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ORIGINAL RESEARCH

Macrophage-associated lncRNA ELMO1-AS1: a novel therapeutic target and prognostic biomarker for hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is a prevalent malignant tumor. Long non-coding RNAs (lncRNAs) have been demonstrated to be abnormally expressed in many tumors and act as crucial regulators in various biological processes. However, the expression and function of the recently identified macrophage-associated lncRNA ELMO1 antisense RNA 1 (ELMO1-AS1) in HCC are unclear.

Methods: The expression of ELMO1-AS1 was determined in HCC tissues and adjacent nontumorous tissues by quantitative real-time polymerase chain reaction (qRT-PCR). The Kaplan-Meier survival analysis and Cox regression analysis were performed to establish the correlation between the expression level and survival of HCC patients in a training set and a validation set, respectively. The overexpression experiments were also conducted to investigate the biological role of ELMO1-AS1 in HCC cells.

Results: We uncovered that ELMO1-AS1 was significantly downregulated in HCC tissues, and high expression of ELMO1-AS1 is correlated with optimistic treatment outcome suggesting its potential as an independent prognostic biomarker for HCC. It was also found that overexpression of ELMO1-AS1 in HCC cells suppressed cell proliferation, migration and invasion and engulfment and cell motility 1 (ELMO1) may be a target of ELMO1-AS1.

Conclusion: Our results suggested that macrophage-associated lncRNA ELMO1-AS1 could be a crucial regulator involved in HCC progression and considered as a potential prognostic biomarker and therapeutic target for HCC.

Keywords: long noncoding RNA, ELMO1-AS1, HCC, prognosis

Introduction

Hepatocellular carcinoma (HCC), as the most common type of primary liver cancer, is one of the most common malignant tumors, which is in the top three leading causes of tumor-related death around the world.[1] HCC is particularly prevalent in Asia,[2,3] and approximately half of the new HCC patients come from China.[4] Although various risk factors of HCC occurrence and development have been revealed,[5] and the concepts of precision medicine (PM)[6] and multidisciplinary team (MDT)[7] have been applied in HCC therapy, poor prognosis and high mortality rate still persist.[8] New approaches to predict the outcome and further study to disclose the mechanism of HCC are urgently needed.

Long non-coding RNAs (lncRNAs), with more than 200 nucleotides in length and barely protein-coding ability, have been identified in the recent years. It is increasingly recognized that lncRNAs interact with DNA, RNA, protein molecules

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and/or their combinations, involve in serial steps of tumor development, and act as the crucial regulators in various biological processes.[9,10] In addition, detection of lncRNAs as the biomarkers in tumors is instrumental to early diagnosis, progression monitoring, prognosis prediction and targeted therapy.[11–13] However, the molecular mechanisms of lncRNAs for the occurrence and development of HCC remain unclear.

Our previous microarray profiling[14] also found that lncRNA ELMO1 antisense RNA 1 (ELMO1-AS1) was significantly upregulated in classically activated macrophages (M1 macrophages) and downregulated in alternatively activated macrophages (M2 macrophages; >10 fold change, P=0.002). To date, the relevance of ELMO1-AS1 to HCC, a novel macrophage-associated lncRNA, has not been investigated. In the present study, we aimed to examine the expression of ELMO1-AS1 in HCC tissues and their adjacent nontumorous tissues and explore its potential for prognosis. The functional studies were also carried out to assess the molecular mechanisms of ELMO1-AS1 in the HCC cells. This study shall offer new insights into the roles of ELMO1-AS1 in the development and progression of HCC.

Materials and methods

Patients and tissue specimens

Two independent sets comprising a total of 222 pairs of surgical specimens, tumor tissues and adjacent nontumorous tissues from HCC patients, were enrolled in this study from the Department of Hepatobiliary Surgery of the Affiliated Tumor Hospital of Guangxi Medical University in China. The training set enrolled 110 cases from January 2014 to December 2014, and the validation set contained 112 patients from January 2015 to August 2016. All patients in the present study underwent radical hepatectomy and had pathologically diagnosed with hepatocellular carcinoma. None of them had received radiotherapy or chemotherapy before surgery. The collected tissue specimens were immediately stored at −80 °C before RNA isolation. In addition, the clinicopathological parameters were retrieved, including gender, age, tumor number, tumor diameter, Alpha-fetoprotein (AFP), portal vein tumor thrombus (PVTT), microsatellite, microvascular invasion (MVI) and TNM stage. The follow-up data were obtained through telephone calls or outpatient visits. This study was approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

Cell culture

Two human liver cancer cell lines, SMMC-7721 and HepG2, were preserved in our central laboratory, and identified by short tandem repeat (STR) profile generated by GENEWIZ Inc. Cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) medium, supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA) with 100 U/ml penicillin and 100 μg/ml streptomycin and cultured in a humidified incubator with 5% CO_2 at 37 °C.

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from the enrolled samples and cell lines was isolated by TRIzol reagent (Invitrogen, CA, USA) according to the protocol of manufacturer. After purity and integrity test, RNA that met the requirements were reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Real-time PCR was performed to detect the expression of RNAs using FastStart Universal SYBR Green Master (ROX) kit (Roche, Germany). Relative expression level was normalized to an endogenous control (β-actin) by relative quantification method ($2^{-\Delta\Delta Ct}$). The primers for ELMO1-AS1 were 5′-TCGGGTGGAAGTCGTTGC-3′ and 5′- CCTCCGCTTGT CTCCCTTT-3′; the primers for ELMO1 were 5′- CCAC GACAGATCCTTTGAGGAG-3′ and 5′- CTCATAACCTG CTCCTTCACCAC-3′; the primers for β-actin were 5′-TGC GTGACATTAAGGAGAAG-3′ and 5′- GTCAGGCAGC TCGTAGCTCT-3′.

Plasmid construction, lentivirus generation and cell infection

Full-length ELMO1-AS1 cDNA was compounded and inserted into the lentiviral expression plasmid pCDH commercially (Sagene, China). HEK293T cells were co-transfected with the lentiviral plasmid packaging system and pCDH-ELMO1-AS1 or pCDH-empty control using Lipofectamine 2000 (Invitrogen, USA). After transfection for 48 hrs, lentiviral particles in the medium were collected and centrifuged at 3000 g for 15 min and then filtered through a 0.22 μm filter. HCC cells were transfected with LV-pCDH-ELMO1-AS1 or LV-pCDH-empty control, respectively. After 48 h, the stable lines were selected by puromycin and used for the functional assay.

Cell proliferation assay

The cell counting kit-8 (CCK-8) assay was employed to detect the proliferation of HCC cells. Approximately 3,000 cells/well HCC cells, ELMO1-AS1 and control, were planked in 96-well plates. The cells were incubated with 100 µl fresh culture medium mixed with 10 µl CCK-8 (7sea, China) for 2 h at 0 h, 24 h, 48 h, 72 h and 96 h after seeded. The viability of HCC cells was detected by the optical density (OD) at a wavelength of 450 nm by using an enzyme-labeled analyzer.

Colony formation assay

500 HCC cells were planked per well in 6-well plates. The culture medium was replaced every 3 days. When macroscopic colonies appeared, the colonies were fixed with methyl alcohol for 30 min and dyed with 0.1% crystal violet solution for 30 min. Colonies in every well were counted independently.

Wound healing assay

HCC cells were seeded into 6-well plates and cultured in the humidified incubator overnight. Artificial wounds were performed by scratching with the 200 µl pipette tips and then incubated with serum-free medium to proceed. The artificial wounds were imaged at 0 h and 24 h by an inverted microscope.

Migration and invasion assays

According to assay protocol, 24-well plate with 8-µm pore size transwell chamber (Costar, USA) with or without Matrigel-coated membrane (BD, USA) was used to measure HCC cell migration or invasion. 5×10^4 and 1×10^5 cells were suspended into the upper chamber per well, respectively. In both assays, HCC cells were suspended in 200 µl of DMEM medium without FBS. 700 µl of DMEM containing 10% FBS was added in the lower chamber. After 18–24 hrs of culture in the incubator, the cells migrated to the bottom surface of the membrane, were fixed with methyl alcohol, stained with 0.1% crystal violet solution for 30 min and photographed and counted under an inverted microscope.

Statistical analysis

All statistical tests for this study were performed using SPSS 22.0 software (Chicago, IL, USA). Graphs were constructed using GraphPad Prism 5 software (La Jolla, CA, USA).

The data about continuous variables from experiments are presented as the mean ± SEM. Student's *t*-test was carried out to evaluate ELMO1-AS1 RNA level between human HCC tissues and the matched non-tumorous samples. Qualitative variables were compared using $\chi2$ tests. The Kaplan-Meier survival analysis and Cox regression analysis test were performed to compare the correlation between the expression level and survival. Two-sided *P*-value less than 0.05 was considered as the statistical significance.

Results

Downregulation of ELMO1-AS1 expression in HCC patients

We measured ELMO1-AS1 in 222 HCC tissue samples and the adjacent nontumorous tissues by qRT-PCR. In order to verify the reliability of the results, a combined set uniting the training set and the validation set was also analyzed. To establish the correlation between ELMO1-AS1 and clinicopathological features, the samples in each set were categorized into two groups (high or low) depending on the median values of ELMO1-AS1 expression in HCC tissues. For the training set, the expression level of ELMO1-AS1 was evidently down-regulated in HCC tissues compared with adjacent nontumorous tissues ($P<0.001$, Figure 1A). For the validation set, ELMO1-AS1 expression was also dramatically reduced in HCC tissues ($P<0.001$, Figure 1B). These results are consistent with the observation from the combined set ($P<0.001$, Figure 1C).

Correlations between aberrant expression of ELMO1-AS1 and clinical pathological features of HCC

Baseline characteristics of HCC patients are shown in Table 1. 110 patients and 112 patients were included in the training set and validation set, respectively. In the training set, the ELMO1-AS1 expression level was strikingly lower in HCC tissues with portal vein tumor thrombus (PVTT, $P=0.039$). In the validation set, the expression level of ELMO1-AS1 was obviously correlated with microvascular invasion (MVI, $P=0.038$).

Association between ELMO1-AS1 expression and outcomes of patients with HCC

We compared the overall survival (OS) and disease-free survival (DFS) between different groups based on ELMO1-AS1

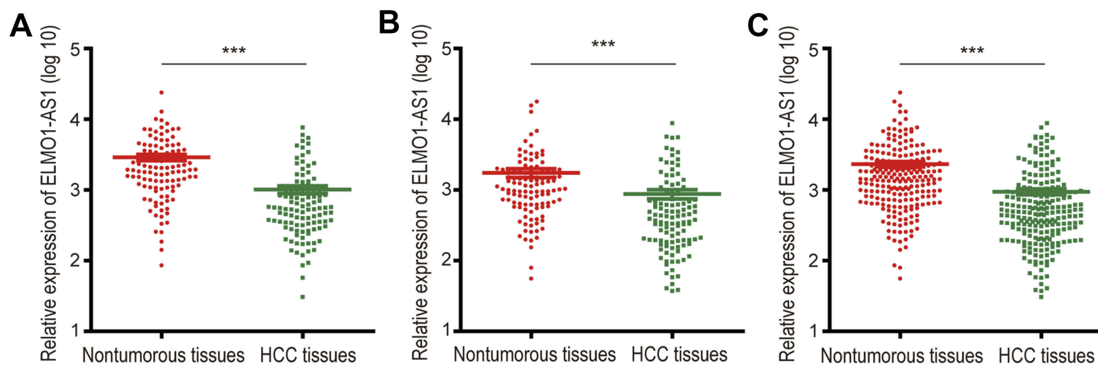

Figure 1 Relative expression of ELMO1-AS1 was frequently downregulated in HCC tissues. ELMO1-AS1 expression, as measured by qRT-PCR, was downregulated in HCC tissues when compared with adjacent nontumorous tissues in the training set (**A**), validation set (**B**) and combined set (**C**). ****P*<0.001.
Abbreviations: HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction.

level. Kaplan-Meier survival analysis suggested that patients with low ELMO1-AS1 expression had a poor OS compared to those with high ELMO1-AS1 expression in the training set, validation set and combined set (46.4% vs 63.2%, *P*=0.021; 47% vs 69.8%, *P*=0.002 and 38.6% vs 60.1%, *P*<0.001, respectively; Figure 2A–C). Consistently, patients with low ELMO1-AS1 showed a poor DSF in all three sets (27.0% vs 50.4%, *P*=0.022; 37.1% vs 51.8%, *P*=0.019 and 23.2% vs 48.7%, *P*<0.001, respectively; Figure 2D–F). Taken together, low ELMO1-AS1 expression is correlated with pessimistic outcomes of HCC.

Univariate and multivariate regression analyses of overall survival and disease-free survival

Univariate and multivariate analysis was implemented for OS (Table 2) and DFS (Table 3) to further investigate the clinical relevance of ELMO1-AS1. In the training set, high ELMO1-AS1 expression was disclosed to be an independent protective factor for OS and DFS (HR: 0.518; 95% CI: 0.277–0.968; *P*=0.039 for OS; HR: 0.557; 95% CI: 0.323–0.960; *P*=0.035 for DFS). In the validation set, high ELMO1-AS1 expression was an independent protective factor for OS (HR: 0.430; 95% CI: 0.225–0.824; *P*=0.011), but not DFS (HR: 0.616; 95% CI: 0.363–1.045; *P*=0.072). Moreover, when combined the two sets, ELMO1-AS1 expression was an independent prognostic factor for both OS and DFS (HR: 0.407; 95% CI: 0.257–0.643; *P*<0.001 for OS; HR: 0.476; 95% CI: 0.324–0.699; *P*<0.001 for DFS). HCC patients with high ELMO1-AS1 expression is likely to have a better prognosis.

Overexpression of ELMO1-AS1 inhibited HCC cell proliferation

SMMC-7721 and HepG2 cells were infected with lentivirus containing the plasmid for stable expression of ELMO1-AS1. After being selected by puromycin, the transfection efficiency of the cells was assessed by qRT-PCR and results showed that the relative expression level of ELMO1-AS1 transfected with LV-pCDH-ELMO1-AS1 is significantly higher compared with LV-pCDH-empty control (Figure 3A). Then CCK-8 assay revealed that upregulated ELMO1-AS1 expression markedly inhibited the growth of both SMMC-7721 and HepG2 cells (Figure 3B and C). Similarly, colony formation assay disclosed that the number of HCC cell colonies were distinctly decreased by overexpression of ELMO1-AS in SMMC-7721 and HepG2 cells (Figure 3D and E).

Overexpression of ELMO1-AS1 inhibited HCC cell migration and invasion

To investigate whether ELMO1-AS1 is involved in HCC cells migration and invasion, wound healing assay and transwell assay were performed. Cell wound healing assay showed that overexpression of ELMO1-AS1 in HCC cells led to slow wound healing rate (Figure 4A and B). Transwell assays without or with Matrigel both found that HCC cells overexpressed with ELMO1-AS1 had less active migration and invasion than control cells (Figure 4C–F). These results indicated that ELMO1-AS1 had an important role in inhibiting HCC cells migration and invasion in vitro.

Prediction of ELMO1-AS1

A co-expression network using Lnc2Catlas[15] indicated that ENST00000419535.1, the transcript of ELMO1-AS1 is

Table 1 Compare between ELMO1-AS1 expression and clinicopathological characteristics in HCC patients

	Training set				Validation set				**Combined set**			
Clinicopathologic Parameters	Total (n=110)	Low (n=55)	High (n=55)	*P*-value	Total (n=112)	Low (n=56)	High (n=56)	*P-value*	Total (n=222)	Low (n=111)	High (n=111)	*P*-value
Gender				0.801				0.341				0.239
Female	19	9	10		11	4	7		30	12	18	
Male	91	46	45		101	52	49		192	99	93	
Age (years)				0.057				0.280				1.000
<55	88	40	48		96	50	46		184	92	92	
≥55	22	15	7		16	6	10		38	19	19	
Tumor number				0.606				0.645				0.304
<3	92	47	45		88	43	45		180	93	87	
≥3	18	8	10		24	13	11		42	18	21	
Tumor diameter				0.189				0.357				0.113
<5 cm	28	11	17		24	10	14		52	21	31	
≥5 cm	82	44	38		88	46	42		170	90	80	
AFP				1.000				0.057				0.178
<400 ng/ml	58	29	29		50	20	30		108	49	59	
≥400 ng/ml	52	26	26		62	36	26		114	62	52	
PVTT				0.039				1.000				0.178
No	97	45	52		94	47	47		191	92	99	
Yes	13	10	3		18	9	9		31	19	12	
Microsatellite				0.185				0.324				0.239
No	100	48	52		92	44	48		192	93	99	
Yes	10	7	3		20	12	8		30	18	12	
MVI				0.340				0.038				0.060
No	55	25	30		55	22	33		110	48	62	
Yes	55	30	25		57	34	23		112	63	49	
TNM stage				0.152				0.287				0.139
I+II	75	34	41		72	34	38		147	69	78	
III+IV	35	21	14		38	22	16		73	42	31	

Abbreviations: AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus; MVI, microvascular invasion.

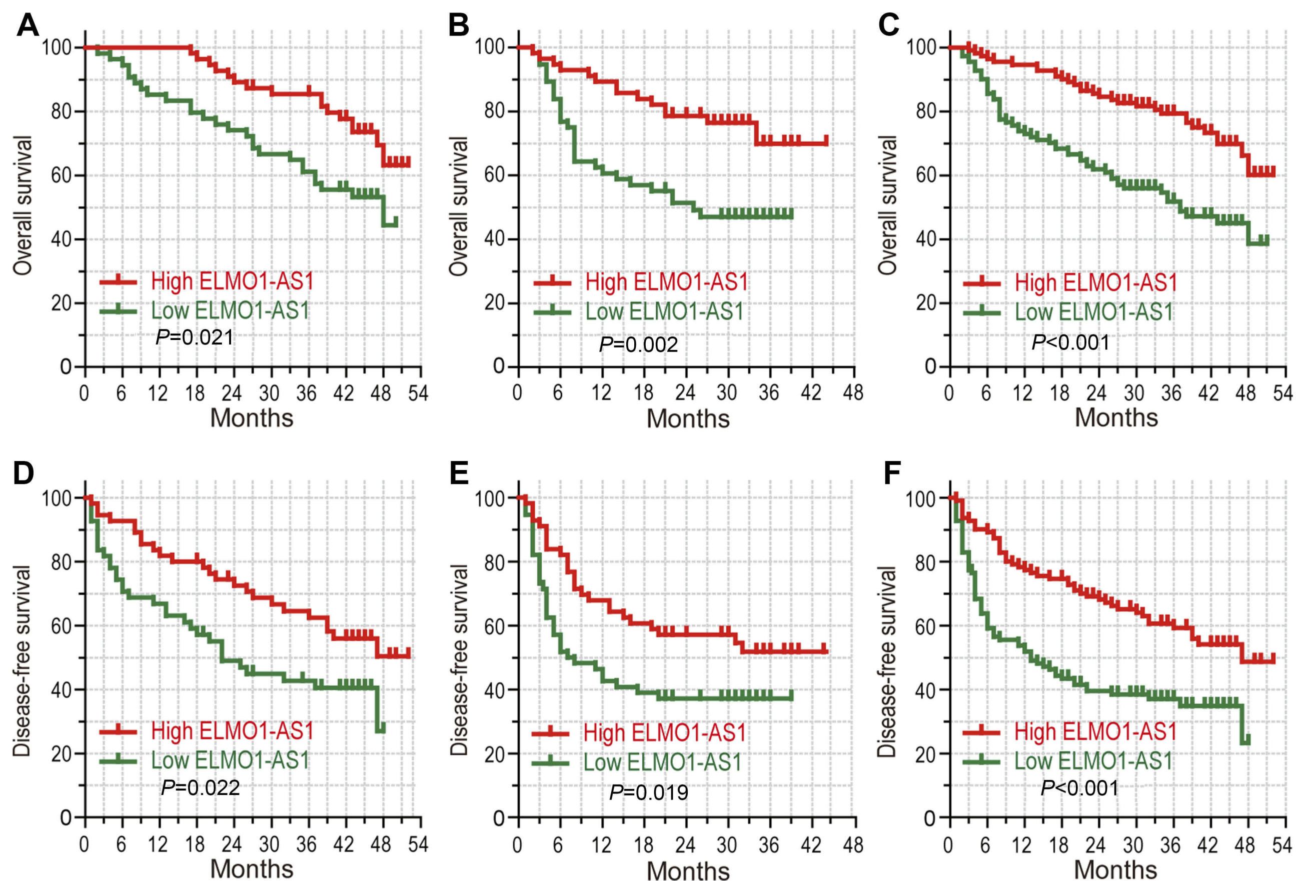

Figure 2 High level of ELMO1-AS1 expression indicated better prognosis. Kaplan-Meier survival analysis comparing time to overall survival and disease-free survival of HCC patients with high or low ELMO1-AS1 expression in training cohort (n=110; **A, D**), validation cohort (n=112; **B, E**) and combined set (n=222; **C, F**).
Abbreviation: HCC, hepatocellular carcinoma.

implicated with various diseases and physiology processes (Figure 5A). Further research by lncLocator[16] discovered that cytoplasm is the dominant subcellular location of ELMO1-AS1, suggesting that ELMO1-AS1 may target miRNAs to modulate genes expression and biological functions as a competitive endogenous RNA (ceRNA). The potential targets of ELMO1-AS1 were identified based on the intersection of DIANA tools-LncBase v.2[17] and miRDB databases.[18] Three miRNAs, hsa-miR-4267, hsa-miR-5096 and hsa-miR-4691-5p, appeared in both sets (Figure 5B). We searched these three miRNAs in TargetScan[19] to find the feasible mRNA targets, based on total context++ score and a cutoff value less than −0.10. Intriguingly, 60 identical mRNAs were found in the intersection of miRNAs targets, including engulfment and cell motility 1 (ELMO1), the neighboring intrachromosomal coding gene of ELMO1-AS1 (Figure 5C). The relationship between ELMO1-AS1 and ELMO1 was assessed in HCC tissues by qRT-PCR and showed a positive correlation (r =0.905, P=0.005, Figure 5D).

Discussion

For the past few years, surgical resection, transplantation, radiotherapy, transarterial chemoembolization and molecular targeted therapy have become the main means of treatment for HCC patients, but the outcome remains pessimistic.[20] It is an enormous clinical challenge to investigate the internal mechanisms of HCC development and excavate novel prognostic indicators.[21] Mounting evidence indicates a vital role for long non-coding RNAs in the development and progression of human tumors. Localizing to chromatin, interacting with protein, modulating RNA metabolism and driving cancer phenotypes are some functions of lncRNAs but not all.[22] Moreover, It also has been used for prediction of tumor prognosis among multiple tumors. Sadek et al[23] suggested in their research that lncRNAs, including ecCEBPA and UCA1, may be helpful as prognostic of HCC. The results of Fang et al[24] showed overexpression of lncRNA DGCR5 inhibits bladder cancer and correlates with better prognosis via transcriptionally facilitating P21 expression. As a matter of fact, the value of lncRNAs on estimating the outcome of

Table 2 Univariate and multivariate Cox regression analyses of overall survival

Characteristic	Training set		Validation set		Combined set	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Univariate analyses						
Gender	1.071 (0.476–2.413)	0.868	5.316 (0.732–38.629)	0.099	1.747 (0.844–3.619)	0.133
Age	0.761 (0.338–1.714)	0.761	0.582 (0.208–1.626)	0.302	0.650 (0.345–1.224)	0.182
Tumor number	1.379 (0.658–2.892)	0.395	2.291 (1.213–4.329)	0.011	1.835 (1.137–2.963)	0.013
Tumor diameter	1.943 (0.863–4.374)	0.109	3.529 (1.259–9.889)	0.016	2.582 (1.371–4.865)	0.003
AFP	1.197 (0.652–2.199)	0.562	2.176 (1.138–4.163)	0.019	1.650 (1.069–2.545)	0.024
PVTT	1.694 (0.749–3.832)	0.205	1.776 (0.876–3.601)	0.111	1.840 (1.081–3.134)	0.025
Microsatellite	4.082 (1.911–8.720)	<0.001	2.357 (1.209–4.594)	0.012	3.201 (1.938–5.287)	<0.001
MVI	1.349 (0.734–2.479)	0.334	2.519 (1.335–4.754)	0.004	1.831 (1.185–2.829)	0.006
TNM stage	2.011 (1.092–3.702)	0.025	2.340 (1.295–4.230)	0.005	2.197 (1.438–3.359)	<0.001
ELMO1-AS1 expression	0.490 (0.262–0.914)	0.025	0.386 (0.206–0.724)	0.003	0.403 (0.258–0.629)	<0.001
Multivariate analyses						
Gender						
Age						
Tumor number			1.514 (0.687–3.336)	0.304	1.438 (0.822–2.514)	0.203
Tumor diameter			2.381 (0.808–7.022)	0.116	1.811 (0.932–3.520)	0.080
AFP			1.293 (0.626–2.669)	0.487	1.367 (0.868–2.152)	0.178
PVTT					1.075 (0.604–1.914)	0.805
Microsatellite	2.794 (1.311–5.954)	0.008	1.337 (0.663–2.912)	0.465	2.013 (1.159–3.496)	0.013
MVI			1.382 (0.663–2.880)	0.387	1.271 (0.782–2.064)	0.333
TNM stage	1.825 (0.987–3.374)	0.055	1.486 (0.764–2.891)	0.243	2.359 (0.828–2.233)	0.225
ELMO1-AS1 expression	0.518 (0.277–0.968)	0.039	0.430 (0.225–0.824)	0.011	0.407 (0.257–0.643)	<0.001

Abbreviations: AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus; MVI, microvascular invasion.

Table 3 Univariate and multivariate Cox regression analyses of disease-free survival

Characteristic	Training set		Validation set		Combined set	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Univariate analyses						
Gender	1.110 (0.543–2.267)	0.775	8.868 (1.228–64.043)	0.030	1.982 (1.036–3.792)	0.039
Age	1.031 (0.544–1.953)	0.925	0.830 (0.394–1.746)	0.624	0.921 (0.569–1.490)	0.736
Tumor number	1.501 (0.774–2.910)	0.229	1.836 (1.058–3.185)	0.031	1.713 (1.124–2.609)	0.012
Tumor diameter	1.745 (0.901–3.378)	0.099	1.637 (0.830–3.230)	0.155	1.732 (1.079–2.782)	0.023
AFP	1.127 (0.667–1.905)	0.655	1.798 (1.064–3.037)	0.028	1.461 (1.013–2.106)	0.042
PVTT	2.162 (1.052–4.443)	0.036	1.656 (0.894–3.065)	0.109	1.905 (1.194–3.039)	0.007
Microsatellite	1.351 (0.578–3.162)	0.488	2.030 (1.143–3.604)	0.016	1.886 (1.182–3.009)	0.008
MVI	1.709 (1.005–2.906)	0.048	1.872 (1.119–3.133)	0.017	1.773 (1.226–2.565)	0.002
TNM stage	1.296 (0.744–2.259)	0.360	1.933 (1.160–3.220)	0.011	1.1623 (1.117–2.356)	0.011
ELMO1-AS1 expression	0.546 (0.320–0.930)	0.026	0.556 (0.334–0.926)	0.024	0.469 (0.323–0.681)	<0.001
Multivariate analyses						
Gender			7.808 (1.066–57.205)	0.043	1.762 (0.916–3.389)	0.090
Age						
Tumor number			1.195 (0.609–2.346)	0.605	1.605 (0.968–2.661)	0.067
Tumor diameter					1.462 (0.884–2.416)	0.139
AFP			1.448 (0.798–2.627)	0.224	1.237 (0.835–1.832)	0.290
PVTT	1.574 (0.741–3.345)	0.238			1.246 (0.745–2.084)	0.403
Microsatellite			1.369 (0.703–2.665)	0.356	1.067 (0.623–1.827)	0.812
MVI	1.642 (0.947–2.849)	0.077	1.072 (0.574–2.002)	0.826	1.466 (0.973–2.210)	0.067
TNM stage			1.535 (0.863–2.730)	0.145	0.999 (0.642–1.556)	0.998
ELMO1-AS1 expression	0.557 (0.323–0.960)	0.035	0.616 (0.363–1.045)	0.072	0.476 (0.324–0.699)	<0.001

Abbreviations: AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus; MVI, microvascular invasion.

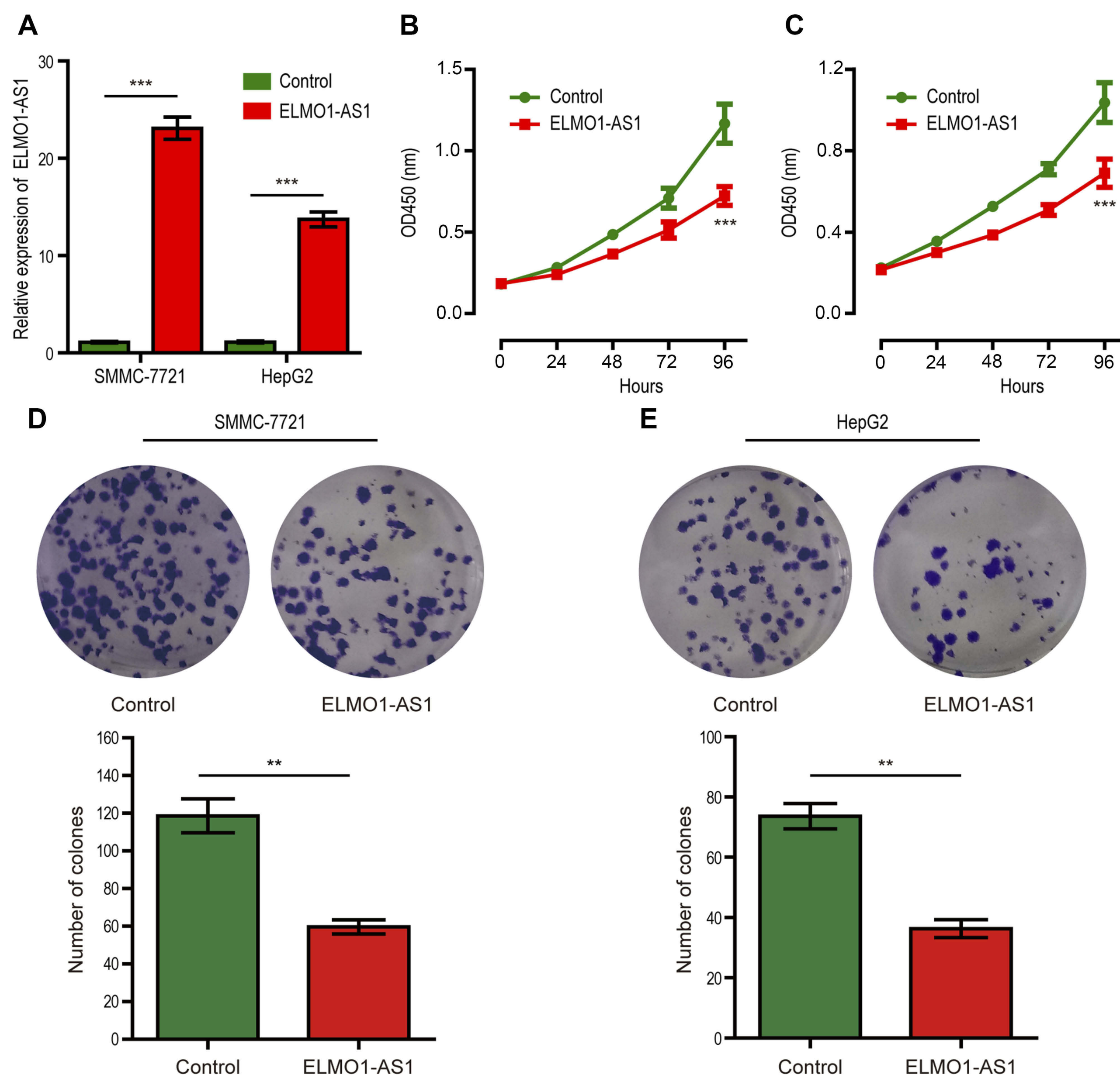

Figure 3 Overexpression of ELMO1-AS1 inhibits HCC cells proliferation. Expression of ELMO1-AS1 in SMMC-7721 and HepG2 cells was significantly upregulated after ELMO1-AS1 lentiviral transfection compared to control (**A**). The CCK-8 assay was performed to determine the proliferation of ELMO1-AS1 or control-transfected SMMC-7721 (**B**) and HepG2 (**C**) cells. Colony formation assay was performed to determine the proliferation of ELMO1-AS1 or control-transfected SMMC-7721 (**D**) and HepG2 (**E**) cells. ** and *** represent $P<0.01$ and $P<0.001$, respectively.
Abbreviations: CCK-8, cell counting Kit-8; OD, optical density; HCC, hepatocellular carcinoma.

HCC patients could provide an accurate direction in the postoperative adjuvant therapy,[25,26] and there are few reports on the relationship between ELMO1-AS1 and HCC.

We investigated the expression of ELMO1-AS1 in two independent sets collected at different times with 222 pairs of tumor tissues and adjacent nontumorous tissues from HCC patients. Evidently, the expression of ELMO1-AS1 is significantly lower in tumor tissues compared to adjacent nontumorous tissues. Statistical analyses suggest that high ELMO1-AS1 expression is closely correlated with a longer overall survival and a longer disease-free survival of HCC patients. Univariate and multivariate analyses of clinicopathological features also show that ELMO1-AS1 expression is an independent protective factor for OS and DFS in patients with HCC, implying that it could be employed as a prognostic biomarker for HCC.

However, the biological role of ELMO1-AS1 in HCC cells remained poorly clear. In this work, we found that

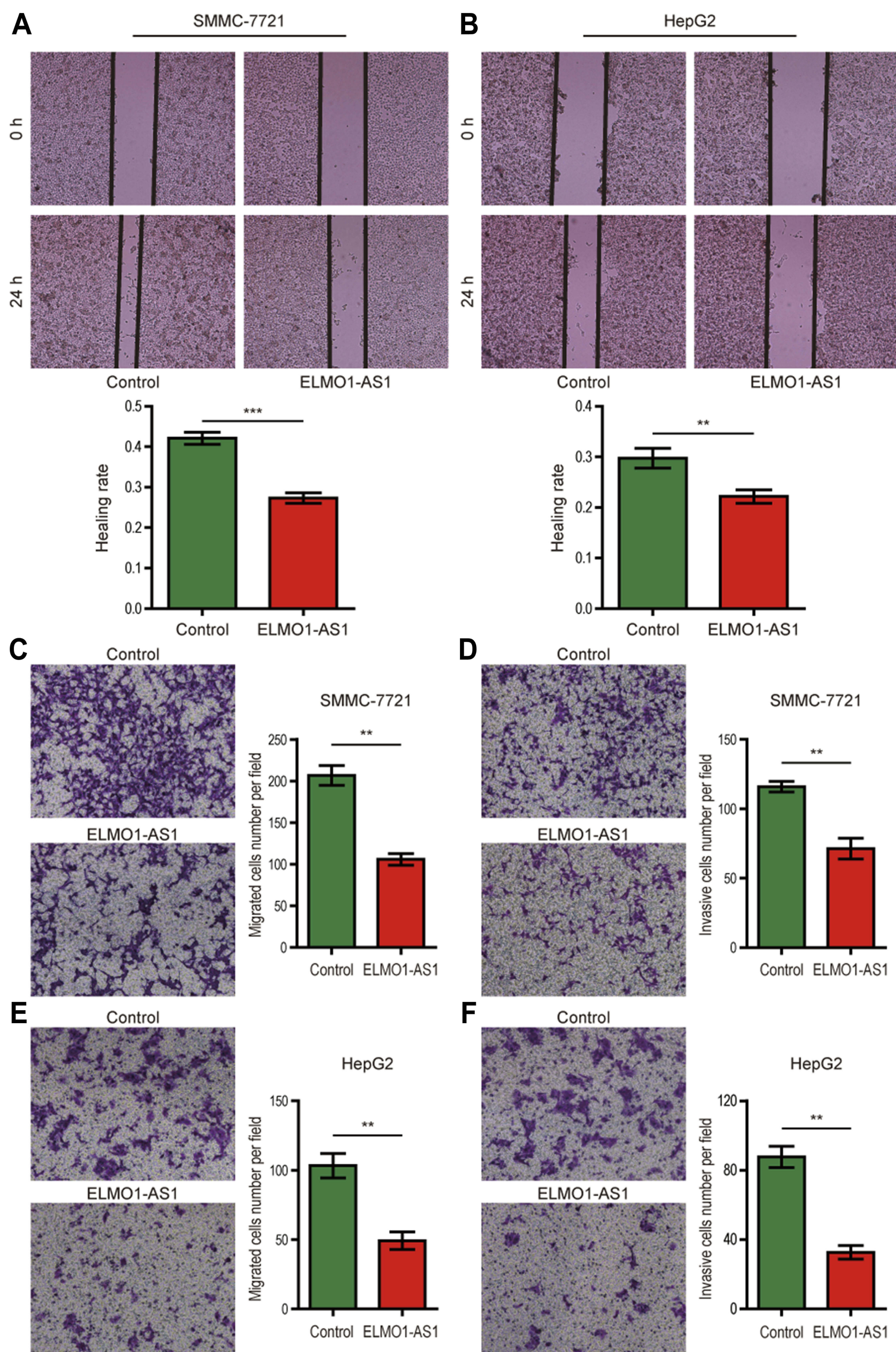

Figure 4 Overexpression of ELMO1-AS1 inhibited HCC cell migration and invasion in vitro. Wound healing assay showed that the migratory potential of SMMC-7721 (**A**) and HepG2 (**B**) cells were significantly reduced in the ELMO1-AS1-transfected group than in the control-transfected group. Transwell migration assay showed that the number of migrated SMMC-7721 (**C**) and HepG2 (**E**) cells was significantly lower in the ELMO1-AS1-transfected group than in the control-transfected group. Transwell invasion assay showed that the number of invaded SMMC-7721 (**E**) and HepG2 (**F**) cells was significantly lower in the ELMO1-AS1-transfected group than in the control-transfected group. ** and *** represent $P<0.01$ and $P<0.001$, respectively.

Abbreviation: HCC, hepatocellular carcinoma.

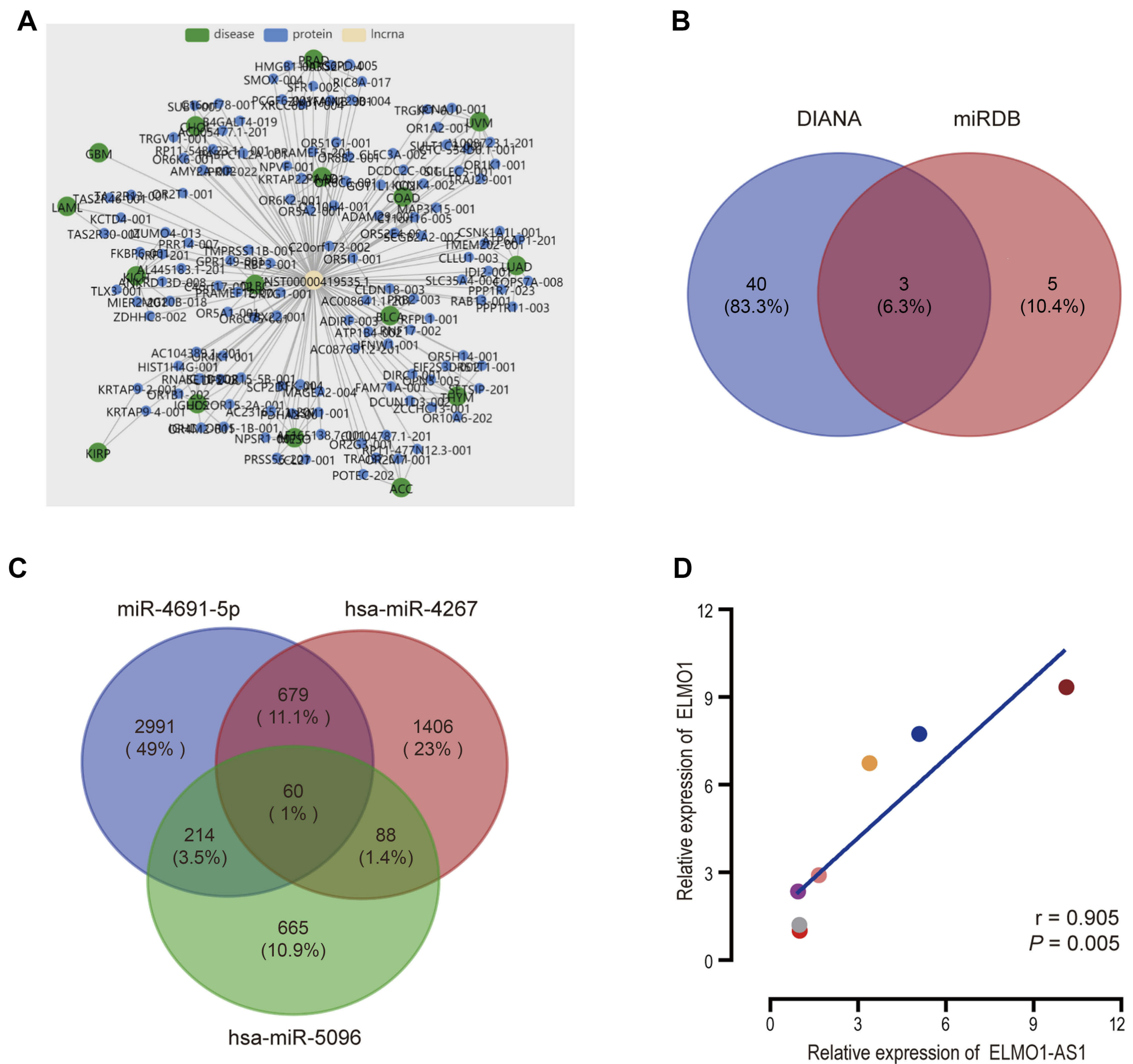

Figure 5 ELMO1 could be one of the potential targets of ELMO1-AS1. The co-expression network showed ELMO1-AS1 associated with multiple diseases and proteins (**A**). Three differently expressed target miRNAs of ELMO1-AS1 were integrated from DIANA and miRDB databases (**B**). ELMO1 was one of the intersections of ELMO1-AS1 associated miRNAs targets (**C**). Pearson analysis of the relationship between ELMO1-AS1 and ELMO1 expression (**D**).

stable upregulated ELMO1-AS1 expression in HCC cells influenced their biological behavior. Cell proliferation assay and colony formation assay showed that overexpression of ELMO1-AS1 inhibited HCC cells growth. On the other hand, the results of wound healing assay and transwell assays indicated that ectopic expression of ELMO1-AS1 suppressed the migration and invasion of HCC cells in vitro. These results suggested that ELMO1-AS1 played an important regulatory role of in HCC.

Moreover, bioinformatics analysis and molecular biology experiment were taken to explore the function and probable molecular mechanism. The co-expression network showed a huge three-dimensional space network among ELMO1-AS1, diseases and proteins, which indirectly imply the importance of ELMO1-AS1 in biology. Accumulating studies have demonstrated that lncRNAs regulate cell functions through interacting with 3′-UTR of miRNAs in the competitive endogenous RNAs (ceRNA) regulatory networks.[27] For example, Huang et al[28] found that lncRNA IGF2-AS affects gastric adenocarcinoma development by SHOX2 via sponging miR-503. Sun et al[29] suggested that high expression of lncRNA HOTTIP is associated with small cell lung cancer

progression through the ceRNA network HOTTIP/miR-574-5p/EZH1. Therefore, we hypothesized that ELMO1-AS1 may also serve as a ceRNA. DIANA tools-LncBase v.2 and miRDB databases were used to predict the potential binding sites between ELMO1-AS1 and miRNAs, indicated that ELMO1-AS1 might target one or more of 43 and 8 miRNAs, respectively. We took the intersection of these two miRNA groups and three miRNAs were identified. TargetScan database was performed to research the connections with ELMO1-AS1. ELMO1, the neighboring intrachromosomal coding gene of ELMO1-AS1, which could function in apoptosis and cell migration,[30] was appeared to be a possible target of miRNAs as described, reminding ELMO1 might be one of the potential target genes of ELMO1-AS1. Moreover, the strong positive correlation between ELMO1-AS1 and ELMO1 shall support this suspect using qRT-PCR array. Multiple studies have shown that neighboring intrachromosomal coding genes can be regulated by lncRNA transcript.[31,32] For instance, Li et al disclosed that lncRNA ZEB1-AS1 is bound up with ZEB1 and positively mediates the ZEB1 expression.[33] Taken together, ELMO1-AS1 shall act as a tumor suppressor in HCC by targeting its neighboring intrachromosomal coding gene ELMO1 thus affect HCC biological behavior. However, the exact mechanism remains to be further studied.

The ELMO1-AS1, located at chromosome 7p14.2, is identified as Homo sapiens engulfment and cell motility 1 antisense RNA 1. The previous microarray profiling experiments, which detecting the differential expression of lncRNAs between diverse phenotypes of macrophage, showed that ELMO1-AS1 was significantly upregulated in M1 macrophages compared to M2 macrophages. Many studies have shown that tumor-associated macrophages (TAMs), mainly M2 macrophages, are recruited to the tumor sites whereby to promote tumor angiogenesis and tumor cell proliferation.[34–36] They also had a close relationship with poor prognosis of patients with different tumors.[37,38] Consistently, our results showed that macrophage-associated lncRNA ELMO1-AS1 was upregulated in adjacent nontumorous tissues compared to tumor tissues and high expression of ELMO1-AS1 could inhibit tumor cell progression and predict favorable prognosis in HCC patients. ELMO1, a possible target of ELMO1-AS1 as demonstrated by us before, have been shown to mediate bacterial internalization and intestinal inflammation in macrophages by Sarkar et al[39] Shi et al[40] disclosed that CC chemokine ligand 18 (CCL18) predominantly secreted by TAMs, could promote migration and invasion of non-small cell lung cancer cells through activating ELMO1-integrin β1 signaling. Those results further suggest that ELMO1-AS1 could affect the occur and development progress of HCC through ELMO1. Moreover, the oncolytic adenovirus expressing tumor suppressor gene, such as SNORD44, GAS5 or TSLC1, could enhance the anti-tumor effect through targeting some crucial signaling pathways.[41,42] Genome editing using clustered regularly interspaced short palindromic repeats-associated endonuclease 9 (CRISPR-Cas9) is an efficient approach to not only delete or block lncRNAs expression by the targeted interruption, but also upregulate lncRNA expression by targeting transcriptional activator complexes or inserting a promoter.[32] These indicated to us that overexpression of ELMO1-AS1 by oncolytic adenovirus or CRISPR-Cas9 provides a hopeful method for HCC targeted therapy. It was found for the first time that macrophage-associated lncRNA ELMO1-AS1 could regulate behaviors of HCC cells and predict the prognosis of HCC patients. However, further studies are required. First, in the validation set, high ELMO1-AS1 expression was an independent protective factor for DFS only under univariate analysis model, but failed to reach significance under multivariate analysis model. That might be explained by patient heterogeneity, because significance was found in the training set and combined set. Second, the exact mechanism of how ELMO1-AS1 regulate its neighboring intrachromosomal coding gene ELMO1 by targeting miRNAs need to be further studied. Third, in vivo experiments are needed to confirm some conclusions drawn from in vitro experiments.

Conclusion

Our results showed that macrophage-associated lncRNA ELMO1-AS1 was significantly downregulated in HCC tissues and high expression of ELMO1-AS1 associated with optimistic outcome in HCC patients. ELMO1-AS1 could inhibit HCC cells proliferation, migration and invasion and ELMO1 may be a target of ELMO1-AS1. ELMO1-AS1 could be a potential prognostic biomarker and therapeutic target for HCC.

Ethical approval and informed consent

All patients provided written informed consent, and that this was conducted in accordance with the Declaration of Helsinki.

This study was approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University.

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Disclosure

The authors report no conflicts of interest in this work.

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