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CICLO XXIII

Characterization of the t(6;11)(q27;q23) in pediatric acute myeloid leukemia

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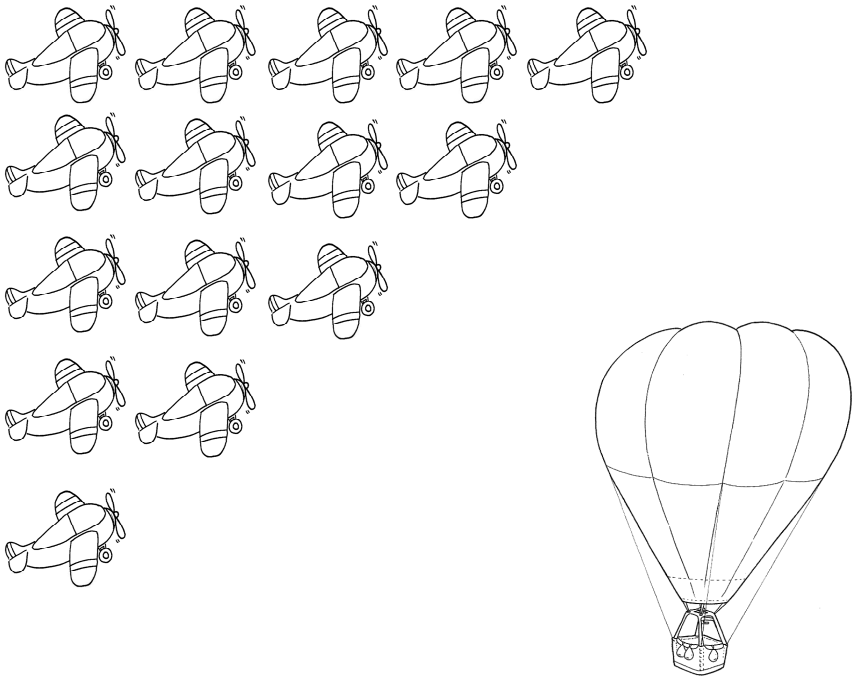

THINK DIFFERENT

Lo scoprire consiste nel vedere ciò che tutti hanno visto

e nel pensare ciò che nessuno ha pensato.

Albert Szent-Györgyi von Nagyrapolt

(Nobel per la Medicina e la Fisiologia 1937)

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SUMMARY

Acute myeloid leukemia (AML) set up for the 10 % of pediatric leukemias. Patient genetic background has been demonstrated to be the most important feature for a correct management of patient diagnosis and prognosis. To date just 40 % of AML shows typical molecular markers with clinical impact, thus for the most of them new genetic alterations might be evaluated in order to achieve a better patients stratification and a deeper understanding of leukemogenesis.

In the AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica) LAM-2002/01 treatment protocol, children affected by acute myeloid leukemia (AML) were stratified by using a risk group classification based on a combination of specific genetic abnormalities and response to treatment. Samples from 741 AML patients collected from 2000 to 2008 were investigated for their biological and clinical significance. *MLL* rearrangements were identified in 77/741 patients (10.4 %), with significantly different clinical outcomes depending on the *MLL* translocation partner. GEP analysis defined significant gene expression signatures dependent on the *MLL*-partner gene. Array-CGH analysis established high levels of DNA amplification (75 %) with respect to deletions (25 %) for *MLL*-AML, and the del(12p) to be considered as a novel feature for leukemia stratification.

The t(6;11)(q27;q23) translocation is characterized by *MLL-AF6* expression, a bad prognostic marker in AML; however, the exact tumorigenic mechanism is still unclear. Patients with *MLL-AF6* displayed an adverse outcome, a specific GEP signature, as well as the highest DNA imbalances with additional recurrent genomic rearrangements in 12p, 11q and 6q. *AF6* gene has no similarities with other *MLL*-partner genes and encodes for a cytoplasmatic protein involved in signal transduction. The chimeric protein, instead, has a nuclear localization, where it can homodimerize to activate transcription. The RAS pathway is often implicated in *MLL*-leukemia and AF6 was shown to inhibit RAS signaling in epithelial cells. Here, by immunofluorescence and immunoprecipitation analyses, the AF6-RAS interaction was demonstrated in bone marrow cells of healthy donors, while in t(6;11)(q27;q23) leukemic cell lines AF6 protein resulted sequestrated into the nucleus. Silencing for *AF6* gene in healthy samples caused the overexpression of the RAF/MEK/ERK pathway proteins, confirming the RAS-inhibiting role of AF6 in hematopoietic cells. Specific silencing of *MLL-AF6* in leukemic cells resulted in AF6

liberation into cytoplasm, where it colocalized with RAS effecting its downstream targets. By reverse phase protein array, cytoplasmic AF6 restoration was shown to improve expression of pro-apoptotic proteins PARP and CASPASE7 in leukemic cells and to diminish levels of P-CREB, mTOR, P-JAK and CYCLINs, involved in cell proliferation. As a result, reduced cell colony formation in semisolid medium was observed, accompanied with an augmented percentage of cell mortality. The same effects were obtained with two specific MEK inhibitors, confirming the RAS pathway implication in *MLL-AF6*-leukemia. These results suggest a possible mechanism by which MLL-AF6 acts in AML: the loss of RAS-inhibition by AF6 nuclear sequestration could be responsible for the proliferation advantage to t(6;11)(q27;q23) cells, enhancing the effect of the chimera in AML development.

SOMMARIO

Le leucemie acute mieloidi (LAM) costituiscono il 10 % delle leucemie pediatriche e l'assetto genetico del paziente è oggigiorno alla base della scelta del percorso terapeutico più adatto, rivestendo un ruolo fondamentale durante la fase diagnostica. Poiché attualmente circa il 40 % dei pazienti LAM presenta un marcatore molecolare riconoscibile, la ricerca in questo campo è tesa verso la scoperta di nuove anomalie che permettano una migliore classificazione dei pazienti e una più profonda comprensione del processo di leucemogenesi.

Secondo il protocollo di trattamento LAM-2002/01 dell'AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica), i bambini affetti da leucemia acuta mieloide (LAM) sono stati stratificati in classi di rischio in base a una combinazione di specifiche anomalie genetiche e risposta al trattamento. I campioni prelevati da 741 pazienti LAM raccolti nel periodo 2000-2008 sono stati studiati per il loro significato biologico e clinico. Riarrangiamenti del gene *MLL* sono stati identificati in 77/741 pazienti (10.4 %), con risultati clinici molto diversi a seconda del partner di traslocazione di *MLL*. Un'analisi di GEP ha definito significativi pattern di espressione genica dipendenti dal gene partner di *MLL*. Mediante un'analisi di Array-CGH è stato stabilito che le LAM caratterizzate da riarrangiamenti del gene *MLL* si riscontra una maggior quantità di amplificazioni del DNA (75 %) rispetto alle delezioni (25 %) e che la delezione del(12p) può essere considerata una nuova caratteristica per la stratificazione delle leucemie.

La traslocazione t(6;11)(q27;q23) è caratterizzata dall'espressione di *MLL-AF6*, un marcatore di cattiva prognosi nella LAM, sebbene l'esatto meccanismo oncogeno non sia ancora chiaro. I pazienti *MLL-AF6* mostrano un esito particolarmente sfavorevole, uno specifico profilo di espressione genica, così come il più alto numero di sbilanciamenti cromosomici con riarrangiamenti genomici aggiuntivi ricorrenti in 12p, 11q e 6q. Il gene *AF6* non ha analogie con altri geni partner di *MLL* e codifica per una proteina citoplasmatica coinvolta nella trasduzione del segnale. La proteina chimerica, invece, ha una localizzazione nucleare, dove può omodimerizzare per attivare la trascrizione. Il pathway di RAS è spesso implicato nelle leucemie caratterizzate da *MLL* ed è stato dimostrato che AF6 è in grado di inibire la trasmissione del segnale a valle di RAS in cellule epiteliali. In questo studio, mediante analisi di immunofluorescenza e

immunoprecipitazione, l'interazione AF6-RAS è stata dimostrata in cellule di midollo osseo di donatori sani, mentre in linee cellulari leucemiche con la traslocazione t(6;11)(q27;q23) la proteina AF6 è sequestrata nel nucleo. Il silenziamento del gene *AF6* in campioni sani ha causato la sovraespressione delle proteine del pathway RAF/MEK/ERK, confermando il ruolo inibitorio di AF6 su RAS nelle cellule ematopoietiche. Il silenziamento specifico di *MLL-AF6* in cellule leucemiche ha comportato la liberazione di AF6 nel citoplasma, dove colocalizza con RAS con un effetto sui suoi effettori a valle. Mediante la tecnica del reverse phase protein array è stato visto che il ritorno di AF6 nel citoplasma in cellule leucemiche ha portato all'aumento dell'espressione delle proteine pro-apoptotiche PARP e CASPASI7 e alla diminuzione dei livelli di P-CREB, mTOR, P-JAK e CICLINE, coinvolte nella proliferazione cellulare. Di conseguenza, è stata osservata una ridotta formazione di colonie in terreno semisolido, accompagnata da una aumentata percentuale di mortalità cellulare. Gli stessi effetti sono stati ottenuti con due inibitori specifici di MEK, confermando l'implicazione del pathway di RAS nella leucemia *MLL-AF6*. Questi risultati suggeriscono un possibile meccanismo attraverso il quale *MLL-AF6* agisce nella LAM: la perdita di inibizione di RAS tramite il sequestro nucleare di AF6 potrebbe essere responsabile del vantaggio proliferativo delle cellule t(6;11)(q27;q23), aumentando l'effetto della chimera nello sviluppo della LAM.

GENERAL INTRODUCTION

Human leukemia: epidemiology and etiopathogenesis

Leukemias are clonal proliferative disorders of hematopoietic stem cells or of a lymphoid/myeloid progenitor blocked at a certain stage of maturation. They are characterized by aberrant proliferative and apoptotic capacity, causing a progressive accumulation of malignant cells in the bone marrow. Leukemias are the most common form of cancer in the pediatric population, covering 35-40 % of all childhood cancers. They are divided into lymphoid and myeloid, both acute or chronic depending on the course of disease, the degree of medullary invasion and the stage of blasts maturation. Among pediatric leukemias, about 77 % is acute lymphatic (ALL), 10 % is acute myeloid (AML), 2-5 % is represented by chronic myelogenous leukemia (CML) and 2 % by juvenile myelomonocytic leukemia (JMML)[1,2].

While in adult patients these tumors are characterized by a highly variable and severe prognosis (mainly related to the age of onset), in the pediatric population both lymphoid and myeloid forms have a survival of more than 75 % for ALL and 50 % for AML. In AML complete remission (CR) is reached in 81.6 % of cases, but in 26.2 % of cases relapse occurs within 5 years (data AIEOP LAM-2002)[1,3-5].

To date, the causes that lead to the development of leukemic disease in pediatric patients are unknown. Although many hypotheses have been developed, the only certainty is that a variable number of factors must contribute to promote the onset of the disease, not a single event[2,6].

There are three categories of factors that are believed to have a dominant role in the development of leukemia:

1) genetic predisposition
2) genetic susceptibility
3) environmental factors.

Genetic predisposition is given by all those familial conditions that increase the risk of developing the disease and that regard mutations or alterations of genes with high penetrance, like chromosomal imbalances (as in the case of Down's syndrome), chromosomal instability syndromes (Fanconi anemia, ataxia-telangiectasia), defects in growth or cell differentiation (Diamond-Blackfan anemia, Noonan syndrome, neurofibromatosis type I) and hereditary immunodeficiencies (such as dyskeratosis congenita). The fact that there is a genetic predisposition to leukemia has been confirmed

by studies on twins which showed that, in the presence of a sick twin, the risk of developing the disease for the other twin was increased compared to the general population. It has been suggested that the first mutational event common to both twins was acquired and transferred from one twin to the other during gestation, while the second event, usually different, would be acquired after birth[7].

Genetic susceptibility refers to the natural presence of nucleotide polymorphisms in genes coding for certain enzymes, and to how these differences affect metabolism. A different efficacy in metabolizing natural compounds or engineered molecules (such as inhibitors of topoisomerase II) is a condition that exposes DNA to the accumulation of errors, predisposing to genetic alterations[8].

Environmental factors include all possible external conditions that could play a role in leukemia development: radiation exposure to electromagnetic fields, cytotoxic substances, abused consumption of substances such as alcohol or tobacco, early contact with the pathogens and the ability to answer of the immune system, immunization and breastfeeding failure. Currently, the only certainty is that prenatal irradiation and high-dose irradiation at an early age cause predisposition to leukemia[6].

The most widely accepted theory implies the cooperation of two classes of genetic abnormalities in promoting the onset of acute myeloid leukemia (Figure 1).

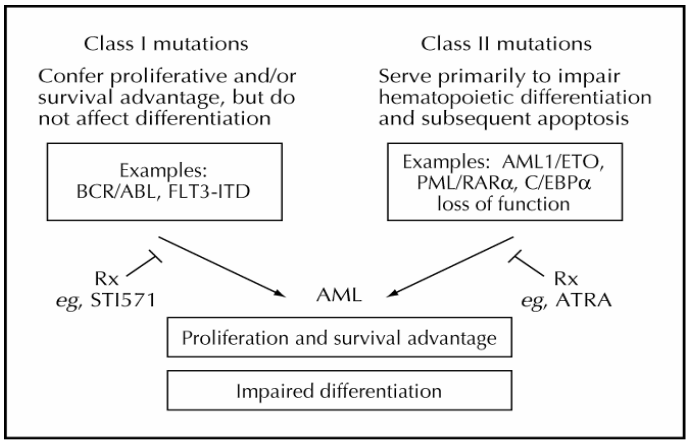

Figure 1. Schematic representation of the model based on the theory of the two mutations[9].

In the first category there are mutations that lead to an increased cell proliferation acting as initiator of the disease, while in the second class there are anomalies that result in abnormal cell differentiation. Mutations of the first class include genes coding for the tyrosine kinases, such as *BCR-ABL* or *FLT3* genes, sufficient to ensure a growth factors-

independent proliferation. The second class is made of mutations affecting genes coding for transcription factors (such as *AML1-ETO* or *PML-RARA*) or promoter sequences that control transcriptional processes, resulting in abnormal cell differentiation and maturation[9,10].

AML classification

The French-American-British (FAB) Cooperative Group established the first and most comprehensive classification of AML that distinguishes myeloid leukemia in different subgroups, according to the morphology of blasts and their responsiveness to specific cytochemical reactions. These two parameters serve to assess the state of blast differentiation. This system allows to distinguish 8 FAB subgroups of myeloid leukemia called from M0 to M7 on the base of the maturational status of the leukemic clone (Table 1). The most common subtypes are M4 and M5, which together form 40 % of pediatric AML and reach up to 80 % of cases below the age of 2 years. These two classes were further subdivided into M4 with a lot of eosinophilic cells (M4eo) and M5a and M5b which differ in the percentage of monocytes[3].

In 2008 the World Health Organization has introduced a new classification which takes into account clinical, immunophenotypic, cytogenetic and molecular-biological criteria, considering together the disciplines involved in the diagnosis and choosing the most appropriate management for every patient[3,11].

The classes are five and include:

CLASS 1. AML with recurrent genetic abnormalities[12].

They are divided in AML with balanced translocations or inversions and AML with gene mutations. The first ones are characterized by the formation of a fusion gene encoding for a chimeric protein necessary (although usually not sufficient) for leukemogenesis. They mainly include AML positive for

- t(8;21)(q22;q22) - *AML1-ETO*
- inv(16)(p13;q22) or (16;16)(p13;q22) - *CBFB-MYH11*
- t(15;17)(q22;q12) - *PML-RARA*
- 11q23 rearrangements - the *MLL* gene is fused with many partner genes.

Gene mutations are mostly point mutations in specific genes controlling cell proliferation or differentiation. Proteins, due to mutations, have altered activity that can be either gain or loss of function.

CLASS 2. AML with multiple dysplasia[13].

They are characterized by different conditions, including

- at least 20 % of blasts with myelodysplastic morphology (such as nuclear fragmentation, megaloblastic aspects of erythrocytes or granulocytic abnormalities)
- a prior history of myelodysplastic syndrome or myeloproliferative disease with signs of myelodysplasia
- cytogenetic abnormalities typical of myelodysplastic syndromes such as monosomy of chromosome 7 (in the absence of alterations that would fall into the category of AML with recurrent genetic abnormalities).

CLASS 3. AML related to treatment[14].

They occur as a late complication of cytotoxic or radiant chemotherapy received for the treatment of neoplastic diseases or not. The main chemotherapeutic molecules responsible of this are alkylating agents and topoisomerase II inhibitors.

CLASS 4. Ambiguous AML[3].

They are divided in biphenotypic and bilinear forms: the first is characterized by the presence of two leukemic clones of different types (for example, one myeloid and one lymphoid), the second one by the simultaneous expression of lymphoid and myeloid markers on the same leukemic clone.

CLASS 5. AML not otherwise specified[15].

This class include all those conditions that do not belong to the groups described above and have the FAB classification as primary basis implemented by immunophenotype data. They comprise all FAB classes (except for M3 which is associated with translocation t(15;17) and belongs to the category of AML with recurrent genetic abnormalities), with the addition of the acute basophilic leukemia, the acute pan-myelosis with myelofibrosis and the granulocytic sarcoma.

SYMBOL	CLASS	MORPHOLOGICAL CRITERIA
M0	Undifferentiated	MPO positivity in < 3 %, NAE negative, characteristic positivity for at least one of the markers CD13 and CD33
M1	Myeloblastic without maturation	MPO positivity in > 3 %, blasts at least 90 % of the bone marrow (excluding erythroblasts) with a monocyte and granulocyte component < 10 %
M2	Myeloblastic with maturation	MPO positivity in > 3 %, blasts equal to 30-90 % of the bone marrow population with a monocytic component less than 20 % associated with granulocytic component of at least 10 %
M3	Promyelocytic	MPO positivity in > 3 %, blast component containing at least 20 % abnormal hypergranular promyelocytes (indicated by the presence of Auer rods)
M3V	Ipogranular promyelocytic variant	MPO positivity in > 3 %, kidney-shaped nucleus and granules with blasts but strongly positive for the MPO
M4	Myelomonocytic	MPO positivity in > 3 %, blasts of at least 30 % of the bone marrow population with a granulocytic and monocytic component between 20 and 80 %
M4eo	Myelomonocytic with hypereosinophilia	As for M4 but with abnormal eosinophils with large granules, basophils and eosinophils
M5	Monocytic	Positive for the NAE, the presence of a monocyte component of at least 80 % of the medullary
M5a	Monocytic without differentiation	As for M5 but with monocytic component at least 80 % monoblasts
M5b	Monocytic with differentiation	As for M5 but with monocytic component till a maximum of 80 % monoblasts
M6	Erythroleukemia	Erythroid cells at least 50 % of the bone marrow, erythroid blasts not > 30 %
M7	Megakaryoblastic	Presence of megakaryoblasts, cytoplasmic blebs and sometimes myelofibrosis, not erythroid blasts > 30 %, necessarily present CD41, CD42, CD61 and factor VIII-related markers

Table 1. Morphologic classification of AML by FAB. MPO (mieloperoxidase) and NAE (Naftil Acetate Esterase) colorations are enzymatic reactions that are usually used in acute leukemia dignosis[85].

Cytogenetics and molecular genetics in the diagnosis of AML

The AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica) includes 27 italian pediatric centers that follow the same therapeutic protocols. The AIEOP LAM 2002 protocol divided the patients into three main risk classes using the response to therapy and the classical cytogenetic analysis/molecular genetics, which are essential to refer the patient to the most appropriate treatment[16]. The promyelocytic AML FAB M3 with t(15;17)(q22;q12) follows a specific protocol AIEOP (GIMEMA), while the remaining AMLs are divided in two classes of risk, standard risk and high risk. The standard risk class is characterized by patients with t(8;21)(q22;q22) or inv(16)(p13;q22) as single abnormalities. The presence of additional chromosome or genetic abnormalities moves patients to the high risk class. In high risk group fall all patients with complex karyotype (in addition to a known translocation), with *MLL*-translocation and those negative for known anomalies. The two risk classes provide a common induction therapy and then different consolidation therapies, with the aim to have hematopoietic stem cell transplantation (HSCT) in first complete remission of subjects included in the high risk category[17].

The cytogenetic and molecular biology play a complementary role in diagnosis[16,18]: the traditional cytogenetics can assess the karyotype of the single subject in its entirety in a single examination, possibly exploring the association of several anomalies, but it needs an adequate number of metaphases, so it is difficult to perform in some patients; the molecular genetics on the other hand is always executable and is able to detect abnormalities not detectable with conventional cytogenetics, such as point mutations or cryptic translocations, but it is unable to identify unknown genetic alterations. An important aspect of molecular genetics is the possibility to monitor minimal residual disease (MRM) in the individual patient once it has been associated with a specific mutation. The ability to verify the recurrence of the molecular alteration with high sensitivity, during and after the therapeutic process, allows the early identification of a possible relapse and improves the prognosis of recurrences[19].

Moreover, it was recently concluded a study conducted by 11 international centers with the aim to create a platform for gene expression able to distinguish the various subtypes of lymphoid and myeloid pediatric leukemia as well as the main classes of dysplastic/proliferative hematological diseases (International Microarray Innovations in

Leukemia Study Group - MILE). The study concluded that this technology is robust and accurate for the diagnosis of hematological malignancies[20].

Recurrent genetic abnormalities and prognostic factors in AML

Among the objectives of the AIEOP LAM 2002 protocol there is the search for new genetic abnormalities for diagnosis and prognosis of new AML subgroups. The *MLL* gene translocations and *FLT3* gene duplication are very frequent genetic alterations with prognostic significance in AML. Their genomic characterization, their incidence and their prognostic impact could help to define additional risk classes in treatment protocol, now all together included within high risk group. The classical screening panel used for diagnosis was enriched with new anomalies (Table 2).

Anomalie genetiche	**Geni coinvolti**
t(4;11)(q21;q23)	*MLL-AF4*
t(9;11)(p22;q23)	*MLL-AF9*
t(10;11)(p12;q23)	*MLL-AF10*
t(11;19)(q23;p13.3)	*MLL-ENL*
t(12;21)(p13;q22)	*TEL-AML1*
t(1;19)(q23;p13)	*E2A-PBX1*
t(8;21)(q22;q22)	*AML1-ETO*
inv(16)(p13;q22) o t(16;16)(p13;q22)	*CBFbeta-MYH11*
t(15;17)(q22;q21)	*PML-RARalfa*
t(1;22)(p13;q13)	*OTT-MAL*
t(6;9)(p23;q34)	*DEK-CAN*
t(16;21)(q24;q22)	*AML1-MTG16*
t(11;19)(q23;p13.1)	*MLL-ELL*
t(6;11)(q27;q23)	*MLL-AF6*
t(X;11)(q24;q23)	*MLL-SEPT6*
t(11;17)(q23;q25)	*MLL-SEPT9*
t(1;11)(q21;q23)	*MLL-AF1*
Internal Tandem Duplication del gene *FLT3*	*FLT3ITD*
Partial Tandem Duplication del gene *MLL*	*MLLPTD*

Table 2. Markers refined the diagnosis of AML according to the screening protocol currently in force in the center of Padova (referent AIEOP).

The search for new molecular abnormalities will allow to assess the incidence and the prognosis in the Italian cases, classifying more correctly apparently very similar leukemias. The current limit in the knowledge of AML in fact resides in the low percentage of patients with a recognizable rearrangement (35-40 %) (Figure 2). This percentage is made up mostly by recurrent translocations, as t(8;21)(q22;q22), inv(16)(p13;q22) and t(15;17)(q22;q21). The incidence of *MLL* gene abnormalities was found in 10 % of AML patients[21]. Currently 84 % of pediatric AML patients is classified as high risk[22], but their clinical course is not uniform, suggesting that genetic characterization is essential to better understand the clinical significance of these AML not yet characterized.

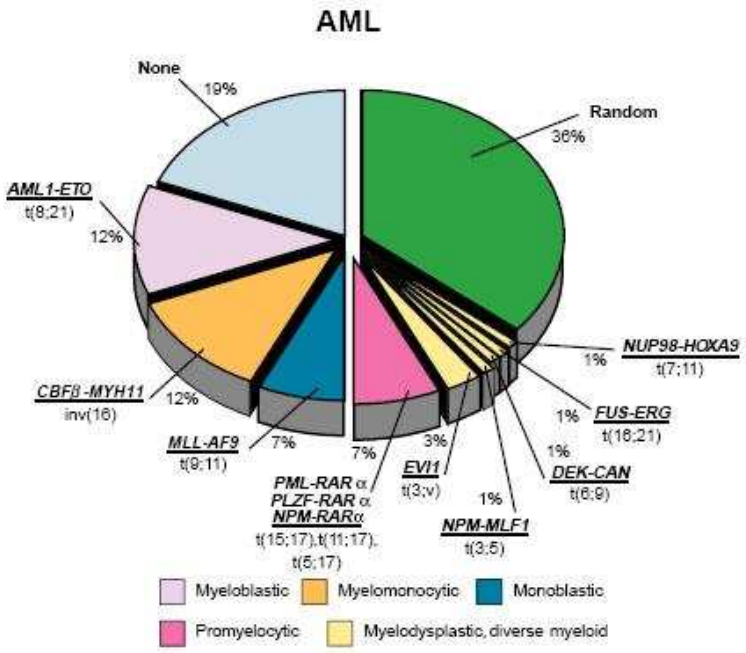

Figure 2. Diagram of the major chromosomal rearrangements involved in AML pediatric patients and young adults[86].

The prognosis of pediatric AML is defined according to the characteristics of three different areas: the patient, the blast population and the therapy response[3,24].

The blast population is evaluated by morphology, presence of genetic abnormalities and leukocyte count at diagnosis. Cytomorphology gives a favorable prognosis to FAB M3 and M3v leukemias, associated with the *PML-RARA* chimeric transcript, and a poor prognosis to the phenotype M7 in the absence of Down syndrome. The cytogenetics and molecular biology allow to identify the isolated anomalies of the Core Binding Factor (CBF) defining the standard risk group. Moreover, the leukocyte count on peripheral blood at the onset of

leukemia allows to distinguish the hyperleukocytic forms (more than 200,000 elements per mm^3), related to premature mortality[3].

Regarding the response to therapy, the early achievement of complete remission seems to be correlated with a better prognosis. However, it can not be considered valid in all cases, as in the translocation t(6;11)(q27;q23) the majority of patients achieved complete remission, but then relapsed with poor prognosis. With the advent of molecular genetics and flow cytometry, the concept of complete remission has changed, so that the presence of molecular or immunophenotypic minimal residual disease has a clear negative prognostic significance as a signal of poor response to therapy[23].

The *MLL* leukemia

Among high risk patients are classified those characterized by *MLL* rearrangements. The strong association of abnormalities in the chromosomal region 11q23 with leukemia has led to the identification of the *MLL* gene, also called *ALL-1*, *HTRX*, *HRX* or *TRX1*. It is the biggest regulator of the expression of the class I *HOMEOBOX* (*HOX*) genes and plays a key role in regulating hematopoietic development: altered pattern of activity of *MLL*, in fact, can cause abnormal expression of *HOX* genes in hematopoietic stem cells, causing a block of maturation and in some cases the development of leukemia[25].

The *MLL* gene is around 89 kb (37 exons)[26] and its cDNA is 11.9 kb[27]. It encodes a multi-domain protein of about 430 kDa (3969 residues)[28,29]. The MLL protein is processed in the cytosol (before entering the nucleus) at post-transcriptional level by TASPASE1, producing two polypeptides (P180 and P300). *In vitro* binding studies indicate physical association between the two segments, comprising residues 1979-2130 and 3613-3876. This therefore implies an association between P300 and P180 in the MLL complex[30]. The cut during the process physically separates the major functions of MLL: P180 contains the SET domain that methylates H3-K4 and a domain capable of activating transcription (TAD); P300 includes the AT-hook domains for the DNA binding, a bromo-domain that binds acetylated lysine on histone H4[31,32], a zinc finger domain (PHD) and a region with homology to DNA methyltransferase (Figure 3).

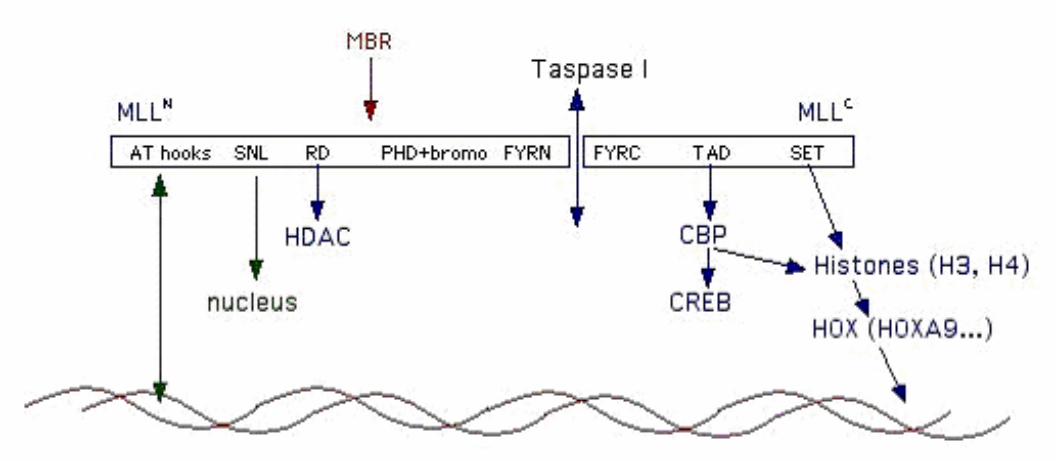

Figure 3. Structure of MLL[87]. MLL^N presents three AT-hook domains that bind DNA, two nuclear localization signals (SNL), two repression domains (or CXXC, cysteine-methyltransferase) and four plants homeodomains and a bromo-domain involved in protein-protein interactions; MLL^C has a transactivation domain (TAD) and a SET domain with methyltransferase activity; MBR: major breakpoint region.

The MLL protein processing allows the formation of a spatial configuration that permits all the interactions of MLL with other proteins and DNA. As mentioned above, MLL is a transcription regulation factor that can interact with more than 30 proteins, including components of the SWI/SNF complex for chromatin remodeling and of the TFIID transcriptional complex. In addition, MLL binds the *HOX* genes promoters through histone acetylation and methylation.

The normal MLL protein is required for the maintenance of a normal gene expression profile and a normal developmental program during embryogenesis. Aberrant proteins resulting from translocations, duplications or amplifications of *MLL* gene cause an abnormal gene expression profile and an aberrant differentiation program, which may result in leukemia development by different mechanisms (Figure 4).

In leukemias with 11q23 translocations are involved the 5-11 exons of *MLL* gene. About 1400 N-terminal amino acids of MLL, including the AT-hook motives and the region of homology to DNA methyltransferase, are fused at the C-terminal portion of various partner genes[33,34] (Figure 5).

To date, more than 50 fusion partners of *MLL* have been cloned and, in any type of leukemia, *MLL* can be fused to a large number of genes with different chromosomal locations (Figure 6), leading to both acute myeloid and lymphoid tumors (*MLL* stands for *"mixed lineage leukemia"*)[35].

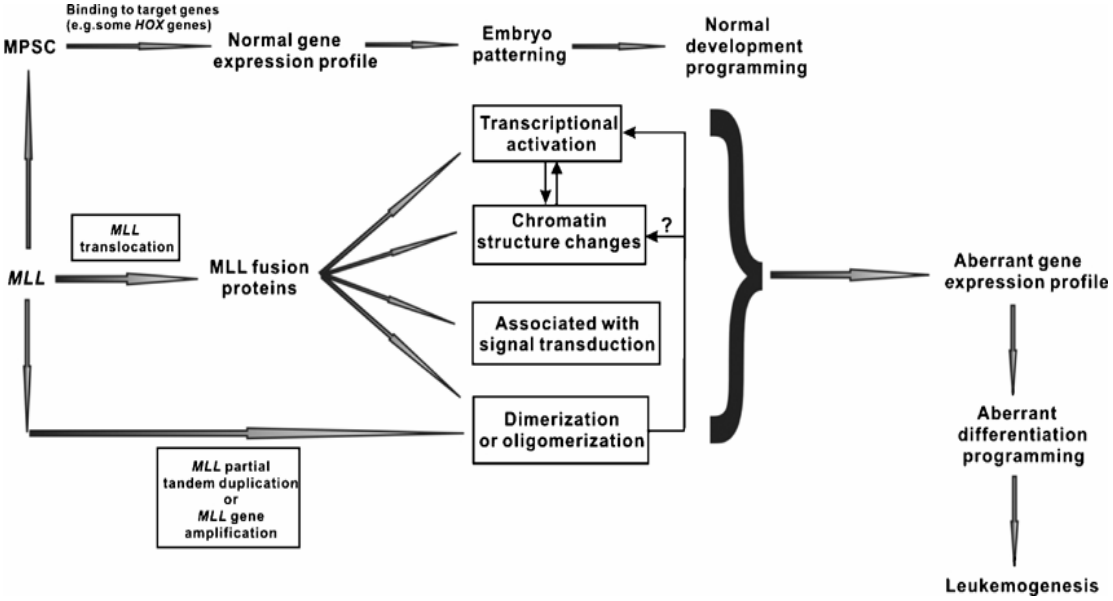

Figure 4. MLL normally regulates gene expression and allows the proper embryonic development. Translocations, duplications and amplifications involving the *MLL* gene lead to aberrant gene expression due to various mechanisms, such as abnormal transcriptional activation, a change in chromatin structure or a wrong translation of the signal. The consequences of these events may result in the development of leukemia[88].

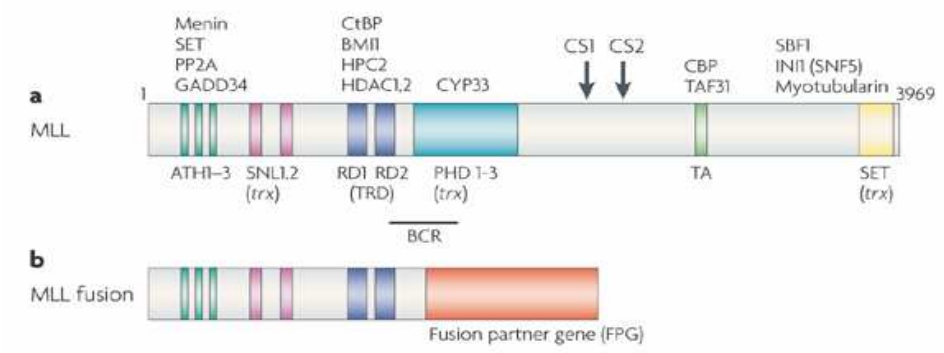

Figure 5. General structure of the *MLL* fusion with one of its partner genes. a) normal *MLL* with all its functional domains, b) example of fusion between the N-terminus of *MLL* and the C-terminal fusion gene[90].

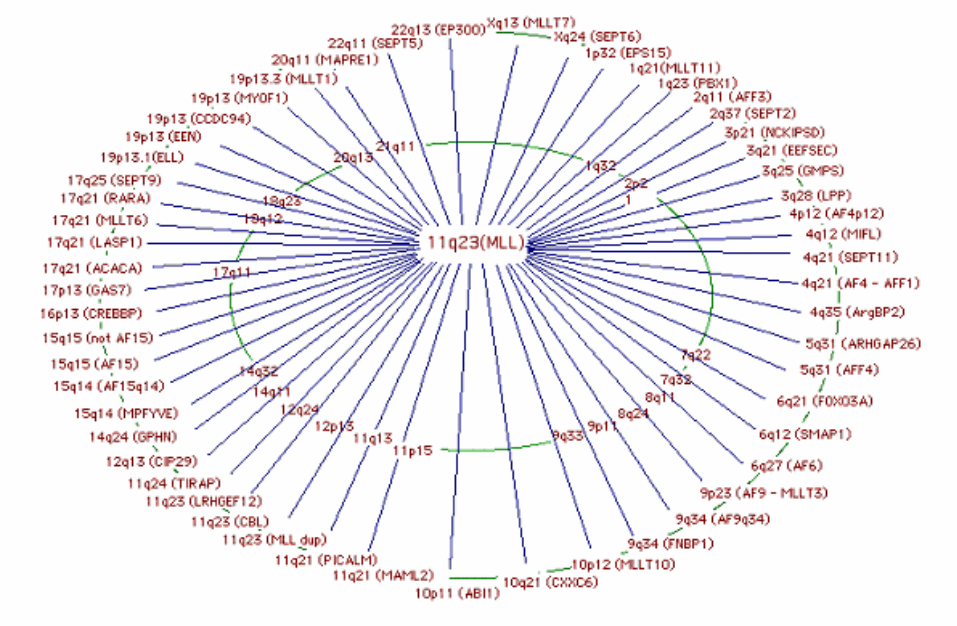

Figure 6. Diagram of all *MLL* partner genes and their chromosomal locations[87].

Patients with *MLL*-rearranged acute leukemia do not exceed 800 cases per year in Europe (about 300 children and 500 adults). The most frequent rearrangements are t(4;11)(q21;q23) involving the gene *MLLT2* (*AF4*), t(9;11)(p22;q23) with the gene *MLLT3* (*AF9*), t(10;11)(p12;q23) with *MLLT10* (*AF10*) and t(6;11)(q27;q23) with *MLLT4* (*AF6*). While t(4;11) is a tipical *MLL*-translocation of ALL with a very low percentage of AML cases, the three most frequent translocations involved *AF6*, *AF9* and *AF10* genes (Figure 7).

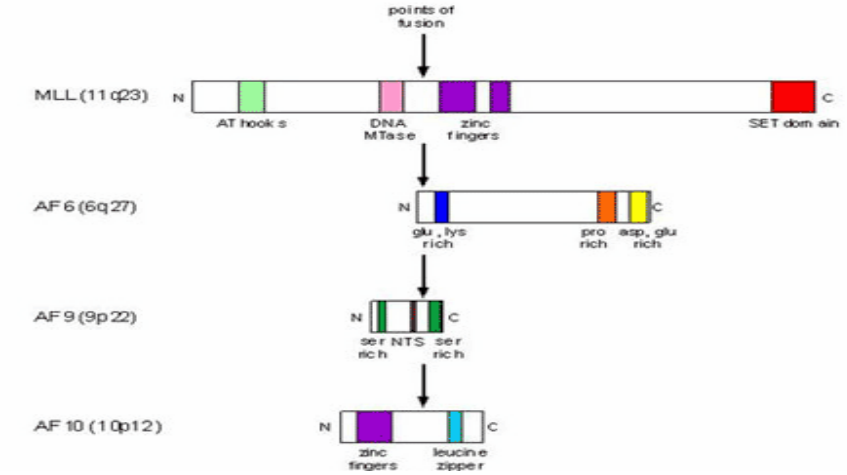

Figure 7. Principal *MLL* fusion genes in AML. Arrows indicate breakpoints[89].

It's interesting to note that the most frequent *MLL* partner genes coding for nuclear proteins (AF4, AF9, AF10) belong to the same cellular network. This indicates that the partner genes are not selected randomly, but selected according to the function.

The analysis of several *MLL* fusion partner genes can help to classify them for subcellular localization, cell function, specific structures of protein domains and ability to interact with other proteins. These classifications must be implemented by functional studies to demonstrate the oncogenicity of the different *MLL* fusions in hematopoietic cells. This will help to classify the wide variety of *MLL* translocations in different risk groups and thus lead to a better stratification and treatment of leukemic patients[35].

The *AF6* gene and the t(6;11) translocation

AF6, a *MLL* fusion partner site on chromosome 6q27[33], covers approximately 140 kb of genomic DNA and consists of 32 exons. It's transcribed into an mRNA of 8 kb, which encodes for a multi-domain protein tumor-suppressor-like of 1612 amino acids. This protein has no similarity with the products of other known partner genes of *MLL*[36] and its N-terminal part contains two RAS-association domains (RA), a forkhead-associated domain and a region of homology to the myosin V class (DIL). In the C-terminus, instead, there are a PSD-95-Dlg1-ZO1 domain (PDZ) and a region rich in proline (Figure 8).

AF6 binds cytoplasmic proteins, such as small GTPases RAS and RAP1, in a PDZ-independent way and colocalizes with tight junctions and adhesion junctions, connecting the junctional complexes to the actin cortical cytoskeleton[37]. It was demonstrated that the BCR kinase phosphorylates AF6, binds its PDZ domain and allows it to interact with RAS to prevent downstream signaling. This mechanism could be responsible for maintaining cells in a non-proliferative state[37].

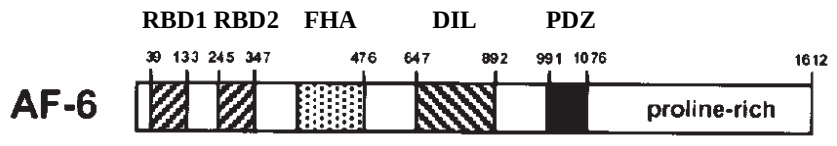

Figure 8. Diagram showing the domain structure of AF6: two RAS binding domains (RBD), a forkhead-associated domain (FHA), a region of homology to the myosin V class (DIL), a PDZ domain, a region rich in proline[91].

In t(6;11)(q27;q23) leukemia the breakpoint is always at the same nucleotide (between nt 27760 and 27761), downstream of the initiation codon and within intron 1[27,38]. In the chimeric transcript, *AF6* exon 2 is fused to exon 6 or 7 of *MLL*[39], therefore it includes the entire *AF6* with exception for the N-terminal 35 amino acids, maintaining both the RAS-interaction domains and the PDZ domain (Figure 9).

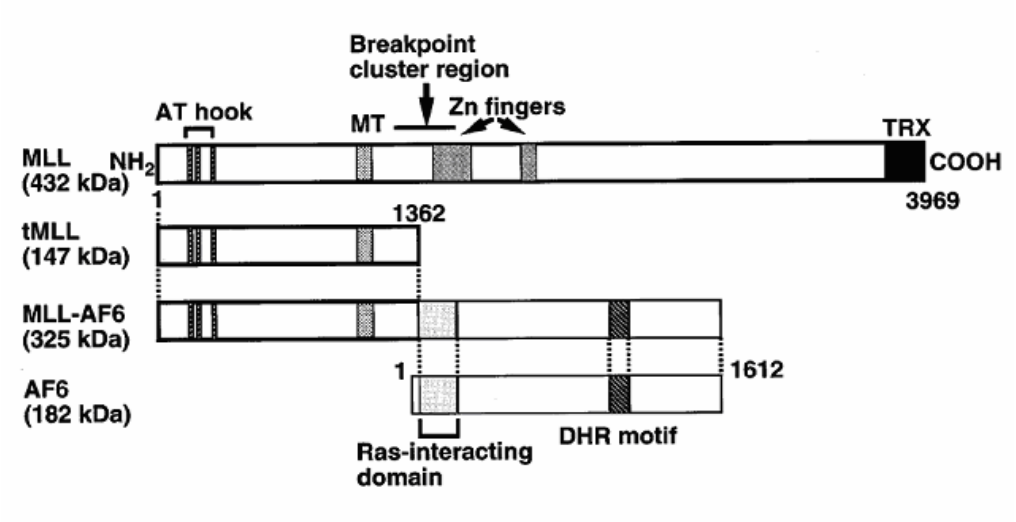

Figure 9. Structure of *MLL*, *tMLL*, *MLL-AF6* and *AF6*[34]. The fusion transcript retains the AT hook domains and the region of homology to the methyltransferase of *MLL* and the RAS-interaction and the DHR motif (Dlg homology repeat) of *AF6*.

Although AF6 is found in the cytoplasm, the fusion protein has nuclear localization. This suggests that the site of action for the product of chimeric *MLL* is in the nucleus and that the N-terminal portion of *MLL* defines their localization[34]. However, it remains unclear if the chimeric product works as an oncogene or as a dominant negative that inhibits the normal function of MLL.

In general, chromosomal abnormalities on 11q23 are indicators of poor prognosis and short event-free survival. Among the rearrangements of *MLL* involved in leukemogenesis, the t(6;11) translocation is one of the less common, but appears in the 10 % of cases. A study published on 26 t(6;11)-positive AML patients (age range 3-72 years) showed that complete remission was achieved in 23 out of 26 patients, the median survival free of events was only 7-8 months and overall survival of 12 months, indicating a poor prognosis for these patients[40].

The Ras pathway in hematopoiesis and leukemia

The mitogen-activated protein kinase (MAPK) pathway can transmit extracellular signals into the cell resulting in a variety of biological functions. Following stimulation, signals are transduced into the cell via a series of protein phosphorylation (Figure 10). These biochemical signals result in physiological cellular responses such as cell proliferation, cellular senescence, cell survival and differentiation[41-43].

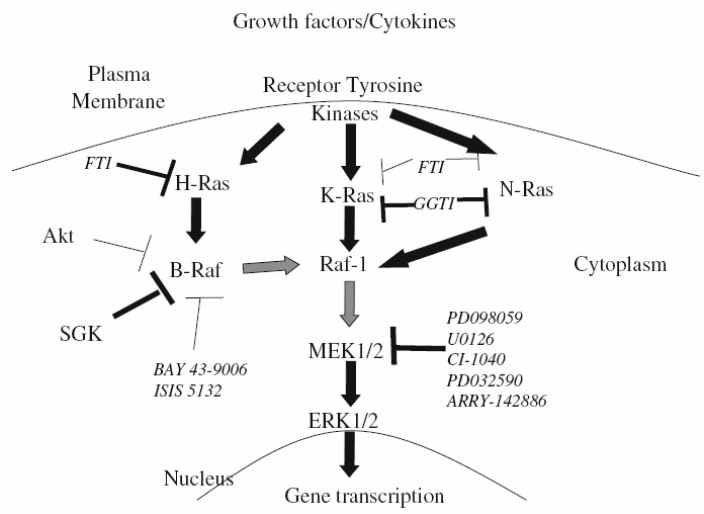

Figure 10. Growth factors stimulation promotes RAS activation and the initiation of the downstream phosphorylating cascade[41].

Stimulation with mitogens, cytokines and growth factors has been shown to activate RAS, a member of the low-molecular weight GTP (guanine triphosphate)-binding family of proteins. There are three *RAS* genes that encode four highly homologous 21 kDa proteins: *H-RAS*, *N-RAS*, *K-RAS4A* and *K-RAS4B*[44]. In the inactive form, RAS is bound to guanine diphosphate (GDP) and is localized to the plasma membrane. Following ligand binding, upstream guanine exchange factors (GEFs) are activated, bind to RAS and trigger the release of GDP. Cellular levels of GTP are approximately ten times higher than GDP, so RAS is more likely to bind GTP following GDP release. Other regulators are GTPase-activating proteins (GAPs) that increase GTPase hydrolyzing ability of RAS (Figure 11). GTP binding triggers a conformational change that increases the affinity for effectors such as RAF[45-48].

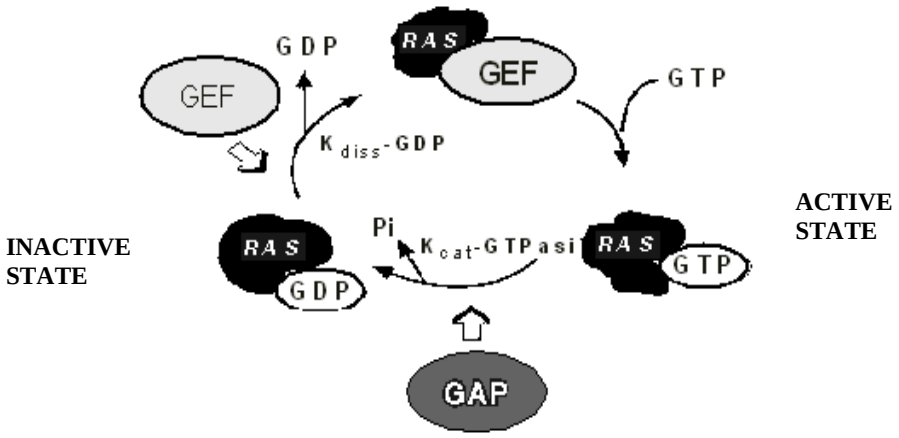

Figure 11. Regulation of RAS activation: guanine exchange factors (GEFs or GNRFs) bind to inactivated RAS and trigger the release of GDP, while GTPase-activating proteins (GAPs) increase GTPase hydrolyzing ability of activated RAS[92]

RAF presents three isoforms (A-RAF, B-RAF and c-RAF) with a RAS-binding domain (RBD) and a catalytic domain for the RAF substrate MEK[49-52]. There are five genes in the *MEK* family (*MEK1*, *MEK2*, *MEK3*, *MEK4*, *MEK5*) and all five are dual specificity kinases, meaning they are able to phosphorylate serine/threonine residues along with tyrosine residues[50]. Of those, MEK1 and MEK2 are activated downstream of RAS and RAF by phosphorylation of two serine residues located within the activation domain. MEK plays a role in promoting cellular proliferation and inhibiting apoptosis through transcriptional mechanisms as well as post-translational mechanisms[53]. Although there are four members of the ERK family (ERK1/2, JNK1/2/3, p38 MAPK and ERK5), ERK1/2 is the only known substrate for MEK1/2[50,54]. Despite all the similarities, ERK1 and ERK2 have been found to have very different functions. The primary function of ERK2 is to promote cellular proliferation, while ERK1 functions as a negative regulator of ERK2[55-57]. However, a recent study showed that ERK1 and ERK2 have redundant roles with regards to proliferation[58].

During hematopoiesis, hematopoietic stem cells (HSCs) gradually loose their self-renewal capacity and commit to either the lymphoid lineage or the myeloid lineage[59]. A fine balance between self-renewal, proliferation and differentiation is critical for hematopoietic homeostasis, so modulating MEK/ERK signaling is important in achieving the correct balance in HSCs[60]. MAPK pathway activation can occur downstream of many cytokine receptors, especially those involved in myelopoiesis resulting in cellular differentiation or proliferation[61,62].

Activating *RAS* mutations have been observed in approximately 30 % of all cancers[63]. Early oncogene studies suggested that mutated *RAS* was insufficient to give rise to cancer and additional oncogenes had to be mutated for a transformation event to occur[64-69]. *K-RAS* is the most frequently activated *RAS* oncogene in human tumors and it have been found in 90 % of pancreatic cancers, 50 % of colon cancers, 25 % of lung adenocarcinomas, 10-15 % of AML, but have not been found in prostate and breast tumors, suggesting that tumor induction is dependent on cell type[63,70]. Mice expressing the mutant form of *K-RAS* developed myeloproliferative diseases (MPD) that closely resemble chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML) in humans[71,72]. While N-RAS has redundant functions during normal development, activating mutations have often been linked to a broad range of hematological cancers. *N-RAS* mutations have been identified in both lymphoid and myeloid malignacies, of which 70 % harbor mutations. Frequency of *N-RAS* mutations in AML is approximately 20-25 % and 30 % in JMML. Approximately 10 % of T lymphoma and T leukemias have *N-RAS* or *K-RAS* mutations. However, 50 % of these diseases have increased RAS signaling, suggesting that RAS pathway plays a very important role in disease[73-76]. Studies on AML patient samples showed that ERK was activated only in 9 % of samples that harbored *N-RAS* mutations, suggesting that oncogenic N-RAS proliferation signaling proceeds through channels other than the canonical MAPK pathway[77,78]. Several studies show that activated ERK has been found in 51-83 % of AML cases, also in patients without *RAS* mutations[79,80]. ERK1/2 has also been shown to be an important prognostic indicator for B- and T-ALL patients. ERK1/2 activation was found to be correlated with higher white blood cell counts and decreased likelihood of complete remission[81].

Beyond RAS, the aberrant function of an increasing list of RAS superfamily proteins has been implicated in human cancer growth and development. The deregulated gene expression and/or deregulated protein function of GEFs and GAPs has been found to have important roles in cancer. As GEF activation is the most common mechanism for signal-mediated GTPase activation, the theme that has emerged is that aberrant signaling from growth factor receptors leads to aberrant GEF regulation, which contributes to persistent RAS activation[82]. Moreover, abnormal activation of GEFs by upregulated gene expression or the loss of GAP activity by mutations allow uncontrolled GTPase activity and can promote cancer development[83,84].

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PART 1

MLL PARTNER GENES DRIVE DISTINCT GENE EXPRESSION PROFILES AND GENOMIC ALTERATIONS IN PEDIATRIC ACUTE MYELOID LEUKEMIA. AN AIEOP STUDY

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Leukemia in press

ABSTRACT

In the AIEOP (Associazione Italiana Ematologia Ed Oncologia Pediatrica) protocol LAM-2002/01 treatment, children affected by acute myeloid leukemia (AML) were stratified by using a risk group classification based on a combination of specific genetic abnormalities and response to treatment. RT-PCR of nine translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene were analyzed in 741 AML patients collected from 2000 to 2008, and their biological and clinical significance has been investigated.

MLL rearrangements were identified in 77/741 patients (10.4 %). Significantly different clinical outcomes depending on the *MLL* translocation partner were confirmed. Gene expression profiling (GEP) and CGH-array discovered novel biological characteristics of *MLL*-AML subgroups. GEP unsupervised analysis clustered *MLL*-AML patients according to translocation partners more strongly than FAB-morphology classification. ANOVA analysis defined significant gene expression signatures dependent on the *MLL*-partner gene. Array-CGH analysis established high levels of DNA amplification (75 %) with respect to deletions (25 %) for MLL-AML, and the del(12p) to be considered as a novel feature for leukemia stratification. Patients with *MLL-AF6* displayed an adverse outcome, a specific GEP signature, as well as the highest DNA imbalances with additional recurrent genomic rearrangements in 12p, 11q and 6q.

Finally, we categorized the pediatric *MLL*-rearranged leukemia for novel biological and clinical aspects.

INTRODUCTION

Childhood Acute Myeloid Leukemia (AML) is a heterogeneous disease with an overall poor treatment outcome compared to Acute Lymphoid Leukemia (ALL). Marked differences in the outcome of AML patients are related to the presence of specific genetic aberrations[1-3]. A subset of high-risk AML patients is characterized by rearrangements involving the *Mixed Lineage Leukemia* gene (*MLL*) on chromosome 11q23[4]. More than 40 different translocation fusion partners of *MLL* have been identified in AML at diagnosis; however, only 5 partner genes account for over 90 % of *MLL*-translocated pediatric AML (*AF9*, *AF10*, *AF6*, *ENL*, *ELL*)[5-7]. Recently, Balgobind et al.[8] published results from a collaborative international study group describing different clinical outcomes for *MLL*-

11q23 translocation partner genes. Here, we investigate the role of various *MLL*-translocations in the Italian AML patients enrolled in recent AML protocols of the AIEOP (Italian Association of Pediatric Hematology and Oncology), currently stratified within the high-risk group. Since genomic aberrations are used to stratify patients, we used gene expression profiling (GEP) to characterize subgroups[9,10]. GEP distinct expression signatures for the major subtypes of AML t(8;21), inv(16) and t(15;17)[11,12], as well as for *MLL*-AML[13-16] were previously reported, but did not involve enough specimens to recognize distinct *MLL*-translocations. Here we used GEP on a large set of *MLL*-AML patients in order to characterize specific *MLL* subtypes of AML and to find specific features of cell biology. Cytogenetic analysis of AML de novo, which is currently used for risk stratification in Italian protocols, is here matched to a-CGH analysis and supports a new interpretation of *MLL*-rearranged AML based on novel features to be considered for risk stratification.

MATERIALS AND METHODS

Patients

Bone marrow (BM) samples of children < 18 years old with newly diagnosed AML were collected from 2000 to 2008. Patients' parents or their legal guardians provided written informed consent following the tenets of the Declaration of Helsinki. The patients analyzed were retrieved from the AIEOP registry that collects data from all children with cancer diagnosed in AIEOP centers in Italy. Patients diagnosed as affected by promyelocitic leukemia, granulocytic sarcoma, secondary AML, myelodysplastic syndrome or Down syndrome, as well as patients with a pretreatment phase longer than 14 days, were excluded from the present study[2].

The initial diagnosis of AML was centrally established according to morphology-FAB classification and immunophenotypic analysis at the laboratory of Pediatric Hematology of the University-Hospital in Padova. Chromosome analysis was performed on BM using standard laboratory procedures. The karyotypes were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 2005). For fluorescence in situ hybridization (FISH) an *MLL* locus specific (LSI) dual color probe for 11q23 (Abbot-Vysis, Downess Grove, IL) was employed. This analysis was performed on metaphases and/or nuclei, when necessary, according to the manufacturer's instructions.

RNA isolation, cDNA synthesis, RT-PCR and sequencing

Total RNA was isolated from BM of all samples using TRIzol following the manufacturer's protocol (Invitrogen, Karlsruhe, Germany); cDNA was synthesized according to the conditions recommended by the European BIOMED-1[17]. Multiplex RT-PCR was used to detect *MLL-AF4*, *MLL-AF9*, *MLL-AF10*, *MLL-AF6*, *MLL-SEPT6*, *MLL-SEPT9*, *MLL-AF1q*, *MLL-ELL* and *MLL-ENL* fusion transcripts. Fusion fragments were purified using a Microcon centrifugal filter (Millipore Corporation, Billerica, MA) and sequenced according to the BigDye terminator v 3.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) on an Applied Biosystems 310 analyzer. BLAST software (http://www.ncbi.nlm.nih.gov/BLAST) was used for the analysis of sequence data. Quantitative real-time PCR was performed using TaqMan Subygreen master mix (Invitrogen) for *AF6*, *TANC1* and *FLT3* expression to validate arrays. All samples were run in triplicate on a 7900 Real-Time PCR system and analyzed using the relative standard curve method.

RNA isolation and microarray analysis

RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The GeneChip Human Genome U133 Plus 2.0 was used for the microarray experiments; in vitro transcription, hybridization and biotin labeling processes were performed according to the Affymetrix GeneChipR 3' IVT Express Protocol. To generate microarray data (CEL files) default Affymetrix GeneChip Command Console Software (AGCC) was used. The percentage of present genes (mean 45.79 +/- 2.33) and the ratio of GAPDH 3' to 5' (mean 1.08 +/- 0.09) indicated an overall high quality of the samples and the assays. CEL files can be found at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/; Series Accession Number GSE19577).

Data analysis

CEL files were normalized using the Robust Multiarray Averaging (RMA) expression measure of an Affy-R package (http://www.bioconducter.org). Wilcoxon's test was applied on the probes that passed filtering by variance to identify differentially expressed genes in two different groups. Analogously, ANOVA analysis was used to compare the major groups of *MLL*-rearranged patients. All p-values (Wilcoxon's tests and ANOVA) were

obtained using a permutation approach. To control the false discovery rate, multiplicity corrections were used; probes with adjusted p-values less than 0.01 Wilcoxon's tests and less that 0.05 for ANOVA were declared significant. Dendrograms were generated to cluster patients using Ward's method and Euclidean distance, and heat maps were used to highlight associations between clusters of patients and clusters of genes. The Ingenuity pathway software (www.ingenuity.com) was used to identify molecular networks between differently expressed genes. Clinical variables were investigated through Fisher's exact test and the Chi-square test, while Holm's corrections[18] were adopted to control the family wise error rate.

Array CGH (Comparative Genome Hybridization)

Molecular karyotyping was performed using an Agilent Human Genome Microarray Kit 244A following manufacturer's instruction (Agilent Technologies, Santa Clara, CA). Arrays were analyzed using an Agilent scanner and Feature Extraction software V.10.1.1.1. Results were obtained using DNA Analytics software V.4.0.76. DNA sequence information refers to the public UCSC database (Human Genome Browser, March 2006 Assembly (hg18). Anomalies present in approximately 30 % (www.chem.agilent.com) of the cells were the detection limit. Gains or losses ≤ 20 kb were not considered because of limits of technical resolution[19,20].

RESULTS

Incidence of *MLL* molecular rearrangements in pediatric AML and EFS

In the series of 741 Italian children with AML consecutively diagnosed between 2000 and 2008, *MLL*-rearrangements were identified by molecular biology in 77 patients (10.4 %). Type t(9;11)(p22;q23) was found in 37/77 *MLL*-positive patients (48 %); t(10;11)(p12;q23) in 18/77 (23 %); t(6;11)(q27;q23) in 10/77 (13 %); t(11;19)(q23;p13.1) in 3/77 (4 %); t(11;19)(q23;p13.3) in 3/77 (4 %); t(x;11)(q24;q23) in 3/77 (4 %); t(1;11)(p32;q23) in 2/77 (2 %); t(11;17)(q23;q25) in 1/77 (1 %). Type t(4;11)*MLL-AF4* was not found in our cohort of 741 AML patients. The outcome of patients with different 11q23/*MLL*-rearrangements varied significantly. One *MLL*-patients subgroup, identified as t(11;other) in tables and figures, grouping together t(11,19)(q23;p13.3), t(11,19)(q23;p13.1), t(x,11)(q24;q23), t(1;11)(p32;q23), and the t(11;17)(q23;q25) had a

good outcome (74.1 % of 3 y EFS). The t(6;11)(q27;q23) group had a very poor prognosis (23.3 % of 3 y EFS). The t(9;11)(p22;q23) and t(10;11)(p12;q23) were confirmed to be *MLL*-subgroups with intermediate and poor prognosis, as previously described (3 y EFS 55.4 % and 27.2 %, respectively)[8]($p < 0.01$, Figure 1S).

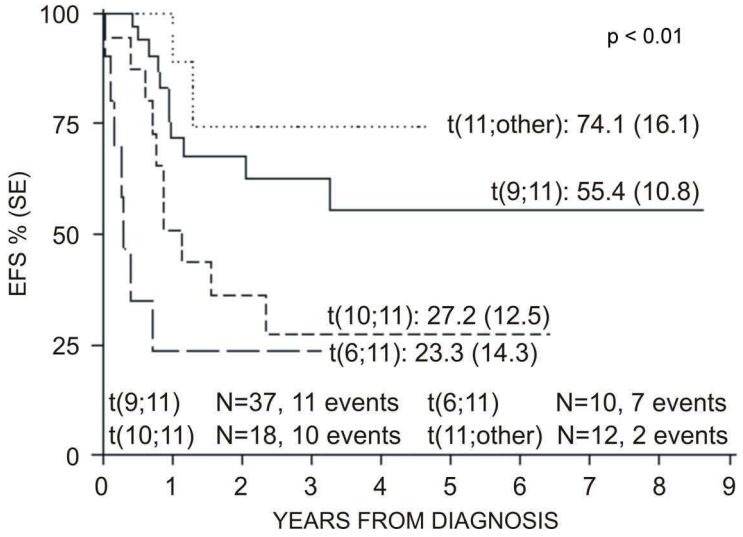

Figure 1S. Event free survival curves of *MLL*-subgroups.

MLL partner gene confers specific clinical and biological features

The identification of a heterogeneous outcome associated with different *MLL*-translocation partners suggested that specific biological features play a distinct role in these subgroups. A complete genome wide analysis was performed on 42 *MLL*-rearranged AML samples for which sufficient stored material was available. Selected patients are described in Table 1. The presence of specific *MLL*-translocations conferred a statistical difference ($p < 0.05$) for age, median WBC, and FAB distribution at diagnosis. Concerning FAB characterization, (67 %) *MLL*-patients had FAB M5 whereas the t(6;11) group was particularly heterogeneous for FAB profiles, including FAB M0, M1, and M4. M7 was found exclusively for t(9;11).

	TOTAL	t(9;11)	t(10;11)	t(6;11)	t(11;19)	11;other	*P
N	42	11	10	11	5	5	
SEX (n= 42) (Male, %)	29 (70 %)	9 (81 %)	5 (50 %)	9 (81 %)	2 (40 %)	4 (80 %)	P=0.488
MEDIAN AGE (years) (N=42)	5.1	2.7	4.7	9.7	3.5	2.3	
<2y, N (%)	22 (52 %)	6 (54 %)	7 (70 %)	1 (10 %)	4 (80 %)	4 (80 %)	
2-9 y, N (%)	7 (16 %)	4 (36 %)	/	3 (27 %)	/	1 (20 %)	
>=10 y, N (%)	13 (32 %)	1 (10 %)	3 (30 %)	7 (63 %)	1 (20 %)	/	
MEDIAN WBC, x109/L (N=37)	26.9	8.2	39.8	76.6	52.3	58	
<20 x 109/L N (%)	12 (32 %)	7 (100 %)	2 (20 %)	2 (20 %)	1 (20 %)	/	
20<100 x 109/L N (%)	18 (49 %)	/	8 (80 %)	5 (50 %)	1 (20 %)	4 (80 %)	
>=100 x 109/L N (%)	7 (19 %)	/	/	3 (30 %)	3 (60 %)	1 (20 %)	p=0.0036
FAB-type, (N=42)							
FAB-M0	1 (2,3 %)	/	/	1 (9 %)	/	/	
FAB-M1	2 (4,7 %)	/	1 (10 %)	1 (9 %)	/	/	
FAB-M2	/	/	/	/	/	/	
FAB-M3	/	/	/	/	/	/	
FAB-M4	5 (12 %)	/	/	2 (18 %)	/	3 (60 %)	
FAB-M5	28 (66,7 %)	5 (45 %)	9 (90 %)	7 (64 %)	5 (100 %)	2 (40 %)	
FAB-M6	/	/	/	/	/	/	
FAB-M7	6 (14,3 %)	6 (55 %)	/	/	/	/	p=0.0038
MEDIAN BLAST IN BM (%), (N=40)	69 %	51 %	73 %	81 %	79 %	65 %	p=1
ADDITIONAL CYTOGENETIC ABERRATION (N=40), N (%)	12 (30 %)	4 (33 %)	3 (25 %)	3 (25 %)	/	2 (17 %)	p=1

Table 1. *MLL*-patients clinical features. *P-values are calculated by Fisher's exact test and Chi-square test with Holm's multiple testing corrections.

Distinct gene expression signatures in 11q23/*MLL*-translocation positive AML

Unsupervised analysis of GEP consistently separated 42 *MLL*-rearranged leukemias with respect to partner gene and FAB-subtype (Figure 1). *MLL-AF9*-positive samples were found divided into two major groups, which were related to distinct FAB classes, M7 and M5. Patients with *MLL*-other rearrangements clustered together for the most part, probably related to outcome or for the shared M5 FAB subtype.

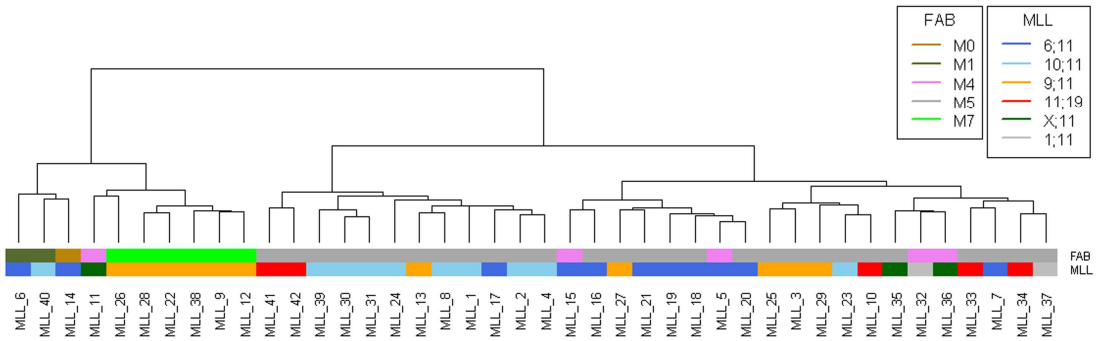

Figure 1. Expression profile analysis of *MLL*-AML positive patients. Unsupervised hierarchical clustering analysis; gene expression profiles of t(6;11) (n = 11); t(9;11) (n = 11); t(10;11) (n = 10) and t(11-other) (t(11;19) n = 5, t(1;11) n = 2 and t(x;11) n = 3) rearranged patients are shown. Dendrogram was obtained using expression data filtered by variance.

The supervised ANOVA analysis identified 229 probe sets that were differentially expressed among the considered *MLL*-subgroups. The apparent homogeneity within *MLL*-rearranged subgroups might be due to FAB affiliation or to the limited number of cases included in the analysis (Figure 2, Table 1Sa). Among the ANOVA predicted genes we highlighted *GAS1* expression, a gene under discussion for its role in stem cell renewal and tumor growth and associated to the two worst prognostic sub-groups, *MLL-AF6* and *MLL-AF10*. Furthermore, heterogeneous expression of *FLT3* in *MLL*-AML needs to be further considered. In particular, t(9;11) cases showed a low *FLT3* expression in patients with FAB M7; whereas it increased significantly for t(9;11) FAB M5 (Figure 3S). These results was confirmed by RQ-PCR (Figure 4S). Considering only the three most prognostically relevant *MLL*-rearranged subgroups (i.e., *MLL-AF9*, *MLL-AF6* and *MLL-AF10*), the supervised ANOVA analysis identified 23 probe sets differentially expressed among these samples (Figure 2S; Table 1Sb).

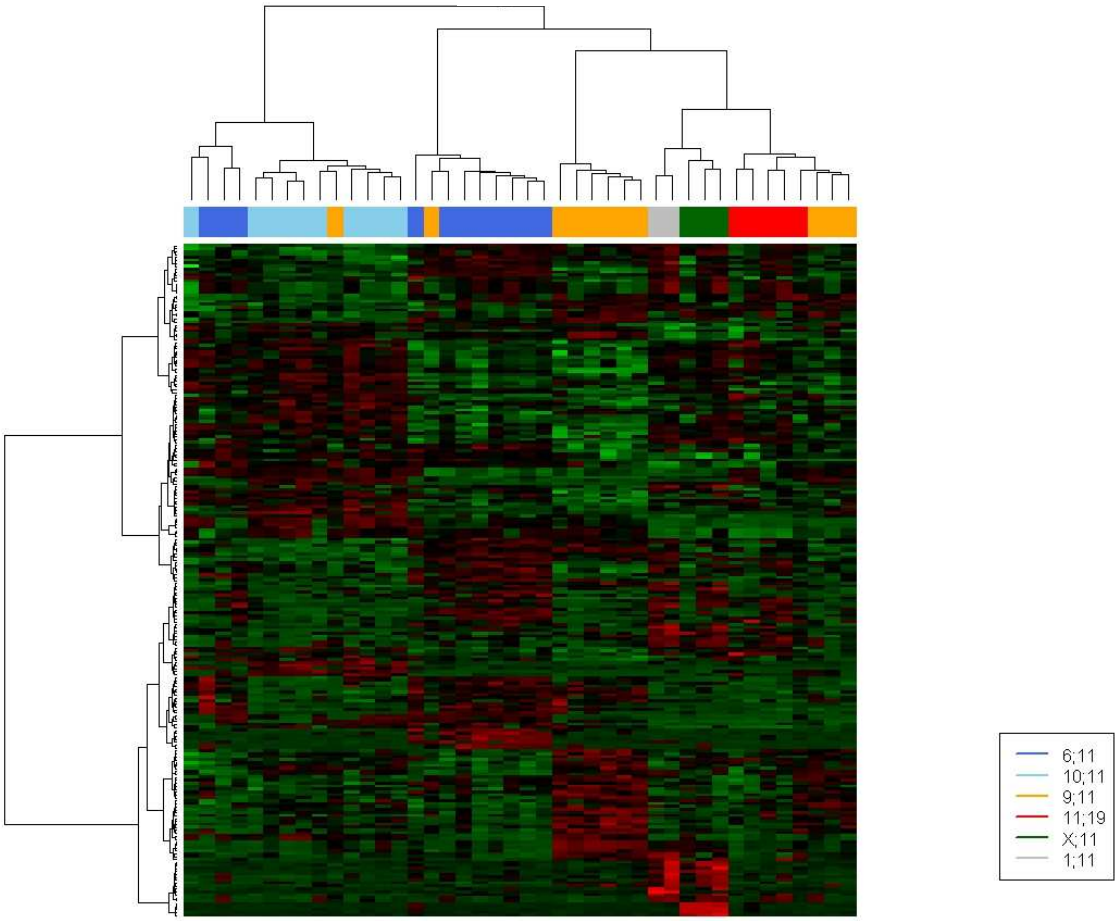

Figure 2. Expression profiles of *MLL*-AML patients. Supervised hierarchical clustering analysis using the 229 probe sets identified by ANOVA analysis among 42 *MLL*-AML patients. The genetic subtypes are indicated below the dendrogram with color codes.

Probe Set ID	Gene Symbol
1553808_a_at	*NKX2-3*
1554876_a_at	*S100Z*
1554910_at	*PRKD3*
1555392_at	*LOC100128868*
1555968_a_at	---
1556064_at	*LOC284926*
1557014_a_at	*C9orf122*
1557543_at	---
1559391_s_at	---
1560999_a_at	---
1561654_at	---
1566557_at	*FLJ90757*
1566558_x_at	*FLJ90757*
1569599_at	*SAMSN1*
1569652_at	*MLLT3*
200602_at	*APP*
200986_at	*SERPING1*
201037_at	*PFKP*
201087_at	*PXN*
201718_s_at	*EPB41L2*
201719_s_at	*EPB41L2*
201904_s_at	*CTDSPL*
202286_s_at	*TACSTD2*
202351_at	*ITGAV*
202718_at	*IGFBP2*
202759_s_at	*AKAP2 /// PALM2 /// PALM2-AKAP2*
202760_s_at	*PALM2-AKAP2*
202762_at	*ROCK2*
202888_s_at	*ANPEP*
202975_s_at	*RHOBTB3*
202976_s_at	*RHOBTB3*
203069_at	*SV2A*
203080_s_at	*BAZ2B*
203355_s_at	*PSD3*
203627_at	*IGF1R*
203817_at	*GUCY1B3*
203948_s_at	*MPO*
203949_at	*MPO*
204070_at	*RARRES3*
204099_at	*SMARCD3*
204214_s_at	*RAB32*
204304_s_at	*PROM1*
204351_at	*S100P*
204429_s_at	*SLC2A5*
204430_s_at	*SLC2A5*
204457_s_at	*GAS1*
204497_at	*ADCY9*
204674_at	*LRMP*
204751_x_at	*DSC2*
204840_s_at	*EEA1*
204917_s_at	*MLLT3*
204918_s_at	*MLLT3*

Table 1Sa. Differentially expressed probe sets among patients harbouring *MLL*-fusion genes.

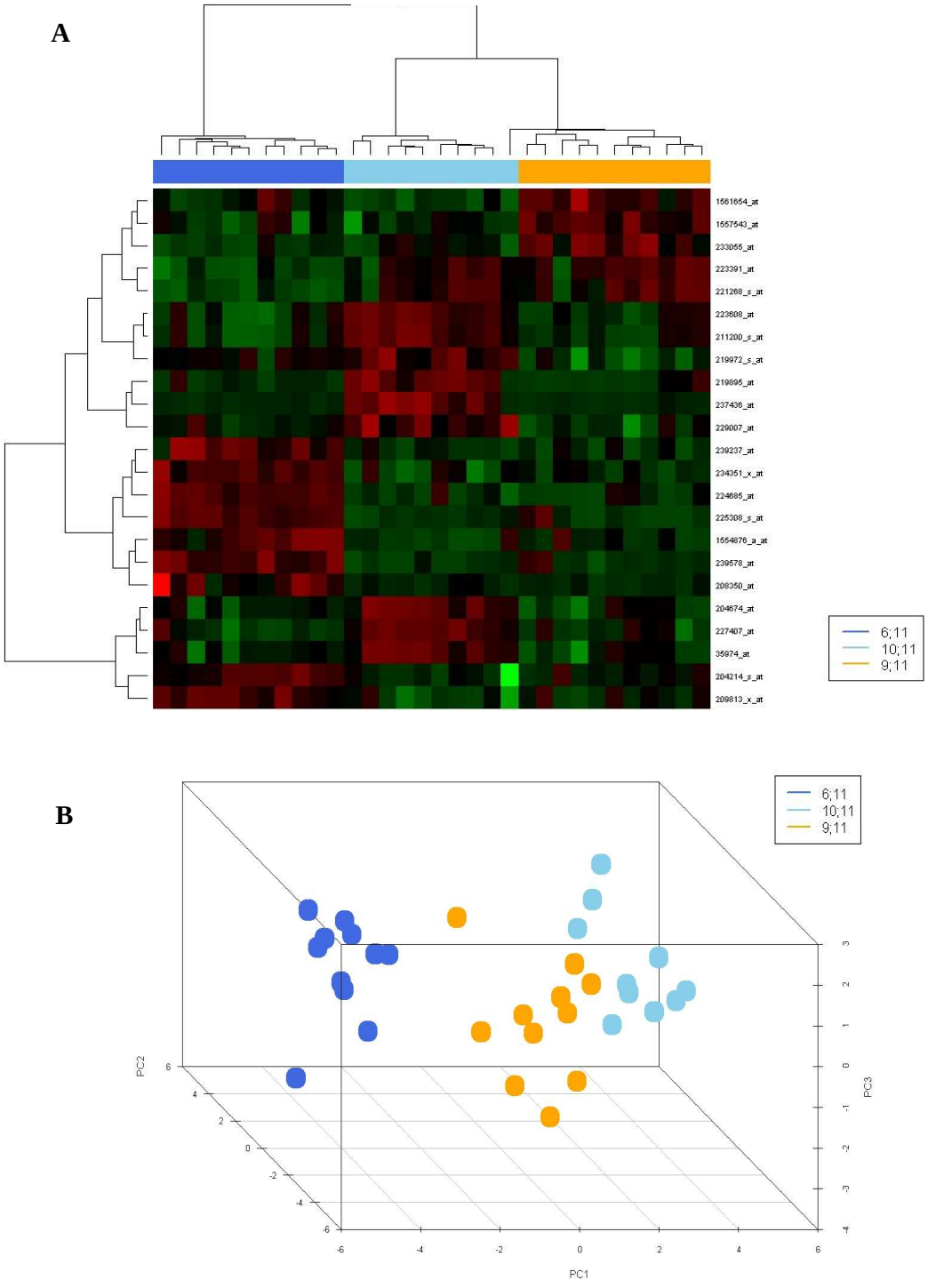

Figure 2S. Hierarchical Clustering Analysis (A) and Principal Component Analysis (B) using 23 probe sets differentially expressed among the three most prognostically relevant *MLL*-rearranged subgroups (i.e., *MLL-AF9*, *MLL-AF6* and *MLL-AF10*) by supervised ANOVA analysis.

Probe sets ID	Gene Symbol
1554876_a_at	*S100Z*
1557543_at	
1561654_at	
204214_s_at	*RAB32*
204674_at	*LRMP*
208350_at	*CSN1S1*
209813_x_at	*TARP*
211200_s_at	*EFCAB2*
219895_at	*FAM70A*
219972_s_at	*C14orf135*
221268_s_at	*SGPP1*
223391_at	*SGPP1*
223608_at	*EFCAB2*
224685_at	*MLLT4*
225308_s_at	*TANC1*
227407_at	*TAPT1*
229007_at	*LOC283788*
233055_at	
234351_x_at	*TRPS1*
237436_at	*LOC100129656*
239237_at	
239578_at	
35974_at	*LRMP*

p value ≤ 0.05 is corrected with muliplicity corrections refer to Benjamini-Hochberg procedure

Table 1Sb. Differentially expressed probe sets among the 3 prognostically most important *MLL* fusion genes (*MLL-AF6, MLL-AF9, MLL-AF10*).

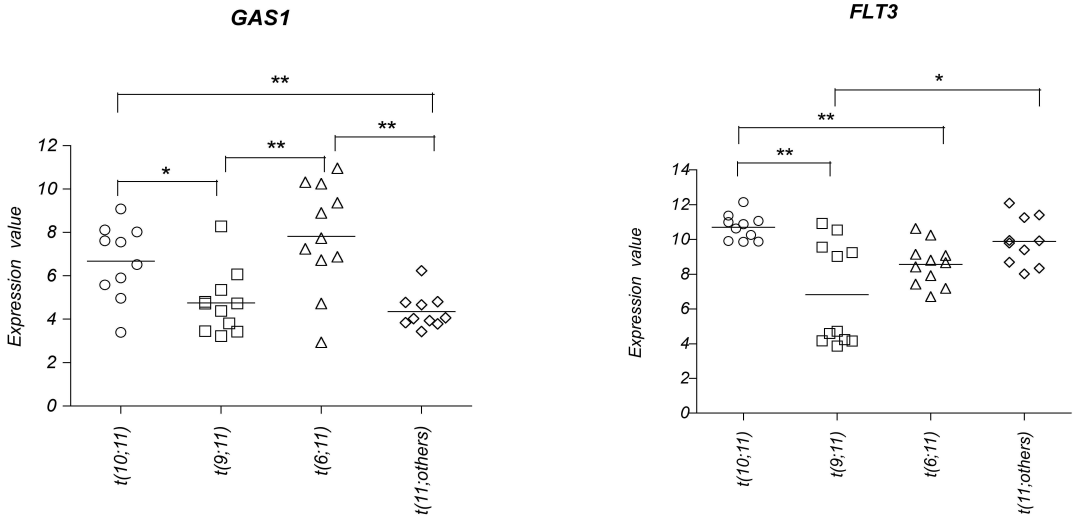

Figure 3S. *GAS1* and *FLT3* expression in *MLL*-subgroups. GAS1 gene is associated to the two worst prognostic sub-groups, *MLL-AF6* and *MLL-AF10*, whereas *FLT3* has an heterogeneous expression among subgroups, showing a low level of expression in t(9;11) patients with FAB M7 and a significantly increased expression for t(9;11) FAB M5.

Gene expression profile of *MLL-AF6*-positive leukemias

The supervised analysis of each *MLL*-partner gene compared to the rest of *MLL*-translocations showed a specific and significant gene expression signature exclusively for *MLL-AF6* (Figure 3), supporting that this translocation might be biologically different. The most frequently selected candidates with higher expression were genes involved in cytokine-cytokine receptor interaction (*AF6*, *TANC1*, *IL12R2*), cell-cell junction and membrane modeling (*MLL2*, *LTK*, *RAB33A*, *NQO*), together with genes involved in typical pathways of nucleotide/RNA binding, transcription factor activity, and protein control, all previously found to be inappropriately recruited by other *MLL* nuclear fusion proteins (Table 1Sc). *AF6* and *TANC1* were confirmed to be upregulated in *MLL-AF6*-positive patients also by real quantitative PCR (Figure 4S).

Despite GEP ability to distinguish *MLL-AF6*, a typical phenotype-related signature by cluster differentiation markers could not be found for t(6;11) rearrangement, although we observed a downregulation of *NG2* expression with respect to other *MLL*-positive patients (data not shown).

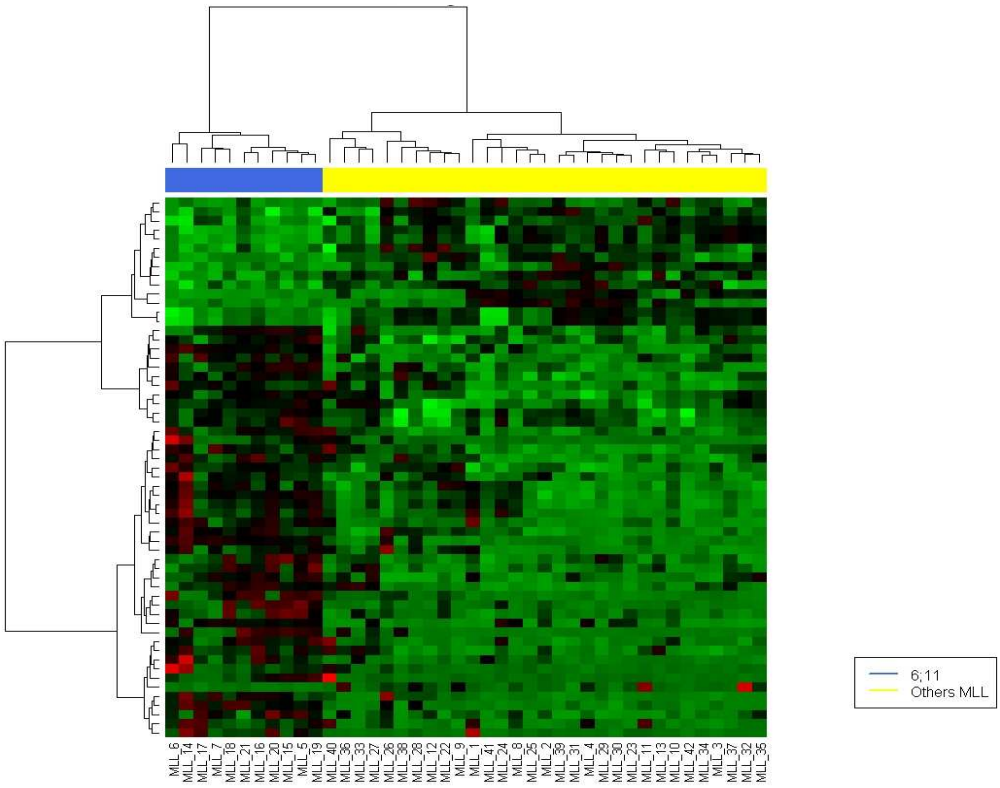

Figure 3. Heat map of the most differentially expressed genes between *MLL-AF6* and all *MLL*-rearrangements studied. The 59 probe sets differently expressed between the two groups were identified by gene selection based on Wilcoxon's test. 44 probe sets (33 known genes) resulted up-regulated and 15 probe sets (10 known genes) resulted down-regulated in *MLL-AF6* patients. The two analyzed groups are indicated with color codes.

Probe stes ID	Gene Symbol	Cytoband	PvalueBHUP	Log2MeanChange DOWN
221268_s_at	*SGPP1*	14q23.2	0,0055	0,6831
223391_at	*SGPP1*	14q23.2	0,0055	0,6026
206039_at	*RAB33A*	Xq25	0,0084	0,3859
234650_at			0,0219	0,3757
244610_x_at			0,0234	0,4227
203819_s_at	*IGF2BP3*	7p11	0,0252	0,4853
216493_s_at	*IGF2BP3*	7p11	0,0257	0,4162
239742_at	*TULP4*	6q25-q26	0,0257	0,3139
203820_s_at	*IGF2BP3*	7p11	0,0258	0,4687
228551_at	*DENND5B*	12p11.21	0,0310	0,3029
220010_at	*KCNE1L*	Xq22.3	0,0385	0,9084
226123_at	*CHD7*	8q12.2	0,0388	0,2992
227173_s_at	*BACH2*	6q15	0,0426	0,2498
207106_s_at	*LTK*	15q15.1-q21.1	0,0441	0,3017
220057_at	*XAGE1D*	Xp11.22	0,0468	0,5536

Probe Sets ID	Gene Symbol	Cytoband	PvalueBHDOWN	Log2MeanChange UP
207455_at	*P2RY1*	3q25.2	0,0055	-0,7229
224685_at	*MLLT4*	6q27	0,0055	-0,6442
225308_s_at	*TANC1*	2q24.1-q24.2	0,0055	-1,2668
231925_at			0,0055	-0,8016
234351_x_at	*TRPS1*	8q24.12	0,0055	-0,5090
239578_at			0,0055	-0,5872
240766_at	*IL23A*	12q13.2	0,0055	-0,6333
241133_at			0,0055	-0,7086
205534_at	*PCDH7*	4p15	0,0084	-1,1811
206999_at	*IL12RB2*	1p31.3-p31.2	0,0084	-0,8266
224218_s_at	*TRPS1*	8q24.12	0,0109	-0,6210
226132_s_at	*MANEAL*	1p34.3	0,0109	-0,4588
218502_s_at	*TRPS1*	8q24.12	0,0129	-0,5657
238599_at	*IRAK1BP1*	6q14-q15	0,0129	-0,5139
230266_at	*RAB7B*	1q32	0,0152	-0,3811
213201_s_at	*TNNT1*	19q13.4	0,0201	-0,6283
208350_at	*CSN1S1*	4q21.1	0,0248	-0,5349
225864_at	*FAM84B*	8q24.21	0,0250	-0,4168
239237_at			0,0250	-0,4564
231310_at			0,0252	-0,4767
226961_at	*PRR15*	7p15.1	0,0254	-0,7136
227522_at	*CMBL*	5p15.2	0,0254	-0,3833
213285_at	*TMEM30B*	14q23.1	0,0257	-0,7918
204429_s_at	*SLC2A5*	1p36.2	0,0257	-0,2474
227134_at	*SYTL1*	1p36.11	0,0257	-0,2944
1553183_at	*UMODL1*	21q22.3	0,0257	-0,5131
218795_at	*ACP6*	1q21	0,0258	-0,2468
220141_at	*C11orf63*	11q24.1	0,0331	-0,3932
204099_at	*SMARCD3*	7q35-q36	0,0341	-0,3245
231851_at	*RAVER2*	1p31.3	0,0341	-0,4404
1554876_a_at	*S100Z*	5q13.3	0,0364	-0,6117
235048_at	*FAM169A*	5q13.3	0,0364	-0,4843
1566557_at	*FLJ90757*	17q25.3	0,0381	-0,3439
228904_at	*HOXB3*	17q21.3	0,0392	-0,6152

Table 1Sc. Down-regulated and Up-regulated probe sets in *MLL-AF6* patients versus other *MLL*-fusion genes.

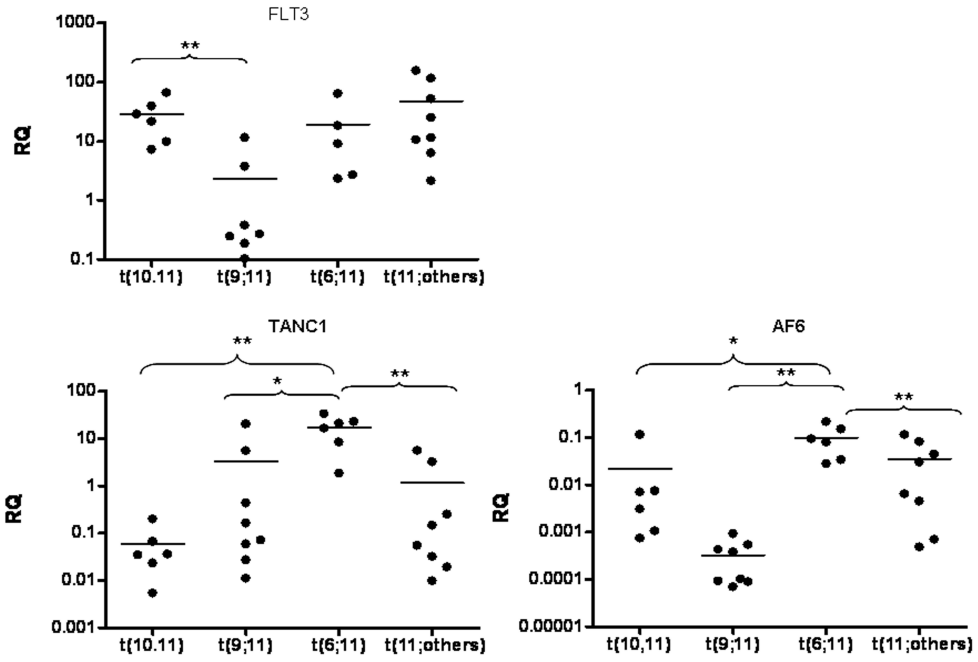

Figure 4S. Expression levels of *FLT3, TANC1* and *AF6* by RQ-PCR. *FLT3* heterogeneous expression in t(9;11) patients was confirmed. *AF6* and *TANC1* were confirmed to be upregulated in *MLL-AF6*-positive patients.

Cytogenetic Analysis and Array based Genomic analysis

Cytogenetic analysis was carried out on BM aspirates from 39 out of 42 patients. Recurrent abnormalities were found in 32 cases. Karyotypes are described in Table 1Sd (Appendix 1). Briefly, the *MLL*-translocation as sole abnormality was seen in 17/32 cases (53 %). The complex karyotype, defined as *MLL*-translocation associated to additional cytogenetic abnormalities, was observed in 13/32 (41 %). Of these, 6 were hyperdiploid karyotypes. In particular, a number of chromosomes ≥ 50 was observed only in the two hyperdiploid t(6;11) patients. Trisomy 19 was observed in 5/6 hyperdiploid patients as a unique numerical anomaly. 2/32 patients had del(12p), one patient carried t(10;11), one t(11;19) translocation. 2/32 patients with complex karyotypes did not show *MLL*-translocation by cytogenetics. FISH analysis was performed in 31/39 patients in order to confirm the presence of *MLL*-rearrangements. 27 cases presented a classic *MLL*-translocation to the partner gene, 3 showed the complete translocation of the *MLL* gene with small split signals in a low percentage of the nuclei, and 1 showed *MLL* gene amplification. Array CGH was performed for 28 *MLL*-rearranged patients. 19/28 cases (68 %) showed genomic abnormalities in this analysis. We identified two recurrent regions of deletion: one at chromosome 12p (4 patients were t(6;11)-translocated), at 11q (5 patients with t(6;11), 1

patient with t(10;11) and 3 patients with t(11;others)), and one at 6q (4 patients with t(6;11), 3 out of 4 were also 12p deleted). Furthermore, one amplified region was found at 11q (Table 2). Among the abnormal cases, the total genomic copy number alteration was 575.8 Mb, 75 % were amplifications and 25 % deletions. Gain and loss of chromosomes were mainly observed in t(6;11)-positive patients, whereas the t(9;11)-cases never showed gain or losses of chromosomes. The group of t(11;other)-translocated patients showed a higher number of Mbs amplified due to a patient with trisomy 8 (146 Mb). In the group of t(10;11) the total loss and gain of Mb was due to a single patient with a complex karyotype. We suggest a minimal common region (mcr) amplified or deleted among the three recurrent abnormalities exclusively in t(6;11)-positive patients (Table 2S) as disease-related regions to be further investigated. We also evaluated the prognosis of novel cytogenetic features here described even if the patient number is too low to perform statistical analysis. Between *MLL*-rearranged patients with del(12p), 5 out of 6 patients relapsed with a median time of 10 monthsand 3 of 5 patients died after relapse. If we excluded the t(6;11) patients with del(12p), the EFS of the t(6;11)-group increased to the 53.3 % (with respect to the 23,3 % discussed above) conferring to the del(12p) an adverse prognosis. Of the 5 patients with hyperdiploid karyotype and trisomy 19, 4 patients relapsed after a median time of 12 months, and 3/4 patients died after relapse.

Translocations	Nº Patients	Abnormal patients	Total genomic deletions (Mb)	Total genomic amplifications (Mb)	Recurrent abnormalities (nº patients)		
					6q	11q	12p
t(6;11)	9	5	112.32 Mb	153.12 Mb	4	5	4
t(9;11)	5	2	0 Mb	81.2 Mb*	0	0	0
t(10;11)	6	5	25.96 Mb**	19.5 Mb**	0	1	1
t(11;other)	8	6	4.68 Mb	179.06*** Mb	0	3	1

Table 2. Copy number changes identified by aCGH. Mb = Megabase; * at chromosome 1(q21.1-q21.2)(q31.2-q44) (57.4 Mb) and 19p (23.8 Mb); ** trisomy 8 (146 Mb); * 97 % in only one patient.**

t(6;11)	mcr 6q27 deleted (chr. 6:167351132-167734788)	mcr 11q23.3 amplified (chr. 11:117762153-117833251)	mcr 12p12.2-12.1 deleted (chr. 12: 20902344-21295433)
Mb mcr	0.38	0.070	0.39
Candidate Genes	*TTLL2* *CCR6*	*MLL* *ATP5L* *UBE4A*	*SLCO1B3* *LST3*
N° patients	3/4	3/5	3/4

Table 2S. Recurrent chromosomal region amplified or deleted in t(6;11) positive cases. Mcr = minimal common region; Mb = megabase.

DISCUSSION

While the role and incidence of *MLL*-rearrangements observed in ALL has been thoroughly investigated, in AMLs they have more controversial features, and their specific biological characteristics are mostly unknown[21-25]. In this study, we demonstrate the potential of the *MLL*-partner gene in delineating unique expression signatures in pediatric AML as well as different prognostic subgroups, as previously reported for AMLs carrying t(8;21), t(15;17) and inv(16)[11,26-27]. The screening of nine different chromosomal *MLL*-translocations has identified that *MLL*-rearrangements occur in 10.4 % of Italian AML patients. We evidenced that *MLL*-rearrangements are the second most frequent genetic lesion after t(15;17) (13 %)[28] in Italian AML. We also improve genomic-based classification in pediatric AML, identifying aberrations at 12p and 6q27 to be further considered for novel risk classes distribution. .

We proved that the *MLL*-AML is an heterogeneous leukemia depending on the *MLL*-partner gene. In particular, in line with the study published by Balgobind et al., we found that t(11;19)(q23;p13.1), t(11;19)(q23;p13.3), t(x;11)(q24;q23), t(11;17)(q23;q25) and t(1;11)(p32;q23) have a good prognosis (74,1 %)[29]. The most frequent translocation in pediatric AML was t(9;11) (48 %), with an intermediate prognosis (55.4 % EFS). We pointed out by GEP that the t(9;11) with FAB-M7 clustered tightly together with respect to the t(9;11) with FAB-M5. The fact that a supervised algorithm assigned a specific gene

expression signature common to all t(9;11)-positive patients despite FAB classification indicates that the translocation drives a specific t(9;11) genetic leukemia signature. Of note, the outcome of M5-t(9;11)-positive patients were not significantly different from the M7-t(9;11). Type t(10;11), another very frequent translocation (23 %), had a poor prognosis (27.2 % EFS)[30,31].

The poor outcome (23.3 % EFS) associated with t(6;11)(q27;q23) reveals the importance of further characterization of this subgroup of patients. These patients showed an older age at diagnosis and a higher WBC count as common clinical features. In these patients clinical adverse events occurring within one year from diagnosis, suggested a very aggressive behavior mediated by the *MLL-AF6* chimera, and adequate investigation of its functional role is urgent in order to deliver an effective therapy. By GEP and cytogenetic analysis we assessed novelties for this subgroup of patients. The *MLL-AF6*-positive patients showed the highest frequency of genomic imbalances, while t(9;11) patients showed the lowest[39]. We found that minimal genomic alterations described for *MLL*-AML in 6q27, 12p12 and 11q23[40,41] chromosome regions were strictly associated to the t(6;11)-positive patients. In particular, 4 out of 6 patients with del(12p) were t(6;11)-translocated suggesting a possible correlation between this translocation and 12p imbalances. Moreover, we describe here for the first time that del(12p) influences the outcome of this subgroup independently from the translocation, even if these data need to be confirmed in a larger patients cohort. In fact, patients positive for t(6;11) and without the del(12p) showed a significant increase in EFS with respect to the t(6;11) patients with del(12p). In an effort to explain these indications, genes in the 12p lost region were listed, and their activity was found to be strictly correlated to *AF6* function[42-44], whose expression was among the upregulated genes in this AML subgroup. The fact that 4 out of 6 del(12p) patients were also deleted in 6q27, opens for further biological and clinical evaluation of the impact of this novel feature in *MLL-AF6*-positive patients. The investigation of *MLL-AF6* fusion protein might be interesting since AF6 is a cytosolic protein that plays a role in signaling regulation, which is very different from nuclear *MLL*-partners, which are for the most part involved in transcription or transcriptional elongation activity (AF9, AF10, ENL, ELL, and others)[7,32,33]. AF6 is a multidomain protein that contains two RAS-binding domains (RA) able to negatively control the RAS-related RAP1 pathway[34-38]. A recent publication demonstrated that one RA of the AF6 protein was sufficient for *MLL* self-association, which might drive myeloid transformation[38]. Novel target genes, *AF6* and *TANC1*, have been highlighted here to be controlled by the *MLL-AF6* chimera, probably through the involvement of the co-

regulatory complexes of the RAS signaling pathway, addressing novel biological aspects that may be involved in this leukemia.

The use a-CGH in *MLL*-AML revealed that genomic gains were found more frequently than losses, suggesting a general role of proto-oncogene activation in this leukemia. The observation that 6 out of 39 (15.5 %) patients had the del(12p), suggested a possible association between the del(12p) to pediatric *MLL*-rearranged AML[49]. We also brought to light novelties for hyperdiploid patients with trisomy of chromosome 19: 4 out of 5 patients died, suggesting an adverse prognosis for this subgroup of patients, to be considered in future AML stratification.

Finally, recognizing the majority of *MLL*-rearrangements in AML, and giving them the right risk assessment related to their partner gene, provides a new approach to accurate diagnosis and risk class distribution. The finding that *MLL*-AML is a distinct biological and clinical disease within the high-risk forms of pediatric AML encourages to reconsider these patients for distinct therapies. The evidence of a predisposition to genomic instability for *MLL*-AML and the role of the del(12p), will benefit from enlarged AML studies of novel genomic imbalances, useful to better define the incidence and the portent of these features. Direct experimentation on listed target genes will be required to determine the mechanistic role of t(6;11)-rearranged leukemia in AML pathogenesis.

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PART 2

AF6 NUCLEAR SEQUESTRATION BY CHIMERIC MLL-AF6 ALLOWS OVEREXPRESSION OF RAS SIGNALING PATHWAY IN t(6;11)(q27;q23) ACUTE MYELOID LEUKEMIA

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ABSTRACT

The t(6;11)(q27;q23) translocation is characterized by *MLL-AF6* expression, a bad prognostic marker in AML; however, the exact tumorigenic mechanism is still unclear. AF6 is a cytoplasmatic protein involved in signal transduction, while chimeric protein has a nuclear localization, where it can homodimerize to activate transcription. The RAS pathway is often implicated in *MLL*-leukemia and AF6 was shown to inhibit RAS signaling in epithelial cells. Here, by immunofluorescence and immunoprecipitation analyses, the AF6-RAS interaction was demonstrated in bone marrow cells of healthy donors, while in t(6;11)(q27;q23) leukemic cell lines AF6 protein resulted sequestrated into the nucleus. Silencing for *AF6* gene in healthy samples caused the overexpression of the RAF/MEK/ERK pathway proteins, confirming the RAS-inhibiting role of AF6 in hematopoietic cells. Specific silencing of *MLL-AF6* in leukemic cells resulted in AF6 liberation into cytoplasm, where it colocalized with RAS effecting its downstream targets. By reverse phase protein array, cytoplasmic AF6 restoration was shown to improve expression of pro-apoptotic proteins PARP and CASPASE7 in leukemic cells and to diminish levels of P-CREB, mTOR, P-JAK and CYCLINs. As a result, reduced cell colony formation in semisolid medium was observed, accompanied with an augmented percentage of cell mortality. The same effects were obtained with two specific MEK inhibitors, confirming the RAS pathway implication in *MLL-AF6*-leukemia. Our results suggest a possible mechanism by which MLL-AF6 acts in AML: the loss of RAS-inhibition by AF6 nuclear sequestration could be responsible for the proliferation advantage to *MLL-AF6*-cells, enhancing the effect of the chimera in AML development.

INTRODUCTION

AF6 was firstly described as a gene located at the 6q27, involved in t(6;11)(q27;q23) leukemia[1] and characterized by the expression of the fusion protein MLL-AF6. The normal MLL protein is required for the maintenance of a normal developmental program during embryogenesis. Aberrant proteins resulting from either translocations, duplications or amplifications of *MLL* gene cause an abnormal gene expression profile, which may result in leukemia through different mechanisms[2]. To date, more than 50 fusion partners of *MLL* have been cloned from different chromosomal locations, resulting in both acute myeloid

and lymphoid tumors[3]. In chimeric *MLL-AF6* transcript, *AF6* is linked-in-phase to *MLL* and includes the entire *AF6* with exception of 35 N-terminal aminoacids, showing no sequence homology to any other *MLL* partner genes[1,4].

A study on 26 t(6;11)-positive AML patients indicated a bad prognosis with short event-free survival for this type of leukemia[5]. Moreover, a recent work on pediatric *MLL*-AML patients showed that the t(6;11)(q27;q23) group had a very poor prognosis and specific characteristics (such as age at diagnosis, WBC count, genomic imbalances and gene expression signature) that could discriminate *MLL-AF6*-patients from the rest of *MLL*-rearranged ones[6]. Given the poor prognosis of t(6;11)(q27;q23) AML, it seems to be very important to characterize the fusion protein function in the development of acute leukemia, in order to understand the mechanism of leukemogenesis in which it is involved. Recently it was proposed a mechanism for transcriptional activation of MLL based on homodimerization of MLL-AF6: AF6 may act as a scaffold protein in the nucleus, mediating the interaction between two chimeras or between MLL-AF6 and other proteins involved in transcriptional activation[7].

AF6 is a cytoplasmatic protein that contains two distinctive features: a PDZ (PSD-95/Dlg/ZO-1) domain and two RA (Ras association) domains. The PDZ domain is thought to drive AF6 at specialized sites of the plasma membrane, where AF6 can interact with many molecules, such as JAM[8], Eph receptor[9], PRR2/nectin[10], SPA-1[11], BCR[12] and c-SRC[13], and where it was seen to have a role in modulating the thresholds of multiple signal transduction pathways (RAS, NOTCH and WNT) *in vivo*[14]. The RAS-interacting domains are homologous to the ones seen in other RAS effectors such as RAF, RALGDS and RIN[11,15-17] and AF6 was found to be a putative target for RAS[16]. Therefore, AF6 is thought to be in competition with RAS effectors for the same binding site on RAS protein. It was demonstrated that AF6 can interact with RAS-GTP in epithelial cells preventing the downstream signal transmission and maintaining cells in a non-proliferative state[12].

Although AF6 is a cytoplasmatic protein, MLL-AF6 has nuclear localization, as the N-terminal AT-hook domains of MLL promotes nuclearization of the chimeric protein[3]. Other studies demonstrated that wild type and chimeric MLL protein presented a nuclear punctate expression pattern because of N-terminal nuclear localization signals (NLS) and domains that promotes the distribution in dots inside the nucleus[18-20].

In this study, AF6 is found to have a role in controlling RAS signaling in hematopoietic cells. MLL-AF6 is shown to sequester AF6 from the cytoplasm to the nucleus in t(6;11)(q27;q23) AML cells, deregulating active RAS-GTP levels. Specific silencing of the

MLL-AF6 chimera restores AF6 protein in the cytoplasm, where it can sequester RAS-GTP causing a lower activation of the downstream proliferating pathway. Thus, the tumorigenic activity of MLL-AF6 is strongly enforced by the loss of physiological role of AF6 in t(6;11)-blasts, giving reasons for the aggressiveness and the adverse outcome of this kind of acute myeloid leukemia.

MATERIALS AND METHODS

Cell culture and transfection

Human AML cell line ML2 and SHI-1 carring the t(6;11)(q27;q23) rearrangement (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were cultured in RPMI 1640 (Invitrogen-Life Technologies) supplemented with 10 % fetal bovine serum (FBS; Invitrogen-Life Technologies).

Primary cultures from healthy bone marrow samples of donors (HBMs) were incubated in RPMI 1640 (Invitrogen-Life Technologies) supplemented with 10 % fetal bovine serum (FBS; Invitrogen-Life Technologies) and human cytokines Flt3-ligand 50 ng/ml, TPO 50 ng/ml, SCF 50 ng/ml, IL3 20 ng/ml, IL6 20 ng/ml (Inalco S.p.A) 24 hours before transfection.

Cell transfection was performed using the Nucleofector systems (Amaxa Biosystems, Lonza Sales Ltd) according to the manufacturer's guidelines.

ML2 and SHI-1 cells were treated with two MEK inhibitors (PD98059 – Calbiochem, part of Merck Chemicals Ltd; U0120 - Sigma-Aldrich) at a final concentration of 40 μM.

RNA interference

MLL-AF6 and *AF6* specific small interfering RNA (sirMA6 and sirAF6) were synthesized by QIAGEN (QIAGEN GmbH). 400 pmols and 600 pmols of sirMA6 were transfected into ML2 and SHI-1 cells, respectively. 600 pmols of sirAF6 were transfected into HBM cells. As control was used a scramble RNA called Control siRNA (sc-RNA) (QIAGEN GmbH).

Immunofluorescence microscopy

Cells were collected onto slides by cytospinning and fixed in 1 % formaldehyde in PBS for 15 minutes. The cells were then permeabilized with 0,1 % Triton X-100 in PBS for 10

minutes and preincubated for 30 minutes in PBS containing 5 % bovine serum albumin (BSA) at room temperature, followed by incubation overnight at 4°C with anti-AF6 antibody or anti-RAS antibody diluited 1:500 in PBS + 5 % BSA + 0,1 % Triton X-100. The proteins were detected by fluorochrome-labeled anti-mouse or -rabbit IgG diluited 1:2000 in the same saturating/permeabilizing solution at room temperature for 1 hour. Cells were stained with DAPI (Sigma-Aldrich) 1:10000 in PBS to visualize the nuclei. The images were taken under a fluorescent microscope at 20X or 60X zoom.

RNA isolation and SYBR Green quantitative real-time reverse transcription-PCR assays

Total RNA was isolated from cell lines and primary cultures (2 to $5*10^6$ per sample) using Trizol (Invitrogen-Life Technologies). RNA was transcribed using the SuperScript II system (Invitrogen-Life Technologies) in 25 µl final volume following the manufacturer's instructions. RQ-PCR was performed with 1 µl cDNA in 20 µl using the SYBR Green method (Invitrogen-Life Technologies) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems-Life Technologies). All PCRs were run in triplicate and gene expression, relative to *GUS*, was calculated by the comparative ΔΔCt method.

Western blot

20 µg from total protein fraction (Cell Extraction buffer Biosource) or from nuclear/citoplasmatic separation (Buffer A: 10 mM HEPES pH 7.8, 15 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM PMSF) were used to perform protein analyses on ML2, SHI-1 and HBMs transiently transfected cells. Protein concentration was determined using the BCA method (Pierce). Samples were subjected to 4-6-10-12 % SDS-polyacrylamide gel electrophoresis and transferred to 0.2 mm polyvinylidene difluoride membranes (GE-Healthcare) for immunodetection with a series of antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Upstate Biotechnology). Antibodies used included anti-β-actin, anti-c-RAF, anti-MEK1/2 (Sigma-Aldrich); anti-HDAC1 (Santa Cruz Biotechnology); anti-AF6 (Becton Dickinson); anti- Phospho-c-RAF (Ser338), anti-Phospho-MEK1/2 (Ser217/221), anti-Phospho-ERK1/2 (Thr202/Tyr204), anti-ERK2 and anti-RAS (Cell Signaling Technology). The specific bands of target proteins were

visualized by enhanced chemoluminescence (ECL advance) according to the manufacturer's instructions (GE-Healthcare).

Ras activation assay

A total of $20*10^6$ ML2 and SHI-1 cells transiently transfected were lysed and 500 μg of protein extract were used for Ras Activation Kit (Assay designs – Tema ricerca S.r.l., Italy) according to the manufacturer's instructions. The assay used a GST-fusion protein containing the RAS-binding domain (RBD) of RAF1 to affinity precipitate active RAS (GTP-RAS) from cell lysates. The GST-pulled-down GTP-RAS was detected by Western Blot using a specific RAS antibody.

Immunoprecipitation

10^6 of HBM, ML2 and SHI-1 cells were lysed in 1 ml of ice-cold 0.1 % CHAPSO lysis buffer (0.1 % CHAPS (Sigma-Aldrich); 137 mM KCl; 5 mM MgCl2; 1 mM EDTA; 20 mM Tris-HCl) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein extracts were immunoprecipitated with 8 μg of antibody anti-RAS and anti-AF6 with 100 μl of Protein A/G MicroBeads (Miltenyi Biotec GmbH) using μ Column and μMACSTM Separator (Miltenyi Biotec GmbH) as manufacturer's instructions. The eluted immunoprecipitate was analysed by polyacrilamide gel electrophoresis and immunoblotting with anti-AF6 and anti-RAS antibodies. The specific bands of target proteins were detected by enhanced chemoluminescence reagent (GE-Healthcare).

ELISA test

10^6 ML2 cells transiently silenced for *MLL-AF6* were used for a PhosphoELISArray Kit (SABiosciences Corporation) to simoultaneously profile the expression level of multiple analytes using the sandwich-based enzyme-linked immunosorbant assay (ELISA) technique. Cell lysates were aliquoted onto a 96-well microplate coated with eight antibodies specific for total p53, phospho-p53, total AKT, phospho-AKT, total ERK, phospho-ERK, total JUN, phospho-JUN. Manufacturer's guidelines were followed and the final colorimetric reaction was measured at absorbance of 450 nm, with a wavelength correction at 560 nm. The values at 560 nm were substracted from readings at 450 nm and the results were normalized on sc-RNA.

Reverse-phase protein array (RPPA)

ML2 and SHI-1 transfected cells were lysated and protein extracts were diluted in a mixture of 2X Tris-glycine SDS sample buffer (Invitrogen-Life Technologies) plus 5 % β-mercaptoethanol and loaded onto a 384-well plate and serially diluted with lysis buffer into four-point dilution curves ranging from undiluted to 1:8 dilution. Samples were printed in duplicate onto nitrocellulose-coated slides (FAST slides, Whatman Schleicher & Schuell) with a 2470Arrayer (Aushon BioSystems). Slides were stained with Fast Green FCF (Sigma-Aldrich) according to the manufacturer's instruction and visualized (ScanArray 4000, Packard). Arrays were stained with antibodies (Cell Signaling Technology) on an automated slide stainer (Dako Autostainer Plus, DakoCytomation) using a Catalyzed Signal Amplification System kit (CSA kit, DakoCytomation) according to the manufacturer's recommendations. Antibody staining was revealed using 3,3'-diaminobenzidine. The TIFF images of antibody-stained and Fast Green FCF–stained slides were analyzed using MicroVigene software (VigeneTech Inc). For each sample, the signal of the negative control array (stained with the secondary antibody only) was subtracted from the antibody slide signal, and then the resulting value was normalized to the total protein value. The results were presented as percentage of protein expression in silenced cells respect to negative controls.

Soft agar colony assay

A total of $2*10^3$ ML2 and SHI-1 cells after *MLL-AF6* silencing were used to test the colony-forming cells. Cells were plated in a minimum methylcellulose semisolid medium (StemCell Technologies) and incubated at 37 °C. Colony evaluation and enumeration was done in situ after 14 days from transduction by light microscopy after 3-(4,55-dimethylthiazol-2-yl)-2,5diphenyltetrazolium incorporation. The number of colonies of silenced samples were normalized to sc-RNA and expressed as percentage of clonogenic capacity.

Apoptosis analysis

Transiently transfected cells were collected and stained with Annexin V/propidium iodide (PI) (Immunostep-ValterOcchiena) and analyzed by flow cytometry using Cytomics FC500 (Beckman Coulter). Relative apoptosis was calculated and expressed as percentage of Annexin-positive/PI-positive cells.

RNA isolation and microarray analysis

RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The GeneChip Human Genome U133 Plus 2.0 was used for the microarray experiments; in vitro transcription, hybridization and biotin labeling processes were performed according to the Affymetrix GeneChipR 3' IVT Express Protocol. To generate microarray data (CEL files) default Affymetrix GeneChip Command Console Software (AGCC) was used. The percentage of present genes (mean 45.79 +/- 2.33) and the ratio of GAPDH 3' to 5' (mean 1.08 +/- 0.09) indicated an overall high quality of the samples and the assays.

Data analysis

Statistical analyses were performed with unpaired two-tailed t test. A P value of < 0.05 was considered significant.

For GEP analysis, CEL files were normalized using the Robust Multiarray Averaging (RMA) expression measure of an Affy-R package (http://www.bioconducter.org). Wilcoxon's test was applied on the probes that passed filtering by variance to identify differentially expressed genes in two different groups. Analogously, ANOVA analysis was used to compare the major groups of *MLL*-rearranged patients. All p-values (Wilcoxon's tests and ANOVA) were obtained using a permutation approach. t-test was used for supervised analysis between t(6;11) patients and HBM samples. To control the false discovery rate, multiplicity corrections were used; probes with adjusted p-values less than 0.01 were declared significant, using Partek Genomic Suite Software. Dendrograms were generated to cluster patients using Ward's method and Euclidean distance, and heat maps were used to highlight associations between clusters of patients and clusters of genes. The Ingenuity pathway software (www.ingenuity.com) and DAVID Functional Annotation Bioinformatics Microarray Analysis were used to identify molecular networks between differently expressed genes.

RESULTS

MLL-AF6 sequesters AF6 from the cytoplasm to the nucleus in t(6;11)(q27;q23) AML cells and RAS is maintained in its active form

In order to assess AF6 expression in healthy bone marrow cells, primary cultures were subjected to immunofluorescence assay for the determination of AF6 protein localization. We found that AF6 was expressed in the cytoplasm of both samples and, interestingly, it colocalized with RAS (Figure 1A). To verify if the same cellular localization implicated also a physical interaction between AF6 and RAS, an immunoprecipitation assay was performed and demonstrated the coimmunoprecipitation of AF6 and Ras in HBM cells (Figure 1B).

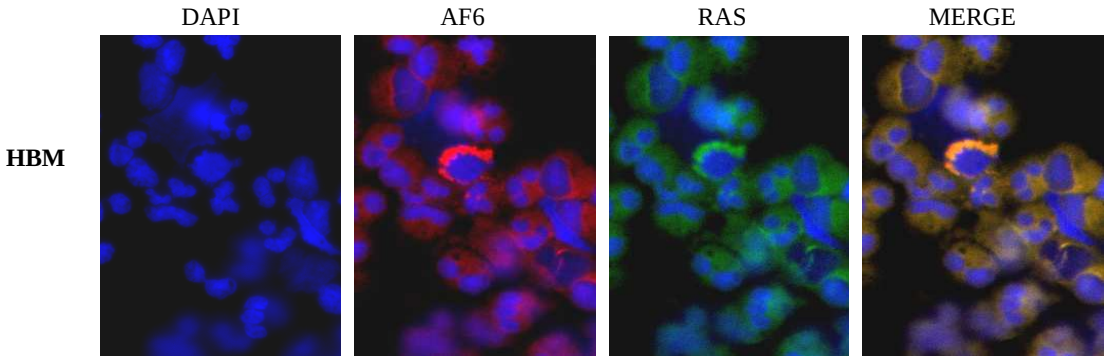

Figure 1A. Colocalization of AF6 (red) and RAS (green) in healthy bone marrow (HBM) cells; cell nuclei stained with DAPI (blue) and merged signals in yellow; 20X zoom.

The same experiments were done for two cell lines characterized by t(6;11)(q27;q23) translocation and expressing the chimera MLL-AF6. Immunofluorescence showed that AF6 was imported from the cytoplasm to the nucleus in a punctuate pattern of expression (Figure 1C). Moreover, by Western blot AF6 protein was established to be nuclear in ML2 and SHI-1 cells, confirming the abnormal localization of AF6 in t(6;11)(q27;q23) cell lines. Immunoprecipitation demonstrated that AF6 was unable to interact with RAS in leukemic cells with t(6;11)(q27;q23) translocation (Figure 1B).

RAS activation assay indicated that ML2 and SHI-1 cells had an elevated amount of RAS in its active GTP-bound status, while in HBM cells AF6 controls the active RAS quantity which was at very low levels (Figure 1D).

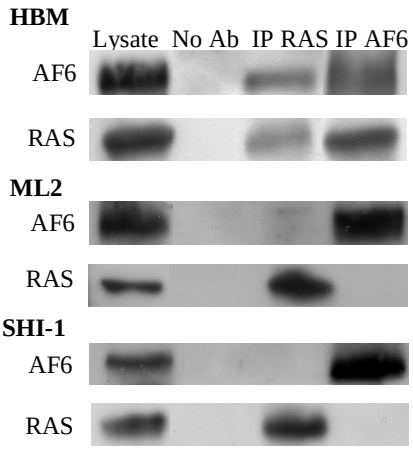

Figure 1B. Coimmunoprecipitation of AF6 and RAS in HBM cells, but no interaction in ML2 nor SHI-1 cells; total lysates were used as positive controls; negative controls were samples immunoprecipitated without an antibody (No Ab).

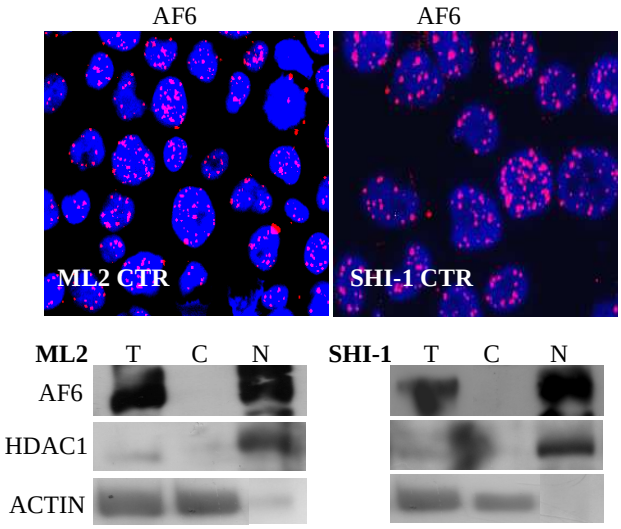

Figure 1C. Nuclear localization of AF6 in ML2 and SHI-1 cells by immunofluorescence assay (AF6 red, nuclei blue, 20X zoom) and by western blot analyses of total (T), cytoplasmatic (C) and nuclear (N) extracts; anti-HDAC1 and anti-ACTIN were used as endogenous controls for nuclear and cytoplasmatic proteins, respectively.

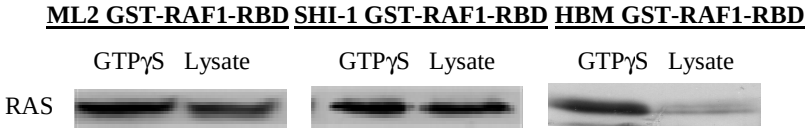

Figure 1D. RAS-GTP levels in HBM (low) and in ML2 and SHI-1 cells (high); samples with an excess of GTP (GTPγS) were used as positive controls.

Citoplasmatic AF6 can sequester RAS-GTP causing a lower activation of downstream pathway

Silencing of *MLL-AF6* in leukemic cell lines ML2 and SHI-1 was done by using specific siRNAs for the fusion transcript. Decreased mRNA and protein expression were confirmed by RQ-PCR (n=3; $p < 0.05$) and Western blot (Figure 2A). Silenced cells were then subjected to immunofluorescence assay to monitor AF6 localization after *MLL-AF6* knockdown. Figure 2B showed that AF6 is completely sequestered at nuclear level, while after *MLL-AF6* silencing the protein returned to cytoplasm in both cell lines. Moreover, we demonstrated that in leukemic cells after silencing of the chimera AF6 colocalized with RAS in the cytoplasm (Figure 2C).

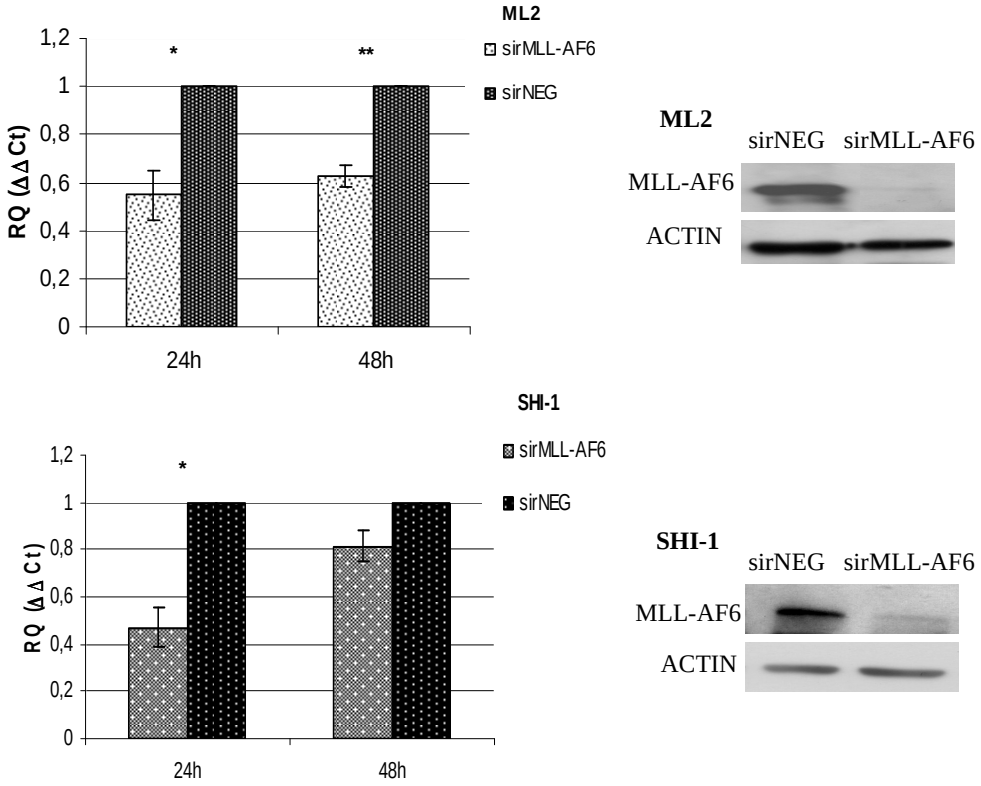

Figure 2A. Decreasing levels of *MLL-AF6* mRNA by Real-Time PCR (ΔΔCt method) on ML2 and SHI-1 cells silenced for *MLL-AF6* (sirMLL-AF6) respect to negative controls (sirNEG) transfected with scramble siRNAs (n=2; $p < 0.05$ signed with a star, *; $p < 0.001$ signed with double star, **). Silencing of the chimera was demonstrated also at protein level by western blot analysis, using anti-ACTIN as endogenous control for protein amount.

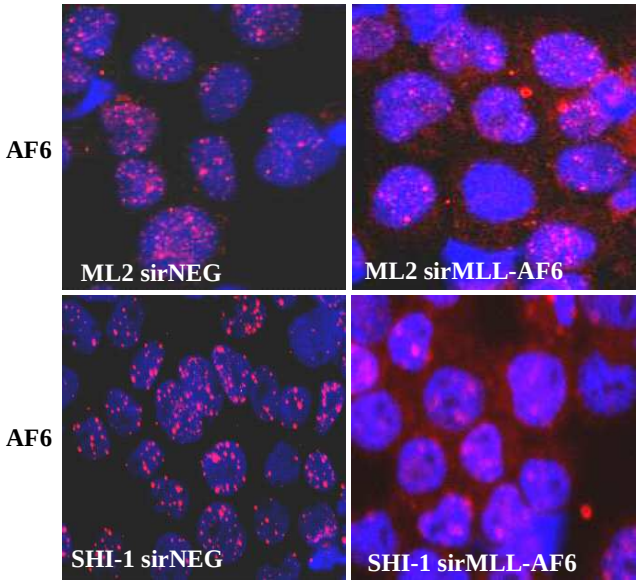

Figure 2B. ***MLL-AF6*** **silenced cells (sirMLL-AF6) showed AF6 in the cytoplasm by immunofluorescence assay (AF6 red, nuclei blue, 20X zoom); as negative controls (sirNEG) were used cells transfected with scramble siRNAs.**

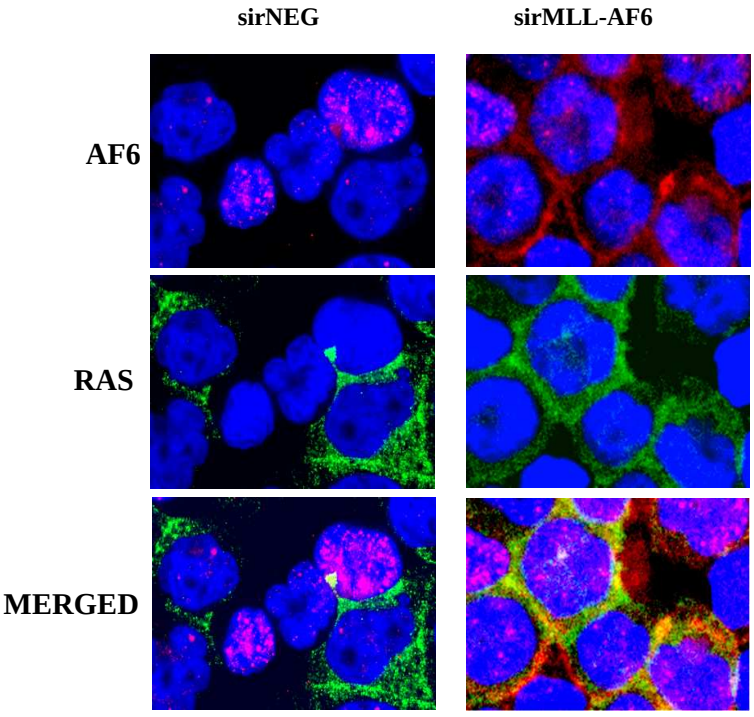

Figure 2C. Colocalization (merged signals yellow, nuclei blue, 60X zoom) of AF6 (red) and RAS (green) by immunofluorescence assay on SHI-1 cells silenced for ***MLL-AF6*** **(sirMLL-AF6); as negative controls (sirNEG) were used cells transfected with scramble siRNAs.**

The cytoplasmic amount of AF6 was able to control RAS-GTP levels, which were found decreased respect to sirNEG in leukemic cells (Figure 3A). To substain the hypothesis of AF6 acting as a controller of RAS signaling we studied the expression of the phosphorylated active form of the proteins downstream of RAS by Western blot. By Western blot it was showed that silencing of *MLL-AF6* in ML2 and SHI-1 cells decreased the expression of the active form of c-RAF, MEK1/2 and ERK1/2 confirming RAS pathway deregulation. Moreover, ML2 cells silenced for *MLL-AF6* were used for an ELISA test that confirmed a decreased level of expression of the two main downstream RAS effectors ERK1/2 and AKT (n=2; $p < 0.05$) (Figure 3B).

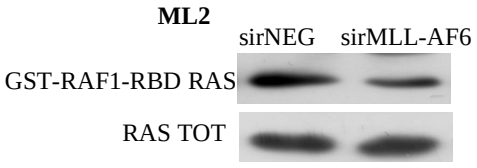

Figure 3A. RAS-GTP levels decreased in ML2 cells silenced for *MLL-AF6* (sirMLL-AF6) respect to negative control (sirNEG) transfected with scramble siRNAs; anti-RAS on total lysates of transfected cells was used as positive control of total RAS amount.

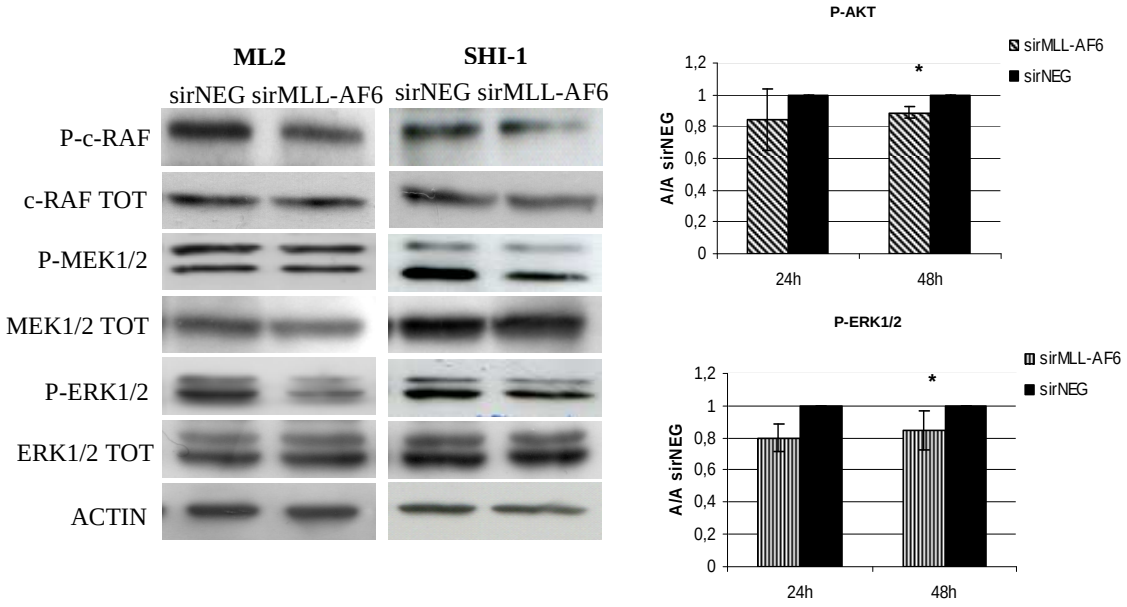

Figure 3B. Western blot analyses of RAF/MEK/ERK pathway on ML2 and SHI-1 transfected cells showed decreasing levels of P-c-RAF, P-MEK1/2 and P-ERK1/2 proteins in cells silenced for *MLL-AF6* (sirMLL-AF6) respect to negative controls (sirNEG) transfected with scramble siRNAs; anti-ACTIN was used as positive control of protein amount. The phopshorylated forms of AKT and ERK1/2 were decreased by ELISA test on ML2 cells after *MLL-AF6* silencing (sirMLL-AF6) respect to negative controls (sirNEG) transfected with scramble siRNAs (n=2; $p < 0.05$ signed with a star, *).

We used HBM cells to confirm AF6 role in RAS pathway regulation in hematopoietic cells, as we had previously demonstrated the interaction between AF6 and RAS in healthy bone marrow cells. We found that silencing of *AF6* (n=2; $p < 0.05$) (Figure 3C) increased the expression of RAF/MEK/ERK pathway proteins in healthy bone marrow cells (Figure 3D).

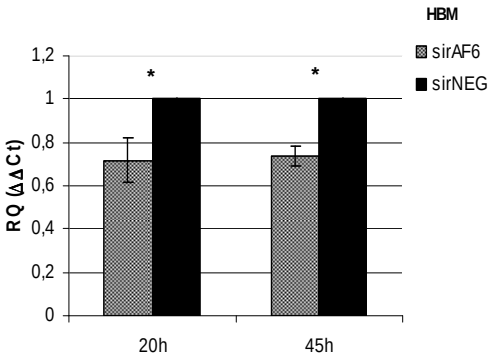

Figure 3C. Decreasing levels of *AF6* mRNA by Real-Time PCR (ΔΔCt method) on healthy bone marrow (HBM) cells silenced for *AF6* (sirAF6) respect to negative controls (sirNEG) transfected with scramble siRNAs (n=2; $p < 0.05$ signed with a star, *).

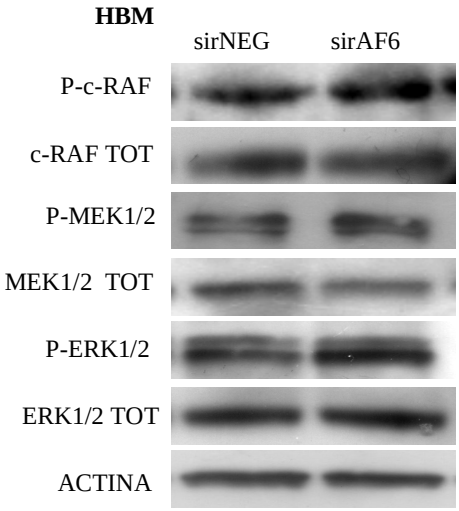

Figure 3D. Western blot analyses of RAF/MEK/ERK pathway on HBM transfected cells showed increasing levels of P-c-RAF, P-MEK1/2 and P-ERK1/2 proteins in cells silenced for *AF6* (sirAF6) respect to negative controls (sirNEG) transfected with scramble siRNAs; anti-ACTIN was used as positive control of protein amount.

Deregulation of RAS pathway by cytoplasmatic AF6 influenced cell proliferation

Functional studies on *MLL-AF6* silenced cells were performed using the Annexin V/propidium iodide (PI) test and the MTT-based cell proliferation assay. We obtained a percentage of cell mortality augmented of about 16 % respect to sc-RNA (n=3; $p < 0.05$) (data not shown) and a colonies formation reduced of about 80 % for ML2 and 55 % for SHI-1 (n=2; $p < 0.05$) (Figure 4A).

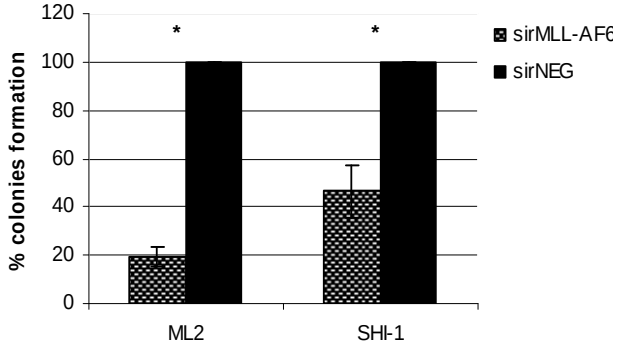

Figure 4A. Percentage of colonies formation on semisolid medium decreased after *MLL-AF6* silencing (sirMLL-AF6) in ML2 and SHI-1 cells (n=2; $p < 0.05$); as negative controls were used cells transfected with scramble siRNAs (sirNEG).

Reverse phase protein array was used for *MLL-AF6* silenced ML2 and SHI-1 cells, with the aim to analize the entire RAF/MEK/ERK pathway and other proteins involved in apoptosis or cell proliferation. It was found a confirmation of ELISA data, with a downregulation of proteins implicated in RAS pathway. Moreover, CYCLIN proteins analized resulted diminished respect to sc-RNA, like P-CREB, mTOR and P-JAK proteins which are known to be implicated in proliferative signals, while there was an augment of proteins involved in apoptosis as Cleaved PARP and Cleaved CASPASE7 (Figure 4B).

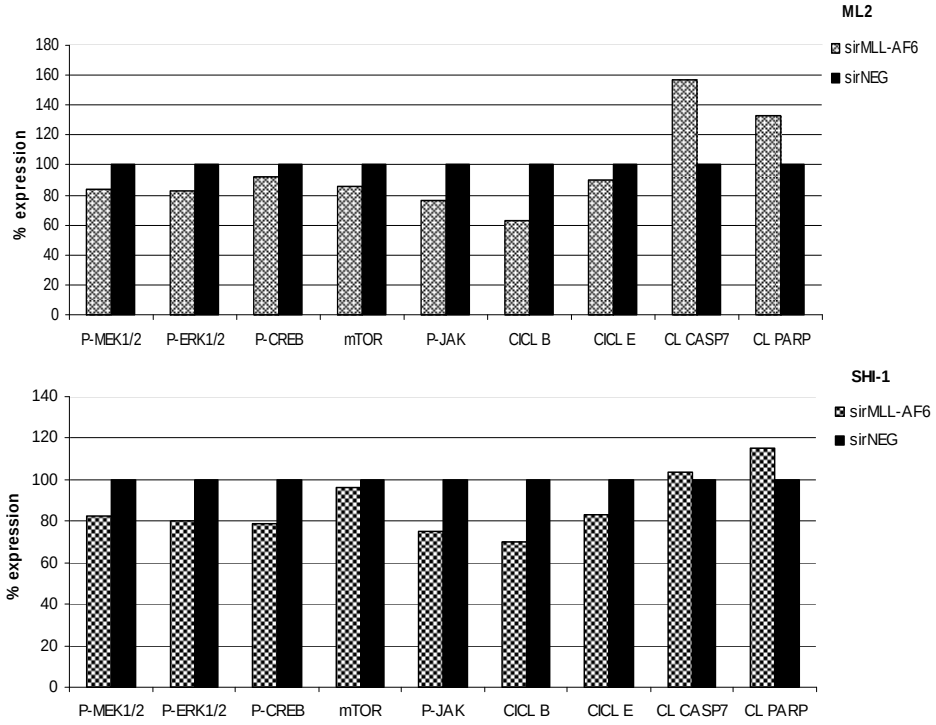

Figure 4B. Reverse phase protein array (RPPA) analysis on ML2 and SHI-1 tansfected cells showed decreased expression of P-MEK1/2, P-ERK1/2, P-CRED, mTOR, P-JAK, CYCLINE, CYCLINB and increased percentage of Cleaved CASPASE7 and Cleaved PARP in *MLL-AF6* silenced cells (sirMLL-AF6) respect to negative controls (sirNEG) transfected with scramble siRNAs.

Finally, to have the confirmation of the RAS via involvement in leukemic cells, we treated ML2 and SHI-1 cells with two specific inhibitors of MEK1/2. We ensure by Western Blot the downregulation of RAS signaling after treatment, detecting expression levels of P-ERK1/2 (Figure 4C).

Then cell mortality and clonogenic ability were measured. We obtained an increased percentage of cell mortality for both cell lines treated with the inhibitor respect to the negative controls treated with DMSO demonstrating that the inhibition of RAS pathway by chemical agent or by the silencing of *MLL-AF6* conferred a diminished proliferation of leukemia cells (n=3; $p < 0.05$) (Figure 4D). For the same cells the clonogenic growth was reduced of about 70 % for ML2 and 30 % for SHI-1 (n=3; $p < 0.05$), as previously found by silencing the chimera *MLL-AF6*.

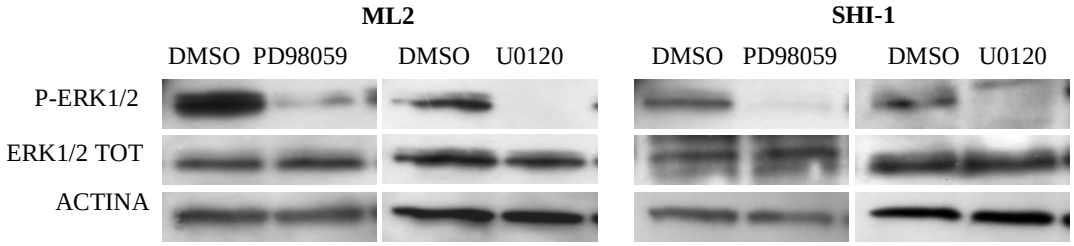

Figure 4C. Western blot analyses of P-ERK1/2 expression on ML2 and SHI-1 after treatment with two MEK inhibitors (PD98059 and U0120) confirmed the interruption of the RAS signaling pathway respect to negative controls treated with DMSO.

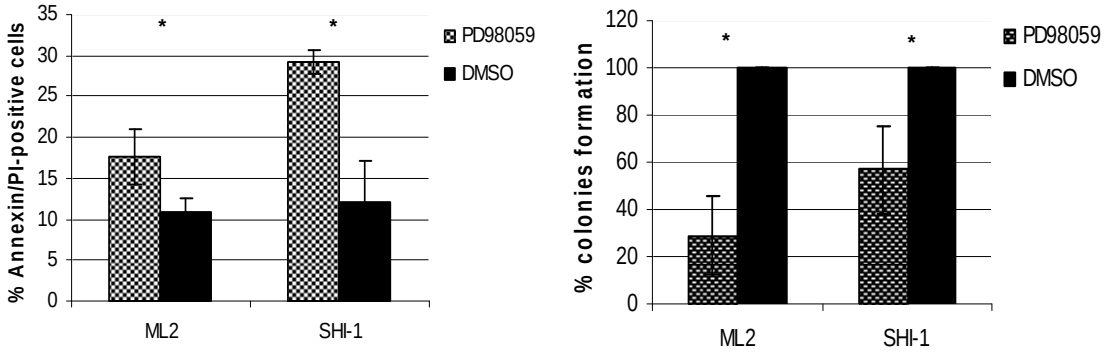

Figure 4D. After treatment of ML2 and SHI-1 cells with a specific MEK inhibitor (PD98059, 40μM) were found increasing percentage of cell mortality and decreasing percentage of colonies formation on semisolid medium respect to negative controls treated with DMSO (n=2; $p < 0.05$ signed with a star, *).

To identify genes and molecular pathways involved in t(6;11)-AML, we performed gene expression analysis using Human Genome U133 Plus 2.0. We analysed 11 t(6;11)-patients and 11 bone marrow samples from healthy donors (HBMs). Supervised analysis between these two groups (t(6;11) vs HBM) identified 435 probe sets differently expressed

(Appendix 2). Among differently expressed genes in t(6;11) samples, we found genes tipically involved in *MLL*-leukemia, such as *MEIS1* and *HOX* genes.

Interestingly, gene ontology analysis by IPA and DAVID softwares using the differently expressed genes identified the RAS pathway as one of the most deregulated pathways in t(6;11)-leukemic patients. Remarkably, we identified *RASA2* (which encodes for a RAS-GAP known to negatively regulate RAS activity) as significantly downregulated in t(6;11)-samples ($p < 0.01$) (Figure 5)

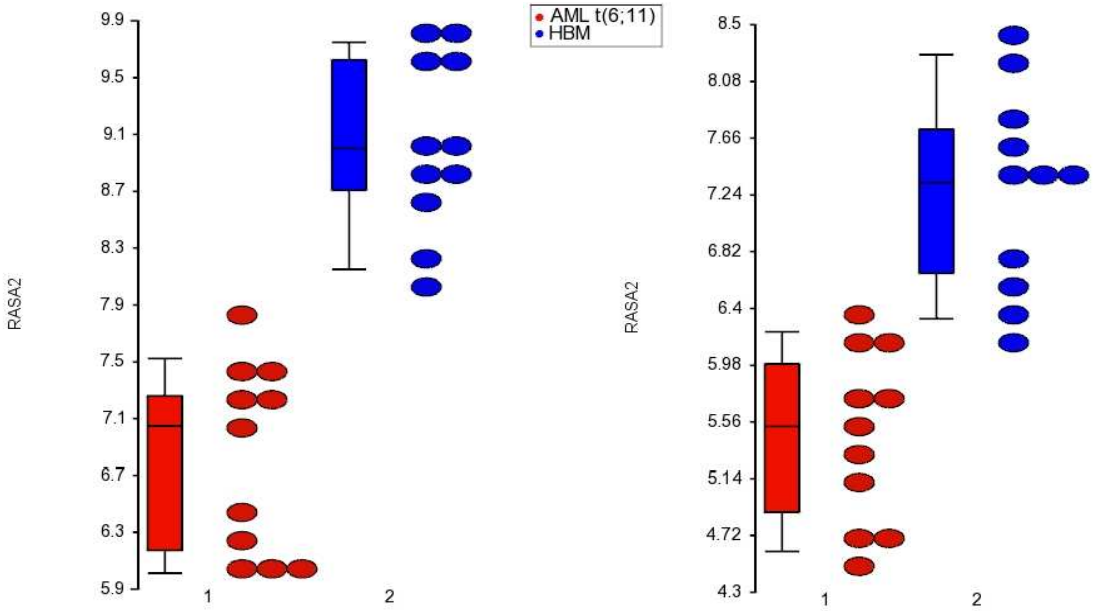

Figure 5. Box-plot of *RASA2* probe sets in t(6;11)-patients vs HBM samples generated using Partek Genomic Suite Software. In the boxes were indicated the expression values median of each group. Y-axis probe sets expression values in log2 scale; X-axis identification ID.

DISCUSSION

AF6 is a protein that usually resides in the cytoplasm to exert its functions. It was demonstrated that AF6 can bind RAS through its RA domain in epithelial cells, sequestering it in its GTP-bound active form[12]. Another study showed that AF6 interacts with activated RAS *in vivo* when activated RAS or AF6 are overexpressed, but the authors were unable to detect the interaction of AF6 with activated RAS induced by natural stimulation with growth factors, so it is unknown what stimuli cause the interaction between these two proteins[21]. In this study we have shown the colocalization and the

interaction of AF6 and RAS in healthy bone marrow cells, where very low levels of RAS-GTP were present, confirming the role of AF6 in RAS signaling control. The silencing of *AF6* in healthy bone marrow cells causes increased expression of the proteins involved in the MAPK pathway, demonstrating that in the cytoplasm of hematopoietic cells AF6 competes with other RAS effectors, maintaining the physiological levels of proliferating signals downstream of RAS.

Although AF6 is a cytoplasmatic protein, in t(6;11)(q27;q23) leukemia MLL-AF6 has a nuclear localization and promotes the sequestration of AF6 in the nucleus[3]. It has been recently demonstrated the nuclear role of AF6 in *MLL-AF6*-positive AML as scaffold protein for dimerization and activation of the transcriptional activity of MLL[7]. Here the ability of MLL-AF6 to subtract AF6 from the cytoplasm had been evaluated in leukemic cell lines. A previous work showed the same tumorigenic mechanism of another *MLL*-mediated leukemia, which implicated the recruitment of a cytoplasmatic protein into the nucleus by MLL-EEN fusion protein[22]. In this study it was shown that, after the silencing of the chimera by RNA interference, AF6 was unable to move into the nucleus and remained in the cytoplasm of t(6;11)(q27;q23) AML cells, colocalizing with RAS and decreasing RAS-GTP levels.

The demonstration of AF6 nuclear localization in t(6;11)(q27;q23) AML cells supported that AF6 can't interact with RAS-GTP, allowing it to bind its downstream effectors. We have shown that restoring AF6 in the cytoplasm of t(6;11)-leukemic cells diminished the activation of the RAF/MEK/ERK pathway, which negative influenced cell proliferation. Proteomic analyses revealed increasing levels of two apoptotic proteins (CASPASE7 and PARP) and decreasing amount of proteins implicated in proliferating pathways (P-CREB, P-JAK, mTOR, CYCLINB, CYCLINE), which reflected the augmented cell mortality and the diminished clonogenic growth of *MLL-AF6* silenced cells. Then the consequence of the AF6 nuclear sequestration in t(6;11)(q27;q23) leukemia is a loss of RAS inhibition with a deregulation of its downstream RAF/MEK/ERK proliferating pathway.

Several studies had already demonstrated the role of deregulated activation of RAS signaling in cancer development and specifically in *MLL*-rearranged leukemias[23-26]. First of all, activating somatic *NRAS* and *KRAS* mutations occur in approximately 20 % of AML, 40 % of chronic myelomonocytic leukemia, and 30 % of juvenile myelomonocytic leukemia (JMML) cases[27]. Alternatively, the loss of the tumor suppressor neurofibromin (*NF1*), which negatively regulates RAS, mimics the effect of an activating mutation of RAS in malignant transformation[28] and germline mutations of the gene *PTPN*[11], which

encodes the protein tyrosine phosphatase (PTP) SHP-2, were identified in pediatric leukemias and were found to promote RAS activation[29,30]. To confirm the implication of the RAS via in t(6;11)(q27;q23) AML cells, we used a specific MEK inhibitor and obtained the same effects on cell mortality and clonogenic ability than *MLL-AF6* silencing, demonstrating that AF6 plays a specific role in hematopoietic cells. Moreover, a GEP analysis on leukemic patients revealed a specific deregulation of genes involved in RAS pathway in t(6;11) patients respect to healthy donors. Interestingly, *RASA2* gene was found significantly downregulated only in *MLL-AF6* patients and not in other *MLL*-translocated patients. It encodes for an important RAS-GAP that enhances the switch of RAS to the inactivated status, so its downregulation in *MLL-AF6*-leukemia could explain the costitutive hyperactivation of RAS and its downstream pathway. Other studies are necessary to confirm this cellular mechanism *in vitro*.

In conclusion, we can assume that in healthy bone marrow cells AF6 is a citoplasmatic protein that interacts with RAS-GTP, preventing an overactivation of the signaling pathway downstream it. In t(6;11)-positive AML cells the formation of MLL-AF6 chimera causes the removal of AF6 from the cytoplasm and its reclusion in the nucleus, thereby preventing its interaction with RAS and its normal function within the hematopoietic cells. In this way, RAS is free to activate its downstream proliferating signals through the RAF/MEK/ERK cascade, enhancing the tumorigenic effect of MLL-AF6 in AML cells. However, it is still unknown what mechanism could be the cause of an elevated amount of RAS-GTP inside t(6;11)-positive AML cells. As we haven't found any mutations by sequencing RAS gene in t(6;11) cell lines and in a set of *MLL-AF6*-positive patients (data not shown), the mechanism by which RAS is hyperactivated in t(6;11)(q27;q23) cells could be caused by the low expression of *RASA2* gene. This mechanism could be an interesting subject for future investigations, which could lead to a complete knowlegde of the leukemogenetic mechanism inside t(6;11)(q27;q23) AML blasts.

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CONCLUSION

CONCLUSION

Leukemias are the most common form of cancer in the pediatric population and a variable number of factors must contribute to promote the onset of the disease, not a single event.

The 10 % of pediatric leukemias are acute myeloid (AML), where *MLL* is translocated very frequently with prognostic significance that stratify patients in high risk group (Zanesco, 2005; Tubergen and Bleyer, 2004; Eden, 2010; Manola, 2009). Altered pattern of activity of *MLL*, in fact, can cause abnormal expression of *HOX* genes in hematopoietic stem cells, causing a block of maturation and in some cases the development of leukemia (Daser and Rabbitts, 2005).

We investigated the role of various *MLL*-translocations in a cohort of AML patients stratified within the high-risk group, demonstrating the potential of the *MLL*-partner gene in delineating unique expression signatures. These data revealed the importance of biologic characterization of patients to ensure a better stratification and treatment of patients within the same risk assessment.

This study delineated a new group of AML patients with a very poor prognosis, the *MLL-AF6*-rearranged, which has been considered in our job. Infact, the *AF6* gene encodes for a cytoplasmatic protein which is able to bind RAS and sequester it in its GTP-bound active form (Radziwill et al., 2003; Yamamoto et al., 1999). Here we have for the first time demonstrated the cytoplasmatic interaction of AF6 and RAS, which maintained low levels of Ras-GTP in hematopoietic cells, while silencing of *AF6* resulted in an upregulation of RAF/MEK/ERK pathway, which might contribute to the aggressiveness of the *MLL-AF6* chimera respect to the other *MLL*-fusions.

Finally, in this PhD course I improve AML knowledge, specifically of 10 % of patients that are destinated to have a poor prognosis and to die. Biological events seem to be the main targets for future therapy to improve AML survival.

APPENDIX

Appendix 1. Cytogenetics and aCGH analysis. ND not done; R MLL classical rearrangement; T MLL complete traslocation; A 5' MLL amplificated; AA 3' MLL amplificated; AAA MLL amplificated; D 3' MLL deleted; NN normal resultes; Mb megabase; G gain and L loss.

PATIENTS	CYTOGENETIC ANALYSIS		aCGH ANALYSIS		
	FISH	KARYOTYPE	GAIN/LOSS	CHROMOSOME BAND	CHANGE,Mb
MLL_14	ND	46, XX	L	5q31.1 - q34	28,27 Mb
			G	6q15 - q27	106 Mb
			G	11q23.3 - q25	16,5 Mb
			L	12p13.33 - p11.23	26 Mb
			L	21q22.11 - q22.3	13,2 Mb
MLL_17	A	46,XX,t(6;11)(q27;q23),ish t(6;11)(MLL-SO cen+,MLL-SG tel+;MLL-SO cen+,MLL-SG tel-)[25]/46,XX[1]	G	11q23.3	0,071 Mb
			G	12p13.33 - p13.32	1,35 Mb
MLL_18	D	46,XX,t(6;11)(q27;q23) . ish t(6;11)(asat 6 +,wcp11+;wcp11+,5'MLL+,3'MLL-)	L	6q27	1,2 Mb
			L	11q23.3 - q24.2	5,9 Mb
			L	12p12.2 - p12.1	0,39 Mb
MLL_6	R	46,XY,t(6;11)(q27;q23),inv(9)(p11q13)[13]/46,idem,dup(2)(q23q33)[7]/46,XY,inv(9)(p11q13)[2]	L	6q24.3 - q27	22 Mb
			L	11q14.1 - q14.3	6,28 Mb
			L	12p13.1 - p12.1	8,7 Mb
			G	Xp22.33 - p21.3	26 Mb
MLL_5	A	46,XY .ish ins(6;11)(q25-27;q23q23) (wcp6+,CEP 6+,5'MLL+,wcp11-), 11q23 (5'MLLx2,3'MLLx2)	L	6q27	0,383 Mb
			G	11q23.2 - q23.3	3,2 Mb
MLL_21	ND	46, XY	N.D.		
MLL_16	AA	47,XY,+Y,t(6;11)(q27;q23)/50,idem,+8,+13,+19	N.D.		
MLL_19	R	46,XY,t(6;11)(q27;q23)[18]/50,XY,+4,+6,t(6;11),+der(6)t(6;11),+7,+18,+19,+21[2]	NN		
MLL_20	R	46 XY, del (11)(q14)	NN		
MLL_15	R	46,XY,t(6;11)(q27;q23)	NN		
MLL_7	ND	46, XY	NN		

MLL_13	R	46,XY,der(1)t(1;?)(q11;?),t(9;11)(p22;q23),add(16)(q22),der(17)t(1;17)(q21;p13)[22]. ish der(1)(1pter-1q11::1q?31-1q?ter)(wcp1+) ; ins(16;1)(q22;q?12q?)(wcp16+,wcp1+)/46,XY[1]	G	1q21.1 - q21.2	1.4 Mb
			G	1q31.2 - q44	56 Mb
MLL_38	ND	46,XY[25]	G	19p	23.8 Mb
MLL_3	R	46,XY,t(9;11)(p21;q23)[24]/46,XY[5]	NN		
MLL_28	ND	46 XY[20]	NN		
MLL_29	R	46,XY,t(9;11;17)(p21;q23;q12)	NN		
MLL_9	ND	47,XX,t(9;11)(p22;q23),+19	N.D.		
MLL_12	ND	46, XY	N.D.		
MLL_22	R	48,XY,+8,t(9;11)(p22;q23),+19[17]/46,XY[2]	N.D.		
MLL_25	ND	46,XX,t(9;11)(p21;q23)[27]46,XY[1]	N.D.		
MLL_26	ND	N.D.	N.D.		
MLL_27	R	46,XX,t(9,11)(p22;q23)[18]/48,idem,+19,+21[4]	N.D.		
MLL_1	R	46,XX,t(3;7;8;10;11)(q21;q34;q13;q11.2;q23)del(10)(p12p14),der(12)t(10;12)(p?;p12)[17]/46,XX[3]	G	10p12.33 - 15.3	19,5 Mb
			L	12p12.2 - p13.33	24,6 Mb
MLL_2	R	46,XY,t(10;11)(p12;q23)[30]/46,XY[3]	L	7q11.22	0.1093 Mb
			L	10p12.1	0.052 Mb
MLL_4	T	46,XX,t(10;11)(p11.2;q23)[16]/ 46,XX,der(10)t(10;11)t(1;11)(q12;q25),der(11)t(10;11)[4]/ 46,XX[4]. ish t(10;11)(wcp11+,5'MLL+,3'MLL+,tel10p-;wcp11+,5'MLL-,3'MLL-,tel10p+), der(10)t(10;11)t(1;11)(wcp1+,wcp11+,5'MLL+,3'MLL+,tel10p-)	L	19q13.31	0.4969 Mb
MLL_30	T	46,XX,t(10;22;11)(p12;q13;q23)[23]. ish t(10;22;11)(wcp11+,3`MLL+,5`MLL+,wcp10+;wcp22+,wcp10+;wcp11+,5`MLL-,3`MLL-)	L	11q23.3	0.0428 Mb
MLL_39	R	46,XY,ins(10;11)(p12;q23q12)[15]/46,idem,del(12)(p12)[8]	L	1p31.1	0.6688 Mb
MLL_40	ND	N.D.	N.D.		
MLL_23	R	46,XX[11]	N.D.		
MLL_24	ND	46,XY,Iinv(10)(p13q24),del(15)((q14q22)[11]/46,XY[6]	N.D.		
MLL_8	R	46,XY,t(10;11)(p12;q23)[1]/46,idem,del(12)(p12)[23] . ish t(10;11)(5' MLL-,3'MLL+;5'MLL+,3'MLL-)	N.D.		
MLL_31	R	46,XY,ins(10;11)(p12;q23q13)	NN		

MLL_33	R	46,XY,t(11;?19)(q23,?)	L	del 12p12.2 - p12.1	0.393 Mb
MLL_41	R	47 XX,+8 t(11;19) (q23;p13)	G	8+	146 Mb
MLL_34	AAA	46,XX,t(11;19)(q23;p13)[20]	G	11q22.3 - q25	31.8 Mb
MLL_42	R	46,XY,1qh+,(11;19)(q23;p13.3)	G	21q22,3	0,06449 Mb
MLL_32	R	46,XY,t(1;11)(q22;q23)inv(1)(p36q22)	L	1q31.1 - q31.2	2.11 Mb
			G	2p24.2 - p24.1	1,2 Mb
			L	13q21.1	0.540 Mb
MLL_11	R	46,Y,der(X)ins(X;11)(q24;q22q23)t(7;11)(q31;q23),der(7)t(X;7)(q24;q31),del(11)(q22q23). ish der(X)(wcpX+,wcp11+.5`MLL+,3`MLL-,wcp7+),der(7)(wcp7+,wcpX+).del(11)(wcp11+, 5`MLL-,3`MLL+)	N.D.		
MLL_35	T	46,Y,t(X;11)(q24;q23). ish t(X;11)(MLL +;MLL -)	L	11q23.1	0,14 Mb
MLL_36	R	46,Y,inv ins(X;11)(q24;q23q13)[15]/46,idem,del(7)(q32)[5]/46,XY[1]	L	11q13.1	1,5 Mb
MLL_10	R	N.D.	N.D.		
MLL_37	R	46 XX,[15]	NN		

Appendix 2. Differently expressed probe sets between t(6;11) patients and HBMs

Probeset ID	Gene Symbol
213150_at	*HOXA10*
235521_at	*HOXA3*
235753_at	*HOXA7*
209905_at	*HOXA9*
213147_at	*HOXA10*
202286_s_at	*TACSTD2*
207072_at	*IL18RAP*
1559266_s_at	*C10orf140 /// LOC730417*
206310_at	*SPINK2*
211833_s_at	*BAX*
206847_s_at	*HOXA7*
214651_s_at	*HOXA9*
204881_s_at	*UGCG*
208478_s_at	*BAX*
200602_at	*APP*
206043_s_at	*ATP2C2*
208557_at	*HOXA6*
221268_s_at	*SGPP1*
229971_at	*GPR114*
225308_s_at	*TANC1*
213272_s_at	*TMEM159*
224391_s_at	*SIAE*
211890_x_at	*CAPN3*
201061_s_at	*STOM*
211889_x_at	*CEACAM1*
209199_s_at	*MEF2C*
206209_s_at	*CA4*
206514_s_at	*CYP4F2 /// CYP4F3*
206576_s_at	*CEACAM1*
223457_at	*COPG2*
226726_at	*MBOAT2*
213288_at	*MBOAT2*
212912_at	*RPS6KA2*
208168_s_at	*CHIT1*
203335_at	*PHYH*
201060_x_at	*STOM*
207802_at	*CRISP3*
206515_at	*CYP4F3*
205472_s_at	*DACH1*
218409_s_at	*DNAJC1*
225898_at	*WDR54*
229269_x_at	*LOC646044 /// SSBP4*
213844_at	*HOXA5*
221764_at	*C19orf22*
208858_s_at	*FAM62A*
232027_at	*SYNE1*
1553605_a_at	*ABCA13*
1552773_at	*CLEC4D*
213935_at	*ABHD5*
212531_at	*LCN2*
209200_at	*MEF2C*
201105_at	*LGALS1*

224967_at	*UGCG*
218224_at	*PNMA1*
202419_at	*FVT1*
207890_s_at	*MMP25*
242838_at	*MAP6D1*
222026_at	*RBM3*
220974_x_at	*SFXN3*
209772_s_at	*CD24*
212768_s_at	*OLFM4*
211883_x_at	*CEACAM1*
205731_s_at	*NCOA2*
226455_at	*CREB3L4*
205129_at	*NPM3*
215450_at	---
206245_s_at	*IVNS1ABP*
209212_s_at	*KLF5*
207384_at	*PGLYRP1*
206272_at	*SPHAR*
222062_at	*IL27RA*
208962_s_at	*FADS1*
244235_at	*IVNS1ABP*
218048_at	*COMMD3*
224685_at	*MLLT4*
1560316_s_at	*GLCCI1*
218847_at	*IGF2BP2*
202472_at	*MPI*
202265_at	*BMI1*
205195_at	*AP1S1*
223894_s_at	*AKTIP*
231955_s_at	*HIBADH*
236075_s_at	---
235291_s_at	*FLJ32255*
214475_x_at	*CAPN3*
212019_at	*RSL1D1*
226822_at	*STOX2*
200835_s_at	*MAP4*
205403_at	*IL1R2*
1553295_at	*ABCA13*
212911_at	*DNAJC16*
217226_s_at	*SFXN3*
204636_at	*COL17A1*
1557411_s_at	*SLC25A43*
222750_s_at	*SRD5A3*
229742_at	*LOC145853*
210944_s_at	*CAPN3*
233010_at	---
209222_s_at	*OSBPL2*
242774_at	*SYNE2*
228113_at	*RAB37*
203168_at	*CREBL1*
203515_s_at	*PMVK*
201944_at	*HEXB*
203627_at	*IGF1R*
1559265_at	*C10orf140 /// LOC730417*

203227_s_at	*TSPAN31*
230179_at	*LOC285812*
212113_at	*LOC552889*
1552302_at	*FLJ77644 /// TMEM106A*
222752_s_at	*C1orf75*
208680_at	*PRDX1*
207717_s_at	*PKP2*
213750_at	*RSL1D1*
229164_s_at	*ABTB1*
208751_at	*NAPA*
203021_at	*SLPI*
205196_s_at	*AP1S1*
218501_at	*ARHGEF3*
221765_at	*UGCG*
206656_s_at	*C20orf3*
229119_s_at	*ZSWIM7*
231771_at	*GJB6*
206208_at	*CA4*
227525_at	*GLCCI1*
210452_x_at	*CYP4F2*
207968_s_at	*MEF2C*
201847_at	*LIPA*
205128_x_at	*PTGS1*
1552772_at	*CLEC4D*
201037_at	*PFKP*
208470_s_at	*HP /// HPR*
204623_at	*TFF3*
230669_at	*RASA2*
215102_at	*DPY19L1P1*
214907_at	*CEACAM21*
1553959_a_at	*B3GALT6*
218176_at	*MAGEF1*
219998_at	*HSPC159*
1555923_a_at	*C10orf114*
208670_s_at	*EID1*
239578_at	---
227195_at	*ZNF503*
209992_at	*PFKFB2*
44040_at	*FBXO41*
228855_at	*NUDT7*
218739_at	*ABHD5*
205315_s_at	*SNTB2*
222457_s_at	*LIMA1*
242397_at	---
225752_at	*NIPA1*
228714_at	---
203420_at	*FAM8A1*
221995_s_at	---
223391_at	*SGPP1*
225936_at	*EID2*
1554834_a_at	*RASSF5*
210004_at	*OLR1*
232797_at	---
212566_at	*MAP4*

222413_s_at	*MLL3*
226676_at	*ZNF521*
211986_at	*AHNAK*
223647_x_at	*HSCB*
220952_s_at	*PLEKHA5*
205732_s_at	*NCOA2*
213908_at	*WHDC1L1*
219669_at	*CD177*
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PARALLEL STUDY

Together with the study of *MLL* and its partner genes in pediatric acute myeloid leukemia (AML), it was conducted a cooperation with other colleagues for the characterization of the oncogenic role of the transcription factor CREB in pediatric acute leukemia. As part of this study, it was demonstrated the mechanism of regulation of CREB protein by its inhibitor ICER. Moreover, this protein has been shown to regulate DUSP1 expression, which is a phosphatase that dephosphorylates the pro-apoptotic p38 MAPK. When ICER is expressed leads to the activation of p38 pathway enhancing chemotherapy sensitivity of leukemia cells. Instead, the improper balance between CREB and ICER caused a high level of CREB protein expression, which was shown to be a common feature in pediatric acute leukemia. The observation that it was produced a large amount of CREB protein, without a corresponding augmented level of its mRNA, led to the consideration of post-transcriptional regulators, such as microRNAs. In particular, it was identificated a specific microRNA, miR-34b, which targets the 3'UTR region of CREB mRNA downregulating its translation. Moreover, it was found that this microRNA is expressed at very low levels in acute leukemia, leading us to the study of the methylation status of the miR-34b promoter. Analyses of miR-34b methylation status in leukemic cell lines and patient samples at the onset of acute leukemia and in remission (as in patients classified as MDS) allowed to establish a direct correlation between the methylation status of the microRNA and the development of leukemia, supporting the hypothesis that a dysregulated expression of miR-34b (and thus of the CREB protein) is of considerable importance in the development of the disease.

During this PhD course, the study of CREB oncoprotein in AML has given good results and led to the following pubblications:

ICER expression inhibits leucemia phenotype and controls tumor progression

Pigazzi M, Manara E, **Baron E**, Basso G

Leukemia 2008 Sep 11

Mir-34b targets cAMP response element binding protein (CREB) in acute myeloid leucemia

Pigazzi M, Manara E, **Baron E**, Basso G

Cancer Research 2009 Mar 3

ICER evokes DUSP1-P38 pathway enhancing chemotherapy sensitivity in myeloid leukemia

Pigazzi M, Manara E, Beghin A, **Baron E**, Tregnago C, Basso G

Clinical Cancer Research in press

Hypermethylation of miR-34b is associated with CREB overexpression and Myeloid Cell Transformation

Pigazzi M, Manara E, Beghin A, **Baron E**, Tregnago C, Gelain S, Giarin E, Bresolin S, Masetti M, Rao D, Sakamoto KM, Basso G

Under revision

About the author

Emma Baron was born on September 29, 1983 in Thiene (VI), Italy. She graduated in 2002 at “Liceo Classico F. Corradini” of Thiene (VI). In 2005 she obtained her Triennal Bachelor in Molecular Biology at the University of Padua. In 2007 she obtained her Specialistic Bachelor in Biology for Human Health at the University of Padua and the qualification of Biologist at the University of Florence. Since 2007 she works at the Onco-hematology laboratory of the Pediatric Department at the University of Padua. Her field of work is the characterization of MLL-AF6 leukemogenic mechanism in t(6;11)(q27;q23) leukaemia as well as the study of the CREB oncoprotein and its regulation in childhood myeloid acute leukaemia.

Publications

Pigazzi M, Manara E, **Baron E**, Basso G. ICER expression inhibits leucemia phenotype and controls tumor progression. Leukemia. 2008 Sep 11.

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Pigazzi M, Manara E, Beghin A, **Baron E**, Tregnago C, Gelain S, Giarin E, Bresolin S, Masetti M, Rao D, Sakamoto KM, Basso G. Hypermethylation of miR-34b is associated with CREB overexpression and Myeloid Cell Transformation. Under revision.

Baron E, Pigazzi M, Basso G. AF6 nuclear sequestration by chimeric *MLL-AF6* allows overexpression of RAS signaling pathway in t(6;11)(q27;q23) acute myeloid leukemia. Under revision.

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