miR-155 regulative network in FLT3 mutated acute myeloid leukemia

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A B S T R A C T
Background: Acute myeloid leukemia (AML) represents a heterogeneous disorder with recurrent chromosomal alterations and molecular abnormalities. Among AML with normal karyotype (NK-AML) FLT3 activating mutation, internal tandem duplication (FLT3-ITD), is present in about 30% of patients, conferring unfavorable outcome. Our previous data demonstrated specific up-regulation of miR-155 in FLT3-ITD+ AML. miR-155 is known to be directly implicated in normal hematopoiesis and in some pathologies such as myeloid hyperplasia and acute lymphoblastic leukemia.

Methods and results: To investigate about the potential influence of miR-155 de-regulation in FLT3-mutated AML we generated a transcription factors regulatory network and combined this with data from multiple sources that predict miR-155 interactions. From these analyses, we derived a sub-network, called “miR-155 module” that describes functional relationship among miR-155 and transcription factors in FLT3-mutated AML. We found that “miR-155 module” is characterized by the presence of six transcription factors as central hubs: four miR-155 regulators (JUN, RUNX1, FOXO, JUNB) and two targets of miR-155 (SPI1, CEBPB) all known to be “master” genes of myelopoiesis. We found, in FLT3-mutated AML, a significant down-regulation of miR-155 target genes CEBPB and SPI1 and up-regulation of miR-155 regulator genes JUN and RUNX1. We also showed that PKC412-related FLT3 inhibition, in MV4-11 cell line, causes down-regulation of miR-155 and increased level of mRNA and protein of miR-155 target SPI1. We showed in experiments of miR-155 mimic in K562 cell line, a high increase of miR-155 and an inverse correlation with the mRNA levels of its targets SPI1 and CEBPB. Moreover silencing of miR-155 in primary AMLs causes mRNA up-regulation of its target SPI1 and CEBPB.

Conclusion: Our results suggest that activating mutation of FLT3 in AML can lead, through the induction of JUN, to an increased expression of miR-155, which then causes down-regulation of SPI1 and CEBPB and consequently may causes block of myeloid differentiation.

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1. Introduction

Acute myeloid leukemia is a cytogenetically heterogeneous disorder with acquired recurrent chromosomal alterations detected in about 55% of adult patients. In the remaining cases showing normal karyotype (NK-AML) a number of novel molecular abnormalities with a prognostic value have been described such as mutations in genes: FLT3, MLL, CEBPA, NPM1, WT1 IDH1, IDH2 DNMT3A [1,2]. FLT3 encodes a receptor tyrosine kinase expressed on hematopoietic progenitor cells involved in stem cell differentiation and proliferation [3]. FLT3 activating mutations such as internal tandem duplication (ITD) are present in about 30% of NK-AML and confer an unfavorable outcome; interestingly FLT3 mutations are also a “drugable” target for molecularly directed therapy [4].

The heterogeneity of AML can also be resolved according to their microRNA signatures [5–10]. MicroRNA (miRNA) are 19–24
nucleotide noncoding RNA which regulate the expression of target mRNAs at transcriptional and translational level; miRNA deregulation has been found in different human diseases, including cancer, leukemia, diabetes, immuno- or neurodegenerative disorders [11–13]. Their role in normal hematopoiesis is elucidated by recent studies, revealing specific variations of the miRNome during the commitment and development of the hematopoietic stem cells in the different lineage [14–16]. FLT3-mutated-associated microRNA expression studies revealed the over-expression of miR-155 [6,10,17]. Human miR-155 is mapped to 21q21.3, its oncogenic role has been demonstrated in transgenic mouse model where its overexpression is sufficient to generate acute lymphoblastic leukemia [18]; functional evidences show that overexpression of miR-155, in human and murine hematopoietic stem cells, induced myeloproliferation with differentiation block [19]. However, the molecular basis for miR-155 effects on the myeloid lineages remains largely unresolved. Transcription factors (TFs) and miRNAs regulate each other and together with their target genes form a complex network that enables the cell to conduct a wide range of biological functions [20]. The aim of this study was to investigate the altered regulatory circuitry between miR-155 and transcription factors underlying the development of AML carrying FLT3 mutations.

2. Methods

2.1. Study schema

Our aim was to integrate data from different sources as GEO gene expression database and MIRINTOP prediction tool to achieve a model about the role of miR-155 in FLT3-mutated AML and to validate the resulting network by experimental analysis. In this study we applied a four steps strategy. Firstly, using gene expression dataset from GEO database, we generated the transcription factors co-regulation network acting in FLT3 mutated AML and at the same time, we predicted the miR-155-TF connections by MIRINTOP. In the second step, we extracted, from the general network, the module of transcription factors connected to miR-155. As a third step, using a new cohort of newly diagnosed AML patients, we verified the expression levels of most intriguing hubs and correlated them to miR-155 expression levels. As final step we verified miR-155 connections by functional studies. Workflow of the experimental strategy is shown in Fig. 1.

2.2. Patients and specimens

100 cases of AML, genetically characterized at “Cervello Hospital” of Palermo, were enrolled. Diagnosis of AML was established according to WHO classification biologically, morphologically, cytogenetic, immunophenotypic and molecular studies [21]. AML patients were selected to obtain comparable genetic groups: 50 FLT3-mutated AML and 50 AML with other genetic alterations; patient’s characteristics are shown in Table 1. Genes and miR-155 expression levels were evaluated in samples from bone marrow obtained at diagnosis or from samples of peripheral blood showing more than 70% leukemic cells. All AML samples were taken for diagnostic purposes from patients registered in the GEMEMA protocols AML12 (SRCTN number 17833622) and AML 1310 (Eudra CT number 2010-023809-36) who gave written informed consent for usage of biological material not necessary for diagnostic purposes for scientific purposes. GEMEMA protocols were approved by Medical Ethics Committee of AOR Villa Sofia Cervello “Comitato Etico Palermo 2”.

2.3. Cell lines, culture conditions

K562 cell line was kindly provided by Dr. P. Vigeneri (University of Catania, Italy) (also available from American Type Culture Collection ATCC, CCL 243). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. MV4-11 cell line, homozygous for the FLT3-ITD mutation, was obtained from American Type Culture Collection (ATCC, CRL-9591) and grown in IMDM medium according to supplier’s information. All other reagents were purchased from Sigma (St. Louis, MO, USA), if not otherwise cited.

2.4. MV4-11 treatment and proliferation assay (MTT assay)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done to test the effect of a FLT3 Inhibitor, PKC412 (kindly provided by Novartis), on cell growth. Briefly, cells were plated in quadruplicate at 1.5 × 10^4 per well and exposed to escalating doses of PKC412 (1–200 nM) for up to 3 days. Means and standard deviations generated from 3 independent experiments are reported as the percentage of growth versus control (untreated cells). Cell proliferation curves were derived from these data by using Microsoft Excel software.

2.5. Cyogenetic and molecular analysis

Cyogenetic studies from bone marrow specimens were performed according standard procedures. For each culture 15–20 metaphases were karyotyped. Chromosomal abnormalities were classified according to the International System for Cyogenetic Nomenclature (ISCN) [22]. Molecular studies from leukemic cells were performed to detect gene fusion-transcripts associated with AML as described by EAC protocols [23]; FLT3 and NPM1 gene mutations analysis were performed according to Meshinchi et al. [24] and to Falini et al. [25] respectively. FLT3/ITD-positive samples were examined using Genescan analysis (Applied Biosystems, Foster City, CA) to determine the ITD allelic ratio ITD-AR was calculated by dividing the peak height of the ITD product to that of the normal WT product.

2.6. miR-155 expression

Total RNA was extracted from blast cells derived from bone marrow at diagnosis by the 6100 Nucleic Acid Prep Station protocol (Applied Biosystems). To study miR-155 expression levels, we used hsa-miR-155 TaqMan assay (4427975, Applied Biosystems). Briefly, reverse transcription reactions were performed for each sample using the High Capacity cDNA Archive Kit (Applied Biosystems) with the specific primer 5′- for miR-155. One microliter of cDNA from each sample was added to a 6.5 μL of TaqMan® Universal PCR Master Mix with 0.65 μL of 20× miR-155 probe in a final volume of 13 μL; Q-PCR amplifications were performed on an ABI 7900HT Q-RT-PCR; data were quantified using the SDS 2.3 software and normalized using the RNU48 as endogenous control. The cycle threshold (Ct) values, which were calculated relatively to the endogenous control, were used for our analysis (ΔCt). The 2−ΔCt method was used to calculate relative changes in gene expression among different samples.

2.7. Transcription Factor Network analysis in FLT3 mutated AML

Data set used consists of gene expression values of 106 FLT3-ITD+ AML cases obtained by Affymetrix Human Genome U133 Plus 2.0 chip and available on the Gene Expression Omnibus database accessing number GSE14468 (http://www.ncbi.nlm.nih.gov/geo/). Using the GO identifier GO:0003700 we limited our analysis to transcription factors (TFs) expression values. To improve the results stability we removed TFs whose expression levels were near to the minimum detection level of the platform. In presence of multiple probe sets for the same gene, we excluded from analysis the non-specific probe identified by the extension “x.at” and “x.at” in
Table 1
Patient’s characteristics.

<table>
<thead>
<tr>
<th>Molecular alteration</th>
<th>Male/female</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients FLT3 mutated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age 55 (range 21–82)</td>
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<td></td>
</tr>
<tr>
<td>24</td>
<td>9/15</td>
<td>16 NK/16 NA</td>
</tr>
<tr>
<td>FLT3 ITD/ NPM1 mutated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>2/1</td>
<td>1 NK/2 NA</td>
</tr>
<tr>
<td>FLT3 ITD</td>
<td></td>
<td>16 NK/1 NA/4 ABN*</td>
</tr>
<tr>
<td>3</td>
<td>2/1</td>
<td>1 NK/2 NA</td>
</tr>
<tr>
<td>FLT3 DB3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>22/28</td>
<td>33 NK/13 NA/4 ABN</td>
</tr>
<tr>
<td>FLT3DB3/ NPM1 mutated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 50</td>
<td></td>
<td></td>
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<tr>
<td>Patients FLT3 unmutated</td>
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<tr>
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<tr>
<td>26</td>
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<td>10 NK/16 NA</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>7/5</td>
<td>7 NK/1 NA</td>
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<td>NPM1 mutated</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>1/1</td>
<td>inv(16)</td>
</tr>
<tr>
<td>CBF3/MYH11</td>
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<td>3</td>
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<td>(8;21)</td>
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<td>5</td>
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<td></td>
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<td>1/1</td>
<td>MLL translocation</td>
</tr>
<tr>
<td>25</td>
<td>25/21</td>
<td>17 NK/17 NA/16 ABN</td>
</tr>
<tr>
<td>Total 50</td>
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</table>

NK, normal karyotype; NA, not available (failed or not done); ABN, abnormal karyotype.
No marker = negative for molecular screening of fusion-transcripts associated with AML and FLT3 and NPM1 mutations.

* One case with trisomy 8, two cases with del20q, one case with del11q.

The Affymetrix ID code. Specific multiple probe sets were summarized by the sample mean. To estimate the TFs network sparse structure in FLT3-ITD+ AML, we applied a Gaussian graphical model (GGM) penalized using the L1-penalty function [26]. The GGM is a graphical model where the genes (vertices) represent the variables and the edges the partial correlation coefficients between two variables given all other variables. To obtain the optimal trade-off between complexity and prediction behavior of the estimated model we developed an original method for the tuning parameter value selection (see supplementary material). To elucidate the TFs biological role inside the estimated sparse network, we used some centrality measures. The simplest centrality measure that we computed is the degree of a vertex that is the number of genes that are adjacent to it. Then we estimated the betweenness: shortest paths between other nodes that run through the node in interest. A further centrality index we measured was the closeness, which is related with the notion of geodesic distance on a graph, namely, the number of transcription factors in the path joining the considered vertex with another vertex. All the analyses were performed using R and Bioconductor software [27,28].

2.8. miR-155 regulator and target prediction analysis

To predict potential regulators and target genes of miR-155 we utilized MIR@NTO@N (http://mironton.uni.lu) which identifies metap-network regulations implicating transcription factors (TFs), miRNAs and target genes. To integrate miRNA-dependent regulations, MIR@NTO@N combine the miBase Targets database and microRNA.org. These resources can be used, through the MIR@NTO@N application, simultaneously with a unified score (ranging from the minimum score of 0 to the maximum score of 1). To predict TF-dependent miRNA regulations on a large scale, MIR@NTO@N use a standard TF binding site (TFBS) detection algorithm and TFBS profiles from the JASPAR database. Position Frequency Matrices (PFMs) were converted into Position Weight Matrices (PWMs) and used to predict potential TFBSs in 10 kb sequences located upstream of mRNA precursors, extracted from Ensembl database, according to pre-miRNA localization provided by miBase. To limit the noise of false predictions, only predicted TFBSs with a score higher than 0.65 were integrated into MIR@NTO@N database.

2.9. Generation and analysis of miR-155 module

To create the miR-155 module we extracted from AML-FLT3-ITD+ TF network all the TFs predicted to be connected with miR-155 together with their connections. Potential connections between genes involved in Transcription Factor Network regulated genes were analyzed by examination of the literature using BiblioSphere (Genomatix SoftwareGmbH, Munich, Germany; http://www.genomatix.de) for uncovering reported regulatory relationships between genes. Relationships defined with this tool were confirmed by manual examination in Pubmed. To uncover the gene pathways that are regulated by miR-155 connected TFs in AML, we performed functional pathway analysis using Genomatix Pathway Systems (GePS). This method categorizes large lists of genes into functionally canonical pathways using informations extracted from public (BioCarta) and proprietary databases. Results from GePS are supported by statistical p-value calculations.

2.10. miR-155 regulator and target gene expression

To determine TFs mRNA expression levels we used Q-RT-PCR with the 7900 TaqMan system and pre-designed available assay (Assay on Demand, Applied Biosystems). Q-RT-PCR data were quantified using the SDS 2.3 software and normalized using the ABL as endogenous control. To calculate relative changes in gene expression among different samples we used the 2–ΔΔCT method.

2.11. SPI1 expression in AML blast cells

Immunophenotype of AML blast cells was assessed by direct immunofluorescence using the appropriate mAb according to standard diagnostic protocol. SPI1 intracytoplasmic staining of AML blast cells was performed using cytokerin/cytospin (BD Pharmingen Catalog # 554714) and labeling with Polyclonal Anti-human SPI1 Fluorescein (R&D System cat. IC8570); as a negative control, samples were labeled with a matched FITC-conjugated isotype. Briefly, 5 × 10^5 cells were resuspended in 0.5 mL of cold cytokerin/cytospin solution and incubated at room temperature for 10 min; cells were then washed twice in saline buffer and after permeabilization, 10 μL of conjugated antibody was added and the cells were incubated for 30 min at room temperature in the dark. The cells were washed twice and then resuspended in saline buffer for final flow cytometric analysis. Flow cytometric analysis was performed by FACS Canto cytometer (BD Pharmingen). Difference in mean intensity fluorescence values was valued by t-test.

2.12. Statistical analysis

Results from each TaqMan run were quantified separately. To increase the accuracy of the results, we applied a filtering criterion that included gene control expression which were reliably quantifiable (cutoff < 35 Ct). Underdefined values of Ct were estimated as 50 Ct (the last cycle of the reaction). Resulting ΔCtS were used for our analysis. Differential expression of miRNA and mRNA targets was defined using the Relative Expression Software Tool (REST) proposed by Pfaffl et al. [29]. Fold change was estimated by REST. Namely, a gene was defined differentially expressed when estimated p-value was <0.05.

To assess correlation between CEBPB/SPI1 and miR-155 we utilized Spearman’s rank correlation test.

2.13. Western blot

MV4-11 were treated or not for 24, 48, 72 h with 1 or 50 μM of PKC412. Total protein cell lysate were obtained and analyzed by SDS-PAGE followed by Western blotting as described in Corrado et al. [30]. Antibodies used in the experiments were: c-Jun, phospho Jun, SPI1, CEBPB, β-actin (all from Cell Signalling Technology, MA, USA).

2.14. miR-155 induction and silencing

K562 cell line was used for in vitro response to the miR-155 overexpression; transfection experiments were performed in triplicates, after short term cultures,
by Lentivirus-GFP-hsa-miR-155 lentivirus (Applied Biological Materials, ABM) as recommended by the manufacturer. GFP-hsa-miR-155 lentivirus is an miR-155 expression vectors that has been tested for functionality and contains a GFP reporter under a constitutive CMV promoter. Infections were performed in a 24-well plate at a density of 5 × 10⁵ cells per well by adding 10 μL of virus (viral titers: 1.0 × 10⁸ IU/mL) in the presence of Polybrene at a concentration of 2 μg/mL. As negative control we used K562 transfected with lentivirus containing scrambled sequence (Lenti-III-mir-Off control virus, ABM).

AML primary cells from patients carrying FLT3 ITD mutation were used for in vitro response to the miR-155 silencing treatments. Transfection experiments were performed in triplicates, after short term cultures, blasts were transfected by lentivirus vector Lentivirus-off-hsa-miR-155 (ABM) as recommended by the manufacturer, in the same condition utilized for K562 cell line. The Lentivirus is a miR-155 inhibitor expression vector that express a short hairpin which is complementary to the mature sequence of miR-155. As negative control we used AML blast cells transfected with lentivirus containing scrambled sequence (Lenti-III-mir-Off control virus, ABM).

Transfection efficiency was measured by GFP fluorescence at cyometry at 48 h after virus infection.

3. Results

3.1. Up-regulation of miR-155 in FLT3-mutated AML

The analysis was finalized to study the expression level of miR-155 in a cohort of 100 AML with different genetic subtype; patient's characteristics are shown in Table 1. We considered as a group all the cases carrying FLT3 mutation (including the double mutants NPM1/FLT3). Values of single AML ΔCt (miR-155 Ct – RNU48 Ct) are reported in Fig. 2A. Statistical analysis performed by REST, showed a 20.8-fold up-regulation of miR-155 (p < 0.0001) in FLT3-mutated AMLs compared with AML without FLT3 mutations (Fig. 2B).

3.2. Network analysis of transcription factor

One of our aims has been to generate a transcription factor regulatory network using mRNA expression levels from newly diagnosed FLT3 mutated AML patients (data obtained from GEO). We identified a network with 273 TFs connected by 926 edges (about 1% of all possible connections) (Fig. 3A). We noticed that a few TFs possess high degree (centrality measure) suggesting that such TFs could play a central biological role inside the genetic network. Centrality measures for 34 transcription factors with a degree > 15 are reported in Table 2.
distance distribution (closeness) the average path length results equal to 2.89 which means that the network satisfy the “small world” property typical of complex biological networks.

3.3. mir-155 regulators and target prediction analysis

To identify the meta-regulation networks involving mir-155 over-expression we used a new computational framework, named MIR@NT@N, which reconstructs such networks of transcription factors and microRNAs. Using MIR@NT@N to predict mir-155 connections we found 33 TFs regulating mir-155 and 151 mir-155 target genes with a score of 0.8 and 0.9 respectively. Next we filtered the targets by “TF targets only” option and we found that only 8 TF are targeted by mirR-155. The predicted regulatory network of mir-155 is shown in Fig. 3B.

3.4. mir-155 module analysis

To estimate the potential influence of mir-155 de-regulation on FLT3-ITD+ AML network we focused on TFs predicted to be targets or regulators of mir-155 that, at the same time, were centrals vertices in the network with a degree > 15. The filtration analysis found six TFs: four regulators (JUN, RUNX1, FOSb, and JUNB) and two targets (SPI1 and CEBPB). Notably, these factors are key components in the orchestration of myeloid cell maturation. Next we extracted from the global FLT3 network a two layers subnetwork in which the first layer are the six TFs directly connected to mirR-155 and the second layer are TFs, that interact with them in the global FLT3 network, which can be, also, influenced by mirR-155 (Fig. 3C). The resulting subnetwork, called “mirR-155 module” consists of 95 vertices connected by 142 edges. Using Genomatix inspector software we verified that 87 of 142 edges are sustained by literature data reporting regulatory relationship or transcription factor binding site prediction or both. To discover functional relationships among TFs present in mirR-155 module and uncover the gene pathways that are potentially influenced by mirR-155 in FLT3-ITD+ AML, we performed functional knowledge-based pathway analysis using GePS. Table 3 lists the category of large lists of genes involved in the most significant biological processes resulting from this analysis. We found, in mirR-155 module, a significant

Fig. 3. Network analysis. (A) Graphical model of the TFs network in FLT3 ITD + AML: the vertices represent the TFs and the edges represent the partial correlation coefficients between couple of TFs. (B) MIR@NT@N computational reconstruction of meta-regulation network involving mirR-155 (octagon) and transcription factors. (circles). Regulatory TFs are connected to mirR-155 with a T shaped arrow, the TFs targeted by mirR155 are connected to mirR-155 with a delta shaped arrow. (C) mirR-155 module: graphical representation of the TFs subnetwork, of most central hubs (circles) predicted to be connected to mirR-155 (octagon) and their line of interactions (no circles). Edges arrow shape describe: activation (arrows), inhibition (blunt arrows), modulation (circles). Edge line style describe: co-regulation (dot), binding site found in promoter (solid) interaction inferred from literature (wider lines).
<table>
<thead>
<tr>
<th>Resource</th>
<th>Functional pathway</th>
<th>p-value</th>
<th>List of observed genes (gene symbols):</th>
<th>List of observed genes (gene IDs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal transduction canonical pathway: AP-1 Transcription Factor Network</td>
<td>p-value: 1.04e−9</td>
<td>FOSL2, CTNNB1, FOSB, NFATC2, ATF3, FOSL1, ETS1, JUN, JUNB, FOX1, HIF1A</td>
<td>2355, 1499, 2354, 4773, 467, 8061, 2113, 3725, 3726, 2353, 3091</td>
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<td>Signal transduction pathway (Genomatix literature mining)</td>
<td>p-value: 1.17e−8</td>
<td>CTNNB1, ETV6, BCL6, ZEB1, SPI1, SOX4, EGR2, HOXA9, RUNX1, SMAD1, MF2C, ZF36L1, ETS1, HOXA10, TSC22D1, CEBPD, STAT4, MYBL2, KLFL, CEBPB, HOX5, NRA42</td>
<td>1499, 2120, 604, 6195, 3688, 6659, 1959, 3205, 861, 4086, 4208, 677, 2113, 3206, 8848, 1052, 6775, 4605, 1316, 1051, 3202, 4929</td>
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</tr>
<tr>
<td>Ccaat enhancer binding protein</td>
<td>p-value: 3.90e−8</td>
<td>NFKB1, SPI1, POU2F1, ATF3, JUN, CEBPD, FO5, CEBPB</td>
<td>4790, 6688, 5451, 467, 3725, 1052, 2353, 1051</td>
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<tr>
<td>Oncogenic</td>
<td>p-value: 8.43e−7</td>
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<td>2305, 1499, 3660, 6688, 4211, 8462, 861, 3725, 1316, 1051, 3091</td>
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<td>27287, 2120, 5425, 6688, 4211, 5451, 3205, 861, 3206, 3725, 3726, 5316, 1052, 2078, 4605, 3202</td>
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<td>Lymphoma non Hodgkin</td>
<td>p-value: 8.04e−16</td>
<td>TCF12, FOXM1, TSC22D3, EGR3, FOSL2, CTNNB1, FOSB, NFkB1, ETV6, BCL6, IRF2, ZEB1, POU2F2, SPI1, MEIS1, POU2F1, NFATC2, ATF3, SOX4, EGR2, SP4, MAFK, ZNF268, RUNX1, TFDP2, SMAD1, RFXAP, KLF2, JDP2, ZF36L1, ETS1, HOXA10, JUN, MYC1, JUNB, TSC22D1, CEBPB, MIR155, ERG, ETV3, NRA41, STAT4, MYBL2, KLFL, FO5, IRF5, CEBPB, HIF1A, NRA42, AHR, IRF1, BZLF1</td>
<td>6938, 2305, 1831, 1960, 2355, 1499, 2354, 4790, 2120, 604, 6355, 5425, 6688, 4211, 5451, 4773, 467, 6659, 1959, 6671, 7975, 861, 7029, 4086, 5994, 10365, 122953, 677, 2113, 3206, 3206, 3725, 4610, 3726, 8848, 1052, 409497, 2078, 2117, 3164, 6775, 4605, 1316, 2353, 3663, 1051, 3091, 4929, 196, 3659</td>
<td></td>
</tr>
<tr>
<td>LAM</td>
<td>p-value: 2.19e−12</td>
<td>TCF12, FOXM1, VENTX, RORA, MXD1, CTNNB1, FOSB, NFkB1, ETV6, BCL6, ST18, IRF2, POU2F2, SPI1, MEIS1, POU2F1, SOX4, EGR2, HOX5, MAFK, RUNX1, SMAD1, FOSL1, MAF, MEF2C, ZF36L1, ETS1, HOXA10, JUN, PKNX1, CEBPB, MIR155, ERG, ETV3, NRA42, STAT4, MYBL2, KLFL, FO5, CEBPB, HIF1A, HOX5, NRA42, AHR, IRF1, BZLF1</td>
<td>6938, 2305, 27287, 6095, 4084, 1499, 2354, 4790, 2120, 604, 9705, 3660, 6935, 5425, 6688, 4211, 5451, 4773, 467, 6659, 1959, 6671, 7975, 861, 7029, 4086, 10365, 122953, 677, 2113, 3206, 3725, 2078, 2117, 3164, 6775, 4605, 1316, 2353, 3663, 1051, 3091, 4929, 196, 3659</td>
<td></td>
</tr>
</tbody>
</table>
overrepresentation of transcription factors involved in crucial cellular process: AP1 Transcription Factor Network (p value: 1.04e−9), “ccaat enhancer binding protein” (p-value: 3.9 e−8), differentiation (p-value: 1.17e−8).

3.5. Expression levels of miR-155 targets in AML blast cells

To validate the findings of miR-155 module in FLT3-ITD+ AML, we tested the expression of TFs directly connected to miR-155; the target genes CEBPB and SPI1 and two regulator genes RUNX1 and JUN in a cohort of 100 AML patients. mRNA levels of target genes were obtained by Q-RT-PCR, ∆Ct (target gene Ct – ABL Ct) values of single AML are reported in Fig. 4A and B. We found that CEBPB and SPI1 genes exhibit a significant inverse correlation with miR-155 up-regulation, being down-regulated in FLT3-mutated AML versus others (respectively 0.354-fold p<0.001, and 0.404-fold p=0.000) (Fig. 4B and D). Moreover we found an inverse correlation between CEBPB/SPI1 and miR-155 with a Spearman’s rank correlation coefficient of −0.13 and −0.19 respectively; we also consider the correlation between the allelic ratio of FLT3-ITD mutated allele and miR-155 expression and we found a positive correlation with a Spearman’s rank correlation coefficient of 0.21.

To analyze SPI1 protein expression in AML blast cells we performed cytometric analysis. Twelve patients were identified by FACS analysis as SPI1-deficient and confirmed by genetic analysis to carry FLT3-ITD activating mutations, and 26 FLT3 negative AML patients were included in this analysis. Remarkably, SPI1 normalized mean intensity fluorescence (MIF) values in FLT3 mutated AML were significant lower than FLT3 negative AML (MIF 81.4 vs 145.7 p<0.05). Fig. 5 shows the FACS profiles of SPI1 from 3 representative FLT3-ITD mutated AML patients in comparison to 3 FLT3 negative AML patients.
3.6. Expression levels of miR-155 regulators in AML blast cells

Using MIR@NT@N to predict TFBSs in 10 kb sequences upstream of miR-155 precursors we found binding sites for 33 TFs, among them JUN and RUNX1 appear not only central hubs in miR-155 module but also actors in signal pathways involved in AML. To verify a connection between these TFs and miR-155 over-expression we tested expression levels of JUN and RUNX1 in two cohorts of respectively 100 and 84 AML patients. ΔCt (target gene Ct – ABL Ct) values of single AML are reported in Fig. 6A and C. We found that JUN and RUNX1 exhibit a significant positive correlation with miR-155 up-regulation being up-regulated in FLT3-mutated AML versus others (2.597-fold p = 0.0210 and 2.64-fold p < 0.0001 respectively) (Fig. 6B and D).

3.7. MV4-11 cell treatment with PKC412 induced miR-155 down-regulation

MV4-11 is an established myelomonocytic leukemia cell line homozygous for FLT3-ITD mutation, this cell line has been used as model of FLT3-ITD AML in numerous studies. The receptor tyrosine kinase inhibitor PKC412 has been shown to inhibit autophosphorylation of mutant FLT3 receptors and disrupt downstream signaling in AML cells [31]. Inhibition of FLT3-ITD signaling was achieved in MV4-11 cell line using PKC412 (1–200 nM) for up to 96 h. Response to treatment was evaluated by proliferation assay. Means and standard deviations generated from 3 independent experiments are reported. A good inhibition of proliferation was obtained after 48 h of treatment using a 50 nM drug concentration (Fig. 7A). To explore the molecular mechanism by which PKC412 down-regulates miR-155 in AML FLT3-ITD mutated cells, we first studied whether PKC412 might modulate miR-155 levels. We show that treatment with 50 nM of PKC412 clearly reduced miR-155 expression (reduction of 50% at 48 h, reduction of 80% at 72 h) compared to untreated MV4-11 control cells (Fig. 7B). We showed that inhibition of FLT3-ITD signaling increases the expression of miR-155 targets such as CEBPB (5-fold at 48 h and 8-fold at 72 h) at mRNA level (Fig. 7C) and SPI1 (1.8-fold at 48 h and 2.1-fold at 72 h) both at mRNA and protein levels (Fig. 7D and E) and decreases phospho-JUN protein level (Fig. 7E), a putative TF regulator of miR-155. We are unable to demonstrate the down-regulation of CEBPB protein (data not shown) probably due to additional genetic aberrations of our cell model MV4-11, our results are in agreement with recent results of Alachkar et al. [32]. Taken together these results suggest that miR-155 is involved in the downstream regulation of FLT3-ITD signaling.
3.8. miR-155 mimic and silencing in AML cell line and fresh blast cells

To assess miR-155 function in the hematopoietic system, we induced the over-expression of miR-155 in K562 cell line by LentimiRa-GFP-hsa-miR-155 lentivirus transduction. Experiments were performed in triplicates. Flow cytometry analysis showed a median of 44% (range 30–52%) of transfection efficiency (Fig. 8A), after 96 h from virus infection, we obtained similar transfection efficiency in negative control (data not shown). After infection we showed an over-expression of miR-155 (>2 log-fold) and a down-regulation of mRNA level of its gene target SPI1 (1.4-fold) and CEBPB (2.2-fold) (Fig. 8B–D). Conversely, fresh leukemic blasts from a FLT3-ITD mutated AMLs were assayed for their in vitro response to the LentimiRa-off-hsa-miR-155 transfection. We obtained, as shown by flow cytometry analysis, a mean 40% (range 30–50) transfection efficiency (Fig. 9A), we obtained similar transfection efficiency in negative control (data not shown). We showed, after 96 h from virus infection, a down-regulation of miR-155 (80%) and an increase of mRNA level of its gene target SPI1 (2.5-fold) and CEBPB (3.8-fold) (Fig. 9B–D).

4. Discussion

In this report, to clarify the role of miR-155 deregulation in FLT3-ITD+ AML, we used and combined among them different sources of interactions and association data, such as in vitro and bioinformatic analysis and functional studies. We described, for the first time, to our knowledge, a bioinformatic regulatory network that connects FLT3-ITD mutation, a poor prognostic marker for AML, to reduced expression of TFs master regulators of myelopoiesis. Our results suggest that activating mutation of FLT3 in AML can lead, to increased expression of miR-155, which then causes down-regulation of SPI1 and CEBPB and consequently causes...
block of myeloid differentiation. More simply, FLT3-ITD → \( \text{mir-155} \rightarrow \) SPI1 ↓ CEBPB → ↓. Myeloid differentiation. The evidence for and implications of this model are discussed below.

In AML, the association of microRNA expression profile and AML genetic categories has been investigated in recent studies [5–11]. In this study we consolidate the knowledge that \( \text{mir-155} \) is up-regulated in FLT3 mutated AML. We used quantitative real-time RT-PCR to study \( \text{mir-155} \) expression in a large cohort of 100 primary AML characterized by common cytogenetic and molecular alterations, including 50 FLT3-mutated AML. According our previous data and other reports on FLT3-ITD+ AML [6,10,17], we found oncogenic \( \text{mir-155} \) to be specifically up-regulated in this category. The mechanisms through which constitutively activated FLT3 associates with increased \( \text{mir-155} \) expression are almost undefined, although during the course of our research, Gerloff et al. have demonstrated that FLT3-ITD signaling induces the oncogenic \( \text{mir-155} \) by its downstream targets NFK-B and STAT5 [33].

Recently emerging regulatory principle is that microRNAs exert their functions interweaving with cellular networks and regulating preferentially most downstream components of cellular signaling networks such as the transcriptional factors [24]. To investigate relevant TFs \( \text{mir-155} \) relationships in FLT3-ITD+ AML we performed a network analysis of transcription factors expression profiles obtained from newly diagnosed FLT3 mutated AML that showed 273 TFs connected by 926 edges with 33 of them characterized by high value of centrality (central hubs). Then, we performed a prediction analysis, by MIR@NT@N, to individuate all the transcription factors (TFs) that are putatively linked to \( \text{mir-155} \) as target or regulator genes; this analysis indicated 33 TFs regulating \( \text{mir-155} \) and 8 TFs targeted by \( \text{mir-155} \). At last to create "\( \text{mir-155} \) module" we extracted from TF network the TFs predicted to be connected with \( \text{mir-155} \) (first line) together with their connections (second line). First line \( \text{mir-155} \) module is represent by 6 TFs: 2 \( \text{mir-155} \) targets, SPI1 and CEBPB, and 4 TFs,
RUNX1, JUN, JUNB and FOSb that are predicted to be regulator of miR-155.

Interestingly, the majority of TFs present in miR-155 module “first line” are indeed “master” regulators of myelopoiesis. Myeloid differentiation is, in fact, orchestrated by a relative small number of TFs; among them are SPI1, CCAAT-enhancer binding protein (CEBP, CEBPB and CEBPE), RUNX1, TL1, JUNB, IKAROS and MYC [34]. These TFs regulate the expression of others myeloid genes such as those encoding receptor for myeloid colony stimulating factor and those encoding for myeloid granule component. We, further, corroborated miR-155 module findings in our cohort of 100 AML samples demonstrating an inverse correlation between the up-regulation of miR-155 and down-regulation of the mRNA level of its targets CEBPB and SPI1. We also demonstrated the down-regulation of the protein SPI1 in FLT3-ITD+ AML fresh blast cells. Our data concerning miR-155 targets observed in fresh leukemic cells are consistent with our functional data; in fact we showed that PKC412-related FLT3 inhibition, in MV4-11 cell line, causes down-regulation of miR-155 and increased level of mRNA and protein of miR-155 target SPI1 and increased level of mRNA of CEBPB. We obtained fitting results in experiment of miR-155 mimic in K562 cell line, in fact we showed a high increase of miR-155 post lentivirus transfection and an inverse correlation with the mRNA levels of its targets SPI1 (0.8-fold) and CEBPB (0.5-fold). Moreover silencing of miR-155 in primary AML blasts causes up-regulation of its target SPI1 (2.5-fold) and CEBPB (3.8-fold). An association between FLT3-ITD mutation and suppression of SPI1 has been also reported by other investigators [35–37]; moreover functional interaction between miR-155 and its target SPI1 was previously reported in lymphoid [38] and myeloid setting [33]. Our results strongly suggest that, in FLT3 mutated AML, miR-155 acts as a potent inhibitor of myeloid differentiation directly targeting master genes, such as SPI1, and CEBPB, involved in myeloid differentiation. The deregulation of these TFs may be responsible of differentiation block observed in FLT3-ITD+ AML, consequently restoring their functions could be an attractive therapeutic strategy.

Although great strides have been made toward understanding the identification of miRNA targets their regulation is a less understood aspect. Recent data suggest that most, if not all, intronic miRNAs contain putative promoters (core promoter) independents from their host gene [39]. To increase our understanding of how the miR-155 is regulated at transcriptional level, we analyzed the 10 kb sequence located upstream of miR-155 precursor and we found potential TFBSs for two important TFs master regulator of myelopoiesis. When we validated the prediction results about TFs regulating miR-155 we observed the up-regulation of JUN in FLT3-mutated AML, as the same also RUNX1 showed a similar expression profile. Moreover we showed that PKC412-mediated
FLT3 inhibition, in MV4-11 cell line, causes down-regulation of both p-JUN and miR-155. Notably, experimental data showed that JUN is strongly induced by FLT3-ITD mutation [40] and is an upstream regulator of miR-155 [41]. FLT3-ITD activates STAT5 and various downstream kinase pathways, like the Ras/mitogen-activated protein kinase (MAPK) pathway [42]; these lead to aberrant gene regulation and abnormal cell growth [42,43] in FLT3-ITD cells. JUN expression is significantly induced by the enhanced activation of the Raf/MAPK kinase pathway [44] and the inhibition of this pathway strongly impairs the growth of FLT3-ITD cells [45]. All these data suggest that JUN may be a functional downstream target of FLT3-ITD.

Our results describe a bioinformatics network that individuates vertices involved in the molecular pathogenesis of FLT3 mutated AML and suggest a molecular pathway that starting from FLT3 activating mutation, through JUN induces miR-155 and alters myeloid differentiation, this model identified some main points described by Gerloff et al. [33] also. Recent study provides evidence that the gene regulation by miR-155 is complex and its function is likely subject to disease context and cell type; in fact miR-155 is reported to induce apoptosis in AML FLT3 WT [46].

There are emerging evidence that miRNAs are part of complex regulatory network involving other miRNAs and transcriptional

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**Fig. 9.** AML primary cells transfection by LentimiRa-off-hsa-miR-155. (A) Efficiency of transfection of primary blasts from FLT3-ITD positive AML patient infected by LentimiRa-off-hsa-miR-155 was evaluated by GFP expression by flow cytometry. (B) Relative expression levels of miR-155 in FLT3-ITD positive AML blasts transfected by LentimiRa-off-hsa-miR-155 compared with that from blasts infected with lentivirus containing scrambled sequence (calibrator sample). miR-155 expression was measured and normalized to RNU48 expression at 96 h post infection; (C) relative m-RNA expression levels of CEBPb in FLT3-ITD positive blasts transfected by LentimiRa-off-hsa-miR-155 compared with that from blasts infected with lentivirus containing scrambled sequence (calibrator sample). CEBPb expression was measured and normalized to ABL expression at 96 h post infection; (D) relative m-RNA expression levels of SPI1 in FLT3-ITD positive AML blasts transfected by LentimiRa-off-hsa-miR-155 compared with that from blasts infected with lentivirus containing scrambled sequence (calibrator sample). SPI1 expression was measured and normalized to ABL expression at 96 h post infection.
regulators that cooperate to govern myelopoesis; miR-155 targets SP1 and CEBPB that in turn have been shown to transcriptionally regulate expression of miR-223 [47]. miR-223 subsequently is involved in CEBPA function, a central transcription factor in myelopoesis, directly repressing its inhibitor NFI-A1. As the same other TFs are indirectly involved by miR-155, in fact the transcription factor pathway analysis of miR-155 global module revealed a overrepresentation of TFs involved in AP1 network, an epicenter for the cellular behavior in different condition like stress, inflammation and malignant cellular transformation [48]. AP1 network involvement is consistent with the knowledge that miR-155 is activated by immune response and inflammation [49]. Furthermore we found the strong presence, in miR-155 module, of transcription factors implicated in "caat enhancer binding protein" signal transduction pathway that is involved in innate and specific immunizations as well as normal and malignant hematopoiesis [50].

5. Conclusions

In conclusion our study consolidates data on miR-155 association with FLT3-ITD+ AML, describes an integration of sequence-based prediction analysis with expression network that individuates vertices involved in the molecular pathogenesis of FLT3 mutated AML, suggests a molecular pathway that starting from FLT3 activating mutation, through miR-155, alters myeloid differentiation. We also suggest that miR-155 deregulation may act as central hub in the multi-steps mechanism of FLT3 mutated leukemogenesis offering new therapeutic strategies.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2015.04.017

References


