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Ranjani Ramamurthy

Maya Hughes

Valerie Morris

Hamid Bolouri

Robert B. Gerbing

See next page for additional authors

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miR-155 expression and correlation with clinical outcome in pediatric AML: A report from Children's Oncology Group

Ranjani Ramamurthy[1], **Maya Hughes**[2,3], **Valerie Morris**[2], **Hamid Bolouri**[4], **Robert B. Gerbing**[5], **Yi-Cheng Wang**[5], **Michael R. Loken**[6], **Susana C. Raimondi**[5,7], **Betsy A. Hirsch**[5,8], **Alan S. Gamis**[5,9], **Vivian G. Oehler**[1,2], **Todd A. Alonzo**[10], and **Soheil Meshinchi**[1,2,3,5]

[1]University of Washington School of Medicine, Seattle, Washington

[2]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington

[3]Seattle Children's Hospital, Seattle, Washington

[4]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington

[5]Children's Oncology Group, Monrovia, California

[6]Hematologics, Inc., Seattle, Washington

[7]Department of Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee

[8]Division of Laboratory Medicine, University of Minnesota Medical Center-Fairview, Minneapolis, Minnesota

[9]Children's Mercy Hospitals & Clinics, Kansas City, Missiouri

[10]Keck School of Medical Department of Preventive Medicine, University of Southern California, Los Angeles, California

Abstract

Background—Aberrant expression of microRNA-155 (miR-155) has been implicated in acute myeloid leukemia (AML) and associated with clinical outcome.

Procedure—We evaluated miR-155 expression in 198 children with normal karyotype AML (NK-AML) enrolled in Children's Oncology Group (COG) AML trial AAML0531 and correlated miR-155 expression levels with disease characteristics and clinical outcome. Patients were divided into quartiles (Q1–Q4) based on miR-155 expression level, and disease characteristics were then evaluated and correlated with miR-155 expression.

Results—MiR-155 expression varied over 4-log10-fold range relative to its expression in normal marrow witha median expression level of 0.825 (range 0.043–25.630) for the entire study cohort. Increasing miR-155 expression was highly associated with the presence of FLT3/ITD mutations ($P < 0.001$) and high-risk disease ($P < 0.001$) and inversely associated with standard-risk ($P = 0.008$)

Correspondence: Soheil Meshinchi, Clinical Research Division, Fred Hutchinson Cancer Research Center, D5-380, 110 Fairview Avenue North, Seattle, WA 98103. smeshinc@fhcrc.org.

Ranjani Ramamurthy, Maya Hughes, and Soheil Meshinchi contributed equally to this manuscript.

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and low-risk disease ($P = 0.041$). Patients with highest miR-155 expression had a complete remission (CR) rate of 46% compared with 82% in low expressers ($P < 0.001$) with a correspondingly lower event-free (EFS) and overall survival (OS) ($P < 0.001$ and $P = 0.002$, respectively). In a multivariate model that included molecular risk factors, high miR-155 expression remained a significant independent predictor of OS (P = 0.022) and EFS (0.019).

Conclusions—High miR-155 expression is an adverse prognostic factor in pediatric NK-AML patients. Specifically, high miR-155 expression not only correlates with FLT3/ITD mutation status and high-risk disease but it is also an independent predictor of worse EFS and OS.

Keywords

AML; children; miR-155

1 Introduction

Pediatric leukemias are the most common childhood cancers in the United States affecting up to 4,100 children per year. Acute myeloid leukemia (AML) accounts for one fourth of pediatric acute leukemias, but leads to nearly half of all deaths. AML is a complex and heterogeneous disease without a unifying disease-causing event.[1,2] Genomic alterations, whether translocations, copy number changes, or somatic mutations contribute to disease pathogenesis. Currently, initial responses to treatment as well as the presence/absence of specific cytogenetic abnormalities and gene mutations are used to prognosticate clinical outcomes and guide therapeutic interventions.[3] For example, the following chromosomal anomalies are routinely used to define AML risk class: t(8;21)(q22;q22), inv(16)(p13.1q22), t(15;17)(q22;q21)/PML-RARA, 11q23/MLL rearrangements, monosomy 7 and monosomy 5/5q deletions.[4] In addition, genomic alterations including FLT3/ITD[5] as well as CEBPA[6] and NPM1 mutations are also utilized as prognostic markers.[7] However, as our understanding of AML leukemogenesis continues to evolve, there has been an effort to identify additional epigenetic/genetic alterations of prognostic significance that could be used for risk-based therapy allocation.

MicroRNAs (miRNAs or miRs) are small regulatory RNAs that control the protein expression of tens to hundreds of targets via mRNA degradation or translational repression.[8] Aberrant miRNA expression has been implicated in the pathogenesis of many cancers including AML.[9–11] Altered miRNA regulation of gene expression in leukemia disrupts many mechanisms important for normal hematopoiesis, including cell proliferation, differentiation, and survival.[12–15] Unique miRNA expression profiles have been demonstrated for specific cytogenetic subsets in AML,[16–18] and altered expression of miR-155, in particular, has been identified as a potential prognostic factor in adult AML.[19] In this report, quantitative expression levels of miR-155 in diagnostic specimens from 198 pediatric normal karyotype AML (NK-AML) patients are analyzed and correlated with clinical characteristics and outcomes.

MiR-155 is encoded by the *MIR155HG* gene located on chromosome band 21q21.3. MiR-155 expression is normally induced in hematopoietic stem cells and myeloid progenitor cells as part of the inflammatory response, under the control of the transcription factors NF-

κß and AP-1.[20] By targeting inflammatory inhibitors, miR-155 regulates the innate immune response.[20–25] Overexpression of miR-155 has been reported in solid tumors like thyroid, breast, colon, cervical, and lung cancers as well as in chronic lymphocytic leukemia (CLL) and lymphomas.[20,26] MiR-155 is considered an oncomiR, promoting tumorigenesis through modulating of target genes involved in proliferation, differentiation, and apoptosis.[27]

2 Methods

2.1 Patients

AAML0531 is the phase III clinical trial in which patients were randomized to conventional chemotherapy +/– Gemtuzumab ozogamicin (GO, Mylotarg).[28] AAML0531 enrolled 1,022 patients from August 14, 2006 to June 15, 2010, including 233 patients with NK-AML. We had 198 diagnostic patient specimens available for analysis, which were prospectively evaluated for miR-155 expression. Normal marrow was obtained from a commercial vendor (All Cells, Alameda, CA). The institutional review boards of all participating institutions approved the clinical protocol and the COG Myeloid Disease Biology Committee approved this research. In accordance with the Declaration of Helsinki, all patients (or guardians on behalf of the pediatric patients) provided written informed consent for the collection and use of their biospecimens for research purposes under studies approved by the British Columbia Cancer Agency Research Ethics Board (Vancouver, BC, Canada) and the Fred Hutchinson Cancer Research Center Institutional Review Board (FHCRC, Seattle, WA, USA). Clinical data were de-identified in compliance with Health Insurance Portability and Accountability Act regulations.

2.2 Methods

MiR-155 expression levels were determined by using TaqMan quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) kit using commercially available primers and probes from Life Technologies, Carlsbad, CA (miR-155 Assay# 000480 and control gene RNU49 Assay# 001006). Specifically, miRNA was extracted from ficollenriched mononuclear diagnostic marrow specimens. Total RNA was extracted from thawed untreated bone marrow cells using Trizol purification method. First stand cDNA synthesis was performed using 100 ng of total RNA in 15 μl of final volume containing 6 μL of primer Pool, 10× RT buffer, 50 U/μl Multiscribe reverse transcriptase and 20 U/μl RNase inhibitor in a 96-well plate. Next, real-time PCR was performed using a standard TaqMan PCR protocol. Specifically, 20 μl PCR reactions included 1 μl of RT product, 1× Universal TaqMan Master Mix, 1× TaqMan probe, and 8 μl of water in a 96-well plate. All RT-PCRs were run in duplicate. Raw data were analyzed using StepOne software version 2.2 from Life Technologies. Expression levels were normalized to control gene expression (i.e., RNU49) and standardized to the normal marrow bone marrow using the *delta - delta* C_T method. miRNA extracted from 10 independent normal bone marrow specimens were pooled for this analysis. The expression level of RNU49 was stable throughout all patient specimens. To evaluate the correlation of miRNA expression and disease characteristics, the patients were divided into four quartiles (Q1–Q4) based on individual miR-155 expression levels, where Q1 consisted of those with the lowest miR-155 expression levels and Q4 consisted of patients with the highest miR-155 expression levels. Expression of miR-155

was correlated with disease characteristics, clinical response, and outcomes across these quartiles. All comparisons and data presented compares high (Q4) versus low (Q1–Q3) expressers.

2.3 Risk stratification

Low-risk patients included those with *NPM1* or *CEBPA* mutations. Patients were classified as high risk if they had *FLT3/ITD* mutations.

2.4 Statistical methods

Clinical outcome data for COG AAML0531 were analyzed. Patients were defined as being in complete remission (CR) if they had 5% or fewer blasts and tri-lineage recovery after one course of induction chemotherapy. Overall survival (OS) was determined both from time of study entry or from end of course 1. Event-free survival (EFS) was defined as the time from study entry until death, induction failure, or relapse. The Wilcoxon rank sum test was used to determine the significance between differences in medians of groups. The Cox proportional hazards model was used to evaluate the impact of miR-155 expression level as a predictor of clinical outcome in the context of prognostic factors (*FLT3/ITD*, *NPM1*, *CEBPA* mutations, treatment arm, risk group, age, WBC) and molecular risk groups using them as covariates in both univariate and multivariate models.

3 Results

3.1 miR-155 expression correlates with high-risk AML

Among 1,022 patients enrolled on AAML0531, 233 patients had NK-AML, and 198 of these patients had diagnostic material available to assess miR-155 expression by quantitative RT-PCR. miR-155 expression varied over 4 $\log_{10}$ (10,000-fold change), with a median expression level of 0.825 (range 0.043–25.630). For purposes of clinical correlation, the study population was divided into four quartiles based on miR-155 expression, and expression levels were correlated with demographics, pretreatment laboratory findings, and disease characteristics across the four quartiles (Table 1). Median expression for quartiles 1–4 was Q1: 0.309 (range 0.043–0.439), Q2: 0.575 (range 0.448– 0.824), Q3: 1.213 (range 0.826–1.636), and Q4: 3.036 (range 1.637– 25.630) (Fig. 1A). MiR-155 expression was highly associated with molecular prognostic factors. *FLT3/ITD*, *NPM1*, *CEBPA*, and *WT1* mutations were detected in 37, 15, 24, and 11% of evaluable samples, respectively. There was a statistically significant increase in *FLT3/ITD* prevalence with increasing miR-155 expression by quartiles (9, 24, 59, and 69% in Q1–Q4, respectively; $P < 0.001$; Fig. 1B), whereas no definitive trend in prevalence was observed for *NPM1*,*CEPBPA*, or *WT1* mutations. Patient samples were stratified into low-risk 67/198 (34%), standard-risk 83/198 (42%), and high-risk 48/198 (24%) according to *FLT3/ITD*, *NPM1*, *CEBPA*, and *WT1* mutations.[29] There was a direct association between miR-155 expression and prevalence of high-risk AML ($P < 0.001$; Fig. 1C). The prevalence of standard-risk disease decreased with each quartile ($P = 0.008$; Fig. 1C). There was no significant trend in prevalence by quartile for low-risk disease ($P = 0.041$; Fig. 1C).

3.2 High miR-155 expression correlates with induction failure and worse survival in NK-AML

Induction response rates to initial chemotherapy was correlated with miR-155 expression level. Patients with high miR-155 expression had induction failure rates of 54% versus 17% for patients with lower miR-155 expression ($P < 0.001$). As *FLT3/ITD* is overrepresented in high miR-155 expressing cases and *FLT3/ITD* is associated with higher induction failure, we evaluated the remission induction rate in patients without *FLT3/ITD*. Similar to the entire cohort, *FLT3/ITD*-negative patients with high miR-155 expression had higher induction failure rates as compared to patients with lower miR-155 expression, 53 versus 14%, respectively ($P = 0.002$; Fig. 2). *FLT3/ITD*-positive patients with lower miR-155 expression had an induction failure rate of 24 versus 55% for patients with high miR-155 expression ($P = 0.008$). These data demonstrate that high miR-155 expression is associated with higher induction failure rates regardless of *FLT3/ITD* mutation status.

We then evaluated the impact of miR-155 expression on survival. Initial evaluation of survival by quartiles demonstrated that patients with miR-155 expression in Q1–Q3 (low expression) had significantly better outcomes as compared with patients with the highest miR-155 expression (Q4) (Fig. 3A). Consequently, OS and EFS were compared for the high miR-155 expression (Q4) versus lower miR-155 expression (Q1–Q3). Actuarial OS at 3 years was 51 ± 14% for patients with high miR-155 expression (Q4) as compared with 75 ± 7% for patients with lower expression (Q1–Q3, $P = 0.002$; Fig. 3C). Corresponding EFS from study entry for those with high and low miR-155 expression was 32 ± 13% versus 59 ± 8% ($P < 0.001$; Fig. 3D).

In order to evaluate whether miR-155 expression might be an independent prognostic factor in NK-AML, we performed a multivariate Cox regression analyses to evaluate the impact of miR-155 expression level as a predictor of clinical outcome in the context of known prognostic factors including *FLT3/ITD*, *NPM1*, *CEBPA* mutation status, age, and total white blood cell count (Table 2). In this multivariate model, high miR-155 expression retained prognostic significance for OS (HR 1.92, 95%CI 1.10–3.36, $P=0.022$) and EFS (HR 1.75, 95%CI 1.10–2.80, $P = 0.019$).

4 Discussion

Pediatric AML accounts for one fourth of acute leukemias in children, but leads to nearly half of all leukemia deaths. While several molecular alterations in AML predict clinical outcomes, there are very few genomic alterations that are used to determine therapy. In normal hematopoietic cells, miR-155 plays an important role in immune cell function by regulating inflammation and immunological memory.[30] MiRNAs control normal myeloid cell development and aberrant expression of miRNAs play key roles in myeloid malignancies.[30] MiR-155 is an oncomiR that contributes to leukemogenesis.[21,22] This study demonstrates the association of high miR-155 expression with poor response to induction chemotherapy and poor EFS and OS in pediatric leukemia patients.

While other investigators have explored the role of microRNA in pediatric AML, none have identified specific microRNA of prognostic significance that could be used to guide therapy

decisions. For example, Daschkey et al.[31] identified clusters of miRNA that were differentially expressed in specific cytogenetic subtypes of pediatric AML, specifically, MLL rearranged and core binding factor AML. In this setting, differential miRNA expression was evaluated solely as a means of distinguishing between various cytogenetic subtypes rather than identifying miRNA expression levels of prognostic significance. Emmrich et al. explored the functional biology of miR-9 in pediatric patients with t(8;21) AML. While differential expression of miR-9 did not seem to have prognostic significance in children with t(8;21) AML, miR-9 expression levels were markedly reduced in t(8;21) patients versus children with other AML subtypes. In addition, when miR-9 was ectopically expressed in both AML cell lines as well as primary patient samples with t(8;21), it was noted that differentiation along the monocytic lineage could be induced.[32] Lastly, while Zhang et al.[33] identified specific miRNA profiles that correlated with a higher incidence of relapse in pediatric acute lymphoblastic leukemia (ALL), to our knowledge, similar findings have not been reported in pediatric AML.

We demonstrate significant variability of miR-155 expression in children with NK-AML. Increased miR-155 expression was directly associated with adverse molecular risk factors (*FLT3/ITD*+) and high-risk disease and inversely associated with low and standard-risk disease. CR rates were consistently low for patients with high miR-155 expression across the entire cohort, which appeared to be unrelated to the presence of *FLT3/ITD* mutations. We further showed an association between miR-155 expression and clinical outcome, and in multivariate analysis that included clinically relevant risk factors, we demonstrate that high miR-155 expression remained an independent predictor of adverse outcome after controlling for FLT3/ITD, NPM1, or CEBPA mutations.

Our findings in pediatric AML patients are similar to observations in adult NK-AML patients where miR-155 expression levels correlated with somatic mutation status and clinical outcome. Specifically, Faraoni et al.[34] examined the expression level of miR-155 in 48 adult patients with newly diagnosed NK-AML and noted increased miR-155 expression in patients with *FLT3/ITD* when compared when patients with wild-type *FLT3*. In a larger study of 363 newly diagnosed adult NK-AML patients, an increased prevalence of *FLT3/ITD* mutations was found in bone marrow and/or peripheral blood blasts of patients with high miR-155 expression.[19] Similar to our findings, this study also demonstrated that high miR-155 expression as compared with low mir-155 expression was associated with shorter disease-free survival (27 vs. 55%, respectively; $P < 0.01$) and OS (28 vs. 57%, respectively; $P < 0.01$), which remained significant after adjustment for *FLT3/ITD* status. In addition, among patients less than 60 years of age with high mir-155 expression, the likelihood of achieving CR was significantly reduced when compared with patients with low miR-155 expression (76 vs. 90%; $P = 0.03$).[19]

While several studies have demonstrated the negative prognostic significance of high miR-155 expression in adults with NK-AML, our analysis is the first study to demonstrate that high miR-155 expression is an independent negative prognostic factor in pediatric AML patients.

Despite strong correlations between increased miR-155 expression and poor outcomes, our mechanistic understanding of how miR-155 contributes to AML remains limited. Recently, signaling downstream of *FLT3/ITD* was shown to directly induce increased miR-155 expression through activation of NF-KB (p65) and STAT5 transcription factors.[35] Whether a similar pathway exists in patients with high miR-155 expression who lack *FLT3/ITD* mutations remains to be determined. HOXA9 regulates miR-155 expression in normal hematopoietic cells[36] and HOXA9 is highly expressed in subsets of adult AML patients, where it contributes to leukemogenesis,[37] consequently HOXA9 may also regulate high expression of miR-155 in leukemia cells.[35,38,39] In *FLT3/ITD* AML cells, miR-155 targets the myeloid transcription factor PU.1 to influence proliferation, differentiation, and apoptosis of leukemic cells.[35] Genes whose expression correlates with miR-155 expression in adult NK-AML have been identified, but their specific role in AML pathogenesis or whether they are directly targeted by miR-155 has not been fully determined.[19] Validated miR-155 targets include genes that impact leukemogenesis, inflammation, and act as tumor suppressors.[40] In myelodysplastic syndromes, miR-155 targets SH2 domain-containing inositol 5′-phosphatase 1 (SHIP-1), which inhibits the PI3K/AKT signaling pathway, and in turn regulates myeloid cell proliferation and survival.[41] Intriguingly, recent observations suggest that while increased miR-155 expression in *FLT3* ITD cases is oncogenic, increased miR-155 in wild-type FLT3 AML cell lines may act as a tumor suppressor to promote cell differentiation and apoptosis. These observations suggest the cellular context of other genomic alterations may influence the effects of miR-155 on leukemogenesis.[42,43] In keeping with this observation, in murine models, miR-155 overexpression in hematopoietic stem cells leads to a myeloproliferative disorder that does not progress to acute leukemia, suggesting additional mutations are involved in AML progression.[22] Nonetheless, our clinical data demonstrate that high miR-155 expression, regardless of mutation status in NK-AML, correlates with poorer EFS and OS.

In conclusion, high miR-155 expression is an adverse prognostic factor in both pediatric and adult NK-AML patients and correlates with *FLT3/ITD* mutation status. High miR-155 expression is an independent predictor of worse EFS and OS. While several miR-155 target genes have been identified, the functional role of miR-155 in AML pathogenesis warrants additional investigations as does the identification of the optimal cut-point for defining high miR-155 expression. The ability to target small noncoding RNAs for therapies in cancers is currently in preclinical development and in early clinical trials.[44–46] Decreasing miR-155 function with antogomirs may provide therapeutic benefit to certain subsets of AML, including *FLT3/ITD*-positive AML patients and high miR-155 expression.[25,26]

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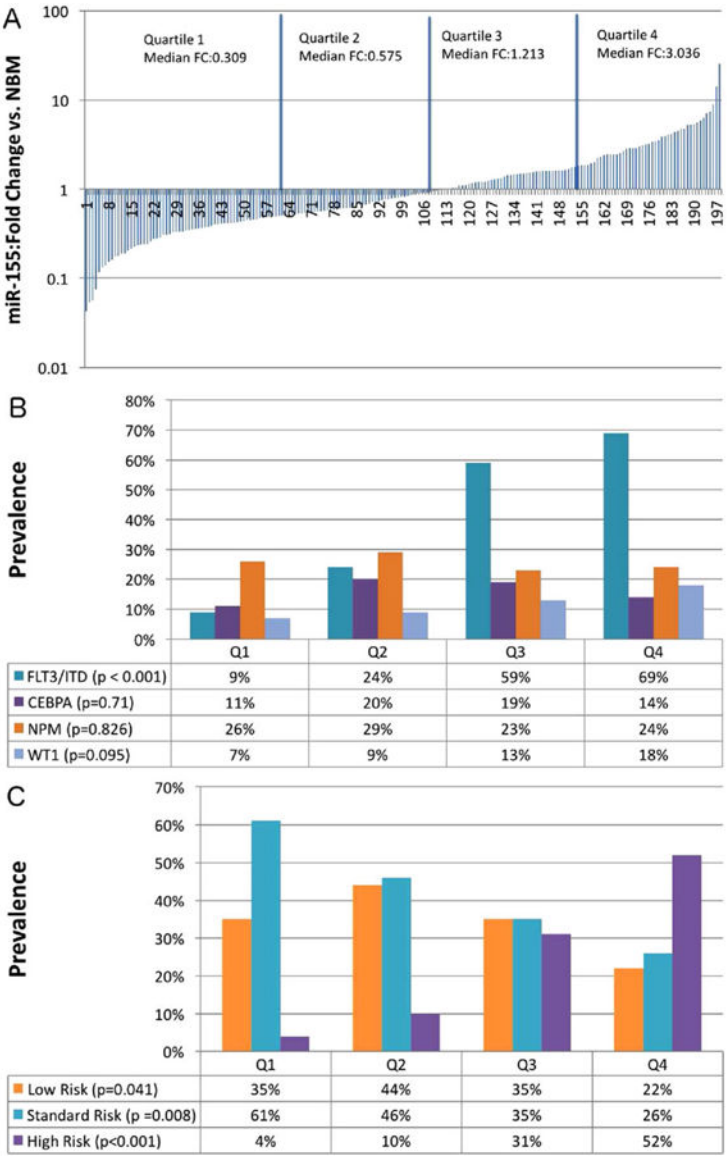
Abbreviations

AML	acute myeloid leukemia
CI	Confidence Interval
CR	complete remission
EFS	event-free survival
FC	Fold Change
HR	Hazard Ratio
miRNA	miR, microRNA
MLL	mixed lineage leukemia
NK-AML	normal karyotype acute myeloid leukemia
OS	overall survival
RT-PCR	Reverse Transcription Polymerase Chain Reaction



	Q1	Q2	Q3	Q4
FLT3/ITD (p < 0.001)	9%	24%	59%	69%
CEBPA (p=0.71)	11%	20%	19%	14%
NPM (p=0.826)	26%	29%	23%	24%
WT1 (p=0.095)	7%	9%	13%	18%

	Q1	Q2	Q3	Q4
Low Risk (p=0.041)	35%	44%	35%	22%
Standard Risk (p =0.008)	61%	46%	35%	26%
High Risk (p<0.001)	4%	10%	31%	52%

Figure 1.

Distribution of miR-155 expression levels per quartile. (A) Log-fold change. (B) Prevalence of mutations. (C) Disease risk group classification

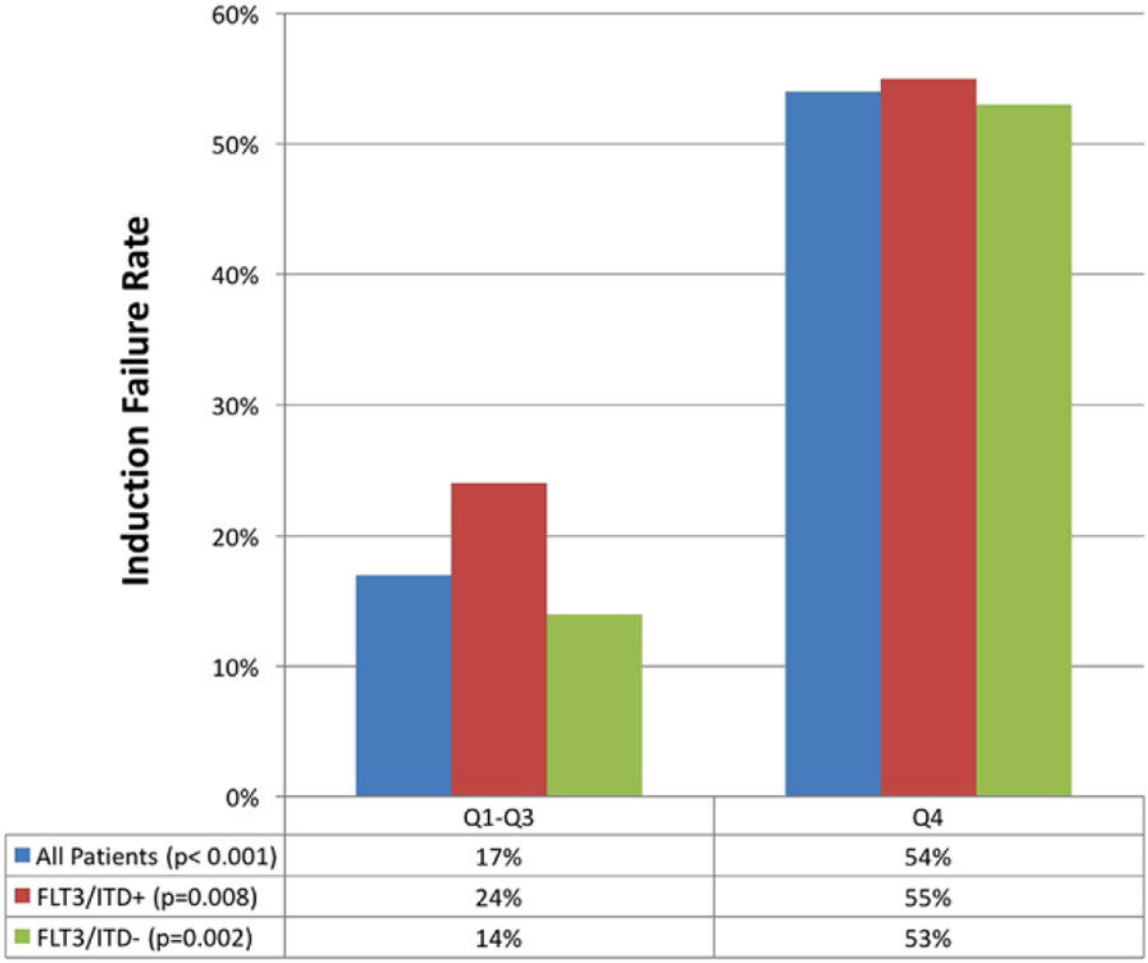


Figure 2. Induction failure rates for all patients, FLT3/ITD-positive patients and FLT3/ITD-negative patients for those with high miR-155 expression levels (Q4) vs. low miR-155 expression levels (Q1–Q3)

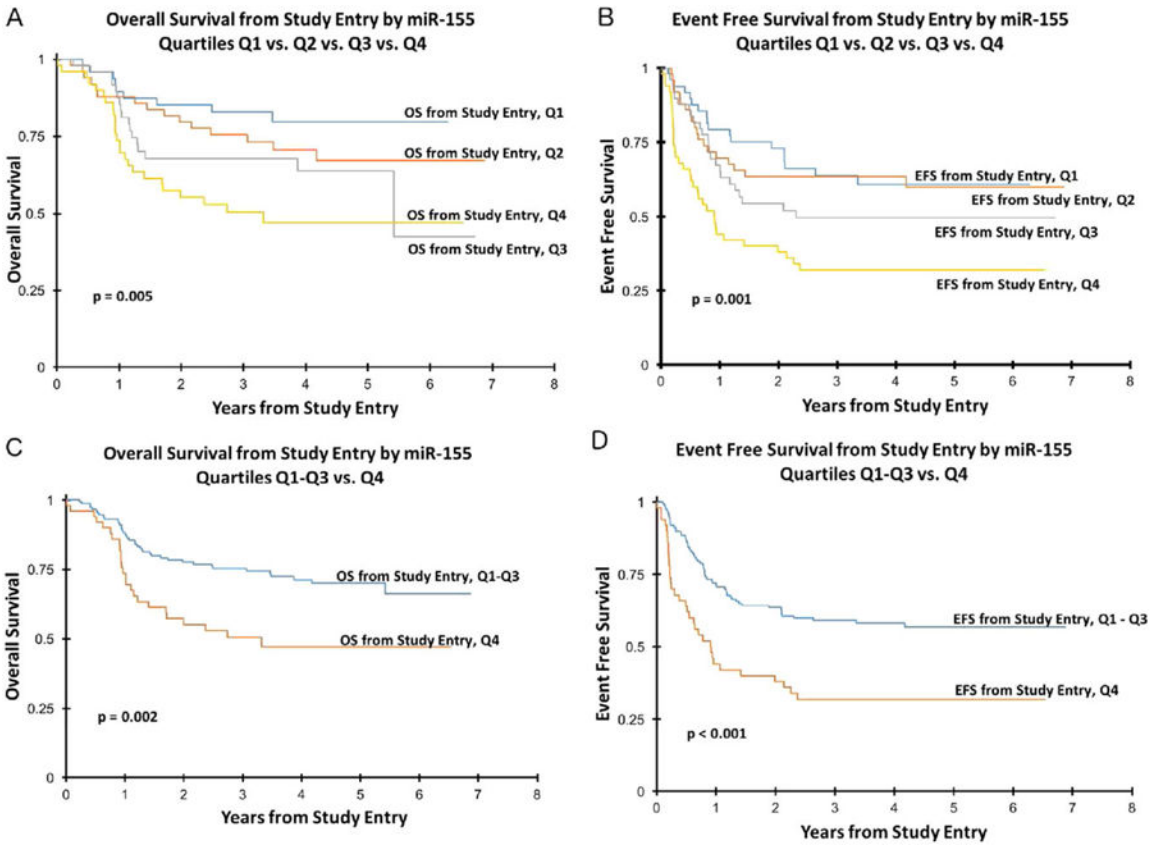


Figure 3.

Association between miR-155 expression levels and long-term clinical outcomes. (A) OS from study entry by miR-155 expression level quartiles Q1 vs. Q2 vs. Q3 vs. Q4. (B) EFS from study entry by miR-155 expression level quartiles Q1 vs. Q2 vs. Q3 vs. Q4. (C) OS for patients with high miR-155 expression levels (Q4) vs. low miR-155 expression levels (Q1–Q3). (D) EFS for patients with high miR-155 expression levels (Q4) vs. low miR-155 expression levels (Q1–Q3)

Table 1

Patient demographics and disease characteristics

	Q1 (N = 49)		Q2 (N = 50)		Q3 (N = 49)		Q4 (N = 50)		Q1-Q3 vs. Q4
Characteristic	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	***P* value**
Gender									
Male	19	39	28	56	24	49	33	66	0.027
Female	30	61	22	44	25	51	17	34	
Age (years)									
Median (range)	14	(0.56–28.7)	12.1 (	1.17–18.7)	11.8	(0.3–20.4)	13.1	(0.27–20.9)	0.771
FAB									
M0	1	2	0	0	2	5	2	5	0.594
M1	12	29	12	25	9	21	14	34	0.261
M4	7	17	9	19	10	24	9	22	0.770
M5	4	10	8	17	4	10	7	17	0.425
M6	0	0	2	4	2	5	0	0	0.574
M7	2	5	1	2	0	0	0	0	1.000
Other	9	22	6	13	4	10	1	2	0.048
Missing	8		2		7		9		
Mutations									
FLT3/ITD	4	9	11	24	27	59	33	69	<0.001
CEBPA Mutant	5	11	9	20	9	19	7	14	0.710
NPM Mutant	12	26	13	29	11	23	12	24	0.826
WT1 mutant	3	7	4	9	6	13	9	18	0.095
Risk groups (cyto/mutation)									
Standard	30	61	23	46	17	35	13	26	0.008
Low	17	35	22	44	17	35	11	22	0.041
High	2	4	5	10	15	31	26	52	<0.001
WBC ($\times 10^3$ μl), median (range)	16.3	(0.9–295.6)	17.4	(0.5–237)	29.5 (	0.2–388.6)	59.4	(1.2–827.2)	0.002
BM blasts, %	65	(0.4–96)	56	(20–94)	75	(3–100)	82	(24.8–99)	<0.001

Characteristic	Q1 (N = 49)		Q2 (N = 50)		Q3 (N = 49)		Q4 (N = 50)		Q1-Q3 vs. Q4
	N	%	N	%	N	%	N	%	*P* value
Platelet count (1,000 μl^{-1}), median	45	(5–865)	67.5	(6–407)	50	(11–365)	58	(7–192)	0.413
Hemoglobin, median (range)	8.8	(3.7–15.4)	8.0 (	2.7–13.2)	7.9	(2.9–13.9)	7.9	(2.4–14.0)	0.945
Course 1 response									
CR	39	87	40	80	39	81	22	46	<0.001
Not in CR	6	13	10	20	9	19	26	54	
Not evaluable	4		0		1		2		

Table 2

Multivariate analysis of OS and EFS

	Multivariate (N = 198)						
		OS			EFS		
	N	HR	95% CI	*P*	HR	95% CI	*P*
miR-155 expression							
Low (Q1–Q3)	148	1			1		
High (Q4)	50	1.92	1.10–3.36	0.022	1.75	1.10–2.80	0.019
Risk groups							
Standard	83	1			1		
Low (NPM or CEBPA mutation)	67	0.34	0.16–0.71	0.005	0.22	0.11–0.43	<0.0001
High (FLT3/ITD)	48	0.85	0.43–1.67	0.633	1.06	0.62–1.82	0.833
Age groups							
0–1 years old	14	3.21	1.42–7.26	0.005	2.12	1.03–4.38	0.042
2–10 years old	63	1			1		
11–21 years old	121	0.92	0.53–1.59	0.757	0.87	0.56–1.36	0.534
WBC							
$<100 \times 10^3$ /μl	160	1			1		
$>100 \times 10^3$ /μl	38	1.94	1.09–3.47	0.025	2.39	1.46–3.90	0.001