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Mohammad Masoud Eslami
Department of Hematology, School of Medicine, Tarbiat Modares University (TMU), Tehran, Iran.

Mina Soufizomorrod
Department of Hematology Applied Cell Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, m.soufi@modares.ac.ir

Mohammad Ahmadvand
Hematology-Oncology and Stem Cell Transplantation Research Center, Tehran University of Medical Sciences, Tehran, Iran

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REASERCH ARTICLE

High Expression of Long Noncoding RNA NORAD is Associated With Poor Clinical Outcomes in Non-M3 Acute Myeloid Leukemia Patients

Mohammad Masoud Eslami [a], Mina Soufizomorrod [b,*], Mohammad Ahmadvand [c]

[a] Department of Hematology, School of Medicine, Tarbiat Modares University (TMU), Tehran, Iran.
[b] Department of Hematology Applied Cell Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[c] Hematology-Oncology and Stem Cell Transplantation Research Center, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Objective/Background: **Dysregulation of long noncoding RNA NORAD has been identified in human solid tumors. However, the expression profile of NORAD and its clinical implications in acute myeloid leukemia (AML) is unclear. The current study aimed to explore the NORAD expression status and its clinical significance in non-M3 AML patients.**

Methods: **NORAD expression was evaluated in 60 de novo non-M3 AML patients and 49 healthy individuals using quantitative reverse transcription−polymerase chain reaction method. The correlation between NORAD transcription levels and clinicopathologic characteristics was statistically studied.**

Results: **Compared with the healthy controls, NORAD was consistently higher in non-M3 AML patients ($p = .01$). Furthermore, initial NORAD upregulation occurred more frequently in patients with unfavorable cytogenetic risk ($p = .02$). The non-M3 AML patients were divided into NORAD high-expressing (NORAD^{high}) and NORAD low-expressing (NORAD^{low}) groups based on the median NORAD expression level. Univariate analyses revealed that patients with high expression levels of NORAD had relatively poor overall survival ($p = .03$) and relapse-free survival (RFS) ($p = .01$). Additionally, multivariate analysis highlighted that NORAD upregulation was an independent risk factor for RFS.**

Conclusion: **Our observations indicate the fact that high expression of NORAD could be an unfavorable risk factor in non-M3 AML patients, and NORAD might be a novel therapeutic candidate for future treatments targeting AML.**

Keywords: Acute myeloid leukemia, Long noncoding RNA, NORAD, Prognosis

1. Introduction

Acute myeloid leukemia (AML) is the most frequent hematologic malignancy described by the clonal growth and accumulation of malignant myeloid blast cells in peripheral blood and bone marrow (BM) [1]. It has become apparent that specific cytogenetic abnormalities, acquired gene mutations, and aberrant gene expression can partly explain the pathogenesis of AML [2]. Karyotype abnormalities found out at diagnosis are the most valuable prognostic factors in predicting the clinical outcomes of the patients [3]. Additionally, dysregulation in the expression of particular genes and several gene mutations have been used for classification and prognostic evaluation of the patients [4,5]. However, accurate prediction of clinical outcomes and chemoresponsiveness of leukemia patients is still difficult, and thus, in order to improve risk assessments and treatment planning process, discovering new diagnostic and prognostic biomarkers are of utmost importance.

Among several types of noncoding RNA (ncRNA), long noncoding RNAs (lncRNAs) are defined as

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* Corresponding author at: Department of Hematology and Blood Transfusion, School of Medicine, Tarbiat modares university (TMU), North Kargar Av, Tehran 14117, Iran.
E-mail address: m.soufi@modares.ac.ir (M. Soufizomorrod).

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transcripts longer than 200 nucleotides without any protein-coding potential. These transcripts could play pivotal roles in every step of cell biological processes such as epigenetic modification, imprinting, cell cycle progression, differentiation, and apoptosis [6]. It had been previously announced that lncRNAs had crucial functions through tumor progression in various types of human malignancies. Furthermore, recent studies hypothesized that lncRNAs could serve as promising diagnosis, prognosis, and therapeutic targets in specific cancers [7–9]. Accordingly, recent evidence indicated the role of the lncRNAs in myeloid differentiation and contribution in regulation of AML-related pathways [10].

Noncoding RNA activated by DNA damage (NORAD, also known as LINC00657) is a novel conserved lncRNA comprising one exon and located on chromosome 20 with a length of 5.3 kb. NORAD is abundantly expressed in mammals with roughly 500–1,000 copies per cell. In the past decade, It has been recently identified that loss of NORAD function results in genome instability by raising PUMILIO (PUM) proteins in the cytoplasm, which have a potential repression function in the process of DNA damage repair and replication [11,12]. In this regard, Munschauer et al. [13] demonstrated that depletion of NORAD or RBMX (RNA-binding motif protein X-linked) causes genomic instability and aneuploidy, while these effects are saved by wild-type NORAD expression. Moreover, dysregulation of NORAD has been elucidated in various types of cancers [14–16]. However, the expression pattern and underlying mechanism of NORAD in AML patients have not been clarified and its clinical relevance is still unknown. The present study investigated the correlation between NORAD expression levels and various clinicopathologic features along with prognosis in non-M3 AML patients. The results may reveal further roles and the clinical implications of NORAD in AML progression.

2. Materials and methods

2.1. Patients' sample

Between September 2018 and February 2021, 60 non-M3 AML patients were included in this study: 29 men and 31 women ranging from 14 to 76 years, with a median age of 42 years at diagnosis. All the de novo non-M3 AML patients were recruited from Hematology/Oncology and Stem Cell Transplantation Research Center, Shariati Hospital, Tehran, Iran. French–America–British (FAB) and World Health Organization systems were used to diagnose and classify AML patients [17,18]. Non-M3 AML patients received standard induction chemotherapy consisting of daunorubicin (60 mg/m^2 daily for 3 days) plus cytarabine (100 mg/m^2 daily for 7 days). Patients who achieved complete remission (CR) received at least one course of 5 + 2 consolidation chemotherapy regimen (100 mg/m^2 cytarabine daily for 5 days plus 60 mg/m^2 daunorubicin daily for 2 days). After the first remission, seven patients experienced allogeneic hematopoietic stem cell transplantation (HSCT). Written informed consent was collected from all individuals. The study has been approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. Clinical characteristics of the non-M3 AML patients are summarized in Table 1. The control group comprised 49 healthy volunteers: 20 men and 29 women ranging from 22 to 74 years, with a median age of 40 years.

2.2. Specimen collection

BM samples were collected from non-M3 AML patients into tubes containing EDTA anticoagulant at diagnosis. BM mononuclear cells were immediately separated by density gradient centrifugation on Ficoll-Paque (GE Healthcare, New York, USA), according to the manufacturer's instruction. Whole-cell pellets were suspended twice with phosphate-buffered saline (pH = 7.4, 0.15 M). Ultimately, newly diagnosed non-M3 AML samples containing 80% or more blast cells were immediately lysed in TriPure Isolation Reagent (Roche Diagnostics, Germany) TriPure Isolation Reagent based on the manufacturer's protocol (Roche Diagnostics, Germany). Yekta Tajhiz, Tehran, Iran Reverse Transcription Kit SYBR Green methodology (Ampliqon, Denmark).

2.3. Molecular cytogenetic analysis

Fresh specimens were directly cultured and harvested following conventional cytogenetic approach. G-banding and fluorescent in situ hybridization techniques were performed to indicate all cytogenetic abnormalities in accordance with the laboratory's validated procedure. Chromosomal disorders were described according to the International System of Human Cytogenetic Nomenclature (ISCN 2013) recommendations [19], and cytogenetic risk statuses were classified based on the current National Comprehensive Cancer Network AML guidelines [20].

Table 1. Clinicopathologic Parameters of 60 Non-M3 Acute Myeloid Leukemia Patients and Expression Status of NORAD.

Patients' parameters	High NORAD expression (n = 30)	Low NORAD expression (n = 30)	p
Sex, male/female	13/17	16/14	0.43
Median age, years (range)	44.50 (14–70)	41 (18–76)	0.62
Median WBC, $\times 10^9$/L (range)	40 (2.91–339.84)	24.49 (1.16–268.88)	0.06
Median hemoglobin, g/L (range)	80 (39–127)	80 (39–150)	0.72
Median platelets, ×109/L (range)	45 (6–309)	36 (6–152)	0.23
BM blasts, % (range)	81 (19–95)	67.5 (20–95)	0.1
FAB, n (%)			
M0	2 (6.6)	0	0.18
M1	5 (16.7)	5 (16.6)	
M2	9 (30)	6 (20)	
M4	10 (33.4)	12 (40)	
M5	2 (6.6)	7 (23.4)	
M6	2 (6.6)	0	
Karyotype classification, n (%)			
Favorable	2(6.6)	0	
Intermediate	13(43.4)	23 (76.7)	0.02
Unfavorable	15(50)	7 (23.3)	
Gene mutation			
NPM1 (+/−)	5/25	2/28	0.22
FLT3-ITD (+/−)	13/17	12/18	0.79
Response to treatment, n (%)			
CR	20 (66.7)	22 (73.3)	0.57
NR	10 (33.3)	8 (26.7)	

Note. Data are presented as range or n (%). BM = Bone marrow; CR = complete response; FAB = French–American–British; NR = no response; WBC = white blood cell.

2.4. RNA purification and cDNA synthesis

Total RNA was extracted from BM sample using TriPure Isolation Reagent based on the manufacturer's protocol (Roche Diagnostics). RNA quantity and quality were assessed using a NanoDrop spectrophotometer. To verify the integrity of total RNA, 5 μL of RNA was run on 1.5% agarose gel to observe 18S and 28S subunits of ribosomal RNA. cDNA synthesis from 1,000 ng of isolated total RNA was performed using the Yekta Tajhiz, Iran Reverse Transcription Kit and stored at −20 °C for future use.

2.5. Quantitative reverse transcription–polymerase chain reaction

The transcription level of NORAD was assessed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) in a StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Green methodology (Ampliqon) under following situations: an initial denaturation step at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. At the end of all runs, melting curve analysis was performed to make sure about the specificity of the primers. The standard curve slope was plotted utilizing a dilution series (5-fold dilutions) of cDNA samples to estimate qPCR efficiency. To normalize the NORAD expression levels, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The primers were as follows: NORAD forward primer: 5′-GCAGACTATTCATTGGGTGTTTGG-3′, NORAD reverse primer: 5′-AACTAATCTCAGGAGCACA-GAACC-3′, GAPDH forward primer: 5′-ACAGCCT-CAAGATCATCAGCAATG-3′ and GAPDH reverse primer: 5′-GCCATCACGCCACAGTTTCC-3′. Relative quantification of NORAD expression levels was calculated by $2^{-\Delta CT}$ method.

2.6. Gene mutation detection

High-resolution melting analysis was conducted to detect NPM1 exon 12 and FLT3/ITD (internal tandem duplications) mutations in all AML patients, as previously described [21]. Amplification and melting analysis of aforementioned mutations were performed using Light Cycler 96 (Roche Diagnostics). Confirmation of mutations was made by capillary electrophoresis–based fragment analysis. All samples were successfully analyzed for NPM1 and FLT3/ITD mutations.

2.7. Statistical analysis

SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8.0

(GraphPad Software Inc., San Diego, CA, USA) were used to perform all statistical analyses. Pearson chi-square analysis or Fisher exact test was employed to measure the association between two categorical variables.

The normality of all continuous variables was examined by the Kolmogorov–Smirnov test. Mann–Whitney *U* test was applied to compare two independent groups with non-normally distributed variables. Spearman rank correlation coefficient was used to analyze correlations between NORAD expression levels and white blood cell (WBC), hemoglobin (Hb), platelets (Plt), and BM blasts. Whether NORAD transcript levels could discriminate non-M3 AML patients from healthy controls was ascertained by receiver operating characteristic curve (ROC) and area under the ROC curve (AUC). The overall survival (OS) was measured from the date of first diagnosis to the date of censoring due to any cause, and relapse-free survival (RFS) was defined as the duration from the date of CR after induction therapy to the date of relapse or death of any cause. Patients undergoing first-line allogeneic HSCT were censored after primary stem cell infusion. Relapse was considered as a re-infiltration of the BM with $\geq$ 5% leukemic blasts or new intramedullary myeloid masses. Definition of CR was according to the European Leukemia Net guidelines [22], and patients who declined to receive CR criteria were named as nonresponse. Subsequently, OS and disease-free survival were plotted by Kaplan–Meier survival curves, and the correlations between the risk factor and prognosis were calculated through log-rank test. To further elucidate the effect of NORAD expression, a Cox proportional hazards model was constructed, with adjusting for other potential confounding covariates. For all analyses, two-tailed p values < 0.05 were considered as the cutoff for significance.

3. Results

3.1. NORAD transcript level was increased in non-M3 AML patients

qRT-PCR was employed to evaluate the expression level of NORAD in BM samples obtained from non-M3 AML patients before induction therapy and healthy controls. As shown in Fig. 1, NORAD transcript levels was significantly upregulated in non-M3 AML patients (median expression value 0.01, range: 0.00045–0.47) compared with healthy controls (median expression value 0.0052, range: 0.00017–0.31; $p = .01$).

In addition, no statistically significant correlation was observed between NORAD expression and WBC, Hb, Plt, and BM blasts.

3.2. NORAD expression as a diagnostic marker of non-M3 AML

The ROC curve analysis was employed to evaluate whether NORAD expression could discriminate non-M3 AML patients from healthy controls. The AUC for NORAD in the diagnosis of patients versus controls was 0.64, and it had an average diagnostic value with 65% sensitivity and 63% specificity (Fig. 2).

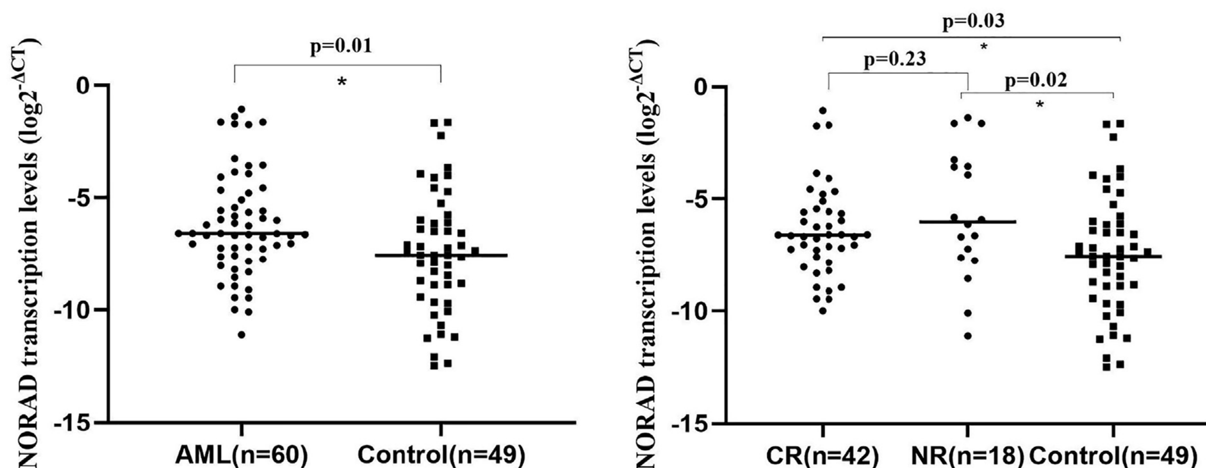

*Fig. 1. NORAD expression in non-M3 AML patients and healthy individuals detected by qRT-PCR. (A) Transcription levels of NORAD between non-M3 AML patients and healthy controls. Each symbol represents a single subject; horizontal lines indicate median values. Independent t test and the associated p value is indicated. (B) Non-M3 AML patients with refractory response to chemotherapies had no higher initial expression levels of NORAD compared with those who achieved CR. Note. AML = acute myeloid leukemia; CR = complete remission; NR = nonresponse; qRT-PCR = quantitative real–time polymerase chain reaction. *p< .05 was considered statistically significant.*

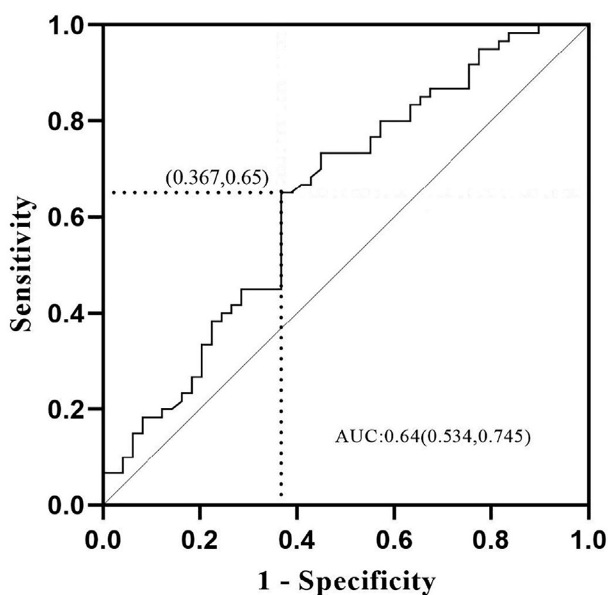

Fig. 2. Discriminative capacity of NORAD expression by receiver operating characteristic curve analysis. Note. AUC = area under the curve.

3.3. Correlation between NORAD expression and clinical/laboratory characteristics of non-M3 AML patients

Based on median level of NORAD expression, non-M3 AML patients were stratified into NORAD high-expressing ($NORAD^{high}$) and NORAD low-expressing ($NORAD^{low}$) groups. The relationships between NORAD expression and clinical characteristics are shown in Table 1. Differences were not deemed statistically significant between $NORAD^{high}$ and $NORAD^{low}$ patients according to the age, sex, blast percentage, peripheral WBCs, Plt count, Hb level, FAB subclassification, NPM1 and FLT3 gene mutations, and the rates of CR after induction therapy ($p > .05$). However, a significant difference was recognized between these two groups of patients and karyotype findings ($p = .02$). Patients in $NORAD^{high}$ group had a higher frequency of unfavorable cytogenetic (50%, 15/30) than patients in $NORAD^{low}$ group (23.3%, 7/30). Besides, Mann–Whitney *U* test indicated that chemotherapy-refractory patients had no statistically significant higher expression of NORAD before treatment ($p = .23$; Fig. 1).

3.4. Association of NORAD expression levels and clinical results of non-M3 AML patients

We performed survival analysis to assess the prognostic impact of NORAD high expression in non-M3 AML patients. The median follow-up time was 9.64 months ranging from 1 month to 16.16 months. Kaplan–Meier survival curves for OS and RFS were classified according to NORAD levels in non-M3 AML patients. The results indicated that patients with elevated expression of NORAD were associated with shorter OS and RFS than those with low expression of NORAD levels ($p = .038$ and $p = .013$, respectively; Fig. 3A and 3B). Patients with high and low NORAD expression levels had median survival times of 4.93 months and 12.5 months, respectively. Regretfully, patients with high NORAD expression levels did not show a significantly shorter OS than patients with low NORAD expression levels among intermediate or favorable cytogenetic subgroup ($p = .28$; Fig. 3C). Subsequently, the multivariate Cox regression analysis identified that there is insufficient evidence to conclude that NORAD was an independent risk factor for OS (hazard ratio [HR] = 2.14; 95% CI = 0.98–4.63; $p = .048$), since the 95% CI includes the null value of 1. High NORAD expression was an independent risk factor for RFS (HR = 2.35; 95% CI = 1.09–5.06; $p = .029$) in non-M3 AML patients. Statistical values stemmed from Cox proportional hazards regression model for NORAD expression, and other clinical risk factors are listed in Table 2.

4. Discussion

It has become evident that lncRNAs are important regulators of gene expression during blood cell formation. Thus, their aberrant expression might block differentiation and proliferation, leading to hematologic malignancies [23]. Also, some of these transcripts have provided diagnostic and prognostic value in hematopoietic cancer and have gained more attention in leukemia, particularly in AML [24]. However, specific expression patterns of some lncRNAs in AML patients and their correlation with clinicopathologic characteristics are not comprehensively understood.

NORAD is a highly conserved lncRNA necessary for maintaining chromosomal stability and proper mitotic divisions by PUM sequestration and interacts with RBMX proteins in human cells [25]. Besides, a vast majority of studies demonstrated that NORAD could regulate microRNAs (miRNAs) through a competitive endogenous RNA (ceRNA) mechanism [25,26]. In this regard, Li et al. [14] reported that NORAD regulates the expression of the small GTP-binding protein RhoA through direct binding to miR-125a-3p, thereby promoting metastasis. Yang et al. [27] found that knockdown of NORAD could yield tumor-suppressing functions in

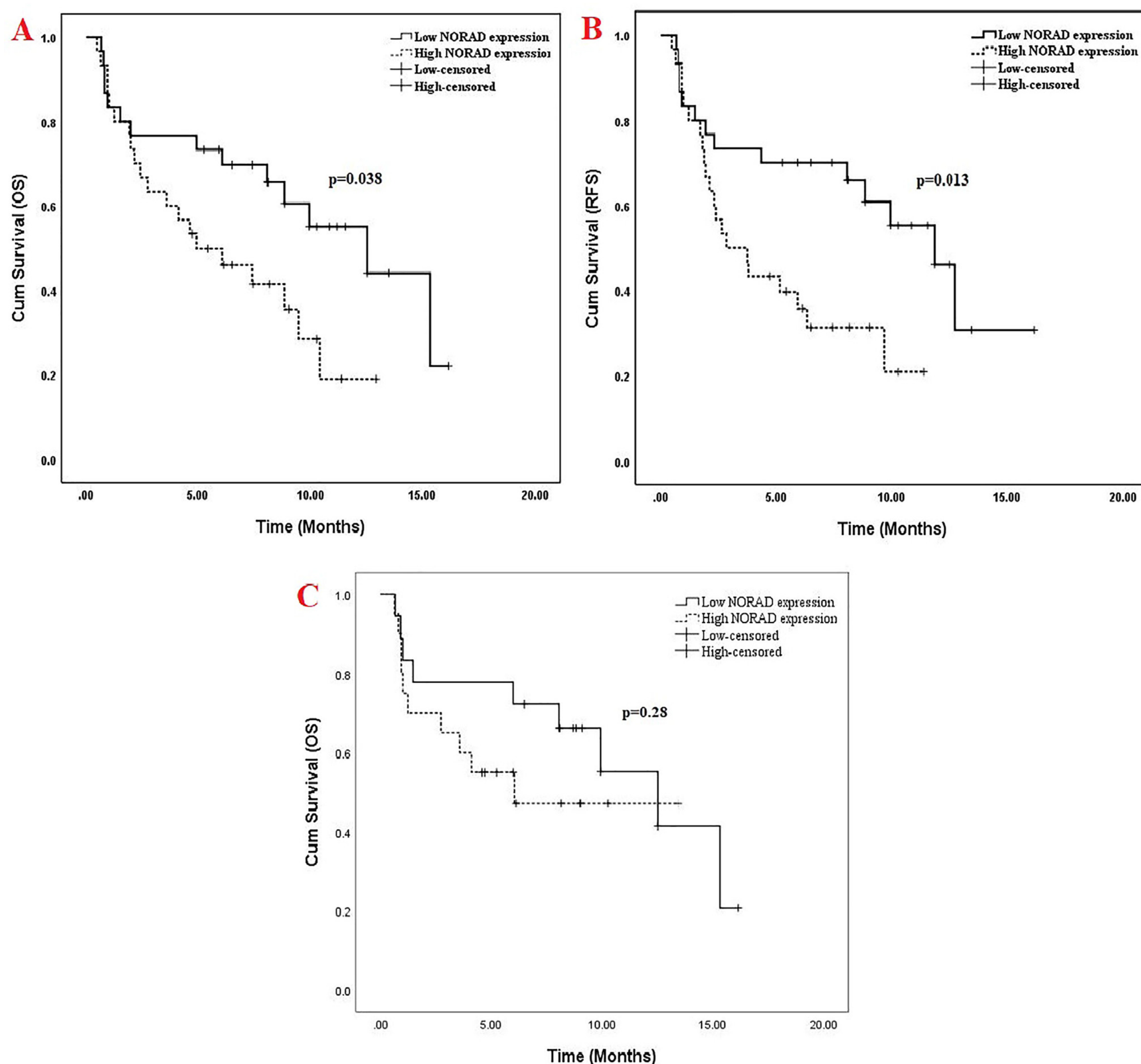

Fig. 3. Kaplan–Meier survival curves according to NORAD transcription levels (A and B) for non-M3 AML patients and (C) for intermediate or favorable cytogenetic subgroup. Patients with high NORAD expression levels had shorter OS and RFS than those with low NORAD expression levels ($p = .038$ and 0.013, respectively). Note. AML = Acute myeloid leukemia; OS = overall survival; RFS = relapse-free survival.

epithelial retinoblastoma through endogenous competition against miR-136-5p. In addition, Chen et al. [28] also observed that NORAD acted as a ceRNA for miR-495-3p to modify TRIP13 expression and play a crucial oncogenic role in prostate cancer patients. The regulatory role of microRNAs, including miR-125a-3p, miR-136-5p, and miR-495-3p, was discovered by functional studies in hematologic malignancies, especially in AML [29–32]. These observations suggest that NORAD lncRNA could play a pivotal role in the modulation of AML progression and may present a novel prognostic or progression marker. However, the expression pattern and prognostic role of NORAD in AML remain unknown.

In order to explore the effects of altered NORAD expression on clinical outcomes and prognosis of non-M3 AML patients, we studied the expression level of NORAD in BM samples. ROC analysis revealed that NORAD transcription levels statistically differentiate non-M3 AML patients from the healthy controls with 65% sensitivity and 63%

Table 2. Univariate and Multivariate Analyses of Variables for OS and RFS in Non-M3 AML.

Factors	OS				RFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
NORAD expression	2.08 (1.02–4.24)	.043	2.14 (0.98–4.63)	.048	2.44 (1.18–5.06)	.016	2.35 (1.09–5.06)	.029
High	Ref.		Ref.		Ref.		Ref.	
Low								
Age	1.82 (0.92–3.62)	.085	1.42 (0.68–2.96)	.34	1.61 (0.82–3.16)	.16	1.16 (0.56–2.40)	.67
Above median	Ref.		Ref.		Ref.		Ref.	
Below median								
Cytogenetic risk group	1.84 (0.96–3.50)	.05	2.25 (1.10–5.08)	.03	1.49 (0.80–2.76)	.20	1.65 (0.86–3.19)	.13
Adverse risk	Ref.		Ref.		Ref.		Ref.	
Favorable and Intermediate risk								
FLT3	2.57 (1.28–5.14)	.007	2.54 (1.27–5.10)	.008	2.49 (1.25–4.92)	.009	2.46 (1.24–4.86)	.01
Mutated	Ref.		Ref.		Ref.		Ref.	
Normal								
NPM1	1.47 (0.50–4.29)	.47	*	*	1.31 (0.45–3.77)	.61	*	*
Mutated	Ref.				Ref.			
Normal								

Note. * = was not calculated; AML = acute myeloid leukemia; CI = confidence interval; HR = hazard ratio; OS = overall survival; Ref. = reference; RFS = relapse-free survival.

specificity, but may not be very applicable in clinical practice. However, NORAD expression levels were not significantly different in patients with diverse FAB classifications. Patients with adverse-risk cytogenetic represent a higher expression of NORAD than moderate- and favorable-risk groups, indicating that NORAD may contribute to the development of AML. In addition, we noticed that elevated expression of NORAD correlates with shorter OS and RFS in univariate analysis. Although, a higher frequency of adverse-risk cytogenetic patients in the high NORAD expression group may be a determinant factor in survival analysis results. Multivariate analysis confirmed that NORAD expression was an independent predicting factor for RFS in non-M3 AML patients.

NORAD function in various malignancies remains controversial. For example, Liu et al. [33] discovered that NORAD was remarkably upregulated in breast cancer cell lines compared with nonmalignant tissues and connected with poor OS. Knockdown of NORAD suppressed breast cancer cell proliferation and tumor size in vitro. By contrast, Hu et al. [34] revealed that NORAD expression was downregulated in clinical specimens and cultured cell lines of hepatocellular carcinoma. Functional assays demonstrated that NORAD could significantly inhibit cell proliferation, migration, and invasion of hepatocellular carcinoma cells, both in vitro and in vivo. From this context, NORAD may contribute to many human malignancies in a cancer-type-dependent manner.

Taken together, the results indicated that higher expression of NORAD could be a poor prognostic biomarker in non-M3 AML patients, which was somewhat similar to the role of lncRNA PANDAR (another DNA damage-activated RNA) in AML [35]. Our report may have a prospective clinical application if confirmed by further studies and higher series of patients. Clinical association of NORAD transcription levels with patients' OS and RFS offers a new risk factor for non-M3 AML patients that could be a helpful tool in patient classification and risk assessment. To establish an optimal cutoff value, quantifying NORAD transcription levels in a validation cohort of patients may be valuable in this direction. A limitation of our study is the missing of additional prognostic mutations (such as TP53, CEBPA, ASXL1) and utilizing ELN-2017 guidelines [36] in risk stratification.

Our study was the first report investigating NORAD expression level and its clinical implications in non-M3 AML patients. However, the precise molecular mechanisms by which NORAD is upregulated in non-M3 AML need to be more thoroughly investigated.

5. Conclusion

Expression of NORAD was significantly upregulated in non-M3 AML patients, and high expression

of NORAD was a poor risk factor for OS and RFS. Therefore, our results suggested that NORAD was a prognostic biomarker for non-M3 AML, and it might effectively predict the outcome of patients.

CRediT authorship contribution statement

Mohammad Masoud Eslami: Conceptualization, Data curation, Formal analysis, Software, Writing – review & editing. **Mina Soufizomorrod:** Funding acquisition, Project administration, Supervision, Validation. **Mohammad Ahmadvand:** Investigation, Methodology, Resources, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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