

lncRNA PAARH impacts liver cancer cell proliferation by engaging miR-6512-3p to target LASP1

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Abstract. Long non-coding (lnc)RNAs serve a pivotal role as regulatory factors in carcinogenesis. The present study aimed to assess the involvement of the lncRNA progression and angiogenesis-associated RNA in hepatocellular carcinoma (PAARH) in liver cancer, along with the associated underlying mechanism. Through the use of reverse transcription-quantitative (RT-q)PCR, differences in the expression levels of PAARH in HepG2, HEP3B2.1.7, HCCLM3, Huh-7 and MHCC97-H liver cancer cell lines and THLE-2 epithelial cell lines were evaluated. The liver cancer cell line with the greatest, significantly different, level of expression relative to the normal liver cell line was selected for subsequent experiments. Using ENCORI database, the putative target genes of the microRNA (miR) miR-6512-3p were predicted. Cells were then transfected with lentiviruses carrying short-hairpin-PAARH to interfere with PAARH expression. Subsequently, HepG2 liver cancer cells were transfected with a miR-6512-3p mimic and an inhibitor, and the expression levels of miR-6512-3p and the LIM and SH3 domain protein 1 (LASP1) in cells were assessed using RT-qPCR analysis. Cell proliferation was subsequently evaluated using colony formation assays, and immunofluorescence and western blotting were used to assess the expression level of LASP1 in transfected cells. The binding interaction between miR-6512-3p and LASP1 was further evaluated using a dual-luciferase reporter gene assay. Liver cancer cells were found to exhibit higher expression levels of PAARH compared with normal liver cells. Following PAARH interference, the expression level of miR-6512-3p was significantly

increased, whereas that of LASP1 was significantly decreased, resulting in a reduction in cell proliferation. In liver cancer cells, miR-6512-3p overexpression led to a significant reduction in the LASP1 level and reduced proliferation, whereas suppressing miR-6512-3p led to a significant increase in LASP1 levels and increased proliferation. Additionally, the inhibition of miR-6512-3p caused the states of low LASP1 expression and reduced cell proliferation to be reversed. LASP1, a recently identified target gene of miR-6512-3p, was demonstrated to be suppressed by miR-6512-3p overexpression, thereby inhibiting liver cancer cell proliferation. Taken together, the findings of the present study demonstrate that the lncRNA PAARH may enhance liver cancer cell proliferation by engaging miR-6512-3p to target LASP1.

Introduction

Liver cancer poses a substantial public health challenge, as 8.3% of patients with cancer worldwide die from liver cancer (1). Within the realm of primary liver cancer, primary hepatocellular carcinoma (HCC) is the predominant histopathological entity (2,3). There have been advancements in therapeutic modalities for liver cancer, encompassing interventions such as surgical resection, chemotherapeutic interventions, radiotherapy and targeted therapies. Nevertheless, due to the high recurrence rates and metastatic dissemination of liver cancer, the efficacy of different types of treatment remains suboptimal (4,5). Therefore, exploring the underlying molecular therapeutic mechanisms specific to liver cancer could result in the refinement of therapeutic approaches against this malignancy.

The function of long non-coding (lnc)RNAs has steadily emerged as a hot topic of study in tumor biology (6). Numerous previous studies have reported that lncRNAs exert marked regulatory roles in tumor processes by acting as 'sponges' of micro (mi)RNAs (miR) or competing endogenous (ce)RNAs (7-10). lncRNAs are engaged in a range of biological processes within liver cancer cells, including cell proliferation, apoptosis, migration, metastasis, cell cycle regulation, tumor stem cell maintenance and modulation of the tumor microenvironment via multiple signaling pathways (11-14). The newly discovered lncRNA, progression and angiogenesis-associated RNA in HCC (PAARH), is associated both with liver cancer

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development and angiogenesis, and with the mechanisms underlying liver cancer progression. A previous study reported that PAARH is overexpressed in liver cancer and exhibits a targeted association with miR-6512-3p (15). However, to the best of our knowledge, the precise mechanistic interplay between PAARH and miR-6512-3p in liver cancer remains unknown.

Therefore, the present study used bioinformatic techniques to analyze the binding sites of lncRNA PAARH and miR-6512-3p, as well as miR-6512-3p and the LIM and SH3 domain protein 1 (LASP1) gene. The present study aimed to assess the functional and regulatory associations between PAARH, miR-6512-3p and LASP1 that are relevant to liver cancer. The purpose of the approach in the present study was to elucidate the underlying mechanism via which the lncRNA PAARH modulates the biological behavior of liver cancer cells through miR-6512-3p-mediated targeting of LASP1. The ultimate goal was to provide a theoretical foundation for the identification of valuable therapeutic targets in liver cancer.

Materials and methods

Cell culture. The HepG2, HEP3B2.1.7, HCCLM3, Huh-7 and MHCC97-H liver cancer cell lines and the THLE-2 epithelial cell lines were used in the present study. All cells were purchased from Aoruisai Biotechnology (Shanghai) Co. Ltd., and underwent STR identification. Cell incubations were performed using DMEM (Wuhan Servicebio Technology Co., Ltd.) supplemented with 100 U.ml⁻¹ penicillin, 10% FBS (Procell Life Science & Technology Co., Ltd.) and 100 mg.l⁻¹ streptomycin (Beijing Solarbio Science & Technology Co., Ltd.). The cells were incubated in a cell culture incubator maintained at 37°C in a humidified atmosphere containing 5% CO₂. Subsequent experiments were performed after the cell confluence had reached 80-90%.

Bioinformatics analysis. The Encyclopedia of RNA Interactomes (ENCORI) database (<https://starbase.sysu.edu.cn/index.php>) was used to predict the binding sites of miR-6512-3p and LASP1. Additionally, gene and clinical data from The Cancer Genome Atlas (TCGA; <https://portal.gdc.cancer.gov/>) LIHC dataset were downloaded to confirm the expression of LASP1 in liver cancer, and to analyze its expression pattern across different tumor (T), lymph node (N) and metastasis (M) stages of liver cancer. Using information retrieved from the Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/>) and TCGA databases, the association between LASP1 expression and the survival of patients with liver cancer was also assessed.

Cell transfection. To evaluate the effects of PAARH interference on liver cancer cell proliferation, lentiviral vectors for PAARH [short-hairpin(sh)-PAARH: (5'-CTCCTCAGCAACCACAGATG-3')] Shanghai GenePharma Co., Ltd., were constructed, with a non-targeting sequence sh-negative control (NC; 5'-TTCTCCGAACGTGTACAGT-3'; Shanghai GenePharma Co., Ltd.) serving as the negative control. The packaging plasmids pGag/Pol, pRev and pVSV-G were utilized (Shanghai GenePharma Co., Ltd.), and the shuttle plasmid was LV3 (Shanghai GenePharma Co., Ltd.). The construction

and packaging of the lentivirus were completed by Shanghai GenePharma Co., Ltd. HepG2 cells were seeded at a density of 1x10⁵ cells per well in 6-well cell culture plates. Transfection was performed using a lentivirus-containing medium (GenePharma, Shanghai, China) after the cell confluence had reached 50-60%. A multiplicity of infection (MOI) value of 50 was chosen for the transfection, which was carried out at room temperature and continued for 24 h. Subsequently, puromycin (2 µg/ml) was used for cell selection and maintenance. Transfection efficiency was assessed 96 h post-transfection using reverse transcription-quantitative (RT-q)PCR.

Furthermore, to assess the impact of miR-6512-3p on liver cancer cell proliferation, miR-6512-3p mimic [sense (S), 5'-UUCCAGCCCUUCUAAUGGUAGG-3' and antisense (AS), 5'-CCUACCAUAGAAGGGCUGGAA-3'] was used to overexpress miR-6512-3p, with miR-NC (S, 5'-UCACAA CCUCCUAGAAAGAGUAGA-3' and AS, 5'-UCUACUCUU UCUAGGAGGUUGUGA-3') serving as the control. An miR-6512-3p inhibitor (5'-CCUACCAUAGAAGGGCU GGAA-3') was used to suppress miR-6512-3p expression, with inhibitor-NC (5'-UCUACUCUUUCUAGGAGGUUG UGA-3') serving as the control. All aforementioned plasmids were acquired from Hanbio Biotechnology, Co., Ltd. For these experiments, cells were seeded at a density of 1x10⁵ cells per well in 6-well cell culture plates. Lipofectamine 3000™ (Invitrogen™; Thermo Fisher Scientific, Inc.) was used as the cell transfection reagent. Transfection was performed with a transfection complex composed of 10 µl Lipofectamine 3000™ and 5 µl of the plasmid, after the cells had reached a confluency of 40-50%. The transfection was conducted at room temperature and lasted for 8 h. Transfection efficiency was evaluated 48 h post-transfection using RT-qPCR.

RT-qPCR analysis. After transfecting cells with different target genes until the specified time, total RNA from each group of cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). An RT kit (Monad Biotech Co., Ltd. and Sangon Biotech Co., Ltd.) was used to synthesize the cDNA according to the manufacturer's instructions. qPCR was performed using SYBR Green method (Shanghai Yeasen Biotechnology Co., Ltd.) on a Light Cycler 96 Real-Time PCR System (Roche Diagnostics). GAPDH was used as the reference gene for PAARH, U6 was used as the reference gene for miR-6512-3p, and β-tubulin was used as the reference gene for LASP1. Analysis of relative gene expression data was performed using the 2^{-ΔΔC_q} method (16). Each experiment set was performed in triplicate. Primer sequences used were as follows: miR-6512-3p S, 5'-CGCGTTCCAGCC CTTCTAAT-3' and AS, 5'-AGTGCAGGGTCCGAGGTA TT-3'; PAARH S, 5'-CCTGAAAGTCTCCAAGGCCA-3' and AS, 5'-TGTGTCTTCAGGCAGCAACT-3'; LASP1 S, 5'-CAA GGGCAAAGGTTTCAGCGTAG-3' and AS, 5'-ATGCGG CTCTTCTCAAACCTCCTC-3'; GAPDH S, 5'-GGACCTGAC CTGCCGTCTAG-3' and AS, 5'-GTAGCCCATAGATGCC CTTGA-3'; U6 S, 5'-GCTTCGGCAGCACATATACTAAA T-3' and AS, 5'-CGCTTCACGAATTTGCGTGTTCAT-3'; and β-tubulin S, 5'-GGAGAACACGGATGAGACCTACTG-3' and AS, 5'-CCAGCTTGAGGGTGCGGAAG-3'. The thermocycling conditions for the RT-qPCR protocol were as follows: Pre-denaturation at 95°C for 5 min, denaturation at 95°C for

10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 20 sec. The aforementioned steps constituted one cycle, and a total of 40 cycles were performed.

Colony formation assay. After the appropriate cell transfections and treatments had been performed, HepG2 cells in the exponential growth phase from each group were seeded at a density of 3×10^3 cells per well in 6-well cell culture plates. During 7 days of cell culture, the medium was replaced every 2 days. Subsequently, the cells were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature, then rinsed twice with PBS (Wuhan Servicebio Technology Co., Ltd.), followed by staining with crystal violet for 10 min at room temperature. Finally, the cells were imaged (IX71 + DP80 microscope; Olympus Corporation) and colonies were counted using ImageJ version 1.54 g (National Institutes of Health), with colonies defined as having >50 cells each.

Immunofluorescence studies. The 6-well cell culture plates were pre-coated with cell crawling tablets (Shanghai Hongwo Biotechnology Co., Ltd.), and HepG2 cells were seeded at a density of 1.5×10^5 cells per well. When the cell density reached 70–80%, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Permeabilization was performed using 0.5% Triton-X100 (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min, with three 5-min PBS washes performed between each step. After the washing steps were completed, PBS was discarded, and the cells were blocked for 30 min at room temperature using 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.), after which the blocking solution was discarded. Subsequently, 500 μ l of anti-LASP1 primary antibody solution (1:100 dilution; cat. no. ab156872; Abcam) was added to each well and the plates were incubated overnight at 4°C (at least 12 h). Fluorescent secondary antibodies (1:500; Fluor594-conjugated; cat. no. S0006; Affinity Biosciences) and DAPI (10 μ g/ml) were added to the solution and the plates were incubated for 1 h at room temperature in the dark. Following the incubation, the coverslips were carefully lifted from the wells and placed onto glass slides, with the addition of 10 μ l of anti-fade mounting medium. Finally, the samples were imaged using a laser confocal microscope (Olympus FV3000; Olympus Corporation) and stored in a refrigerator at 4°C.

Western blotting analysis. A 30-min incubation on ice was performed to lyse the HepG2 cells using pre-chilled RIPA lysis buffer (Invent Biotechnologies, Inc.). The lysates were then centrifuged for 20 min at 4°C and $14,000 \times g$. The protein levels in the supernatants were quantified using a BCA protein quantification kit (Monad Biotech Co., Ltd.). Protein concentrations were adjusted before being mixed with SDS protein loading buffer, followed by denaturation at 95°C for 5 min and western blot analysis.

After separating the proteins (30 μ g protein per lane) using 12% SDS-PAGE (using the WIX-easyPRO4 Easy Mini Vertical Electrophoresis Cell System; Interscience Sdn Bhd), an equivalent amount of denatured proteins was transferred to a PVDF membrane (MilliporeSigma) using a transfer apparatus (WIX-easyPRO4). After blocking for 1 h with 5%

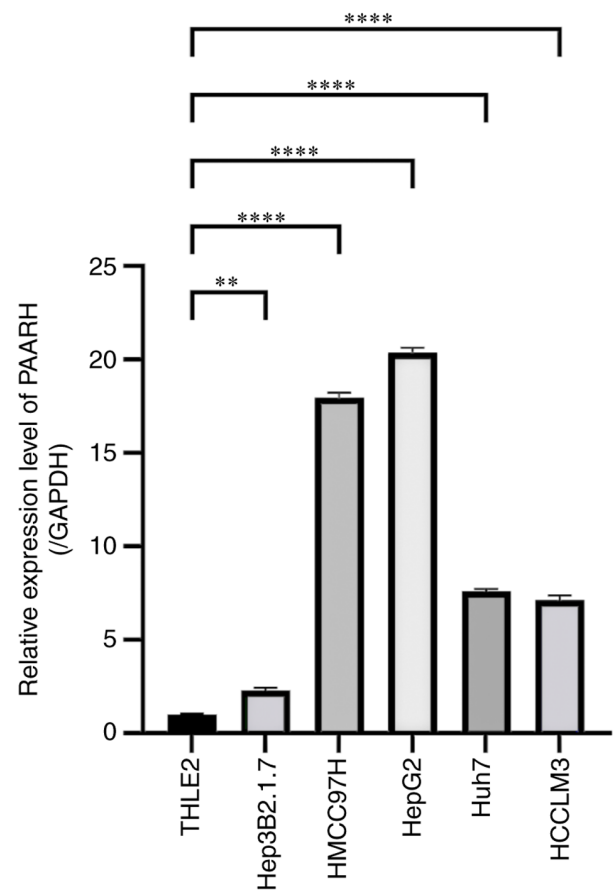


Figure 1. Expression levels of PAARH in each group of cells. Expression levels were normalized to those of GAPDH. ** $P < 0.01$ and **** $P < 0.0001$ vs. THLE2. PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma.

skimmed milk, the membrane was incubated overnight at 4°C with primary antibodies, including anti-LASP1 (1:20,000; cat. no. ab156872; Abcam) and anti- β -tubulin (1:50,000; cat. no. S0001; Affinity Biosciences), diluted in TBST containing 0.05% Tween-20 and 3% BSA. The following day, the samples were washed three times with TBST for 5 min each. Following the washes, the membrane was incubated for 1 h at room temperature with secondary antibodies (1:50,000; 488 Fluorescent Dye; cat. no. 66240-1-Ig; Proteintech Group, Inc.). An enhanced chemiluminescence kit (Monad Biotech Co., Ltd.) was used to visualize the protein signals. ImageJ software (version 1.54d; National Institutes of Health) was used to semi-quantify the band intensities. The loading control was β -tubulin, and the relative expression of the target protein was calculated as the ratio of the intensity of the target protein band to the intensity of the β -tubulin protein band.

Luciferase reporter assay. Luciferases test-human-LASP1-3UTR-wild-type (LT-h-LASP1-3UTR-WT) and Luciferases test-human-LASP1-3UTR-mutant (LT-h-LASP1-3UTR-MUT) plasmids were constructed by Hanbio Biotechnology, Co., Ltd. These plasmids were co-transfected into 293T cells with miR-6512-3p mimic and miR-NC using Lipofectamine 3000™ (Invitrogen; Thermo Fisher Scientific, Inc.) transfection reagent. At 48 h post-transfection, the luciferase activity in each cell group was assessed using the Dual-Luciferase

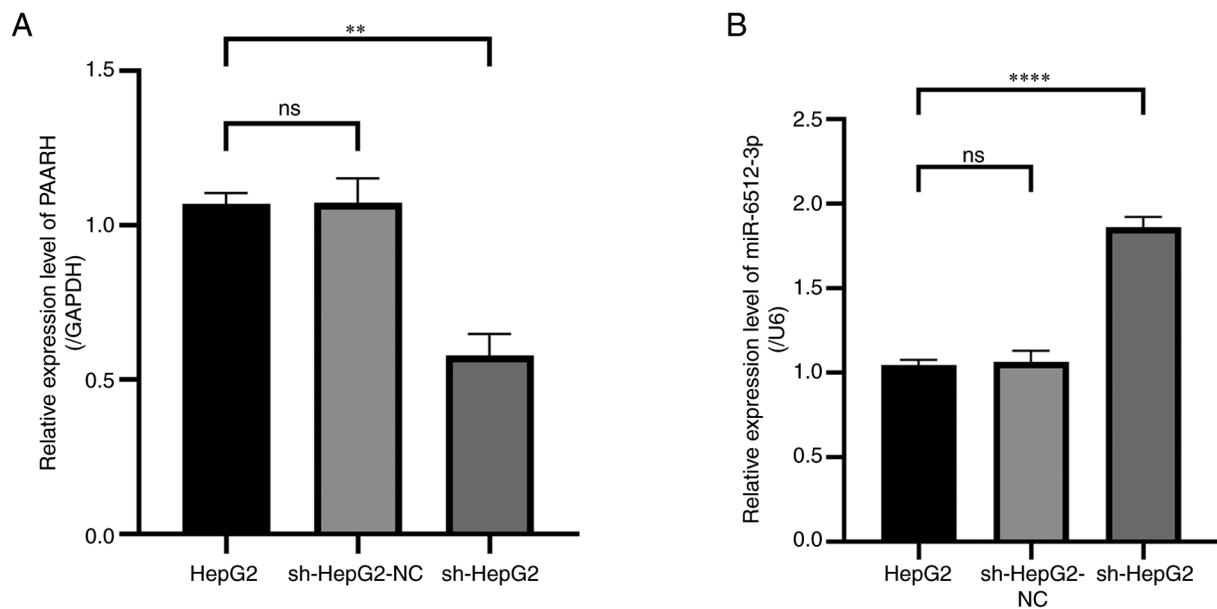


Figure 2. Differences in expression levels among liver cells transfected with sh-PAARH. Differences in (A) PAARH and (B) miR-6512-3p expression levels in HepG2 cells transfected with sh-PAARH, which were normalized to GAPDH and U6, respectively. ** $P < 0.01$ and **** $P < 0.0001$ vs. HepG2. PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma; miR, microRNA; sh, short-hairpin; NC, negative control; ns, not significant.

Reporter Assay Kit (Hanbio Biotechnology, Co., Ltd), with *Renilla* luciferase activity used as the normalization reference. All the aforementioned experiments were performed in triplicate.

Statistical analysis. For data analysis, the statistical software GraphPad Prism version 9 (Dotmatics) and SPSS version 27 (IBM Corp.) were used. The mean \pm standard deviation was used to present quantitative data. One-way analysis of variance was used to compare multiple groups of data, followed by the Least Significant Difference post hoc test for datasets containing three groups and the Tukey post hoc test for datasets with >3 groups. The Kruskal-Wallis test was used for non-normally distributed data, and Dunn's post hoc test was applied to datasets containing more than three groups. $P < 0.05$ was used to indicate a statistically significant difference.

Results

Liver cancer cells exhibit an elevated level of PAARH expression. RT-qPCR was performed to evaluate the expression level of PAARH in the HEP3B2.1.7, HepG2, HCCLM3, Huh-7, MHCC97-H and THLE-2 cell lines to assess the differential expression of PAARH between the liver cancer cells and the normal liver cell line (THLE-2). The expression of PAARH was demonstrated to be significantly upregulated in HepG2, HEP3B2.1.7, HCCLM3, Huh-7 and MHCC97-H cells compared with the normal liver cell line, THLE-2 (Fig. 1). Notably, the HepG2 cell line exhibited the highest level of PAARH expression, and therefore HepG2 cells were chosen for use in future experiments.

PAARH modulates the proliferation of liver cancer cells via miR-6512-3p. In the initial phases of the present study, an interaction between PAARH and miR-6512-3p was observed (15).

To elucidate the influence of PAARH on the proliferation of liver cancer cells via miR-6512-3p, a lentiviral vector was constructed (sh-PAARH) to knock-down PAARH expression in liver cancer cells. RT-qPCR analysis revealed that, after sh-PAARH transfection, there was a significant reduction in PAARH expression in sh-HepG2 cells compared with that in HepG2 cells (Fig. 2A), with a corresponding significant increase in the level of miR-6512-3p expression (Fig. 2B), which indicated that PAARH may regulate miR-6512-3p as a ceRNA.

Subsequently, miR-6512-3p mimic and miR-6512-3p inhibitor plasmids were transfected into HepG2 and sh-HepG2 cells, respectively (Fig. S1). Colony formation assays showed that, compared with that in HepG2 cells, cell proliferation was significantly reduced in sh-HepG2 cells following PAARH knockdown (Fig. 3A). miR-6512-3p overexpression led to a significant reduction in cell proliferation in both HepG2 and sh-HepG2 cells, whereas suppression of miR-6512-3p expression in these cells led to a significant increase in cell proliferation (Fig. 3B and C). These results revealed that silencing miR-6512-3p in sh-HepG2 cells partially compensated for the decreased proliferation observed after PAARH silencing. Taken together, these experiments demonstrated that PAARH may promote liver cancer cell proliferation through competitively inhibiting miR-6512-3p expression.

Increased levels of LASP1 expression suggest a poor prognosis in patients with liver cancer. Using ENCORI database analysis, the binding region between LASP1 and miR-6512-3p was predicted. TCGA and GEPIA databases were used to access the gene and clinical data for 424 samples from patients with liver cancer, including 374 liver cancer tissues and 50 adjacent non-cancerous tissues. The LASP1 expression level was demonstrated to be substantially and significantly increased in liver cancer tissues, compared with normal tissues (Fig. 4A). Across several stages of liver cancer, the expression level of

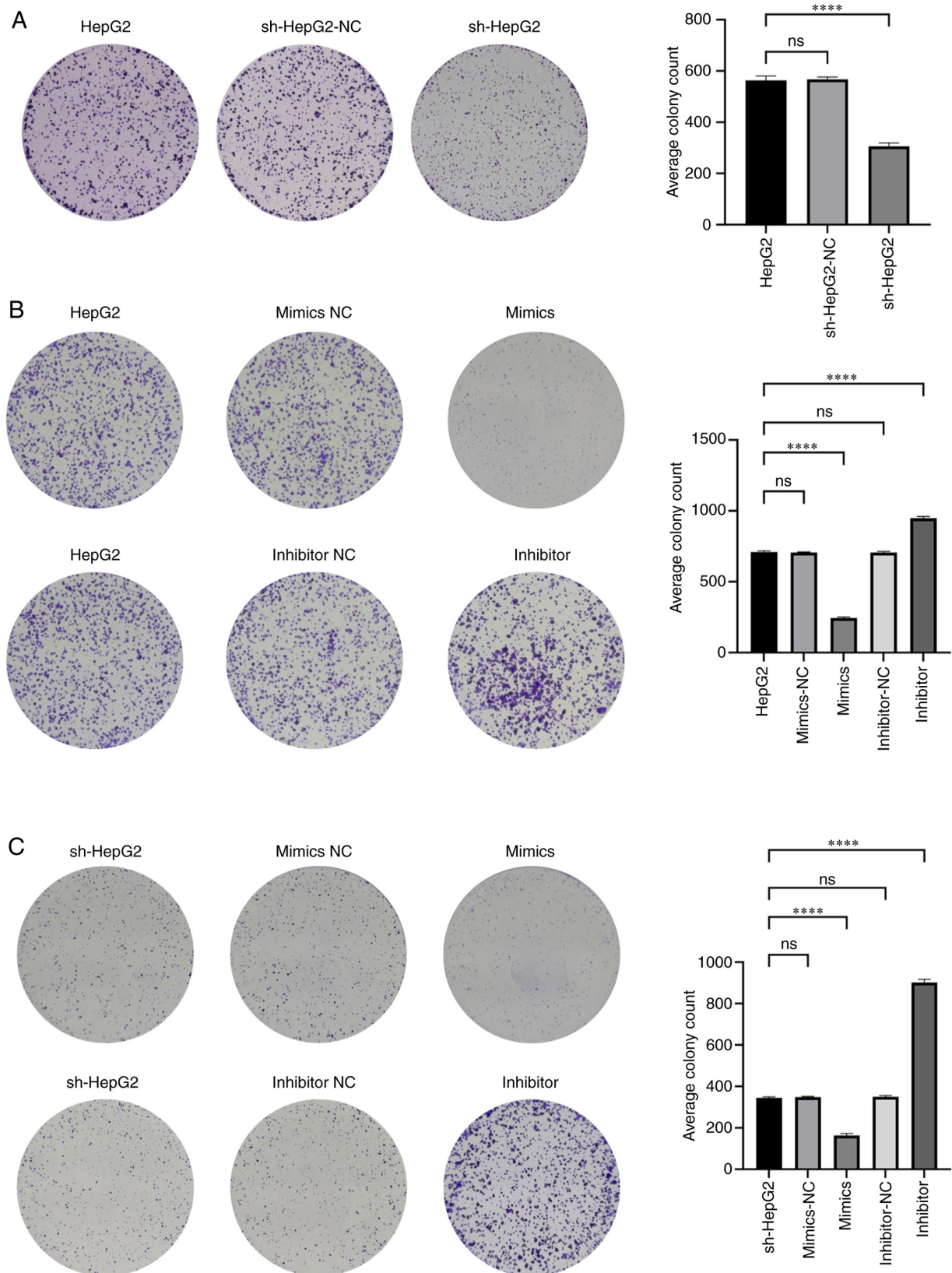


Figure 3. Variations in cell proliferation among different groups. Changes in the cell proliferation of (A) HepG2 cells transfected with sh-PAARH, (B) HepG2 and sh-HepG2 cells transfected with a miR-6512-3p mimic and (C) HepG2 and sh-HepG2 cells transfected with a miR-6512-3p inhibitor. **** $P < 0.0001$. PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma; miR, microRNA; sh, short-hairpin; NC, negative control; ns, not significant.

LASPI was not demonstrated to be statistically different in terms of TNM staging, lymph node involvement or distant metastasis (Fig. 4B-E). However, compared to patients with

a low expression level, those with a high expression level of LASPI in cancer exhibited significantly lower disease-free survival rates (Fig. 4F).

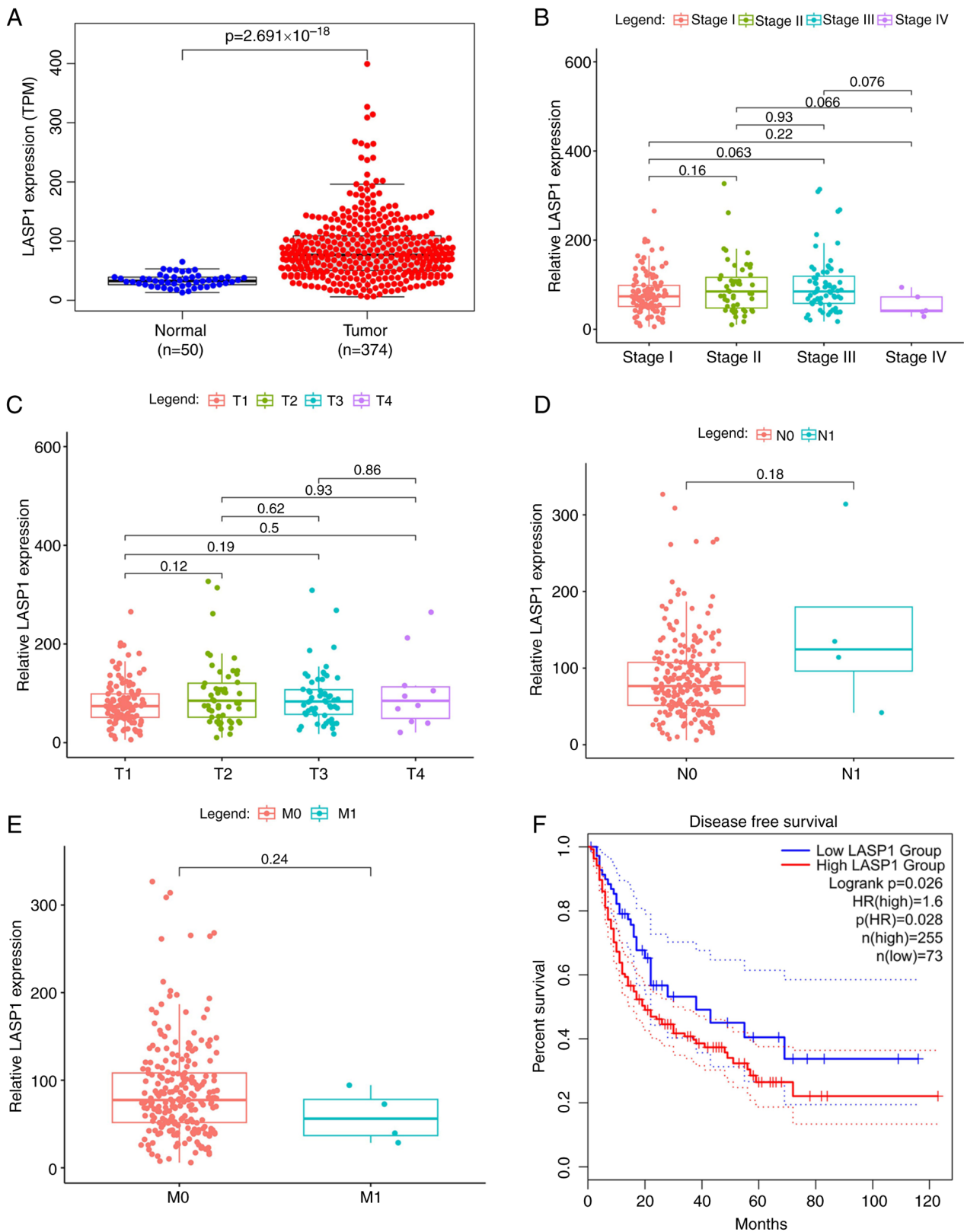


Figure 4. High expression of LASP1 indicates poor prognosis in patients with liver cancer. (A) Gene Expression Profiling Interactive Analysis database was used to assess the expression of LASP1 in 374 liver cancer tissues and 50 normal tissues. (B) Relationship between LASP1 expression levels and liver cancer stages. Association between LASP1 expression levels and (C) T, (D) N and (E) M stages. (F) Disease-free survival in patients with liver cancer. LASP1, LIM and SH3 domain protein 1; T, tumor; N, node; M, metastasis; TPM, transcripts per million.

Regulation of LASP1 expression via miR-6512-3p. To assess changes in the LASP1 expression level following miR-6512-3p regulation, LASP1 expression was initially evaluated in

HepG2 cells and HepG2 cells transfected with sh-PAARH. RT-qPCR analysis revealed that, compared with that in HepG2 cells, LASP1 expression was significantly reduced in HepG2

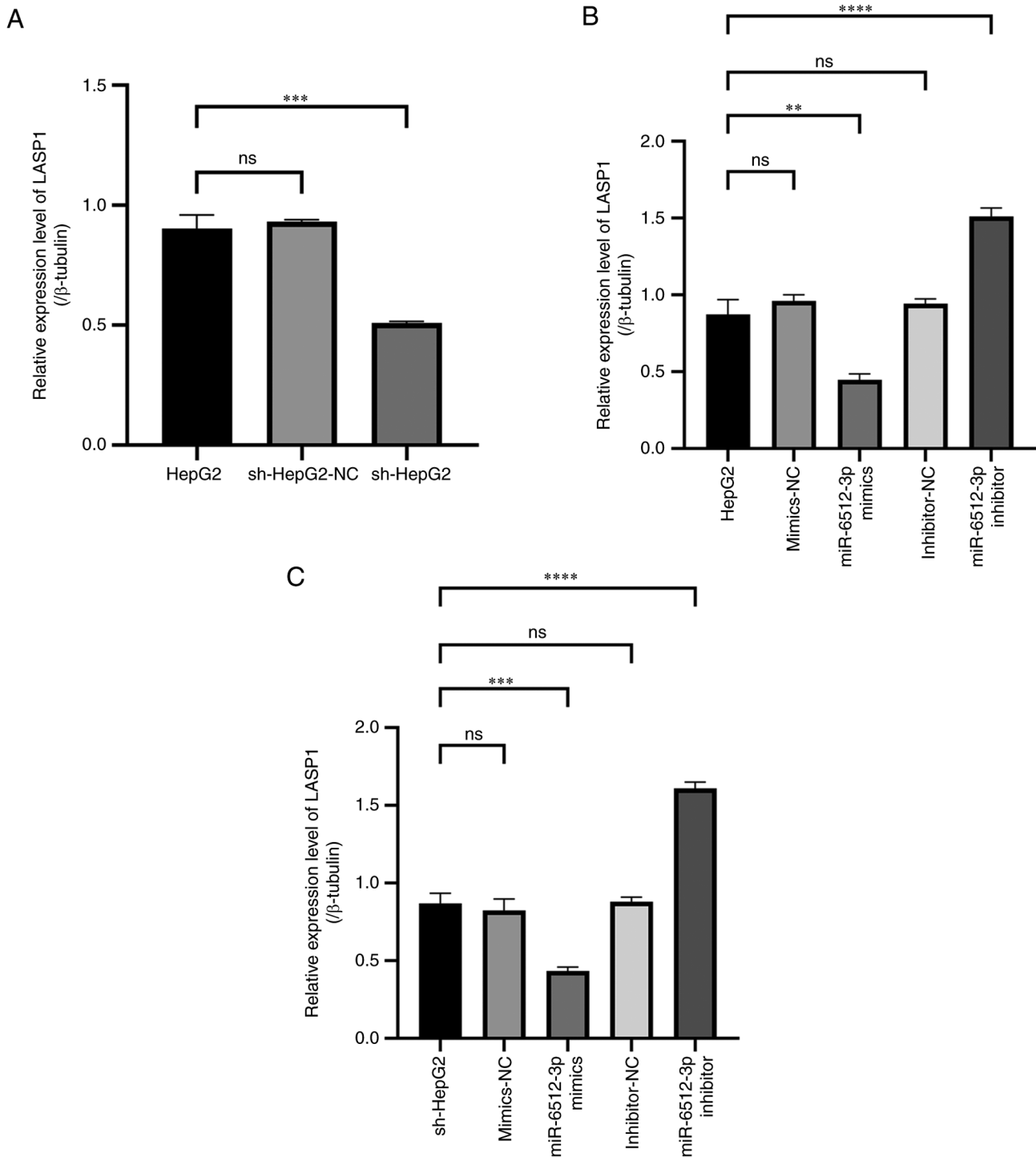


Figure 5. LASP1 expression assessed in several HepG2 cell groups using reverse transcription-quantitative PCR. LASP1 expression in (A) HepG2 cells transfected with sh-PAARH, (B) HepG2 cells transfected with a miR-6512-3p mimic or inhibitor, and (C) sh-HepG2 cells transfected with a miR-6512-3p mimic or inhibitor. Expression levels were normalized to those of β -tubulin. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. LASP1, LIM and SH3 domain protein 1; sh, short-hairpin; PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma; miR, microRNA; NC, negative control; ns, not significant.

cells transfected with sh-PAARH (Fig. 5A). In HepG2 cells, transfection with a miR-6512-3p mimic led to a significant decrease in LASP1 expression, whereas transfection with a miR-6512-3p inhibitor significantly increased LASP1 expression (Fig. 5B). Similarly, in sh-HepG2 cells, transfection with a miR-6512-3p mimic led to a significant decrease in LASP1 expression, whereas transfection with a miR-6512-3p inhibitor significantly reversed the lowered expression level of LASP1

caused by sh-PAARH interference (Fig. 5C). The immunofluorescence (Fig. 6) and western blot (Fig. 7) experiments that were subsequently performed also confirmed these findings. Overall, the results collectively indicated that miR-6512-3p could suppress LASP1 expression.

miR-6512-3p directly targets LASP1. ENCORI database analysis revealed that miR-6512-3p bound to the 3'-untranslated

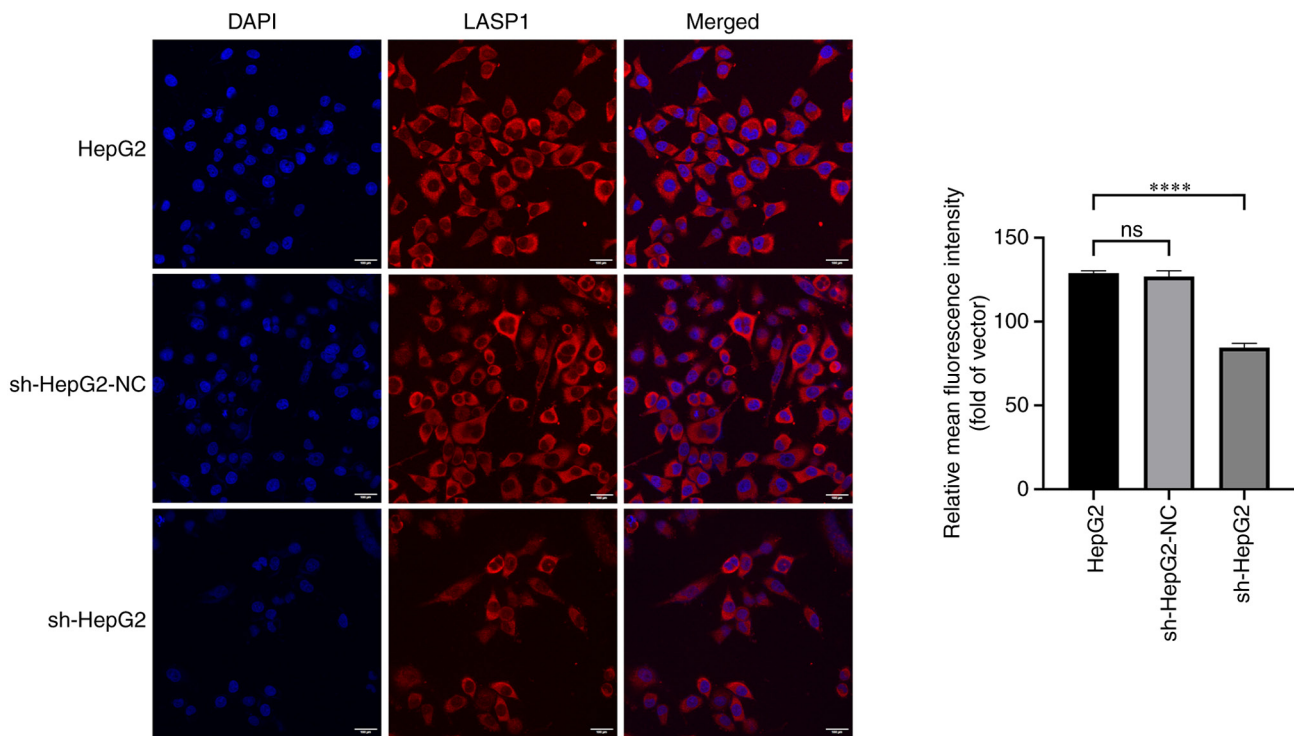


Figure 6. LASP1 expression in cells was assessed using immunofluorescence staining. The expression level of LASP1 is shown in HepG2 cells transfected with sh-PAARH and in non-transfected HepG2 cells. **** $P < 0.0001$. Scale bar, 100 μm . LASP1, LIM and SH3 domain protein 1; sh, short-hairpin; PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma; NC, negative control; ns, not significant.

region (UTR) of LASP1 at nucleotide positions 261-268, 823-830, 2,685-2,691 and 1604-1610 (Figs. 8 and 9). To assess the binding between miR-6512-3p and LASP1, a luciferase reporter gene assay was performed. In the h-LASP1-3UTR-WT-transfected cells, hsa-miR-6512-3p led to a significant reduction in the level of luciferase expression compared with the NC group, demonstrating their interaction during binding. In contrast, after mutation, hsa-miR-6512-3p was not demonstrated to significantly suppress luciferase expression in h-LASP1-3UTR-MUT-transfected cells compared with the NC group, demonstrating that the mutation was successful (Figs. 10 and S2). Taken together, these experiments demonstrated that there was a targeted association between miR-6512-3p and LASP1.

Discussion

Cancer metastasis and recurrence are major contributing factors in the low survival rates of patients with liver cancer (17). The role of lncRNAs in the proliferative processes of several types of human cancer, including liver cancer, has been confirmed (18,19). In both our previous and present studies, an increased expression in the lncRNA PAARH was observed in liver cancer cells and this increased expression has been reported to facilitate the proliferation of HCC cells (15). The mechanistic basis for this effect involves the previously identified role of lncRNA PAARH in promoting the progression and angiogenesis of liver cancer through HOXA transcript at the distal tip upregulation and hypoxia-inducible factor-1 α /VEGF signaling pathway activation (15). In the present study, it was identified that the mechanistic basis for the proliferative

effect of lncRNA PAARH in liver cancer cells may also be achieved through competitively inhibition of the expression of miR-6512-3p. These findings indicated that PAARH served a diverse, complex and notable regulatory role in the occurrence and development of liver cancer, representing a potential prognostic biomarker and therapeutic target for liver cancer. However, several crucial functions of PAARH have not yet been elucidated, including its roles in epigenetic regulation, control at the transcriptional level, modulation of signaling pathways and structural contributions, among others; therefore, an in-depth exploration of the role and significance of PAARH in tumor initiation and progression is still required.

The mechanism of lncRNA action involving ceRNAs refers to the process whereby lncRNAs share common miRNA response elements with miRNAs, thereby inhibiting miRNA binding to other target mRNAs. This subsequently leads to the regulation of the expression of these mRNAs (20). Within tumor cells, miRNAs are able to bind to the 3'-UTR of their target genes, leading to mRNA degradation or translational repression, thereby suppressing the expression of the target genes. However, lncRNAs can also competitively bind to miRNA binding sites, forming lncRNA-miRNA complexes that prevent miRNAs from binding to other target genes. This alleviates miRNA-mediated inhibition and upregulates these genes. This action can lead to changes that may either promote or inhibit cancer (21). Numerous studies have reported that lncRNAs exert an influence on the functions of HCC cells through the aforementioned ceRNA mechanism. For example, according to Kong *et al* (22), lncRNA AC006329.1 acts as a ceRNA by inhibiting miR-127-5p and stimulating the SHC3/ERK signaling pathway. This promotes the progression,

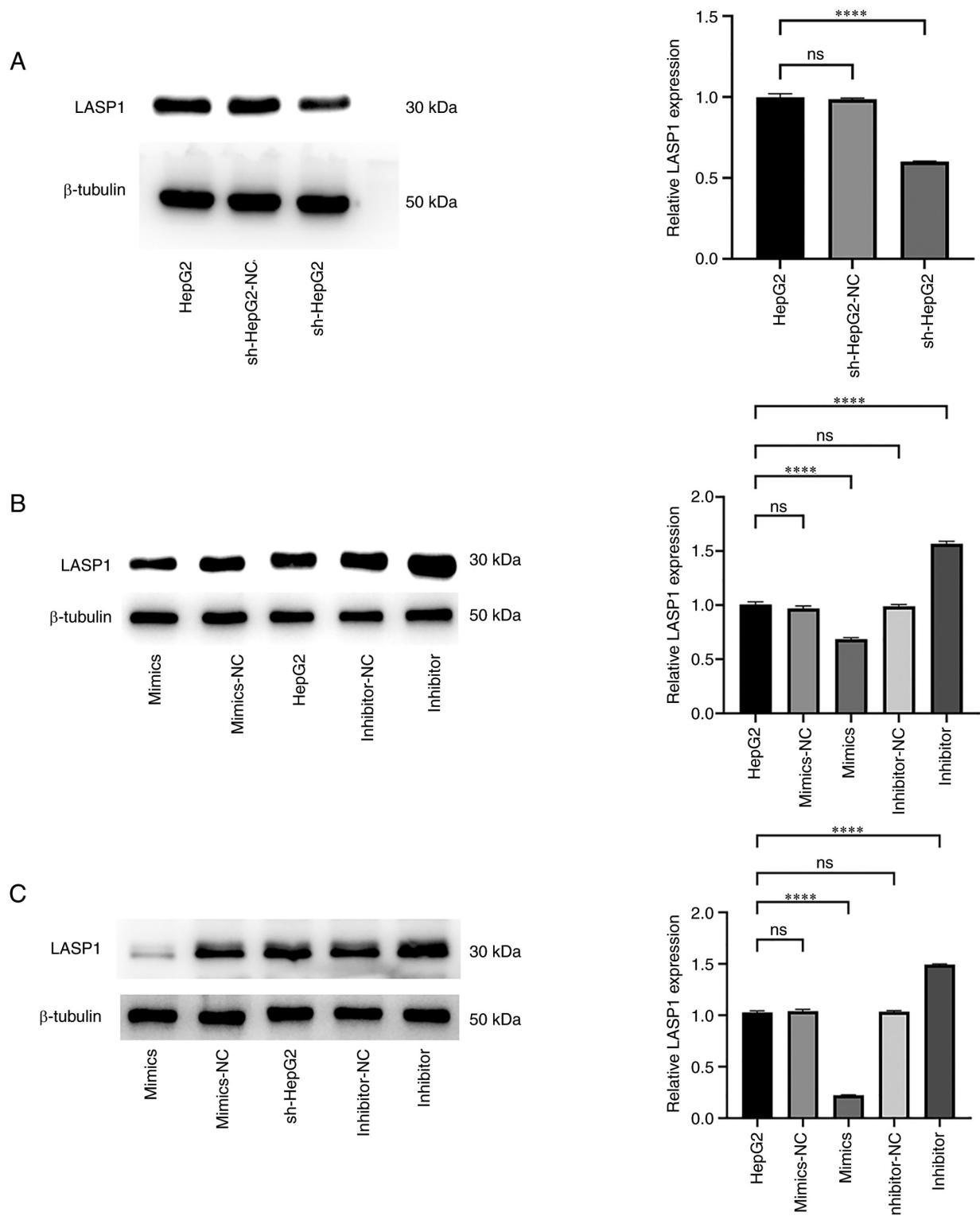


Figure 7. LASP1 expression in cells was assessed using western blot analysis. (A) LASP1 expression in HepG2 cells transfected with sh-PAARH. (B) LASP1 expression in HepG2 cells transfected with miR-6512-3p mimics and miR-6512-3p inhibitor. (C) Transfection of sh-HepG2 cells with miR-6512-3p inhibitor. Expression levels were normalized to those of β-tubulin. **** $P < 0.0001$. LASP1, LIM and SH3 domain protein 1; sh, short-hairpin; PAARH, progression and angiogenesis-associated RNA in hepatocellular carcinoma; NC, negative control; ns, not significant.

metastasis and epithelial-mesenchymal transition (EMT) of HCC cells. According to Yao *et al* (23), phosphatidylinositol transfer protein α -antisense RNA 1 functions as a ceRNA to sequester miR-363-5p, thereby regulating the expression of platelet-derived growth factor-D. This then promotes carcinogenesis. Similarly, Wang *et al* (24) reported that lncRNA 02027

served as a ceRNA by competitively binding to miR-625-3p to regulate the expression of PDZ and LIM domain protein 5, thereby suppressing HCC cell invasion, proliferation, EMT and migration.

lncRNA PAARH, also known as CMB9-22P13.1 with a gene ID of 101927789, is a lncRNA located on the long arm

Poorly conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type
Position 261-268 of LASP1 3' UTR hsa-miR-6512-3p	5' ...CUCUGGGGAGGCAGGGCUGGAA... 3' GGAUGGUAUUCUCCGACCUU	8mer
Position 261-268 of LASP1 3' UTR hsa-miR-6720-5p	5' ...CUCUGGGGAGGCAGGGCUGGAA... 3' GCGCCGCGGAUGGUCCCGACCUU	8mer
Position 823-830 of LASP1 3' UTR hsa-miR-6720-5p	5' ...CUUGCCUGCCUCCUA-GGCUGGAA... 3' GCGCCGCGGAUGGUCCCGACCUU	8mer
Position 823-830 of LASP1 3' UTR hsa-miR-6512-3p	5' ...CUUGCCUGCCUCCUAGGCUGGAA... 3' GGAUGGUAUUCUCCGACCUU	8mer
Position 2685-2691 of LASP1 3' UTR hsa-miR-6720-5p	5' ...GGGCCUUUUUUUAUAGCUGGAAA... 3' GCGCCGCGGAUGGUCC---CGACCUU	7mer-A1
Position 2685-2691 of LASP1 3' UTR hsa-miR-6512-3p	5' ...GGGCCUUUUUUUAUAGCUGGAAA... 3' GGAUGGUAUUCUCCGACCUU	7mer-A1

Figure 8. Binding site of miR-6512-3p with LASP1 identified through Targetscan database search. miR, microRNA; LASP1, LIM and SH3 domain protein 1; UTR, untranslated region.

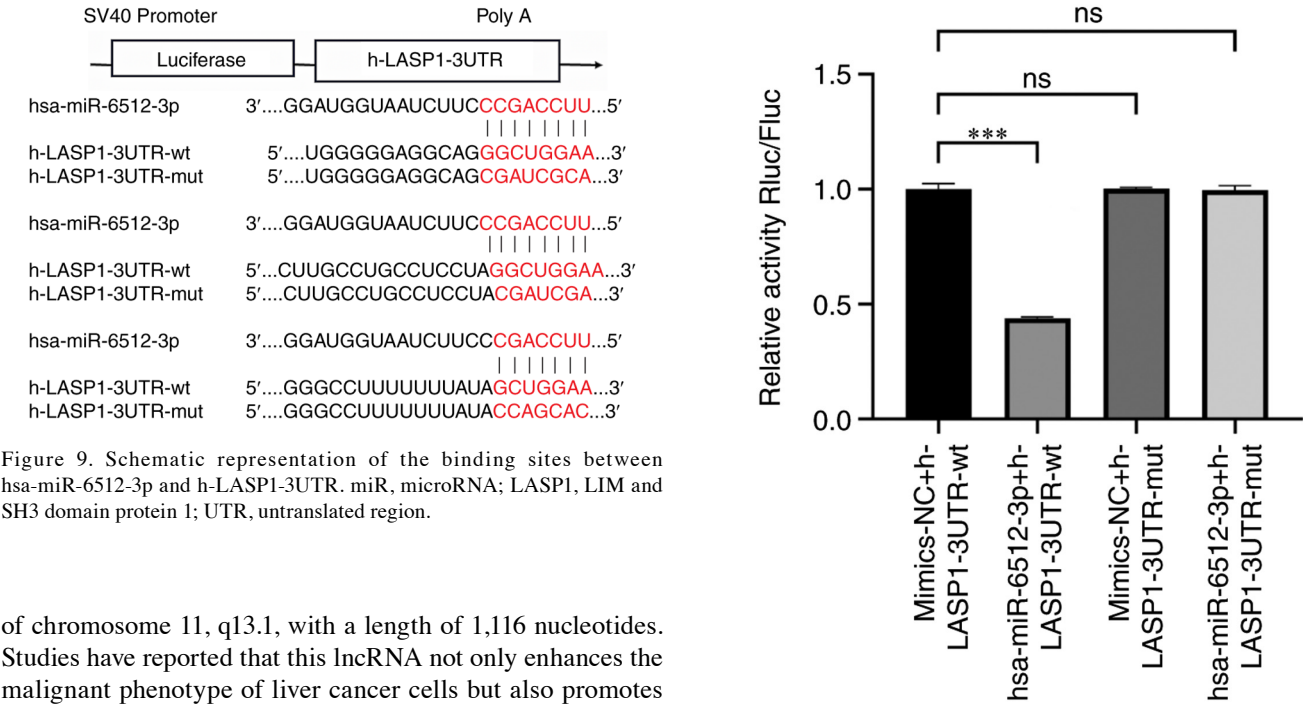


Figure 9. Schematic representation of the binding sites between hsa-miR-6512-3p and h-LASP1-3UTR. miR, microRNA; LASP1, LIM and SH3 domain protein 1; UTR, untranslated region.

of chromosome 11, q13.1, with a length of 1,116 nucleotides. Studies have reported that this lncRNA not only enhances the malignant phenotype of liver cancer cells but also promotes angiogenesis (15). In earlier investigations, it was demonstrated that lncRNA PAARH functions as a sponge, recruiting miR-6512-3p, miR-6760-5p and other miRNAs (15). In the present study, to elucidate the mechanism of action between PAARH and miR-6512-3p in promoting liver cancer cell proliferation, a lentiviral vector was constructed for PAARH knockdown to reduce its expression in liver cancer cells. The results demonstrated a significant reduction in PAARH expression in HepG2 cells following transfection with sh-PAARH (Fig. 2A), accompanied by an elevation in miR-6512-3p expression (Fig. 2B). This suggested a negative association between PAARH and miR-6512-3p, indicating

Figure 10. Luciferase reporter assay reporter gene assay detecting the interaction between hsa-miR-6512-3p and h-LASP1-3UTR. ***P<0.001. miR, microRNA; LASP1, LIM and SH3 domain protein 1; UTR, untranslated region; NC, negative control; Rluc, Renilla luciferase; Fluc, Firefly luciferase; WT, wild type; mut, mutant.

that PAARH may regulate miR-6512-3p through a ceRNA mechanism. Subsequently, HepG2 and sh-HepG2 cells were separately transfected with miR-6512-3p mimics and miR-6512-3p inhibitors, respectively, following PAARH silencing. Proliferation was significantly decreased following

PAARH silencing (Fig. 3A). miR-6512-3p overexpression in HepG2 and sh-HepG2 cells also led to a decrease in proliferation (Fig. 3B), whilst miR-6512-3p expression inhibition in these cells resulted in an increase in proliferation (Fig. 3C). This indicated that the inhibition of miR-6512-3p expression in sh-HepG2 cells counteracted the decrease in proliferation observed following PAARH silencing. Therefore, PAARH may promote liver cancer cell proliferation by competitively inhibiting miR-6512-3p expression.

In addition, the present study further assessed the ceRNA mechanism of lncRNAs. To evaluate the ceRNA network mechanism of PAARH, bioinformatics tools were used to predict miR-6512-3p target genes. An increased expression level of LASP1 was associated with a poor prognosis in patients with liver cancer. LASP1 is a protein extensively expressed within cells, capable of promoting cellular cytoskeletal remodeling and migration by binding with cytoskeletal proteins, such as actin. This interaction enhances the invasive and metastatic capabilities of tumor cells, demonstrating that LASP1 is crucial for cellular adhesion, proliferation, migration and transformation (25). LASP1 also interacts with extracellular matrix receptors such as integrins, thereby regulating cell adhesion and infiltration (26). In tumor tissues, the overexpression of LASP1 may alter cell adhesion properties to the extracellular matrix, which has the effect of facilitating the invasion of tumor cells into surrounding tissues and promoting tumor development (27). Previous studies have also reported an aberrant expression of LASP1 in several types of cancer, with its expression closely associated with tumor prognosis. For example, a study by Ke *et al* (28) reported that LASP1, regulated by the miR-181c-5p/LASP1 axis, experienced changes in expression levels that affected the proliferation, invasion and migration of squamous cell carcinoma cells. Furthermore, a study by Herrmann *et al* (29) suggested that there was an association between LASP1 and chemotherapy resistance in chronic myeloid leukemia. Bioinformatics analyses in the present study were able to confirm the association between the upregulation of LASP1 expression in liver cancer and liver cancer prognosis. Further studies have reported that LASP1 is involved in cellular migration, invasion, proliferation and apoptosis through several mechanisms, such as participation in signaling pathways, m6A methylation modification or influencing the tumor microenvironment (30-34). In addition, progress has been made in terms of ongoing therapeutic research seeking to target LASP1 (35). Therefore, LASP1 is widely regarded as a prognostic marker for determining tumor prognosis (36-38).

In our previous study, the binding sites between lncRNA PAARH and miR-6512-3p were identified (16). In the present study, to elucidate the regulatory role of PAARH via the action of miR-6512-3p on LASP1 expression, the expression levels of LASP1 in HepG2 cells and PAARH-knockdown HepG2 cells were initially assessed. A significant decrease in LASP1 expression was observed in the PAARH-knockdown cells, with synchronized expression patterns between PAARH and LASP1. Furthermore, miR-6512-3p was demonstrated to suppress LASP1 expression; miR-6512-3p overexpression in liver cancer cells reduced LASP1 expression levels, whilst the inhibition of miR-6512-3p enhanced LASP1 expression, indicating a negative regulatory relationship between

miR-6512-3p and LASP1. lncRNA PAARH deletion increased miR-6512-3p expression in liver cancer cells, concomitant with a decrease in LASP1 expression. In addition, interference with miR-6512-3p expression suppressed the low expression of LASP1 in PAARH-knockdown liver cancer cells, suggesting that PAARH may regulate LASP1 expression via miR-6512-3p. Subsequent dual-luciferase reporter gene assays confirmed their binding relationship, where miR-6512-3p overexpression led to a reduction in LASP1 expression. These findings collectively confirmed the existence of the PAARH-miR-6512-3p-LASP1 ceRNA network.

The present study exhibits some limitations, including an insufficient exploration of the cellular biological functions and the absence of animal trials, which constitute considerable deficiencies. In conclusion, the present study demonstrated that lncRNA PAARH may regulate the expression of LASP1 by competitively sequestering miR-6512-3p, thereby promoting the proliferation of liver cancer cells. These findings further suggest that PAARH may serve as a potential prognostic biomarker and therapeutic target in liver cancer.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GL and ZiH conducted the analysis and interpretation of the experimental data for reverse transcription-quantitative PCR. JN analyzed and interpreted the experimental data for immunofluorescence and western blotting, and was responsible for creating and modifying all figures. YH, ZhH and LH performed bioinformatics analysis and interpretation. QW led the design and implementation of the experiments and was the primary contributor to manuscript writing. JP was the principal investigator, conceived and designed the experimental content, supervised the entire experimental process, made substantial revisions to the discussion section of the manuscript to ensure the accuracy or completeness of the research work, and undertook the task of drafting responses to reviewer comments. QW and GL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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