Original Paper

Circulating miR-30d Predicts Survival in Patients with Acute Heart Failure

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Key Words

Circulating microRNAs • miR-30d • Acute heart failure • Survival

Abstract

Background/Aims: Identification of novel biomarkers to identify acute heart failure (AHF) patients at high risk of mortality is an area of unmet clinical need. Recently, we reported that the baseline level of circulating miR-30d was associated with left ventricular remodeling in response to cardiac resynchronization therapy in advanced chronic heart failure patients. However, the role of circulating miR-30d as a prognostic marker of survival in patients with AHF has not been explored. **Methods:** Patients clinically diagnosed with AHF were enrolled and followed up for 1 year. Quantitative reverse transcription polymerase chain reactions were used to determine serum miR-30d levels. The univariate logistic regression analysis and multivariate logistic regression analysis were used to determine the predictors for all-cause mortality in AHF patients. Kaplan–Meier survival analysis was used to analyze the role of miR-30d in prediction of survival. **Results:** A total of 96 AHF patients were enrolled and followed up for 1 year. Serum miR-30d was significantly lower in AHF patients who expired in the one year follow-up period compared to those who survived. Univariate logistic regression analysis yielded 18 variables that were associated with all-cause mortality in AHF patients, while the multivariate logistic regression analysis identified 4 variables including heart rate, hemoglobin, serum sodium, and serum miR-30d level associated with mortality. ROC curve analysis showed that hemoglobin, heart rate and serum sodium displayed poor prognostic value for AHF (AUCs not higher than 0.700) compared to miR-30d level (AUC = 0.806). Kaplan–Meier survival analysis confirmed that patients with higher serum miR-30d levels had significantly lower mortality (P=0.001). **Conclusion:** In conclusion, this study shows evidence for the predictive value of circulating miR-30d as 1-year all-cause mortality in AHF patients. Large multicentre studies are further needed to validate our findings and accelerate the transition to clinical utilization.

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Introduction

Acute heart failure (AHF) constitutes a leading public health burden in both developed and developing countries [1-3]. In the global context, AHF is responsible for over 1,100,000 admissions and 60,000 deaths annually [4, 5]. AHF is a major cause of hospital admission in patients older than 65 years old, and is associated with increased morbidity and mortality [5, 6]. Despite utilization of guideline-mandated therapy, clinical outcomes of AHF patients after hospitalization remain poor [2]. Identification of novel prognostic biomarkers will assist in tailoring of appropriate therapy for at-risk AHF patients [1, 2, 7].

MicroRNAs (miRNAs, miRs) are a class of endogenous, non-coding RNAs of 19-25 nucleotides in length [8, 9]. As central regulators of gene networks, miRNAs participate in several biological processes including proliferation, hypertrophy, inflammation, apoptosis and stress response [10-13]. Deregulation of miRNAs play a critical role in many cardiovascular diseases including heart failure [14-16]. Interestingly, miRNAs are present in the systemic circulation in a stable form, and the profile of plasma miRNAs can reflect cardiovascular disease [17-21]. A circulating miRNA signature for AHF patients has been reported, and more recently, the admission level of one of these AHF-associated miRNA miR-423-5p, has been shown to predict 1-year mortality in AHF patients [22, 23].

We have recently described that baseline levels of circulating miR-30d in patients with advanced chronic HF, was associated with left ventricular remodeling in response to cardiac resynchronization therapy (CRT) [24]. Interestingly, miR-30d appears to play a functional role in remodeling, activating protective signaling pathways associated with adaptive hypertrophy of primary cardiomyocytes and could protect against tumor necrosis factor- α (TNF- α)-induced cardiomyocytes apoptosis [24]. However, if levels of circulating miR-30d could translate to meaningful clinical outcomes in patients with AHF is unclear.

Here a total of 96 patients with clinically diagnosed AHF were enrolled in this study and followed for up to 1 year. Serum miR-30d levels were determined by quantitative reverse transcription polymerase chain reactions (qRT-PCRs) and correlated to clinical outcomes. Our results suggest that levels of serum miR-30d in AHF correlated with mortality at 1 year even after adjustment for important clinical variables in multivariate logistic models. Collectively, this study suggests a prognostic role for circulating miR-30d in predicting 1-year mortality in AHF patients. Large multicentre investigations to validate these findings may expedite the clinical utilization of circulating miR-30d to identify at-risk AHF patients.

Materials and Methods

Study cohort

All human investigations conformed to the principles outlined in the Declaration of Helsinki and were approved by the institutional review committees of Nanjing Medical University. The AHF cohort used in this study consisted of 96 patients (60 male and 36 female) admitted to the cardiac care unit between March 2012 and April 2015.

The diagnosis of AHF was confirmed by two senior physicians in the Department of Cardiology based on the clinical and biological parameters (including N-terminal pro-B-type Natriuretic Peptide (NT-proBNP)) according to the guidelines of American Heart Association [25, 26], and defined as a rapid or gradual worsening of the signs and symptoms of heart failure. Eligible patients were those hospitalized with new-onset or worsening preexisting heart failure as the primary cause of admission. The exclusion criteria are as follows: 1) Patients diagnosed with malignant tumors, cognitive dysfunction or dementia, severe mental illness, or other uncontrolled systemic disease. 2) Patients had a history of PTE/DVT, or anti-coagulation therapy within 3 months. All participants gave written informed consent before enrollment in the study. This study was registered at <http://www.chictr.org/cn/>. (ChiCTR-ONC-12001944).

Follow-up and endpoints

Typical clinical follow-up was performed in the outpatient setting during monthly visits or via telephone interview. The endpoints of the study were defined as all-cause mortality within 1 year from

the time they were diagnosed with AHF. During the 1-year follow-up, all enrolled AHF patients received standard treatment and management. Information concerning the death was obtained from the hospital medical record, the patient's physician, or family members.

Measurement of circulating miRNAs

Venous blood was collected in serum tubes and after a two-step centrifugation (4°C at $820 \times g$ for 10 min, then 4°C at $16000 \times g$ for 10 min), the supernatant was transferred to RNase/DNase-free tubes and stored at -80°C. Total RNA was isolated from the serum samples using the mirVana PARIS isolation kit (Ambion, Austin, Texas) according to the manufacturer's instructions for serum samples without enrichment for small RNAs. *Caenorhabditis elegans* miR-39 (cel-miR-39) of 50 pmol/L was added as the spike-in control after the equal volume of denaturing solution was added. The Bulge-Loop™ miRNA qPCR Primer Set (RiboBio) was used to determine the expression levels of miRNAs by qRT-PCRs with Takara SYBR Premix Ex Taq™ (TliRNaseH Plus) in ABI-7900 Real-Time PCR Detection System. Cel-miR-39 was used as an internal control. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 20.0, Chicago, IL) was used in all statistical analysis. Quantitative variables in normal distribution were expressed as mean $\pm$ SD, and categorical variables as count (percentage). miR-30d was dichotomized into 2 categories with categorical analysis including higher than median and lower than median. Groups were compared using two independent sample *t* test. To calculate the predictive value of variables including miR-30d for AHF, miR-30d relative expression values were calculated with the ΔCt method (mean Ct cel-miR-39—Ct target miRNA) and log-transformed by taking the base 2 logarithm, and logistic regression models using forward stepwise were constructed. Unordered categorical variables were analyzed by applying dummy variables, odds ratios and 95% confidence interval (CI)'s were calculated for the parameters. The survival curve was drawn by the Kaplan-Meier method and survival analysis used the log-rank test. Receiver-operator characteristic (ROC) curve was established to calculate the area under the curve (AUC) of miR-30d level for diagnosing AHF patients. MedCalc software (MedCalc Software, Mariakerke, Belgium) was used to compare the areas under every ROC curves. A difference of $P < 0.05$ (two-sided) was considered statistically significant.

Results

Baseline characteristics

A total of 96 patients with clinically diagnosed AHF were enrolled in this study and followed for up to 1 year. During the 1-year follow up, 17 patients died (17.7%). Patients that died within 1 year after AHF had faster heart rate, lower levels of systolic blood pressure (SBP) and diastolic blood pressure (DBP), and more co-morbidities such as atrial fibrillation and congenital heart diseases (Table 1).

Serum miR-30d level and all cause of death within 1 year

Serum miR-30d levels in the AHF patients who survived was compared to the patients who met the primary end-point of all-cause mortality in the follow-up period. The expression level of miR-30d in serum was significantly lower in those patients that expired compared to patients who survived during the 1 year follow up (Fig. 1) (average miR-30d Ct for survival: 29.83 ± 0.16 ; average cel-miR-39 Ct for survival: 15.55 ± 0.18 ; average miR-30d Ct for those expired: 30.63 ± 0.60 ; average cel-miR-39 Ct for those expired: 14.53 ± 0.33), indicating that serum miR-30d level may correlate with survival in AHF patients.

Fig. 1. Expression of serum miR-30d in acute heart failure patients survive during 1 year follow up comparing to those died. Y axis: -delta CT. **, $P < 0.01$.

Table 1. Acute heart failure patient characteristics with different clinical outcomes (No. or mean $\pm$ SD)

	Survival (n=79)			Death (n=17)			P value
Age (years)	61	$\pm$	16	62	$\pm$	17	0.919
Gender (No.)							0.173
Male			52			8	
Female			27			9	
Heart rate (beats/min, mean $\pm$ SD)	78	$\pm$	17	89	$\pm$	16	0.017
NT-proBNP (ng/mL)	1934.00	$\pm$	2331.59	2847.60	$\pm$	2117.09	0.794
CK-MB (IU/L)	14.73	$\pm$	9.17	59.76	$\pm$	229.38	0.287
ASA class (No.)							0.282
I			1			0	
II			15			2	
III			44			7	
IV			19			8	
Ejection Fraction (%)	47	$\pm$	16	48	$\pm$	18	0.797
HFpEF(No.)			35			8	0.986
SBP (mmHg, mean $\pm$ SD)	129	$\pm$	26	113	$\pm$	17	0.015
DBP (mmHg, mean $\pm$ SD)	77	$\pm$	15	67	$\pm$	6	0.001
Co-morbidities (No. (%))							
Atrial fibrillation			26(32.91%)			10(58.82%)	0.045
Hypertension			41(51.90%)			6(35.29%)	0.214
Acute coronary syndromes			8(10.13%)			0(0)	0.343
Ischemic heart disease			16(20.25%)			2(11.76%)	0.622
Cardiomyopathy			27(34.18%)			4(23.53%)	0.572
Valvular disease of the heart			17(21.52%)			5(29.41%)	0.701
Pulmonary heart disease			3(3.80%)			0(0)	1.000
Hyperthyreosis			2(2.53%)			1(5.88%)	1.000
Congenital heart diseases			4(5.06%)			4(23.53%)	0.044
Diabetes mellitus			20(25.32%)			5(29.41%)	0.965
Renal insufficiency			2(2.53%)			1(5.88%)	1.000

Serum miR-30d level in relation to baseline characteristics

The AHF cohort was divided into 2 groups according to the median of serum miR-30d levels. These two groups of AHF patients had similar age and gender characteristics, and received standard treatment and management for the whole course from admission to discharge. Lower incidence of atrial fibrillation and higher incidence of cardiomyopathy were found in miR-30d high level group (Table 2). Expect for these two variables, binary logistic regression showed that miR-30d was not associated with other baseline clinical characteristics (Table 3), suggesting that serum miR-30d was not a surrogate marker of other comorbidities in this cohort.

Serum miR-30d as a prognostic indicator for AHF

In univariate logistic regression analysis, the variable selection process yielded 18 variables that closely related to the outcomes of AHF (Table 4). Of the 18 variables, factors including heart rate, serum sodium, blood urea nitrogen, hemoglobin, cystatin C, uric acid and serum miR-30d level were chosen as independent variables. These variables were then included in the multivariate logistic regression analysis. Our results showed that higher hemoglobin, serum sodium and miR-30d level were associated with a reduced risk of death caused in AHF patients (OR: 0.962 [0.930 - 0.995], 0.759 [0.610 to 0.944] and 0.610 [0.409 to 0.911], respectively, all $P < 0.05$), while higher heart rate correlated with increased mortality after AHF (OR: 1.048 [1.000 - 1.098], $P = 0.048$) (Table 5).

We further evaluated the predictive power of these 4 variables to the outcomes of AHF by ROC curve analysis. Hemoglobin, heart rate and serum sodium displayed only moderate prognostic values for AHF (AUCs ≤ 0.700) compared to miR-30d level (AUC = 0.806) (Fig. 2). Combination of two or three variables (excluding miR-30d) also did not improve the

Table 2. Patient characteristics in groups with different miR-30d level (No. or mean±SD). NT-proBNP: N-terminal B-type natriuretic peptide; CK-MB: creatine kinase isoenzyme; ASA: American society of anesthesiologists; HFpEF: heart failure preserved ejection fraction; SBP: systolic blood pressure; DBP: diastolic blood pressure

	miR-30d low level (n=52)		miR-30d high level (n=44)		P value
Age (years)	63	± 17	60	± 15	0.402
Gender (No.)					0.290
Male	25		35		
Female	27		9		
Heart rate (beats/min, mean±SD)	82	± 20	78	± 15	0.272
ASA class (No.)					0.537
I	1		0		
II	6		11		
III	25		26		
IV	12		15		
Ejection Fraction (%)	51	± 16	45	± 17	0.126
HFpEF(No.)	21		22		0.275
SBP (mmHg, mean±SD)	127.70	± 28.32	124.98	± 22.58	0.601
DBP (mmHg, mean±SD)	75.80	± 16.26	74.71	± 12.16	0.710
Co-morbidities (No. (%))					
Atrial fibrillation	23(44.23%)		13(29.55%)		0.006
Hypertension	20(38.46%)		27(61.36%)		0.528
Acute coronary syndromes	4(7.69%)		4(9.09%)		1.000
Ischemic heart disease	10(19.23%)		8(18.18%)		0.330
Cardiomyopathy	9(17.31%)		22(50%)		0.023
Valvular disease of the heart	13(25%)		9(20.45%)		0.155
Pulmonary heart disease	1(1.92%)		2(4.55%)		1.000
Hyperthyreosis	2(3.85%)		1(2.27%)		0.883
Congenital heart diseases	4(7.69%)		4(9.09%)		1.000
Diabetes mellitus	14(26.92%)		11(25%)		0.235
Renal insufficiency	1(1.92%)		2(4.55%)		1.000
NT-proBNP (ng/mL)	1808.41	± 2510.08	2973.69	± 4773.67	0.135
D-Dimer (mg/L)	1.10	± 3.61	2.05	± 3.69	0.223
Plasma potassium(mmol/L)	3.98	± 0.64	3.98	± 0.56	0.974
Plasma sodium(mmol/L)	140.19	± 3.28	139.51	± 5.18	0.457
CK-MB (IU/L)	14.73	± 9.17	59.76	± 229.38	0.287
Blood urea nitrogen (mg/dL)	7.63	± 3.01	10.30	± 6.68	0.018
Scr (μmol/L)	87.75	± 28.83	105.84	± 57.82	0.052
Hemoglobin (g/L)	135.06	± 17.33	126.82	± 28.19	0.096
RDW (%)	14.02	± 2.46	15.07	± 2.06	0.028
ALT (U/L)	90.47	± 290.49	71.12	± 171.68	0.702
AST (U/L)	135.73	± 515.48	84.77	± 244.20	0.554
Cystatin C (mg/L)	1.35	± 0.61	1.64	± 1.01	0.114
Uric acid (μmol/L)	457.02	± 155.46	518.91	± 179.40	0.078
Ejection Fraction (%)	45.17	± 16.54	50.54	± 16.26	0.126
LVDD (mm)	60.18	± 14.29	56.48	± 13.24	0.210

Table 3. Association of miR-30d serum levels with baseline characteristics. ASA: American society of anesthesiologists

Variable	Odds ratio	95% CI	P value
Age	0.989	0.963-1.015	0.398
Gender	0.565	0.681-3.594	0.291
ASA class	0.972	0.549-1.722	0.923
Heart rate	0.987	0.964-1.010	0.271
Pneumonia	0.707	0.247-2.022	0.518
Atrial fibrillation	0.304	0.128-0.721	0.007
Ischemic heart disease	0.600	0.213-1.686	0.333
Cardiomyopathy	2.852	1.141-7.128	0.025
Acute coronary syndromes	0.833	0.196-3.546	0.805
Hyperthyreosis	0.412	0.036-4.700	0.412
Hypertension	0.998	0.986-1.010	0.742
Congenital heart diseases	0.833	0.196-3.546	0.805
Diabetes mellitus	0.575	0.229-1.442	0.238
Renal insufficiency	1.720	0.151-19.632	0.662

clinically prognostic value (Fig. 3A, Fig. 3B and Fig. 4). While AUC for combination of all four variables reached 0.872, which was significantly improved over the AUC areas of the single variables; this combination was not statistically different compared to serum miR-30d ($P>0.05$) (Fig. 4), indicating that serum miR-30d could be a prognostic indicator for AHF. Moreover, we correlated serum miR-30d to the established conventional marker NT-

Table 4. Prediction of death (univariate analysis). NT-proBNP: N-terminal B-type natriuretic peptide; CK-MB: creatinine kinase isoenzyme; Scr: serum creatinine; ALT: alanine aminotransferase; AST: aspartate transaminase; LVDD: left ventricular end-diastolic dimension

Variable	Odds ratio	95% CI	P value
Age	1.002	0.969-1.036	0.918
Gender	0.444	0.154-1.286	0.135
NT-proBNP	1.000	1.000-1.000	0.569
D-Dimer	0.981	0.819-1.173	0.830
Heart rate	1.038	1.005-1.072	0.025
Serum potassium	1.606	0.662-3.899	0.295
Serum sodium	0.826	0.724-0.941	0.004
CK-MB	0.998	0.986-1.010	0.742
Blood urea nitrogen	1.104	1.009-1.208	0.032
Scr	1.003	0.992-1.013	0.622
Hemoglobin	0.968	0.945-0.991	0.007
ALT	1.003	1.000-1.007	0.053
AST	1.003	0.999-1.006	0.160
Cystatin C	2.189	1.117-4.291	0.022
Uric acid	1.003	1.000-1.006	0.047
Ejection Fraction	1.004	0.972-1.037	0.803
LVDD	0.967	0.926-1.009	0.118
miR-30d	0.472	0.316-0.705	<0.001

Table 5. Prediction of death (multivariate analysis)

Variable	Odds ratio	95%CI	P value
Hemoglobin	0.962	0.930-0.995	0.025
Heart rate	1.048	1.000-1.098	0.049
Serum sodium	0.759	0.610-0.944	0.049
miR-30d	0.610	0.409-0.911	0.016

Fig. 2 The receiver-operator characteristic (ROC) curves for acute heart failure patients survive during 1 year follow up comparing to those died with single variable. The receiver-operator characteristic (ROC) curves for distinguishing acute heart failure patients survive during 1 year follow up from to those died by hemoglobin, heart rate, serum sodium and serum miR-30d level, respectively. AUC, area under the ROC curve.

proBNP, and though the AUC area reached 0.850, no statistical difference was observed when compared to miR-30d (Fig. 3C).

Serum miR-30d and mortality

Kaplan-Meier survival analysis confirmed that patients with higher serum miR-30d levels had significantly lower mortality ($P=0.001$) (Fig. 5). Taken together, these results extend our previous studies that suggested an association between circulating miR-30d and cardiac remodeling to suggest a potential association with a clinically meaningful endpoint (mortality) in patients with AHF.

Fig. 3. The receiver-operator characteristic (ROC) curves for acute heart failure patients survive during 1 year follow up comparing to those died with two combined variables. The receiver-operator characteristic (ROC) curves for distinguishing acute heart failure patients survive during 1 year follow up from to those died by combining two variables with (A) or without (B) miR-30d and combining miR-30d with NT-pro BNP (C), respectively. AUC, area under the ROC curve.

Fig. 4. The receiver-operator characteristic (ROC) curves for acute heart failure patients survive during 1 year follow up comparing to those died with three or four combined variables. The receiver-operator characteristic (ROC) curves for distinguishing acute heart failure patients survive during 1 year follow up from to those died by hemoglobin + heart rate + serum sodium, hemoglobin+serum sodium+ serum miR-30d, heart rate + serum sodium+ serum miR-30d, and hemoglobin +heart rate + serum sodium+ serum miR-30d, respectively. AUC, area under the ROC curve.

Fig. 5. Kaplan–Meier cumulative survival analysis according to serum miR-30d levels. The acute heart failure patients in this study were divided into two groups according to the median of miR-30d (lower than median vs higher than median). Survival rate free of all cause of death based on serum miR-30d level.

Discussion

Identification of AHF patients who are at high risk of mortality following discharge is of critical importance to guide therapeutic decision making for physicians [1]. Identification of high-risk AHF patients could facilitate allocation of resources including ventricular assist device support, cardiac transplantation, and enrollment into AHF management programs [2, 27]. Nevertheless, accurate risk assessment remains a challenging task [28]. Thus, exploring novel biomarkers for prediction of survival in AHF patients is greatly needed [27].

Several biomarkers measured during an AHF admission have been reported to independently predict death after discharge, including soluble suppression of tumorigenicity

2 (sST2), galectin-3, copeptin, and mid-regional pro-adrenomedullin etc. [1, 29-31]. miRNAs have been found to exist in circulation in a consistent and reproducible manner, making them attractive candidates for biomarker development in AHF patients [22, 23]. Growing evidence has showed that several miRNAs can predict outcome in AHF patients, including miR-423-5p, miR-18a-5p, and miR-652-3p [22, 23]. Recently, we have reported that circulating miR-30d was associated with response to cardiac resynchronization therapy in HF [24]. miR-30d was found to have a higher diagnostic value than cTnI for the early diagnosis of acute myocardial infarction (AMI), but it was not associated with 6 month cardiovascular events in AMI patients [32]. In the present study, we have found that AHF patients with higher serum miR-30d levels had significantly lower mortality even after adjustment for clinical and biochemical variable, thereby providing evidence that circulating miR-30d may be a novel prognostic biomarker for survival in AHF patients. It would be interesting to investigate how other biomarkers (sST2, galectin-3, copeptin, etc.) may relate to circulating levels of miR-30d as a novel biomarker in the future.

Our previous study reported a cardiac origin for circulating miR-30d, and cardiomyocyte as the possible source of the extracellular miR-30d [24]. In another study, miR-30d was reported to be absorbed by the failing heart [33]. We have previously shown that miR-30d acts as a 'protecteomir', with its upregulation associated with molecular features of adaptive hypertrophy, and cardioprotection against TNF- α mediated apoptosis [24]. Although in other models of cardiovascular diseases, miR-30d has been associated with cardiomyocyte pyroptosis [34], this study supports our findings that higher levels of circulating miR-30d are likely cardioprotective. Moreover, we also found lower incidence of atrial fibrillation in miR-30d high level group and as atrial fibrillation is a major cause of cardiovascular morbidity and mortality, this relationship was in accordance with our findings. We speculated that miR-30d might also be protective for AHF in animal models. Nonetheless, definitive proof of the functional role of miR-30d can only come from gain-of-function and loss-of-function experiments in animal models. Besides that, we previously have shown that miR-30d was induced to release by increased mechanical stress [24], which might be similar in AHF patients.

Several limitations of the present study should be highlighted. Importantly, rather than performing microarrays or RNA sequencing, we only measured miR-30d based on our previous study [24]. As has been conventionally accepted, we normalized miR-30d levels to an exogenous spike-in. The issue of normalization of biofluid extracellular RNAs remains a hotly debated topic in the field as no commonly internal house-keeping control has been accepted, and we agree that this remains a limitation in the field. Nonetheless, we took care to use non-hemolyzed specimens and measured miR-30d in the same amount of sample for each patient. In addition, our sample size was limited to 96 patients, and undoubtedly our findings will need to be validated in larger studies and other populations. Besides that, the standard deviations for NT-proBNP and CK-MB are high, which is due to the heterogeneous nature of the AHF populations and the inclusion of outlier values as well. Moreover, it remains to be determined if miR-30d could response to treatment and if miR-30d guided therapy in AHF could achieve additional benefits. Finally, whether miR-30d could be a therapeutic target for AHF remains to be determined.

In conclusion, our study provides novel insights into the predictive value of circulating miR-30d for 1-year mortality in AHF patients. Large multicentre investigations to validate our findings would be highly desired to expedite the transition to clinical utilization.

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Disclosure Statement

The authors declare there are no conflicts of interest.

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