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Original Article

Circulating Bmi-1 mRNA as a diagnostic and prognostic biomarker for non-small cell lung cancer

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Abstract: Bmi-1, a key member of the Polycomb group complex, has been shown in non-small cell lung cancer (NSCLC) tumorigenesis and progress. The aim of this study was to detect the levels of circulating Bmi-1 mRNA in plasma of NSCLC patients and evaluate its diagnostic and prognostic value. Levels of circulating Bmi-1 mRNA were detected by reverse transcription quantitative real-time PCR in plasma of 108 NSCLC patients and 40 healthy controls. The results showed that circulating Bmi-1 mRNA levels were significantly upregulated in plasma of NSCLC patients when compared with healthy controls (P<0.001), and significantly correlated with tumor size (P=0.038) and clinical stage (P=0.025). The receiver operating characteristics (ROC) curve analyses revealed that circulating Bmi-1 mRNA had considerable diagnostic accuracy, yielded an AUC (the areas under the ROC curve) of 0.805 with 72.9% sensitivity and 69.1% specificity in discriminating NSCLC from healthy controls. Moreover, Kaplan-Meier analysis demonstrated a correlation between increased circulating Bmi-1 mRNA level and reduced overall survival (P=0.028). Cox analysis indicated that it was an independent prognostic factor for NSCLC. In conclusion, our findings suggested that circulating Bmi-1 mRNA might be a promising non-invasive diagnosis and prognosis biomarker for NSCLC.

Keywords: Non-small cell lung cancer, circulating Bmi-1 mRNA, diagnosis, prognosis

Introduction

As one of the most common malignant tumors in the world, lung cancer is responsible for the majority of cancer-related deaths [1]. Lung cancer is histologically classified as either small cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC), with the latter accounting for over 80% of all lung cancers. Despite recent significant advances in the management of NSCLC, the disease has a very low survival rate, with a 5-year overall survival of only approximately 15%, primarily attributable to late diagnosis when curative surgery is no longer an option [2]. Early diagnosis represents one of the most effective strategies in improving survival and prognosis of NSCLC patients. Among the available NSCLC diagnostic methods, fiberoptic bronchoscope is recommended as the most reliable tool for high-risk patients. However, the invasive nature and cost incurred have hampered its wide application. Computed tomography provides excellent anatomic information and can detect some NSCLC cases at an early stage, but the diagnostic procedures and the hazards of the associated radiation may outweigh the potential benefits [3]. Other methodologies, such as bronchoalveolar lavage and sputum cytology, have not been proven to be effective screening tools. Thus, the identification of novel noninvasive biomarkers is urgently required to complement and improve on current strategies for NSCLC detection.

Since first found in nasopharyngeal carcinoma and melanoma patients in 1999 [4, 5], various circulating RNAs species have been detected in the plasma/serum of patients with different types of cancer, including telomerase components, viral RNA transcripts and other tumor-associated transcripts [6]. Some investigators have reported that circulating RNA could be more sensitive and tissue-type specific compared with conventional tumor biomarkers [7,

Table 1. Correlations between circulating Bmi-1 mRNA expression and clinicopathological characteristics of patients with NSCLC (n=108)

Characteristics	Number of patients	Circulating Bmi-1 mRNA levels		
		Low	High	*P* value
Gender				0.318
Male	82	39	43	
Female	26	14	12	
Age				0.406
≤50	46	21	25	
>50	62	30	32	
Smoking status				0.192
Non-smoker	28	15	13	
Smoker	80	38	42	
Histotype				0.205
Squamous cell carcinoma	52	28	24	
Adenocarcinoma	46	24	22	
Large cell carcinoma	10	4	6	
Cell differentiation				0.118
Well and Moderate	76	40	36	
Poor	32	15	17	
Tumor size				0.038*
≤3 cm	21	15	6	
>3 cm	87	26	61	
Lymph node metastasis				0.072
No	46	22	24	
Yes	62	29	33	
Distant metastasis				0.085
No	92	48	44	
Yes	16	7	9	
Clinical stage				0.025*
I-II	60	47	13	
III-IV	48	9	39	

*$P<0.05$.

8]. Moreover, in some cases, the increased concentrations of circulating RNA have been shown to correlate with poor prognosis [9-11]. Therefore, circulating tumor-associated mRNAs open up a new and interesting field in the diagnosis and prognosis of cancer patients.

B cell-specific moloney murine leukemia virus integration site 1 (Bmi-1) was originally identified as a c-myc cooperating oncogene in murine lymphomas [12]. Bmi-1 is one of the core subunits of the Polycomb group (PcG) complex, which can modify chromatin structure and thereby regulates the transcription of a number of important genes, such as the Ink4a/ARF locus, a gene code of two important tumor suppressor proteins p16Ink4a and p14ARF [13]. Bmi-1 alone or in coordination with other molecules can induce malignant transformation in various types of non-tumorigenic immortalized cell lines [14], whereas silencing endogenous Bmi-1 expression can cause apoptosis and/or senescence of tumor cells [15]. Recent studies have shown that Bmi-1 was overexpressed in NSCLC tissues, and moreover, the level of Bmi-1 was closely associated with the development of NSCLC [16, 17]. This suggests that Bmi-1 might be one of the most promising tumor biomarkers for the diagnosis and prognosis of NSCLC. Although circulating Bmi-1 mRNA has been detected in plasma/serum of patients with breast cancer [9], gastric cancer [18] and colorectal cancer [19], little is known about the relationship between circulating Bmi-1 mRNA and clinical features in NSCLC. In order to gain further insight about the significance of circulating Bmi-1 mRNA in NSCLC, Bmi-1 mRNA levels were detected in plasma from patients with NSCLC and healthy controls, and its diagnostic and prognostic values for NSCLC were analyzed.

Materials and methods

Patients and samples

Between May 2007 and December 2010, a total of 108 cases of newly diagnosed NSCLC were enrolled in this study from department of Thoracic Surgery in Shandong Provincial Chest Hospital (Jinan, China). Histological diagnosis and tumor grading was established according to the World Health Organisation classification of lung tumors. Tumor staging was performed in agreement with the 7th lung cancer TNM classification and staging system. Details of the backgrounds and clinicopathological characteristics of the patients with NSCLC are summarized in **Table 1**. A total of 40 sex- and age-matched healthy subjects were collected as the healthy controls and each subject had no prior diagnosis of any other malignancy. Blood samples were collected before any therapeutic proce-

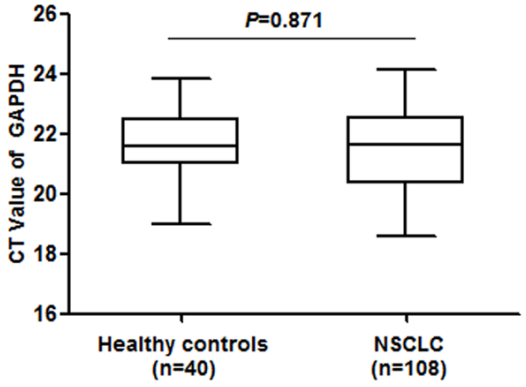

Figure 1. Ct values of GAPDH between NSCLC (n=108) and healthy controls (n=40).

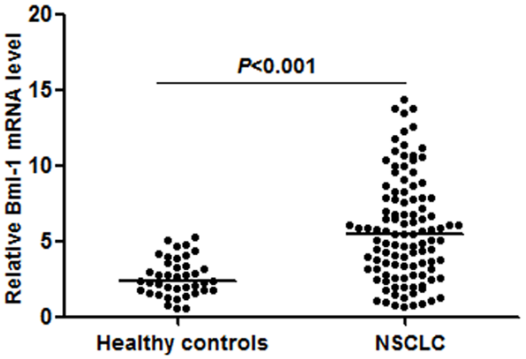

Figure 2. Comparison of plasma levels of circulating Bmi-1 mRNA in NSCLC (n=108) and healthy controls (n=40). Expression levels of Bmi-1 are normalized to GAPDH. The line represents the median value.

dures, including surgery, chemotherapy and radiotherapy. This study was approved by the Ethics Committee of Shandong Provincial Chest Hospital (Jinan, China), and each participant provided signed informed consent.

All NSCLC patients have been followed up at intervals of 3 months during the first 2 years and 6 months up to the fifth year, and the date of latest record retrieved was January 31, 2016.

Samples processing and RNA extraction

Blood samples were obtained by vena puncture from all participants, then collected into EDTA tubes and processed within 1 hr. The plasma was separated by centrifugation at 1,200 g for 10 min at 4°C followed by a second centrifugation at 12,000 g for 10 min at 4°C to completely remove cellular components, and then stored at -80°C until use. Total RNA was extracted from 400 μl of plasma using the mirVana PARIS kit (Ambion, Austin, TX, USA), and eluted into 100 μl of pre-heated (95°C) elution solution according to the manufacturer's directions.

Reverse transcription quantitative real-time PCR (RT-qPCR)

First-strand cDNA was generated from 50 ng of RNA in triplicates using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Subsequently, qPCR was performed in triplicate on a 7500 real-time PCR system (Applied Biosystems, USA) and the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, melting curve analyses were performed in order to validate the specific generation of the expected PCR product. The expression levels of Bmi-1 were normalized relative to the expression of GAPDH, and were calculated using the $2^{-\Delta\Delta Ct}$ method [20].

Serum CEA and CYFRA21-1 assay

The serum levels of CEA and CYFRA21-1 are generally used in the diagnostics of NSCLC. In this study, CEA and CYFRA21-1 were measured by the chemiluminescent enzyme immunoassay using Roche Cobas e601 Analyzer (Roche AG, Germany). The upper limits of 5 ng/ml for CEA and 3.3 ng/ml for CYFRA21-1 were used to define normal values as recommended by the manufacturers.

Statistical analysis

The Mann-Whitney U test or the Kruskal-Wallis test was performed to compare circulating Bmi-1 mRNA levels between groups. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the diagnostic performance of circulating Bmi-1 mRNA for the detection of NSCLC. Survival curves were carried out by the Kaplan-Meier method and compared with the log-rank test. The influence of each variable on survival was examined by the Cox proportional hazards regression model analysis. All statistical analyses were carried out by using SPSS 13.0 software (IBM, USA). A *P* value less than 0.05 was considered statistically significant.

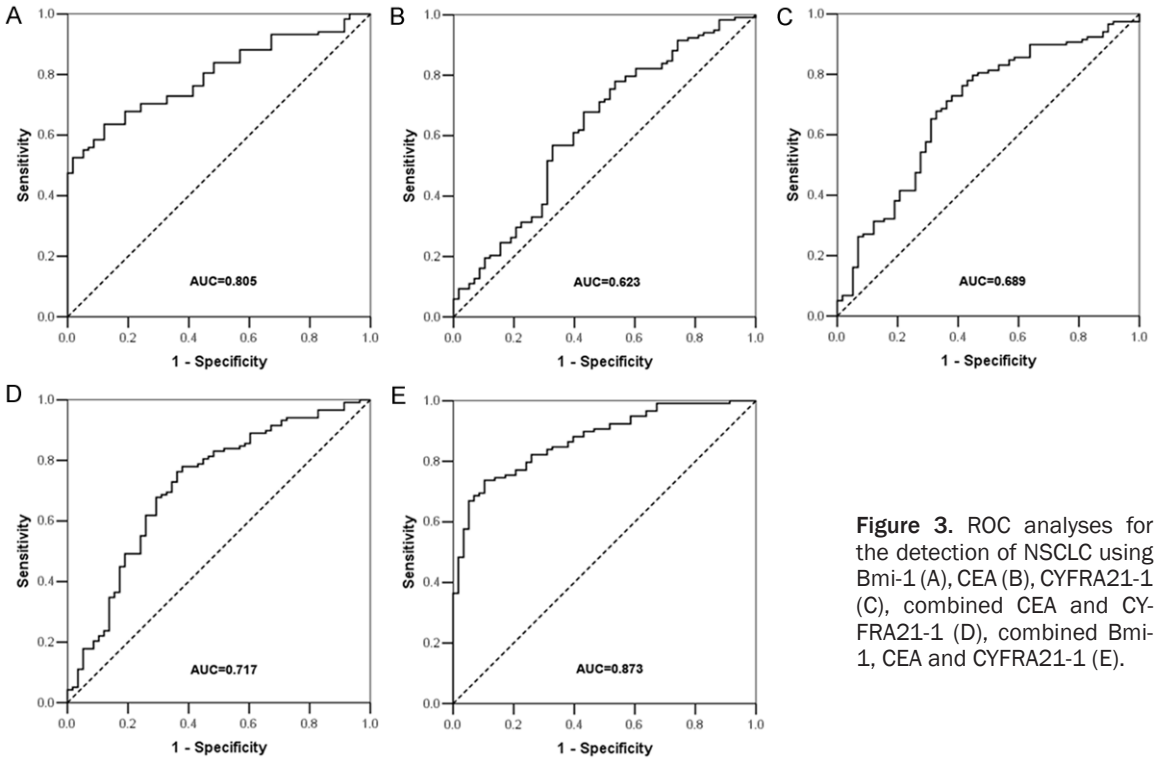

Figure 3. ROC analyses for the detection of NSCLC using Bmi-1 (A), CEA (B), CYFRA21-1 (C), combined CEA and CYFRA21-1 (D), combined Bmi-1, CEA and CYFRA21-1 (E).

Results

Quantitative analysis of circulating Bmi-1 mRNA in plasma

Circulating GAPDH mRNA was readily detectable in plasma of all subjects involved in this study, and no significant difference was observed in terms of Ct values of GAPDH (**Figure 1**, *P*=0.871) between patients with NSCLC (n=108) and healthy controls (n=40), indicating that GAPDH constitutively expressed in plasma regardless of different disease conditions and could be used as a suitable internal control for normalizing target circulating mRNAs. Levels of circulating Bmi-1 mRNA were detected by RT-qPCR in plasma of 108 NSCLC patients and 40 healthy controls. Our data indicated that the levels of circulating Bmi-1 mRNA were significantly higher in NSCLC compared with healthy controls (**Figure 2**, *P*<0.001).

Circulating Bmi-1 mRNA correlates with clinicopathological features of NSCLC

The correlation between circulating Bmi-1 mRNA and various clinicopathological features in NSCLC was evaluated. These data are summarized in **Table 1**. The level of circulating Bmi-1 mRNA expression was observed to be closely correlated with tumor size (*P*=0.038) and clinical stage (*P*=0.025). In contrast, there was no correlation between circulating Bmi-1 mRNA expression and other clinical factors, such as gender, age, smoking status, histological type, cell differentiation, lymph nodes metastasis and distant metastasis (all at *P*> 0.05, respectively). Larger tumor size or advanced clinical stage correlated with higher levels of circulating Bmi-1 mRNA.

Diagnostic value of circulating Bmi-1 mRNA for NSCLC

ROC curve analyses illustrated that circulating Bmi-1 mRNA was a potential biomarker for differentiating NSCLC from healthy controls with an AUC of 0.805 (**Figure 3A**). At the optimal cutoff value of 3.12 (relative expression in comparison with GAPDH), the sensitivity was 72.9% and the specificity was 69.1%. To compare the diagnostic value between circulating Bmi-1 mRNA and the other tumor markers on the diagnosis of NSCLC, two conventional tumor markers, CEA and CYFRA21-1, were also measured in all subjects. At a cutoff value of 5 for CEA expression level, the sensitivity and specificity for NSCLC were 65.3% and 56.9%, respec-

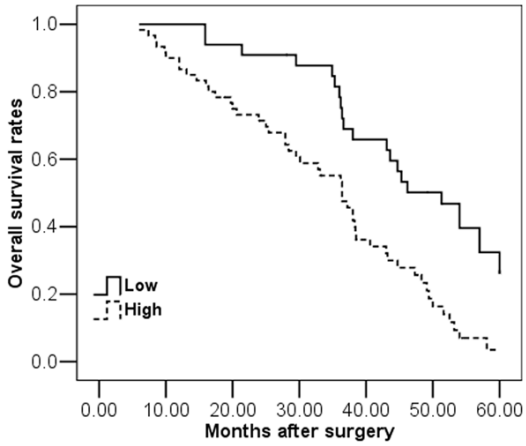

Figure 4. Kaplan-Meier curves for overall survival according to the circulating Bmi-1 mRNA levels. The optimal cutoff value of circulating Bmi-1 mRNA (3.12) was used to categorize the NSCLC patients into high or low level group.

tively. At a cutoff value of 3.3 for CYFRA21-1 expression level, the sensitivity and specificity for NSCLC were 69.5% and 63.8%, respectively. The AUC for circulating Bmi-1 mRNA in the detection of NSCLC was significantly larger than that for CEA (0.623) (**Figure 3B**) or CYFRA21-1 (0.689) (**Figure 3C**), or combination (0.717) (**Figure 3D**), indicating that circulating Bmi-1 mRNA might be a more reliable biomarker than CEA or CYFRA21-1 in discriminating between NSCLC and cancer-free cases. Moreover, ROC curves analysis showed that the AUC for the combination of circulating Bmi-1 mRNA, CEA and CYFRA21-1 was 0.873 (**Figure 3E**), which was significantly larger than that for circulating Bmi-1 mRNA alone (P=0.013).

Correlation between circulating Bmi-1 mRNA and prognosis in NSCLC patients

All 108 patients with NSCLC were received follow up analysis, with a median follow-up duration of 35 months (range 6-60 months). The optimal cutoff value of circulating Bmi-1 mRNA (3.12) was used to categorize patients with NSCLC into high or low level group. The prognostic value of circulating Bmi-1 mRNA expression was investigated using the Kaplan-Meier method and log-rank test. The results indicated that the cumulative 5-year overall survival rate of NSCLC patients with high circulating Bmi-1 mRNA expression was significantly lower than that of those patients with low circulating Bmi-1 mRNA expression (**Figure 4**, P=0.028, log-rank test). Univariate Cox proportional hazards regressions model analysis revealed a statistically significant correlation between overall survival and circulating Bmi-1 mRNA level (P=0.011), cell differentiation (P=0.045), lymph node metastasis (P=0.021), distant metastasis (P=0.009) and clinical stage (P=0.035) (**Table 2**). No significant correlations were found for gender, age, smoking status, histological type, tumor size and patient outcome. Subsequently, parameters significantly related to survival in the univariate analysis were put into the multivariate analysis to identify the independent factors for prognoses. It confirmed that circulating Bmi-1 mRNA level (P=0.027), distant metastasis (P=0.018) and clinical stage (P=0.043) were independent prognostic factors for patients with NSCLC (**Table 2**).

Discussion

In the present study, we found that the levels of circulating Bmi-1 mRNA were significantly elevated in the plasma of patients with NSCLC. Compared with conventional tumor markers, such as CEA and CYFRA21-1, circulating Bmi-1 mRNA demonstrated as a more appropriate marker for the detection of NSCLC. Moreover, circulating Bmi-1 mRNA level was identified as an independent factor for poor prognosis of NSCLC.

The development and progression of NSCLC involves a series of genetic events including abnormal activation of oncogenes and metastasis-related genes as well as inactivation of tumor suppressors. As a key member of the PcG complex, Bmi-1 acts as a transcriptional repressor by organizing the chromatin into an inaccessible structure that cannot bind transcription factors, which participates in axial patterning, hematopoiesis, cell cycle regulation, and senescence [21-23]. Studies showed that Bmi-1 expression was significantly upregulated in NSCLC tissues and cell lines and that Bmi-1 may be involved in the carcinogenesis of NSCLC by repressing the INK4a/ARF pathway [24, 25]. Down regulation of Bmi-1 could inhibit the proliferation, colony formation, invasiveness and migration of NSCLC cell lines [26], suggesting Bmi-1 might play an important role in the tumorigenesis and progression of NSCLC. In the present study, our results demonstrated

Table 2. Univariate and multivariate analysis of overall survival in NSCLC patients (n=108)

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender	1.698 (0.677-2.233)	0.455		
Age	1.239 (0.605-1.864)	0.628		
Smoking status	1.841 (0.852-2.293)	0.186		
Histotype	0.959 (0.697-1.485)	0.378		
Cell differentiation	1.633 (0.853-2.243)	0.045*	1.227 (0.572-2.186)	0.282
Tumor size	1.375 (0.799-2.161)	0.069		
Lymph node metastasis	3.413 (2.392-6.285)	0.021*	2.653 (0.976-5.395)	0.137
Distant metastasis	1.992 (0.873-2.764)	0.009*	1.376 (0.731-2.403)	0.018*
Clinical stage	3.251 (1.687-5.452)	0.035*	1.921 (0.974-3.056)	0.043*
Circulating Bmi-1 mRNA level	2.231 (1.158-4.476)	0.011*	2.405 (1.686-4.517)	0.027*

HR hazard ratio, CI confidence interval, *P<0.05.

that the levels of circulating Bmi-1 mRNA in patients with NSCLC were significantly elevated compared with healthy controls, which was in line with the results found in NSCLC tissues based on immunohistostaining [16]. In addition, the increased expression of circulating Bmi-1 mRNA was significantly associated with larger tumor size and advanced clinical stage, suggesting that the dysregulated expression of Bmi-1 might be an early event of NSCLC malignancy, and that a higher level of Bmi-1 expression might be related to advance NSCLC. Nonetheless, the precise mechanisms of Bmi-1, which induce adverse clinical characteristics in NSCLC, need further study.

Numerous soluble tumor-associated markers in plasma/serum have been used in the diagnosis of NSCLC, with CEA and CYFRA21-1 being the most widely used ones [27, 28]. However, their low sensitivity and specificity in blood tests limited the further application in screening of high-risk individuals and early detection of NSCLC. In the present study, we describe that circulating Bmi-1 mRNA can be used to effectively discriminate NSCLC patients from healthy controls, with 72.9% sensitivity and 69.1% specificity. Furthermore, the AUC for circulating Bmi-1 mRNA showed higher diagnosis capability than that for CEA or CYFRA21-1, alone or combined. Based on these findings, circulating Bmi-1 mRNA could possibly be used as a less expensive and more reliable alternative to the combination CEA and CYFRA21-1 in the diagnosis of NSCLC. We were also tempted to analyze combined circulating Bmi-1 mRNA, CEA and CYFRA21-1 to improve the detection capability of NSCLC. The AUC for this combination was significantly higher than that for circulating Bmi-1 mRNA alone, indicating the combination of circulating Bmi-1 mRNA, CEA and CYFRA21-1 might improve the diagnostic performance in NSCLC, indicating circulating Bmi-1 mRNA might be an effective complement to current NSCLC detection strategy.

Previously, several clinical studies provided the proof that increased Bmi-1 expression in situ was significantly associated with poor prognoses in various types of human cancers, such as pancreatic cancer [29], cervical cancer [30], gastric carcinoma [31] and nasopharyngeal carcinoma [32]. However, Hayry et al. showed that negative Bmi-1 expression might serve as a marker of poor prognosis in oral tongue carcinoma patients [33], suggesting that Bmi-1 might play variable roles in the pathogenesis of different kinds of cancers. To identify the potential prognostic value of circulating Bmi-1 mRNA for NSCLC, the correlations between circulating Bmi-1 mRNA level and survival were firstly evaluated. From Kaplan-Meier survival analysis, patients with NSCLC who had high circulating Bmi-1 mRNA levels had poorer overall survival rate compared with those with low levels. Taking a further step, Cox proportional hazards regression model analyses revealed that circulating Bmi-1 mRNA level was an independent factor influencing overall survival. Our results are consistent with the previous studies that had demonstrated the potential of tissue Bmi-1 as a prognostic marker for NSCLC [16, 17]. In this respect, circulating Bmi-1 mRNA level may serve to identify patients at higher risk or lower

risk in a similar way to classical prognostic factors such as distant metastasis or clinical stage. Furthermore, technically speaking, plasma is more convenient and less invasive, which appears to be an ideal source of biomarkers for detecting diseases.

In conclusion, our results provide evidence for the potential usefulness of circulating Bmi-1 mRNA in plasma as a noninvasive diagnosis and prognostic tool in patients with NSCLC. Further multi-center study that included a higher number of patients collected from several hospitals, or even diverse ethnic populations are needed to validate whether it can be incorporated into routine clinical practice.

Disclosure of conflict of interest

None.

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