Role of MiR-155 Expression in Acute Myeloid Leukemia and Correlation with Nucleophosmin 1 (NPM1) Mutations

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Original Article

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ABSTRACT

Background: Acute myeloid leukemia (AML) is a highly heterogeneous disease marked by the inability of progenitor cells in the bone marrow to differentiate terminally from developing to mature blood cells . Furthermore, microRNAs (miRNAs) are short, non-coding RNAs that regulate multiple gene expressions that regulate hematopoietic stem/progenitor cell commitment and differentiation.

Materials and Methods: The present study involved 40 patients newly diagnosed with AML in addition to 40 matched controls. A quantitative reverse transcription real-time PCR (qRT-PCR) assay was performed to quantify miR-155. The NPM1 mutations in a new cohort of Egyptian AML patients were assessed using RT-PCR assays. The aim was to examine the correlation between these mutations and microRNAs, as well as to determine their expression in relation to demographic, clinical, and laboratory data.

Results: For Mir-155, there were a statistically substantial differences between the mean CT values of patients and controls. NPM1 results exhibited marked differences between controls and patients.

Conclusion: In conclusion, the value of 155 did not accurately predict cases of AML. Additionally, no correlation was observed between NPM1 and miR-155.

Key Words: AML, miR-155, NMP, qRT-PCR.

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INTRODUCTION

AML is a highly heterogeneous disease marked by the inability of progenitor cells in the bone marrow to differentiate terminally from developing to mature blood cells. AML is characterized by various genomic and genetic abnormalities like inversions, chromosomal translocations, mutations, and gene deletions. Additionally, there have been reports of miRNAs causing changes in posttranscriptional regulation in AML[1].

Moreover, miRNAs compromise a large group of small non-coding RNAs regulating target gene expression negatively through attachment to mRNAs' 3' untranslated region (UTR), leading to degradation or translation inhibition. Additionally, miRNAs are involved in a wide range of biological processes, including differentiation, carcinogenesis, angiogenesis, hematopoiesis, apoptosis, metastasis, and proliferation. However, there is a consistent identification of miRNA aberrant expression in the chemoresistance and pathogenesis of hematopoietic malignancies[2].

MiR-155 has significant functions in hematopoiesis, cancer, inflammation, and immunity[3]. It is one of the most commonly upregulated miRNAs in cancer. It has also been implicated in solid tumors of the lungs, colon, and breast, along with various human lymphomas and leukemias[4].

NPM1 is an essential gene encoding a nucleolar protein involved in numerous functions, such as stabilizing P14 alternate reading frame tumor suppressor protein (p14 ARF) as well as controlling centrosome duplication and ribosome biogenesis[5].

The vast majority of mutations, specifically 50 different molecular variations that have been discovered until now, occur in the NPM1 gene's exon 12[6]. These mutations all result in substantial alterations in NPM1 protein's C terminus[7].

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Alterations in NPM1 transport properties induce NPM1 variants' accumulation in AML cells' cytoplasm, which is considered essential for leukemogenesis[8]. NPM1 mutations are potentially an early event in leukemia development[9].

The objective of this study was to elucidate the involvement of miR-155 in the pathogenesis and prognosis of AML, as well as to test the correlation between both miRNAs and the NPM1 gene.

SUBJECTS AND METHODS

This study included two study groups: The patient group consisted of 40 de novo AML patients (19 men and 21 women), aged from 20 to 70 years and (41.63 ± 13.26 years) mean age.

Besides the control group, the study recruited 40 age- and sex-matched controls (16 men and 24 women). The age of the subjects varied between 19 and 50 years and (32.45 ± 7. 33 years) mean age.

AML diagnosis was determined on the presence of ≥20% blasts within the BM, myeloperoxidase-positive staining, and immunophenotypic patterns consistent with an AML diagnosis.

Each patient underwent a comprehensive clinical examination, a complete medical history, and a complete medical examination, including renal function tests, Leishman-stained PB, complete blood count, liver function tests,coagulation tests, BM smears, and LDH.

We also performed BM/PB samples' immunophenotyping (IPT) through whole blood lysis using EPICS XL flow cytometer (Coulter-USA), in addition to routine monoclonal antibody panel (for acute leukemia). This panel includes myeloid markers (CD 13, CD33, CD 117, HLA-DR, MPO), B cell lymphoid markers (CD10, CD 19, CD 20), T-cell lymphoid markers CD7, CD5, CD3, CD2), monocyte marker (CD 14) and stem cell marker (CD 34).

Markers were deemed positive if the expression was observed in ≥20% of cells, with the exception of CD34 and MPO.

Real-time PCR with Light Cycle probe design software (Roche Diagnostics, GmbH, Germany) was utilized for quantification and detection of miR-155 expression levels and NPM1 mutations.

Sampling

All samples were collected after research ethics committee approval. In addition, all subjects provided ,formal written consent.Data confidentiality was maintained following the revised Helsinki Bioethics Declaration.

Three ml of venous blood was collected using a sterile vacutainer and placed on ethylenediaminetetraacetic acid (EDTA), and then it was used to perform CBC and IPT. Subsequently, 3 ml of BM was aspirated. A few drops were used for immediate BM testing using Leishman and MPO-stained films. One ml was collected in a heparinized tube for FISH analysis. The remaining samples were collected on EDTA for IPT.

For miRNA expression by PCR, 300-500 microns of anticoagulated PB/BM samples were added to 1.3 miRNA stabilization solution (RNAlater®) in a sterile RNase-free microcentrifuge tube.

To fully penetrate the cells and stabilize the RNA, they were stored at 4 °C overnight and then at −20 °C for RNA extraction. Additional serum samples were collected for further analysis.

RT-PCR Total RNA Isolation

The RiboPure™ Blood Kit (supplied by Applied Biosystems, Ambion catalog number AM1560, AM1556) was used to isolate total RNA from anticoagulated whole blood. The isolated RNA was then mixed with RNA solution and processed per manufacturer's instructions.

Two-Step RT-PCR: The TaqMan® MiRNA Reverse Transcription Kit (P/N 4366596) and TaqMan® miRNA Primers and Hybridization Probes for miR-155 and RNU6B (P/N 4427975) were supplied by Applied Biosystems. They were used to detect and quantify miR-155 over a dynamic range of more than 6 logs.

TaqMan® miRNA Reverse Transcription Kit included RT buffer, dNTPs, RNase inhibitors, and Multiscrib™ RT enzyme.TaqMan® miRNA Assay (2 sets) contained miRNA-specific RT primer, forward PCR primer, reverse PCR primer (#4366596), TaqMan® MGB probe, Taqman® Universal Master Mix (#4440043), and TaqMan® miRNA control. Additionally, (# 4427975) was used to normalize the results.

For NPM1,a pre-built TaqMan® SNP real-time PCR assay consisting of the Pure Link DNA kit (#K182001), Universal Taqman Master Mix (#4440043), and SNP Assay (#4351379) were utilized.

For each 15 µL RT reaction, 1–10 ng of total RNA was combined with RT master mix in a ratio of 5 µL total RNA: 7 µL RT master was mixed with RT master mix in a ratio of 5 µL total RNA to 7 µL RT master mix. A volume of 12.0 µL of this mixture was added to the reaction tube. Subsequently, 3 µL of RT primers (from each assay set) were added to the corresponding RT reaction tubes, increasing the total volume per well to 15 µL. The reaction vessels were sealed, subjected to brief centrifugation, and then placed onice until loaded into a thermocycler.

Primer and probe sequences were as follows

The sequence of primers and probes was as follows:

miR- 155 Primer: F:(5'ACACTCCAGCTGGGTTAATGCTAATCG TGAT-3')

R:(5'-TGGTGTCGTGGAGT CG-3')

Primers for NPM1 (rs35979859) C/T: F: (5'TTTTTCTTCATTTACAGGTTGTGAA-3')

R (5'TAAAGGCCGACAAAGATTATCACTT-3')

The temperature cycling conditions for reverse transcription runs were set at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

Quantitative PCR (qPCR) Amplification

Each qPCR reaction included 1.00 µL (TaqMan®-Small RNA Assay (20X), 7.67 µL (nuclease-free water), and 10.00 µL (TaqMan®-Universal PCR Master Mix) added to 1.33 µL (RT-PCR reaction product) until reaching 20 µL total volume.

The reaction components were mixed in a microcentrifuge tube, then briefly centrifuged, and subsequently transferred to the wells of a reaction plate. The plate was sealed and briefly centrifuged.

PCR runs were established utilizing the specified thermal cycling conditions. The enzyme was activated by exposing it to a temperature of 95°C for a period of ten minutes. The PCR procedure comprised 40 cycles, with each cycle compromising denaturation at 95°C for 15 seconds, followed by annealing and extension at 60°C for 60 seconds.

The experiment was subsequently scrutinized, and the outcomes were interpreted by analyzing the amplification diagram in order to identify base values and thresholds.

Data analysis

The measurement of miRNA expression levels was conducted utilizing the CT method as well as the comparative CT method (ΔΔCT). Each miRNA's expression level was determined by subtracting its CT value from the average CT value of a reference gene for each sample in a specific sample set[10].

The reference gene utilized was RNU6B. Subsequently, we computed the relative (removed) fold change of each candidate miRNA (in each group) by employing the formula 2-(ΔΔCT)[11].

Each miRNA's ΔCT (in each sample) was determined as ΔCTsample = CT target miRNA – CT RNU6B, whereas ΔΔCT was estimated based on the equation ΔΔCT = (CTmiRNA – CRNU6B) patient − (CT miRNA – CRNU6B) control.

Statistical methods

The 21st V of the SPSS software was utilized for analyzing data (SPSS Inc.Chicago IL ,USA).

For descriptive data , quantitative data were expressed as the mean ± standard deviation (mean ± SD) or range (median if appropriate). Qualitative data were described in terms of percentages and numbers.

For analysis statistics , the tests employed for the comparison of two median values were Pearson's chi-square test, Fisher's exact test, Mann-Whitney test, and Student's t-test.

Patients whose expression values exceeded the median of all samples were categorized as having an elevated expression of both mir-155. The Pearson correlation coefficient was utilized to determine the correlations between various variables, specifically for linear relationships. *P-values* below 0.05 are deemed statistically significant, while *P-values* below 0.01 are highly statistically significant.

RESULTS

This study was conducted on 40 de novo AML patients recruited from the Department of Medical Oncology, Qasr Al Aini Medical College, Cairo University. These patients were considered as the patient group for the study. Furthermore, a control group consisting of 40 healthy volunteers of matching sex and age was included.

Regarding clinical data, weight loss was reported in 32.5% of cases, fever/infection in 40% of cases, bleeding tendency in 27.5%, hepatomegaly in 30%, splenomegaly in 55% of cases, and splenomegaly in 15% of cases.

Regarding the laboratory data of patients, the total white blood cell count ranged from 12.40 to 153.00 x 109/L (median = 34.75). Hemoglobin levels ranged from 3.40 to 13.40 Hb gm/dl (median = 7.65). Platelet counts ranged from 10.00 to 160.00 x 109/L (median = 36.50).

Peripheral blood blast percentage ranged from 20.00 to 96.00% (median = 61%).Blast percentage in the bone marrow ranged from 22.00 to 92.00% (median = 65.5%).

The immunophenotyping results showed that CD13, CD33, and CD34 were positive in 100% of cases, HLA-DR in 90% of cases, CD14 in 22.5% of cases, and lymphocyte markers in 100% of cases.

Twenty-one patients underwent cytogenetic and molecular analysis, revealing the presence of t (8;21) in 14 (35%), 46xx del6 in 1, and t (15,17) were detected in four cases. One patient had t (16,16), and one had normal 46xy.

Regarding FAB classification, 3/40 cases (7.5%) of the examined cases were diagnosed as M0, 8/40 cases (20%) as M1, and 14/40 cases (35%) as M2.,4/40 cases (10%) were diagnosed as M3, 4/40 cases (10%) were diagnosed as M4, 5/40 cases (12.5%) were diagnosed as M5, and 1/40 cases (2.5%) were diagnosed as M6, and 1/40 cases (2.5%) were diagnosed as M7.

The values of miR-155 expression in both patients and controls are depicted in (Table 1,2).

Table 1: MiR- 155 in AML patients and controls

Variable		Patients (N=40)		Controls (N=40)	
		Count	%	Count	%
miR-155	Detectable	38	95.0%	40	100%
	undetectable	2	5.0%	0	0%

Table 2: The real-time values of the studied genes expressed by CT method according to the formula 2-(ΔΔCT).

	Patients(no=40)	Controls(no=40)	*P value*
Mir-155	Median(range) 23.885 (0-39.443)	Median(range) 28.395 (25.45-30.49)	0.03(S)
RNU6B	Median(range) 31.05 (24.67-41.17)	Median(range) 32.45 (28.11-34.49)	0.79(NS)

Regarding Mir-155, there were substantial differences between the mean CT values according to the formula 2-(ΔΔCT)of patients and controls (*p-value* = 0.03).

For RNU6B, there were substantial differences between the mean CT values of patients and controls (p-value = 0.79 for both groups).

As previously stated, patients were categorized into two groups based on these values: an upregulated group and a downregulated group. The median and range of Mir-155 values were 62.834 (2.303 to 10697.6) in the upregulated group and 0.186 (0.0009 to 2.205) in the downregulated group (Table 3).

Table 3: Results of Mir-155 in patients group

	Mir-155	
	Upregulated	Downregulated
Median	62.834	0.186
Range	2.303- 10697.6	0.0009- 2.205

There was no statistically significant correlation between demographic or laboratory data & miR-155 in patients or control groups (Tables 4,5).

Table 4: Correlation between demographic & laboratory data miR-155 among patients

parameter	miR-155	
	R	p
Age	-0.176	0.289
Initial Hb(g/dl)	-0.049	0.768
Initial TLC(x109/L)	-0.253	0.125
Initial platelets(x109/L)	-0.035	0.833
Intial blast%(PB)	-0.212	0.201
Intial blast%(BM)	-0.014	0.932
LDH(u/l)	-0.012	0.952

Table 5: Relationship between demographic and laboratory data and miR-155 data among controls

parameter	miR-155	
	R	p
Age	-0.001	0.993
Initial Hb(g/dl)	0.224	0.164
Initial TLC(x109/L)	0.069	0.670
Initial platelets(x109/L)	0.063	0.700

NPM1 polymorphism in AML patients and controls

Patient group: The AA (wild type) was detected in 0/40 cases (0%), and the AG (heterozygous) genotype was detected in 31/40 cases (77.5%), while the GG genotype (homovariant) was detected in 9/40 subjects (22.5%).

Control group: The AA (wild type) was detected in 5/40 control subjects (12.5%)

Comparison of frequencies of NPM1 genotype in patients with AG genotype (heterozygotes) in 40 controls (77.5%), and GG genotype (homomutant) detected in 4/40 controls (10%) Comparison of NPM1 genotype frequencies in patients based on gender (P = 1.000) AML onset age showed no statistically significant difference (P = 0.358) in patients' symptoms and signs.

No substantial differences were detected between NPM1 genotypes (p = 0.749 and 0.215, respectively). Furthermore, no marked differences between NPM1 genotypes were detected in the patient's hematological findings. The NPM1 genotype and miR-155 (P = 0.946) demonstrated no substantial differences (Table 6).

Table 6: Relation between NPM1 mutant genotypes among AML patients and miRNA

Variable	Heterozygous (GG)		Homozygous mutant (AG)		*P value*
	Median	Range	Median	Range	
miR-155	7.70	0.03-10.54	7.40	12.60-153.00	0.946(NS)

A logistic regression analysis was conducted to identify the most significant predictor of AML: age, sex, NPM1, and miR-155. All these variables were included in the regression model. Table 7 shows prognostic predictors for AML among the study group. Sex had no prognostic significance, indicating that there was no preference for poor prognosis based on sex. Old age (>60 years) and higher miRNA-155

and NPM gene mutation (G allele) have been identified as significant negative predictors. Each of these factors, when considered independently, is an independent prognostic factor that can elevate AML risk (Table 7).

Table 7: Prognostic predictors for AML among the studied group

	Odds ratio(OR)	95.0% C.I.for (OR)	*P-Value*
Age	1.116	1.046-1.191	0.001(HS)
Sex(f/m)	0.824	0.239-2.843	0.759(NS)
G allele (AG+GG)	5.249	1.008-27.322	0.049(S)
miR-155	1.545	1.050 -2.274	0.027(S)

DISCUSSION

The prevalence of hematopoietic and lymphoid cancers in Egypt has increased by approximately 11-fold in 2001 compared to 1972[12].

Leukemia ranked as the fourth most prevalent cancer in Egypt in 2010, accounting for 7.2% of cases. In 2014, the WHO reported that Egypt had an age-standardized mortality rate of 5.55 per 100,000 inhabitants for leukemia, which ranked 19th [13].

The precise etiology of leukemia remains unknown despite comprehensive investigations into its underlying mechanisms. AML is a heterogeneous disease in which recurrent cytogenetic changes were found in approximately 55% of cases, while the remaining 45% do not show any visible genetic abnormalities. This latter subgroup, cytogenetically normal AML (CN-AML), is distinguished by a range of subtle mutations that impact multiple genes. Alternations in Nucleophosmin (NPM1) contribute to roughly 60% of CN-AML cases[14].

The NPM1 gene is located on 5q35.1 and compromises a total of 12 exons.It is primarily located in the nucleolus and is hypothesized to act as a molecular chaperone for proteins. It facilitates the movement of ribosomal proteins across centrosome duplication and nuclear membrane[15].

NPM1 disruption occurs due to chromosomal translocation or mutation, leading to the translocation of NPM1 to the cytoplasm. The inability of cytoplasmic NPM1 to carry out its typical roles as a binding partner and transport protein implies that NPM1 mutations might be an early event in leukemogenesis[9].

The presence of NPM1 in multiple fusion proteins resulting from chromosomal translocations strongly indicates its significant involvement in leukemia and lymphoma. The chromosomal translocation t(2;5) (p23;q35) is responsible for 8% of non-Hodgkin lymphomas in young adults as well as children. It also results in a chimeric fusion of NPM1 and ALK (anaplastic lymphoma kinase). The t(3;5) (q25.1;q34) chromosomal translocation is uncommonly detected in cases of myelodysplastic syndromes. Additionally, AML leads to NPM1 a fusion transcript and myeloid leukemia factor 1 (MLF1)[16]. Additionally, specific miRNA expression signatures can be utilized to accurately categorize different leukemia types in humans[17].

The potential of circulating miRNAs as cancer biomarkers relies on their high stability and capacity to accurately indicate tumor status and predict treatment response.They are characterized by tissue-specific expression, and their signatures can be used to classify different types of cancer. Additionally, their presence in body fluids enables analysis using less invasive methods, thereby rendering biomarker analysis more feasible in clinical settings[18].

Furthermore, tumors secrete exosomes, which aid in cell-to-cell communication and protect against the activity of endogenous ribonucleases within these bioactive vesicles. Of these miRNAs, the ones that are increased in expression are primarily linked to faster cell cycle progression and reduced apoptosis, which are typical features of cancer cells.

Moreover, oncomiR is widely recommended[19] since MiR-155 plays essential roles in hematopoiesis, immunity, inflammation, and cancer[20]. This miRNA is frequently overexpressed in cancer. It has been linked to different types of lymphomas and leukemias in humans, as well as solid tumors the lungs, colon, and breast[21].

The hypothesized mechanism of apoptosis induced by mir-155 involves targeting anti-apoptotic factors, similar to how mir-15/1629 targets BCL2[22]. In this study,a qRT-PCR assay was applied to relatively quantify miR-155.

Our objective was to determine the function of these miRs in AML and establish a relationship between the expression levels of these miRs and NPM1 RT-PCR. Therefore, we conducted RT-PCR on a cohort of 40 adults recently diagnosed with AML attending the medical oncology unit (patient population). Volunteers who were 40 years old and of good health served as controls.

Age is significant independent prognostic factor in AML patients. Our logistic regression analysis suggested that the risk of developing AML would increase with each additional year of age (Table 6). This finding is consistent with the American Cancer Society's 2017 estimation of leukemia in the United States, which stated that AML is generally a disease of the elderly and is common after age 45[23].

Kouchkovsky and Abdul-Hay, 2016 also stated that the incidence of AML increases with age. Despite the existing treatments, a significant proportion of patients aged 65 and above, up to 70% of patients over the age of 65, die from the disease within a year of diagnosis, and the prognosis remains poor for older people, who make up the majority of new cases[24].

Our study revealed that miR-155 was present in the majority of the cases analyzed (38 out of 40 cases, or 95%), while it was absent in 2 out of 40 cases (5%). Out of the total of 40, 20 were upregulated, 18 were downregulated, and two were undetectable.

Gee HE *et al*., 2011 discovered that the expression of miR-155 is typically low in normal hematopoietic cells and often upregulated in AML, and 140 mRNA targets of miR-155 were identified. Most of these are regulatory proteins involved in inflammation, leukemogenesis, myelopoiesis and transcription factors[25].

Qiong *et al*., 2017 also observed that when miR-155 is overexpressed in human hematopoietic stem cells, it enhances myeloid progenitor cell proliferation. This overexpression also results in extramedullary hematopoiesis and is accompanied by megakaryocyte and erythroid cell differentiation, as well as the presence of morphological dysplasia. It was reported to inhibit the growth of granulocytic monocytic cells[1].

Another study by Jihane Khalife and colleagues from 2015 found that miR-155 overexpression leads to PI3K-AKT signaling pathway activation, leading to increased tumor growth and proliferation. The primary targets of miR-155, which have pathological implications in AML, are SHIP1 (Src homology two domain inositol 5-phosphatase 1) and CEBPβ (CCAAT/enhancer binding protein beta gene)[26].

The current study identified miR-155 as a potential independent prognostic factor in the prognosis of her AML, as determined by logistic regression. Consistent with our results, Marcucci *et al*., 2013 found that miR-155 expression serves as an independent prognostic indicator in CN-AML patients. Moreover, patients with elevated miR-155 expression exhibited a notably reduced rate of complete remission and were linked to the progression of the disease. The study demonstrated that both the disease-free and overall survival rates were reduced[27].

We showed no substantial differences between miR-155 and NPM1 mutations. This finding aligns with Jongen-Lavrencic *et al*. They found that oncogenic microRNA-155 wassubstantially upregulated in AML with FLT3-ITD, whereas microRNAs (-10a, -10b, -196a, -196b) were upregulated in AML with mutant NPM1[28].

We observed a notable increase in the expression of miR-155 in FLT3+ AML samples. No significant differences were observed in the analysis of miR-155 expression levels in CN-AML in relation to NPM1[1].

This found that miR-155 was upregulated in the bone marrow of AML patients in whom mutations were observed in (NPM1) Garzon *et al*., 2008[29]. and Qiong *et al*., 2017[1].

The G allele was considered a poor predictor of his AML development among NPM1 genotypes.

CONCLUSION

At the time of diagnosis, the measurement of a single miRNA expression can enhance and broaden our existing understanding of AML. This measurement is relatively simple to quantify and can be used to assess the molecular risk of individual patients. The comprehension of the functional correlation between miRNAs and AML is currently in its early stages.

This study provides a comprehensive understanding of the diagnostic and prognostic value of mir-155, its role in determining therapeutic strategies, and its potential as a future therapeutic agent in AML. MiR-155 was found to be a poor predictor for AML cases. In addition, no correlation was observed between NPM1 and miR-155.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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