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Original Article

Expression of eEF2 is associated with prognostic outcome in acute myeloid leukemia patients

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Abstract: Background: Eukaryotic elongation factor 2 (eEF2) has been shown to be overexpressed in multiple types of cancer. Whether this association is also true in AML is unknown. The purpose of this study is to investigate the expression status of eEF2 and its prognostic significance in AML patients. Methods: Total RNA was extracted from the bone marrow or plasma of 87 AML patients and 21 healthy volunteers. The expression of eEF2 was measured by real-time quantitative PCR. The association of eEF2 expression with the patients' clinicopathological features and its prognosis significance were statistically analyzed. Results: The expression of eEF2 was significantly up-regulated in AML patients compared to healthy volunteers (P<0.001), and the eEF2 expression level was much lower in patients who had already achieved CR compared with newly diagnosed AML patients (P=0.029). Furthermore, the expression of eEF2 was significantly associated with BM blast count (P=0.023) and complete remission (P<0.001). Patients with high eEF2 expression had a relatively poor overall survival (OS) (P<0.001) and a shorter disease-free survival (DFS) (P<0.001). Moreover, eEF2 expression was an independent prognostic factor for both OS (P=0.010) and DFS (P=0.005). Conclusions: eEF2 was significantly over-expressed in AML patients. The over-expression of eEF2 was correlated with poor prognosis and may serve as an independent prognostic factor for both OS and DFS in AML patients.

Keywords: eEF2, acute myeloid leukemia, bone marrow, prognosis

Introduction

Acute myeloid leukemia (AML) comprises an epigenetically and genetically heterogeneous group of hematopoietic malignancy characterized by uncontrolled rapid expansion and accumulation of immature myeloid blasts in the bone marrow (BM), blood, and other organs [1, 2]. With regard to biological behavior of the leukemic cell and its responses to current treatment, AML is similarly highly heterogeneous [3]. A large proportion of patients with AML fail to respond to standard first-line chemotherapy, and the existing salvage therapeutic regimen seldom yields persistent remissions, with relapse being common [4, 5]. Only 35-40% of patients under 60 years of age and less than 10% of patients over 60 years are curable [6, 7]. The long-term disease-free survival of AML patients is only 50% even in young patients who have received standard chemotherapy. As for most of the aged patients who have only received leukemia-stabilizing therapy, the median survival is no more than 1 year [8]. To improve patient management and help identifying novel therapeutic regimens with a higher efficiency and lower toxicity, the discovery of novel biomarkers related to AML is of critical importance.

Eukaryotic elongation factor 2 (eEF2) is one of the three protein factors involved in the elongation of the polypeptide chain during the process of protein translation [9]. The activity of eEF2 in translation is regulated by protein phosphorylation. Cells control the translation levels at the elongation step through modulation of eEF2 activity during multiple biological processes such as genotoxic stress [10, 11] and cell cycle progression [12] or in response to endogenous carbon monoxide, which induces anti-proliferative effects [13]. Recently, eEF2 was found to

Table 1. Correlation of eEF2 expression with the clinicopathological features of AML patients

Characteristics	Total (n=87)	eEF2		*P* value
		Low (n=48)	High (n=39)	
Gender				0.404
Male	47 (54.0%)	24 (51.1%)	23 (48.9%)	
Female	40 (46.0%)	24 (60.0%)	16 (40.0%)	
Age (years)				0.189
≤60	64 (73.6%)	38 (59.4%)	26 (40.6%)	
>60	23 (26.4%)	10 (43.5%)	13 (56.5%)	
FAB subtype				0.604
M 1	15 (17.2%)	9 (60.0%)	6 (40.0%)	
M 2	22 (25.3%)	9 (40.9%)	13 (59.1%)	
M 3	9 (10.3%)	5 (55.6%)	4 (44.4%)	
M 4	12 (13.8%)	8 (66.7%)	4 (33.3%)	
M 5	29 (33.3%)	17 (58.6%)	12 (41.4%)	
WBC ($\times 10^9$/L)				0.755
<10	44 (50.6%)	25 (56.8%)	19 (43.2%)	
≥10	43 (49.4%)	23 (53.5%)	20 (46.5%)	
HGB (g/L)				0.484
<80	46 (52.9%)	27 (58.7%)	19 (41.3%)	
≥80	41 (47.1%)	21 (51.2%)	20 (48.8%)	
PLT ($\times 10^9$/L)				0.258
<10	46 (52.9%)	28 (60.9%)	18 (39.1%)	
≥10	41 (47.1%)	20 (48.8%)	21 (51.2%)	
Blasts in BM				0.023
<50%	43 (49.4%)	29 (67.4%)	14 (32.6%)	
≥50%	44 (50.6%)	19 (43.2%)	25 (56.8%)	
Complete remission				0.000
Yes	40 (46.0%)	34 (85.0%)	6 (15.0%)	
No	47 (54.0%)	14 (29.8%)	33 (70.2%)	
Living Status				0.000
Alive	52 (59.8%)	37 (71.2%)	15 (28.8%)	
Dead	35 (40.2%)	11 (31.4%)	24 (68.6%)	
Relapse				0.000
Yes	44 (50.6%)	13 (29.5%)	31 (70.5%)	
No	43 (49.4%)	35 (81.4%)	8 (18.6%)	

be overexpressed in multiple types of cancer, such as head and neck squamous cell carcinoma (HNSCC), esophageal carcinoma, lung cancer, breast cancer, pancreatic carcinoma, gastric and colorectal cancer, prostate cancer, non-Hodgkin lymphoma (NHL), and glioblastoma [14-16]. Knockdown of eEF2 by shRNA could apparently inhibit the growth of cancer cells [14]. However, whether this association is also true in AML is unknown. To analyze the clinical significance of eEF2 in AML, we compared the expression of eEF2 in patients with AML and healthy volunteers and investigated its clinical and prognostic significance.

Materials and methods

Patients and samples

Between June 2010 and January 2014, bone marrow samples were obtained from 87 newly diagnosed AML patients without treatment and 7 patients who had achieved complete remission (CR) at the First Affiliated Hospital of Guangzhou Medical University. CR was defined as having a cellular bone marrow that contains less than 5% blasts and other marrow elements that show evidence of normal maturation. There were 47 male patients and 40 female patients, with a medium age of 45 years (range 13-82 years). Peripheral blood samples were obtained from 12 of the above patients and 21 unrelated healthy adult donors as controls. The control cohort was matched with the patient cohort in terms of age and sex. All of the patients have received standard induction chemotherapy. Details of the clinical features of these patients are summarized in **Table 1**. The follow-up time of the AML cohort ranged from 1 to 119 months, and the median follow-up time was 35 months. All of the recruited patients were diagnosed according to the morphologic, cytochemical, immunological and cytogenetic evaluation criteria of the French American British (FAB) classification and the World Health Organization (WHO) criteria [17-20]. Informed consent to the use of these clinical samples for research purposes was obtained beforehand from all of the patients, and all the protocols of this study were approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the bone marrow of the recruited patients using TRIzol reagent (Transgen, China) according to the manufactur-

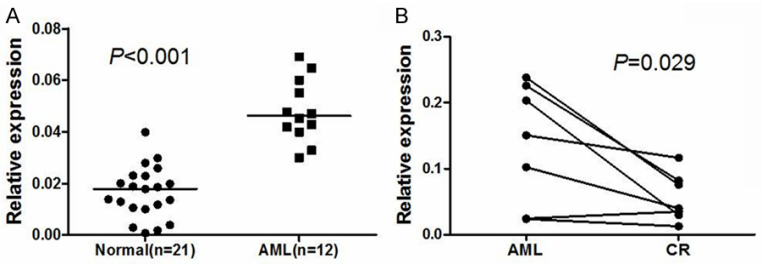

Figure 1. eEF2 expression in newly diagnosed AML patients and in healthy controls detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. A. Expression levels of eEF2 in newly diagnosed AML patients (median value 0.016, range: 0.003-0.039) and healthy controls (median value 0.048, range: 0.029-0.069). Data shown are the median and range. Each symbol represents an individual AML patient and healthy control. The horizontal lines indicate the median values. B. Expression levels of eEF2 in 7 AML patients before (median value 0.138, range: 0.024-0.238, P=0.029) and after complete remission (CR) (median value 0.056, range: 0.012-0.117). Each symbol represents an individual AML patient at diagnosis and at CR. The paired t test and associated *P* value are indicated.

er's instructions. One microgram of RNA from each sample was reverse transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit #K1622 (Thermo Scientific, USA). cDNAs were subjected to quantitative real-time PCR (qRT-PCR) analysis. Briefly, each reaction mixture consisted of 2 μL cDNA, 10 μL SYBR Green PCR Master Mix (BIO-RAD), 1 μL EEF2 primers (5 nmol/mL) or 1 μL GAPDH primers (5 nmol/mL), and deionized water, making up to a total volume of 20 μL. The sequences of primers were as follows: 5'-TGCCATGGGCATTAAAAGCTG-3' (forward primer) and 5'-GTCCAAAGGCGTAGAACCGA-3' (reverse primer) for *eEF2*; 5'-TGTTGCC ATCAATGACCCCTT-3' (forward primer) and 5'-CTCCACGACGTACTCAGCG-3' (reverse primer) for *GAPDH*. qRT-PCR was performed on a CFX96 Real-Time System (BIO-RAD). The amplification cycle was performed with a denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s, and 72°C for 45 seconds. A melting program was performed after each reaction to validate the specificity of the expected PCR product. Each sample was analyzed in triplicate, and the mean expression level was calculated. The relative expression level of eEF2 was calculated by the 2-ΔΔCT method.

Statistical analysis

The time interval between the date of each patient's diagnosis of AML and the date of death from any cause was defined as overall survival (OS). The interval between the date of the achievement of complete remission and the date of the hematological relapse or death was defined as disease-free survival (DFS). All statistical analyses were performed with the Statistical Program for Social Sciences 20.0 (SPSS, Chicago, IL, USA). Graphs were plotted using Graph Pad Prism 5.0 (Graph Pad Software, La Jolla, CA, USA). The Mann-Whitney U-test was used to evaluate the difference in eEF2 expression between the AML patients and healthy donors. A paired t test was performed to assess the difference in eEF2 expression between patients with newly diagnosed AML and patients who have already achieved CR. The Chi-square test or Fisher exact test was utilized for categorical variables. The cumulative survival rate was calculated by the Kaplan-Meier method, and the statistical significance was analyzed by the log-rank test. Multivariate Cox proportional hazard model was used to evaluate the prognostic significance of the clinical features. A *P*-value less than 0.05 was regarded as statistically significant, and all the reported *P*-values were two-sided.

Results

eEF2 is overexpressed in the peripheral blood and bone marrow of AML patients

We evaluated the expression levels of eEF2 in the peripheral blood samples of 12 newly diagnosed AML patients and 21 unrelated healthy donors by qRT-PCR. A statistically significant difference was found in the expression level of eEF2 between these two groups (P<0.001, **Figure 1A**). Newly diagnosed AML patients exhibited significantly higher expression levels of eEF2 (median value 0.016, range: 0.003-0.039), compared with the healthy control cohort (median value 0.048, range: 0.029-0.069). Moreover, we investigated the expression levels of eEF2 in the bone marrow of 7 newly diagnosed AML patients without any treatment and 7 AML patients who had already achieved CR. The expression level of eEF2 was much lower in the patients who had already achieved CR (median value 0.056, range: 0.012-0.117) compared with the newly diag-

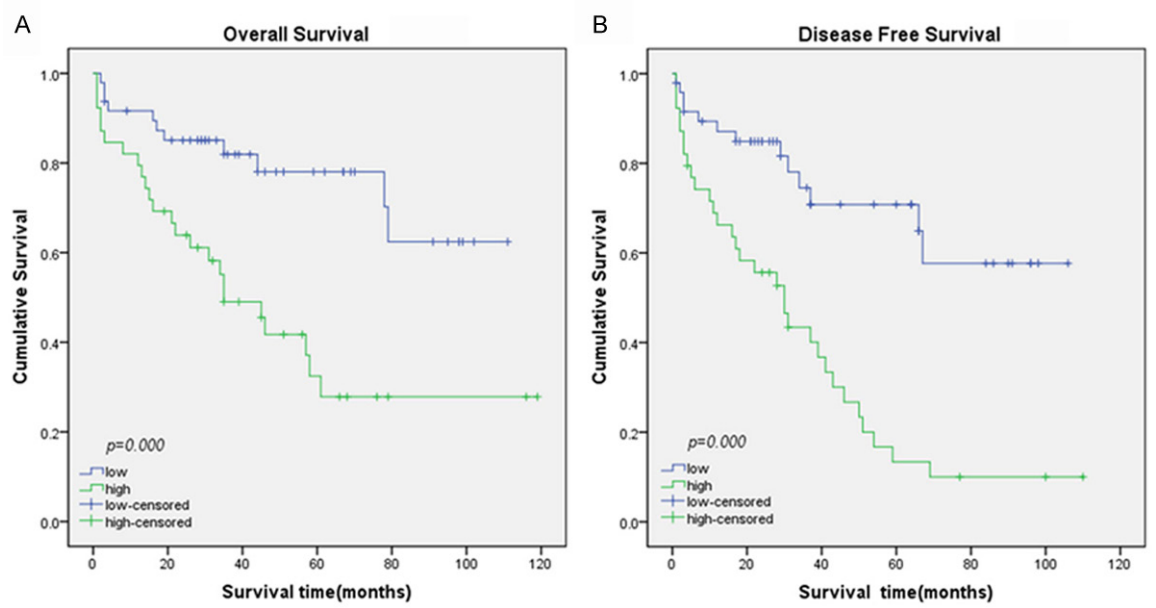

Figure 2. Kaplan-Meier curves for the overall survival (OS) and disease-free survival (DFS) of 87 AML patients according to their high or low level of eEF2 expression. A. The OS rate of AML patients with high or low levels of eEF2 expression. B. The DFS rate of AML patients with high or low levels of eEF2 expression. The *P*-value was calculated using the log-rank test.

nosed cohort (median value 0.138, range: 0.024-0.238, P=0.029) (**Figure 1B**).

Correlation of eEF2 expression with clinical characteristics of AML patients

To better understand the potential functions of eEF2 in AML occurrence and progression, qRT-PCR was performed to detect the expression of eEF2 in the bone marrow of 87 patients with newly diagnosed AML. The patient samples were divided into two cohorts according to the median expression of the gene (high vs. low expression). Among these samples, high eEF2 expression was observed in 39 samples (44.83%) and low eEF2 expression was detected in 48 samples (55.17%, **Table 1**). We further investigated the relationship between eEF2 expression and other clinical indexes. As summarized in **Table 1**, the expression of eEF2 was significantly associated with BM blast count (P=0.023) and complete remission (P<0.001). In terms of gender, age, FAB subtype, WBC count, platelet count, and HGB count, no significant correlations with eEF2 levels were observed.

eEF2 is negative correlated with OS and DFS

To determine the prognostic value of eEF2, we explored the association between eEF2 expression and the clinical outcomes of AML patients. Patients with a high expression level of eEF2 had a relatively poor overall survival (OS) (P<0.001) and a short disease-free survival (DFS) (P<0.001) (**Figure 2**). Among 87 cases, the CR rate after two cycles of chemotherapy was 70.8% (34/48) in the low-expression group, compared with 15.4% (6/39) in the high-expression cohort. Univariate analyses indicated that blasts in BM (P=0.001) and the eEF2 level (P=0.001) were prognostic factors for OS (**Table 2**), while age (P=0.003), blasts in BM (P<0.001) and eEF2 level (P<0.001) were significantly associated with DFS (**Table 3**). The multivariate Cox regression analysis indicated that eEF2 expression was an independent prognostic factor for both OS (Hazard ratio [HR]=0.383, 95% confidence interval [CI], 0.184-0.795, P=0.010) and DFS (HR=0.387, 95% CI, 0.198-0.755, P=0.005) of AML patients.

Discussion

eEF2 is a GTP-dependent translocase that catalyzes the translocation of the nascent peptidyl-tRNAs from the A-site to the P-site of the ribosome, which is an essential function in different types of cells. It plays an essential role in the elongation of the polypeptide chain during the process of protein translation and is highly conserved in eukaryotes. A functional homo-

Table 2. Cox regression analysis of parameters associated with overall survival of AML patients

Factor	Univariate			Multivariate		
	HR (95% CI)	*B value*	*P* value	HR (95% CI)	*B value*	*P* value
Gender						
Male	Reference					
Female	0.826 (0.423-1.613)	-0.191	0.575	-	-	-
Age (years)						
≤60	Reference					
>60	0.502 (0.252-1.001)	-0.688	0.050	-	-	-
FAB subtype						
M 1	Reference		0.555			
M 2	1.134 (0.428-3.003)	0.126	0.800			
M 3	0.199 (0.024-1.623)	-1.613	0.132			
M 4	0.790 (0.229-2.722)	-0.235	0.709			
M 5	0.832 (0.332-2.087)	-0.184	0.695	-	-	-
WBC (*10^9/L)						
<10	Reference					
≥10	0.721 (0.369-1.408)	-0.327	0.338	-	-	-
HGB (g/L)						
<80	Reference	0.375	0.278	-	-	-
≥80	1.455 (0.739-2.865)					
PLT (×10^9/L)						
<10	Reference	-0.495	0.154	-	-	-
≥10	0.609 (0.309-1.203)					
Blasts in BM		-0.141	0.001	Reference		
<50%	Reference			0.302(0.134-0.681)	-1.197	0.004
≥50%	0.244 (0.110-0.543)					
eEF2						
Low	Reference	-1.205	0.001	0.382(0.184-0.795)	-0.961	0.010
High	0.300 (0.146-0.615)					

logue, named elongation factor G (EF-G), exists in prokaryotes [9]. Cells control the translation levels at the elongation step through modulation of eEF2 activity during multiple biological processes such as genotoxic stress [10, 11] and cell cycle progression [12], or in response to endogenous carbon monoxide, which induces anti-proliferative effects [13].

From yeast to humans, there are several known post-translational modifications that modulate the activity of eEF2, such as the constitutive formation of diphthamide residue through sequential modifications of histidine residue [21] or the inhibitory phosphorylation at threonine 56 (T56) by an eEF2-specific kinase [22]. Diphthamide is the target region of a number of bacterial toxins that inactivate eEF2 through ADP-ribosylation and result in the interruption of protein translation and induction of cell death [23, 24]. Another mechanism that modulates the activity of eEF2 is the phosphorylation at T56, which falls within the GTP-binding domain of eEF2 and prevents eEF2 from binding with the ribosome [25, 26]. Ca^{2+}/calmodulin-dependent kinase III, also termed eEF2 kinase (eEF2K), phosphorylates eEF2 on T56 [27-29]. Several biological signals induce the phosphorylation of eEF2K on residues that suppress or enhance its activity [30-32]. Furthermore, eEF2 has been discovered to be proteolytically cleaved in different types of cells at different stages of cell growth [33]. The generation of cleaved fragments of eEF2 is generally induced by irradiation, aging and oxidative stress [34, 35]. However, the exact function of these small cleaved fragments is still obscure.

Recently, eEF2 was found to be overexpressed in multiple types of cancer, such as HNSCC, esophageal carcinoma, lung cancer, breast cancer, pancreatic carcinoma, gastric and

Table 3. Cox regression analysis of parameters associated with disease-free survival of AML patients

Factor	Univariate			Multivariate		
	HR (95% CI)	*B value*	*P* value	HR (95% CI)	*B value*	*P* value
Gender						
Male	Reference					
Female	0.751 (0.411-1.370)	-0.287	0.351	-	-	-
Age (years)						
≤60	Reference			Reference		
>60	0.398 (0.218-0.730)	-0.920	0.003	0.481 (0.261-0.888)	-0.732	0.019
FAB subtype						
M 1	Reference		0.375			
M 2	1.286 (0.529-3.123)	0.251	0.579			
M 3	0.168 (0.021-1.347)	-1.783	0.093			
M 4	0.856 (0.277-2.642)	-0.156	0.786			
M 5	0.909 (0.392-2.111)	-0.095	0.825	-	-	-
WBC (*10^9/L)						
<10	Reference					
≥10	0.667 (0.366-1.216)	-0.405	0.186	-	-	-
HGB (g/L)						
<80	Reference	0.202	0.507	-	-	-
≥80	1.224 (0.673-2.224)					
PLT (×10^9/L)						
<10	Reference	-0.071	0.816	-	-	-
≥10	0.932 (0.512-1.693)					
Blasts in BM						
<50%	Reference	-1.401	0.000	0.331 (0.164-0.668)	-1.107	0.002
≥50%	0.246 (0.124-0.491)					
eEF2						
Low	Reference	-1.326	0.000	0.387 (0.198-0.755)	-0.949	0.005
High	0.265 (0.138-0.509)					

colorectal cancer, prostate cancer, NHL, and glioblastoma [14-16]. The overexpression rate of eEF2 was reported to be over 70% in lung cancer, esophageal carcinoma, breast cancer and prostate cancer, and in NHL, gastric carcinoma and colorectal cancer, the rate was greater than 90% [14, 15], which indicated that eEF2-targeted therapy may be a new therapeutic strategy against malignant carcinoma. However, the precise molecular mechanism of eEF2's function in the generation and development of tumor still remains poorly understood.

It was proposed that eEF2 may facilitate the growth of tumor cells through inhibiting apoptosis and that it also participated in the cell cycle progression of tumor cells [15, 16]. Another potential mechanism of eEF2's impact on cell transformation was that eEF2 could be cleaved into small functional fragments, which accumulated in the nucleus and induced nuclear deformation and aneuploidy [36]. The instability and aneuploidy of the nucleus are directly associated with malignant transformation of cells [37].

Nakamura [15] and colleagues have reported that the up-regulation of eEF2 in gastrointestinal cancers promoted the progression of G2/M of the cell cycle in association with the activation of Akt and a G2/M regulator, named cdc2, and led to the enhancement of cancer cell growth *in vitro* and *in vivo*. Another study from Sun et al [38] showed that the expression levels of eEF2 in the serum of NSCLC patients were markedly higher than in the healthy donors, particularly in patients at late stages and with a tumor size larger than 2 cm. Patients after surgery showed a trend for declining eEF2 levels. Chen et al [16] reported that patients

with high expression of eEF2 usually had a higher incidence of early recurrence and a lower survival rate. Consistently, the present study showed for the first time that the expression of eEF2 in patients with newly diagnosed AML is much higher than that in healthy donors and that eEF2 expression was much lower in patients who had achieved CR, compared with newly diagnosed AML patients. We further investigated the association of eEF2 expression with other clinical characteristics in AML patients. The expression of eEF2 was significantly associated with BM blast count and complete remission. In terms of gender, age, FAB subtype, WBC count, platelet count, and HGB count, no significant correlations with eEF2 levels were observed.

In our study, the patients with high eEF2 expression had a relatively poor OS and short DFS. Among 87 cases, the CR rate after two cycles of chemotherapy was 70.8% in the low-expression group, compared with 15.4% in the high-expression cohort. Cox regression revealed that eEF2 expression was an independent prognostic factor for both OS and DFS in AML patients. This finding indicates the possibility of using eEF2 expression as a predictor for AML prognosis and survival.

To our knowledge, this is the first report addressing eEF2 expression and its clinical and prognostic significance in AML patients. Our findings suggest that eEF2 is over expressed in AML and may be a valuable molecular biomarker for the prediction of the prognosis and survival of AML patients. The expression status of eEF2 may be useful for stratifying AML patients at high risk, and the development of eEF2-targeted immunotherapy could be an effective therapeutic strategy applicable to patients with AML. However, the precise function of eEF2 in the tumorigenesis and development of AML remains obscure, and further investigation is required to clarify the mechanisms by which eEF2 is involved in its development and progression.

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Disclosure of conflict of interest

None.

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