

Oxidored-nitro domain-containing protein 1 expression is associated with the progression of hepatocellular carcinoma

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Abstract. Hepatocarcinogenesis is a stepwise process during which multiple genes are altered. Understanding the molecular mechanisms that induce hepatocarcinogenesis may improve the screening, prevention and treatment of patients with hepatocellular carcinoma (HCC). In recent years, the oxidored-nitro domain-containing protein 1 (NOR1) gene has been identified to have an important role in the development of HCC *in vitro* experiments. The current study aimed to examine the expression of NOR1 mRNA and protein expression in specimens of normal liver, hepatitis, cirrhosis and HCC, together representing the process of HCC development. Furthermore, the association between NOR1 expression and clinicopathological parameters of HCC patients was analyzed. Tissue microarrays containing the specimens of human normal liver, hepatitis, cirrhosis and HCC were purchased, and *in situ* hybridization and immunohistochemistry were used to detect the expression of NOR1 mRNA and protein expression, respectively. It was revealed that the positive rate of NOR1 protein and mRNA expression in the specimens of hepatitis and cirrhosis were not significantly different from that in the normal liver samples. However, the specimens of HCC exhibited an increased positive rate of NOR1 protein and mRNA expression in comparison with the normal liver samples. In addition, a higher positive rate of NOR1 protein expression was observed in HCC patients with a poor pathological

differentiation grade and high tumor node metastasis (TNM) stage. In conclusion, the present study provides evidence, for the first time, of the increased expression of NOR1 in human HCC tissues, and its correlation with the pathological stage and TNM status. These findings indicate that NOR1 may be involved in the progression of HCC and it could be employed as a predictive biomarker in HCC development.

Introduction

Hepatocellular carcinoma (HCC) accounts for 85-90% cases of primary liver cancer (1). It is the fifth most common malignancy and the third leading cause of cancer-related mortality worldwide (1-3). The epidemiological characteristics of liver cancer differ between various countries; for example, ~85% of cases of liver cancer occur in developing countries and 54% occur in China (4). Furthermore, >80% of patients with HCC have a history of hepatitis or cirrhosis, and HCC generally has a poor prognosis (1,5-7). The majority of patients with HCC are present and are diagnosed in the advanced stages of the disease (5), and no effective chemotherapy or radiotherapy exists for the advanced disease. Surgical resection is effective only in the early stages of HCC, however, the 5-year survival rate is as low as 25-39% following surgery (8). It has been demonstrated that multiple genes are altered during the process of hepatocarcinogenesis (9). Understanding the molecular mechanisms that induce hepatocarcinogenesis may improve the screening, prevention and treatment of patients with HCC (10).

Oxidored-nitro domain-containing protein 1 (NOR1) gene is a nitroreductase (NTR) gene that was initially isolated from nasopharyngeal carcinoma (NPC) (11). The NOR1 gene encodes two transcripts and acts as a candidate tumor repressor gene associated with NPC. It has a similar activity to bacterial NTR, which converts 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), a monofunctional alkylating agent, into a toxic form. A previous study supported the hypothesis that NOR1 is involved in the chemical carcinogenesis of hepatic cancer. NOR1 overexpression increased the expression levels of growth factor receptor-bound protein 2 (Grb2) mRNA and protein in HepG2 cells, and activated mitogen-activated protein kinase (MAPK) signal transduction, thus leading to enhanced CB1954-induced cell killing in HepG2 cells (12).

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Furthermore, DNA microarray data suggested that overexpression of NOR1 protein results in altered gene expression profiles in HepG2 cells, including 59 upregulated genes and 103 downregulated genes (13). These findings indicate that the NOR1 gene may have an important role in the development of HCC.

The present study examined, for the first time, the expression levels of NOR1 mRNA and protein in specimens of human normal liver, hepatitis, cirrhosis and HCC, together representing the process of HCC development. In addition, the association, between NOR1 expression and clinicopathological parameters of HCC patients was investigated. The present study may facilitate in elucidating the role of NOR1 expression in liver carcinogenesis. The study was approved by the Ethics Committee of The Third Xiangya Hospital, Central South University (Changsha, Hunan, China).

Materials and methods

Tissue microarrays. Sections (5 μ m) of formalin-fixed, paraffin-embedded tissue microarrays (cat. no. LV20812) were purchased from US Biomax, Inc. (Rockville, MD, USA). The tissue microarray contained 16 samples of normal human liver, 24 samples of hepatitis, 32 samples of cirrhosis and 32 samples of HCC. Sections were arranged in duplicate cores per sample.

In situ hybridization (ISH). The formalin-fixed paraffin-embedded sections were baked at 60°C for 30 min, then deparaffinized by immersing in xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 15 min twice. After immersing in 100% ethanol (Sinopharm Chemical Reagent Co., Ltd.) for 5 min, the slides were air-dried and then incubated with pepsin (Coolaber Technology Co., Ltd., Beijing, China) at 37°C for 30 min. Digoxigenin-labeled RNA probes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences were as follows: 5'-CTAAGTTCTTTGATGATCCAGACATGAGTTTCCACAGGCTGATTCACGCT-3'dig. The probes were diluted in prewarmed hybridization buffer (Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China) to a concentration of 10 ng/ μ l and added to the slides for incubation at 37°C overnight. The slides were washed with saline-sodium citrate and Tris-buffered saline (both Coolaber Technology Co., Ltd.), then incubated with mouse anti-digoxigenin monoclonal antibody (1:200 dilution; cat. no. ab119345; Abcam, Cambridge, MA, USA) at 4°C overnight. For signal development, nitroblue tetrazolium and bromochloroindolyl phosphate were used for staining at 37°C for 30 min, and nuclear fast red dye served as the counterstain at 37°C for 1-2 min (all Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry (IHC). The sections were baked at 60°C for 30 min followed by deparaffinization in xylene and rehydrated with graded ethanol. Antigen retrieval was performed by heating the sections in 0.01 M citrate buffer (Coolaber Technology Co., Ltd.) for 2 min, then adding 3% H₂O₂/methanol solution (Sinopharm Chemical Reagent Co., Ltd.) to quench endogenous peroxidase. The sections were

subsequently incubated with goat anti-NOR1 polyclonal antibody (1:50 dilution; cat. no. sc-161980; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. After washing with 0.1% Tween-20/phosphate-buffered saline (Coolaber Technology Co., Ltd.), Polymer Helper (ZSGB-BIO, Beijing, China) was added to the sections and incubated at room temperature for 15 min. The sections were washed for three times and then incubated with horseradish peroxidase-conjugated anti-goat IgG (1:2,000 dilution; cat. no. PV-9003; ZSGB-BIO) at room temperature for 15 min. The slides were stained using a DAB staining kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) and counterstained with hematoxylin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C for 3-5 min.

Scoring method. The present study used the same semiquantitative scoring method for ISH and IHC (14,15). The sections were semiquantitatively analyzed independently by two pathologists. All staining was observed using an E200 microscope (Nikon, Tokyo, Japan). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The staining density score was based on the percentage of positive cells, as follows: 0 (0%), 1 (1-10%), 2 (11-50%), 3 (51-80%) and 4 (81-100%). The staining intensity and density scores were multiplied and used as the overall score. An overall score of >3 was considered to indicate positive NOR1 expression; scores of \leq 3 indicated negative NOR1 expression.

Statistical analysis. Statistical analysis was performed using SPSS software (version 19.0; IBM SPSS, Chicago, IL, USA). Data are representative of at least three independent experiments. Pearson's χ^2 test and Fishers exact test were performed to compare differences between groups. $P < 0.05$ was considered to indicate a statistically significant.

Results

Expression of NOR1 mRNA expression in normal liver, hepatitis, cirrhosis and HCC tissues. NOR1 mRNA ISH was performed on all sections representing human normal liver, hepatitis, cirrhosis and HCC. Representative staining patterns for NOR1 mRNA are indicated in Fig. 1. Positive signals were detected in the cytoplasm and/or nucleus. Table I indicates the expression of NOR1 mRNA in normal liver, hepatitis, cirrhosis and HCC. The positive rate of NOR1 mRNA expression in normal liver, hepatitis and cirrhosis was 43.8, 58.3 and 65.6%, respectively. The expression of NOR1 mRNA in hepatitis and cirrhosis did not appear to be significantly different from the normal liver ($P > 0.05$). By contrast, positive expression of NOR1 mRNA was exhibited in 25 (78.1%) HCC cases and negative expression of NOR1 mRNA was observed in the remaining 7 cases (21.9%). The positive rate of NOR1 mRNA in patients with HCC was significantly higher compared with the normal control ($P = 0.017$).

Expression of NOR1 protein expression normal liver, hepatitis, cirrhosis and HCC tissues. IHC was performed to examine the expression of NOR1 protein in human normal

Table I. Expression of NOR1 mRNA in normal liver, hepatitis, cirrhosis and HCC.

| Specimen type | n | NOR1 mRNA expression, n (%) | | χ^2 | P-value ^a |
|---------------|----|-----------------------------|-----------|----------|----------------------|
| | | Negative | Positive | | |
| Normal | 16 | 9 (56.2) | 7 (43.8) | | |
| Hepatitis | 24 | 10 (41.7) | 14 (58.3) | 0.819 | 0.366 |
| Cirrhosis | 32 | 11 (34.4) | 21 (65.6) | 2.100 | 0.147 |
| HCC | 32 | 7 (21.9) | 25 (78.1) | 5.672 | 0.017 |

^aP-value vs. normal tissue. NOR1, oxidored-nitro domain-containing protein 1; HCC, hepatocellular carcinoma.

Table II. Expression of NOR1 protein in normal liver, hepatitis, cirrhosis and HCC.

| Specimen type | n | NOR1 protein expression, n (%) | | χ^2 | P-value ^a |
|---------------|----|--------------------------------|-----------|----------|----------------------|
| | | Negative | Positive | | |
| Normal | 16 | 16 (100.0) | 0 (0.0) | | |
| Hepatitis | 24 | 21 (87.5) | 3 (12.5) | 2.162 | 0.141 |
| Cirrhosis | 32 | 27 (84.4) | 5 (15.6) | 2.791 | 0.095 |
| HCC | 32 | 11 (34.4) | 21 (65.6) | 18.667 | <0.001 |

^aP-value vs. normal tissue. NOR1, oxidored-nitro domain-containing protein 1; HCC, hepatocellular carcinoma.

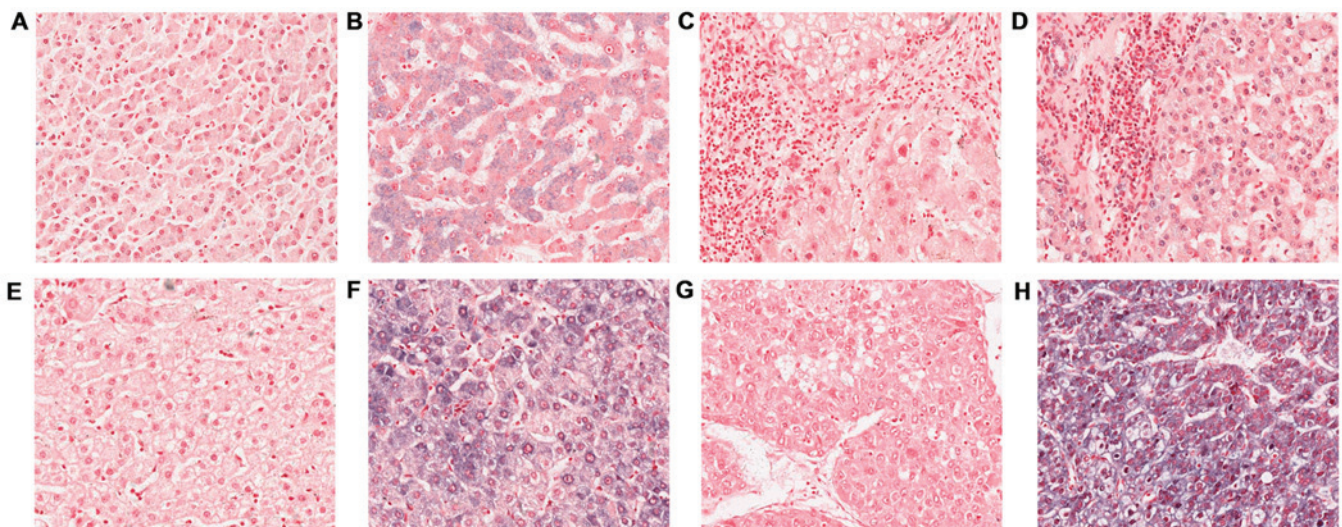


Figure 1. Expression of oxidored-nitro domain-containing protein 1 (NOR1) mRNA in normal liver, hepatitis, cirrhosis and HCC. (A) Negative and (B) positive expression of NOR1 mRNA in normal liver. (C) Negative and (D) positive expression of NOR1 mRNA in hepatitis. (E) Negative and (F) positive expression of NOR1 mRNA in cirrhosis. (G) Negative and (H) positive expression of NOR1 mRNA in hepatocellular carcinoma. Nitroblue tetrazolium, bromochloroindolyl phosphate and nuclear fast red staining; magnification, x400.

liver, hepatitis, cirrhosis and HCC. Fig. 2 shows representative staining patterns of NOR1 protein. The results indicated that NOR1 protein was expressed at variable levels, and localized within the cellular nuclei and cytoplasm.

Negative expression for NOR1 protein was observed in all normal liver samples, while the positive rate was 12.5% in hepatitis and 15.6% in cirrhotic liver samples. However, no significant difference in positive expression rate was observed

between normal liver and hepatitis/cirrhotic liver samples ($P>0.05$).

NOR1 protein was expressed differentially between the normal liver and HCC. In a total of 32 cases of HCC, the expression of NOR1 protein was positive in 21 (65.6%) cases and negative (34.4%) in the remaining 11 cases. The positive rate of NOR1 protein expression was significantly higher in HCC tissues compared with the normal liver tissues ($P<0.001$; Table II).

Table III. Association between NOR1 mRNA expression and clinicopathological parameters of patients with HCC.

| Feature | n | NOR1 mRNA expression, n (%) | | χ^2 | P-value |
|--------------------|----|-----------------------------|-----------|----------|---------|
| | | Negative | Positive | | |
| Age, years | | | | | |
| <50 | 15 | 4 (57.1) | 11 (44.0) | 0.379 | 0.678 |
| ≥50 | 17 | 3 (42.9) | 14 (56.0) | | |
| Gender | | | | | |
| Male | 24 | 4 (57.1) | 20 (80.0) | 1.524 | 0.327 |
| Female | 8 | 3 (42.9) | 5 (20.0) | | |
| HBsAg | | | | | |
| (+) | 16 | 3 (27.3) | 13 (61.9) | 3.463 | 0.135 |
| (-) | 16 | 8 (72.7) | 8 (38.1) | | |
| Pathological stage | | | | | |
| I | 3 | 2 (28.6) | 1 (4.0) | 4.355 | 0.113 |
| II | 19 | 4 (57.1) | 15 (60.0) | | |
| III | 10 | 1 (14.3) | 9 (36.0) | | |
| TNM status | | | | | |
| I | 2 | 1 (14.3) | 1 (4.0) | 1.234 | 0.745 |
| II | 19 | 4 (57.1) | 15 (60.0) | | |
| III | 10 | 2 (28.6) | 8 (32.0) | | |
| IV | 1 | 0 (0.0) | 1 (4.0) | | |

NOR1, oxidored-nitro domain-containing protein 1; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

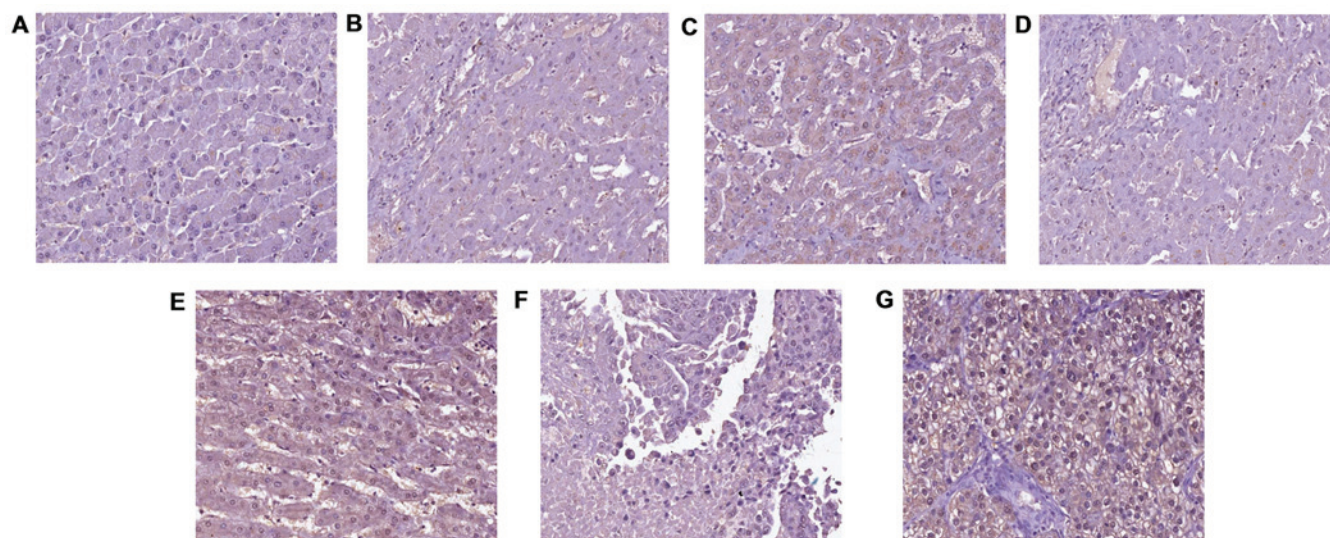


Figure 2. Expression of oxidored-nitro domain-containing protein 1 (NOR1) protein in normal liver, hepatitis, cirrhosis and HCC, as determined by immunohistochemistry. (A) Negative expression of NOR1 protein in normal liver. (B) Negative and (C) positive expression of NOR1 protein in hepatitis. (D) Negative and (E) positive expression of NOR1 protein in cirrhosis. (F) Negative and (G) positive expression of NOR1 protein in hepatocellular carcinoma. 3,3'-diaminobenzidine and hematoxylin staining; magnification, x400.

Correlation between NOR1 expression and clinicopathological parameters of patients with HCC. The present study also analyzed the association between NOR1 expression and clinicopathological parameters of patients with HCC. The results in Table III indicate that there was no statistically significant association between NOR1 mRNA expression

and any of the clinicopathological parameters investigated ($P>0.05$). However, Table IV demonstrates that NOR1 protein expression was correlated with the differentiation degree and tumor-node-metastasis (TNM) (16) tumor stage of patients with HCC. The NOR1 protein positive protein expression rate was significantly higher in HCC patients

Table IV. Association between NOR1 protein expression and clinicopathological parameters of patients with HCC.

| Feature | n | NOR1 protein expression, n (%) | | χ^2 | P-value |
|--------------------|----|--------------------------------|-----------|----------|---------|
| | | Negative | Positive | | |
| Age, years | | | | | |
| <50 | 15 | 4 (36.4) | 11 (52.4) | 0.744 | 0.472 |
| ≥50 | 17 | 7 (63.6) | 10 (47.6) | | |
| Gender | | | | | |
| Male | 24 | 6 (54.5) | 18 (85.7) | 3.740 | 0.088 |
| Female | 8 | 5 (45.5) | 3 (14.3) | | |
| HBsAg | | | | | |
| (+) | 16 | 4 (57.1) | 12 (48.0) | 0.183 | 1.000 |
| (-) | 16 | 3 (42.9) | 13 (52.0) | | |
| Pathological stage | | | | | |
| I | 3 | 3 (27.3) | 0 (0.0) | 6.709 | 0.035 |
| II | 19 | 6 (54.5) | 13 (61.9) | | |
| III | 10 | 2 (18.2) | 8 (38.1) | | |
| TNM status | | | | | |
| I | 2 | 2 (18.2) | 0 (0.0) | 11.002 | 0.012 |
| II | 19 | 9 (81.8) | 10 (47.6) | | |
| III | 10 | 0 (0.0) | 10 (47.6) | | |
| IV | 1 | 0 (0.0) | 1 (4.8) | | |

NOR1, oxidoreduced-nitro domain-containing protein 1; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

with a low differentiation degree and a high TNM stage ($P<0.05$).

Discussion

The NOR1 gene was initially identified in NPC by Nie *et al* in 2003 (11). The expression of NOR1 was downregulated in the CNE1 NPC cell line in comparison to normal nasopharyngeal epithelial cells; however, enzymatic activity was higher in the CNE1 cells compared with the normal nasopharyngeal epithelial cells. NOR1 is regulated by heat shock factor 1 and nuclear respiratory factor 1 (17), which are two stress-responsive transcription factors that have important roles in carcinogenesis (18,19). During the last decade, research regarding the association between NOR1 expression and human cancer has predominantly focused on NPC (20-22). It has been demonstrated that NOR1 is able to regulate NPC cell proliferation, apoptosis, autophagy and metabolism (20,21). In addition, a small number of studies have reported that NOR1 overexpression is associated with prostate (23) and cervical (21) cancer.

Certain *in vitro* studies also suggest that NOR1 may be a candidate gene for hepatocarcinogenesis. NOR1 overexpression may induce Grb2 and E-selectin expression in HepG2 cells, and activate MAPK signal transduction (12,13,24). These studies indicated that NOR1 may be important in the formation of chemical carcinogens and carcinogenesis of HCC.

The development of HCC is a multistep process with the involvement of a multifactorial etiology. It is well-established that HCC frequently arises in the setting of cirrhosis, and

cirrhosis is attributable to chronic hepatitis B or C virus infection. This trend has been observed in cases in almost all countries (25-27).

In the current study, the expression of NOR1 was determined in specimens of normal liver, hepatitis, cirrhosis and HCC obtained as tissue array slides. ISH was used to detect the expression of NOR1 mRNA, whereas the expression of NOR1 protein was examined by IHC. The results indicated that NOR1 was primarily localized within the nuclei and cytoplasm.

By performing a western blot assay, Xiang *et al* identified that human livers exhibit an absence of NOR1 protein. Furthermore, tissue sections from the liver did not stain positive for NOR1 in an IHC assay (28). Consistent with the results in the literature, the IHC results of the present study demonstrated negative expression for NOR1 protein in all 16 normal liver specimens analyzed. However, the ISH assay revealed that the positive rate of NOR1 mRNA was 43.8% in normal liver specimens.

In a previous study by Xiang *et al*, the expression of human NOR1 protein expression was examined in various normal and cancerous tissues. It was observed that NOR1 expression was weak or negative in the majority of malignant cells, however, moderate to strong expression of NOR1 was displayed in liver cancer cells (29). Similarly, the present study identified a trend for increased positive rate of NOR1 protein and mRNA expression from normal liver samples to hepatitis, cirrhosis and HCC samples. However, positive NOR1 protein and mRNA expression observed in the normal liver samples was not significantly different to the hepatitis and cirrhotic liver samples. By contrast,

the positive rate of NOR1 mRNA and protein expression in patients with HCC was significantly higher in comparison to the normal control. The aforementioned findings indicate a possible role of NOR1 in the progression of HCC. Furthermore, the current study identified that NOR1 protein expression correlates with certain clinicopathological parameters of HCC, including pathological stage and TNM status. The positive rate of NOR1 expression was higher in HCC patients with poor pathological differentiation grade and high TNM stage.

In conclusion, the present study examined the mRNA and protein expression of NOR1 in normal human liver, hepatitis, cirrhosis and HCC specimens, which together represent the process of HCC development. NOR1 expression was increased in HCC and its expression was correlated with clinicopathological parameters of patients with HCC. Thus, NOR1 may be involved in HCC progression and could be employed as a predictive biomarker in HCC development.

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