LYMPHOID NEOPLASIA

Gene profiling of Graffi murine leukemia virus induced lymphoid leukemias: identification of leukemia markers and Fmn2 as a potential oncogene

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The Graffi murine leukemia virus induces a large spectrum of leukemias in mice and thus provides a good model to compare the transcriptome of all types of leukemias. We analyzed the gene expression profiles of both T and B leukemias induced by the virus with DNA microarrays. Given that we considered that a four fold-change in expression level was significant, 388 probsets were associated to B, to T, or common to both leukemias. Several of them were not yet associated with lymphoid leukemia. We confirmed specific deregulation of Fmn2, Arntl2, Bfsp2, Gfra2, Gpm6a, Gpm6b in B leukemia, of Nln, Fbln1 and Bmp7 in T leukemias and of Etv5 in both leukemias. More importantly, we show that the mouse FMN2 induced an anchorage-independent growth, a drastic modification in cell shape with a concomitant disruption of the actin cytoskeleton. Interestingly, we found that human Fmn2 is overexpressed in about 95% of pre-B acute lymphoblastic leukemia with the highest expression levels in patients with a TEL/AML1 rearrangement. These results, surely related to the role of Fmn2 in meiotic spindle maintenance, suggest its important role in leukemogenesis. Finally, we propose a new panel of genes potentially involved in T and/or B leukemias.
Introduction

To understand the mechanism of induction of leukemia in humans, the mouse has been considered to be an ideal model given the extent of genetic similarity between these two species. We have already shown that the Graffi murine leukemia virus (MuLV), when inoculated into newborn mice, induces a broad spectrum of leukemias of lymphoid (T or B) and non lymphoid (myeloid, erythroid or megakaryoblastic) origins.\(^1\) We also demonstrated that the Graffi MuLV directly targets the \(c\text{-}myc\), \(Fli\text{-}1\), \(Pim\text{-}1\) and \(Spi\text{-}1/PU.1\) oncogenes\(^2\) but also the \(Gris\text{-}I\) locus that encodes an oncogenic truncated form of cyclin D2.\(^3,4\) We took advantage of the large spectrum of leukemias induced by the Graffi murine leukemia virus to analyze and compare the transcriptome of these leukemias with a DNA microarray approach. One clear advantage of microarray analysis is that genes that are not targeted through retroviral integration but act as oncogene upon deregulation can be equally identified. Using this approach, we recently identified several genes that are directly involved in erythroid and megakaryoblastic leukemias in both mouse and human.\(^5\) In this report, we have specifically compared the transcriptomes of T and B lymphoid leukemias induced by Graffi MuLV to their corresponding controls. We identified new relevant signatures that highlight many potential markers or oncogenes for T and B leukemias (especially for pre-B-leukemia), some of which were common to both types of lymphoid leukemia. Among the selected genes, we validated the modulation of the expression of 10 genes out of 12, which functions remained poorly elucidated in lymphoid leukemias. Furthermore, for the first time, we provide data supporting a role for \(Fmn2\), a member of the formin family in leukemogenesis in pre-B-ALL and particularly, in pediatric pre-B-ALL harboring the t(12;21) TEL/AML1 translocation.
Materials and Methods

Human sample collection

For this study, samples from 12 pediatric pre-B-ALL were obtained from Dr. Daniel Sinnet (Sainte-Justine hospital, Montreal) while samples from 13 adult patients with different types of B leukemia were obtained from the Quebec Leukemia Cell Bank. As a control (CH), we pooled peripheral blood mononuclear cells isolated from ten healthy adults. Information relative to each patient is provided in supplemental Table S1. The research protocol was approved by Ethic Committees of all concerned institutions.

Mice sample collection

Newborn (≤ 24 Hrs) NFS mice were injected intraperitoneally with viral particles of Graffi MuLV variant GV-1.4 (1. 10^6 PFU) or GV-1.2 (3. 10^6 PFU). Lymph nodes, thymus, bone marrows and spleens were harvested from moribund mice for flow cytometry analysis and RNA extraction. All the experimental procedures were approved by the Animal Care Committee of Université du Québec à Montréal.

Flow cytometry and cell isolation

Flow cytometry was performed as previously described. Cell populations (tumor or control) were purified from the hematopoietic organs by positive selection using magnetic beads coated with the chosen antibody. Leukemic cells were sorted as follow: T-cells from the thymus, B-cells from the enlarged lymph nodes, erythroid and megakaryoblastic cells
from the infiltrated spleen. Non leukemic control cells were sorted from a pool of 12 non-infected NFS mice: T-cells from the thymus, B-cells from the spleen, erythroblasts and megakaryoblasts from the bone marrow.

**RNA isolation, Microarray hybridization and Data set normalization**

Total RNA was directly isolated from spleen, thymus, lymph nodes, bone marrow samples or after cell-sorting with the Trizol reagent (Invitrogen, Burlington, ON, Canada) and purified using RNeasy Mini Kit (Qiagen, Mississauga, Canada). Total RNA (2 ug) from each sample was prepared for hybridization to Affymetrix Gene Chip® Mouse Genome 430 2.0 arrays (Genome Quebec Innovation Centre, Montreal, Canada). The Affymetrix MicroArray Suite version 5.0 was used to scan and quantify the arrays. Normalization of gene expression data were performed using the Bioconductor implementation of RMA (Robust Multi Array, B. Bolstad, University of California, Berkeley) available from the Flexarray software (1.2, R 2.7.2,6).

**Microarray data analyses**

Using the gene expression profiles obtained from selected leukemias, the RMA values of the 45000 probsets were used to identify differentially expressed genes. Genes with signal intensity significantly higher (up-regulated), or lower (down-regulated) in leukemias versus control cells, i.e. with a 4 fold-change in expression levels were selected. To group microarrays and/or genes based on the high degree of their expression patterns, hierarchical clustering (complete linkage clustering, correlation uncentered) was constructed using GeneCluster software.7 Prosets selected were further analyzed and the results were treated
with Tree View program. The NetAffx website (Affymetrix, Santa Clara, CA, USA) was also used to retrieve gene ontology (GO) annotations, probe sequences, and utilized as a link to Unigene (NCBI) for further functional studies. The microarray dataset was deposited at Gene Expression Omnibus under the accession number GSE12581.

**Semi-quantitative RT-PCR**

Reverse transcription reactions were performed with oligo(dT) as primer using the Omniscript enzyme (QIAGEN, Mississauga, Canada) and 100 ng of total RNA. Using a RT reaction corresponding to 10 ng of tumor RNA samples for each selected gene and to 2 ng for actin, the PCR reactions were performed with the Taq polymerase kit (Qiagen) and the following conditions: 94°C for 3 min, 94°C for 45 s, 56°C for 45 s, 72°C for 30 s with a final extension at 72°C for 10 min. Annealing temperature and number of cycles were optimized for each of the selected 12 genes with specific primers (Table S2) for semi-quantitative analysis. PCR products were analyzed on agarose gel and band density was quantified with Quantity One Image Software, using the actin gene as an internal control.

**Cellular localization of FMN2**

NIH/3T3 fibroblasts, obtained from ATCC (Rockville MD), were grown in DMEM medium supplemented with 10% calf serum, 50U penicillin and 50 ug of streptomycin (Gibco, Invitrogen, Frederick, MD), 16h prior to transfection. Cells were respectively transfected with p-EGFP-N1 (control vector) and GFP-tagged *Fmn2* using the polyfect reagent (Qiagen). *FMN2* localization was analyzed by confocal microscopy 48h after transfection. For co-localization, cells were plated on glass coverslips, grown at 50%
confluency, and transfected as described above. Co-localization with the cell surface membrane was determined after cell staining for 5 min with 2.5 ug/ml of CellMask™ Plasma Membrane Stains (Invitrogen) and washing with PBS. Actin filament staining was performed after cell fixation for 20 min with 4% paraformaldehyde, followed by PBS washes and permeabilization for 5 min with 0.1% Triton X-100 in PBS. Cells were incubated 1h in PBS with 1% BSA, washed twice with PBS and the coverslips and then incubated with 0.3 uM of phalloidin coupled to the Alexa Fluor 555 (Invitrogen) for 20 min. After two washes with PBS, coverslips were mounted onto slides using Prolong Gold antifade reagent (Invitrogen) and observed within 24h by confocal microscopy. For alpha-tubulin staining, the primary antibody used was a mouse monoclonal anti-alpha-tubulin (1:2000) (Sigma).

Colony formation in soft agar

To determine the anchorage-independent growth, colony formation was tested in soft agar as previously described. Briefly, NIH/3T3 cells were transiently transfected with 2.5 ug of empty vector (pCMV), a Ras EJ 6.6 or pCMV-Fmn2 expression vector. After 48h, 1 x 10^4 cells were mixed with melted 0.3% agarose in DMEM medium and seeded in six-well plates on top of a 0.6% agarose base layer containing the same medium. The top layer was covered with 1.5 ml of DMEM. Cells were fed twice a week for 4 weeks and observed with an optical microscope (Ernst Leitz, 6MBH Wetzlar) at 40X. Colonies whose size was at least twice larger than that of control colonies were counted.

Results
To better elucidate the cancer signatures of B and T leukemias induced by the murine Graffi virus and to identify new oncogene candidates, a microarray analysis was performed on different types of B and T leukemias induced by this virus in comparison to non leukemic B (CB1 (CD45R⁺CD19⁺)) and T (CT1 (CD4⁺CD8⁺)) cell populations. Three B leukemias (B1 and B2 (CD45R⁺CD19⁺), B3 (CD45R⁺lowCD19⁺Sca1⁺)) and 3 T leukemias (T1 (CD4⁺CD8⁺), T2 (CD4⁺CD8⁺) and T3 (CD4⁺CD8⁺)) were chosen for the microarray experiments (NCBI GEO: GSE12581). We were especially interested to identify genes commonly deregulated in these tumors despite their heterogeneity and different stage of differentiation. Hierarchical clustering analyses of genes with a four fold-change in expression levels compared to control samples were used to obtain a general trend (Figure S1). This analysis allowed us to group leukemia samples (columns) and/or genes (rows) based on the similarity of their expression level. As a result, T leukemias and B leukemias were clustered separately, making 2 distinguishable groups (Figure S1). According to these data, Graffi-induced T and B leukemias showed both distinct and common gene expression profiles. Indeed, clustering led to the formation of 6 subgroups representing probsets either specifically deregulated in B leukemias (188 overexpressed and 86 down-regulated), specifically deregulated in T leukemias (9 overexpressed and 48 down-regulated) or commonly deregulated in both types (8 overexpressed and 32 down-regulated). The complete list of genes presenting these lymphoid signatures is available at: www.biomed.uqam.ca/rassart/microarray2.html.

Gene expression profile specific for pre-B leukemias

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We first analyzed the expression profile of genes that could determine the stage of differentiation of the B leukemias. For all B leukemias, \textit{Rag2}, \textit{Vpreb1}, \textit{Igll1}, \textit{Enpep}, \textit{Ebf1}, \textit{Il7R}, \textit{Bst1} and \textit{Foxp1} were overexpressed compared to control cells (Table 1). Results in this table correspond to the average expression calculated from all samples analyzed in the microarray analysis (B and T controls, B lymphoid, T lymphoid, myeloid, erythroid and megakaryoblastic tumors). Strong expression of these genes indicated that the three Graffi MuLV-induced B tumors were most likely at a pre-B differentiation stage.\textsuperscript{5,9-15} In all B leukemias, we also detected high levels of \textit{CD79a} and \textit{CD79b} mRNAs and low levels of \textit{CD20/Ms4a2} mRNA (Table 1). A total of 274 probsets, corresponding to at least 218 genes, were found highly deregulated in all three B leukemias compared to control B lymphocytes. Among these genes, 72 (86 probsets) were down-regulated and 146 (188 probsets) were up-regulated (Figure S1). Several of them had already been associated with B leukemias and are listed in Table 2.

In order to identify new potential oncogenes associated with the development of pre-B leukemias, we further focused our analysis on 191 probsets (Table S3), mostly because they were not yet associated with lymphoid leukemias. Some of them (70 probsets including \textit{Crisp3} and \textit{Lphn2}) were already involved in cancer but never associated with B leukemia. Others (72 probsets) were never associated with cancer and 36 probsets had no assigned function. Finally, the 13 remaining probsets were involved in leukemias other than B leukemias (Table S3).

\textbf{Gene expression profile specific for T leukemias}
For all analyzed T leukemias, the expression levels of CD3, CD4 and CD8 markers, in comparison to the other tumor samples (B lymphoid, myeloid, erythroid and megakaryoblastic tumors), were in accordance with a T lineage immunophenotype (Table 1). Other markers known to be present at the T-cell surface were also detected (Table 1). Among these, Cd6 and Cd28 were the most specific to Graffi-induced T leukemias. We also observed that Cd160 was expressed in all 3 T leukemias but not in double positive control T-cells. Moreover, the two CD8+ tumors (T1 and T2) also expressed Cd7 and Dntt (TdT) (Table 1).

We found that 57 probsets corresponding to 48 genes showed the same pattern of deregulation in all three T leukemias in comparison to control T lymphocytes (Figure S1; Table S3) but not in the B leukemias. Most of them (40 genes) were down-regulated while only 8 were up-regulated.

Nine probsets were already associated with T leukemias (Table 2), thus validating our approach. Among the remaining 48 probsets, 15 were associated with other cancers, 5 were related to leukemias other than T leukemias, 20 were neither associated with leukemias nor with other types of cancer and 8 had no assigned function (Table S3).

**Expression profiles common to both B and T leukemias**

The RMA analysis also revealed that 57 probsets (corresponding to 48 known genes) were modulated in both T and B leukemias compared to controls (Figure S1). These genes may be associated with common characteristics of lymphoid leukemias or common oncogenic features and/or directly related to Graffi leukemogenesis.
Several genes already known to be associated with B and T leukemias were identified (Table 2). Moreover, among the 57 selected probsets, 36 had never been simultaneously associated to both types of lymphoid leukemias although some were already associated with one type (T leukemia: 10; B leukemia: 4; Table S3). Among the remaining probsets, 8 were identified in other cancers, 9 had never been associated with any types of cancer and 4 remain uncharacterized. Matr3 was the only gene already associated with leukemia (acute myeloid leukemia).

RT-PCR validation
To validate our microarray data, the expression levels of 12 genes specific to either B, T or to both leukemias (Fmm2, Arntl2, Bfsp2, Gfra2, Gmp6a, Gmp6b, Bmp7, Fbhn1, Nln, Mettl1, Etv5 and Celsr1 (Tables 3 and S3)) were measured by semi-quantitative RT-PCR in several Graffi MuLV induced tumors (pure cell populations (Figure 1) or unsorted cell suspensions (Figure S2)). Significant over-expression of Fmm2, Arntl2, Bfsp2 and Gfra2 was observed in the majority of B leukemias while being absent in T leukemias confirming their specificity to B leukemias (Figure 1A; Tables 3 and S3). Gmp6a and Gmp6b, expected to be B leukemia-specific, showed significant overexpression in these tumors when compared to the control samples albeit in only 2 and 3 out of the 5 tested B leukemias, respectively. As expected, these two genes were not expressed in T leukemias or control samples. High expression levels of Bmp7 were significantly observed in all T leukemias while Fbhn1 and Nln were overexpressed in 3 out of 5 T leukemias (Figure 1B). Mettl1, expected to be T leukemia-specific gene, was expressed in both T and B leukemias (including controls) (Figure 1B). For, Etv5, significant higher levels of expression were observed in 4 out of 5 B
leukemias and 3 out of 5 T leukemias (Figure 1C). Finally, Celsr1 was significantly overexpressed in most T leukemias compared to normal control (CT2) but, in contradiction with the microarray data, was poorly expressed in all B leukemias (Figure 1C). Similar validation results were obtained when using CD19+ splenic cells as normal control instead of CD45R+ cells (not shown).

**FMN2 induces anchorage-independent growth**

We further focused our interest on Fmn2, a gene specifically overexpressed in B leukemias. This gene is a member of the Formin family (FH proteins) and its product is highly conserved among the multidomain proteins involved in a growing range of actin-based processes. Most importantly, they may promote cancer cells to become invasive and metastatic through their actin remodeling function.

We first studied the impact of Fmn2 over-expression on the anchorage-independent growth of NIH/3T3 cells, a classical assay to demonstrate the oncogenic potential of proteins. As shown in Figure 2, the number of larger colonies formed in soft agar was significantly higher in Fmn2-expressing cells in comparison to control cells and similar in cells expressing the human Ras oncogene (Ras EJ 6.6) (Figure 2). These results suggest that the FMN2 protein confers anchorage independence to NIH/3T3 cells.

**FMN2 colocalizes with the plasma membrane and disrupts the actin network**

We next determined the subcellular localization of FMN2. When GFP-tagged Fmn2 was transiently expressed in NIH/3T3, the protein was localized at the plasma membrane and within the cytoplasm (Figure 3A). This was further confirmed by co-localization of FMN2
with a cell membrane marker (CellMask™ Plasma Membrane Stains) (Figure 3B).

We also observed that NIH/3T3 cells expressing Fmn2 showed a reduced size and an abnormal morphology compared to control cells (Figure 3). Moreover, Fmn2-expressing NIH/3T3 cells appeared rounded with long extensions (Figure 3A-D). Actin cytoskeleton is one of the cellular components that maintain cell shape and oncogenic transformation causes profound modifications linked to cell architecture. Since the overexpression of Fmn2 in NIH/3T3 cells altered their morphology, we first tested the effect of its overexpression on actin filaments and on the microtubule network due to its reported action on actin cables. As illustrated in figures 3C and D, cells overexpressing Fmn2 showed a drastic disruption of the actin and microtubule network and a reduced number of stress fibers compared to control cells.

**Fmn2 gene overexpression in human pre-B leukemia**

We measured the expression levels of human Fmn2 in patients with different types of B leukemias (Figure 4). No expression was detected by RT-PCR in control samples, in Mantel cell lymphoma, follicular lymphoma, B-cell prolymphocytic leukemia and chronic lymphocytic leukemia. In contrast, Fmn2 expression was easily detected in almost all pre-B-ALL patients (L1 and L2) (18/19) and in one out of two Burkitt leukemias. Interestingly, the highest levels of Fmn2 were detected in all L1 pediatric pre-B-ALL samples bearing a t(12;21) translocation (lanes 7, 9, 11, and 12). Another tumor (lane 3) also showed high levels of Fmn2 expression suggesting that it could harbor the translocation as well although this remained to be confirmed.
Discussion

Graffi MuLV is a good model to gain new insights on leukemia development and progression and to identify new oncogenes. Gene expression profiling of each type of leukemia (T lymphoid, B lymphoid, myeloid, erythroid, megakaryoblastic) served to identify the cancer signatures of these specific leukemias. In this paper, we determined the expression profile of 3 B and 3 T leukemias induced by this retrovirus in comparison to non leukemic cell controls (CB and CT) (Tables 2, 3 and S3) and to non lymphoid leukemias induced by the same virus (Table 1, www.biomed.uqam.ca/rassart/microarray2.html). Setting the minimal acceptable change in expression levels to four folds, we selected 388 probsets corresponding to 305 genes: 48 genes specifically modulated in T leukemias, 218 in B leukemias and 40 in both types in comparison to their respective controls.

Phenotypic properties and cancer signature of B leukemias

Our analysis suggests that B leukemias induced by Graffi MuLV are arrested at the pre-B stage of differentiation based on the high expression of pre-B specific markers (Table 1). As in human pre-B-ALL, these leukemias overexpressed surface markers such as CD79a and CD79b and lacked CD20/Ms4a2 (Table 1). These results suggest that data retrieved from the gene profiling analysis of B leukemias should be especially relevant for human pre-B-ALL. Indeed, the results obtained for Fmn2 expression in human leukemias are in total agreement with the mouse data.

We selected 218 genes that were specifically deregulated in all 3 B leukemias and as presented in Table 2, several of these genes were already known to be involved in lymphoid leukemia. This not only validates our microarray analysis but also further defines the cancer
signature of these B lymphoid leukemias.

**Phenotypic properties and cancer signature of T leukemia**

Three distinct T leukemias (T1 (CD4+CD8+), T2 (CD4-CD8+), T3 (CD4+CD8-)) were chosen for the microarray experiments. These three different phenotypes are frequently observed in Graffi-induced T leukemias and are also representative of normal T cell types in healthy mice.¹ The retrovirus may have targeted slightly different T-cell blasts or transformed the targeted cells through these slightly different lineages. Based on the analysis, all T leukemias expressed markers specific for the T lineage (Table 1) as well as T-ALL associated markers such as *Cd7* and *Dntt (TdT).*²³ However, we also found that all 3 T leukemias expressed high levels of *Cd160.* This marker, normally expressed on human NK and only on a subtype of human CD8+ cells,²⁴ seems to be overexpressed in almost all cases of B-cell chronic lymphocytic leukemia (CLL).²⁵

The analysis reveals that a total of 48 genes were strictly modulated in all T leukemias in comparison to control T-cells (Tables 2, 3 and S3), including 9 probsets already associated with leukemias. The overall data certainly reveals the existence of a common leukemic gene signature between T leukemias induced by the virus despite heterogeneity and phenotypic differences. We can speculate that similar deregulation patterns observed in these leukemias are associated with common characteristics of T leukemias or common oncogenic properties and are likely to be found in human T leukemias as well.

**Cancer signature common to both B and T leukemias**

Since we simultaneously analyzed gene expression profiles of B and T leukemias induced
by Graffi MuLV, regardless of lineage differences and tumor heterogeneity, we found that 57 others probsets modulated in T leukemias were similarly modulated in B leukemias, including 21 previously reported genes (Table 2). These results strongly reinforce the potential of the remaining 36 probsets to be important for the development of B leukemias. Among these, 23 probsets had already been associated with either T or B leukemias, with myeloid leukemia or other cancers (Table S3). These genes are certainly part of the common cancer signature of the T and B lymphoid leukemias and may be relevant for human lymphoid leukemias.

New potential markers and candidate oncogenes from B and T leukemias

The combination of our Graffi-induced tumor model and DNA microarrays allowed us to identify potential new markers of B and T leukemias that could also play an oncogenic role. Among the selected 388 probsets (at least 305 genes), we further identified 275 genes not yet associated with lymphoid leukemias (Table S3). Some were specific to B, T leukemias or common to both types and 124 were obvious oncogene candidates since they had been already associated with non lymphoid leukemia or other cancers. More interestingly, we identified a total of 103 genes that had never been associated with any type of cancer and 32 probsets corresponding to uncharacterized genes or genes with unknown function.

By RT-PCR, we have validated changes in the expression levels of 10 out of the 12 genes selected according to their specificity for lymphoid leukemias (Figures 1 and S2). Our analysis revealed deregulation of the expression of several genes associated with Graffi MuLV-induced B leukemias (Table 1; Figures 1A and S2). These genes are \textit{Fmm2}, \textit{Arntl2} (from the bHLH-PAS superfamily involved in regulating cell growth and differentiation\textsuperscript{26}),
**Bfsp2** (a member of the intermediate filament family and component of cytoskeleton proteins in the lens cells, maintaining their morphology and promoting their motility\(^\text{27}\)), **Gfra2** (from the glial cell line-derived neurotrophic factor receptor alpha family\(^\text{28}\) and associated with primary neuroblastomas\(^\text{29}\) and with some medullary thyroid carcinoma (MTC) tumor cells\(^\text{30}\)), **Gmp6a and Gmp6b** (members of the myelin proteolipid protein family and neuronal homologues of PLP/DM2\(^\text{31}\)).

We also identified several genes that were associated with T leukemias (Figure 1B), namely, **Nln** (from the metallopeptidase M3 family and involved in the metabolism of neurotensin\(^\text{32}\)), **Bmp7** (a cytokine from the TGF\(\beta\) superfamily and expressed in various types of cancer including prostate and breast cancers and melanoma\(^\text{33-35}\)) and **Fbln1** (involved in heart development and in cell signalling involving growth factors\(^\text{36}\) and associated with human neoplasia especially breast and ovarian cancers\(^\text{37}\)).

**Etv5** (a member of the Ets family of transcription factors) was already described in association with B leukemias\(^\text{38}\) but, according to our data, it appears also overexpressed in T leukemias (Figure 1C). In contrast, the modulation of expression of **Celsr1** (involved in the regulation of cell polarity and in convergent extension\(^\text{39}\) and expressed in gastrointestinal tumors\(^\text{40}\)), expected to be specific to both B and T leukemias based on the microarray analysis, was validated in all T leukemic samples (Figures 1C and S2) but in only one B tumor (Figure S2).

Finally, the high level of expression of **Mettl1** (highly expressed in lung cancer\(^\text{41}\)) observed by the microarray analysis was not confirmed by RT-PCR (Tables 3 and S3; Figure 1B) and, as for **Celsr1**, may reflect a certain degree of tumor heterogeneity as often observed in human leukemias as well.
We also compared gene expression between B tumors and normal B cells from the bone marrow sorted with an anti-CD45R antibody. This control includes B cells at different stages of maturation. The majority of the tested genes showed results similar to those obtained with spleen the B cells as control except for \textit{Fmn2}, which was highly expressed in the bone marrow-derived cells (data not shown). Although this had not yet been reported, \textit{Fmn2} seems to be normally expressed at an early stage of B-cell lymphopoiesis in the bone marrow. Its expression decreases when B-cells move from the bone marrow to the spleen for maturation. This inverse correlation between the expression level of \textit{Fmn2} and B-cell maturation does not necessarily contradict its involvement in carcinogenesis. This is exemplified with \textit{GATA-2} which is essential for the maintenance and the proliferation of hematopoietic progenitors during normal hematopoiesis,\textsuperscript{42} while having been implicated in tumorigenesis.

Overall, these results strongly suggest that the majority of the 275 selected probsets, in particular \textit{Fmn2, Arntl2, Gpm6a, Gpm6b, Bfsp2, Gfra2, Nln, Bmp7, Fbln1} and \textit{Etv5}, are potentially new specific markers or oncogenes for B, T or B and T leukemias. Our analysis also identified several down-regulated genes such as \textit{KLK6} and \textit{TGFβI} (Table 2) that are already identified as tumors suppressors. Further studies are required to determine whether the modulation of expression also correlates with changes at the protein level and most importantly to determine their potential role in human leukemias. Regarding FMN2, our attempts to measure levels of this protein in mice tumors was hampered by the lack of specific antibodies.
Fmn2 gene is a good candidate oncogene

Among the 10 genes validated by RT-PCR, we further characterized Fmn2, which was specifically associated with B leukemias. Fmn2 is expressed in the developing and mature central nervous system and in oocytes. It was identified as a formin homology (FH) gene and the protein contains 2 characteristic FH protein domains: FH1 (proline-rich region) and FH2. The latter is responsible for actin nucleation. The comparison of the mouse and human Fmn2 showed 74.7% sequence homology. Members of the formin family are implicated in cytokinesis, organogenesis and normal tissue homeostasis but are also involved in the invasive potential of cancerous cells and metastasis. The implication of Fmn2 in the development of tumors had not yet been demonstrated even though human Fmn2 ESTs were found in several human tumors (parathyroid tumor, glioblastoma, retinoblastoma and chondrosarcoma).

In this paper, we report for the first time that Fmn2 is not only specifically overexpressed in B leukemias induced by the Graffi virus in mice (Figure 1; Tables 3 and S3) but more importantly in human pre-B-ALL (Figure 4).

Moreover, we demonstrate that ectopic expression of Fmn2 confers anchorage-independent growth to NIH3T3 cells (Figure 2). This anchorage-independent growth conferred by FMN2 is probably related to its ability to induce the disruption of the actin and microtubules network and a reduction of the number of stress fibers. The biological role of FMN2 in actin and microtubule network disruption in B leukemia is not quite clear. However, we are convinced that the up-regulation of Fmn2 expression could disturb the dynamic of the actin network of ALL cells accompanied by the reorganization of their cytoskeleton which in turn could contribute to their abnormal behaviours. Some examples
highlight the fact that even B cells change form depending upon their state of development or their abnormal behaviours. Indeed, it was reported that during spreading, the B lymphocyte cyto-architecture is converted from a semirigid (before migration) to a more flexible state (during migration). This migration seems to involve up-regulation of CD44 adhesion molecule on activated B lymphocytes and to require the rearrangement of many cytoskeleton components (actin, microtubules and vimentin).\textsuperscript{45} Also, Caligaris-Cappio et al. demonstrated that cells from B-CLL or hairy cell leukemia (HCL) showed an aberrant cytoskeleton organization.\textsuperscript{46} Additionally, Schmitt-Gräff et al. observed changes in the F-actin in B-cells of patients with ALL.\textsuperscript{47}

**Implication of Fmn2 in human pre-B-ALL**

To determine the possible contribution of human *Fmn2* to leukemogenesis, we measured its expression in 25 different B leukemia samples. We showed that this gene was specifically overexpressed in L1 and L2 pre-B-ALL (18/19 of cases) (Figure 4) thereby agreeing with our microarrays data (Table 1). More importantly, we show that four pediatric pre-B-ALL samples with a t(12;21) translocation produced the strongest signals. Such t(12;21) rearrangement involving the *TEL/AML1* genes is more frequent in childhood-ALL (25-30\%) with a B-precursor phenotype than in adult-ALL (3-5\%).\textsuperscript{48} Despite the fact that this translocation is associated with a favorable outcome and a good response to conventional chemotherapy, 25\% of relapses occur off-therapy and require additional therapeutic strategies. The strong expression of *Fmn2* in pediatric pre-B-ALL with the t(12;21) translocation suggests that the TEL/AML1 fusion protein could directly or indirectly upregulate *Fmn2* gene expression. Together, these results suggest that very high
expression of $Fmn2$ in pre-B-ALL could be correlated with a t(12;21) translocation and
could be used as marker although this has to be confirmed with a larger panel of samples.

In conclusion, we identified a set of genes that are specific markers for B and T
leukemias induced by the Graffi MuLV and thus may also serve as potential markers in
human lymphoid leukemias. Some of these genes may have oncogenic properties as
revealed with the mouse $Fmn2$ gene. For the first time, we show that $Fmn2$ is up-regulated
in human pre-B-ALL and more specifically in pediatric pre-B-ALL with the t(12;21)
translocation. Additional investigations are necessary to further characterize its function in
tumor induction.

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Authorship

Contribution: E.R. and V.V. designed the microarray experiments; V.V. performed the
microarray experiments; C.C. analyzed the microarrays data of the lymphoid leukemias, and
performed the experiments; L.C.L. contributed to some experiments and helpful discussion; C.C., E.E. and E.R. wrote the manuscript; E.E and E.R. supervised the overall project.

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References


Table 1. B and T leukemias immunophenotype

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**B-cell phenotype**

**T-cell phenotype**

Results are presented in log base 2. CT: CD4+CD8+; T1: CD4+CD8-; T2: CD4+CD8-; T3: CD4+CD8-; CB: CD45R+CD19-; B1-B2: CD45R+CD19-; B3: CD45R-CD19-; M: myeloid leukemia (CD11b+CD123+); CE: control erythroid cells (Ter119+CD71+); E1-E3: erythroleukaemias (Ter119+CD71+); Mk1-Mk3: megakaryoblastic leukaemias (Mk1 (Kit-CD41+); Mk2-Mk3 (Kit+CD41+))

* amplitude of deviation from the mean calculated from the RMA values

** ratio T-CT and B-CB: mean of the deviation of T1, T2 and T3 / T control value and mean of the deviation of B1, B2 and B3 / B control value
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</table>
Results are presented in log base 2. T1, T2, T3: T leukemias; B1, B2, B3: B leukemias. CT: T control; CB: B control.

* ratio T-CT: mean of the deviation of T1, T2 and T3 / T control value
** ratio B-CB: mean of the deviation of B1, B2 and B3 / B control value
<table>
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<tr>
<th>ProbesetIDs</th>
<th>T1-CT</th>
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Results are presented in log base 2. A positive deviation of 4 and above and a negative deviation of 4 and below means a fold-change of 4. Values comprised between +0.585 and -0.585 are considered to be not significant.

T1, T2, T3: T leukemias; B1, B2, B3: B leukemias; CT: T control; CB: B control.

* ratio T-CT and B-CB: mean of the deviation of T1, T2 and T3 / T control value and mean of the deviation of B1, B2 and B3 / B control value.
Figure Legends

Figure 1. Analysis of selected genes differentially expressed in sorted lymphoid leukemia samples. Semi-quantitative RT-PCR analysis in 5 T (T4: CD4+CD8+, T5: CD4+CD8+, T6: CD4+CD8+, T7: CD4+CD8+, T8: CD4+CD8–) and 5 B (B4: CD45+CD19+Sca1+, B5: CD45R+CD19+Sca1+, B6: CD45R+CD19+Sca1+, B7: CD45R+CD19+Sca1+, B8: CD45R+CD19+Sca1+) leukemias. RT-PCRs were performed in triplicate for each gene. The actin gene was used as internal control in specific conditions described in Materials and Methods and expression level in each leukemia is presented as a selected gene/actin density ratio. (A) B leukemia-specific genes. (B) T leukemia-specific genes. (C) Genes common to both leukemias. Statistical analysis was performed using One-way ANOVA and p <0.05 was considered to be significant (* p<0.05, ** p<0.01, *** p<0.001) compared to the respective control (B-cells from normal spleen (CB2) for the B leukemias and T-cells from normal thymus (CT2) for the T leukemias).

Figure 2. Effect of FMN2 protein expression on anchorage of NIH/3T3 cells. Cells were transiently transfected with pCMV empty vector, with the pCMV-Fmn2 vector and the Ras (EJ 6.6) expression vector. Transfected or control cells (10^4) were plated in soft agar as described in Materials and Methods. After 4 weeks, the number of colonies (at least twice larger than colonies from controls) was scored (A). The results represent the average of three independent experiments. Statistical analysis was performed using One-way ANOVA, p<0.05. Cells were observed with an optical microscope (Ernst Leitz, 6MBH Wetzlar) and representative fields were photographed using a numerical camera (Nikon coolpix 4500) (original magnification X 40). Cells were either left untransfected (B) or transfected with
Ras EJ 6.6 (C), empty vector (D) or pCMV-Fmn2 (E).

Figure 3. Subcellular localization of FMN2 and its effect on cytoskeleton. The GFP-tagged FMN2 protein was localized in NIH/3T3 cells with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) (original magnification X60) (A) and tested for its effect shows on the shape of the cells. (B) Plasma membrane labeling with CellMask. (C) Actin labeling with Alexa Fluor 555-conjugated phalloidin. (D) Alpha-tubulin labeling with anti-alpha-tubulin antibody. Data are representatives of three independent experiments. The GFP vector alone was used as a control. Ovals and arrows indicate transfected and non-transfected cells, respectively.

Figure 4. Expression analysis of human Fmn2 gene in different types of B leukemic samples. Semi-quantitative RT-PCR analysis was performed on 12 pediatric pre-B-ALL (lanes 1-12), 7 adult pre-B-ALL (13-19), 2 Burkitt leukemias (20-21), 1 mantel cell lymphoma (22), 1 follicular lymphoma (23), 1 B-cell prolymphocytic leukemia (24) and 1 chronic lymphocytic leukemia (25). RT-PCRs were performed in triplicate for each gene. The actin gene was used as an internal control as described in Materials and Methods and expression level in each leukemia is presented as a selected gene/actin density ratio. Statistical analysis was performed using One-way ANOVA and p <0.05 was considered to be significant (* p<0.05, ** p<0.01, *** p<0.001) compared to the respective control (CH).
Figure 1

A

B

C

Fmn2

Gpm6a

Arnt2

Gpm6b

Bfsp2

Gfra2

Fbln1

Bmp7

Mettl1

Nln

Etv5

Celsr1
Figure 2

A

![Bar chart showing colonies number for different groups: NIH/3T3, Ras EJ 6.6, pCMV, pCMV-Fmn2.](chart)

B, C, D, E: Images of colonies for different groups.
Figure 3

A

GFP

GFP-Fmn2

B

CellMask

GFP-Fmn2

Merge

C

Phalloidin

GFP

Merge

D

a-tubulin

GFP

Merge
Figure 4
Gene profiling of Graffi murine leukemia virus induced lymphoid leukemias: identification of leukemia markers and Fmn2 as a potential oncogene

Cyndia Charfi, Véronique Voisin, Louis-Charles Levros, Jr., Elsy Edouard and Eric Rassart