Implication of IRF4 Aberrant Gene Expression in the Acute Leukemias of Childhood

Maria Adamaki¹*, George I. Lambrou¹, Anastasia Athanasiadou¹, Marianna Tzanoudaki², Spiros Vlahopoulos¹, Maria Moschovi¹

¹Pediatric Hematology/Oncology Unit, First Department of Pediatrics, University of Athens, “Aghia Sofia” Children’s Hospital, Athens, Greece, ²Department of Immunology and Histocompatibility “Aghia Sofia” Children’s Hospital, Athens, Greece

Abstract

The most frequent targets of genetic alterations in human leukemias are transcription factor genes with essential functions in normal blood cell development. The Interferon Regulatory Factor 4 (IRF4) gene encodes a transcription factor important for key developmental stages of hematopoiesis, with known oncogenic implications in multiple myeloma, adult leukemias and lymphomas. Very few studies have reported an association of IRF4 with childhood malignancy, whereas high transcript levels have been observed in the more mature immunophenotype of ALL. Our aim was to investigate the expression levels of IRF4 in the diagnostic samples of pediatric leukemias and compare them to those of healthy controls, in order to determine aberrant gene expression and whether it extends to leukemic subtypes other than the relatively mature ALL subpopulation. Quantitative real-time RT-PCR methodology was used to investigate IRF4 expression in 58 children with acute leukemias, 4 leukemic cell lines and 20 healthy children. We show that aberrant IRF4 gene expression is implicated in a variety of leukemic subtypes; higher transcript levels appear in the more immature B-common ALL subtype and in T-cell than in B-cell leukemias, with the highest expression levels appearing in the AML group. Interestingly, we show that childhood leukemia, irrespective of subtype or cell maturation stage, is characterised by a minimum of approximately twice the amount of IRF4 gene expression encountered in healthy children. A statistically significant correlation also appeared to exist between high IRF4 expression and relapse. Our results show that ectopic expression of IRF4 follows the reverse expression pattern of what is encountered in normal B-cell development and that there might be a dose-dependency of childhood leukemia for aberrantly expressed IRF4, a characteristic that could be explored therapeutically. It is also suggested that high IRF4 expression might be used as an additional prognostic marker of relapse at diagnosis.

Introduction

Acute leukemia (AL) is regarded the most common type of malignancy in children. Despite the good overall response of childhood patients to current chemotherapeutic treatments and the long-term event-free survival rates exceeding 80% [1,2], a significant percentage of the population show resistance to therapy and/or relapse, often with devastating consequences. This has highlighted the importance of devising new therapies that will target the resistant clones and increase overall survival. Of significant interest are the genetic alterations that might affect the characteristics of hematopoietic stem cells (HSCs). For example, it has already been demonstrated that a subset of genes expressed in normal HSCs are highly reactivated in leukemic stem cells (LSCs), thus indicating that the LSCs express the self-renewal-associated programme normally characterising the HSCs [3]. Such findings suggest that leukemic transformation of progenitor cells is associated with ectopic reactivation of the genes responsible for self-renewal. Indeed, the presence of MLL rearrangements in both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) seems to relate with high level expression of genes normally expressed in HSCs during the early stages of hematopoiesis, such as FLT3 and HOXA9 [4–10]. Consequently, the identification of specific genetic aberrations that characterise leukemic cells, such as abnormal gene expression, can lead to a better understanding of what constitutes their unlimited self-renewing properties and subsequently reveal potential molecular targets for successful therapy.

The most frequent targets of genetic alterations in human leukemias are transcription factor genes with essential functions in normal blood cell development. The Interferon Regulatory Factor 4 (IRF4) gene encodes a transcription factor important for hematopoietic development and immune responses. It is essentially involved in all developmental stages within the B-cell lineage (except during the germinal center (GC) reaction), with known critical functions in at least 3 key developmental processes: the termination of the GC B-cell transcriptional program, immunoglobulin class-switch recombination, and plasma cell development [11–13]. IRF4 is induced by mitogenic stimuli, such as antigen receptor engagement, lipopolysaccharides, and CD40 signaling [14,15], which activate the Nuclear Factor-kappa B (NF-κB) pathway, ultimately leading to IRF4 promoter activation [16–18]. Despite functioning as a tumor suppressor gene in early B-cell
**Gene Expression in Childhood Leukemia**

**RNA Extraction**

Total RNA was extracted from BM leukocytes/myelocytes, using TRIzol reagent (Invitrogen, Inc.) according to the manufacturer’s protocol. Extraction of RNA from the 4 cell lines was performed with the same protocol, only without the addition of RBC Lysis Buffer, for obvious reasons. RNA was treated with amplification grade DNase I to eliminate any residual genomic DNA from the sample and further purified with the RNAeasy® Micro Kit (Qiagen Inc.). RNA quantification measurements were performed on the NanoDrop® ND-1000 Spectrophotometer (NanoGen Inc.).

**Flow cytometry and Immunophenotype**

Blastic cell immunophenotyping was performed by direct immunofluorescence and 5-colour flow cytometry, on a FC500 instrument (Beckman Coulter, Brea CA). Apoptosis and necrosis measurements were performed as described previously [30].

**Sample handling**

Red Blood Cell Lysis Buffer (RBC Lysis Buffer) was added to every BM sample (2:1 ratio), in preparation for RNA extraction. Every sample was vortexed and left at room temperature for 3–5 minutes to ensure sufficient lysis of red blood cells, then centrifuged at 3,000 rpm for 7 minutes, so that the white blood cells, intact, collected at the bottom of the tube, while the lysed red blood cells remained in the liquid supernatant. For immunophenotyping, samples were collected in EDTA tubes containing 1 ml of Hank’s Balanced Salt Solution for every 1 ml of BM specimen. Specimens were analyzed within 3 hours post collection.

**Results**

**Materials and Methods**

**Patient and control samples**

Fifty eight (58) children were diagnosed with AL (52 with ALL and 6 with AML) at the Hematology/Oncology Unit of Athens University, at “Aghia Sofia” Children’s Hospital, Athens, Greece. Bone marrow (BM) samples were collected at diagnosis and all patients were classified morphologically and immunologically. Patient data were as follows: males: 37, females: 21, median age at diagnosis (years): 4.72±4.32, median male age (years): 4.77±4.48, median female age (years): 4.07±3.89, median ALL age: 4.39±4.03, median AML age: 10.48±5.36. Patient clinical data are summarized in **Table S1**. BM samples were also collected from 20 healthy children to be used as negative controls for the qRT-PCR studies. Four childhood leukemia cell lines were also included in the study: REH (pre-B ALL), CCRF-CEM (T-cell ALL), CCRF-SB (T-cell ALL), and THP-1 (acute monocytic leukemia). All cell lines were obtained from the European Collection of Cell Cultures (ECACC, UK).

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**DNA Extraction**

Sample handling

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**Reverse transcription**

**Flow cytometry**

**Real Time Quantitative RT-PCR**

Gene expression was investigated with the Real-Time Reverse Transcription PCR (qRT-PCR), using the Plexor™ One-Step qRT-PCR System kit (Promega Inc.) and the LightCycler 2 Instrument (Roche Diagnostics GmbH). Both the reverse transcriptase and DNA polymerase were added during the initial reaction setup, and the thermal cycler was programmed to perform the reverse transcription step, immediately followed by the thermal cycling program.

For each 25 µl PCR reaction, a 12.5 µl aliquot from the Plexor® Master Mix (2×) was added to a LightCycler® glass capillary (Roche Diagnostics GmbH), followed by 0.5 µl of RNasin® Plus RNase Inhibitor, 0.2 µl of ImPROM-II™ Reverse Transcriptase, 2 µl of primers (1 µl Forward primer and 1 µl Reverse primer), 1 µl of RNA (20 ng/µl) and 8.8 µl of nuclelease-free water. For the housekeeping gene reaction only 1 µl of primer was added, as both the Reverse and Forward primers were provided in the same tube by the manufacturer (Eurogentec S.A.).

The thermal cycling programme was as follows: 1 cycle of 45°C for 5 minutes (reverse transcription), 1 cycle of 95°C for 2 minutes (initial denaturation and inactivation of the reverse transcriptase), 40 cycles of 95°C for 5 seconds (denaturation) and 63°C for 35 seconds (annealing and extension), and 60°C to 95°C, ramp 0.4°C/second intervals (melt temperature curve). The specificity of
the PCR products was verified by gel analysis and products were shown to consist of only a single band.

The sequences of the primers in the 5’ to 3’ direction were as follows: IRF4F: 5’-AG-CGC-ATT-TCA-GTA-AAT-GTA-AAAC-ACA-T-3’ and IRF4R: 5’-TCT-TGT-GTT-CTG-TAG-ACT-GCC-ATG-A-3’. Housekeeping genes GAPDH and b-actin were used for normalization purposes. In their majority experiments were performed with the GAPDH housekeeping gene.

Initially, method sensitivity was tested by creating a standard curve with kanamycin control RNA as provided by the manufacturer. The standard curve included six points at a final RNA concentration ranging from 50 ng to 0.0005 ng (ie. 0.5 pg) in 10-fold increments, therefore it was decided that for the patient and control samples a total of 20 ng would be sufficient for each reaction.

During the first 6 experiments (6 patients) reactions were performed in duplicates and experiments were performed in triplicates to assess intra- and inter-assay variability (no significant variability found). In all other experiments the patient and control samples were tested in triplicate reactions and experiments were performed once. The average value for all three reactions was used for quantification. Non-template negative control reactions were included with each run. All PCR products were electrophoresed on a 2% agarose gel to confirm successful amplification of the desired products.

Real-Time Data pre-processing and Analysis

Real-time data were collected and pre-processed using the LightCycler Software Version 3.5 (Roche Diagnostics GmbH). Following this, data were exported to the PlexorTM Analysis Software (www.promega.com/plexorresources) (Promega, Inc.) and gene expression was obtained in the form of Ct values; this refers to the PCR cycle number during exponential amplification at which the product (measured in real-time by fluorescence emission) crosses an arbitrary threshold. Data pre-processing was performed with Microsoft® Excel.

Statistical Analysis

First of all an independent t-test was performed between patients and controls to assess whether there were any differences in the mean GAPDH expression levels (Ct values) between the two groups. The t-test failed to reveal a statistically reliable difference (p = 0.068), so we accepted all GAPDH Ct values as reliable normalization constants. A second t-test was then performed to compare the mean IRF4 expression levels between the two groups, and a statistically significant difference was revealed (p = 0.003). The Ct value of IRF4 (for every patient and healthy control) was then normalized against the Ct value obtained for the housekeeping gene, GAPDH (ie. ΔCt = Ct of IRF4 – Ct of GAPDH) and the corrected gene expression levels were defined as the expression level for each gene divided by the GAPDH expression level. Taking the corrected expression levels (ΔCt) into account, each patient was tested for significance in differential expression using a z-test. Patients were regarded as significantly differentially expressing the gene if their z-score had a corresponding p-value of ≤0.05. T-tests were performed using the IBM SPSS Statistics program (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.), whereas all other analyses were performed with Microsoft® Excel. Raw Data are included in Table S2 (Raw Data for IRF4) and all statistical analyses performed on SPSS are included in File S3 (Independent t-Tests for IRF4).

Results

Immunophenotypic and cytogenetic subtypes

Immunophenotypes, karyotypes and cytogenetics are also presented in Table S1. Our results are in agreement with other studies regarding the frequencies of the immunophenotypic groups [31,32]. Similarly, our calculated cytogenetic frequencies also seem to be in agreement with those of other western countries [33].

Quantitative Real Time RT-PCR analysis

A quantitative real-time RT-PCR assay was established for the gene of interest. IRF4 was found to be differentially over-expressed, as compared to the healthy controls, in 30 patients (51.7% of total cohort): 26 ALL (18 B-common, 3 pre-B, 4 T-cell and 1 infant leukemia of unknown immunophenotype), 4 AML (1 with M1 and 3 with M5 maturation), and 3 of the 4 cell-lines: REH (pre-B ALL), CCRF-CEM (T-cell ALL) and THP-1 (AML) (Figure 1). The patients over-expressing IRF4 are also highlighted in bold in Table S1. Five (5) of these 30 patients and the 3 cell lines mentioned above revealed a much higher expression ratio (R > 11.6) than the rest (p = 0.000). In addition, the gene was found to be differentially down-regulated in 6 patients (~10%) (Table 1). Therefore, patients were divided into 4 groups: subnormal ratio (ratio < 2.4), normal (healthy) ratio (2.4 ≤ ratio ≤ 7), high ratio (7 < ratio < 11.6) and very high ratio (11.6 < ratio). In the subnormal ratio group, the mean IRF4 expression was significantly lower (p = 0.000) than in the healthy controls group (File S5). Overall, the gene was found up-regulated in a broader spectrum of leukemic subtypes and maturation stages than previously thought, with the highest transcript levels appearing in the AML group (Figure 1).

Correlation Analysis Between Gene Expression and Cytogenetic Abnormalities

From our group of pediatric patients found to over-express IRF4, only 8 (26.7%) appeared to have one of the commonly found cytogenetic translocations, and that was either TEL-AML1 or MLL rearrangement, with the exception of one patient who was BCR-ABL+ve (Table S1). The majority of patients with high transcript IRF4 levels (n = 22) did not have a detectable cytogenetic translocation at diagnosis or had additional cytogenetic abnormalities, such as extra gene copy numbers, deletions, etc. No apparent correlation was found between high gene transcript levels and specific cytogenetic abnormalities.

Correlation Analysis Between Gene Expression and other diagnostic variables

No apparent correlation was found between abnormal IRF4 expression and age, gender, or karyotype (data not shown).

Correlation Analysis Between Gene Expression and MRD levels

No apparent correlation was found between abnormal IRF4 expression and resistance to therapy (as measured in MRD level on day 30). Only 3 patients had detectable MRD on day 30 since initiation of treatment (NYII protocol for ALL and AML-BFM 2004 protocol for AML), one of whom relapsed, along with another patient who relapsed but who had no detectable MRD on day 30 (Table S2).
Correlation Analysis Between Gene Expression and Overall Survival

No significant correlation was found between abnormal IRF4 expression and overall survival (OS) (as measured in years of survival from the diagnosis) on comparing patients with normal and patients with high IRF4 expression (Figure 2, A–H). Please note that Patients 56 and 57 were diagnosed at a much earlier date (2003) and so were regarded as outliers and were not included in the analysis. Even though we have observed twice as many deaths (4 versus 2) in the patient group over-expressing IRF4 (Table S2), the Kaplan Meier curves have not produced a statistically significant difference (p>0.05). The results for estimated leukemia-free time (i.e. time to relapse) were marginally significant, as it can be seen in Figure 2 (E & G) (p = 0.08, CI = 90% and

**Table 1. IRF4 expression levels in leukemia patients and cell lines.**

|                          | Subnormal ratio R<2.4 | Normal ratio 2.4≤R<7 | High ratio 7≤R<11.6 | Very high ratio 11.6≤R | p-value* |
|--------------------------|------------------------|----------------------|---------------------|------------------------|----------|
| Healthy donors (n = 20)  | –                      | 4.72±1.17 (n = 20)   | –                   | –                      | –        |
| All patients & cell lines (n = 62) | 0.89±0.73 (n = 6) | 4.99±1.2 (n = 23)   | **8.90±1.11** (n = 27) | **12.6±0.44** (n = 6) | **0.000** |
| Patients & cell lines with ALL (n = 55) | 0.89±0.73 (n = 6) | 5.02±1.2 (n = 21)   | **8.89±1.16** (n = 25) | **12.72±0.20** (n = 3) | **0.000** |
| Pro-pre-B ALL (patients) (n = 2) | 0.55 (n = 1) | 4.80 (n = 1)       | –                   | –                      | –        |
| B-common ALL (patients) (n = 35) | 0.81±0.62 (n = 3) | 4.69±1.28 (n = 14) | **8.77±1.11** (n = 17) | **12.8±(n = 1) | **0.000** |
| Pre-B ALL (patients) (n = 9) | 1.17±1.27 (n = 2) | 5.64±0.99 (n = 4)   | **8.23±0.49** (n = 3) | –                      | –        |
| Pre-B ALL (cell lines) (n = 1) | – | – | – | 12.50 (n = 1) | **0.000** |
| T-cell ALL (patients) (n = 5) | – | 6.90 (n = 1) | **10.27±0.47** (n = 4) | – | **0.000** |
| T-cell ALL (cell lines) (n = 2) | – | 5.40 (n = 1) | – | **12.87±(n = 1) | **0.000** |
| Unknown (N/A) ALL Patient (n = 1) | – | – | 7.35 (n = 1) | – | **0.041** |
| Patients & cell lines with AML (n = 7) | – | 4.77±0.85 (n = 2) | **8.98±0.07** (n = 2) | **12.54±0.64** (n = 3) | **0.001** |
| Patients with AML (n = 6) | – | 4.77±0.85 (n = 2) | **8.98±0.07** (n = 2) | **12.17±0.14** (n = 2) | **0.000** |
| Cell lines with AML (n = 1) | – | – | – | 13.27 (n = 1) | **0.000** |

*the p-value has been calculated by taking into account both groups over-expressing IRF4 (i.e. both high and very high ratio), as compared to the healthy controls ratio.

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p = 0.05, CI = 95% respectively). Interestingly, when leukemia-free
time was estimated within a specific time frame of 3-years (i.e. only
the patients diagnosed in the years 2008, 2009 and 2010 were
included in the analysis), a significant correlation appeared to exist
between high IRF4 expression and relapse (p = 0.03) (Figure 2, I).

Discussion

In the present study we have found high IRF4 transcript levels in
a significant number of patients (51.7%) and in 3 of the 4 cell lines,
as compared to the healthy controls. This up-regulation seems to
associate with a variety of leukemic subgroups, possibly reflecting
the many roles of IRF4 in the immune system [12]. Considering
that IRF4 is essential for several stages of normal B- and T-cell
activation and differentiation, it is only logical to assume that
aberrations in the IRF4 transcriptome would also reflect in
lymphoid-cell associated malignancy.

A significant difference in IRF4 transcript levels was observed
between the pre-B and B-common cohorts, as well as between the
total B- and T-cell ALL groups (Figure 1), with higher transcript
levels appearing in the B-common and T-cell subtypes, respecti-
vely. In the past, however, IRF4 mRNA expression has been
found low in adult samples of T-cell ALL [34]. At this point it is
difficult to validate the reasons for this difference, besides possible
age-related dissimilarities in the biology of ALL, yet it points out
that aberrant IRF4 transcriptional activity is developmental-stage-
specific as well as cell-type-specific. It has already been demon-
strated that IRF4 is expressed at varying levels throughout B-cell
development, with expression peaking in plasma cells, therefore
varying transcript levels in human malignancies could mirror IRF4
expression in normal lymphoid activation and differentiation
[35,36]. However, in contrast to the Gene Expression Omnibus (GEO)
data profiles from healthy tissues [37] and a recent study from le Viseur
et al [28], in our leukemia samples we have observed that the greater the distance from a differentiated B-cell,
the higher the level of IRF4 expression. In other words,
significantly higher transcript levels were observed in the B-
common subgroup, than in the more mature pre-B subtype
(CD34dim−CD19+). This suggests that, at least for the particular
patient cohort studied here, ectopic expression of IRF4 does not
exact expression and OS (Figure 2, A-H). This could be due to the small number of patients included in the analysis or perhaps to the fact that for most of these patients OS could not be calculated in terms of a 3-year or a 5-year survival. Whereas a marginally significant correlation appeared to exist between abnormal IRF4 expression and estimated leukemia-free time (p = 0.08 and p = 0.05) (Figure 2, E & G), a significant correlation appeared (p = 0.03) when leukemia-free time was estimated within a specific time frame of 3 years (Figure 2, I). This implies that high IRF4 expression might be used as an additional prognostic marker of relapse at the diagnosis of childhood acute leukemias. Further analyses on a larger number of patients over-expressing IRF4 over a longer period of time (i.e. 5-year survival), should be able to confirm this observation in the near future.

Additional efforts should be made in order to identify the putative targets of IRF4 and to determine the ones directly regulated by it. A number of proteins have already been identified as repressors in B-cells and found to interact with IRF4: PU.1, Spi-B, E47, NFAT, Stat6, Bcl-6 and Blimp-1 [14,38,48–50]. In addition, CD40 signaling has been found to directly induce the expression of IRF4 in GC B-cells which, in turn, binds to the promoter region of the BCL-6 gene to repress its expression [51]. We are currently investigating the expression patterns of other hematopoietic progenitor genes in a larger cohort of pediatric patients in an attempt to gain a better understanding of how deregulated transcription on key signaling pathways affects the malignant phenotype and disease progression in childhood leukemia.

Conclusions

In summary, our study shows that aberrant IRF4 gene expression is implicated in a variety of leukemic subtypes, possibly reflecting the many roles of the gene in the immune system. In contrast to other studies, we observe higher transcript levels in patients characterised by a more immature immunophenotype, and in T-cell leukemias than in leukemias of the B-lineage, implying that in childhood leukemia, IRF4 gene expression is not subject to the feedback regulation mechanisms that operate in normal tissues. In addition, ectopic expression of IRF4 does not seem to be a specific feature of lymphoid leukemia, but it also extends to myeloid leukemia, as it was in our AML group that the highest expression levels were observed. Again, our results are in contrast to other studies documenting a down-regulated IRF4 expression in adult myeloid leukemias [34,42,43], even though this is most probably due to age-related dissimilarities and a different pathogenetic leukemia mechanism. Interestingly, we show that childhood leukemia, irrespective of subtype or cell maturation stage, is characterised by a minimum of approximately twice the amount of IRF4 gene expression encountered in healthy children.
This implies that there might be a dose-dependency of the disease for aberrantly expressed IRF4, a characteristic that could be explored therapeutically. In addition, a statistically significant correlation was found between high IRF4 expression and relapse, suggesting a prognostic value of IRF4 in the clinical course of childhood leukemias. Data from the past few years indicate that many important regulators of HSC development are also implicated in the abnormal self-renewal capacity of the LSCs. Only through the identification of these regulators, and of their downstream targets, will we gain a better understanding of what constitutes their unlimited self-renewing properties and subsequently reveal potential molecular targets for successful therapy.

Supporting Information

**File S1 Independent t-Tests for patients and cell lines over-expressing IRF4 (both high and very high expression) and for patients under-expressing IRF4.**

(DOC)

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**Table S1 Clinical data.**

**Table S2 Raw data for IRF4.**

(XLSX)

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**Author Contributions**

Conceived and designed the experiments: MA GI. Performed the experiments: MA MT. Analyzed the data: MA GI SV. Contributed reagents/materials/analysis tools: AA MT MM. Wrote the paper: MA GI. Contributed in collecting patient samples and clinical data: AA. Reviewed the manuscript: SV MM.
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