UNIVERSITA DEGLI STUDI DI MILANO-BICOCCA

DIMET

Unveiling the heterogeneity within childhood Ph+ acute lymphoblastic leukemia

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"Faz bem aquele que ama o que faz"

(Portuguese proverb)

To my family, my special friends and my love Choco

For all the support, motivation, ideas and patience

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Chapter I. Introduction

I.1. *Philadelphia* chromosome positive acute lymphoblastic leukemia (Ph+ ALL)

In the past two decades, childhood acute lymphoblastic leukemia (ALL) cure rate has reached over 80% due to treatment advances, but some resistant ALL subtypes, such as Ph+ ALL, still don't respond to therapy. [1, 2]

The t(9;22)(q34;q11) translocation, which generates the *Philadelphia* (Ph) chromosome, is found in about 3–5% of childhood ALL compared to 25-40% of adults and is associated to a dire prognosis, with high risk of relapse (EFS rates of 25-30%, even worse in adults, less than 20%). [2, 3] The translocation results from a rearrangement between the *c-ABL* proto-oncogene (on chromosome 9) and the *BCR* gene (on chromosome 22) generating a BCR-ABL fusion protein with an enhanced tyrosine kinase (TK) activity, which causes changes of multiple signal transduction pathways and has been demonstrated to be a primary cause of leukemia formation *in vivo*. It is involved in the malignant transformation of hematopoietic cells, by enhancing proliferation, reducing apoptosis, and deregulating cell adhesion.[4] Although BCR-ABL is known to activate some important pathways (such as JAK/STAT, RAS, PI-3 kinase and SCR family), the detailed mechanisms behind BCR-ABL-induced leukemogenesis remain unknown.[5]The breakpoints in *c-ABL* occur in the same region, but *BCR* can be broken in different clusters, generating distinct proteins. More than 85% of children with Ph+ ALL show a break in the "minor" point cluster region between the *BCR* exons e1 and e2, forming a fusion protein of 190 kDa (called p190). When the rearrangement of *ABL* happens with the "major" breakpoint cluster region of *BCR*, is formed a fusion protein of 210 Kda (p210), which is characteristic of chronic myeloid leukemia (CML) and is found in 10% of childhood Ph+ ALL. There is also a rare isoform that originates a p230 protein, and affects an uncommon subtype of CML that commits neutrophils (CNL).[5, 6] (Figure I.1)
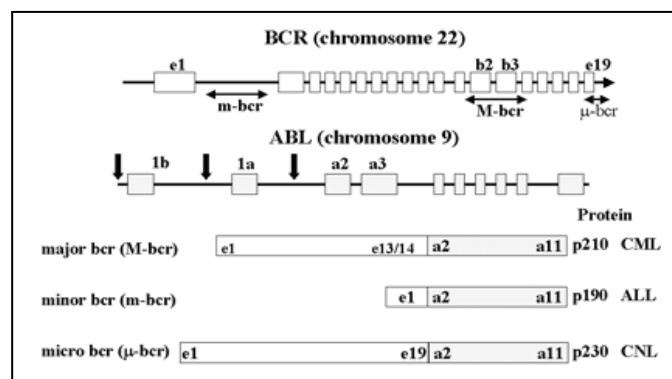

Figure I.1: Different isoforms of BCR-ABL.

The *BCR-ABL* rearrangement can be detected at diagnosis by cytogenetic (classical and FISH) and by reverse transcription-polymerase chain reaction (RT-PCR). [7]

During treatment, the molecular remission can be investigated by quantitative real-time PCR looking for BCR-ABL transcripts or for immunoglobulin/T-cell receptor (Ig/TCR) gene rearrangements, which are characteristics of leukemia clonal proliferation. These methods can detect minimal residual disease (MRD) prior to clinical relapse to drive clinical intervention.[8, 9, 10]

Although several have been the medical efforts, this disease is still the ALL subgroup with the worst prognosis. Nevertheless, several long-scale studies have shown that Ph+ ALL is heterogeneous in terms of clinical parameters such as leukocyte count, age at diagnosis and initial steroid response. A set of international studies with large series of patients have shown that an earlier remission after induction with glucocorticoids and intrathecal methotrexate (IT MTX) is correlated to a better outcome. [2, 11, 12]

Patients that present less than 1×10^9 lymphoblasts/L after the first 7 days of prednisone therapy and after one IT injection of an age-adapted dose of MTX are considered prednisone good responders (PGR). Conversely, those who present blast count higher than 1×10^9/L are considered prednisone poor responders (PPR). The collaborative group from Associazione Italiana di Ematologia Pediatrica and Berlin-Frankfurt-Munster (AIEOP-BFM ALL) showed in a study with Ph+ ALL children diagnosed between 1986 and 1995 that patients PGR have a significant lower risk of treatment failure than those PPR, when treated with an intensive chemotherapy protocol, with or without hematopoietic stem cell transplantation - HSCT [13] (Figure I.2).
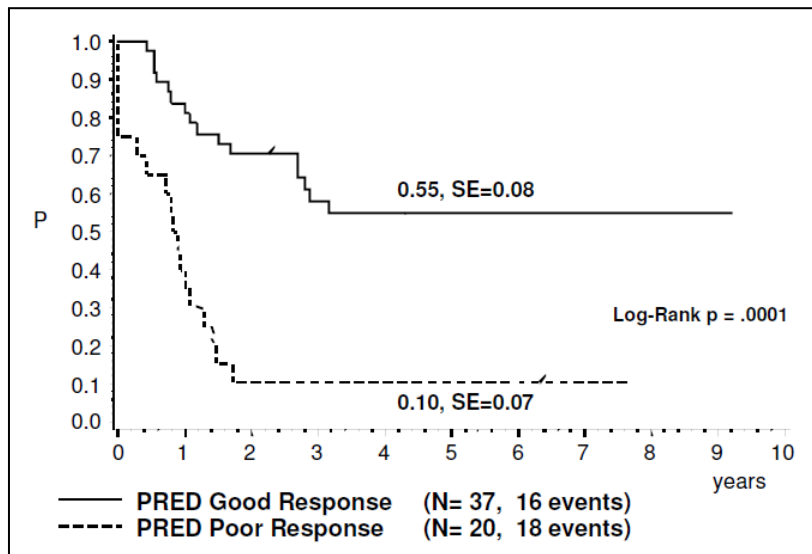

Figure I.2: Results from BFM/AIEOP (1986-95) showing event-free-survivor (EFS) according to prednisone response in childhood Ph+ ALL. Removed from Schrappe M *et al*, Blood 1998[13]

In addition, the kinetic of Ph+ leukemic blasts analyzed by PCR during and after induction chemotherapy might reflect the different propensities of Ph+ ALL to achieve complete remission. Recent studies have shown that MRD monitoring at early treatment phases can identify patients with potentially different risks of relapse. [14, 15, 16]

After monitoring molecular response in two different time points during the first month of induction, patients that presented a standard/intermediate risk MRD (SR/MR) had significantly better outcomes than those with high risk MRD (HR).[17] (Figure I.3)

Together these results show that the initial response to prednisone and the MRD risk can be used to predict responsiveness to early conventional treatment, and can be beneficial for the accurate stratification of children to guide new clinical strategies. [9, 14, 15, 16, 17]
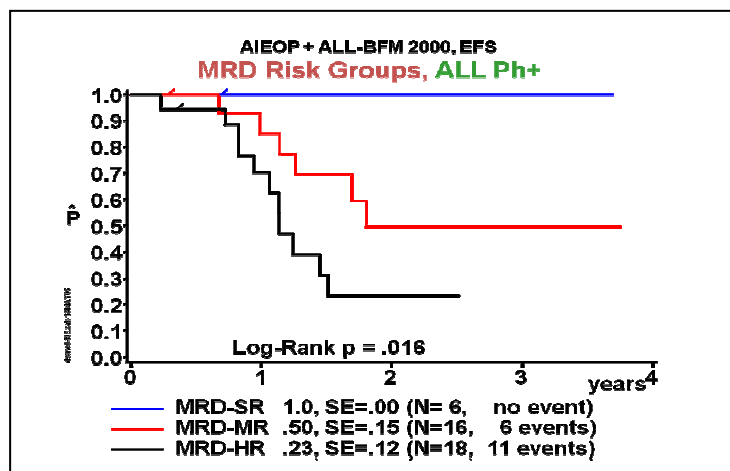
Figure I.3: 4 years EFS of children Ph+ ALL under AIEOP-BFM 2000 protocol

Given the role of BCR-ABL in the leukemogenesis of Ph+ ALL, current treatments have focused on inhibition of this oncogenic TK. The development of Imatinib mesylate (Novartis), inhibitor of TK proteins such as BCR-ABL, PDGF receptor and c-KIT, has enhanced the survivor of Ph+ patients, especially in CML. Imatinib acts blocking the biding-site of ATP, blocking the phosphorilation of downstream proteins and the malignant signal pathway[18] (Figure I.4).

Figure I.4: Imatinib mechanism of BCR-ABL blockage. Adapted from Novartis website.

Although Imatinib improved a lot the survivor rates in CML, it doens't have the same effect in ALL Ph+. [6, 18] In adults, imatinib improved remission and 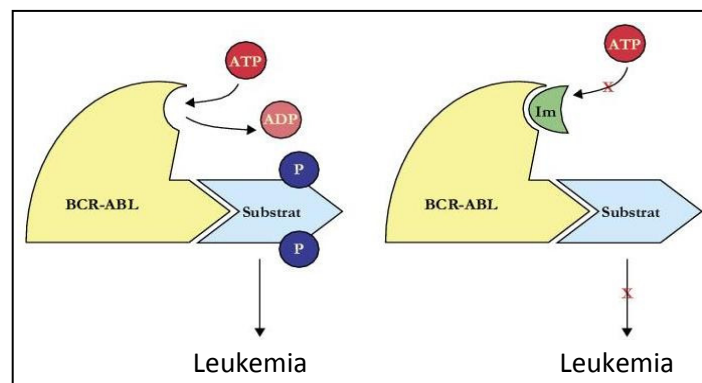DFS when combined with standard chemotherapy regimens, even in the absence of HSCT. [19] In children, imatinib has been recently introduced in the American therapy protocol and is being tested in Europe.[20] The American Children's Oncology Group (COG) have shown that imatinib can be safely used in combination to chemotherapy, without toxic effects in children.[21]   Recent results showed an increase of EFS in children ALL Ph+ that were treated with imatinib in combination with chemotherapy and HSCT, in comparison to normal chemo-protocol alone, with no appreciable increase in toxicity (figure I.5). HSCT plus imatinib offered no advantage over transplantation alone.[22] Additional follow-up is required to determine the impact of this treatment on long-term EFS and determine whether chemotherapy plus imatinib can replace HSCT.
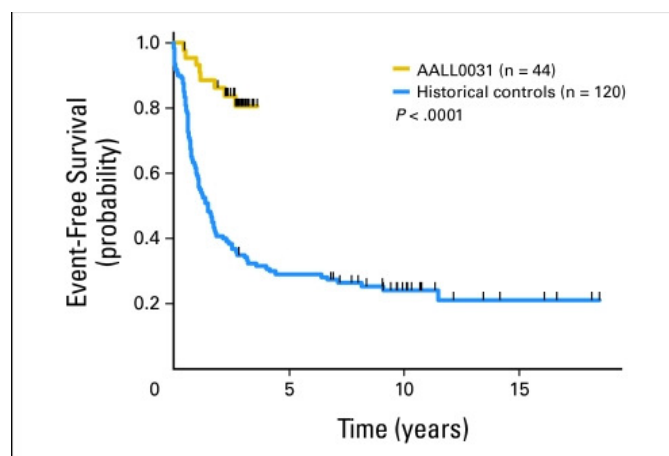

Figure I.5: 3-years EFS of children Ph+ALL treated with imatinib+ chemo compared to EFS of classical chemotherapy Ph+ALL.[22]

Since 2004 a randomized clinical trial phase II/III named EsPhALL was being tested in different European countries, trying to improve Ph+ ALL children survivor by randomicaly adding Imatinib to the chemotherapy protocol to verify its toxicity and efficacy. Imatinib was delivered according to a BFM backbone, in all PPR, and randomicaly in PGR (who account for 65–70% of the total Ph+ ALL). The trial was changed in late 2009 in response to the publication of the findings of the COG, so that all patients, independent of risk category, receive imatinib in an open label phase II study design (Biondi A, personal communication). As patients still have the option to undergo HSCT, then this study will afford an important comparison with the COG trials.[20]

Despite the good remission rates shown by different groups, many children relapse and the mechanisms underlying resistance in Ph+ ALL are multifactorial.[23] Novel TK inhibitors are

being developed and have shown efficacy in some patients with imatinib-resistant disease. The dual BCR-ABL/SRC family kinase inhibitor, dasatinib, has shown promising activity in the treatment of Ph+ patients after imatinib failure and has recently been approved for clinical trials in USA. [24, 25]

Other TKI-based therapies, such as nilotinib and bosutinib, are being tested too. These drugs are showing potential effect in CML imatinib-resistant patients but little information in children Ph+ ALL has been reported.[26, 27]

Although *BCR-ABL* is the initiator of both Ph[+] ALL and CML, numerous differences exist between these diseases both clinically and at a molecular level. It seems that *BCR-ABL* may be sufficient for the development CML, but this is not the same in Ph[+] ALL. Murine models of CML and Ph[+] B-ALL showed that although *SRC* kinases are not required for the development of CML, they are important for the development of Ph[+] ALL. [28] In Ph[+] ALL, there are many additional epigenetic changes, copy number abnormalities, and mutations downstream of *BCR-ABL* that contribute to the very aggressive clinical course. [29]

Recent gene expression studies have identified a heterogeneous pattern of expression associated with *BCR-ABL* status when comparing Ph+ to other subtypes of ALL. [30, 31] Other results showed differences also inside Ph+ ALL group that may be related to therapy response, which may be useful for developing novel prognostic markers and future patient stratification procedures. [32, 33]

Further characterization of molecular subtypes of Ph+ ALL may help to distinguish those few patients with a potentially good outcome from the majority who face inevitable relapse. Understanding these genetic lesions will be relevant both to awareness of the possible limitations of responses to current "targeted" agents for Ph[+] ALL and to design novel therapies in the future.

In order to find additional genetic lesions that could explain these biological differences among Ph+ ALL children, we designed a study that applies combined techniques to analyze specific gene and micro-RNA (miRNA) expression patterns in diagnostic samples that can be related to aggressiveness and clinical response.

I.2. Aim of the project

To dissect the Ph+ ALL heterogeneity by studying patterns of gene/miRNA expression associated to leukemogenesis.

I.3. Patients in study

Children (0-18 years) diagnosed as ALL between 2000 and 2010 in Italy were sent to Fondazione Tettamanti as reference center for molecular and genetic analysis. They were analyzed by citogenetics and RT-PCR and 78 (59M/19F) were confirmed as Ph+. BCR-ABL was 73% p190, 26% p210 and 1% both breakpoints. The median age was 9,5 years, 74% do the children were considered PGR and 62% had a non-HR MRD. Their clinical and biological features are described on table I.1.

Table I.1. Characteristics of patients in study

Patients	overal (n=78)	range/ %
WBC (median .10^9/L)	34.6	1,4-580
Sex (M/F ratio*)	59/17	76%
Age (median/years)	9.5	1-17.9
BCR-ABL		
p190	57	73%
p210	20	26%
both	1	1%
MRD		
non-HR	41	62%
HR	25	38%
Prednisone response		
PGR	39	74%
PPR	14	26%

*% corresponds to male proportion.
WBC, white blood cells; PGR, prednisone good response;
PPR, prednisone poor response.

I.4. Scope of the thesis

The following chapters will be focused in three arguments:

1- Identification of *Ikaros* deletions in childhood Ph+ ALL

2- Micro-RNA expression in childhood Ph+ ALL

3- Sumary, conclusions and future perspectives

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Chapter II. Identification of *Ikaros* deletions in childhood Ph+ ALL

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II.1. Introduction

The *Philadelphia* chromosome (Ph), a chromosomal abnormality that encodes BCR–ABL, is the defining lesion of chronic myelogenous leukemia (CML) and a subset of very aggressive acute lymphoblastic leukemia (ALL).

The expression of BCR–ABL in hematopoietic stem cells can alone induce CML, but cooperating oncogenic lesions are required for the generation of an acute leukemia.[1]

Recent studies have detected deletions on *Ikaros* gene (*IKZF1*) in 84% of Ph+ ALL cases and in 66% of blast crisis CML, but not in chronic-phase CML, suggesting that loss of Ikaros is an important step in leukemia aggressiveness.[2,3] Ikaros is a member of the Kruppel-like zinc finger transcription factor subfamily and is required for normal hematopoietic differentiation and proliferation, particularly in lymphoid lineages. Ikaros is involved in the differentiation of pro-B cells into pre-B cells and, together with the same family member Aiolos, downregulates pre-BCR function. [4, 5]

The *Ikaros* gene is located on the 7p12 chromosome and is normally transcribed in several isoforms as the result of alternative splicing, essentially altering the expression of exons 3 through 5 that encode the N-terminal DNA-binding domain. The long isoforms (Ik1 through Ik3) can bind efficiently to DNA, different from the shorter isoforms (Ik4 through Ik10), which lack the N-terminal zinc fingers and lose the capacity of DNA-binding [2, 6] (Figure II.1). The physiological relevance of these isoforms in normal hematopoiesis remains unclear. [5, 6]
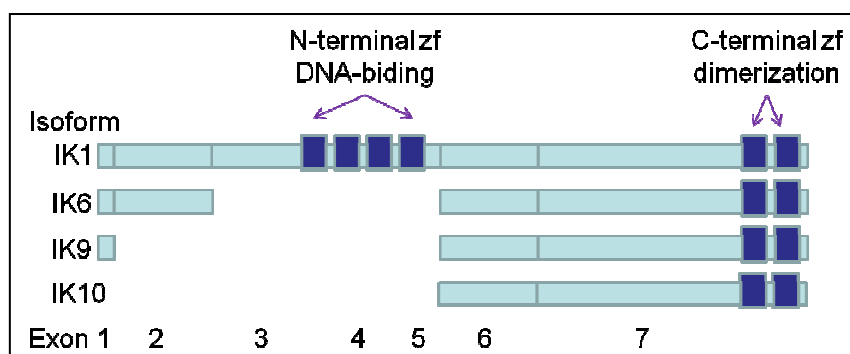

Figure II.1: IKZ1 isoforms. Adapted from Mullighan *et al*, Nature 2008[2]

In the end of the 90's some murine studies correlated the presence of these splicing variants to abnormalities in B and T cell development and to the formation of different types of leukemia. [7, 8] Germline mutant mice expressing only non–DNA binding dominant-negative leukemogenic Ikaros isoforms developed an aggressive form of lymphoblastic leukemia. [7, 8, 9] Many years later, studies with patients showed that in fact mutations in *IKZF1* gene resulted in the expression of different isoforms that seem to be involved in leukemia progression. [10] Gene expression studies revealed that chromosomal deletions involving *IKZF1* are present in 30% of high-risk B-cell precursor ALL[11] and are highly prevalent in ALL with *BCR-ABL1* fusions.[12]

Two big studies with genome-wide arrays of ALL samples showed that *IKZF1* deletions are correlated to the presence of BCR-ABL, being present in 75% of children and more than 80% of adults [10, 11] The most frequent alteration involving the *IKZF1* gene is a deletion of an internal subset of exons from 4 through 7 (d4-7). Since this isoform keeps the the C-terminal-dimerization domain, it seems to act as a dominant-negative and blocks the action of Ikaros, but little is known about the functioning mechanism of the other deleted isoforms. [12]

Authors have also showed that heptamer recombination signal sequences (RSSs) recognized by RAG enzymes during V(D)J recombination were located immediately internal to the deletion breakpoints, suggesting that the *IKZF1* d4-7 deletion was the result of aberrant RAG-mediated recombination. [12, 13]

A variable number of additional nucleotides have been described as present between the flanking breakpoint regions in the *IKZF1* gene and were specific for each patient. The gene breakpoints and the additional nucleotides inserted at the junction were maintained with fidelity at relapse and could be exploited for monitoring MRD. [12]

Later studies showed that *IKZF1* alterations are associated with poor outcome in Ph+ALL adults an also in negative ALL cases lacking recurrent chromosomal alterations. [14,15,16] There was an interesting similarity between the gene expression profiles of BCR-ABL positive ALL and the BCR-ABL negative cases with deletion of IKZF1 and poor outcome. This subtype of 'BCR-ABL-like' cases harbor additional genetic alterations, such as IKZF1 deletions, that result in activation of tyrosine kinase signaling pathways similar to those downstream of BCR-ABL, but it is not clear how Ikaros works with and without the presence of BCR-ABL.[18]

A study performed by the Children's Oncology Group, in which high-risk ALL cases were analyzed for DNA copy number alterations and gene expression differences, identified IKZF1 alterations in one-third of the cases, and found that these alterations were associated with a tripling in the risk of treatment failure. These findings have been confirmed in multiple cohorts

and testing for IKZF1 alterations at diagnosis is being evaluated in prospective ALL clinical trials. [19]

Here, we analyzed *Ikaros* status in a group of children with Ph+ ALL diagnosed in Italy between 2000 and 2010, in collaboration to the group of Dr Martinelli from Instituto Seragnoli di Ematologia e Oncologia Medica at Bologna University.

We used genomic polymerase chain reaction (PCR) and direct sequencing for the most common deletions identification. The negative PCR cases were analyzed by high-resolution interrogation of genomic copy number alterations (Affymetrix GeneChip Human Scanner 3000 and Whole Genome 2.7M Array). The breakpoint cluster regions with patient-specific additional nucleotides were sequenced and primers were designed for MRD evaluation using quantitative PCR.

Our goal was to identify lesions on the *Ikaros* gene that escape standard cytogenetic observations and combine with *BCR-ABL* to induce ALL and may be relevant in therapy response.

II.2.Patients and Methods

II.2.1. Ph+ALL in study

Between September 2000 and September 2010, a total of 78 patients younger than 18 years were diagnosed as Ph+ ALL in Italy and enrolled in the high risk group of AIEOP-ALL protocol (later EsPhALL). Seventy five out of 78 (96%) were elegible for this study and were effectively analyzed. Some BCR-ABL negative ALL patients were used as control samples. The diagnosis of ALL cases was made on the basis of morphologic, biochemical, and immunologic features of the leukemic cells, followed by cytogenetic analysis and RT-PCR. In addition, the human SD1, TOM1, SUPB15 and BV173 cell lines were included in the study. The cell lines were obtained from DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and maintained in culture following DMSZ recommendations. Bone marrow samples were obtained at diagnosis, at the two consecutive follow-up time points day 33 (TP1) and day 78 (TP2) during the therapy and at relapse.

Mononuclear cells were obtained after centrifugation on a Ficoll–Hypaque gradient. Part of the cells was frozen for posterior analysis, such as protein extraction. Other amount had the genetic material extracted. Total RNA for BCR-ABL analysis was extracted following standard procedure protocol using guanidinium isothiocyanate [20] and DNA for Ikaros analysis was extracted using Promega purification kit following the manufacturer instructions. DNA

and RNA were quantified using a NanoDrop spectrophotometer, and quality was assessed by the NanoDrop and by agarose gel electrophoresis.

II.2.2. RT-PCR for BCR-ABL

The t(9;22) translocation was detected by a single-round RT-PCR, following the BIOMED-1 protocol derived from the report of the European collaborative group on standardization analysis of fusion gene transcripts in childhood ALL. [21] Briefly, 0.5 µg of total RNA were reverse transcribed using 200 unities of Superscript II enzyme (Invitrogen), with random hexamers (5 µmol/µl) and dNTP (1 mmol/µl), in a final volume of 20 µl, at 42°C for 45 min. The PCR was performed in a final volume of 25 µl containing 2 µl of cDNA, 400 nmol/µl random primers, 200 µmol/µl of dNTP and 1 unit of Taq enzyme (Roche Diagnostics).

PCR temperatures and cycle times were: initial melting at 95°C for 30 s, followed by 35 cycles at 94°C for 30 s, 65°C for 60 s, 72°C for 60 s. PCR products were separated by 2% agarosis gel and isoforms of BCR-ABL were identified.

II.2.3. PCR and sequencing analysis of *Ikaros* deletions

PCR conditions were performed as described by Iacobucci *et al*. [6] The primers sequence are described in the figure II.2. PCR was performed using 100 ng of genomic DNA, 20 pmol/µl of primers, 200 µmol/µl of dNTP, 2mM of Cl_2Mg and 1 unit of Taq DNA Platinum (Gibco) in a final volume of 50 µl. The PCR cycling parameters were: 95℃ for 5 min (complete denaturation), followed by 35 cycles at 95℃ for 30 s, 62℃ for 30 s, extension at 72℃ for 70 s, with an additional extension at the end for 7 min (for d2-7 and d4-8 reactions the extension time was 110s).

> **Primers for IKZF1 deletions**
> **Δ4-7**
> S: GGTTACTTTTGCACCAACCTAATAGATGG
> AS: CAACTGGTATCCCTCAACAGAGATCAC
> **Δ2-7**
> S: GTTGGTTCTTGTCATATTCTAAGGGAG
> AS: AGGGACTCTCTAGACAAAATGGCAGGA
> **Δ4-8**
> S: TTCTTAGAAGTCTGGAGTCTGTGAAGGTCA
> AS: CTGTGTCTTGCCTGTGCCTA

Figure II.2: Primers used for analysis of most common *IKZF1* deletions

The positive PCR products were purified with QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol and were directly sequenced using the Big Dye Terminator DNA sequencing kit (Applied Biosystems) and the refereed primers, in the sequencer machine ABI PRISM 3130 Genetic analyzer (Applied Biosystems). The sequences were analyzed using the Sequencher software.

II.2.4. SNP array for 7p12 analysis

Cases considered negatives for *Ikaros* deletions by PCR were genotyped with GeneChip Human Scanner 3000 and Whole Genome 2.7M array (Affymetrix) according to the manufacturer's instructions. For all samples, 100 ng of DNA were amplified, fragmented, and hybridized to human Version 2.7M arrays.

The total DNA previously extracted was diluted in TE buffer and quantified. It was applied in a 96 well-plate with a denaturation and a neutralization solutions, in addition to the amplification buffer, all provided by the manufacturer. The whole genome amplification was performed overnight in a termal-cycler using the parameters indicated by Affymetrix. PCR products from each set of reactions were pooled, purified and fragmented. Fragmented products were then labeled, denatured and hybridized to the arrays for 16 hours at 50^0C and 60 RPM. Arrays were washed using Affymetrix fluidics stations and scanned using the Gene Chip Scanner 3000. Array image data was decodified using using Affymetrix genechip command console (AGCC) v3.1, exported as cel files and analyzed using ChAS (Chromosome Analysis Suit, Affymetrix) software v1.0.

II.2.5. Western blotting

The western blotting (wb) for Ikaros isoforms was performed in collaboration with the group of Dr Marina Pitto, from the proteomic laboratory at the department of Experimental, Environmental Medicine & Biotechnology of Milan-Bicocca University. Frozen cells were thawed and lysed with Ripa buffer (Sigma). Cell lysates ($5x10^6$ cells/250 µl) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels and then transferred to nitrocellulose membranes (Amersham Biosciences). The blots were incubated for 60 minutes in blocking buffer before incubation overnight at 4°C with polyclonal anti-Ikaros antibody H100 (Santa Cruz Biotechnology), followed by one hour of incubation with the secondary antibody. Blotted proteins were detected and quantified by use of the imaging system KODAK.

II.2.6. RSS-flanked *IKZF1* genomic breakpoints

Junctional regions of *IKZF1* deleted PCR products for were sequenced as described previously. Upon sequencing, patient-specific junctional region sequences of potential MRD PCR targets were identified using Vbase (http://vbase.mrc-cpe.cam.ac.uk) database and commonly distributed hardcopy sequences. At least one allele-specific primer was designed complementary to the junctional region sequence using the Primer Express software (Applied Biosystems).

Briefly, ten fold serial dilutions of the diagnostic leukemic DNA sample were made in normal mononuclear DNA (pooled MNC DNA of peripheral blood from six healthy donors) and used for RQ-PCR amplification in duplicate to generate a standard curve and to determine the sensitivity of the primer. After receiving the follow-up samples, a dilution series was used to quantify the tumor load in triplicate in the patient's remission/relapse samples.

The PCR was performed in a final volume of 25 µl containing 5 µl of diluted DNA, 1.5µl of primers, 0.5 µl of probe, 1 µl of BSA and 12.5 µl of master mix (Applied Biosystems).

RQ-PCR were performed in a 7500 Real-Time system (Applied Biosystems) using the conditions: 95°C for 10 minutes, 40 cycles of 95°C for 1 minute and 60°C for 1 minute.

II.3. Results

The RT-PCR analysis detected different BCR-ABL isoforms, being 73% of p190 and 27% of p210. One patient presented both p210 breakpoints (b2a2 and b3a3) and one presented p190 and p210. (Figure II.3)
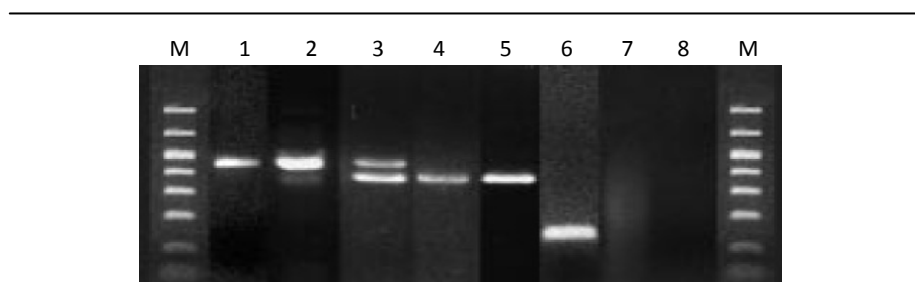

Figure II.3: RT PCR to detect BCR-ABL isoforms

M: molecular weight marquer 1000 bp; 1 to 6: Ph+ ALL patient samples, being 1 and 2 p210 b2a2,

3 shows both p190 and p210,4 and 5 p210 b3a2 and 6 p190; 7 and 8: Ph Negative ALL and H_2O

The PCR for *Ikaros* mutations revealed 31 patients positives for the deletions between the exons 4 and 7 (d4-7), 9 for the d2-7, 3 for the d4-8 and one patient harboring both d2-7 and d4-7. These cases were confirmed by direct sequencing. (Figures II.4 and 5).
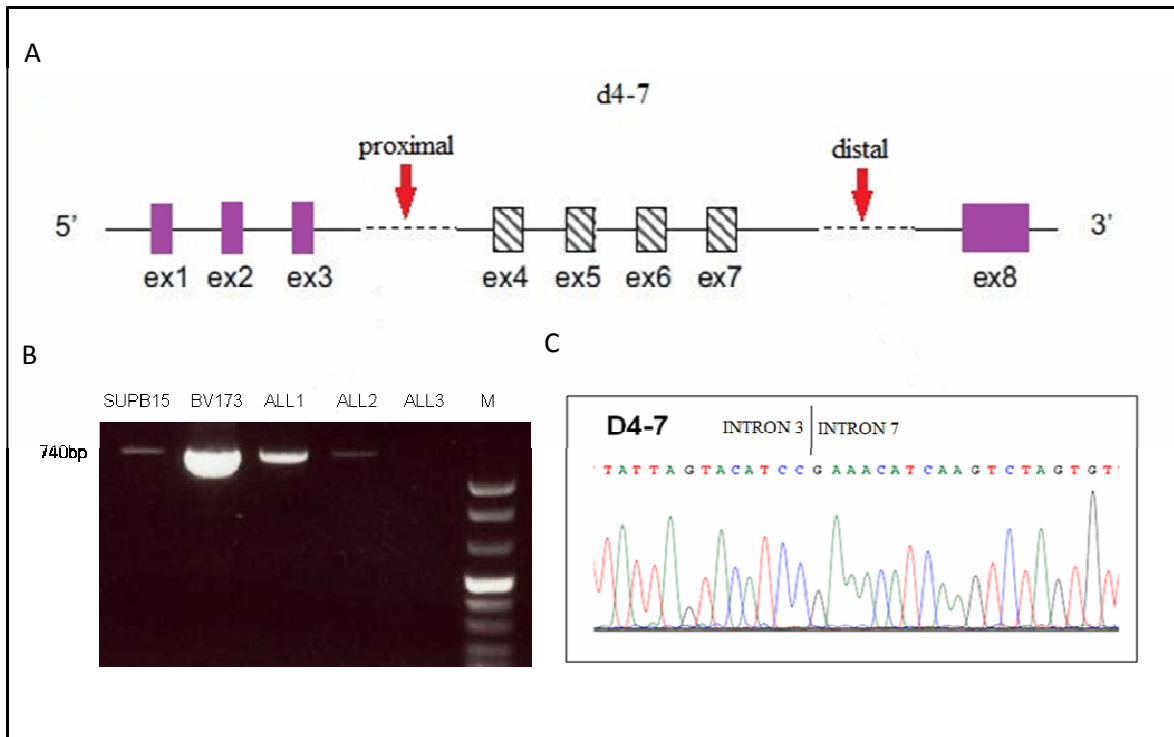

Figure II.4: Detection of *IKZF1* d4-7 (a) Flanking regions for d4-7; (b) agarosis gel showing PCR detection of d4-7 in 2 cell lines and 2 Ph+ patients. One patient is negative for the deletion; (c) Sequencing of 1case positive for d4-7.
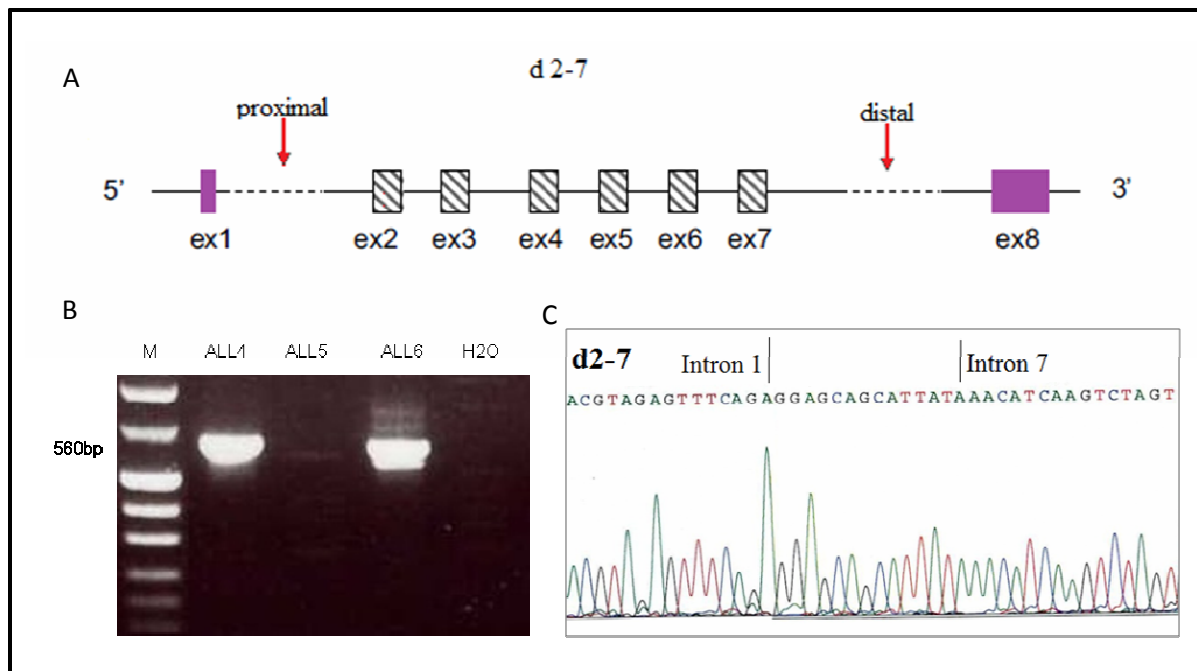

Figure II.5: Detection of *IKZF1* d2-7 (a) Flanking regions for d2-7; (b) agarosis gel showing PCR detection of d2-7 in 2 Ph+ ALL patients; (c) Sequencing of one case positive for d2-7.

The negative cases were re-analyzed by whole genome array and 7 other abnormalities were detected, being 3 cases with chromosome 7 monosomy and 4 rare deletions on the chromossome 7 that started or ended outside the *Ikaros* gene, so they could not be reconized by PCR with the used primers. (Figure II.6)
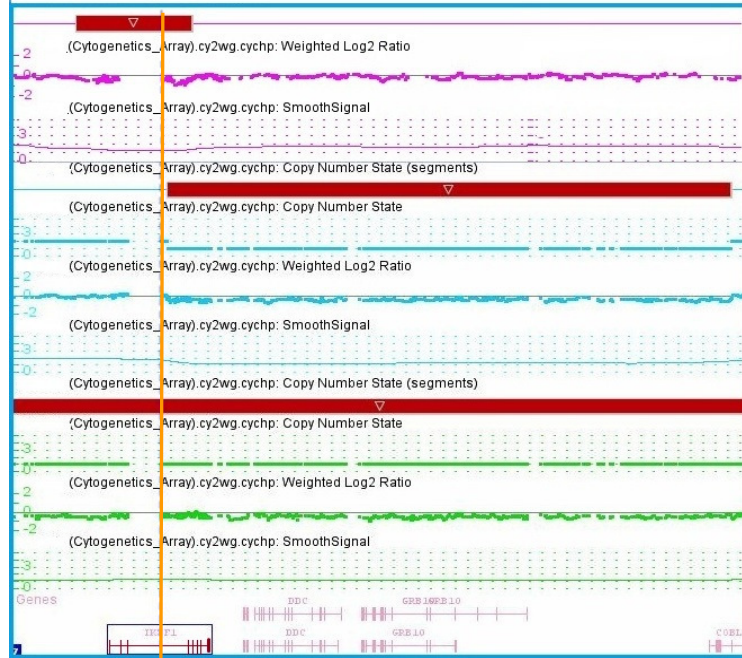

Figure II.6: Rare 7p12 abnormalities detected by SNP array.

The western blot was performed to detect Ikaros isoforms at protein level. Ph+ cell lines were used as controls, since SD1 is not mutated (wild type, wt), BV173 is homogygous for d4-7, SupB15 is heterozygous for the same deletion and Tom1 is heterozygous for d4-8 . SD1 and Tom1 showed expression of only the DNA-binding isoforms, whereas BV173 and SupB15 were positive for Ik6, this last presenting a light band for the wt isoforms. Although a background was present in the run due to the antibody policlonality, Ik1/2 and IK6 were well visualyzed. (Figure II.7)
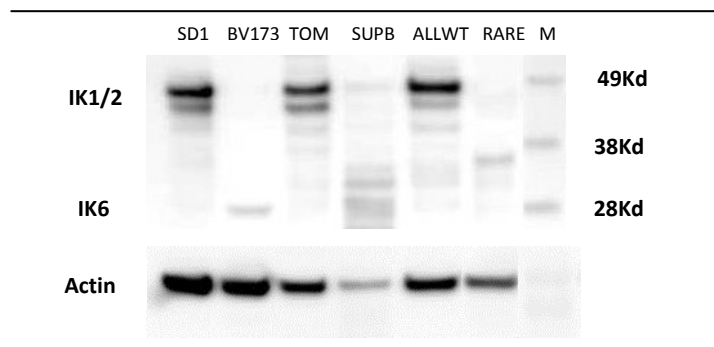

Figure II.7: Wb for Ikaros proteins. Ik1/2, IK6 ans Actin detected in Ph+ cell lines, one patient wild tipe (wt) for the mutation and another with chr 7 monosomy

A total of 67% of the patients had an abnormality on the locus 7p12 (figure II.8) at diagnosis. Our findings from PCR and sequencing analysis clearly demonstrated elevated expression of the Ik6 isoform in patients with Ph+ ALL, associated with a decrease in expression of the functional predominant long isoforms (Ik1 and Ik2).

IKZF1	n	(%)	type	n	(%)
wt	24	32%			
mut	51	68%	D2_7	9*	18%
			D4_7	32*	62%
			D4-8	3	6%
			monosomy 7	3	6%
			del(7)	4	8%

Figure II.8: Frequency of *IKZF1* mutations. * one patient presented both deletions

The relapse samples were analysed and two of the patients that were negative for *IKZF1* mutations at diagnosis presented a mutation at relapse, one the d2-7 and other the d4-7. The positive d4-7 and d2-7 patients had their breakpoint flanking regions analysed for RQ-PCR primers synthesis. For each patient, two or three set of primers were tested and the most efficient primers were used to the absolute quantification of *IKZF1* mutation. A dilution curve (figure II.9) was performed for each case in order to define the sensitivity of detection at diagnosis/MRD points/relapse/new remission. Some of the patients flanking sequences are described at figure II.10.
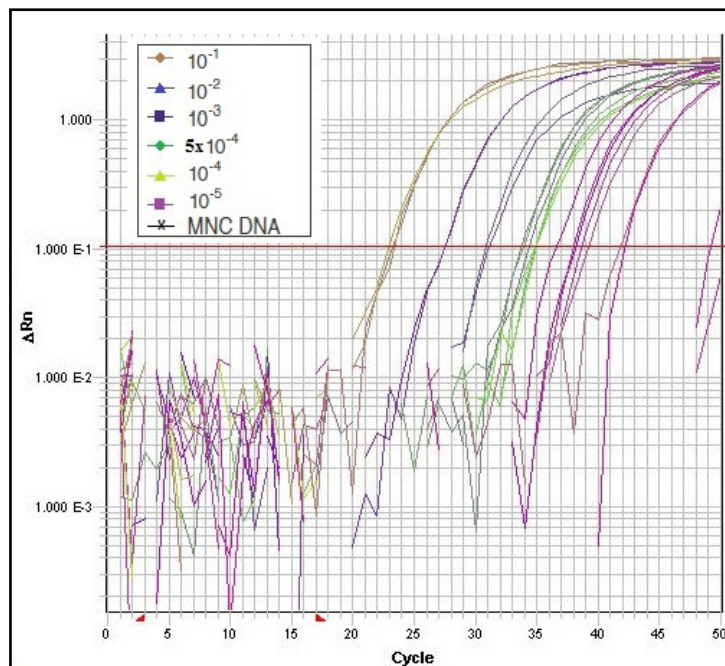

Figure II.9: Sensitivity of IKZF1 RQ-PCR experiment showing maximal sensibility.
The 10^{-5} dilution shows good amplification with reproducible CT values
(difference between replicates <1.5); the maximal sensitivity is 10^{-6}

Figure II.10: *IKZF1* wild-type and deleted sequences detected in Ph+ ALL children

II.4. Discussion

ALL is the most common childhood malignancy, and has long been one of the best characterized tumors at the genetic level.[1] ALL may be of B or T lymphoid lineage, and comprises a group of disorders characterized by recurring chromosomal alterations including aneuploidy (hyper- and hypodiploidy) and chromosomal rearrangements that commonly deregulate hematopoietic transcription factors and tyrosine kinases.[1,2] These alterations are important initiating events in leukemogenesis, and influence treatment outcome, yet usually do not alone cause leukemia in experimental models.[19] The *Ph* chromosome encodes the oncogenic BCR-ABL kinase and defines the subgroup of ALL with the worst prognosis. The reasons for the aggressive nature of Ph+ ALL are still under investigation and have not been elucidated yet.[1,2,15,19]

Recently, mutations on *IKZF1* gene have been highly associated to the presence of BCR-ABL and to a poor outcome in ALL.[11-18] *IKZF1* encodes a protein required for lymphoid lineage differentiation, proliferation, and function.[7,8] Alternative splicing can generate multiple functionally different Ikaros isoforms that lack variable numbers of internal exons.

Isoforms that lack the N-terminal ZnFs are unable to bind transcriptional targets normally but retain the C-terminal ZnFs and the ability to dimerise and act as dominant negative inhibitors of Ikaros function.[9]

Ikaros transgenic and mutant mouse models have clearly demonstrated the important role of Ikaros in both normal hematopoiesis and tumor suppression. Mutant Ikaros -/- mice have severe lymphoid cell defects, but heterozygous Ikaros DN -/- mice invariably develop T-cell malignancies.[4,5]

Recent studies demonstrated that intragenic deletions in the *IKZF1* gene were responsible for the generation of different aberrant Ikaros isoforms associated to ALL.[6,11-19]

In order to identify additional oncogenic lesions involved in the generation of Ph+ ALL that could be working together with BCR-ABL, we analyzed the *IKZF1* mutation status in a group of 76 Ph+ ALL children, diagnosed between 2000 and 2010. We found that homozygous or heterozygous deletions in the *IKZF1* gene are very frequently in our group of Ph+ ALL children (68%), as previously demonstrated by Mullighan *et al.*[11]

Here, we characterized and mapped all breakpoints in *IKZF1* gene, demonstrating that 2 frequent deletions occur in *Ph+ ALL*: d*4-7* (62%), removing exons 4 through 7, and d*2-7* (18%), resulting in a transcript with only exons 1 and 8.

An elevated frequency of genomic aberrations could be directly caused by an abnormally high incidence of DNA double-strand breaks. In normal cells, DNA lesions are detected and repaired by sophisticated physiologic machinery and a system of cell cycle checkpoints, preventing cells that have sustained DNA damage from proliferating further.

In this study, we also identified heptamer RSSs in the flanking breakpoint regions, suggesting that *IKZF1* deletions may arise from aberrant RAG-mediated recombination, as reported previously.[11,12,22] The gene breakpoints and the additional patient-specific nucleotides inserted at the junctions were conserved at relapse and can be used for monitoring MRD.[22] Our RQ-PCR for Ikaros MRD reached a high sensitivity and a prospective patient monitoring will enable *IKZF1* mutation analysis to be used as a prognostic marker. Further studies comparing *IKZF1* mutation status to the overall response will be performed to evaluate its role in therapy resistance.

II.5. Conclusion

In conclusion, our results confirmed, as previously showed by Mullighan et al, [11] that *IKZF1* deletion is present in around 70% of childhood Ph + ALL, and they provided details on breakpoint sequences enabling the use of *IKZF1* mutations for MRD monitoring. It is likely that Ikaros loss combines with *BCR-ABL1* to induce lymphoblastic leukemia, arresting B lymphoid maturation.

Future studies will be important to explain how Ikaros is regulated in normal cells, both at transcriptional and by post-translational levels, in order to clarify its role in leukemogenesis.

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Chapter III. Micro-RNA expression in childhood Ph+ ALL

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III. I. Introduction

MicroRNAs: novel genome regulators

The microRNAs (miRNAs) are a family of small noncoding RNAs that has been increasingly associated to the pathogenesis of malignant diseases.[1] These single-stranded RNAs of 18–24 nucleotides (nt) can regulate many genes through direct biding to complementary sites in the untranslated regions (UTRs) of messenger RNAs (mRNAs) to down-regulate translation and protein synthesis.[2] MiRNA targeting results in either degradation of mRNA or translation repression (figure III.1). [3] Since their discovery in Caenorhabditis elegans by Lee and collaborators in 1993[4], almost 4000 miRNAs have been identified among different species of animals, plants and viruses. By now more than 700 miRNAs have been found in the human genome and they are thought to regulate more than 30% of all human genes.[5] Most of the miRNAs are conserved across species and exhibit tissue-specific expression, suggesting that they help to control critical cellular processes, such as proliferation, apoptosis and differentiation. But miRNAs are frequently located in polycistronic clusters, which are often located in fragile chromosomal regions and are potentially subjected to deletions, amplifications, and other genome-damaging events.[5,6] Deregulation in expression of miRNAs in several diseases and their frequent location in genomic fragile regions supports their relevance to the development of malignancies.[7]
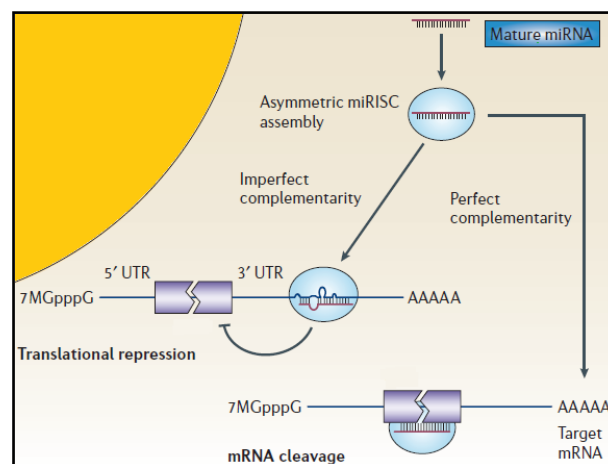
FigureIII.1: miRNA targeting mechanism in the cytoplasm. Adapted from Nature Reviews 2006 [3]

Many studies showed specific alterations in miRNA expression profiles that correlate with particular human tumor phenotypes.[7] In certain cancers miRNAs have been shown to act as tumor suppressors and in others as oncogenes (figure III.2). For example, miR-15a and miR-16-1, negative regulators of the pro-apoptotic protein Bcl-2, are not expressed in half of all B-cell chronic lymphocytic leukemia (CLL) due to the presence of a deletion at 13q14 that codes the polycistronic miRNA cluster.[8] The same happens with let-7 family members, which directly down-regulate the expression of Ras and other proto-oncogenes, and have the expression reduced in lung cancer.[9] On the other hand, some miRNAs can act as oncogenes, as miR-221 overexpressed in melanoma and hepatocarcinoma,[10, 11] or in the case of miR-17-92 cluster and miR-155 increased expression in B-cell lymphomas.[12]
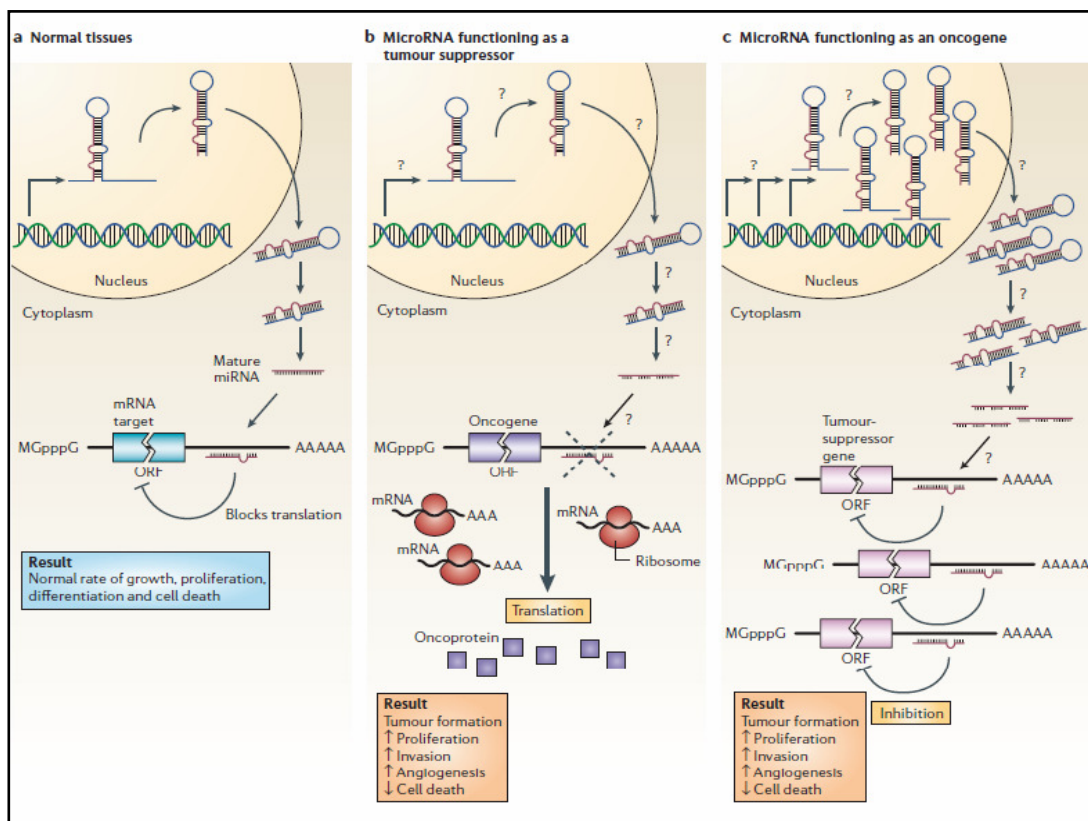
Figure III.2: miRNA in normal and malignant tissues. Removed from Nature Reviews 2006[3]

MiRNAs and acute lymphoblastic leukemia (ALL)

High-throughput studies have experimentally identified functional targets and roles to miRNAs in hematopoiesis.[13] *In vitro* experiments have shown the importance of different miRNA through the differentiation of hematopoietic precursors.[14, 15] To understand their mechanisms of action may help to explain the growing of leukemic cells. MiRNAs have first been related to leukemia in studies with CLL,[16] as mentioned before.

Recently, specific miRNAs have also been associated to different subtypes and prognosis in acute myeloid leukemia (AML). [17]

By now, the results with ALL patients are not very conclusive. This disease, which is the most common cancer in childhood and very aggressive in adults, can commit T or B-lineages and is characterized by several different cytogenetic abnormalities, including *TEL-AML1*, *MYC*-related translocations, *TAL1*, E2A-PBX; 11q23 translocations (*MLL*); *BCR-ABL*, and others.[18]

The first miRNA expression abnormalities characterized in ALL was performed by Zanette *et al.*,[19] comparing samples of 7 ALL patients grouped by cell lineage and genetic abnormalities to CD19+ normal cells. ALL showed 5 upregulated miRNAs and 5 downregulated miRNAs (Table III.1).

Later, the miR-17-92 cluster was found to be upregulated also in ALL, as previously reported for some types of lymphomas, and was shown by Nagel *et al.* to repress E2F1 in T-lineage ALL.[20]

Mi *et al.* compared the miRNA profile expression in B-lineage ALL, all with 11q23 translocations, to AML that carried some recurrent cytogenetic abnormalities and to normal bone marrow cells. They found differential expression of 27 miRNAs in ALL, with 6 upregulated and 21 downregulated (Table III.1). They proposed that the deregulation of the 4 most important miRNAs could be used to distinguish ALL and AML leukemias, with miR-128a and miR-128b indicating ALL, and let-7b and miR-223 indicating AML. [21]

More recently, Fulci *et al.* compared miRNA expression between T-cell and B-cell lineages ALL. They performed a clustering analysis of several adult ALL (without known molecular abnormalities). Statistical analysis showed several genes differentially expressed between both groups. [22] (Table III.1)

Schotte et al. showed that 14 microRNAs were upregulated and five were downregulated in ALL compared with normal CD34+ cells (table III.1). The authors also showed that MLL-related ALLs presented an additional subset of deregulated miRNAs compared to other ALL subtypes, including upregulation of miR-196b and downregulation of miR-708 and let7-b. [23]

The same group recently published the association of 14 of these miRNAs with good prognosis in pediatric ALL and reported the let7-b downregulation in MLL-leukemia patients to be linked to the upregulation of the oncoprotein c-Myc. [24]

In a study with Ph+ adult patients Bueno *et al.* showed that miR-203, which directly targets ABL, is lost in many cases of BCR-ABL-related leukemias by genomic instability and CpG methylation. CML, Ph+ALL and Ph+AML presented low levels of miR-203 compared to

normal tissues and the authors showed that the use of demethylation agents in Ph+ cell lines can re-establish the miRNA expression and induce cell-death. [25]

Finally, two different groups reported cases of ALL containing a translocation involving the complete miR-125b sequence fused to the IGH gene locus, causing overexpression of this miRNA that could imply in the leukemogenesis of precursor B-ALL cells.[26,27] Subsequent *in vivo* studies from Bousquet *et al.* showed that miR-125b overexpression promotes malignant transformation of different hematopoietic lineages. (Table III.1)

The authors also showed cooperation between miR-125 and BCR-ABL, once both co-expression increases leukemic cell-proliferation in mice.[28]

Taken together, these results begin to identify miRNA signatures that are distinctly expressed in different ALL subtypes. However, further clarification is needed, as only some subtypes have been characterized by multiple studies, and few have concretely shown an impact on translational regulation of target genes. The relevance of these classifications to prognosis also remains to be determined.

The most recent results suggest that the miRNA may play an important role in Ph+ leukemia pathogenesis. Since there is a huge heterogeneity inside Ph+ ALL children implying in different therapy responses, there is still a lot to be studied to understand and fight against this terrible disease. It is known that treatment outcome is associated to response to the pre-phase treatment with prednisone and intrathecal methotrexate, and to the minimal residual disease (MRD) evaluation. Patients with prednisone good response (PGR) and standard risk MRD (SR) had a significantly lower risk of treatment failure compared to those with prednisone poor response (PPR) and high risk MRD (HR). [29]

In order to find additional genetic lesions that could explain these biological differences among Ph+ ALL children, we investigated the miRNA expression profile in two different groups of patients based on the first response to therapy. Understanding the miRNA role into aggressiveness will be relevant both to awareness of the resistance mechanisms to current targeted agents for Ph[+] ALL and to design novel therapies that can increase survivor in the future.

Subject	Compared	Upregulated	Downregulated	Reference
ALL	CD19+ normal	miR-128b, -204, -218, -331,-181b	miR-135b, -132,-199, -139, -150	Zanette et al.[19]
T ALL	other cancers	miR-17-92 cluster		Nagel et al.[20]
ALL	normal CD34+	miR-128a, -142-3p, 142-5p, -150, -151-5p, - 181a, -181b,-181c, -193, -30e-5p, -34b, -365, -582, -708	miR-100, -125b,-99a, -196b, let-7e	Schotte et al.[23]
t(11q23) ALL	AML	miR-128a, - 128b,-130, -151, -210, -1	miR-223, -125a, -221, -222, -23a, -23b, 24, - 27a, -27b, -199b, -26a, -335, -21,-22, -424, - 451, let-7a, let-7b, let-7c,let-7e	Mi et al.[21]
B ALL	T ALL	miR-151	miR-148a and miR-424	Fulci et al. [22]
t(11q23) ALL	other ALL	miR-196b	miR-193, -151-5p,-30e-5p, -34b, -582, -708, let-7e	Schotte et al.[24]
Ph+ leukemias	Ph-		miR-203	Bueno et al.[25]
IgH translocated /BCR-ABL ALL		miR-125b-1		Bousquet et al.[28]

Table III.1: Literature review - miRNA and acute lymphoblastic leukemia

III.2. Patients and Methods

III.2.1. Ph+ALL in study

Between September 2000 and September 2010, a total of 78 patients younger than 18 years were diagnosed as Ph+ ALL in Italy and enrolled in the high risk group of AIEOP-ALL protocol (later EsPhALL). The diagnosis was made on the basis of morphologic, biochemical and immunologic features of the leukemic cells, followed by cytogenetic analysis and RT-PCR for BCR-ABL identification. Two groups of patients were chosen for this study: PGR/non HR MRD (including those SR or MR) vs. PPR/HR MRD (n=10 vs. 10), as shown in table III.2. In addition, the human Ph+ cell lines SD1, TOM1, SUPB15 and BV173 were included in the analyses. The cell lines were obtained from DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and maintained in culture following DMSZ recommendations.

Patients' diagnosis bone marrow aspirates were processed and mononuclear cells were obtained after centrifugation on a Ficoll–Hypaque gradient, having their genetic material extracted as described ahead.

NI	Age (years)	WBC	MRD risk	pdn_resp	Fusion protein
1	9,5	14300	SR	PGR	p190
2	13,2	115050	MR	PGR	p210
3	6,9	NA	MR	PGR	p190
4	10,4	1440	MR	PGR	p190
5	4,0	63500	MR	PGR	P190
6	14,3	48600	MR	PGR	p210
7	8,3	17670	MR	PGR	p190
8	14,1	26700	MR	PGR	p190
9	9,5	34610	MR	PGR	p190
10	13,6	144100	MR	PGR	p210
11	13,9	212000	HR	nk	p210 + p190
12	10,7	104000	HR	PPR	p210
13	13,9	307000	HR	PPR	p210
14	3,0	106000	HR	PPR	p190
15	13,9	NA	HR	PPR	p190
16	7,0	10800	HR	PPR	p210
17	12,5	259000	NA	PPR	p190
18	3,0	580400	HR	PPR	p210
19	8,2	NA	HR	PPR	p190
20	3,2	146900	NA	PPR	p190

Table III.2: Two groups of patients in study based on their prednisone response and MRD evaluation.

NA= not available/nk=not known

III.2.1. RNA extraction and microRNA microarray experiments

A. miRNA array

The total RNA was extracted following standard procedure protocol using guanidinium isothiocyanate[30] , quantified using a NanoDrop spectrophotometer, and quality was assessed by 0.8% agarose gel electrophoresis. Briefly, 700ng of total RNA were reverse-transcribed with the megaplex RT primers human pool A (Applied Biosystems). This pool contains specific stem-loop primers for 377 human miRNAs, 3 small RNAs, and 1 negative control, and are all based on miRBase [31]. The resulting cDNA was transferred to a TaqMan Human MicroRNA A Array v2.0 (Applied Biosystems) and QPCR was performed on an Applied Biosystems 7900HT Sequence Detection system. Cycling conditions were 50°C for 2 min, 94.5°C for 10 min, and 45 cycles of 97°C for 30 s and 59.7°C for 1 min. Quantification cycle values (Cq; standard name for Ct or Cp value) were recorded with SDS version 2.3 software.

Cq values ≥40 were considered beyond the limit of detection (a Cq value of 39 represents a single molecule template detection) and were removed.

b. Data analysis

Raw data extracted from SDS files were normalized using Stat Miner v4.2 (Integromics). The GCRMA procedure was used for background subtraction, $\log_2$-transformation and a second normalization on Microsoft excel using BRB Array-Tools (available at http://linus.nci.nih.gov/BRB-ArrayTools.html). MiRNA differentially expressed between the two-groups were identified by the Significance Analysis of Microarrays (SAM, available at http://www-stat.stanford.edu/,tibs/SAM/ index.Html). Array data were clustered using Cluster 3.0. and visualized using Java TreeView 1.0.

III.2.2. Bioinformatics for target prediction

MiRNA target prediction of the differentially expressed miRNAs was performed by using TARGETSCAN(http://www.targetscan.org),MIRANDA(http://www.microrna.org/microrna/home.do), and PICTAR (pictar.bio.nyu.edu).

III.2.3. Quantitative Real-Time Polymerase Chain Reaction

a. MiRNA single assays

MiRNAs differently expressed between the two groups were re-analyzed using single assays specific for each significant miRNA. Quantitative real-time PCR assay for miRNAs were performed using TaqMan MicroRNA assays (Applied Biosystems). All the reagents for performing reverse transcription reaction and the real-time PCR assays were specific to mature miRNAs. The reverse transcription reaction (RT) and real-time PCR were carried out using the ABI Prism 7900 HT Sequence Detector System (Applied Biosystems). Total RNA previously extracted was synthesized into miR-cDNA using RT-mirRNA kit (Applied Biosystems) following the manufacturer's instructions. Briefly, 250 ng of total RNA in 7.5 µl of final volume containing 50nM of stem-loop primer, 1µl RT buffer, 3.33 U/ml MultiScribe reverse transcriptase and 0.25 U/ml RNAse inhibitor. The mix was incubated at 16^0C for 30 min, 42^0C for 30 min, 85^0C for 5 min and then held at 4^0C. Q-PCR was performed using a standard TaqMan PCR protocol. The 10µl PCRs reactions included 2 µl of RT product, 6 µl Universal TaqMan Master Mix and 2µl TaqMan probe/primer mix.

The reactions were incubated in a 96-well plate at 95^0C for 10 min followed by 45 cycles of 95^0C for 15 s and 60^0C for 1 min. All PCR reactions were run in triplicate and RNU-48 was used as endogenous control.

b. Target mRNA expression

In addition, target mRNAs expression was measured by RQ-PCR using Universal Probe and primers from Roche following their instructions. Briefly, 0.5 µg of total RNA were reverse transcribed using 200 unities of Superscript II enzyme (Invitrogen), with random hexamers (5 µmol/µl) and dNTP (1 mmol/µl), in a final volume of 20 µl, at 42^oC for 45 min. The PCR was performed in a final volume of 25 µl containing 2 µl of cDNA, 400 nmol/µl specific primers (p27, p57, Kit, Jak), 200 µmol/µl of dNTP, 1U of Taq polimerase enzyme and 1U specific Universal probe (both from Roche Diagnostics). PCR temperatures and cycle times were: 95^oC for 30 s, followed by 35 cycles at 94^oC for 30 s, 65^oC for 60 s, 72^oC for 60 s.

c. Statistical analysis

The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.[32] TaqMan CT values were converted into absolute copy numbers using a standard curve with the median of expression from the good responders group. Fisher's exact test, t-test, and chi-square were used to compare baseline clinical features and average miRNA expression between the two groups of patients. All reported *p* values were 2-sided and obtained using the SPSS software package (SPSS 15.0, IBM). Some graphics were performed using GraphPad prism software (www.graphpad.com).

III.2.4. miRNA up/down-regulation

The Ph+ cell lines described were used for *in vitro* assays designed to knock-down/overexpress the miRNA using different transfection vectors.

a. Liposome vectors

The antagomirs are molecules with anti-sense sequences against the interest miRNA capable to inhibit its synthesis. Anti-miR 221/222 share seed sequences and are capable to reduce both miRNA expressions. Following literature advices we used both molecules together to increase inhibition efficacy. The liposome vectors carrying 100ng of locked nucleic acid antagomiRs (LNA-siRNAs, Exigon) were gently provided by Dr Maria Silvia Sesana, from Professor Masserini's group (Department of Experimental Medicine, Milano-

Bicocca University). Fluorescein isothiocyanate (FITC) formula of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP): 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in a ratio of 1:1 (w/w) carrying LNA molecules where added to TOM1 cells in order to analyze miR-221/222 downregulation effects. A million cells were cultivated in triplicate on 6-well plates on the presence of lip-anti221, lip-anti222, lip anti221+222, lip-scrambled and empty liposomes. After 24, 48, 72 and 96 hours cells were counted using trypan blue (BD Biosciences), analyzed by FACS (to measure efficacy of liposome transfection), the miRNA was extracted and evaluated by RQ-PCR and target proteins were searched by western-blot, as described ahead.

b. Nucleoporation

ALL Ph+ cell lines were transfected using Basic Nucleofector kit using Amaxa Nucleofector II device (Amaxa Biosystems). MiR-125b-2 mimics and controls were obtained from Ambion and LNA-based miR-221/222 inhibitors and controls were obtained from Exiqon. To validate the best solution and program for our cells a test with the Amaxa optimization kit was performed, according to manufacturer's instructions. Once we found the best settings, a total of 5 million of Tom1/SupB15/BV173 and SD1 cells were nucleoporated using Amaxa (Solution V, Program X001) with 5 µg of precursor oligonucleotide and 0.5 µg green fluorescent protein (GFP) plasmid, in a total volume of 10 ml. Transfected cells were cultured into t25 plates cells for 24, 48 and 72 hours, then were counted using the ViCell counter (Beckman Coulter) and analyzed by FACS for viability and GFP expression. Remaining cells were tested for miRNA expression or tested for proliferation by MTS assay, as described ahead.

c. Lentiviral vector production and transduction

The pre-miR-125b lentivirus and the miRzip-221/222 are immunodeficiency virus-based construct purchased from Systems Biosciences (SBI). The first construct consists on the stem loop structure of *miR-125b* and 300 bp of upstream and downstream flanking genomic sequence cloned into the lentimiR plasmid (figureIII.3.a) that results in the synthesis of the pre-miR-125b, which will be processed by cellular machinery and originate both miR and miR* isoforms. The miRzip vector is based upon SBI's pGreenPur™ lentivector (figure III.3.b) an improved third generation of HIV-based expression lentivector. Packaging of lentimiR and

miRzip constructs in pseudoviral particles was performed using the pPACKH1 packaging plasmid mix, according to the manufacturer's instructions (SBI). Both vectors express cop-GFP, a GFP-like reporter that express a stronger fluorescent signal.

HEK293T cells were maintained in Dulbecco's Modified Eagle Media (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and a mixture of antibiotic–antimycotic. 6.0×10^5 HEK 293T cells were plated on t75 plates in 10 mL of DMEM/10% FBS without antibiotics and were transfected at 80% for production of the lentiviral vector. After 48 hours the virus was precipitated and concentrated overnight using PEG-it (SBI) at 4^0C.

Target ALL Ph+ cells were plated in 48-well plates with virus and polybrene (hexadimethrine bromide, Sigma–Aldrich) at a final concentration of 5 µg/ml. Cells were incubated at 37°C in a humidified atmosphere for 24 hours, after which medium containing the virus library was replaced with RPMI (Invitrogen) with 10% FBS and antibiotics. Infected cells were monitored for cop-GFP by fluorescence microscopy, expanded and sorted by FACS. Lentivirus rapid titer PCR Kit (SBI) was used to measure the copy numbers and determine the multiplicity of infection to perform experiments. Empty lentivirus was used as a control for the experiments.
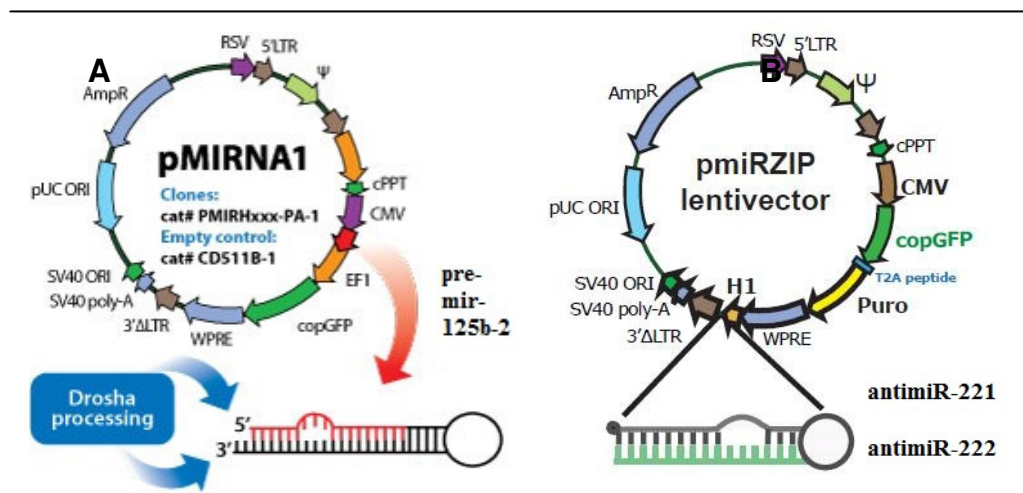
Figure III.3: lentivirus vectors for miRNA (a) lentimiR for miR-125b and (b) miRzip for miR-221 and 222. Adapted from SBI website

III.2.5. *In vitro* ALL treatment

ALL Ph+ cell lines were treated with chemotherapy drugs to evaluate miRNA deregulation effects. The drugs tested were Prednisone (Sigma-Aldrich), Dexamethasone (Sigma-Aldrich) and Gleevec (Novartis). A curve for toxicity was performed using a million cells per well

searching the concentration capable of generating 50% of mortality. Cells were counted and viability was measured by an apoptosis assay, as described below.

III.2.6. Apoptosis assay

Apoptosis was evaluated by FACS for the detection of annexin V (Biolegend)/ propidium iodide (PI, Sigma) positive cells. Transfected and treated cells were labeled with annexin V conjugated with FITC and PI. Briefly, cells were washed once in PBS and once in 1x binding buffer, then 5 µL of annexin V-FITC and 1 µL of PI were added to the cells. Cells were incubated at room temperature for 15 min, after which 400 µl of 1x binding buffer was added and cells were analyzed by FACS. Early apoptotic cells were defined as weakly annexin V positive and PI negative, while late apoptotic cells were those strongly annexin V positive including those cells that were both annexin V and PI positives.

III.2.7. Proliferation assay by incorporation of 3H thymidine

The number of viable cells in proliferation was measured with a MTS/PMS assay (Cell Titer MTS, Promega). Twenty-four hours after transfection, 20×10^3 cells were seeded into 96-well culture plates for 24, 48, 72 and 96 hours. The cells were then incubated with 20 µL of MTS for 4 hours at 37℃. The optical density was determined with a spectrophotometer (Spectra Max M2, Molecular Devices Corporation) at a wavelength of 490 nm. Each experiment was performed in quadruplicate.

III.2.8. Cell cycle assay

For cell cycle analysis, 48 hours after transfection, cells were pelleted at 1000 rpm for 5 min and washed with 10ml of PBS. Then 5µl of PI (Sigma) and 1µl of RNAseA (Sigma) were added to the cells and samples were analyzed 30 min after staining with the use of flow cytometry -BD FACSCalibur (BD) and CellQuest software.

III.2.9. Western blotting

A western blotting (wb) for the proteins that have been previously reported as targets of our miRNAs was performed in collaboration with the group of Dr Marina Pitto, from the Proteomic

laboratory at the department of Experimental, Environmental Medicine & Biotechnology of Milan-Bicocca University. Patients' cells/cell lines were lysed with Ripa buffer (Sigma). Cell lysates (5×10^6 cells/250 μl) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels and then transferred to nitrocellulose membranes (Amersham Biosciences). The blots were incubated for 60 minutes in blocking buffer before incubation overnight at 4°C with anti-target antibody a-p27. P57, Kit, Jak (Santa Cruz Biotechnology), followed by one hour of incubation with the specific secondary antibody. Blotted proteins were detected and quantified using the image system KODAK.

III.2.10. In vivo experiments

a. Subcutaneous injection

Animal studies were performed according to Ohio State University institutional guidelines. Different concentrations of viable SD1/SupB15/Tom1 and BV173 cells were injected subcutaneously into the back of 5-week-old female NUDE mice (Charles River Breeding Laboratories). Once the best cell-concentration was determined, cells were injected into both flanks of new mice. The tumors size was measured daily until the tumor reached 50 mm^3. Then, three different experiments were performed: (a) 5 mice received miR-125b in one flank and scrambled (scr) in the other, (b) 5 mice received miR-221+222 in one flank and scr in the other and (c) 5 mice received pbs in one flank (mock) and scrambled (scr) in the other. A total of 5 μg of synthetic miR-125b-2, LNA-anti221+222 or scrambled oligonucleotides diluted in Lipofectamine 2000 (Invitrogen) solution (100 μL total volume) were injected directly into the tumors (right flank = miR-125b-2 or miR-221+222 or mock; left flank = scrambled) at baseline and after 3, 7, and 10 days (figure III.4). Tumors were measured with caliper on the day of the injections and 4 days after the last injection. At that time, the mice were killed, necropsies were performed, and tumors were weighted. Tumor volumes were calculated using the equation V (in mm^3) = A × B^2/2, where A is the largest diameter and B is the perpendicular diameter.
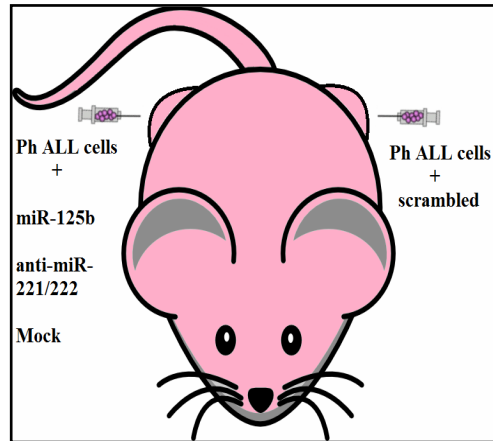
Figure III.4: Nude mice received subcutaneous injection of Ph+ cell lines.
After tumor formation were treated with miRNA molecules

b. Intraperitonial injection

A total of 50 million viable SD1/SupB15/Tom1/BV173 cells were injected into the peritoneum of 5-week-old male NOD/SCID mice (Charles River Breeding Laboratories). Mice conditions were observed daily for over a month and after death the organs were analyzed to check leukemia presence. MiRNA deregulation experiments were not performed yet.

III.3. Results

III.3.1. miRNAs related to preliminary response in Ph+ALL

The miRNA analysis by array cards revealed eight miRNA genes differentially expressed between the two groups of Ph+ patients separated according to the first response to therapy (figure III.5). Their significant different expression was confirmed by single assays experiments: miR-221, miR-222, miR-222*, miR-125b and mi-125b-2* were overexpressed in the PPR/HR group (figure III.6), whereas mi-135b, miR-27a, miR-193a and let-7c were overexpressed in the PGR/SR group (figure III.7).

Figure III.5:

(a) MDS for miRNAs expression according to preliminary response

PGR/SR-MR   PPR/HR

(b) Heatmap showing significant miRNAs differently expressed
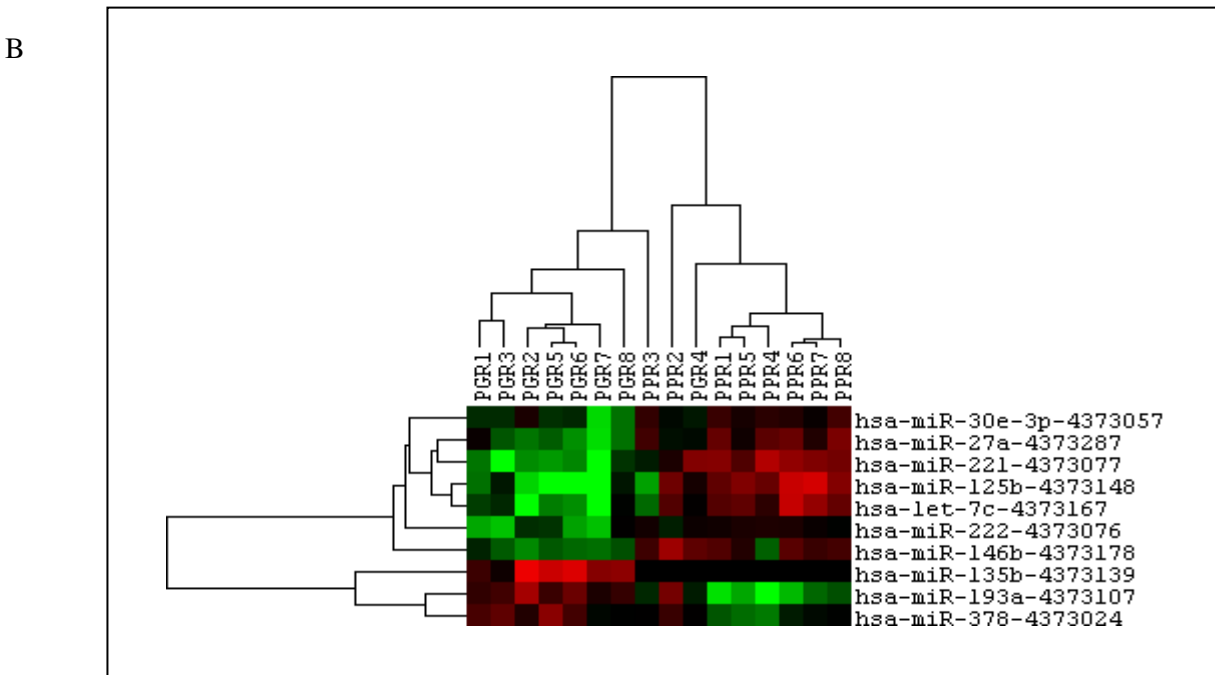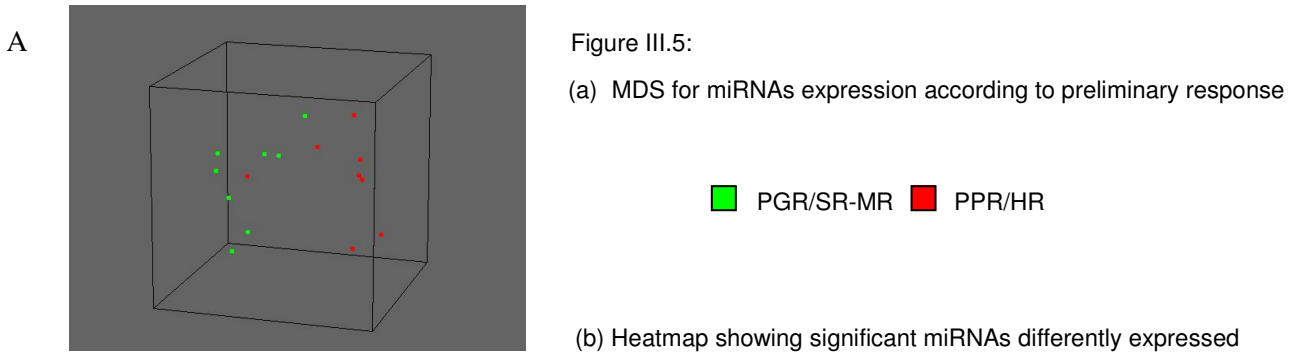
C

	Parametric p-value	t-value	% CV support	Geom. mean of intensities in class 1	Geom. mean of intensities in class 2	Fold-change	Unique id
1	0.0001386	-5.184	100	1325.9	4969.61	0.27	hsa-miR-27a
2	0.0008965	-4.197	100	685.93	4569.92	0.15	hsa-miR-221
3	0.0017322	-3.86	100	140.98	1461	0.096	hsa-miR-125b
4	0.003437	-3.514	100	411.35	905.26	0.45	hsa-miR-30e-3p
5	0.0034399	-3.514	100	91.08	533.11	0.17	hsa-let-7c
6	0.0035999	-3.491	100	5186.04	14485.17	0.36	hsa-miR-146b
7	0.0036541	-3.483	100	18760.5	46931.68	0.4	hsa-miR-222
8	0.0089578	3.032	38	195.46	96.11	2.03	hsa-miR-378
9	0.0020858	3.766	100	883.64	197.71	4.47	hsa-miR-193a
10	1.12e-05	6.639	100	25.81	1.99	12.97	hsa-miR-135b

(c) Data analysis of miRNA array in BRB after Stat Miner normalization.

Class 1 = Good responders group
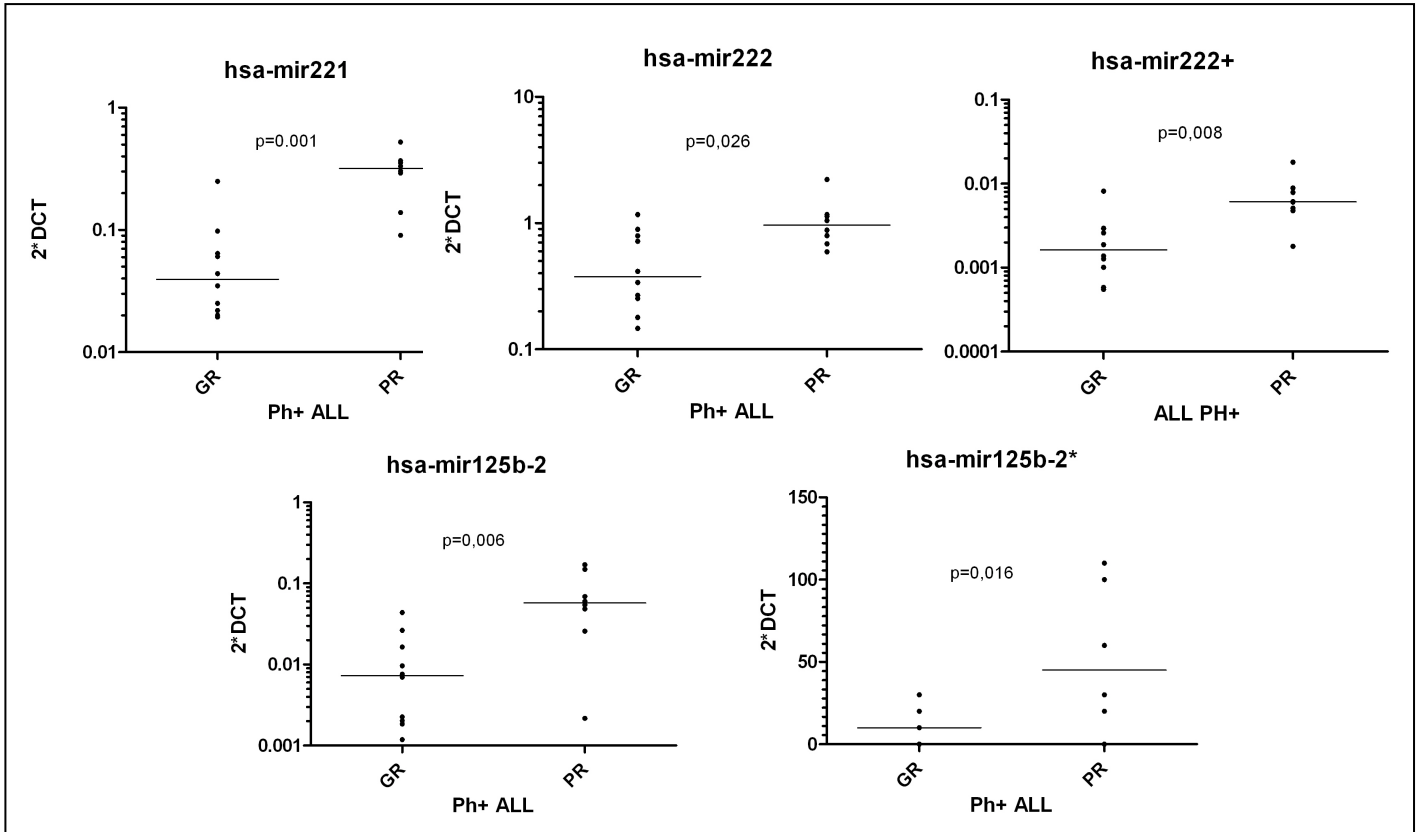
Class 2= Poor responders group

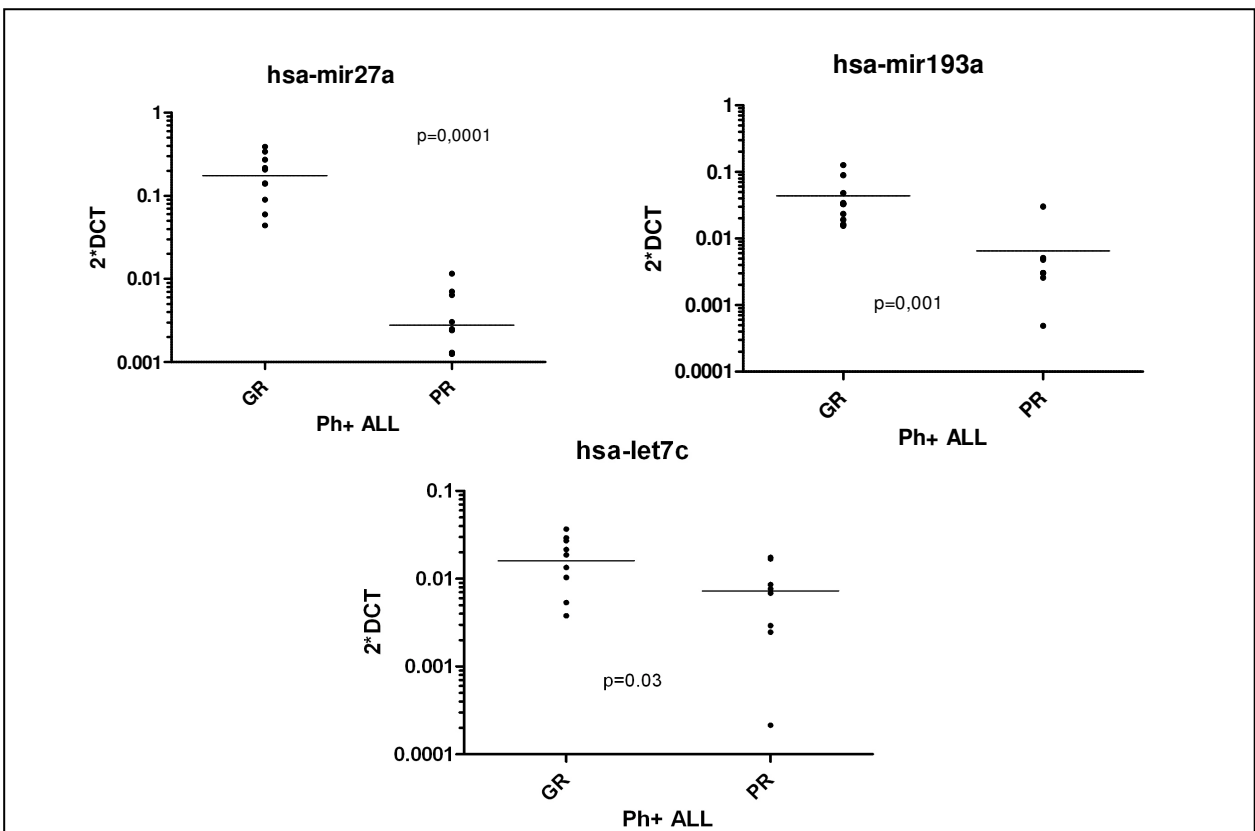Figure III.6: miRNAs overexpressed in Ph+ ALL PPR/HR

Figure III.7: miRNAs downregulated in Ph+ ALL PPR/HR

The miRNA target prediction and literature review revealed important targets for the miRNA found (figure III.8).

miRNA	chrom	localization	cluster	Important targets
221	X	intergenic	222, PAX3	
222	X	intergenic	221, PAX3	p27,p57,c-KIT [33, 34,]
222+	X	intergenic	PAX3	
125b	125b1- 11	intergenic	PAX4, GATA1	
	125b2- 21	intronic		p53, BAK1, MAPK3, RAF1[35, 36]
125b-2+	125b1- 11	intergenic	PAX4, GATA1	
	125b2- 21	intronic		IKZF1
135b	1	intronic		JAK2, PIK3CA, HRAS, RAF1[37]
miR 27a	19	intergenic		Oncogenes [38]
let7c	21	intronic		Oncogenes [39]
193a-5p	17	intergenic	PAX4b	Oncogenes

Figure III.8: miRNA target prediction and literature review

III.3.2. MiRNA expression in Ph+ cell lines

Liposome vector for miR-221/222 knock-out

The most significant miRNAs overexpressed in the PPR/HR group were miR-221 and 222 which are located in the same cluster and share seed sequences. These two miRNAs were reported to be associated to the worst therapy response in different cancers. [33, 34, 39] We developed an *in vitro* knock-down experiment in order to test whether overexpression of these miRNAs was responsible for chemotherapy resistance and aggressiveness.

LNA-anti-miR-221/222 liposome vectors were used to transfect TOM1 cell line (which over express both miRNAs). After 48 and 72 hours of treatment the cells reached over 80% of silencing (figure III.9).
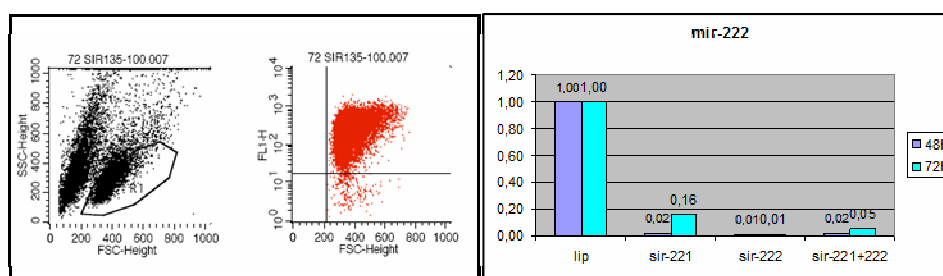

Figure III.9: miRNA Knock-down with lipossome vectors
(A) Facs showing fitced cels after 72hs (B) RQ-PCR for miR-222

No increase of mortality or proliferation by miR-221 and 222 were observed in the presence or absence of LNA. The published miR-221 or 222 targets, p27, p57 and c-KIT, were tested by western blot but no significant changes were seen after miRNAs silencing (figure III.10a, b). This could be related to the decreasing levels of miRNA inhibition after 4 days of culture (figure 6c). The mechanisms by which the mRNA targets are inhibited are unknown. If in TOM1 the miRNAs act degrading the mRNA targets, we do not know how long after miRNA silencing the cell starts to synthesize these targets again, or after how long the cellular effects of target inhibition can be seen.
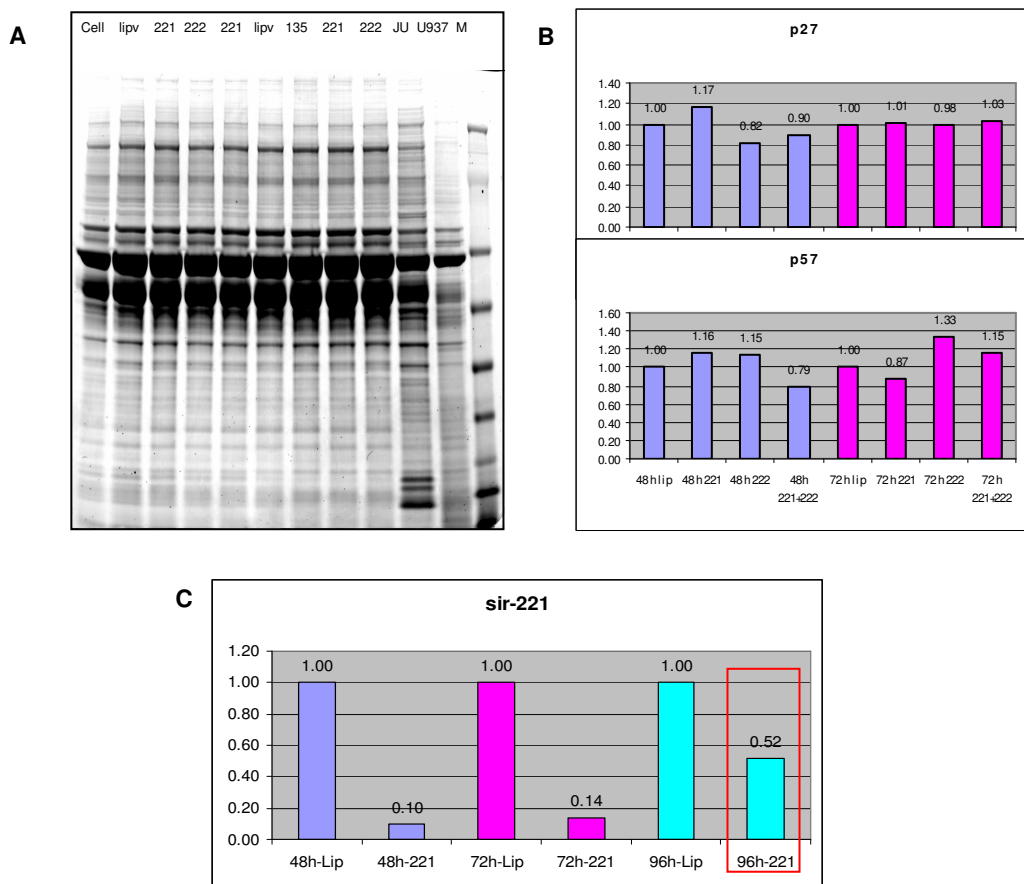
Figure III.10: (A) monodimensional gel for overall protein expression;
(B) wb quantification of p27 and p57;
(C) mi-221 expression after 48, 72 and 96hs of silencing

Nucleoporation using Amaxa system

Bettering order to improve the transfection assay, we performed nucleoporation with the Amaxa kit. Two groups of miRNA were found as the most significant differentially expressed: (1) miR-221, -222 and 222*, and (2) miR-125b-2 and 125b-2*.

In patients PPR/HR there is a high expression of miR-221,222,222*, 125b and 125b*. Differently, the Ph+ ALL cell lines in study express high levels of miR-221,222 and 222* but do not express miR-125b and 125b*.

In order to test these miRNA effects on restoring chemosensitivity in the presence of anti ALL- drugs in our cell lines, we knocked down the expression of miR-221,222 and 222* and overexpressed miR-125b and 125b*. In a preliminary experiment we optimized the solution and program for nucleoporation by using an optimization kit. Cells were tested with different programs according to the manufacturer's indications. Solution V and program X-001 were the most efficient, expressing 43% of GFP after 24hours (Figure III.11).
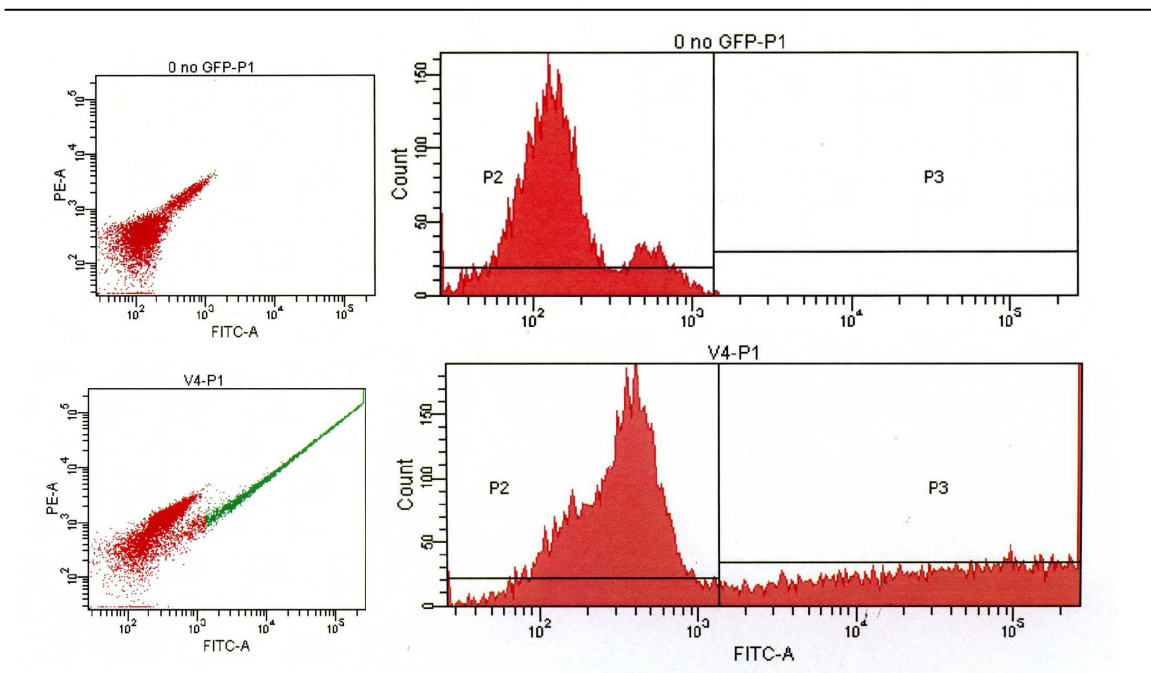
Figure III.11: GFP expression on SupB15 cells in the control sample (P3=0%) and with V solution + X001 program (P3=43%)

All the four Ph+ cell lines were transfected only with GFP plasmid and tested for delivery efficiency and viability (on the cell counter) using the same optimized conditions. GFP transfection in Tom1 and SupB15 reached 43% of efficiency, with a viability of 91 and 76% respectively. In the other cell lines the transfection was not so efficient, in BV173 cells the GFP reached 15% of the cells with 83% viability, while in SD1 line the GFP got only in 1.7% of the cells, with 84% of viability. (Figure III.12)

	Viability % (cell counter)		GFP expression % (FACS)	
A (chart area)				
	TOM empty	TOM GFP	TOM empty	TOM GFP
	72	91	0	43
	BV173 cell	BV173 GFP		BV173 GFP
	77	83	0	15.8
	SD1 cell	SD1 GFP		SD1 GFP
	86	84	0	2
	SUP cell	Sup GFP		Sup GFP
	70	76	0	43.1
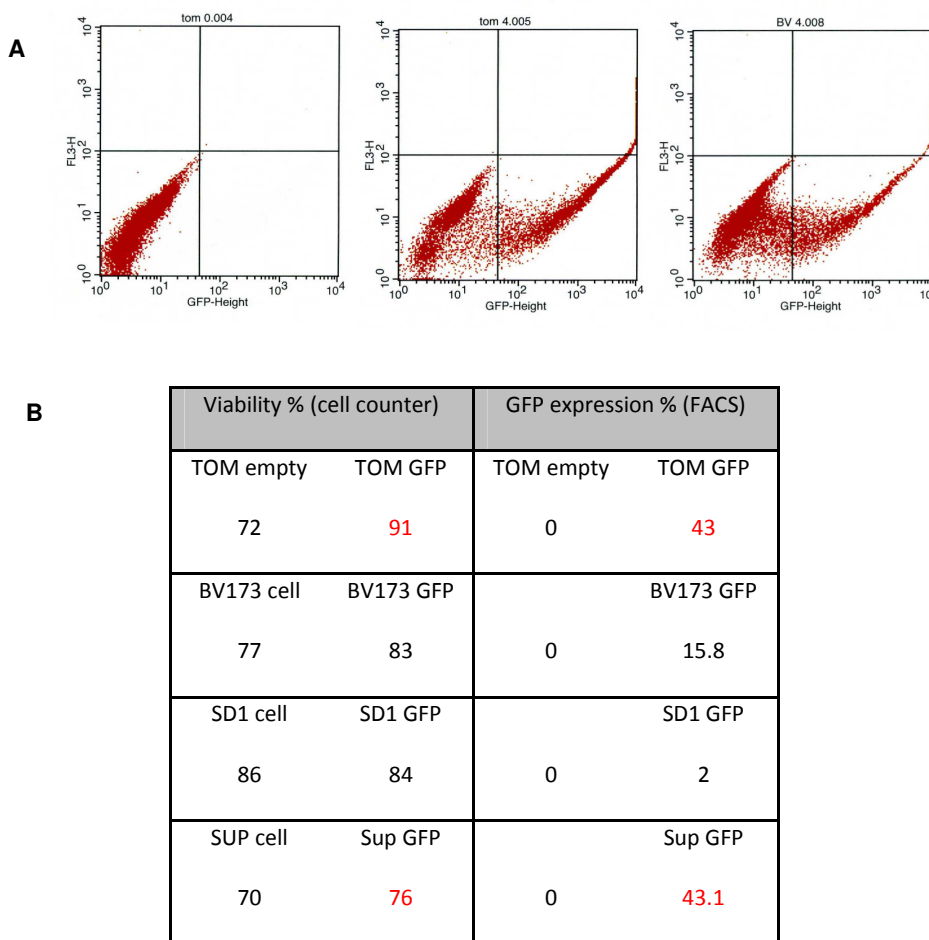
Figure III.12: Nucleoporation in Ph+ cell lines. (A) FACs for Tom 1 and BV173 after Amaxa
(B) Viability and GFP % expression on all transfected cell lines

After the GFP transfection assay, the miRNAs knock down and up-regulation were tested. It was expected to have better transfection rates using the oligos carrying the pri-miR and the antagomiR since they are easier to get into the cells than the GFP plasmid. An experiment using two different concentrations of anti-miR-221 (50 and 100 nM) was performed. SupB15 and SD1 cells had a good miRNA knock down (99 and 86% of inhibition, respectively) while the TOM1 and BV173 had lower inhibition rates (70 and 46), as seen in figure III.13. The viability was evaluated on the cell counter and it was always more than 70%, as shown in the figure III.14.

Figure III.13: RT-PCR showing mi-221 knock down after anti-221 transfection using Amaxa nucleoporation kit

TOM1	viability (%)	Cell count (million/mL)	mi-221 inhibition (%)
Scr	72	0,62	100
50 nM	91	0,8	57,00
100 nM	82	1	70,00
BV173			
Scr	77	0,7	100
50 nM	83	0,7	16
100 nM	75	0,5	46
SD1			
Scr	86	0.9	100
50 nM	84	1,1	85
100 nM	77	0,9	86
SUPB15			
Scr	70	0,72	100
50 nM	76	0,71	95
100 nM	83	0,7	99

Figure III.14: viability, cell count and % of mi-221 inhibition after Amaxa transfection

The Amaxa transfection showed to be the best method since cells kept cell viability and in two lines the miRNA expression could be efficiently knocked down. A new experiment was performed looking for the best anti/pri-miRNA concentration to completely knock out/over express the miRNAs.

All miRNAs of interest were deregulated in the SupB15 and BV173 cells. The anti-miR concentration tested on BV173 cells was increased to 150 nM and one condition putting all the anti-miRNAs together was tested. The miR-221 expression was 97 and 99 % reduced in BV173 and SupB15 anti-221 treatment, respectively. The anti-222 lead to 76 and 84% reduced expression and anti-222* was efficient only in BV173, with 91% of inhibition.

Anti-221 was capable to reduce not only mi-221 expression but also mi-222 in about 50%. The co-transfection of all three anti-miRNAs 221,222 and 222* was able to reduce mi-221 and 222 in more than 90% but not the miR-222* expression. On the other hand, pri-125b* increased miRNA expression from a 30 ct to a 14 ct in both cell lines. The figure III.15 shows the results.

Figure III. 15: Graphs showing miRNAs percentage of expression based on 2-DCT values obtained by RT-PCR after Amaxa transfection of anti/pri-miRNA in BV173 and SupB15 cells.

The over expression of miR-125b alone was the only one that showed difference on cell viability using the apoptosis assay. Cells treated with pri-125b died 30% less than cells treated with scrambled. However, a high mortality rate was observed with the scrambled miRNA, probably being an unspecific effect of the nucleoporation procedures (figure III.16)
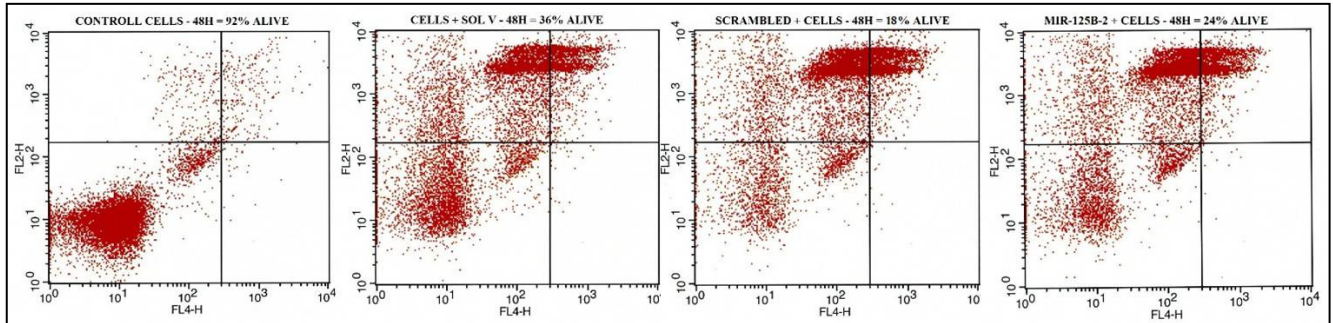

Figure III.16: Apoptosis assay showing increased viability with miR-125b(24%) compared to scrambled (18%) but high mortality caused by Amaxa procedures.

No effect on cells mortality was referred to neither miR-221 nor miR-222 upregulation. Cell cycle analysis by propidium iodide staining was performed to check if the miRNA have any role on cell progression control (figure III.17).

BV173 cell cycle	apoptotic cells (%)	G0/G1 (%)	S (%)	G2/M (%)
cells	5	58	21	17
scr miRNA	19	54	13	13
anti-221	16	57	13	14
anti-222	12	56	13	17
anti-222*	17	53	14	15
125b+125b*	19	53	14	15
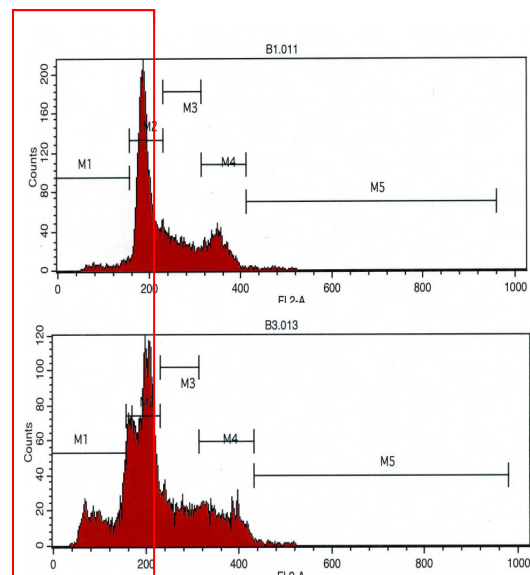
Figure III.17: cell cycle staining by FACs showing increasing of M1 area (apoptotic cells) in all the Amaxa cells

The cell cycle test showed that after Amaxa more cells appeared in the M1 area corresponding to the apoptotic cells (lower PI expression) and fewer cells appear in M2 pick that corresponds to the cells in G0/G1 phase. This result shows that there was no difference on the cell cycle related to the miRNA deregulation, but there was a "stress" effect by the transfection method that induces apoptosis.

Drug effects after transfection

Since the miRNAs in study seem to be related to the response to chemotherapy, it is important to show that their deregulation *in vitro* can affect drug response. A new experiment was performed using SupB15 cells under established Amaxa conditions with all the miRNA molecules. After 24 hours the control/ transfected SUPB15 cells were treated with two different concentrations of prednisone (220 and 400nM) and imatinib (3 and 10 µM), following the literature indications to have 50% of death after 48 hours of treatment. The results showed that drugs concentrations used to kill the cells were not efficient (figure III.18), since the cells did not die even in the control samples cultivated with the drugs alone (without transfection of miRNA molecules).
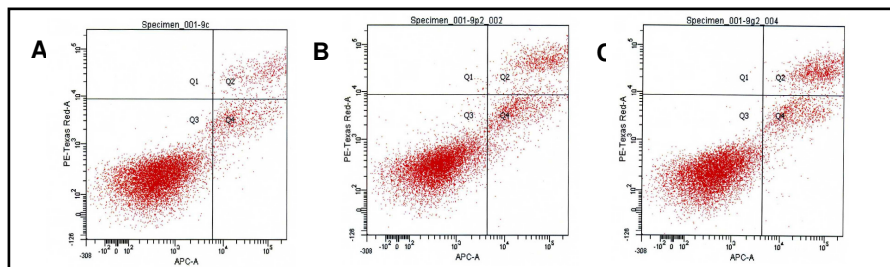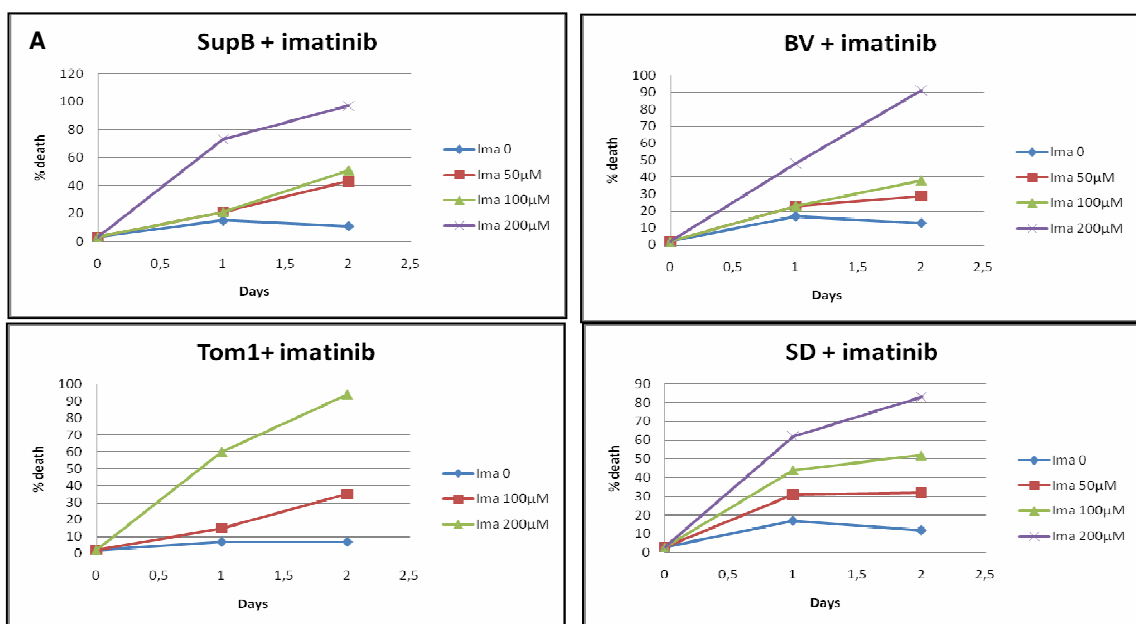

Figure III.18: FACs for SupB15 + Amaxa not treated (A) or treated with the higher concentrations of prednisone (B) and imatinib (C).

Since the used imatinib and prednisone concentrations did not kill the cells, a curve of toxicity for these drugs was established after treatment with different concentrations for each cell line (figureIII.19).
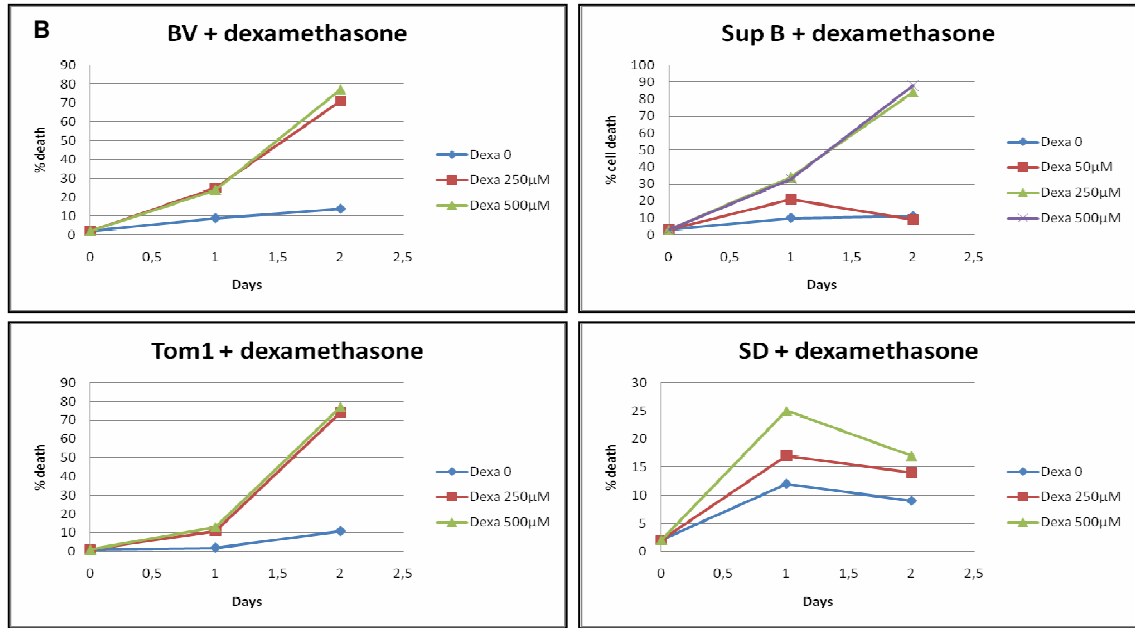
Figure III.19: Curve of imatinib (A) and dexamethasone (B) treatment in Ph+ ALL cell lines

We decided that the drug that should be used to test the first chemo effects would be dexamethasone instead of prednisone, first because prednisone needs to be broken into prednisolone by digestion to affect the cells in normal therapy; and also because dexamethasone have the same corticoid effects in the cells but was previously shown to be more efficient *in vitro* than prednisolone.

Once the drug conditions were established, TOM1 cells were transfected with 100nM of pri-miR-125b or scrambled. The cell death was measured after 24 and 48 hours of transfection but no change was seen when compared to the scramble sample. However, in the presence of dexamethasone killing the cells, the miR-125b treated cells seem to be dying less than the control cells (figure III.20).

Amaxa	day2 (cell %)			
	anexin+pi	anexin only	apoptotic cells	alive
tom 0	0	8	8	79
tom 125*	14	5	19	74
tom 125* + dexa	50	10	**60**	34
tom scr	14	5	19	74
tom scr + dexa	58	14	**72**	22

Figure III.20: Cell-death assay for Tom1 transfected with pri-miR-125b treated or not with dexamethasone

The experiment was repeated using SupB15 cells. Although the cells seem to be good according to the viability shown on the cell counter (minimum of 70%), the apoptosis assay showed that the cells were dying even without the drugs in the presence of scramble-miR. This compromised the whole experiment because the death rate was already high in the controls, so the pri/anti-miRNA effect could not be seen. (Figure III.21/ 22)
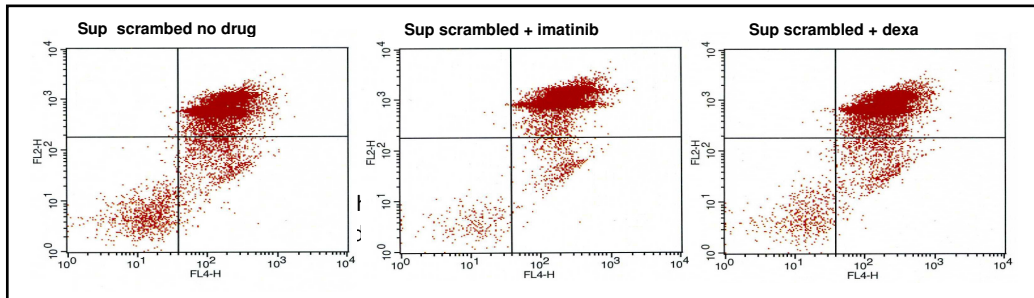
Figure III.21: Cell death assay showing Amaxa high toxicity in SupB15 cells in the absence or presence of drugs
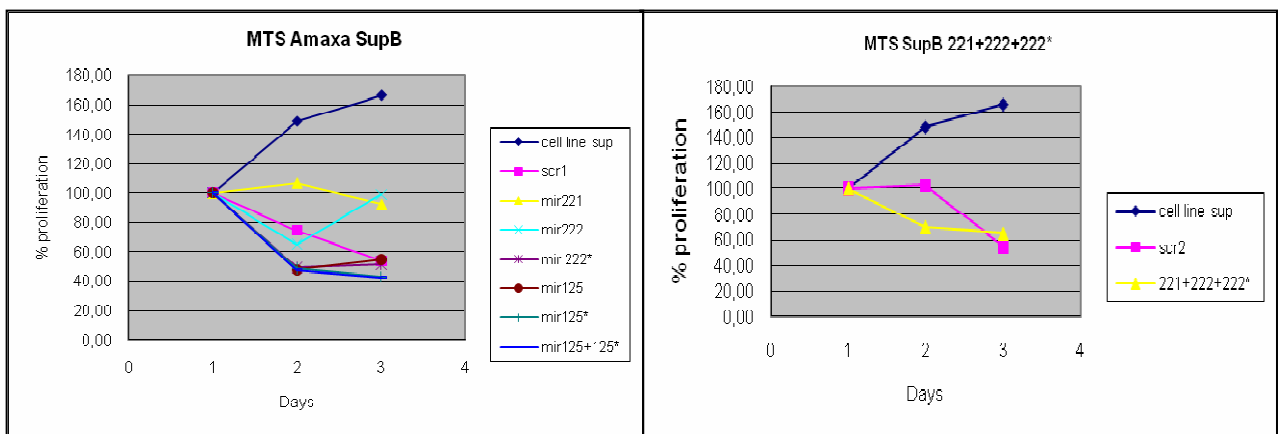
Figure III.22: Proliferation assay showing SupB15 cells that do not grow when treated with Amaxa

In order to verify the toxicity of Amaxa, that could be hidden by the viability results measured in the cell counter in the previous experiments, a test was performed with all the cell lines using only the solution V and the scramble pri-miRNA. Three conditions were established: cells not treated, cells chocked only with solution V and cells chocked with scramble pri-miR. Proliferation, viability and apoptosis were measured after 24, 48 and 72 hours. The results finally showed that although the cell counter says the viability is good, the cells are dying even without any pri/anti-miR. (Figure III.23/24)

Amaxa	Day 1		Day 2	
	apoptotic cells	viability	apoptotic cells	Viability
Tom	21%	79%	25%	75%
Tom solV	73%	27%	80%	20%
Tom scr	78%	22%	93%	7%
BV173	54%	46%	55%	45%
BV173 solV	84%	16%	96%	4%
BV173 scr	84%	16%	95%	5%
SD1	24%	76%	18%	82%
SD1 solV	45%	55%	53%	47%
SD1 scr	46%	54%	54%	46%
Sup	44%	56%	53%	47%
Sup solV	77%	23%	86%	14%
Sup scr	77%	23%	85%	15%

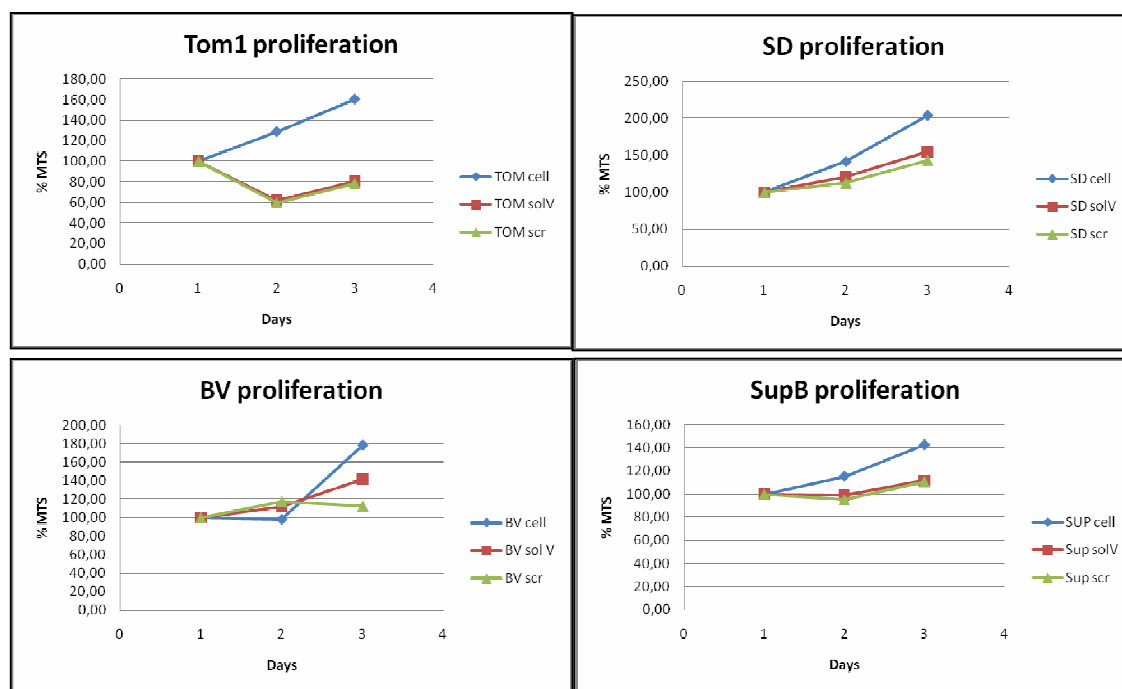Figure III.23: Viability according to anexin/PI assay after Amaxa nucleoporation

Figure III.24: Proliferation assay shows that after Amaxa chock the cells grow less than the non-treated cells

These results suggest the use of a new protocol of infection to be performed with lentiviral vectors in order to have a stable miRNA silencing and to study their possible targets by proteomics approaches.

Lentivirus infection

Using 293TN cells the virus were produced (figure III.25) using scrambled miR-zip, anti-miR 221 and 222, scrambled miR and miR- 125b.
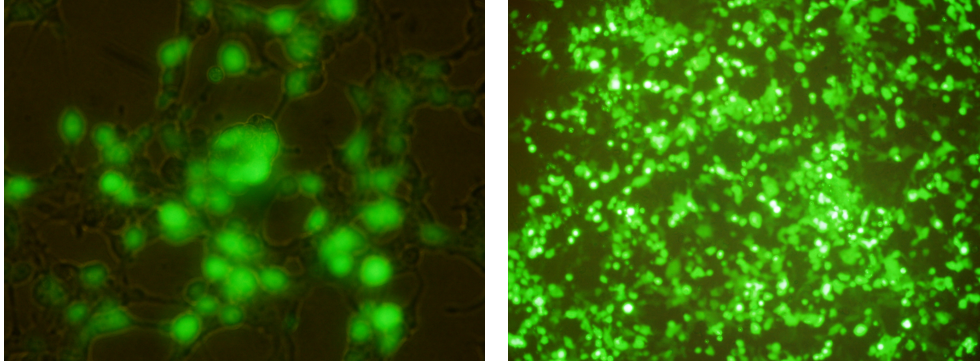
Figure III.25: 293TN lentivirus production

Ph+ lines infections were very low, in the first experiments 1 million cells were used reaching a MOI of 6, but less than one percent of the cells were green. Infected SupB15 cells were sorted but only 0.2% was GFP positive According to the FACs facility personal, the sorter machine is capable to recognize only cells with a high intensity green signal, what explains the difference between the cells seen on the microscopy and sorted.

Several tests were done and a reduction of cell concentration together an ultra dosage of virus resulted in about 10% of green cells, which are been grown to be sorted. (Figure III. 26). The effects of miRNA constant deregulation will be tested *in vivo,* in NOD/SCID mice.

Figure III.26. Tom1 (A) and SD1 (B) zip-scrambled infected cells

Two potential target expressions which came out on literature and on our statistical analysis (BAK-1 and BCLGL14) were tested by RQ-PCR but the mRNA, which was not expressed by the untreated cells, was not re-expressed after mi-125b up-regulation.

III.3.3. In vivo experiments

In order to analyze the interest miRNA roles *in vivo* two different xenograft models were tested using the four ALL Ph+ lines Tom1, SupB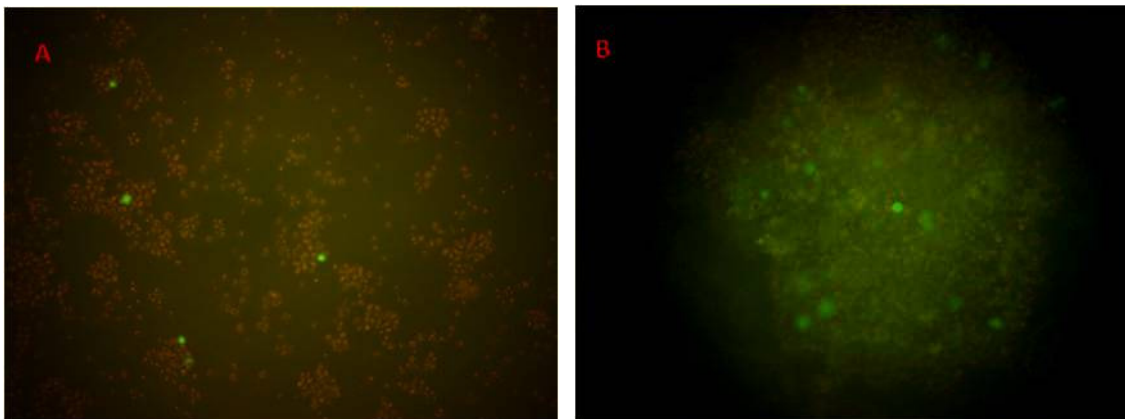15, SD1 and BV173 (1) subcutaneous injection of cells in NUDE mice to be locally treated in vivo with LNA/miR molecules and (2) intraperitonial injection (IP) of normal and in-vitro infected cells in NOD/SCID mice.

Subcutaneous injections

The first experiments searched for the best amount of cells to be injected in order to create a tumor with treatable dimensions. Twenty four NUDE mice were divided in 4 groups (according to each cell line) and received four different concentrations of cells. Three mice received 5 millions cells in the right flank and 15 in the left. The other 3 mice received 10 million cells in the right and 20 in the left flank.

Only with SD1 the situation was different: three mice received a unique back injection of 15 million cells because the total amount of on-culture cells was not sufficient for the test. After eleven days mice SD1 10/20 started to develop tumors in both flanks. They were sacrificed and in the autopsy there was no systemic disease.

All the other lines didn't show changes until the 30st day, when more cells (50 million) were re-injected in the mice back. They developed tumors after 40 days of the second injection. Tom1 and BV173 tumors did not grow enough to be treated at this concentration. SD1 and SupB15 injections were repeated in new mice to test miRNA deregulation. Mice SD1 have a faster tumor development and started to be treated after 2 weeks.

The tumors were injected every 3 or 4 days in a total of 4 times. As shown in figure III.27, at 7, 10 and 14 days after the first injection, tumors injected with synthetic miR-125b (n = 7) were significantly bigger than the scrambled oligonucleotide (n = 9) and mock controls (n = 4) (p =0.04). The mice that received scrambled and zip-221+222 were not different in size or weight. Mice SupB15 are still being analyzed.
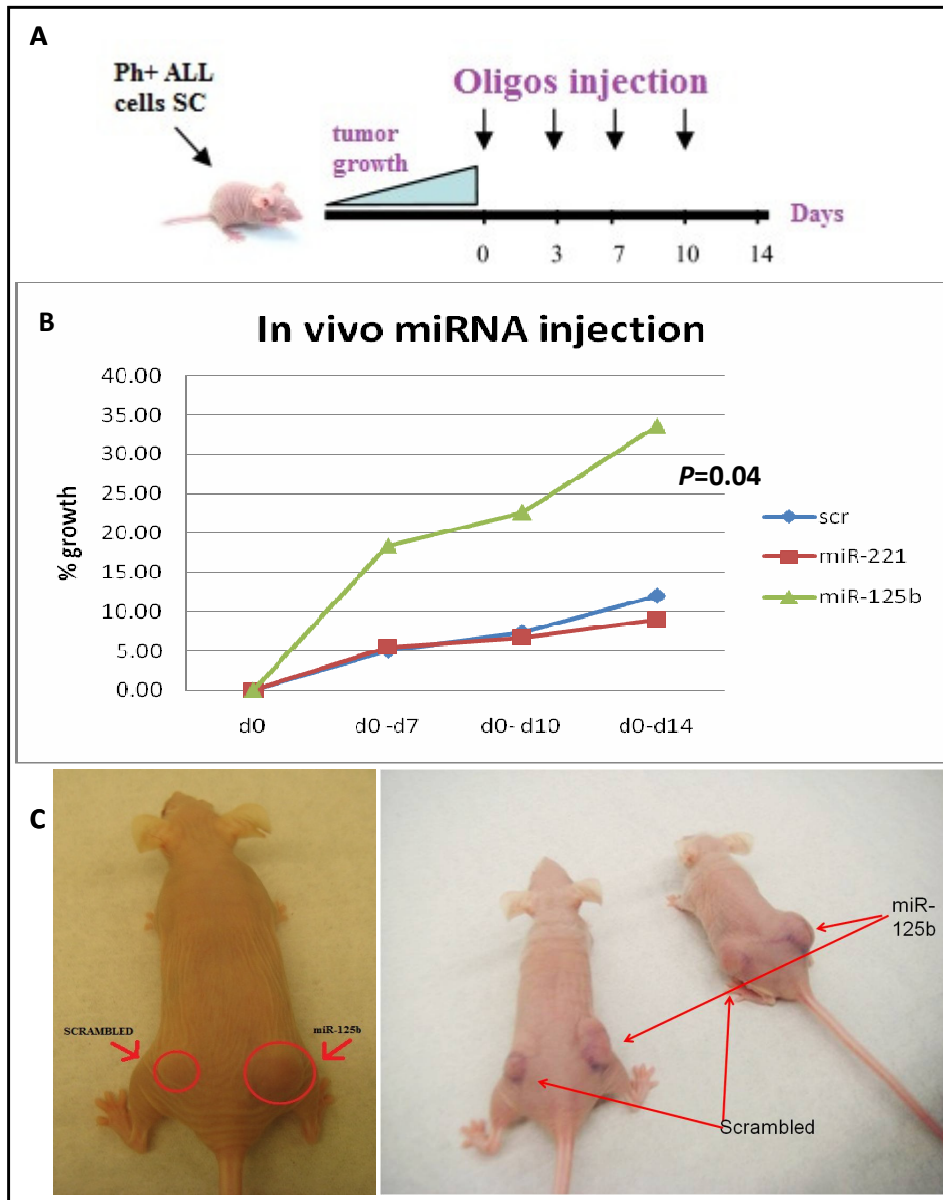
Figure III.27: miR-125b and anti-221 treatment in SD1 mice. (A) Illustration of experimental design of mice xenograft experiment (B) Graphic representing % of tumor growth for the 3 groups: Scrambled, miR-125b and anti-miR-221. (C) Mice injected with scrambled (left) and miR-125b (right) tumors.

IP injections

The NOD/SCID animals received 2 different concentrations: in 3 mice were injected 10 million cells and in 3 others 50 million. After 2 weeks one mouse SD1 10 started to develop a bad aspect and died in the day +30. Another mouse from the same cadge died after 45 days of injection. A week later 2 mice SD1-50 died. The bodies were analyzed and presented increased spleen, characteristic of leukemia. We are now growing lentivirus infected cells to be injected in new mice. The next experiment will compare normal/scrambled/miRNA cell lines effects in leukemia formation/response to drugs.

III.4. Discussion

Several long-scale studies have shown that Ph+ ALL is a heterogeneous disease in terms of clinical features and overall therapy response.[41] Despite the disease aggressiveness, it has been shown that an earlier remission after induction with glucocorticoids and a low risk MRD are correlated to a better outcome, serving as a prognostic parameter.[42, 43]

Our study demonstrated the presence of a miRNA signature in Ph+ ALL children that are resistant to the first-phase of therapy. Particularly the overexpression of two different miRNA clusters miR-221, -222 and mir-125b-2, -125b-2* seem to be related to disease aggressiveness and poor prognosis.

MiR-221 and 222 are among the most deregulated miRNAs implicated in cancer.[44] Their expression is highly upregulated in a variety of tumors, including thyroid cancer[45], hepatocarcinoma[46], melanoma[47], breast cancer[48] and other tumors. Elevated miR-221 and 222 expressions have been associated to the progression of several cancers, by increasing proliferation[48, 49, 50], migration[47] and decreasing apoptosis[51]. However, the molecular mechanisms mediating miR-221 and 222 function in cancer seems to involve different pathways, since target deregulation varies from disease to disease. For example, many targets have been described, such as p27 in melanoma and prostate cancer[47,50], p57 in colorectal and hepatocarcinoma[46], PTEN in lung and liver cancers[51], KIT[10,52] and others.

Recently, Galardi *et al*. described NF-kB and c-Jun to be responsible for the overexpression of these miRNAs in prostate carcinoma and glioblastoma cell lines, by binding the upstream region of the miR-221 and 222 promoters.[53] NF-KB is constitutively activated by BCR-ABL in Ph+ leukemias[54] and although its role in leukemogenesis is unclear, the blocking of its activation in NUDE mice experiments has emerged as good alternative against imatinib resistance. [55]

In the present study we found miR-221 and 222 overexpressed in the group of poor responder patients. Our analysis could not show the *in vitro* increasing of apoptosis by knocking down miR-221 and 222, probably due to the high mortality during transfection procedures. The *in vivo* blocking of miR-221 and 222 did not lead to tumor reduction, indicating that these miRNA may be acting in collaboration to other mechanism to promote therapy resistance in leukemia. Another hypothesis is that these miRNAs upregulation could be just a consequence of upstream pathway activation (such as NFkB). Additional experiments testing the antimiRNA effect in the presence of the drugs *in vivo* may identify miR-221 and 222 roles in Ph+ ALL.

Our study also found the miR-125b-2 and 125b-2* overexpressed in the group of poor responder patients.

MiR-125b deregulated expression has been related to many cancers, but the mechanisms of action are still controversies. The literature supports the notion that miR-125b plays different roles depending on cell context, acting as oncogene or tumor suppressor according to cancer type. [28]

MiR-125b can confer increased chemoresistance of breast cancer cells by inducing antiapoptotic pathways.[56] In colorectal cancer miR-125 overexpression is related to poor prognosis. [57] And miR-125b has been demonstrated in animal models to promote tumor growth of prostate cancer.[58] On the other hand, miR-125b also acts as a tumor suppressor in melanoma [59], glioma [60], liver [61] and ovarian [62] cancers. In addition, SNPs in the 3′ UTR of BMPR1B, a direct target of miR- 125b, confer a good prognosis in breast cancer by abrogating miR-125b down-regulation of BMPR1B. [63]

In the immature hematopoietic system, miR-125b is antiapoptotic and promotes expansion of hematopoietic stem cells; its specific effect is determined by context, as different cell populations have different levels of baseline apoptosis during homeostatic hematopoiesis.[64] This specific property may explain why miR-125b overexpression is associated with multiple hematologic malignancies. [64,65] Two different groups reported cases of ALL containing a translocation involving the complete miR-125b sequence fused to the IGH gene locus, causing overexpression of this miRNA that could imply in the leukemogenesis of precursor B-ALL cells.[26,27]

Some authors though have described miR-125 overexpression in concomitancy to other genetic alterations, indicating a probable synergism during leukemogenesis. This is the case of the pro-proliferative effect of miR-125b-2 accentuated by *GATA1* mutation in megakarioblastic leukemia[66], or the resistance to therapy when miR-125b is overexpressed in the presence of *TEL-AML1* fusion gene in childhood ALL. [67]

Bousquet *et al*. showed that miR-125b overexpression promotes malignant transformation of different hematopoietic lineages *in vivo*.[28] Furthermore, co-expression of miR-125b and the BCR-ABL fusion gene in transplanted cells accelerated the development of leukemia in mice, compared with control mice expressing only BCR-ABL, suggesting that miR-125b confers a proliferative advantage to the leukemic cells.

Finally, Schotte *et al* showed a higher expression of miR-125b in ALL patients that are resistant to glucocorticoids. [24]

The data presented here also indicate the miR-125b-2 increased expression in patients that are resistant to induction therapy, specifically against Ph+ALL. The effects of miR-125b-2 overexpression in the Ph+ cell lines couldn't be seen *in vitro*, probably due to the high mortality during transfection procedures. Nevertheless, the forced expression of miR-125b-2 *and* -125b-2* in Ph+ ALL cell lines induced tumor growth in mice. After a week until the end of treatment the tumors have grown three times more than those treated with scrambled miRNA. This result demonstrates that miR-125b-2 conferred a proliferation effect to the cells and accelerated the tumor development in cooperation to BCR-ABL. Further studies to test miRNA deregulation in the presence of drugs *in vivo* may explain better the oncogene mechanism by which these miRNAs induce therapy resistance.

III.5.Conclusion

In conclusion, we demonstrated the presence of a miRNA signature in childhood Ph+ ALL according to first-therapy response. Particularly miR-221,222 and miR-125b-2 are highly overexpressed in the poor responder group of patients. We showed *in vivo* that miR-125b-2 acts as an oncogene and increases proliferation and leukemia aggressiveness. Further studies will be necessary to understand how miR-221, 222 and 125b-2 act in cooperation with BCR-ABL to develop leukemia. In the future an antimiRNA-based therapy can emerge as a novel pro-apoptotic approach to be added to the tyrosine kinase therapies in order to improve response and survivor in Ph+ ALL.

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IV. Chapter 4: Conclusions and future perspectives

IV.I. Summary

In 1960, Nowell and Hungerford identified an abnormal small chromosome in patients with chronic myeloid leukemia (CML).[1] This chromosome, which is known as the Philadelphia (Ph) chromosome, was later identified as the result of a reciprocal translocation between the *BCR* gene on chromosome 22 and the *ABL* gene on chromosome 9. The fusion of the *BCR* and *ABL* genes produces a chimeric protein known as BCR-ABL.[2] Three different fusion sites result in the formation of different BCR-ABL protein products named p190, p210, and p230, each differing in the amount of *BCR*-encoded sequences they contain. p190 has been observed in patients with a very aggressive, short latency leukemia known as acute lymphoblastic leukemia (ALL). The p210 form of BCR-ABL is the causative mutation in 95% of cases of CML, a less aggressive and longer latency disease is the most recent form of BCR-ABL discovered and is associated with a chronic neutrophilic leukemia.[3] The ability of BCR-ABL to initiate leukemia has been established through extensive studies in cell culture and animal models. BCR-ABL is a deregulated tyrosine kinase that transforms both fibroblasts and hematopoietic cells in culture and cells transformed by BCR-ABL can form tumors in NUDE mice.[3] BCR-ABL is involved in the malignant transformation of hematopoietic cells, by enhancing proliferation, reducing apoptosis, and deregulating cell adhesion.[4] This fusion gene is known to deregulate important pathways (such as the JAK/STAT, the RAS and the PI-3 kinase),but the detailed mechanisms behind BCR-ABL-induced leukemogenesis remain unknown.[5] The *Ph* chromosome is present in 3–5% of children and in 25-40% of adults with ALL. Although treatment advances in the past decades, this disease is still the ALL with the worst prognosis, with a high risk of relapse. [6] Even so, several long-scale studies have shown that Ph+ ALL is heterogeneous in terms of biological features and initial steroid response. [6] A set of international studies with large series of patients have shown that the response to initial therapy and the MRD risk can be used to predict responsiveness to early conventional treatment, and can be beneficial for the accurate stratification of children to guide new clinical strategies. [7-10] The intrinsic biological characteristics from the patients at diagnosis can reveal a lot about the tumor aggressiveness that promotes therapy resistance. [8,9] Once all the Ph+ ALL carry the same molecular translocation, there may be other mechanisms that work together to BCR-ABL to worse

leukemia. [10] Looking for unravel these hidden characteristics that led to different clinical responses among Ph+ ALL, we designed a study that applies combined techniques to analyze specific gene and miRNA expression patterns in diagnostic samples. The study analyzed 78 children diagnosed with Ph+ ALL between 2000 and 2010. First, the samples were tested for the presence of *IKZF1* mutations by PCR, SNP array and gene sequencing. *IKZF1* encodes a protein required for lymphoid lineage differentiation and proliferation, and deletions have been highly associated to the presence of BCR-ABL and to a poor outcome in ALL.[11-18]

The results revealed 68% of deletions/loss of chromosome 7, with mutations being conserved at relapse. The frequency is similar to that previously found by Mulligham *et al* in a cohort of Ph+ ALL children (75%). Our study also showed that *IKZF1* mutations can be used as MRD markers, reaching a very high sensitivity. Although our results did not found association between *IKZF1* deletions and the initial therapy response, the high mutation incidence suggests that Ikaros loss combines with *BCR-ABL1* to induce ALL, arresting B lymphoid maturation. The following experiments were focused on miRNA analysis to identify possible therapy-resistance markers. Several studies have associated miRNA deregulated expression to leukemia development[19-24] , but little is known in childhood Ph+ ALL. The patients were divided in two groups according to the first-therapy response: PGR/non-HR MRD and PPR/HR MRD. The analysis of overall miRNA expression found a miRNA profile that is related to the poor response. The most significant miRNAs have already been described in other cancers to be related to tumor development.[25-34] MiR-221, 222 and 222*, all in the same cluster, and miR-125b-2 and 125b-2* were confirmed by RQ-PCR with a larger number of patients to be overexpressed in the HR/PR group. *In vitro* tests using Ph+ cell lines faced a high technique-intrinsic mortality and did not show any important change in cell apoptosis/proliferation/response to drugs. The downregulation of miR-221/222 and 222* in mice did not altered tumor sizes compared to scrambled/mock injections. One possible explanation is that these miRNAs overexpression in patients is just a consequence of BCR-ABL activation of NF-KB that would be increasing the synthesis of miR-221/222 cluster.[35-37] Other hypothesis is that miR-221/222 may be acting in cooperation with other mechanisms to promote aggressiveness, so miRNA deregulation by itself may not be sufficient to promote cell changes. On the other hand, *in vivo* experiments demonstrated that upregulation of miR-125b-2 *and* -125b-2* in Ph+ ALL cell lines induce tumor growth in mice (p=0.04). This result shows that miR-125b-2 acts as oncogene and increases leukemia aggressiveness. Further

studies testing the tumor growth in the presence of anti-ALL drugs in combination to miR-125b-2 studies may explain better the mechanism by which these miRNAs cooperate with BCR-ABL and induce therapy resistance.

In conclusion, we confirmed the high incidence of IKZF1 deletions in our children with Ph+ ALL and the presence of a miRNA signature in childhood Ph+ ALL according to first-therapy response. Particularly miR-221, 222 and miR-125b-2 are highly overexpressed in the poor responder group of patients. We showed *in vivo* that miR-125b-2 acts as oncogene and increases leukemia aggressiveness. Further studies will be necessary to understand how Ikaros, miR-221,222 and 125b act in cooperation with BCR-ABL. Our group will continue this study, Ikaros status will be compared to the overall outcome and further mice are already being tested the presence of anti-ALL drugs and the miRNAs of our interest. These results can help to understand the mechanism by which deregulation of these genes work in hematopoietic cells and promote leukemia. In the future other gene target therapies can emerge as novel anti-apoptotic approaches to be added to the tyrosine kinase therapies and improve survivor in pediatric Ph+ ALL.

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