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Serum microRNA-16 as a potential biomarker for HCV-induced hepato-cellular carcinoma in Egyptian patients

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in the world. Two risk factors that cause 80–90% of HCC cases globally are chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV). The diagnostic value of circulating microRNAs (miRNAs) in numerous tumors has been described. Our research assessed microRNA-16 (miR-16) as a novel biomarker in patients with HCV-induced HCC. The study included three groups. Group 1 included 55 individuals with cirrhosis caused by liver HCV infection in addition to HCC. Group 2 included 55 individuals with cirrhosis brought on by HCV infection. Group 3 included 55 normal control individuals. Expression of miR-16 in blood was assessed by real-time polymerase chain reaction (RT-PCR). The mean level of miR-16 was significantly different in the three groups, with group 1 having the greatest value (1.098 ± 0.647), followed by group 2 (1.1035 ± 0.8567) and group 3 (control subjects) having the lowest value (0.3842 ± 0.21485). The receiver operating characteristic (ROC) curve analysis showed that miR-16 had a higher diagnostic value at area under the curve (AUC) of 0.935 than alpha-feto protein (AUC of 0.859) to differentiate between HCC and control subjects. MiR-16 has a sensitivity of 81.82 % and a specificity of 69.09%, to distinguish between patients with liver cirrhosis and HCC patients. Our findings illustrated that circulating miR-16 can be proposed as a marker for detection of patients with HCV-induced HCC.

Keywords: hepatocellular carcinoma, microRNA-16, biomarker, HCV

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common forms of cancer in people around the globe.[1] Due to both its morbidity and mortality, HCC has grown more and more dangerous.[2] Chronic liver inflammation brought on by hepatitis C virus (HCV) infection causes a number of changes, including the creation of steatosis, fibrosis, cirrhosis, and ultimately

HCC.[1] Alpha-feto protein (AFP) is utilized as a marker for diagnosis of HCC and to predict prognosis; however, when used alone, it has a 40% false-negative rate for patients with preliminary HCC. Even in patients with advanced HCC, 15–30% of patients may still have normal results.[3,4]

Only one-third of patients with new diagnoses can get curative therapy, despite significant advancements in the diagnostic management of HCC. The development of novel biomarkers has been facilitated by technological advancements and a better understanding of the biology of HCC.[5] A significant type of non-coding RNAs known as miRNAs is thought to play a role in the development of HCC by serving as tumor oncogenes or suppressors.[6] They are naturally occurring nucleotides which can be found in intracellular and extracellular areas such as the blood, saliva, and urine.[7,8]

Numerous research studies have recently found that miR-16 plays a role in HCC, but the results are often debatable.[9,10] MicroRNA-16 (miR-16) is thought to function as a tumor suppressor and is down-regulated in a range of human malignancies, including HCC.[9] However, in a different investigation, the scientists reported that in the HCC studied group the blood level of miR-16 was significantly greater than its level in healthy subjects.[10]

The role of this circulating miRNA in liver illnesses is currently receiving more attention from researchers, yet the reported findings of diverse studies are contradictory. Additionally, the majority of the examined indicators can be utilized to distinguish between HCC patients and healthy individuals, but not for the distinction between HCC and high-risk populations, such as individuals with liver cirrhosis or other liver diseases.[11-13] Therefore, further experimental data should be gathered to support the role of miR-16 as a biomarker. Consequently, our study aimed to investigate the likelihood of serum miR-16 as a diagnostic biomarker by assessing miR-16 expression levels in the serum of HCC patients, patients with cirrhosis and normal participants and analyzing the diagnostic effectiveness of this miRNA.

Subjects and Methods

This case-control study included 165 Egyptians. The participants were divided into 3 groups: Group 1 (HCV-induced HCC group) included 55 patients; group 2 (HCV-induced liver cirrhosis group) included 55 patients. The diagnosis of liver cirrhosis and the identification of HCC were made using abdominal ultrasonography. Triphasic computed topography was used to verify HCC. And group 3 (control group) included 55 apparently healthy individuals. We excluded people who had hepatitis B virus infection, significant alcohol use, liver cirrhosis caused by factors other than HCV infection, hepatorenal syndrome, hepatic encephalopathy, spontaneous bacterial peritonitis, or coinfection with human immunodeficiency viruses.

All patients received a thorough screening process that included a medical history check, a physical exam, and a biochemical profile that included complete blood count, a coagulation profile, liver and kidney function tests and a serum AFP level. Such data were obtained from patients' records at the hospital.

Assessment of miR-16 in serum samples

A peripheral blood sample, approximately 10 ml was obtained from each study subject by venipuncture. To separate the serum, a blood aliquot was spun at 1000 x g for 10 min. The separated serum was stored at -20 ºC. Another blood aliquot was centrifuged for 10 minutes at 14,000 x g, and the separated serum was stored at -80 °C pending for RNA extraction.

The reverse transcription polymerase chain reaction (RT-PCR) was used to identify and measure miR-16 in serum samples from all participants. The miRNA was extracted by using the miRNA Serum/Plasma Kits (Qiagen, Germany), according to the manufacturer's instructions. The extracted RNA was used to generate complementary DNA (cDNA) using the reverse transcription process.

The extracted microRNA was reversely transcribed using commercial kits (catalog number 4366596, TaqMan® MicroRNA Reverse Transcription kits, Applied Biosystems, Thermo

Fisher Scientific, USA), according to manufacturer's instructions, with the following conditions: incubation at 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and then maintained at 4°C. Real-time PCR was conducted using MicroRNA Assay Kits and TaqMan Universal Master Mix II, (catalog number 4440040, Applied Biosystems), with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles each of 95°C for 15 seconds and 60°C for 1 minute. The internal control used was the hsa-miR-21-5p.[14] The 2−ΔΔCt (cycle threshold) method was used to determine the relative miRNA expression.[15]

Statistical Analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 20.0. (Armonk, NY: IBM Corp). To confirm the distribution's normalcy, the Shapiro-Wilk test was used. The mean, standard deviation, and range were the metrics used to present quantitative statistics. Frequency and percentages were used to define qualitative factors. Independent sample t-tests and Mann-Whitney tests were used to evaluate the independent groups. The link between clinical variables was also examined using the Pearson chi-square test. The correlation number r was calculated using Pearson correlation. The receiver operating characteristic (ROC) curve was used to determine the best cutoff value for the parameters under study. The threshold for statistical significance was considered at a *p*-value of ≤0.05.

Results

Demographic and laboratory data of the study participants

There were 20 females and 35 males in the HCC group (group 1), and their mean age was 55.28 ± 4.07 years. In the cirrhosis group (group 2), there were 20 males and 35 females. Their mean age was 55.23 ± 5.68 years. The control group (group 3) consisted of 30 males and 25 females, and their age was 54.48 ± 7.89 years.

The lowest mean hemoglobin value was observed in group 1 (9.57 ± 1.53), followed by group 2 (9.63 ± 0.86) and the highest level in group 3 (12.5 ± 1.83). The mean level of hemoglobin was significantly lower in the HCC group as opposed to the cirrhosis group ($p< 0.001$). The mean level of the platelet count was significantly lower in cirrhosis group as opposed to HCC group ($p < 0.001$). The lowest platelet count was observed in group 2 (77.61 ± 14.4), followed by group 1 (99.41 ± 20.5) and the greatest value in group 3 (354.21 ± 47.2) as shown in Table 1.

Our findings revealed that the HCC group had considerably higher levels of alanine transaminase (ALT) and aspartate transaminase (AST) compared to the other groups (Table 1). However, levels of total bilirubin, direct bilirubin, and the international normalized ratio (INR) were significantly higher in the cirrhosis group than in the other groups. However, the mean albumin level in the cirrhosis group was much lower than in the other groups.

The mean alpha-fetoprotein was significantly greater among HCC patients as opposed to both patients with cirrhosis and the control subjects (44.1871± 53.94705, 6.9182± 3.09863 and 4.9971± 1.13465, respectively) as shown in Table 2. However, when performing the ROC curve analysis (Figure 1), the sensitivity of AFP in HCC patients compared with the control group was 76.36 % and the specificity was 100 % with an area under the curve (AUC) of 0.859 (95% CI 0.780 to 0.918, $p< 0.001$).

Table 1. Laboratory data of the studied patients.

Variable	Group1 (mean ± SD)	Group2 (mean ± SD)	Group3 (mean ± SD)	*p* value (F test: ANOVA)	LSD
Hemoglobin (g/dl)	9.57 ± 1.53	9.63 ± 0.86	12.5 ± 1.83	<0.001	>0.05[1] <0.05[2] <0.05[3]
WBCs (/cmm)	7.27 ± 1.40	7.59 ± 1.52	7.21 ± 1.91	NS	>0.05[1] >0.05[2] >0.05[3]
Platelets (/cmm)	99.41 ± 20.5	77.61 ± 14.4	354.21 ± 47.2	<0.001	>0.05[1] <0.05[2] <0.05[3]
Total bilirubin (mg/dl)	3.581 ± 0.87	4.18 ± 0.90	1.16 ± 0.15	<0.001	>0.05[1] <0.05[2] <0.05[3]
Direct bilirubin (mg/dl)	2.51 ± 0.50	2.91 ± 0.40	0.241 ± 0.05	<0.001	>0.05[1] <0.05[2] <0.05[3]
Total Protein (g/dl)	5.95 ± 0.41	6.02 ± 0.55	6.61± 0.32	<0.001	>0.05[1] <0.05[2] <0.05[3]
Albumin (mg/dl)	2.591 ± 0.54	2.51 ± 0.48	3.75 ± 1.30	<0.001	>0.05[1] <0.05[2] <0.05[3]
ALT (IU/L)	58.17 ± 13.4	37.15 ± 9.5	22.16 ± 6.48	<0.001	>0.05[1] <0.05[2] >0.05[3]
AST (IU/L)	66.15 ± 12.5	42.17 ±9.70	19.11 ± 4.40	<0.001	<0.05[1] <0.05[2] <0.05[3]
INR	1.29 ± 0.26	1.47 ± 0.18	1.08 ± 0.12	<0.001	<0.05[1] <0.05[2] <0.05[3]

Group 1 (HCV-induced HCC group); group 2 (HCV-induced liver cirrhosis group); group 3 (control group); F test: ANOVA; LSD: Least-significant difference post-hoc test. LSD1: HCC group versus cirrhosis group; LSD2: HCC group versus control group; LSD3: cirrhosis group versus control group; $p > 0.05$ is not significant (NS).

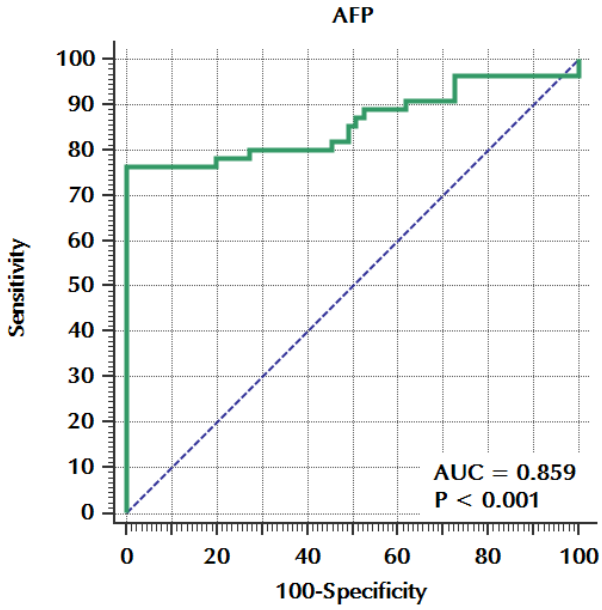

Figure 1. Receiver operating characteristic (ROC) curve analysis using alpha-fetoprotein (AFP) for differentiation between hepatitis C virus (HCV)-induced Hepatocellular carcinoma (HCC) group and the control group.

Differential expression of miR-16 between the studied groups

Our findings demonstrated that the mean serum expression level of miR-16 in HCC patients was significantly greater than in cirrhotic patients and controls ($p < 0.001$). The highest value was observed in the HCC group 1 (1.098 ± 0.647), then in the cirrhosis group 2 (1.1035 ± 0.8567) and the lowest in the control group 3 (0.3842 ± 0.21485) as shown in Table 2.

Table 2. Comparison of alpha-fetoprotein (AFP) and miRNA-16 between the studied groups.

	Group 1 (N=55)	Group2 (N=55)	Group3 (N=55)	*p1*	*p2*	*p3*	*p4*
AFP							
Mean±SD	4.99±1.13	6.91±3.09	44.18± 43.94	<0.001[a]	<0.001[b]	<0.001[b]	<0.001[b]
Median (Range)	4.99 (3.21–6.92)	5.87 (2.78 – 21.91)	50.06 (3.02 – 295.86)				
miRNA-16							
Mean±SD	0.38±0.21	1.10±0.85	1.09±0.64	<0.001[a]	<0.001[b]	<0.001[b]	<0.001[b]
Median (Range)	0.34 (0.13 – 1.09)	0.83 (0.29 – 3.98)	1.05 (0.30 – 5.36)				

Group 1 (HCV-induced HCC group); group 2 (HCV-induced liver cirrhosis group); group 3 (control group); N=Total number of patients; Qualitative data were expressed as a mean±SD and Median (Range); *p*-value1: between the three groups; *p*-value2: between the control group and HCV-induced HCC group; p-value3: between HCV-induced liver cirrhosis group and HCV-induced HCC group; *p*-value4: between the control group and HCV-induced liver cirrhosis group; a: Kruskal Wallis H test; b: Mann Whitney U test; *p*-value<0.05 is significant.

Analysis of the miR-16's diagnostic utility in hepatocellular carcinoma patients

The ROC curve analysis showed that the miR-16 had a sensitivity of 87.27%, and specificity of 92.73%, to differentiate between control individuals and HCC patients with an AUC of 0.935 (Figure 2 and Table 3). While for differentiation between HCC group and liver cirrhosis group, the miR-16 had a sensitivity of 81.82%, specificity of 69.09%, at AUC of 0.700 (Figure 3).

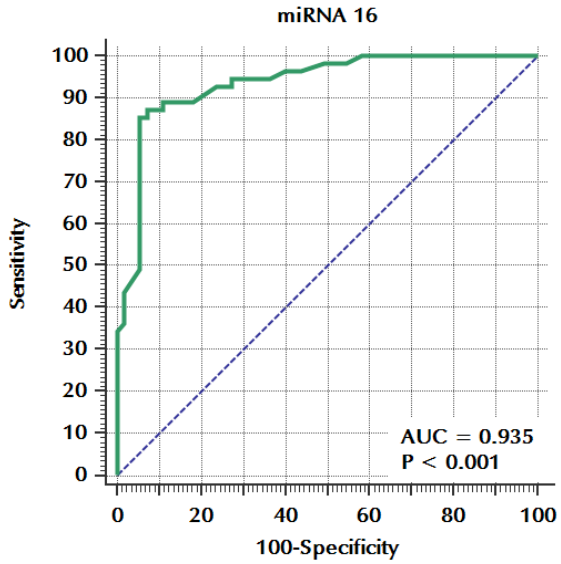

Figure 2. Receiver operating characteristic (ROC) curve analysis using miRNA-16 for differentiation between hepatitis C virus (HCV)-induced Hepatocellular carcinoma (HCC) group and the control group.

Table 3. Area under curve (AUC) and corresponding sensitivity and specificity of miRNA-16 and alpha-fetoprotein (AFP) in HCC patients compared with the control group.

Variable	AUC	Sensitivity	Specificity	SE	*p* value	95% Confidence Interval	
						Lower Bound	Upper Bound
MiR-16	0.935	87.27	92.73	0.0233	<0.001	0.872	0.973
AFP	0.859	76.36	100.00	0.0392	<0.001	0.780	0.918

AUC, area under curve; SE, standard error; *p*-value<0.05 is significant.

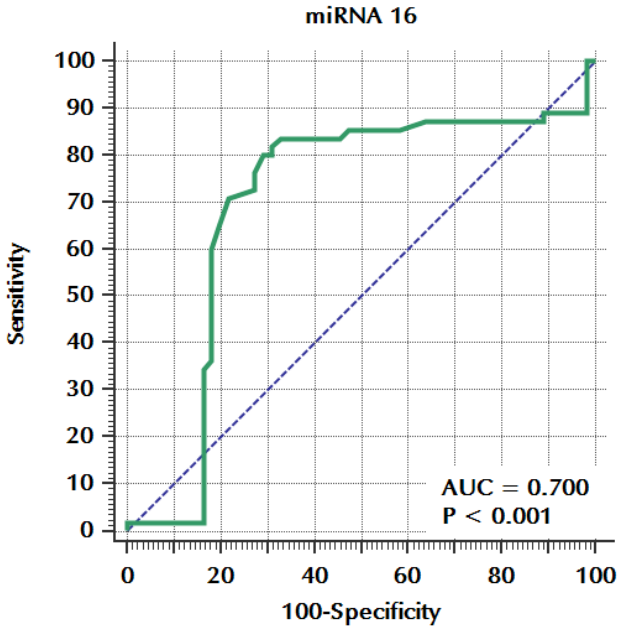

Figure 3. Receiver operating characteristic (ROC) curve analysis using miRNA-16 for detection of hepatocellular carcinoma (HCC) group from liver cirrhosis group.

Discussion

Infection with the HCV is regarded as a severe universal health hazard. In addition to the 350,000 HCV-related disorders, it has been estimated that more than 80 million people globally have chronic HCV infection and that 3–4 million new instances of infection occur each year.[16] Egypt is recognized as the nation with the greatest prevalence of HCV infection worldwide, with genotype 4 being the most prevalent.[16,17] HCC is typically brought on by HCV infection and develops after liver fibrosis and cirrhosis.[18]

HCC is a significant global health problem that is characterized by a wide range of prognoses as well as biochemical and clinical variability which resulted in various therapeutic philosophies.[19] HCC is still one of the most aggressive types of human cancer worldwide with a very dismal prognosis. Since an early surgical treatment can improve the patient's prognosis, an early identification of HCC is very desirable clinically. AFP is currently one of the principal biomarkers used in clinical practice to diagnose primary HCC, however its sensitivity is unsatisfactory.[20] Consequently, it is vital to determine novel non-invasive biomarkers for the early identification of HCC.

The vast majority of studies emphasized the detection of polypeptides and whole proteins.[21-23] However, due to their relatively low sensitivity and overly complex processes, protein detection techniques are more challenging for clinical analysis when compared to qPCR-based analysis.[24] Recent research studies indicated that serum miRNA has a great level of stability and tissue specificity.[25] [26] Our findings revealed that HCC patients had higher levels of miR-16 than apparently healthy control subjects. Such findings agreed with those of Mourad et al., 2018,[10] Fang et al. 2022,[27] and Huang et al. 2009,[28] who reported that miR-16 had significantly higher level in patients with HCC as opposed to control individuals.

However, the study by Qu et al. 2011 stated that miR-16 was diminished in HCC patients, even though HCV infection was the primary cause of HCC in their research participants.[29] The study by Ge et al., 2014, also demonstrated that miR-16 was significantly decreased in the blood of HCC patients. Together with let-7f and miR-21, these biomarkers could be utilized to estimate the size of the tumor as well as its propensity to recur.[30] In his study, Cheng et al., 2019, stated that miR-16 inhibited the malignant cells from invasion and migration, via suppressing the insulin-like growth factor 1 receptor (IGF1R) protein expression.[31]

According to our findings, miR-16 has a diagnostic value in separating HCC from the control subjects. The ROC curve analysis showed that miR-16 has a great diagnostic efficacy (AUC = 0.935 with sensitivity of 87.27% and specificity of 92.73%). These results are consistent with those of the study by Fang et al., 2022,[27] who found that it has a valuable diagnostic value in distinguishing HCC from control subjects with sensitivity of 91% and specificity of 58%.

International recommendations stated that those who have liver cirrhosis are at a higher risk of getting hepatic cancer. They must undergo abdominal ultrasound surveillance every six months, with or without AFP.[32] Through our analysis, miR-16 levels in patients with liver cancer and patients with liver cirrhosis were considerably different. The effectiveness of miR-16 in monitoring the risk of hepatic cancer in individuals with liver cirrhosis was investigated using ROC curve analysis. According to our findings, miR-16 can efficiently differentiate between individuals with liver cirrhosis and HCC as it has sensitivity of 81.82% and specificity of 69.09%, with an AUC value of 0.700 and 95% CI values of 0.605 to 0.783 (p=0.001) (Figure 2). These results agreed with those of a study by Fang et al., 2022,27 who reported that miR-16 has a strong diagnostic value for differentiating HCC from liver cirrhosis patients at AUC of 0.936.

Regarding the mechanism by which miR-16 can mediate the occurrence of HCC, Reis et al., 2020, reported that miR-16 is anticipated to regulate pathways with significant functions in cancer, such as PI3K/AKT signaling pathway.[33] Additionally, the study by Fang et al., 2022, stated that miR-16 is very likely to target the PI3K/Akt, mitogen-activated protein kinase (MAPK), forkhead box O (FOXO), and transforming growth factor (TGF)-beta signaling

pathways in order to cause HCC.[27] However, more research is needed to confirm the relation between miR-16 and such signaling pathways.

Our results demonstrated a statistically significant difference between the study groups concerning AFP which was found to be significantly higher among HCC patients as opposed to healthy individuals and patients with liver cirrhosis (p<0.001). These findings matched those of other research studies carried out by Tsai et al., 2014, Sacco et al., 2014, Changchien et al., 2008, and Chen et al., 2006.[34-37] For AFP to distinguish HCC cases from control subjects, the ROC curve analysis showed that at AUC of 0.859, the sensitivity and specificity were 76.36% and 100% (95% CI 93.5-100, p< 0.001.).

Our results demonstrated that the serum miR-16 has a significant AUC over AFP. Many HCC patients were found not to have elevated serum AFP levels during the clinical diagnosis and treatment processes.[8] In our study, data in Table 3 shows that AFP had a sensitivity of 76.36% for diagnosing HCC, which was lower than miR-16's sensitivity (87.27%). These findings may demonstrate the significance of circulating miR-16 for the diagnosis of liver carcinoma.

In our study, HCC patients had significantly higher platelet counts than patients with liver cirrhosis. This difference could be attributed to the para malignant syndrome that is associated with HCC or the notion that HCC can produce thrombopoietin, a key component in platelet production.[39] According to a previous report, platelets interact with cancer cells and aid in their proliferation.[40] In their work, Yilmaz and Erdal, 2016, concluded that platelet count has a valuable predictive value in HCC and can be employed in screening programs as well as to predict the prognosis of the disease.[41]

We found a statistically significant variation in the mean value of AST and INR in the HCC group compared to the liver cirrhosis group. The ongoing damage to the liver tissue brought on by the growth, invasion, and eventual loss of functional liver cells in HCC patients can be explained by the elevation of AST. According to Carr et al., 2016,[42] patients with HCC had significantly greater aggressiveness scores with higher levels of gamma-glutamyl transferase (GGT), ALP, AST, total bilirubin, and platelets. Additionally, higher aggressiveness score for HCC was found to be connected to higher AFP levels, tumor multifocality, and a larger incidence of portal vein thrombosis.[43]

In conclusion, our study observed that miR-16 has a higher sensitivity and lower specificity than alpha-fetoprotein. Such findings indicate that miR-16 could be a potential biomarker for the detection of patients with HCC linked to HCV.

Author Contributions

ATEH; Study design, Sample processing and Manuscript review. FN; Manuscript preparation and Manuscript review. REH; Sample processing and Manuscript review. HKAM; Sample collection and Manuscript review. GAA; Manuscript preparation and Manuscript review. MA; Sample collection and Manuscript review.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University (ZU-IRB#9684/14-8-2022).

Informed consent

Each participant signed a written informed consent form before being included in the study.

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