RESEARCH ARTICLE

Prognostic Value of Plasma miR-29a Evaluation in Chronic Lymphocytic Leukemia Patients

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Abstract

   Objective: Dysregulation of microRNA expression could attenuate the course of chronic lymphocytic leukemia (CLL). Therefore, the aim of our study is to address the association between miR-29a expression and other prognostic markers in CLL patients. **Methods**: miR-29a expression was determined by quantitative real-time PCR in the plasma of 158 CLL patients at diagnosis beside 21 healthy controls in a prospective study. **Results:** The levels of miR-29a expression were found to be significantly higher in CLL patients as compared to healthy controls (P<0.001). Moreover, a significant association between high miR-29a expression and poor prognostic markers (high expression of CD38 and ZAP70, high LDH levels, Stage III Rai stage, unfavorable cytogenetic finding, time to first treatment (TTFT) and patients outcome (P<0.001 for All). Using ROC curve, we have reported that miR-29a expression levels (29a<0.76 vs >0.76) is able to discriminate severity subgroups of CLL patients. **Conclusion:** Up regulation of miR-29a expression at CLL diagnosis was detected. Determination of miR-29a expression concentration levels at diagnosis could be demonstrated as a prognostic biomarker in CLL patients.

Keywords: CLL- miR-29a- Prognosis

Introduction

   Chronic lymphocytic leukemia (CLL) is an indolent and heterogeneous neoplasm. This heterogeneity makes prognostication and therapeutic decisions is challenging. The detection of new biomarker is the focus of current research in order to improve CLL risk stratification (Aref et al., 2020; Aref et al., 2022). Recently, miRNA profiles in serum of CLL patients, have attracted attention for both risk stratification as well as target therapy (Katsaraki et al., 2021; Grenda et al., 2022). Continous efforts are carried out in current research to detect new biomarkers in order to improve risk stratification.

   MicroRNAs (miRNA) are small non-coding RNAs, act as potent regulators of multiple genes expression through down regulation of translational or destabilization of target genes through binding to the 3′-untranslated region (UTR) of mRNAs. miRNAs are associated with the development of several diseases and the their dysregulation is a cardinal feature of cancer. miRNAs play important roles in several biological processes, such as cell proliferation, apoptosis, differentiation and cell cycle. Thus, abnormal expression or dysfunction of miRNAs are associated with the development of diseases, including cancer (Zhao et al.,

2010; Jiang et al., 2014; Anelli et al., 2021).

   Small RNAs are frequently dysregulated in cancer, circulate in body fluids in a stable form, representing interesting candidates for non-invasive biomarkers. Soluble miRNAs can be identified as cell-free entities or as a composition in extracellular vesicles (EVs). They are secreted by many cell types, including cancer cells, and can be assessed in the peripheral blood, making them an ideal source of tumor biomarkers (Anelli et al., 2021).

   In recent years, miR-29 has emerged as a critical miRNA in various cancers, and it has been shown to regulate multiple oncogenic processes. Although *miR-29*a has been thoroughly documented as a tumor suppressor in the majority of studies, some controversy remains with conflicting reports of miR-29 as an oncogene (Kwon et al 2019; Raeisi et al., 2020). Recently, Nguyen et al., (2022) and Cao (2023) stated that *miR-29*a-3p is a promising biomarker and prospective therapeutic target for the diagnosis and prognosis of colorectal cancer (CRC).

   The aim of our study is to evaluate the expression levels and the prognostic relevance of *miR-29*a expression in CLL patients

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Materials and Methods

The current study included 158 newly diagnosed patients with CLL (100 male; 58 female) with mean age 61.39 ± 10.07 year at Mansoura university oncology center, Egypt (2019-2022) he study protocol was approved by the IRB of Mansoura Faculty of Medicine. The CLL diagnosis was based on morphological assessment, Lymphocytes ≥ 5.0X10⁹/L lymphocytes, Immunophenotyping results (CD5/CD19; CD23, FMC7,s IgM, CD200, kappa/Lambda restriction). For All included patients both clinical (age, sex, Rai staging) as well as laboratory prognostic markers (Lymphocytes doubling time, ZAP70%, CD38%, Beta-2 microglobulin, Serum LDH, FISH cytogenetic findings for 17pdel, 11qdel, 13qdel, Trisomy 12). Time to first treatment (time from diagnosis till the patients required therapy) was recorded for every patient included in this study.

Plasma RNA extraction from CLL patients

From each CLL patient included in this study 5 ml EDTA blood sample was obtained at time of the diagnosis. Fresh plasma samles were separated by centrifugation at 3,000 rpm/ minute for 10 minutes. The separated patients plasma samples were stored in aliquots at -80°C. RNAs extraction were obtained using the miRNA isolation kits (Qiagen, Inc). The quality of miRNA was assessed by Nano drop (Thermo Fisher Scientific, Inc., USA) at wave length 230, 260, and 280; The ratio of 260/230 equal 1.8-2.1 which pointed for high purity of RNA

Complementary DNA (cDNA) Synthesis

According to the manufacturer's instructions, cDNA was synthesized using a Taqman microRNA Reverse Transcriptase(RT) kit (Applied Biosystems, USA). In brief, for synthesis of cDNA we use 2~20 ng of total RNA. The RT reaction mixture contained 0.15 μL of 100 mM dNTP mix (100 mM each dATP, dGTP, dCTP, and dTTP at a neutral pH), 1 μL of 50 U/μL RT, 1.5 μL of 10x RT buffer, 0.19 μL of 20 U/μL RNase inhibitor, and the total volume of the reaction was adjusted to 15 μL with nuclease-free water. The run program for cDNA synthesis experiment was done as follows: 16°C for 30 min then 42°C for 30 min, and 85°C for 5 min. RT reactions were performed using a 96 test thermal cycler (Arkitik, Thermo Scientific, USA).

miRNA analysis using qPCR

According to manufacturer instruction, TaqMan miRNA small RNA assay (Applied Biosystems, USA) was used to detect and quantify miRNA expression using miRNA-specific primers. In brief, 2 μL of cDNA was added to 10 μL of probe qPCR mix and 8 μL of nuclease water. The following TaqMan small RNA assay (Applied Biosystems, USA) primers were used: hsa-*miR-29*a and hsa-miR-16-5p. All analyzed miRNAs are of human origin. qPCR reactions were performed using DT prime 4 real time PCR (DNA Technology, Russia). The experiments were done in duplicate. Data analysis were done by using the 2-delta Ct method . The has-miR-16-5p, was used as an endogenous control.

Statistical analysis

Statistical analysis was performed using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.)was used for statistical analysis. The quantitative data are presented as median and range in non-parametric parameters, or mean and SD in parametric parameters, while non-quantitative data was presented as percentage. Comparing miRNA expression results in CLL patients and controls was done using Mann-Whitney-U test. Correlations studies between continuous data were done by Spearman rank test. In order to compare clinical as well as demographic data we used the χ^2 and Fisher's exact tests. The optimal cut off value that predict shorter time to the first treatment (TTFT) was identified by ROC curve. COX regression analysis was applied for prediction of TTFT. Kaplan-Meier curve was used to address the impact of miR-29a expression levels on TTFT in CLL patients. The statistical significant level was considered when p value less than 0.05.

Results

CLL patients demographic Parameters

This study was carried on 158 patients beside 21 healthy controls. Individuals in the study were 100males (63.3%)and 58 females (36.7%) aged (61.39 ± 10.07). Rai staging was diagnosed as stage 1, stage 2 and stage 3 (44(27.8), 74(46.8%) and 40 (25.4%) respectively). Bone marrow lymphocytes presented in the table1. The percentage of ZAP70+, CD38+, high ß2- microglobulin were recorded in (70 (44.3%), 53 (33.5%) and 57 (36.1%) respectively in CLL patients. The most frequent cytogenetic finding was 13qdel (found in 85 (53.8%) of patients). Median WBCs, smudge cell, LDH, *miR-29*a and TTFT are presented in the Table 1. *miR-29*a expression in CLL patients versus controls

The median and range of *miR-29*a expression in CLL patients[0.76 (0.10-3.3)] versus control group. It is evident that the expression of *miR-29*a was significantly higher in CLL patients as compared to controls[0.26 (0.0-0.75)] (P<0.001) (Figure 1).

Expression pattern of miR-29a in CLL patients as compared to healthy subjects

The CLL patients group was subdivided into two subgroups based on miRNA levels (*miR-29*a <0.76 vs >0.76). Comparing patient's characteristics and laboratory parameters among CLL subgroups revealed that WBCS, smudge, LDH were significantly elevated in *miR-29*a >0.76 group compared to *miR-29*a <0.76 group. On the other hand TTFT was significant reduced in *miR-29*a >0.76 group compared to *miR-29*a <0.76 group. There was significant increase in the frequency of ZAP70+, CD38+ and high ß2- microglobulin in subgroup of CLL patients have *miR-29*a >0.76 group compared to those with subgroup of patients with miRNA29a <0.76 . There was significant difference as regard Rai staging, pattern of BM lymph, and FISH findings among groups (Table 2).

In order to address the optimal cut off value that could predict shorter TTFT, we used ROC analysis. The results

Table 1. Baseline Clincopathological Data of Studied CLL Patients

Parameter		Results
Mean age		61.39 ± 10.07
Gender	Male	100 (63.3%)
	Female	58 (36.7%)
WBCs ($\times 10^9$ /L) at diagnosis, median (range)		74.0 (32.0-320.0)
Smudge cell (%), median (range)		7.0 (1-30)
LDH (IU/L) at diagnosis, median (range)		334 (120-4200)
Rai staging	1	44 (27.8%)
	2	74 (46.8%)
	3	40 (25.4%)
ZAP70%	Negative	88 (55.7%)
	Positive	70 (44.3%)
CD38%	Negative	105 (66.5%)
	Positive	53 (33.5%)
ß2- microglobulin (mg/L)	Negative	101 (63.9%)
	Positive	57 (36.1%)
FISH	Normal	31 (19.6%)
Cytogenetic Findings, no (%)	17p del	11 (7.0%)
	11q del	15 (9.5%)
	Trisomy 12	16 (10.1%)
	13q del	85 (53.8%)
Median time to first treatment (TTFT) (/month)		15.0 (1-25)
Median miR-29a		0.76 (0.1-3.3)

indicated that the best discriminating *miR-29a* expression value was 0.79. The area under the curve (AUC) was 0.742 (p=<0.001) (Figure 2).

Cox regression analysis was carried out in order to identify factors that could predict time to the first treatment. This was done using age, gender, WBCs, smudge cells percentage, LDH, *miR-29a*, ZAP70, CD38, ß2- microglobulin, Rai staging as covariates. In multivariate analysis, miRNA29a, positive CD38, Beta2 microglobulin and high Rai staging were significant risk factors for shorter TTFT (Table 3).

Survival analysis

Using Kaplan Meier curve, we have evaluated the impact of *miR-29a* expression levels on TTFT in CLL patients. The analysis revealed that CLL patients with high miRNA 29a (>0.79) expression showed shorter TTFT as compared to those with low miRNA 29a expression (< 0.79) (P <0.001) (Figure 3).

Discussion

miRNAs play various roles in pathogenesis and evolution of cancer as well as initiation, tumor growth, and metastasis. Moreover, miRNAs may be used as diagnostic biomarker and as therapeutic target An optimal biomarker must have several characteristics (Califf, 2018): allow early disease diagnosis, accurate minimal residual disease identification, and to be specific to disease , and non-invasive. Previous studies indicated that *miR-29a* can act as either oncogene or tumor suppressor gene. The expression patterns of *miR-29a* in CLL patients is controversial (Jiang et al., 2014; Anelli et al., 2021).

In this study our findings revealed that *miR-29a* was significantly increased in CLL patients as compared to healthy controls. This finding is consistent with that reported by Raeisi et al., (2020) and Li et al (2011). In contrast, Calin et al., (2005) found that *miR-29a* was down regulated in CLL patients. In a previous study, miR-29c was reported to be elevated in AML patients and was found to be correlated with a higher frequency of disease relapse (Butrym et al., 2016).

A recent report found that all miR-29 family members were consistently decreased in the CLL microenvironment and correlated in a significant manner with shorter overall
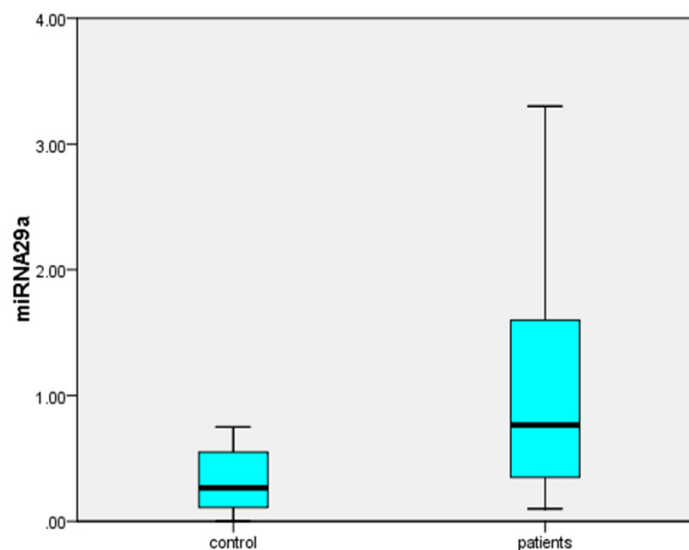

Figure 1. The Median and Range of miR-29a Expression in CLL Patients[0.76 (0.10-3.3)] versus Control Group. It is evident that the expression of miR-29a was significantly higher in CLL patients as compared to controls[0.26 (0.0-0.75)] (P<0.001).

Table 2. Comparison of Patient's Characteristics and Laboratory Parameters among Studied Groups

Parameter		miR-29a <0.76 (n=79)	miR-29a >0.76 (n=79)	P value
Age (years)	Mean ± SD	62.5 ± 9.7	60.4 ± 10.3	0.192
Gender	Male	56 (70.9%)	52 (65.8%)	0.494
	Female	23 (29.1%)	27 (34.2%)	
WBCs (×10^9 /L)	Median (Min-Max)	65.0 (32-115)	80.0 (54-320)	<0.001
Smudge cells (%)	Median (Min-Max)	6.0 (1-16)	12.0 (3-30)	<0.001
LDH (IU/ml)	Median (Min-Max)	276.0 (120-820)	600.0 (134-4200)	<0.001
miRNA29a	Median (Min-Max)	560.0 (36-2800)	880.0 (50-5200)	<0.001
Rai stages	1	25 (31.6%)	19 (24.1%)	<0.001
	2	53 (67.1%)	21 (26.6%)	
	3	1 (1.3%)	39 (49.3%)	
ZAP70 (%)	Negative	53 (67.1%)	35 (44.3%)	0.004
	Positive	26 (32.9%)	44 (55.7%)	
CD38 %	Negative	68 (86.1%)	37 (46.8%)	<0.001
	Positive	11 (13.9%)	42 (53.2%)	
ß2- microglobulin (mg/L)	Negative	64 (81.0%)	37 (46.8%)	<0.001
	Positive	15 (19.0%)	42 (53.2%)	
Cytogenetic findings	Normal	16 (20.3%)	15 (19.0%)	<0.001
	17pdel	0 (0.0%)	11 (13.9%)	
	11qdel	2 (2.5%)	13 (16.5%)	
	Trisomy 12	0 (0.0%)	16 (20.3%)	
	13qdel	61 (77.2%)	24 (30.4%)	
TTFT (months)	Median (Min-Max)	18.0 (2-25)	3.0 (1-24)	<0.001
CLL outcome	Live	79 (100.0%)	52 (65.8%)	<0.001
	Dead	0 (0.0%)	27 (34.2%)	

survival of CLL patients, they attributed this to B cell receptor activation. Moreover, in this study the authors demonstrated that Tumor-Necrosis Factor Receptor-Associated Factor 4 (TRAF4) as a new direct target of miR-29a, and increased TRAF4 levels lead to NFkB activation in downstream signaling pathway (Sharma et
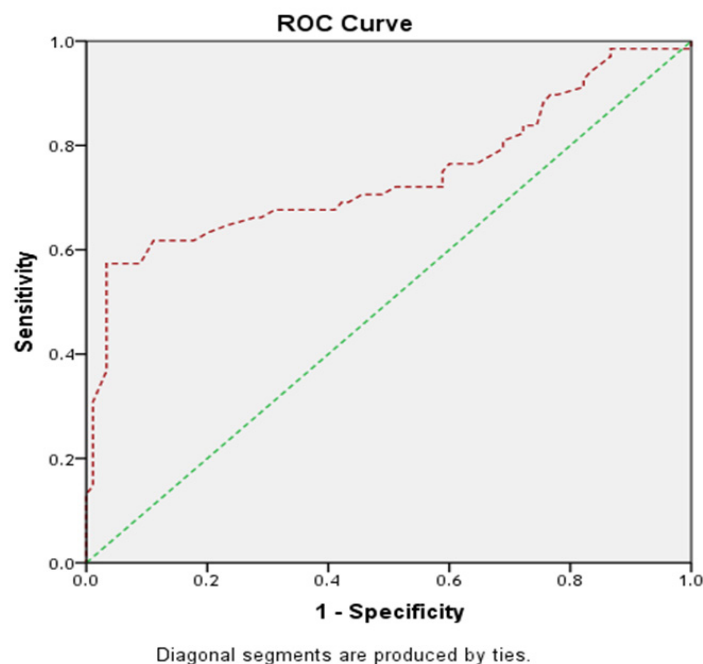

Figure 2. ROC Analysis was Conducted to Identify the Optimal Cut off Levels for Prediction of Shorter TTFT. miR-29a best cut-off value was 0.79. The area under the curve (AUC) was 0.742 (p=<0.001).

Table 3. COX Regression Analysis for Prediction of TTFT in Studied CLL Patients

		Univariate analysis				Multivariate analysis		
		P	HR	95% CI		p	HR	95% CI	
Age (years)		0.032	0.975	0.952	0.988	0.966	0.999	0.972	1.028
Gender		0.325	1.281	0.782	2.097				
WBCs ($\times 10^9$ /L)		<0.001	1.015	1.011	1.019	0.595	1.002	0.995	1.009
Smudge cells%		<0.001	1.145	1.108	1.184	0.876	0.995	0.936	1.058
LDH(U/L)		<0.001	1.002	1.001	1.003	0.817	1.001	0.999	1.002
miR-29a expression		<0.001	2.957	2.318	3.773	0.037	1.525	1.026	2.266
ZAP70+		<0.001	5.806	3.36	10.03	0.593	1.255	0.546	2.882
CD38+		<0.001	17.471	9.759	31.27	<0.001	6.622	2.554	17.164
ß2- microglobulin (mg/L)		<0.001	10.41	6.00	18.07	0.032	2.419	1.079	5.423
Rai staging	2 vs 1	0.773	0.894	0.419	1.909				
	3 vs 1	<0.001	21.023	14.395	35.576	0.019	18.846	1.627	21.251

HR, hazard ratio; CI, confidence interval

al., 2020).

High *miR-29a* expression was significantly associated with CLL poor prognostic markers namely high WBCs count, low smudge cells count, high LDH levels , short TTFT, ZAP70+, CD38+ and high ß2-microglobulin, and advanced Rai stage. These findings could be attributed to significant association between up-regulation of miRNA29a and p53 deletions (Grenda et al., 2022).

ROC analysis was conducted to identify the optimal Cut off levels for prediction of shorter TTFT. The analysis revealed that the best cutoff value for *miR-29a* was 0.79. The area under the curve (AUC) was 0.742 (p<0.001). Furthermore, Cox regression analysis was conducted to address the independent factor that could predict CLL patients TTFT, using age, gender, WBCs count, smudge cells count, LDH concentration levels, miR29a expression, Positive ZAP70%, CD38%, ß2- macroglobulin, Rai staging as covariates. In multivariate analysis, miRNA29a, positive CD38, ß2-microglobulin and high grade Rai stage were significant risk factors for shorter TTFT. Previous report stated that there is significant association between *miR-29a* expression levels and p53 deletion in CLL patients (Grenda et al., 2022). Moreover, a recent report by Sharma et al., (2020) showed that a miRNA- miR-29 dependent mechanism acts to activate CD40 signaling/T-cell interactions in a CLL microenvironment and described a novel miR-29–TRAF4–CD40 signaling axis modulated by B-cell receptor activity.

In conclusion, Up regulation of *miR-29a* expression at CLL diagnosis was detected. Determination of *miR-29a* expression levels at diagnosis could be demonstrated as a prognostic biomarker in CLL.
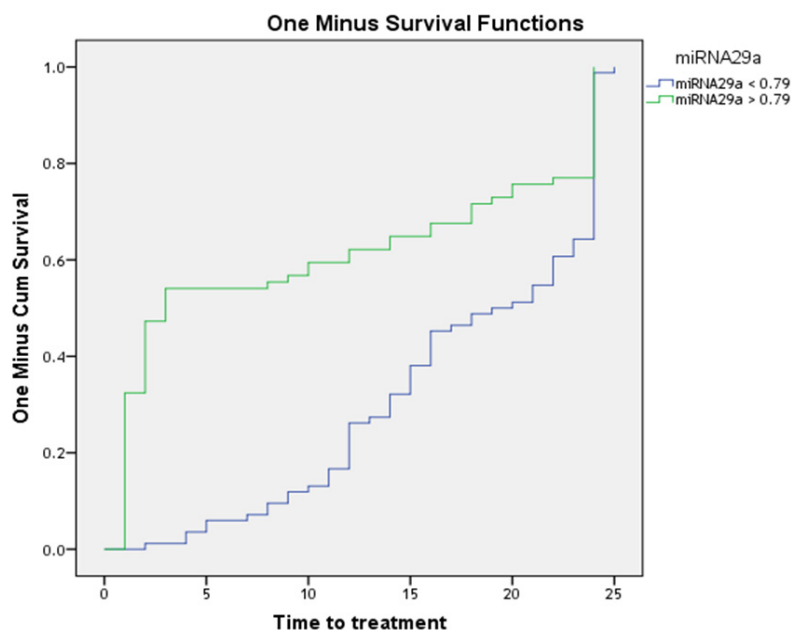

Figure 3. Impact of miR-29a Expression Levels on TTFT in CLL Patients. CLL patients with high miR-29a expression showed shorter TTFT as compared to those with low miR-29a expression .

Author Contribution Statement

Acknowledgements

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