the T-ALL series, 26% of the cases showed pro/pre-T-cell immunophenotype, while in T-LBL only 16% of the cases were classified as pro/pre-T-LBL. Interestingly, the mature T-cell immunophenotype with surface CD3 expression, CD1a negativity and either CD4 or CD8 expression was equally
frequent in T-ALL and T-LBL [2].

Early T-cell precursor leukemia

Early T-cell precursors (ETPs) are a subset of thymocytes representing recent immigrants from the bone marrow to the thymus; they retain multilineage differentiation potential suggesting their direct derivation from haemopoietic stem cells. In particular, Campana et al identified a unique biological subtype of childhood leukemia, ETP-ALL, associated with a high risk of remission induction failure or relapse in patients treated with contemporary protocols of intensive chemotherapy for ALL. They used gene expression profile of normal ETP to identify the leukemic counterparts and define the immunophenotype. ETP-ALL cases are characterized by a specific gene-expression profile, an increased number and size of genomic lesions, denoting genomic instability, and distinct cell-surface features that readily enable diagnosis: absence of CD1a and CD8 expression, weak CD5 expression, and expression of one or more myeloid-associated or stem-cell associated markers [5]. Chiaretti and co-workers recently identified by gene expression analysis a subset of adult T-ALL patients that appeared to resemble the pediatric cases of ETP-ALL in terms of an active myeloid and/or stem cell program.
coupled, in some case, to co-expression of myeloid antigens; the poor clinical outcome and the similar incidence. By contrast, at immunophenotypic level, this new identified subset of T-ALL patients expressed CD5, CD8 and CD1a.

Figure 2: Schematic overview of T-ALL genetic subgroups in relation to their developmental stage based on EGIL or TCR classification system.

**Molecular genetics features**

Leukemic transformation of immature thymocytes is caused by a multistep pathogenesis involving numerous genetic abnormalities that permit uncontrolled cell growth. In particular there are four pathways that could be affected: cell cycle, differentiation, apoptosis and self-renewal resulting in a
developmental arrest in nearly all stages of T-cell maturation in the thymus. The most common aberrations are chromosomal translocations that juxtapose transcription factors under the control of the of T-cell receptors (TCRs) enhancer elements causing their ectopical over expression. Besides other non TCR gene-mediated translocations that may produce fusion products, many other genes become activated or inactivated due to the presence of specific point- or insert/deletion mutations, or are affected by somatic copy number variations (amplifications and/or deletions). Many of these genes normally play important roles in T-cell commitment and differentiation or control important checkpoints in T-cell development that warrant for the selection of proper antigen specificity. In the last years many groups have been tried to unraveling the molecular-genetic abnormalities in T-ALL including chromosomal translocations, deletions, amplifications duplication and point mutations. A number of these genetic events delineate distinct molecular-cytogenetic T-ALL subgroups, including defects that result in the ectopic expression of TAL1, TAL2, LMO1, LMO2, LYL1, TLX1 (HOX11) TLX3 (HOX11L2), MYB and HOXA genes, or defects that generate specific fusion products including
PICALM-MLLT10 (CALM-AF10), MLL-MLLT1 (ENL) and SET-NUP214. Various microarray studies led to the establishment of specific expression signatures for several of these abnormalities. In particular, Ferrando et al (2002) demonstrated the common expression profiles for T-ALL subgroups with TAL1, TAL2, LMO1 and LMO2 abnormalities. Also patients with PICALM-MLLT10, MLL rearrangements and SET-NUP214 share a similar expression profile that is characterized by the activation of HOXA genes. Other types of mutations including TLX1, TLX3 and MYB have been associated with a unique gene expression profile (figure 3) [6-9].

Gene expression profiling and array-CGH contributed to the classification of T-ALL patients into unique subgroups. Type A genetic abnormalities that may dictate T-ALL subgroups are thought to cause arrest at specific stage of normal T-cell differentiation. Different type A mutations that belong to different subgroups are mutually exclusive, although within TAL/LMO subgroups the simultaneous presence of TAL1 and LMO1 translocation have been described for some T-ALL cases that proved synergistic in mouse leukemia models [10]. In contrast to these type A mutations type B genetic abnormalities are shared by several T-ALL subgroups and targeted cellular processes including cell cycle, NOTCH1 pathway and TCR
signaling [9].

“Type A” mutations

<table>
<thead>
<tr>
<th>Rearrangement</th>
<th>Gene(s)</th>
<th>T-cell arrest*</th>
<th>Outcome</th>
<th>Frequency† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAL/LMO subgroup</td>
<td>TAL1</td>
<td>Pre-ALL/TCR-αβ</td>
<td>Good†</td>
<td>15</td>
</tr>
<tr>
<td>ip2 deletions</td>
<td>STILL/TAL1</td>
<td>Pre-ALL/TCR-αβ</td>
<td>Good†</td>
<td>4</td>
</tr>
<tr>
<td>il7 deletions</td>
<td>TAL1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(11;14)(pl5q13;11q23)</td>
<td>LMO1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>&lt;1</td>
</tr>
<tr>
<td>(11;14)(pl5q13;11q23)</td>
<td>LMO2</td>
<td>Immature/TCR-αβ</td>
<td>Unknown</td>
<td>7</td>
</tr>
<tr>
<td>(11;14)(pl5q13;11q23)</td>
<td>LMO2</td>
<td>Immature/TCR-αβ</td>
<td>Unknown</td>
<td>5</td>
</tr>
<tr>
<td>TLE/LMX1 subgroups</td>
<td>TLE/LMX1</td>
<td>Immature/TCR-αβ</td>
<td>Good†††</td>
<td>8</td>
</tr>
<tr>
<td>TLE/LMX1 subgroups</td>
<td>TLE/LMX1L2</td>
<td>Immature/TCR-αβ</td>
<td>Poor†§§§§§</td>
<td>24</td>
</tr>
<tr>
<td>HOXA subgroup</td>
<td>TLE/LMX1</td>
<td>Immature/TCR-αβ</td>
<td>Poor†§§§§§</td>
<td>5</td>
</tr>
<tr>
<td>t(17;19)(q23;13q34)</td>
<td>TLE/LMX1</td>
<td>TCR-αβ or TCR-γδ</td>
<td>Undefined</td>
<td>4</td>
</tr>
<tr>
<td>t(10;12)(p13;q14)</td>
<td>TLE/LMX1</td>
<td>TCR-αβ or TCR-γδ</td>
<td>Undefined</td>
<td>4</td>
</tr>
<tr>
<td>t(11;16)(p13;q13)</td>
<td>TLE/LMX1</td>
<td>TCR-αβ or TCR-γδ</td>
<td>Undefined</td>
<td>1</td>
</tr>
<tr>
<td>t(16;17)(p13;q22)</td>
<td>TLE/LMX1</td>
<td>TCR-αβ or TCR-γδ</td>
<td>Undefined</td>
<td>3</td>
</tr>
<tr>
<td>MYB subgroup</td>
<td>MYB</td>
<td>Unknown</td>
<td>Unknown</td>
<td>3</td>
</tr>
<tr>
<td>TLE/LMX1</td>
<td>TLE/LMX1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>3</td>
</tr>
<tr>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>3</td>
</tr>
</tbody>
</table>

*Based upon TCR-classification (van Gouw et al. 2006, 2008; Van Vlierberghe et al. 2007).
†Van Gouw et al. 2008.
‡A number of alternative LMX translocations have been described.
§MLLT1 is the most common NLL translocation partner in T-ALL. Nevertheless, other NLL translocations also occur in T-ALL.
*Care et al. 2006.
**Schneider et al. 2008.
††Foss et al. 2002.
‡‡Fernando et al. 2006.
§§Fernando et al. 2003.
¶¶Billet et al. 2002.
†††Maertens et al. 2002.
||Valovirta et al. 2003.

Figure 3: Classification of genetic type A abnormalities in T-ALL

TAL/LMO

Basic helix-loop-helix (bHLH) transcription factors share a 60 amino acid HLH motif that includes a dimerization domain and a DNA binding domain for specific E-box sites. Class A bHLH proteins, such as E2A and HEB, control the expression of various genes that are involved in V(D)J recombination of TCR genes. Class B includes TAL1, TAL2, LYL1 and bHLH1 proteins.
that act as transcriptional co-factors forming complex with E2A/HEB. Class B members are usually targeted by chromosomal rearrangements in T-ALL mostly due to miss rearrangements of the TRA@/TRD@ or TRB@. For function, bHLH family members also bind to members of the LIM-domain only (LMO) gene family. Class B bHLH and LMO proteins participate in a single transcription complex that inhibits E2A function. The LIM-domain only genes LMO1 and LMO2 are also frequently targeted in T-ALL by chromosomal translocations leading to high activation of LMO1 or LMO2 [9]. The TAL1 gene is normally expressed in a subset of haematopoietic cells, endothelial cells and cells of central nervous system. It is constitutively activated in up to 25% of T-ALL cases, but only in 3% of these does the activation result from the translocation to the TAL1 to the TCR loci. The remaining cases have a deletion that replaces the 5’ regulatory sequence of TAL1 with that of the upstream gene that is known as SIL, and this leads to dysregulation of TAL1 expression [11].

**TLX1**

TLX1 is a class II homeobox gene that is normally involved in spleen development but is not express during normal T-cell development. In about 5-10% of pediatric T-ALL cases, TLX1 is
activated through the translocation t(7;10)(q34;q24) or t(10;14)(q42;q11) in which TRA@/TRD@ or TRB@ regulatory sequences drive TLX1 expression. TLX1 positive T-ALL cases share a highly similar gene expression profile that shows arrest at the early cortical, CD1-positive thymocyte stage. TLX1 positive T-ALL has been associated with a favorable outcome, possibly due to a low expression of anti-apoptotic proteins characteristic for this early cortical differentiation stage or a high expression of genes involved in cell growth and proliferation. In addition, this subset has also been associated with elevated expression level of the glucocorticoid receptor that may explain the enhanced sensitivity towards dexamethasone. TLX1 expression has occasionally been reported in T-ALL cases in the absence of TLX1 rearrangements and it was proposed that alternative mechanisms, such as promoter demethylation or trans-activating mechanisms might be responsible for this aberrant gene expression [9].

**TLX3**

TLX3 is a homeobox gene essential for the ventral medullary respiratory center [12]. Ectopic expression of TLX3 is present in about 20% of childhood T-ALL patients. TLX3 expression is
mostly due to the cryptic translocation t(5;14)(q35;q32), juxtaposing TLX3 to BCL11B, a gene expressed during T-cell maturation. Like TLX1, TLX3 is not expressed during normal T-cell development. TLX3 positive T-ALL has been associated with poor outcome in some studies, but this effect was not confirmed in other studies, possibly due to differences in treatment protocols or differences in additional cooperating genetic aberrations [13-15].

**HOXA T-ALL**

The PICALM-MLLT10 fusion gene is caused by a recurrent translocation, t(10;11)(p13;q14), which is found in both T-ALL and AML (acute myeloid leukemia) patients. The PICALM-MLLT10 fusion is detected in about 10% of pediatric T-ALL and has been associated with poor prognosis. All of these patients have an immature or γδ-positive T-cell immunophenotype. HOXA gene activation by PICALM-MLLT10 depends on the recruitment of the histone H3 methyltransferase hDOT1L by AF10. hDOT1L was shown to be a crucial factor for cellular transformation that prevents nuclear export of CALM-AF10 and activates HOXA5 expression through specific histone H3 methylation on residue K79 [9].

The interstitial deletion on chr 9(q34.11 –q34.13) originates the
SET-NUP214 fusion gene. SET-NUP214 functions as a transcription co-factor that binds in the promoter regions of specific HOXA genes, i.e. HOXA9 and HOXA10, where it interacts with CRM1 and the H3K79 methyltransferase hDOT1L. Methylation of the histone H3 backbone is accompanied by histone H3 acetylation in the promoter regions of all HOXA genes members. This possibly results in an “open state” chromatin structure that allows binding of other transcription regulators eventually leads to the activation of HOXA®. From a therapeutic point of view, the recruitment of hDOT1L by SET-NUP214 is of particular interest, because this methyltransferase has also been implicated in HOXA activation by PICALM-MLLT10 and MLL-MLLT1 mediated leukemias. Given that hDOT1L seems to be the common factor in leukemogenic HOXA activation for these different fusion genes, further research could focus on the potential of hDOT1L as a therapeutic target in the treatment of SET-NUP214, PICALM-MLLT10 and MLL-MLLT1 mediated leukemias [9].

MYB

The MYB transcription factor is the cellular counterpart of the v-Myb oncogene of the acutely transforming avian myeloblastosis virus, which causes a rapid fatal monoblastic
leukemia in chickens. *Myb* is essential for hematopoietic, as well as T-cell development, and when overexpressed in thymocytes, \( \nu-Myb \) causes T-ALL [16]. The MYB gene encodes a nuclear transcription factor that is implicated in proliferation, survival and differentiation of hematopoietic progenitor cells. Proper levels of MYB are important during hematopoietic cell development, and overexpression of MYB is implicated in mouse leukemogenesis [17].

Myb duplication and translocation was very recently demonstrated in T-ALL [6, 17]. The most important mechanism underlying MYB copy number alteration in T-ALL occurs somatically by homologous recombination between Alu elements, providing a means by which an evolving T-ALL clone develops an increased dosage of MYB during leukemic transformation [16].

The t(6;7)(q23;q34) was recently identified as a novel recurrent translocation in T-ALL that result in the activation of the MYB oncogene through rearrangement with the TRB@. This MYB translocation was predominantly identified in very young children and may define a new and unique T-ALL subgroup based upon gene expression profiling [9].

*“Type B” mutations*
Various genetic abnormalities that have been identified in T-ALL are found irrespective of the major T-ALL subgroups specified by type A mutations. These shared abnormalities will be denote as type B mutations (figure 4), and include deregulation of genes that are involved in cell cycle, self renewal, TCR signaling pathway, T-cell differentiation or lead to the activation of tyrosine kinases [9].

<table>
<thead>
<tr>
<th>Rearrangement</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Outcome</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9p21 deletion/hypomethylation</td>
<td>CDK6-FAF1</td>
<td>Cell cycle</td>
<td>Unknown</td>
<td>70-85%</td>
</tr>
<tr>
<td>t(12;20)(p13;q11)</td>
<td>CCND2</td>
<td>Cell cycle activator</td>
<td>Unknown</td>
<td>&lt;3</td>
</tr>
<tr>
<td>t(7;12)(p25;q14)</td>
<td>NOTCH1</td>
<td>Self-renewal differentiation</td>
<td>Unknown</td>
<td>&lt;3</td>
</tr>
<tr>
<td>t(7;12)(p25;q14)</td>
<td>NOTCH1</td>
<td>T cell commitment</td>
<td>Unknown</td>
<td>&lt;3</td>
</tr>
<tr>
<td>t(11;14)</td>
<td>ABCD7</td>
<td>Progenitor degradation</td>
<td>Good***</td>
<td>8-30%***</td>
</tr>
<tr>
<td>t(11;14)</td>
<td>ABCD7</td>
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<td>&lt;3</td>
</tr>
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<td>RAS</td>
<td>Signal transduction</td>
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<td>&lt;3</td>
</tr>
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<td>Signal transduction</td>
<td>Unknown</td>
<td>&lt;3</td>
</tr>
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<td>Signal transduction</td>
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<td>&lt;3</td>
</tr>
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<td>Tyrosine kinase</td>
<td>Unknown</td>
<td>&lt;3</td>
</tr>
<tr>
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<td>FLT3</td>
<td>Receptor signaling</td>
<td>Unknown</td>
<td>&lt;3</td>
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</table>

Figure 4: Classification of genetic type B abnormalities in T-ALL

Genetic abnormalities affecting the cell cycle
The cell cycle is a tightly regulated process, in which a number of checkpoints control the coordination between cell growth, cell division and differentiation. Major regulators of the cell cycle are summarized in figure 5. Homozygous or heterozygous inactivation of the genomic CDKN2A and CDKN2B loci, that are located in tandem at chromosome 9p21, is the most frequent abnormality identified in T-ALL. In up 90% of cases, the CDKN2A/B loci are inactivated through cryptic deletions, promoter hypermethylation, inactivating mutations or (post-)transcriptional modifications. The CDKN2A and CDKN2B loci encode for p16 and p15, respectively, and act as inhibitors of the cyclinD/cyclin-dependent kinase CDK4 proteins (INK4). The CDKN2A locus also encodes for the alternative p14ARF product, which is negative regulator of HDM2, as part of the p53-regulatory circuitry. Therefore deletion of CDKN2A/B not only promotes uncontrolled cell cycle entry, but also disables p53 controlled cell cycle checkpoint and apoptosis machinery. CDKN2A/B deletions are not restricted to T-cell leukemia but reflect a general mechanism in cancer [9].
Activation of the NOTCH1 pathway

More than 50% of cases of T-ALL have activating mutations that involve NOCTH1, a gene encoding a transmembrane receptor that regulates normal T-cell development [18]. NOTCH1 receptors become activated when ligands of the Delta-Serrate-Lag2 family of proteins bind to the extracellular portion of the transmembrane molecule. This interaction initiates a cascade of proteolytic cleavages, terminating in γ-secretase generation of intracellular NOTCH1 (ICN1), which translocates to the nucleus and regulates by transcription a diverse set of responder genes, including the MYC oncogene [19] (figure 6).
NOTCH1 receptors play a critical role in cell fate specification during development and participate in multiple biological processes. In the hematopoietic system NOTCH1 activation plays a critical role at multiple stages of T-cell development.

Figure 6: Notch1 signaling in normal thymocytes

Ablation of NOTCH1 function in hematopoietic progenitors results in a complete block at the earliest stages of T-cell lymphopoiesis due to a failure in the specification of T-cell lineage. In addition to this early role in T-cell lineage specification, NOTCH1 signaling is continuously required at different stages at T-cell development and plays role in progression through the early DN1, DN2 and DN3 stages of
thymocytes maturation; participates in the regulation of TRB gene rearrangements; and regulates lineage decision between αβ and γδ lineages and, at least in some systems, between CD4 and CD8 lineages. Constitutive NOTCH1 activation in T-ALL was first identified in rare leukemia cases harboring the t(7;9)(q34;q34.3) translocation, which juxtapose a truncated NOTCH1 gene next to TRB@ locus [20]. The strong oncogenic activity of NOTCH1 was demonstrated by the rapid development of T-ALL in mice transplanted with hematopoietic progenitors infected with retroviruses driving the expression of ICN1 [21]. Similarly, transgenic mice expressing ICN1 in hematopoietic progenitor cells or in immature thymocytes developed T-cell tumors [22]. However, the role of NOTCH1 in the pathogenesis of T-ALL was not fully realized until the identification of activating mutations in NOTCH1 in many T-ALL patient samples. Activating mutations in NOTCH1 result in ligand-independent activation of the receptor or increased ICN1 protein stability in over 50% of T-ALL [23]. In particular, the majority of mutations found in the NOTCH1 locus are located in two regions, the hetero-dimerization (HD) and PEST (proline P, glutamic acid E, serine S and threonine T rich) domains. Mutations found in HD region result in ligand-indipendent proteolityc cleavage of NOTCH1,
leading to constitutive activation of the NOTCH signaling pathway. Whereas, mutations in the PEST domain appear to increase the half-life of the intracellular domain by preventing Fbxw7 interaction and, thereby, targeting of Notch to proteasome [24].

**FBXW7**

Fbxw7 is the F-box component of an SCF-E3 ubiquitin ligase complex. F-box proteins recognize substrates that are phosphorylated at sequence termed degrons and provide substrate recognition for the SCF complex. Recognition of a specific substrate by Fbxw7 results in ubiquitination of the substrate by the E2 ubiquitin-conjugating enzyme. Several proto-oncogenes have been identified as substrates of Fbxw7, such as Myc, Jun and Notch. The Fbxw7-mediated degradation of Notch requires phosphorylation of multiple residues. The first phosphorylation event at S2514 mediates Fbxw7 binding, and the second at T2512 is required for polyubiquitination of the protein by Fbxw7 that results in rapid turnover of NIC by the proteasome (figure 7).
Figure 7: Mechanism of FBXW7 recognition and degradation of its targets

Mutations in FBXW7 involving the residues that mediate the interaction of this F-box protein with phosphodegron moiety in the NOTCH1 PEST domain, also extend NOTCH1 signaling by impairing the proteasomal degradation of intracellular domains of NOCTH1 in 15% of T-ALL cases. Importantly, ~15% to 25% of these leukemias harbor two concurrent lesions activating NOTCH1; the first one inducing ligand-independent activation of NOTCH1 (an HD or juxtamembrane allele) and a second one leading to increased protein stability and extend duration of NOCTH1 signaling (a PEST or FBWX7 mutations) [25].

*NOTCH1 and prognosis*
The correlation between NOTCH1 mutations and prognosis is still unclear and controversial even though many groups in different contest tried to find it. Breit et al. in a study with 157 T-ALL patients enrolled to ALL-BFM 2000 protocol determined that NOTCH1 mutations predict a more rapid early treatment response and a favorable long-term outcome in children with T-ALL [26]. On the other hand, Zhu YM and coworkers affirm that NOTCH1 mutations are associated with poor prognosis in a set of 77 patients (adults and pediatric patients treated with a different protocol) [27]. Recently Larson Gedman et al reported the absence of any significant correlation between NOTCH1 and FBXW7 mutations and prognosis in pediatric T-ALL in the Children’s Oncology Group contest [28].

In the DCOG (Dutch Childhood Oncology Group) and the COALL (German Cooperative study group for childhood acute lymphoblastic leukemia) contest mutations in Notch1, identified in 63% of patients, were associated with a good initial in vivo prednisone response but were not associated with a superior outcome in both cohorts [29]. Finally Clappier and colleagues investigated the prognostic implication of Notch1 hyperactivation in 134 children with T-ALL enrolled in EORT-CLG (Children Leukemia Group of the European Organization for Research and Treatment of Cancer) trials. Even though
Notch1 mutated patients showed a better early response to chemotherapy, their outcome evaluated as 5-years event-free survival was similar to Notch1 unmutated patients [30]. These data suggest that differences in prognostic value of NOTCH1 and FBXW7 between studies may reflect the inclusion of T-ALL specimens from patients treated with different chemotherapy protocols and the possibility that aberrant NOTCH1 signaling may impact sensitivities to various chemotherapy drugs to different extent.

Targeting NOTCH1 signaling in T-cell Lymphoblasts

A fundamental mechanistic aspect of the oncogenic receptors encoded by NOTCH1 mutant alleles is that are dependent on the γ-secretase-mediated cleavage of the receptor for activation. Importantly, the presenilin γ-secretase complex has gained much attention as therapeutic target because of its role in the generation of amyloid deposits in the brain of Alzheimer’s disease patients [31]. Consequently, small molecule γ-secretase inhibitors, or GSIs, originally developed for the treatment of Alzheimer’s disease could be exploited for the inhibition of oncogenic NOTCH1 signaling in T-ALL [32]. Inhibition of NOTCH1 signaling with GSIs in T-ALL results in rapid clearance of ICN1 and in the transcriptional down regulation of
NOTCH1 target genes. These biochemical changes are coupled with decreased growth and proliferation characterized by G0/G1 cell cycle arrest and decrease in cell size in some cell lines. Unfortunately the phase I study showed very limited antitumor activity and major gastrointestinal toxicity presumably resulting from the inhibition of NOTCH1 signaling in the gut [33]. The poor results of this clinical trial highlight several major difficulties in the clinical development of anti-NOTCH1 therapies for T-ALL. In particular, GSIs seems to exert only a cytostatic effect against human T-ALLs, in contrast with mouse model in which inhibition of NOTCH1 signaling resulted in dramatic responses and high level of apoptosis [18, 34]. Moreover, primary resistance to NOTCH1 inhibition is highly prevalent in human T-ALL cell lines, suggesting that this may also be significant clinical problem in the treatment of primary T-ALL. Finally the intestinal epithelium seems to be very sensitive to systemic inhibition of NOTCH1 signaling and GSIs treatment is associated with dose limiting gastrointestinal toxicity resulting from the accumulation of mucus secreting goblet cells in the gut. Improved strategies for the clinical application of GSIs and NOTCH1 inhibition therapies will probably include:

✓ the use of combination therapies that will increase the
antileukemic effects of these drugs and improve their therapeutic window;

✓ the use of biomarkers to predict the response of T-ALL tumors cells to GSI treatment;

✓ new parenteral drug formulations aiming to avoid the toxic effects of inhibiting NOTCH1 signaling in the gut;

✓ new classes of NOTCH1 inhibitory molecules such as anti-NOTCH1 inhibitory antibodies, which could be engineering to specifically block the NOTCH1 receptor in the leukemic clone [35].

Figure 8: Schematic representation of targeting of NOTCH1 signaling pathway in T-ALL

Genetic defects in the (pre)TCR signaling pathway

Multiple components of the (pre)TCR signaling pathway are targeted by either mutations or chromosomal rearrangements
in T-ALL, indicating that the (pre)TCR pathway or downstream components play an important role in T-cell leukemogenesis.

LCK is a member of the SRC family of tyrosine kinases which is highly expressed in T-cells and plays a central role in the initiating events of (pre)TCR signaling. In a few T-ALL cases, a translocation, t(1;7)(p34;q34), has been described causing ectopic LCK expression through rearrangements with TRB [9].

The RAS protein is involved in the transmission of TCR signaling from the membrane receptor molecules to the ERK protein. However, RAS is also involved in a variety of other signal transduction pathways and is commonly mutated in a wide variety of malignancies. In T-ALL, activating RAS mutations have been identified in 4-10% of cases [9].

The PTEN phosphatase has been identified as an important regulator downstream (pre)TCR signaling, that directly opposes the activity of the phosphor-inosital-3 kinases (PI3K). PTEN dephosphorylates PIP₃ into PIP₂ thereby functioning as a negative regulator of the AKT pathway. Independent from activation following (pre)TCR stimulation, PTEN is negatively regulated by NOTCH1. Homozygous PTEN inactivation through frameshift and nonsense mutations have been found in 17% of primary T-ALL patients [9].
Activation of other tyrosine kinases

ABL1 is involved in various molecular-cytogenetic abnormalities in T-ALL including the episomal NUP214-ABL1 amplification that is identified in about 6% of cases, and results in the formation of a variable number of ABL1 copies. Other variant ABL1 aberrations with a very low incidence have been identified in T-ALL, including the gene fusions ETV6-ABL1, EML1-ABL1 and BCR-ABL1. For the most T-ALL patient samples, NUP241-ABL1 is only detected in a limited percentage of leukemic cells, indicating that it may represent a relatively late genetic event in T-ALL that probably reflects disease progression. The various ABL1 fusion products are constitutively phosphorylated and lead to an aberrant tyrosine kinase activation and excessive proliferation. This constitutively ABL1 activation can be reverted by imatinib, a tyrosine kinase inhibitor, and these patients may therefore benefit from additional imatinib treatment [9].

Others

In the last two years other abnormalities were described with a notably percentage in T-ALL patients. In particular deletions and/or mutations were found in single genes with different functions: protein tyrosine phosphatase (PTPN2), PHD finger-
containing protein (PHF6) and DNA-binding transcription factor (LEF1). Even though these lesions contribute on one side to better characterize the T-cell leukemia, on the other side make more complex the T-ALL scenario.

PHF6. Since T-ALL has a threefold higher incidence in males compared with females, Van Vlierberghe and colleagues tried to identify a possible X-linked tumor suppressor in T-ALL performing an X-chromosome-targeted mutational analysis in tumor DNA samples from 12 males with T-ALL by next-generation sequencing using SOLiD3 platform. This analysis identified 66 candidates previously uncharacterized nonsynonymous single-nucleotide variants and 7 positions with high confidence calls for containing complex variants such as insertions or deletions. Sequence of paired DNA samples obtained at time of clinical remission showed that most of these variants corresponded to previously unreported germline polymorphisms. However they also identified three somatically acquired changes corresponding to two nonsynonymous single-nucleotide substitutions and a frameshift-creating insertion of five nucleotides in the PHF6 gene. Array comparative genome hybridization (array-CGH) primary for X-chromosome was performed on 246 T-ALL samples (179 male and 67 female). The common minimal region was narrowed

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down to an area of 80 kb containing PHF6 gene, confirmed on positive cases by quantitative PCR analysis. PHF6 encodes a plant homeodomain (PHD) factor containing four nuclear localization signals and two imperfect PHD zinc-finger domains with a proposed role in controlling gene expression [36]. Quantitative RT-PCR analysis demonstrated ubiquitous expression of PHF6 transcripts in human tissue, with highest level of expression in thymus, ovary and thyroid, and moderate level of expression in spleen, testes and adipose tissue. Mutational analysis of PHF6 in an extended panel of pediatric and adult T-ALL primary samples identified truncating or missense mutations in PHF6 in 38% (16/42) of adult and ~16% (14/89) of pediatric T-ALL samples. Finally, no mutations in PHF6 were identified in DNA samples from B-lineage ALL (n=62), suggesting that mutational loss of PHF6 in lymphoid tumors could be restricted to T-ALL [37].

PTPN2 (protein tyrosine phosphatase type 2, also known as TC-PTP) is a cytosolic tyrosine phosphatase that function as a negative regulator of a variety of tyrosine kinases and other signaling proteins. In agreement with its role in the regulation of the immune system, PTPN2 was identified as a susceptibility locus for autoimmune disease. PTPN2 is highly expressed in thymocytes and was described as a phosphatase for both JAKs
and STATs, which are important signaling proteins downstream of cytokine receptors. Kleppe and colleagues showed that inactivation of PTPN2 occurs in approximately 6% of individuals with T-ALL, associated with intermediate age (range 4-49 years, median 24 years) and the TLX1-positive subgroups. Deletion of the entire PTPN2 gene is the most common inactivation mechanism. Taken together, the data obtained in human and mouse T-cell leukemias indicate that loss of PTPN2 sensitizes T-ALL cells to cytokine stimulation resulting in enhanced activation of cytokine receptor pathways, which may support T-ALL development and proliferation under limiting cytokine concentration in vivo [38].

LEF1 is a member of the LEF/TCF family of DNA-binding transcription factors, which interact with nuclear β-catenin in the WNT signaling pathway. LEF1 has also been shown to mediate key aspects of TGFβ signaling during craniofacial morphogenesis in the mouse. The intracellular domain of NOTCH1 has also been shown to function as coactivator of LEF1, leading to the upregulation of target genes distinct from those activated by β-catenin binding. Interestingly, LEF1 has been shown to function in vivo as either an oncogene or a tumor suppressor in different cellular context. Gutierrez et al, recently identified a new molecular subtype of human T-ALL defined
by inactivation of LEF1 through a combination of mono- or biallelic microdeletions and gene-specific mutations that are predicted to lead to the premature termination of translation. In particular the authors performed array CGH on genomic DNA extracted from the diagnosis specimens of 47 pediatric patients with T-ALL; the analysis of data revealed recurrent LEF1 microdeletions in 11% (5 of 47) of the primary clinical samples. These deletions were highly focal and involved no other known genes. Moreover the analysis of gene expression microarray data suggested that LEF1 inactivation was generally mutually exclusive of the overexpression of the known T-ALL oncogenes TAL1, HOX11 and HOX12L2, or gene in the HOXA cluster which were used to define the currently recognized molecular subtypes of T-ALL. Furthermore LEF1-inactivated cases had increased level of MYC expression, even when compared with cases with intact LEF1 and activation of NOTCH1, suggesting that LEF1 inactivation may promote the transcriptional upregulation of MYC by NOTCH1 [39].

**Unravelling the genetic bases of T-ALL: from DNA to gene and microRNAs expression**

As mentioned above T-ALL/LBL is a very complex disease. T-ALL is one of the best examples of multistep pathogenesis
where mutations that cause arrest at differentiation stage cooperate necessarily with mutations causing proliferative advantages or self-renewal capacity to the cell. By now the wide panel of alteration documented let us understand that a single investigative method is not the right approach. Since the alterations placed at the base of the pathogenetic mechanism of T-ALL/LBL involve four different pathways: differentiation impairment, cell-cycle defects, self-renewal and proliferation and survival the investigation of this pathology have to cover different cellular mechanisms from genomic to transcriptomic levels.

**DNA alterations**

The DNA of patients with T-ALL/LBL is affected by many alterations such as deletions, amplifications and translocations. The development of genome-wide copy number arrays had permitted to study the whole DNA finding known and unknown aberrations. In the last years this technology has rapidly improved going from about 100.000 SNP markers of coverage with a resolution of 25kb (SNP-array 100K, Affymetrix) to a 294 bp for the cancer gene loci with the new released Affymetrix Cytogenetics Whole-Genome 2.7M Array. At present this array provides the greatest power to detect
known and novel aberrations across the entire genome, as the best available proxy to the high throughput whole genome sequences, although very small abnormalities cannot be found. With unbiased, whole-genome coverage and the highest density of 2.7 million markers, including not only 400,000 SNPs, but also 2.3 million of non-polymorphic markers, this array provides dense coverage of the annotated genes, cancer genes, microRNA regions and haploinsufficiency genes [40]. So the SNP-array analysis provides highly sensitive platform to detect large and small copy-number changes, as well as copy number neutral loss-of-heterozygosity (CNN-LOH), which is represented by one allele being deleted and the other allele duplicated. The latter cannot be detected by either karyotypic or comparative genomic hybridization analyses [41].

Besides the advantages to detect very small structural changes, these types of technologies are not able to detect balanced translocations; so the integration with other methods is necessary. Fluorescence in situ hybridization (FISH) is a consolidate technique used in diagnostic and in research field. In our study we introduce a newly designed strategy defined as combined interphase FISH (CI-FISH) that by an initial screening panel of 26 genomic clones and the successively integration of other 10 probes, is able to detect all the known
translocations and some deletions/amplifications occurring in T-ALL.

**Gene expression profile**

Gene expression analysis using oligonucleotide or cDNA microarrays offers a tool for delineating molecular pathways that drive the malignant transformation of developing thymocytes. Ferrando et al. identified previously unrecognized molecular subtypes of T-ALL and linked the activation of particular oncogenes to defined stages of normal thymocytes development. Highly favorable clinical outcomes were observed for the cluster of patients with an overexpression of TLX1, whose cell samples showed a pattern of gene resembling that of early thymocytes. Apoptosis is a major regulatory process during T-cell development that regulates the elimination of normal thymocytes unable to express functional TCR. Thus the lack of expression of BCL2 and other antiapoptotic genes in the TLX1 positive lymphoblasts could explain the better therapeutic responsiveness in this T-ALL subgroup. Less favorable outcomes were observed in subgroups defined by gene expression profiles characteristic of TAL1 and LYL1 positive samples, which resemble late cortical and early pro-T thymocytes, respectively. Drug resistance in
these patients may be explained by the high levels of BCL2 and BCL2A and the increased resistance to apoptosis [12]. The importance of gene expression to define novel subtype of pediatric T-ALL and to correlate them with prognosis was also confirmed by others. In particular in the DCOG and COALL contest, the CALM-AF10 translocation was associated with an immature immunophenotype and poor outcome. Moreover the TLX3 translocation was associated with both immunophenotypically immature cases as well as cases committed to the γδ-lineage and was associated with poor outcome [15].

Recently the individuation in adult T-ALL patients by gene expression analysis of a subgroup “AML-like” (10%) and the correlation of those patients with a poor outcome underlines the importance of gene expression profile in the better characterization of T-ALL [42].

**MicroRNAs**

MicroRNAs are small non-coding RNAs of ~22 nucleotides that post-transcriptionally regulate mRNA expression levels. Currently the Sanger Institute has listed 1344 mature miRNAs in humans (release 16; www.mirbase.org).

MiRNA involvement in leukemia provides an additional layer
of complexity to understanding the development and progression of the disease state. Just as miRNA involvement has been investigated in developmental processes including normal hematopoiesis, the roles of disregulated miRNAs in the development and progression of leukemias has been the subject of multiple studies. As with hematopoiesis, investigators have approached this task using the dual strategies of high-throughput analysis and functional analysis of specific disregulated miRNAs. Collectively, the goals of these types of studies are three-fold: 1) to understand the role of microRNAs in the pathogenesis of the disease, 2) to determine differentially expressed miRNAs that might prove useful diagnostically, and 3) as starting points for developing novel therapies. While high throughput methods may provide clear signatures of miRNA disregulation correlating to karyotypic abnormalities and/or disease prognosis, functional analysis of individual miRNAs is necessary to understand the altered cellular processes.

The emerging portrait of miRNA involvement in leukemia has provided both signatures of consistently deregulated miRNAs and specific functional information of the processes controlled by miRNAs. While the signatures themselves may prove valuable in diagnosis, functionally critical miRNA may prove useful as targets for therapeutics. The ability to target miRNA
for downregulation and for replacement in cancer treatment will depend on the ability to address key points, mainly delivery systems and the biochemical nature of oligonucleotide modifications. Therapeutic treatments to downregulate and to replace miRNA share some common elements with current therapeutic designs for small interfering RNA (siRNA) with regard to oligonucleotide modifications and delivery systems [43].

An example of correlation between microRNAs expression and prognosis was provided by a recent work on a dataset of adult patients with T-ALL. In particular those patients that showed a signature “AML-like”, defined by gene expression analysis, had an overexpression of miR-223 compared with the other counterpart of T-ALL patients. This subgroup appeared to be associated with unfavorable outcome suggesting that a different treatment approach for these patients could be improve their prognosis [42].

Recently Shotte and co-workers found that different genetic subtypes of ALL have unique miRNA expression profiles that point to several miRNAs with potential oncogenic and tumor suppressive activity. Moreover, their study indicates that specific miRNAs are associated with resistance to vincristine, daunorubicin and L-asparaginase, but not with resistance to
prednisolone. Finally, they were also able to correlate with outcome of pediatric ALL patients the expression levels of specific miRNAs. All together their data forms a comprehensive repository of miRNAs being aberrantly expressed in genetic subtypes of ALL that can be used to explore the functional role of miRNAs in the corresponding types of pediatric ALL [44].
Scope of the thesis

The project developed during these last three years is quite ambitious and concerns the deep study of a very complex pathology such as T-cell lymphoblastic leukemia/lymphoma. The first question we wondered about was: T-ALL and T-LBL are usually considered the same disease with a different localization so, are we sure that they are the same disease with the same biological base or do we have to consider them as two different diseases with a different biological potential?

To try to answer this question we performed genomic and transcriptomic analysis on T-ALL and T-LBL pediatric patients analyzing differences and similarities resulting from their comparison. The aim of a better characterization of these two pathologies is to improve the treatment for patients with T-LBL that could not benefit from the prognostic tools used for T-ALL. Even though the introduction of the ALL-type chemotherapy in the treatment of T-LBL patients has improved their outcome the prognosis for these patients remain poor.

On the other side the evaluation of MRD as a "surrogate" marker to measure the response to treatment in T-ALL patients offers unique opportunities to study the pathology for a potential clinical application. So the second question we
addressed here was: are there any biological features that could explain the different prognostic impact in the treatment of T-ALL? The integration of data from high throughput technologies applied to the same patients within MRD-based subgroups will allow having better insights into the pathogenesis of the disease and the early response to treatment. More specifically, the aim of this part of project is the identification of biological correlates of MRD kinetics and clinical outcome. Moreover, the investigation of specific targets will potentially allow to better select appropriate treatments for T-ALL patients. This requires the integration of all different and various abnormalities found in T-ALL as well as new information from CNA and microRNA and gene expression.
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Chapter 2 - T-CELL ACUTE LYMPHOBLASTIC LYMPHOMA SHOWS DIFFERENCES AND SIMILARITIES WITH T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA BY GENOMIC AND TRANSCRIPTOMIC ANALYSES
T-CELL ACUTE LYMPHOBLASTIC LYMPHOMA SHOWS DIFFERENCES AND SIMILARITIES WITH T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA BY GENOMIC AND TRANSCRIPTOMIC ANALYSES

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Submitted
Introduction

Childhood T-cell malignancies include T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL), the latter representing about one third of non-Hodgkin’s lymphomas (NHL) of childhood. T-ALL and T-LBL are often considered to be different manifestations of the same disease [1-3]. T-LBL is morphologically and immunophenotypically identical to T-ALL although T-LBL shows minimal marrow involvement (<25% bone marrow blasts) and often large mediastinal mass [4]. Treatment of childhood T-LBL was based on NHL therapy, but recently the use of T-ALL type therapy has improved the outcome [5]. Despite the many similarities that exist between T-ALL and T-LBL, interesting differences in the patterns of disease distribution occur. Initial central nervous system (CNS) involvement is more frequent in T-ALL compared with T-LBL, and the typical sites of relapse are predominantly local relapse in T-LBL patients [5] and systemic relapse in T-ALL [5]. Furthermore, relapses in T-LBL occur in general earlier than in T-ALL.

T-cell lymphoblastic malignancies are thought to rise from transformation events occurring in crucial steps of thymocyte
development [6]. Indeed, de-regulation of specific oncogenes has been linked to the arrest at particular stages of thymocyte differentiation. In about half of pediatric T-ALL patients have been identified recurrent chromosomal translocations, which juxtapose promoter and enhancer elements of the T-cell receptor (TCR) loci next to genes involved in thymocyte development such as TLX1, TAL1 and LYL1 [7]. The study of chromosomal abnormalities in T-LBL is hampered by the paucity of vital specimens, however, based on the very limited available data, the typical chromosomal aberrations reported in T-ALL can also be found in T-LBL [8]. In addition to chromosomal translocations, other mechanisms lead to de-regulation of specific oncogenes expression which results in the acquisition of characteristic transcriptional signatures associated to T-ALL subgroups [9].

Constitutive activation of NOTCH1 has been shown to play a role in the pathogenesis of T-ALL. NOTCH1 is targeted by rare translocations and very common (in more than 50% of cases) activating mutations [10]. A limited number of T-LBL cases has been studied for NOTCH1 activating mutations and the results suggest the presence of similar mutational patterns both in T-ALL and T-LBL [11-12].
A comprehensive analysis of the biological differences and similarities between T-ALL and T-LBL requires the implementation of genome-wide approaches. An early attempt based on the use of genome expression profiling revealed clear differences between T-ALL and T-LBL transcriptional profiles [13]. However, a recent study performing genome-wide analysis of copy number alterations (CNA) in pediatric lymphoblastic lymphomas failed to detect genetic lesions not previously identified in T-ALL [14].

Toward a better understanding of the biological relationship between T-ALL and T-LBL we performed genomic and transcriptional profiling analyses on a pediatric panel of patients characterized for NOTCH1 mutational status. The results confirmed that T-LBL and T-ALL share a large number of features but also display distinct transcriptional signatures. Moreover, several recurrent genetic lesions were associated only with one of the two malignancies. As expected, NOTCH1 mutations were identified in both T-ALL and T-LBL, however the mutational status distinguished two subgroups based on their transcriptional profiles and genetic aberrations.
Methods

Cases

The tumor tissue was material exceeding the diagnostic specimens obtained from pediatric patients affected by de novo T-LBL (20), T-ALL (10), B-cell lymphoblastic lymphoma (B-LBL, 7) and common (CD10+) lymphoblastic leukemia (common-ALL, 6). Patient data are reported in Table SI. In all cases histological and morphological analysis was centrally reviewed. The normal control DNA used for the SNP analysis was obtained from bone marrow aspirates and/or from peripheral blood obtained at remission. Informed consent was obtained from the patients and the tissue collection was approved by each institutional ethics committee.

Gene expression and single nucleotide polymorphism profiling

Total RNA was isolated using Trizol Reagent (Invitrogen) from diagnostic bone marrow aspirates or tumor tissue of 10 T-ALL, 20 T-LBL, 7 B-LBL and 6 common-ALL. The gene expression profiles (GEPs) were generated starting from 5ug of total RNA using one-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA) to generate double-strand cDNA, IVT Labeling Kit (Affymetrix, Santa Clara, CA) to generate biotinylated cRNA and the HG-U133Plus2.0 GeneChip® arrays following the
manufacturer’s indications (Affymetrix, Santa Clara, CA). The gene expression values were determined by Affymetrix Microarray Suite 5.0 (MAS 5.0) software, using the Global Scaling option. Paired (normal and tumor) genomic DNA of 9 T-ALL patients was extracted from freshly isolated BM-MNC by a commercial procedure (Puregene Blood kit, Gentra Systems, Minneapolis, MN), and genomic DNA from 9 T-LBL patients was obtained from nucleated cells isolated by differential lysis using the QIAamp tissue kit (Qiagen, Hilden, Germany). The genome-wide single nucleotide polymorphism (SNP) profiles were generated by GeneChip Human Mapping 100K Array Set (GeneChip Human Mapping 50K Array Xba 240 and the GeneChip Human Mapping 50K Array Hind 240) (Affymetrix, Santa Clara, CA). Washing and staining of the GeneChip Mapping arrays were performed automatically in 450/250 Fluidics Station (Affymetrix, Santa Clara, CA). The GeneChip Scanner 3000 was used to acquire the array images.

Copy Number Alteration (CNA) analysis

The SNP arrays raw data were acquired by the Affymetrix GCOS software (version 1.4) and SNP calls were generated by the GTYPE software (version 4.0).

The recommended QC and SNP call rate thresholds were used
The median SNP call rate per array was 97.95% for XbaI 50K arrays and 97.26% for HindIII 50K arrays. The overall mean SNP call rate was 97.13% (0.02% SD) (96.48% for tumor cells, 97.78% for normal cells). Paired copy number analysis was performed using three different algorithms: PartekGS, CRMA and CNAG. The aberration segments provided by Partek Genomics Suite 6.08 software were generated using the genomic segmentation algorithm at a p-value of 0.001 and a signal to noise ratio of 0.3 and a minimum of ten genomic markers. The segmentation data were then filtered for p-value ≤ 0.01. Copy-number values obtained by Robust Multichip Analysis (CRMAv.2) were generated by normalizing each tumor’s probe set intensity to that of its paired normal reference. Raw copy number was estimated by using the CRMA algorithm [16]. Copy number segmentation of log2 ratios was performed in R using the DNA copy number package v1.16.0 which applies CBS (Circular Binary Segmentation) [17]. Genomic copy numbers were also calculated using the CNAG v2.0 software package which relies on the Hidden Markov Model (HMM) analysis [15]. Copy number values ≤ 1.7 and ≥ 2.3 were considered losses and gains, respectively. Minimal common regions (MCR) of aberration were identified as the smallest overlapping aberrant regions (BRLMM algorithm) [15].
detected by at least two algorithms in two or more patients.

**Gene expression profiling data analysis**

MAS 5.0 normalized GEPs were used in the analyses. Unsupervised clustering was performed using an algorithm based on the average-linkage method [18-19] and Pearson’s correlation. Probe sets with expression mean $\mu < 50$ and standard deviation $\sigma < 0.3\mu$, were considered uninformative and were thus excluded, leaving 40,517 probe sets for the analysis. To identify genes differentially expressed between phenotypes we performed T-test on log2-transformed data using a threshold of Bonferroni-corrected $p<0.05$. Gene set enrichment was measured by GSEA (Gene Set Enrichment Analysis) [20].

**Copy number alteration and gene expression profiling correlation analysis**

The analysis was performed for all genes which mapped (based of NCBI RefSeq database, hg18) to recurrent regions of aberration. GEP from T-ALL and T-LBL samples were normalized by GCRMA [21] as separate or combined datasets. For each gene, CNA and corresponding gene expression signal matrices were created. Spearman correlation was measured 100 times per gene between its copy number value and
corresponding gene expression signal. The correlation coefficient was calculated as the mean of the coefficients. The samples were randomly permuted and the correlation was run 10,000 times to generate a null distribution. The p-value of the correlation was calculated as the number of times the absolute value of the coefficient was greater than the coefficient generated by random permutation. All probe sets for a gene were analyzed and the set that had the lowest p-value was considered.

**Mutational analysis**

NOTCH1 and FBXW7 mutational analysis was performed on 11 T-LBL and 7 T-ALL. Polymerase chain reaction was performed with 200ng DNA, PCR Expand Long Template Kit (Roche) for exon 26 (HD-N terminal), exon 27 (HD-C terminal), exon 34 (PEST domain) of NOTCH1, exon 9, 10 and 12 of FBXW7 and PCR G-C Buffer (Roche) for exon 28 (juxtamembrane domain) and exon 34 (transactivation domain TAD) of NOTCH1. Primers used for PCR were the same used for direct sequencing. Primer sequences were as follow: exon 26 FW:

GCTGAGGGAGGACCTGAACCTTGG; exon 26 RV:

CCTGAGCTGGAATGCTGCCTCTA; exon 27 FW:

CATGGGACCTCAGTGTCCCT; exon 27 RV:
TAGCAACTGGCACAACACAGC; exon 28 FW:
GCGTAGCCGCTGCCTGAT; exon 28 RV:
CAGACTCCGGGTAGGATGC; exon 34TAD FW:
GCTGGCCTTTGAGACTTG; exon 34TAD RV:
CTCCTGGGGCAGAATAGTGT; exon 34PEST FW:
ACAGATGCAGCAGCAGACC; and exon 34PEST RV:
CCTGGGGCCAGATAAACAGTACA [22]; exon 9 FW:
ACATACAATAATGCCTTTGTGC; exon 9 RV:
ATTTAAGAGCAGACTGTAC; exon 10 FW:
ACCAAGCATGCAGCATTCTAG; exon 10 RV:
TGGACTGTACTGGACCGC; exon 12 FW:
AGCATCAGAGCTGTGA; exon 12 RV:
CAAGCCAACATCCTGCACCAC [11]
Results

*T-LBL and T-ALL show distinct gene expression profiles*

In order to investigate the common and unique transcriptional characteristics of T-LBL and T-ALL, gene expression profiles (GEPs) were generated from diagnostic tumor tissue or bone marrow aspirates representative of T-LBL (20 patients) and T-ALL (10 patients), respectively. The different tumor localization (nodes versus bone marrow) and consequent diverse contamination from normal cells affected greatly the gene expression analyses. Therefore, in order to control for the normal cell components associated with the nodal biopsies (T-LBL) and with the bone marrow aspirates (T-ALL), GEPs of B-cell lymphoblastic lymphoma (B-LBL; 7 patients) and common (CD10+) acute lymphoblastic leukemia (ALL; 6 patients) were included in the analyses. Indeed, unsupervised clustering performed on the complete dataset confirmed that each tumor type shows a distinct gene expression profile and that the main discriminators were represented by the localization (bone marrow versus nodes), followed by the cell of origin (T versus B) (Fig 1).
Figure 1. T and B cell malignancies show distinct gene expression profiles driven by the tumor localization and the cell of origin. Unsupervised hierarchical clustering performed on T-LBL (20), T-ALL (10), ALL-common (6) and B-LBL (7) gene expression profiles. The analysis includes the most informative 358 genes. The vertical bars on the right of the matrix point to genes associated with the contaminating normal tissue (bone marrow or nodal tissue) or with the cell of origin (B or T).
In order to identify genes differentially expressed between T-LBL and T-ALL, regardless of the tumor localization, a subtractive approach was used. First, we identified a gene signature distinguishing the lymphomas (T-LBL and B-LBL) from the leukemias (T-ALL and common-ALL). Then, this signature, enriched in genes differentially expressed between nodal tissue and bone marrow, was subtracted from the list of genes differentially expressed between T-LBL and T-ALL. The remaining signature (78 genes) was still able to distinguish in an unsupervised fashion T-LBL from T-ALL, but not B-LBL from common-ALL (Fig 2), suggesting that the differentially expressed genes were not representative of the tissue localization.
Figure 2. Identification of a gene signature discriminating T-ALL and T-LBL regardless of the tumor localization. Unsupervised hierarchical clustering of the complete dataset including B- and T-cell malignancies using a gene expression signature identified to discriminate T-ALL from T-LBL after filtering the genes differentially expressed because of the nodal or bone marrow contamination.
Overall, the tumor localization and the cell of origin were identified to be the leading differences in the GEP of T-LBL and T-ALL, nevertheless a signature independent from these features was able to distinguish between the two phenotypes.

*The expression of genes involved in the chemotactic response and angiogenesis discriminates T-LBL and T-ALL*

Upon elimination of genes differentially expressed in nodal-versus bone marrow-derived samples, we were able to identify a subset of genes which appeared to be differentially expressed between T-LBL and T-ALL. This signature included genes involved in chemotactic responses and angiogenesis which may play a role in the different tumor cell localization (Fig 2).

T-LBL and T-ALL responsiveness to CXCL12 is likely to be differentially influenced by the consistent down-regulation of β-arrestin 2 (ARRB2) detected in T-LBL. Indeed, T-lymphocytes genetically deficient in β-arrestin 2 are impaired to chemotactic responses to CXCL12 despite an increase of G protein stimulation as assessed by ligand activated GTPase [23].

T-ALL displayed high expression of SIGIRR, an orphan receptor belonging to the IL-1R/TLR family. SIGIRR appears to act as a negative regulator of ILR and TLR [24-25], suggesting that a negative modulation of ILR and TLR pathways may have
a role in T-ALL pathogenesis.

The T-LBL growth in the nodal compartment implies that the tumor cells acquired the ability of promoting angiogenesis and surviving in hypoxic conditions. Consistent with these requirements, T-LBL showed up-regulation of EPAS1 (HIF-2α) which was discovered as a hypoxia inducible factor involved in angiogenesis [26-27] and PTPRB, a receptor-type phosphotyrosine phosphatase with an essential role in angiogenesis [28-29]. SLIT2, a secrete glycoprotein that activates ROBO receptors to initiate a cell signaling pathway shown to promote tumor angiogenesis [30], was expressed in T-LBL, but not in T-ALL. Interestingly, epigenetic inactivation of SLIT2 has been recently reported in T-ALL and concordantly lack of SLIT2 expression was observed in most T-ALL here analyzed [31].

The signature discriminating T-ALL and T-LBL included also a few genes (FMNL2, ESAM) which have been previously reported to correlate in solid tumors with nodal metastasis [32-33] and, consistent with these observations, were up-regulated in T-LBL.

In conclusion, T-LBL and T-ALL displayed distinct gene expression profiles characterized by differences in the expression of genes involved in the response to cyto- and
chemokines and in angiogenesis.

**Copy number alteration analysis in T-LBL and T-ALL reveals common and unique genetic alterations**

In order to investigate the common and unique genetic aberrations of T-LBL and T-ALL, copy number alteration (CNA) analysis was performed on a subset of the samples analyzed by GEP. Paired (normal and tumor) genomic DNA was interrogated using single nucleotide polymorphism (SNP) arrays (Human Mapping 100K arrays, Affymetrix) for 9/20 T-LBL patients and 9/10 T-ALL patients. The CNA analysis was performed using three methods: the PartekGS platform and the CRMA and the CNAG algorithms (Fig 3). The large number of segments displaying CNA was filtered retaining only segments concordantly identified by at least two algorithms. Furthermore, the analysis was restricted to CNA detected recurrently in at least two patients and the minimum common region (MCR) was identified based on the shortest segment of aberration. Overall, the CNA analyses identified 42 regions which showed aberrant copy number (22 gains and 20 losses) in at least two patients.

The most common aberration found both in T-LBL and T-ALL was represented by a deletion affecting chromosome 9p21.3; as
previously reported, the minimum deleted region pointed to CDKN2A/B genes consistent with their tumor suppressor function. Overall, 8 patients showed CDKN2A/B deletions including three affected by lymphoma and five by leukemia (Fig 4). Interestingly, the deletion of CDKN2A/B in T-LBL was detected only in patients who at diagnosis showed a tumor stage III (3/4 stage III; 0/5 stage IV). Within the T-ALL group loss of CDKN2A/B was a common feature even though the extension of the deletion was different among T-ALL samples. In particular, the deletion of the CDKN2A/B flanking region including MLLT3 gene, has been found only in the two patients with early-T immunophenotype.

In three patients (one T-LBL and two T-ALL) were detected focal deletions of chromosome 12p13 (confirmed by FISH, data not shown), a region previously investigated as tumor suppressor host for being frequently deleted in pre-B-ALL [34-35] (Fig 4). Several tumor suppressor candidates are embedded in this genomic region including ETV6, an ets family transcription factor frequently rearranged in leukemias [36] and BCL2L14, an apoptosis related gene [37-38].

Among the few focal regions of deletions the loss of chromosome 4p16.2 in three patients was restricted to the
homeobox gene MSX1 which was recently reported to be often methylated in T-ALL [31]. Two distinct regions of loss on chromosome 9p24.3 and 9p23 affected DOCK8 and PTPRD genes, respectively. DOCK8 is a recent member of the DOCK180-related regulators of morphology whose deletion or inactivating mutations have been associated with a combined immunodeficiency characterized by hyper-IgE syndrome and impaired activation of T cells [39-40]. PTPRD is a protein phosphatase very similar to the leukocyte common antigen related molecule (LAR), whose function in T cell has not been studied yet [41].

A gain on chromosome 5p12 pointed to a MCR which includes the genes coding for the mitochondrial ribosomal protein MRPS30 and for the fibroblast growth factor 10 (FGF10). Interestingly, thymus development is impaired in FGF10-deficient mice suggesting that aberrations of this locus may influence thymocytes development [42]. Another gene whose deficiency has been associated with impairment of thymus formation is EYA1 [43], which was identified as the only target gene in a MCR of gain on chromosome 8q13.3.

On chromosome 8q21.12 a focal gain affects the interleukin 7 (IL7) and the not yet characterized FAM164A genes. The role of
IL7 on thymic development has been reported [44] and more recently it has been shown that it may cooperate with NOTCH in promoting proliferation and survival of thymocyte precursors [45].

Several recurrent MCR of gain were detected on chromosome 8, including whole chromosome gains in 2 T-LBL cases. In addition at least 2 cases showing whole arm gains were identified for chromosome 5p, 14q and 21q in combination with a variable number of patients showing focal amplifications in the same regions (Figures 3 and 4).

The analysis was then focused toward the identification of aberrations recurrently detected only in T-LBL or T-ALL and several focal MCR appeared to be specific to the lymphoma or to the leukemia subset in this panel of patients (Fig 4). Two focal MCR of loss restricted to T-LBL were identified on chromosomes 4q32.3 and 12p11.21 affecting the DDX60L and FGD4 genes, respectively. DDX60L is a not well characterized gene belonging to the DEAD-like helicases superfamily and FGD4 is a GDP/GTP exchange protein for CDC42 found mutated in Charcot-Marie-Tooth disease [46-47]. A MCR affecting 12 genes on chromosome 17q11.2 includes NF1, whose mutations and micro-deletions have been showed to be the
cause of neurofibromatosis type 1 [48-49]. NF1 has been previously reported to be deleted and to function as tumor suppressor in approximately 3% of pediatric T-ALL but no information has been reported in T-LBL [50-51]. In this study we identified NF1 deletions in two T-LBL patients but in none of the T-ALL, suggesting that NF1 aberrations may be more common in lymphoma than in leukemia.

A MCR of gain on chromosome 22q13.32-q13.33 was identified as a specific aberration in the T-ALL subgroup. Among the 9 genes included in this region is Pim-3, belonging to a family of protooncogenes that encode serine/threonine protein kinases. Pim-3 has been reported to promote cell cycle progression by phosphorylating and down-regulating CDKN1B and to inhibit apoptosis leading to tumorigenesis [52].

In conclusion T-LBL and T-ALL appeared to share a number of genetic aberrations. Nevertheless, the results here reported on a limited number of cases suggest that a subset of genetic lesions could be relevant for T-LBL but not for T-ALL and viceversa.
Figure 3. Summary of the genomic copy number alterations. CNA were identified using three distinct analysis approaches (CNAG, CRMA and PartekGS). All aberrations identified by each algorithm are displayed in gradient of red and blue representing gains and losses, respectively. Only aberrations concordantly detected by at least two methods were considered further.
Figure 4. Recurrent aberrations detected in T-LBL and T-ALL. Heat map summarizing the copy number gains (red) and losses (blue) recurrently detected in T-LBL and T-ALL. The aberrations detected only in T-LBL or T-ALL are labeled with an orange and green bar, respectively. For the CNA affecting less than 15 genes, the gene symbols are reported in parenthesis. The details on genomic coordinates and gene lists are reported in Table S3.
Copy number alterations affect the gene expression levels

In order to identify the relationship between CNA and GEP data we used an approach based on the Spearman correlation (see Methods). More than 38% of the tested genes showed a positive correlation (Rho ≥ 0.3) between the genetic lesion and the gene expression in at least one dataset (T-LBL, T-ALL or combination of the two), while only 3.2% showed a negative correlation (Table SIV), suggesting that the gene expression data could be informative on the effects of genetic aberrations (p-value<5.7E-72 by Fisher Exact Test).

A significant enrichment for genes displaying a positive correlation between CNA and GEP was found in multiple MCR of gain including chromosome 1p36.33-p33, 4p16.3-p16.2, 5p15.33, 17q25.1-q25.3, 19q13.32-q13.33, 21q22.3 and in two region of loss (17q11.2 and 5q33.2). The enrichment analysis was feasible only for the largest MCR affecting one or more cytobands. The results showed that most of the MCR affecting 12 or more genes were significantly enriched in genes displaying at the mRNA level the effects of the genetic lesion.

The largest MCR of gain covers chromosome 1p36.33-p33 and includes 614 genes, 235 of which showed a correlation with changes in their expression levels. We investigated the
functional annotations of the responsive genes involved in the chromosome 1p36.33-p33 gain and using DAVID (Database for Annotation, Visualization and Integrated Discovery) we found significant enrichment for genes involved in cellular nitrogen compound and nucleic acid metabolic processes (data not shown).

Overall, the integration of CNA and GEP data obtained from the same patients led to the identification of a subset of genes for which the genetic aberrations are directly linked to their transcriptional de-regulation.

*A subset of genetic aberrations segregates with the presence of activating mutations in NOTCH1*

Mutational analysis was performed for NOTCH1 and FBXW7 genes in order to characterize NOTCH1 aberrant activation in the tumor cases reported in this study [10, 53]. The analyzed panel included 11 T-LBL and 7 T-ALL of which 7 and 4, respectively, showed mutations in the NOTCH1 gene. None of the NOTCH1 wild type samples carried mutations of the FBXW7 gene. The mutational analysis results were summarized in Table SV.

Gene Set Enrichment Analysis (GSEA) was applied to check if a previously reported NOTCH signature, identified in T-ALL cell
lines upon treatment with γ-secretase inhibitors [54], was enriched in NOTCH1-mutated versus –unmutated T-LBL and T-ALL. The results confirmed that NOTCH1 activity was traceable in the samples carrying activating NOTCH1 mutations but not in the samples with a wild type NOTCH1 gene (Fig 5A).

A GEP-based approach was then applied to identify a gene signature able to discriminate NOTCH1-mutated from NOTCH1-unmutated samples in our dataset. Ten genes previously identified to be transcriptionally responsive and bound in their promoter by NOTCH1 [54], were used in a supervised analysis to identify the most significant discriminating genes in our T-ALL and T-LBL dataset (Fig 5B).
Figure 5. NOTCH1 activation is associated with a transcriptional signature and correlates with a subset of genetic aberrations. A) Measurement of the enrichment in NOTCH1-mutated and -unmutated T-LBL and T-ALL cases of a NOTCH1 gene signature (51 genes) generated in T-ALL cell lines upon NOTCH1 signaling inhibition [54]. The mutated cases show significant enrichment for the NOTCH1 activation gene signature. B) Supervised analysis applied on previously published direct targets of NOTCH1 identifies a gene signature discriminating NOTCH1-mutated and -unmutated cases. C) Unsupervised clustering based on the NOTCH1-activation gene signature was used to predict NOTCH1 mutational status in T-ALL and T-LBL. The two major clusters segregate with the unmutated (left branch) and mutated (right branch) NOTCH1. D) Copy number alterations correlating with NOTCH1 mutational status. The samples for which the NOTCH1 mutational status was validated by direct sequencing are displayed in bold. The aberrations detected only in T-LBL or T-ALL are labeled with an orange and green bar, respectively.

The identified gene signature was then used to cluster the whole dataset, in order to predict the NOTCH1 mutational status for the samples which could not be subjected to the
mutational analysis because of biological specimen limitation. The results showed that the dataset included 18 NOTCH1-mutated (10 tested, 8 predicted) and 12 NOTCH1-unmutated (6 tested, 6 predicted) samples (Fig 5C). Overall, we observed aberrant NOTCH1 activation in approximately 60% of the cases, in concordance with previously reported data [10].

The results of the CNA analysis were then revaluated to identify among the recurrent regions of aberration, those that were affected only in NOTCH1-mutated or NOTCH1-unmutated samples (Fig 5D). Amplifications affecting chromosome 1 and 5p appeared to be a feature of NOTCH1-unmutated cases. Interestingly, the gained regions on chromosome 1p36.33-p33 and 1q32.2 includes the NOTCH targets HES5, HEYL and CR2, suggesting that in absence of NOTCH1 mutations, some critical targets may be affected by genetic alterations aiming to deregulate their expression [54-56]. NOTCH1-mutated cases displayed loss of chromosome 6q16.2-16.3 and of a gene-desert region on chromosome 4q35.2. These aberrations did not segregate with the type of T-cell malignancy but only with the NOTCH1 mutational status. Additional aberrations (chromosome 1, 5, 12 and 13) restricted to NOCH1-mutated or –unmutated cases were identified, however they also associated with the lymphoma versus
leukemia phenotype (Fig 5D).

Further validation in a larger panel of cases is needed; nevertheless these results suggest that distinct genetic aberrations could be detected in T-cell malignancies with or without constitutive NOTCH1 activation.
**Discussion**

This study aimed to the transcriptomic and genomic analyses of pediatric T-cell malignancies including both lymphomas and leukemias in order to dissect molecular similarities and differences between T-LBL and T-ALL. At the best of our knowledge this represents the first T-LBL dataset for which genomic and transcriptomic data have been generated from the same samples. The paucity of bioptic material obtained for diagnosis from pediatric T-LBL patients implies very limited possibilities to save any excess of diagnostic tissue for molecular studies. Therefore the dataset size reflects the limitations faced in the study of this disease. Nevertheless, even if limited in number, the samples here analyzed showed to be informative in this genome-wide study, pointing to several molecular characteristics which will require further validation on larger datasets using classic low throughput techniques.

The T-LBL cohort (20 patients) reported here is the largest investigated so far by genome-wide gene expression profiling. Two previous reports showed, on smaller datasets, that T-LBL and T-ALL display different gene expression profiles [13, 57]. A major limitation of both these studies was represented by the fact that the normal cell contamination issue, which is the main
driver of differences between T-ALL and T-LBL (Fig 1), was not fully addressed. We included in our analysis different types of leukemia and lymphoma toward the identification of specific markers differentiating T-LBL from T-ALL regardless of their normal cell contaminations. The T-LBL/T-ALL discriminating signature was largely depleted of genes differentially expressed between malignancies located in the nodes or in the bone marrow (Fig 2) indeed providing the specific differential transcriptional characteristics of T-LBL and T-ALL. Given the widely shared pathogenetic events that lead to the rise of these T cell malignancies, the identification of differences in their transcriptomes implies that the malignant T cells have unique molecular characteristics which drive them to colonize predominantly either the bone marrow or the nodes. The most relevant differences affected genes involved in the chemotactic response and in angiogenesis suggesting that the two malignancies have different ability to respond to several cyto- and chemokines and that, as expected, T-LBL need to modulate transcription to promote angiogenesis as well as to deal with hypoxic conditions. Of interest, a few genes reported to correlate with nodal metastasis in solid tumors, were identified to be up-regulated in T-LBL suggesting that these molecules could have a role in the nodal homing of malignant cells.
Regardless of the limited dataset, extensive CNA analysis was performed using multiple algorithms. The identified recurrent aberrations recapitulated the known CNA previously associated with T-ALL and pointed to several loci that appeared to be preferentially affected in one of the two malignancies.

Focal deletions of CDKN2A/B have been reported as the most frequent deletion in cancer [58] and losses affecting chromosome 9p21.3 were indeed the most common aberration in our dataset (detected in 44% of the patients). Both T cell malignancies displayed loss of the CDKN2A/B locus and among the T-LBL the deletion appeared to be restricted to stage III tumors. None of the 5 stage IV T-LBL displayed CDKN2A/B deletions, while 3/4 of the stage III did. The correlation observed in T-LBL between disease stage and CDKN2A/B deletions, suggests that this aberration may be a relatively early event in disease progression.

The LOH of a region spanning chromosome 6q14-q24 has been reported as negative prognostic factor in T-LBL, but not in T-ALL, with 6q16 being the most frequently affected region (13/21 cases with LOH in 6q) [1]. In this study we detected a deletion on chromosome 6q in two patients (1 T-LBL and 1 T-ALL) with
the MCR of aberration (6q16.2-q16.3) defined by the T-LBL patient. Of interest the same MCR has been previously detected in 10% of T-ALL [51]. Noteworthy, in our dataset this aberration correlated with NOTCH1 mutational status, suggesting a potential association between activating mutations of NOTCH1 and 6q16.2-q16.3 deletions, an observation which needs to be corroborated in a larger number of patients.

NF1 deletions have been reported in approximately 3% of T-ALL but never investigated in T-LBL. Our study showed that NF1 was deleted in 2/9 (22.2%) of T-LBL and in none of the T-ALL patients suggesting that aberrations affecting NF1 may be more relevant in the lymphoma than in the leukemia pathogenesis. Further studies on deletions and/or inactivating mutations of NF1 in a larger panel of T-LBL are needed to support this finding.

In this study we were able to show that CNA correlates with gene expression, suggesting that for a sizable fraction of genes the genetic aberration is a mechanism of gene expression deregulation. This type of data will be instrumental toward the identification of the subset of genes whose expression is affected by a genetic alteration and it may drive attention to the most relevant targets of a given aberration. For example, nine
genes appeared to be targeted by the 6q16.2-q16.3 deletion, however only 4/9 displayed an effect on their expression and one of them (CCNC) has been shown to be involved in the control of the G0-G1 transition [44], a function that should grant further investigations of its role in the pathogenesis of T-cell malignancies. Investigating the gene expression changes associated with a genetic aberration adds a critical layer of information, nevertheless it is relevant to consider the lack of a full correlation between CNA and gene expression. This may be due to several reasons, the most likely represented by the fact that the acquisition of a genetic lesion is not the only way to affect gene expression. Indeed, epigenetic gene silencing is a common mechanism of transcriptional regulation which may impair expression of structurally normal genetic loci. NOTCH1 mutations were identified uniformly across T-LBL and T-ALL in approximately 60% of the cases, corroborating previous reports suggesting that constitutive activation of the NOTCH pathway is a common pathogenetic mechanism in both malignancies [11-12]. Several CNA were identified to correlate with NOTCH1 mutational status, and interestingly gains of chromosome 1p36-p33 and/or 1q32.2, which include a few NOTCH1 targets, were identified in 4/7 unmutated cases. These results suggest that some critical NOTCH1 targets may be
affected by genetic aberrations leading to their induction regardless of NOTCH1 activation.

Overall this study represents the first comprehensive genomic and transcriptomic analysis of pediatric T-LBL in comparison with T-ALL. The data showed that behind a largely common pathogenesis these T-cell malignancies represent two distinct biological entities with unique transcriptional and genetic characteristics. The complete understanding of the molecular characteristics of T-LBL and T-ALL represents the driving element toward the design of fully successful therapeutic approaches.
References


Chapter 3 - GENETIC CHARACTERIZATION OF THE DIFFERENT RESPONSE TO TREATMENT IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA SUBGROUPS
GENETIC CHARACTERIZATION OF THE DIFFERENT RESPONSE TO TREATMENT IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA SUBGROUPS

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RATIONALE

T-cell acute lymphoblastic leukemia (T-ALL) is a malignant disease of thymocytes, accounting for 10-15% of pediatric cases. Patients with T-ALL tend to present with very high blast cell counts in the bone marrow, mediastinal masses and central nervous system involvement. Leukemic transformation of immature thymocytes is caused by a multistep pathogenesis involving numerous genetic abnormalities that permit uncontrolled cell growth. A wide variety of genetic events affecting cellular processes, such as the cell cycle, differentiation and survival have thus far been identified in T-ALL and results in developmental arrest in nearly all stages of T-cell maturation in the thymus. Many of these genes that are involved in chromosomal translocations in T-ALL become activated due to disturbances in the rearrangements process of the TCR genes. Besides other non-TCR gene-mediated translocations that may produce fusion products, many other genes become activated or inactivated due to the presence of specific point- or insertion/deletion-mutations, or are affected by somatic copy number variations, such as amplifications or deletions. Many of these genes normally play important roles in T-cell commitment and differentiation or control important checkpoints in T-cell development.
Even though many efforts have been done to improve the treatment of T-ALL patients, many of them still have a poor prognosis, or encounter drugs toxicity. The early kinetic of the response to the chemotherapy, evaluated by Minimal Residual Disease (MRD) monitoring, is considered as the most relevant prognostic factor and used in clinical treatment protocols. MRD is defined as the percentage of blasts that persist in the bone marrow of the patients after any therapy. MRD monitoring, performed through the investigation of tumor-specific markers of disease, allows stratifying patients in three different risk groups (standard risk SR, medium risk MR and high risk HR) based on the number of residual leukemic cells at the end of the Induction therapy. In the ongoing AIEOP-BFM ALL2000 clinical protocol, while the 5 years EFS of HR T-ALL cases is around 50%, overall SR and MR (non-HR) patients have an outstanding EFS of more than 70%. However, there is no evidence of biological features which could explain the different prognostic impact in the treatment of T-ALL.
MATERIALS AND METHODS

Cases

For this study 22 MRD-HR and 35 MRD non-HR (SR + MR) T-ALL patients from the AIEOP LAL2000 protocol have been enrolled. Selection was independent on the early prednisone response. Patients were matched for immunophenotype and age.

Copy number alteration analysis

The copy number alteration analysis was evaluated by Whole-Genome Cytogenetic 2.7M array (Affymetrix). Briefly, for each sample, 100ng of genomic DNA was amplified, purified by beads, fragmented and labeled. Then the labeled DNA was loaded into the chip and hybridized over night. The chip was washed and stained in the Affymetrix GeneChip Fluidic Station 450 and scanned by Affymetrix GeneChip Scanner 7G. The results were analyzed by Chromosome Analysis Suite (ChAS) Software (Affymetrix).

Each diagnostic sample was compared with its own remission. The segmentation file was generated by ChAS; amplifications/duplications and deletions were identified by copy number state (≥3 or ≤1), number of markers in the region ≥
20 and size of the region $\geq 30$kb for losses and $\geq 50$kb for gains. The software is also able to detect loss of heterozygosity copy number neutral; segments considered as long continuous stretch of homozygosity (LCSH) have a copy number (CN) = 2, size $\geq 2$Mb and a number of markers $\geq 20$.

All abnormalities detected in diagnosis and remission samples were considered as constitutive copy number alterations (CNA). Abnormalities detected in at least two patients were considered as recurrent.

**Combined interphase FISH technology (CI-FISH)**

Clones were labeled with spectrum orange and spectrum green (Vysis, IL, USA) for double-color assays in either break-apart tests or two combined split FISH tests. Quick-FISH probe preparation: After re-suspension in a formamide hybridization mixture with cot-1 DNA (Invitrogen, Milano, Italy), directly-labelled clones were ready. Slides were prepared using diagnostic cytogenetic pellets. At least six assays were spotted and the corresponding hybridization areas delimited by a round 12 mm coverslip stick with rubber cement to avoid cross-contamination. Slides and probes were co-denatured on a plate at 75°C for 10 minutes, hybridized over-night at 37°C, and washed with nonidet P-40 at 75°C and at room temperature.

'106
Analysis of 120-200 nuclei and/or 5 abnormal metaphases was done with a fluorescence microscope (Provis, Olympus, Milano, Italy) equipped with a CCD camera (Sensys, Photometrics, Tucson, AZ) run from an image analysis software (Vysis, Stuttgart, Germany). Abnormal hybridization patterns were: a) split signal (1 fusion signal and separate green and red signals), b) duplication/trisomy (3 fusion signals), c) deletion/monosomy (1 fusion signal), and d) partial deletion (1 fusion signal and 1 orange or 1 green signal). Cut-offs were assumed at the upper limit value obtained in 500 normal peripheral blood nuclei for each assay: split, duplication/trisomy patterns were considered positive when found in >5% of interphase cells; monosomy/deletion/partial deletion was considered positive when found in >10% [1].

**TAF_Iα/NUP214 and TAF_Iα/NUP214 fusion transcripts.** To detect TAF_Iα and TAF_Iβ isoforms the NUP_2916R primer (exon 20) was used with, respectively, forward primers TAFa_283F (5’-GAAACCAAGACCACCTCCTG-3’) and TAFb_38F (5’-AGCTCAACTCCAACCACGAC-3’) in the first amplification round. The common reverse primer NUP_2601R (exon 18) and either TAFa_283F or TAFb_38F were used for the second amplification round. PCR products were cloned in pGEM-T easy vector (Promega) and sequenced [1].
NOTCH1 mutational analysis

NOTCH1 mutational analysis was performed by polymerase chain reaction (PCR). In particular PCR was performed with 200ng DNA, PCR Expand Long Template Kit (Roche) for exon 26 (HD-N terminal), exon 27 (HD-C terminal), exon 34 (PEST domain) of NOTCH1 and PCR G-C Buffer (Roche) for exon 28 (juxtamembrane domain) and exon 34 (transactivation domain TAD) of NOTCH1. Primers used for PCR were the same used for direct sequencing. Primer sequences were as follow: exon 26 FW: GCTGAGGAGGGACTGAACTTGG; exon 26 RV: CCTGAGCTGGAATGCTGCCTA; exon 27 FW: CATGGCCTCAGTGCTGCTCTA; exon 27 RV: TAGCAACTGGAATGCTGCTCCT; exon 28 FW: GCGTAGGGCTGCCTGAGCT; exon 28 RV: CAGACTCCCGGAGTGGCTGCTGCTCCTA; exon 34TAD FW: CAGACTCCCGGAGTGGCTGCTGCTCCTA; exon 34PEST FW: ACAGATGCAGCGACAGAACC; and exon 34PEST RV: CCTGGGAGCTGACATCTGTTAC [2].

Gene expression profiling

RNA preparation

RNA was isolated from bone marrow or peripheral blood
mononuclear cells separated by Ficoll-Hypaque technique (Pharmacia, Uppsala, Sweden), extraction were performed either from fresh cells at time of diagnosis or from stored frozen diagnostic material. Total RNA were isolated by TRIZOL following manufacturer instruction (Invitrogen, Paisley, UK), quality control was done with the 2100 Bioanalyzer using “Eukaryote total RNA Nano Assay” (Agilent Technologies). All material was stored at -80°C.

Gene expression arrays

Gene expression profile was performed on 54 T-ALL patients by HG-U133 Plus 2.0 GeneChip® (Affymetrix, Santa Clara, CA, USA). Patients were processed starting from 100 ng of total RNA using GeneChip® 3’IVT express kit and protocol (Affymetrix, Santa Clara, CA, USA). Hybridization, staining and washing were performed using protocols as recommended by the manufacturer, stained chip were scanned on GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, California). Expression files (Affymetrix .CEL files) were generated using the GCOS 1.4 or 1.2 and Affymetrix® GeneChip® Command Console® Software (Affymetrix).

Data analysis
Arrays have been normalized using robust multiple-array average (RMA). When data belonged to different protocols batch effects were removed using Combat. Unsupervised analyses were based on hierarchical clustering (with Euclidean distance and Ward’s method). Heatmaps were used to highlight the associations between the clusterings and the expression levels of the genes. The shrinkage approach was used to assess differences in gene expression levels between two groups of interest, using local false discovery rate as method to control false positives. When this approach had been believed inaccurate, we used a permutation approach on filtered probe sets (filtering out probe sets with small variance across samples; 90% of the probe sets removed) with tests based on standardized rank sum Wilcoxon statistics and we control false positives with the method of Benjamini and Hochberg. Results from these two approaches were considered significant if they had local false discovery rate < 0.05 or adjusted p-value < 0.05, respectively.

**MicroRNA profiling**

MicroRNA expression profile was initially evaluated in 10 HR and 11 nonHR patients by TaqMan® Human MicroRNA Arrays Set A and B v2.0. 100ng of total RNA was pre-amplified and loaded into the card (A or B).
To identify putative miRNA-mRNA functional pairs miRNA and mRNA expression data were integrated using Partek®
Genomics SuiteTM software, version 6.5 Copyright © 2010
(Partek Inc., St.Louis, MO, USA). The “combine microRNA
with their mRNA targets” function were applied to identify the
predicted target of most deregulated miRNAs between the
most deregulated mRNA. TargetScan (Release 5.1) was choosen
as algorithms for miRNA target prediction. Functional gene
interactions were investigated using Gene Set Enrichment
Analysis (http://www.broadinstitute.org/gsea/index.jsp) [3].
RESULTS

Copy number and SNP analyses

15 MRD nonHR patients and 14 MRD HR patients were analyzed by Cytogenetics 2.7M arrays.

In all patients at least one aberration was detected. The total number of lesions was 168 (95 losses, 28 gains and 45 LCSH). The average of abnormalities for the HR group was 6 and for the nonHR group was 5.6. The most common alteration was the mono-or bi-allelic deletion of CDKN2A on chr9 (26/29). Known aberrations were detected as recurrent abnormalities, such as, gain of MYB (2/29), interstitial deletion of SIL-TAL1 (5/29), deletion of PTEN (3/29), deletion of FBXW7 (2/29), loss of LEF1 (3/29), loss of RB1 (2/29). Other known alterations were found in single patients such as loss of PTPN2, PHF6, LMO2 and WT1.

Most aberrations were indistinctly detected in the two groups, but some peculiar alterations were also found:

- deletion of a 3Mb region on chr14 in 2/14 HR patients;
- deletion of LEF1 (chr4q25) in 3/16 nonHR patients;
- focal deletion of IKZF1 (chr7p12.2) in 2/16 nonHR patients.
- deletion of RB1 (chr13q14.2) in 2/16 nonHR patients.
<table>
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<th>minimal common region</th>
<th>cytoband</th>
<th>HR</th>
<th>nonHR</th>
<th>genes</th>
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</table>

Table 1: Recurrent copy number abnormalities in T-ALL patients. In red gains, in blue losses and in pink CNN-LOH
Translocation analysis.

The analysis of CALM-AF10 t(10;11) and MLL-t(11;19) translocations has been performed in 21 HR patients and in 26 nonHR patients by Dr. Lo Nigro (Catania). In the HR group, two patient resulted to be positive for t(10;11) (9.5%) and another patient (4.8%) has been found to be positive for t(11;19). In contrast, in the nonHR group no patients are positive for t(11;19) but 5 patients have t(10;11) (19.2%). The screening for the SIL-TAL1 fusion gene was performed at DNA level to detect the intergenic deletion. In the HR group 5/22 (22%) patients show the fusion gene while in nonHR group the SIL-TAL1 gene is present in 7/35 (20%) patients. All patients were negative for t(4;11); t(9;22) t(12;21) and t(1;19).

Combined-interphase FISH

CI-FISH analyses were performed in 31 T-ALL patients (13 HR and 18 nonHR). CI-FISH was abnormal in 30/31 patients, detecting ≥2 genetic changes in 20.

In particular 6/31 (19.3%) patients showed rearrangements of TCRB: 2 inv(7)(p15;q34)TCRB-HOXA; 2 t(7;9)(q34;q34)TCRB-TAL2; 1 t(7;11)(q34;p15)TCRB-LMO1 and 1 not characterized. 3/31 (9.7%) T-ALL patients presented rearrangements of TCRA/D: 1 TCRA/D-unknown partner, 1
t(10;14)(q34;q11)TCRA/D-HOX11 and 1 t(11;14)(p13;q11)TCRA/D-LMO2.

6/31 (19.3%) patients resulted to carry the TLX3 translocation; the TLX3 partner remain still unknown.

Furthermore some common lesions were tested as shown in the table 2.

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<tr>
<th>Pt.</th>
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<th>TCRB</th>
<th>TCRAD</th>
<th>TAL2</th>
<th>TAL1</th>
<th>OCI1</th>
<th>MYB</th>
<th>IKAROS</th>
<th>JAK2</th>
<th>CDKN2A</th>
<th>CDKN2B</th>
<th>LEF1</th>
<th>PAX5</th>
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</table>

Table 2: Recurrent lesions tested by CI-FISH. In yellow translocations, in blue losses and in red gains.

115
The most frequent alterations found were the deletion of CDKN2A/B, the TLX3 translocation and the translocations involving the TCRB. The CI-FISH results confirm the alterations found by copy number analysis in those patients tested for both techniques and improve the T-ALL patients characterization with the informations about translocations.

**NOTCH1 mutations**

30 T-ALL patients (13HR and 17nonHR) were analyzed for the exon 26 (HD-N), exon 27 (HD-C), exon 28 (JME) and exon 34 (TAD and PEST domains). On the whole, 19/30 (63%) patients resulted to be mutated for NOTCH1, in line with the data of the literature. The most frequent exon mutated was the HD-N in 12/19 patients (63%). Moreover 76% of nonHR (13/17) resulted to be mutated while only 46% of HR (6/13) had a mutation in Notch1. None patients carried more than one mutation. Even though the Notch1 mutation was until now tested in few patients our results seemed to confirm the positive correlation between Notch1 mutations and good outcome. The complete results for Nocth1 mutations were showed in table 3.
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<tr>
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Table 3: Notch1 status in the 30 T-ALL patients studied
Gene expression analysis

Gene expression profile was performed on 54 T-ALL patients. The unsupervised analysis of all T-ALL patients enrolled the study was performed in order to see if HR and nonHR group would have a different expression pattern. The analysis revealed that the patients in HR and non-HR groups didn’t cluster in a significant statistically manner (figure 1).

Then we evaluated the differences in the expression between the two groups of specific genes, such as TAL1, LYL1, LMO1, LMO2, TLX1, TLX3 and HOXA genes in order to delineate specific subtypes (already known in literature) and correlate their over-expression or down regulation to outcome in our patients. No differences in the expression levels of these genes were found in HR vs nonHR group (data not shown).

A supervised analysis between HR and nonHR group was performed. The figure 2 shows the heatmap for the first 66 probes resulting significant different, by T test analysis, for the two groups. While the HR and the SR risk groups are well divided, the MR group is distributed equally within the HR and the SR patients.
Figure 1: Unsupervised analysis of all 54 T-ALL patients

Figure 2: Supervised analysis of all 54 T-ALL patients
Since MR patients do not have a specific expression pattern but their profile cluster together with HR or SR patients, we supposed that this distribution could be due to the different outcome for MR. In particular we hypothesized that MR patients in the HR group should have a greater frequency to relapse than MR patients in the SR group. So we performed an unsupervised analysis between relapsed vs not relapsed patients but the result was not significantly different (data not shown).

In order to understand if HR and SR patients have a significant difference in their expression pattern, we performed an unsupervised analysis of HR vs SR, excluding the MR patients.

Figure 3: Unsupervised analysis of HR and SR patients
A gene set enrichment analysis was performed on the 65 probes most significantly differentially expressed. We used these genes to perform by the free online GSEA software. The GSEA software is able to determine if a group of genes enrich a specific pathway. We performed the analysis with 200 genes differentially expressed between HR and SR group, the only two pathways resulted to be enriched were: miR-215/miR-192 pathway and methylation pathway (figure 4).

Figure 4: Pathways identified by GSEA using the 200 genes most differentially expressed between HR and nonHR group.

Furthermore supervised analyses for specific features were
performed independently from the response to therapy of the patients. In particular we tested whether our patients presented specific signature patterns for clinical and biological characteristics. No significant differences were found between the different immunophenotype and no significant differences were detected for the different early response to treatment with prednisone.

Figure 5: Gene expression profile of T-ALL patients for immunophenotype (a) and prednisone response (b). GPR: good prednisone responders; PPR: poor prednisone responders.

Moreover, to better characterized T-ALL patients in terms of phenotype we performed an unsupervised gene expression analysis between our T-ALL patients and a data set of pediatric
AML patients (figure 5). As expected by literature [4] a subgroup of 18 T-ALL cluster together with AML.

![Figure 6: Unsupervised gene expression analysis of T-ALL and AML patients](image)

On the base of data obtained by copy number analyses, CI-FISH experiments and the study of Notch1 mutations, we evaluated the expression pattern of specific lesions. In particular we found distinct pattern of expression for Notch1 mutated patients (figure 7), patients presenting the SIL-TAL1 fusion gene, CALM-AF10 (figure 8) and TLX3-translocated patients and patients carrying the deletion of PTEN and LEF1 (figure 9).
Figure 7: Gene expression profile of T-ALL patients for CDKN2A deletions (a) and Notch1 mutations (b).

Figure 8: Gene expression profile of T-ALL patients for SIL-TAL1 deletions (a) and CALM-AF10 translocation (b).
Figure 9: Gene expression profile of T-ALL patients for TLX3 translocation
(a) LEF1 deletion (b) and PTEN deletion (c)

In order to understand whether the distribution of MR and the different signature of HR and SR were correlated with specific gene lesions, for each patient the genomic information about the most relevant and recurrent abnormalities has been included in the analysis. In particular starting from the heatmap of the supervised analysis between HR and nonHR group were considered SIL-TAL1 and CALM-AF10 fusion genes, TLX3-translocation, deletion of CDKN2A, the deletion of PTEN and
LEF1 and the mutational status of NOTCH1. Even though many patients were not characterized for all abnormalities it was possible to note that some lesions seem to be specific for the HR or nonHR group (figure 10).

![Figure 10: Supervised gene expression analysis based on MRD response (first line). The others lines show the distribution of some abnormalities tested.](image)

In particular 11/51 patients tested, were positive for SIL-TAL1 fusion gene and belong to HR group.

Also the deletion of LEF1 seems to be specific for the HR group (3/0) although not all patients (30/54) were tested for this lesion.
In order to overcome this problem, using the signature specific for LEF1 we tried to predict whether other patients could carry the LEF1 deletion (data not shown). By the gene expression analysis no other patients cluster together with LEF1 deleted patients.

Moreover 3/28 T-ALL patients positive for the focal deletion of PTEN were in the HR group; by gene expression profiling other 3 patients were predicted as PTEN deleted and belong to the HR group as well. As regard the nonHR group it seems to be characterized by the TLX3 translocation, indeed 3/4 patients were positive for the translocation and other 2 patients have a signature “TLX3-translocated-like”.

**MicroRNAs expression**

The use of TaqMan microRNAs set cards v2.0 in 9 HR_MRD patients and 11 nonHR_MRD allowed to test about 700 different microRNAs as an initial screening. By statistical analysis (t test), 26 microRNAs resulted to be significantly differentially expressed (p<0.05) between the two groups. In particular we focused on hsa-mir-215 and hsa-mir-107, resulting down-regulated in the HR patients compared with nonHR group, since they showed the most significantly
difference in the expression with a p value < 0.001.

Figure 11: box-plot representing the expression of microRNA-107 and 215

Moreover we performed a combined analysis between gene expression profile and microRNAs expression comparing the microRNAs most differentially expressed between HR and non HR group and the genes most differentially expressed between
the same groups. The results were shown in table 3.

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Table 3: Combination between microRNAs and genes most differentially expressed

The down regulation of microRNAs means a possible up regulation of their target genes. None of the genes we found correlated to miR-215 and miR-107 have been yet validated in literature. But it's known that mir-215 is up-regulated by p53 in response to genotoxic stress; it also has a common seed region with mir-192 that is known to induce G1 and G2-M cell cycle arrest [5-6]. The validation by single assays and the computational analysis for gene targets will be performed.
DISCUSSION

Aim of this comprehensive project was to integrate data from different high throughput technologies applied to the same patients selected based on MRD subgroups, to have better insights into the pathogenesis of the disease and the early response to treatment.

Gene expression analyses revealed no differences in the signature pattern of HR vs nonHR (SR plus MR) patients. But differences in the expression rose up when MR patients were subtracted from the nonHR. By definition MR patients had an intermediate behavior between HR and SR even though their kinetic of response to therapy, evaluated by MRD, is more similar to SR than HR patients. The unsupervised analysis revealed the presence of more than 200 genes differentially expressed (p<0.05) between SR and HR patients. The gene set enrichment analysis delineated two different pathways: a pathway involving microRNAs 215 and 192 and the methylation pathway. We still didn’t perform any experiments about methylation mechanisms, although this result opens another complex point of view in the pathogenesis of T-ALL and in the possible involvement of epigenetic in drugs metabolism.
As regard to microRNAs, even if the results are preliminary, we identified miR-215 as one of the most differentially expressed between the two T-ALL subgroups. From literature it’s known that miR-215 is involved in the regulation of cell cycle by targeting many proteins of this pathway, as a consequence of p53 overexpression caused by genotoxic stress [5-6]. In our patients this microRNA resulted to be downregulated in HR compared with nonHR patients. We could suppose that in HR patients the amount of miR-215 is not sufficient to efficacy target genes of cell cycle causing the growth arrest. Thus the DNA could not be repaired, the cell cycle go on accumulating genetic defects and, probably, the cells becoming resistant to chemotherapy.

The expression of miR-192 didn’t differ between HR and nonHR patients, but the limited number of patients and the necessity to confirm the results by single assay could not made conclusive our results.

We also verified the expression of microRNA-223 in order to see whether its expression was correlated with a poor outcome, as shown for adult T-ALL patients [4]. But, as for mir-192, we didn’t find any differences in the expression of this microRNA between T-ALL and “AML-like” patients.

As regard to microRNA 107, we found a downregulation in its
expression in the HR group compared to the nonHR group. In a recent work on gastric cancer cell the authors identified mir-107 as a potential regulator of CDK6 [7]. CDK6 is a cyclin-D1-dependent kinase, which phosphorylates the retinoblastoma protein, thereby removing the repression of E2F transcription factor activity and influencing cell cycle progression [8]. Inhibition of CDK6 prevents murine erythroid leukemia cells from undergoing differentiation upon stimulation [9]. Moreover high levels of CDK6 expression have been reported in pancreatic cancer suggesting a role of CDK6 in cancer [10]. Feng and colleagues demonstrated that the ectopic expression of miR-107 reduced both mRNA and protein expression levels of CDK6, inhibited proliferation, induced G1 cell cycle arrest, and blocked invasion of the gastric cancer cells [7]. Consistent with these data the downexpression of miR-107 could reflect an upregulation of CDK6 in HR patients compared with nonHR suggesting a role of this microRNA in the worst outcome of these patients.

We also found a correlation between microRNAs differentially expressed and genes differentially expressed between HR and nonHR patients, and we identified those genes as probable targets for miR-215 and miR-107. Deeper studies are necessary
to confirm the effective difference in the expression of these microRNAs and the direct correlation with the targets genes we found.

The supervised gene expression analysis revealed that MR patients were equally distributed within HR and SR group. In order to understand whether the distribution of MR and the different signature of HR and SR were correlated with specific gene lesions, we annotated to the gene expression results some of the most recurrent abnormalities we found by copy number, translocation and mutation analyses.

The deletions of PTEN seemed to be specific of the HR group. It’s known that mutations in PTEN cause the loss of function of the gene and a probable consequent upregulation of AKT, conferring resistance to standard chemotherapy to the patients carrying the mutation [11]. Consistent with these data we could suppose that the deletion of PTEN could contribute to the poor outcome for HR patients.

Specific for the HR patients we found also the deletion of LEF1 gene. It has been hypothesized that the inactivation of this gene could lead to a developmental arrest at cortical stage of the T-cells; moreover, the analysis of the available clinical data showed that LEF1 inactivation was not a significant predictor of event-free survival in children with T-ALL treated in the
Children’s Oncology Group or Dana-Farber Cancer Institute studies [12]. In contrast with these results we didn’t find any correlation with immunophenotype (two patients were early-T and one thymic-T). Even though we tested few patients, in our contest the deletion of LEF1 seemed to be associated to a poor outcome.

Also the fusion gene SIL-TAL1 resulted to be peculiar of HR patients. Consistent with our results, in literature the overexpression of TAL1 is usually associated to a less favorable outcome in pediatric T-ALL[13].

As regard to the nonHR group, the translocation involving TLX3 appeared to be a distinctive feature for this group. The overexpression of TLX3 has been associated with poor outcome [13-14]; it cannot be excluded that the discrepant results are probably due to the different protocol of treatment.

By gene expression we were also able to individuate specific genetic subgroups, as already described in literature, for SIL-TAL1, TLX3, CALM-AF10 [12-14], for PTEN and forNotch1 mutated patients. However, none of these signature correlates with prognosis in our patients.

Even though performed in few patients, the constitutive activation of Notch1 by mutations seemed to correlate with nonHR patients, similar to what reported by the BFM group.
The copy number and the translocation analyses provided a genomic characterization for the T-ALL patients studied. All patients presented at least two abnormalities, with an overall average of 5.8 lesions detected by copy number analysis. Many lesions resulted to be common features between the HR and nonHR patients. But we also identified some anomalies that seemed to be specific for each group.

We found that the deletion of LEF1 was specific for the nonHR-MRD group, even though these patients in the gene expression analysis cluster together with the HR patients. More studies are necessary to confirm the correlation of lesions to prognosis. The deletion of IKZF1 in two patients of nonHR group resulted to be in contrast with the data that reported in BCP-ALL an association between IKZF1 deletion and poor outcome. Of course the increase of number of patients and a deeper study of the involvement of these genes in the pathogenesis of T-ALL could clarify their correlation with outcome [15].

Overall, our study revealed the great heterogeneity of T-ALL patients and confirms that it’s indeed not sufficient to consider a single lesion as specific for a group but the cooperative effects of the many lesions that affect the HR or nonHR patients. We could hypothesize that the contribution of different lesions acts
on specific pathways, justifying the different expression pattern between HR and nonHR patients.

Modeling these abnormalities in \textit{in vitro} and \textit{in vivo} is also necessary to functionally test the consequences of those lesions and to demonstrate whether they can be responsible for the different early response to treatment measured by MRD. This could potentially open the way for testing specific drugs that could have efficacy in these subgroup of patients that have, although limited in numbers, the worst prognosis.
References

11. Larson Gedman, A., et al., The impact of NOTCH1, FBW7 and PTEN mutations on prognosis and downstream signaling in pediatric T-cell acute lymphoblastic leukemia: a report from the


Chapter 4 – Summary, conclusion and future perspectives
In the first part of our work we focused our attention on the biological question about the differences between two pathologies: T-cell lymphoblastic leukemia and T-cell lymphoblastic lymphoma. These two diseases share many features such as immunophenotypic features, lymphoblast morphology and clinical characteristics and are differentially diagnosed only on the base of bone marrow involvement. We tried to understand whether T-cell leukemia and lymphoma are a unique pathology with a different manifestation or whether they are two different diseases. The results obtained by gene expression profiling revealed an intrinsic difference in the expression of 78 genes between T-ALL and T-LBL. In particular since these genes belong to the angiogenesis and the chemotactic response we supposed that the two malignancies have different ability to respond to several cyto- and chemokines and that T-LBL need to modulate transcription to promote angiogenesis as well as to deal with hypoxic conditions. Also by analysis of copy number we were able to identify some abnormalities that seemed to be specific for each group regardless the limited data set of patients. Although this work provides additional elements in the characterization of these two pathologies, many studies have yet to be done, especially on the comprehension of the different capability of
cells to migrate and invade the bone marrow compartment.

For the future, we will increase the number of patients, especially for the T-ALL group, to perform new expression profiling and copy number analyses in order to confirm the results obtained. Moreover, the genes resulted to be differentially expressed by GEP will be functionally tested to confirm their role in the pathogenesis and in the different manifestation of T-ALL and T-LBL.

The complete understanding of the molecular characteristics of T-LBL and T-ALL represents the driving element toward the design of fully successful therapeutic approaches.

The second part of the study focused on the genetic characterization of two different groups of T-ALL patients on the basis of MRD response. With the aim to find biological correlates with the outcome for HR and nonHR patients, we performed many analyses, starting from copy number analysis to microRNAs expression profiling. Furthermore, we tried to integrate all the data in order to delineate common characteristics for each group of patients. First of all, the study of copy number revealed the presence of multiple abnormalities in all patients: we found known and unknown lesions, and in
some cases we were able to associate them with HR or nonHR patients. The improvement of copy number results was obtained by the study of translocations. Also in this case we found one or more translocation in the majority of patients and we identified that the most recurrent were SIL-TAL1 and TLX3 translocations. Moreover, we tested the Notch1 mutations and, as expected, about 60% of patients were mutated for Notch1, with a tendency for Notch1 mutations to be more frequent in the nonHR group.

The second step was the analysis of gene and microRNAs expression. The initial unsupervised analysis between HR and nonHR group failed to distinguish the two groups; but the successively supervised analysis revealed the distribution of MR patients in an equal manner between HR and SR patients. Thus an unsupervised analysis without MR patients showed a specific pattern of expression for each group (SR and HR). The GSEA analysis performed highlighted the enrichment of two specific pathways: the mir-215/192 pathway and the methylation pathway. The results obtained by the expression profile of about 700 microRNAs in the HR and nonHR group and those achieved by the combined analysis of GEP and microRNAs suggested miR-215 and miR-107 as the most differentially expressed and provided some possible target
genes of these microRNAs.

Moreover, to delineate specific pattern of expression not driven by MRD but by other alterations, we used the data derived from copy number, translocation and mutational analyses to supervise genetic subgroups. Significant results were obtained for Nocth1 mutated vs non-mutated, TLX3 translocated vs non-translocated, PTEN and LEF1 deleted vs non-deleted.

We also tried to integrate all the data provided by both genomic and transcriptomic analyses to understand whether the distribution of MR and the different signature of HR and SR were correlated with specific gene lesions. SIL-TAL1 fusion gene and the deletion of LEF1 and PTEN seemed to be specific for the HR group while the TLX3-translocation seemed to be peculiar for the nonHR group of patients.

In conclusion HR and nonHR patients seemed to show some peculiar lesions and patterns of expression that could be justify the different response to therapy.

In the future, we intend to increase the number of patients tested for Notch1 mutations and to study, for the same patients, also the Fbxw7 gene in order to find solid correlation between Notch1 activation status and prognosis.

The genes resulted to be differentially expressed by GEP
between HR and SR will be analyzed by other methods to confirm the results and then functionally characterized in order to find the correlation between their differential expression and the different MRD-response of our patients.

We hypothesized that microRNA could play a role in T-ALL and that a different expression signature of microRNAs could predict prognosis for T-ALL patients. The validation by single assays of miR-215, miR-107, miR-192 and miR-223 and the computational analysis for gene targets is ongoing. The subsequent genetic characterization and the comparative analysis of different results will be necessary in order to comprehend the biological mean of the different expression pattern and to associate it to clinical outcome. Moreover we will perform a deep study of target genes of microRNAs resulting differentially expressed between the two groups. We expect to identify target proteins that have a role in proliferation, apoptosis or drug metabolism pathways in order to explain their involvement in clinical outcome in the two groups of patients.

Therefore, future experiments will consist in silencing or increasing the expression of microRNAs of interest in cells (preliminarily in cell lines and then in blasts from patients) observing the effects on target genes and proteins. Afterwards,
using the same cellular model, chemo-resistance testing will be performed, in order to correlate the different expression pattern of microRNAs in HR and nonHR groups to the different response to treatment of T-ALL patients.

In summary, several high throughput methodologies have been applied to the selected subgroup of patients to study the biological correlates of the different response to therapy. As soon as data are completed, they will be analyzed in a comprehensive way, and target mechanisms will be tested in vitro and in vivo models of the disease. Of course there are some issues that remain still open such as the presence or not of point mutations in genes not yet tested, the presence of single nucleotide polymorphism in genes involved in drugs metabolism or the role of methylation in those pathways involved in the T-ALL pathogenesis or in others.

By this work we tried to provide a better characterization of T-ALL and to give a way of interpretation for the different outcome of T-ALL patients.
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