Sp1 cooperates with Sp3 to upregulate MALAT1 expression in human hepatocellular carcinoma

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Abstract. Long non-coding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as nuclear-enriched transcript 2 (NEAT2), is highly conserved among mammals and highly expressed in the nucleus. It was first identified in lung cancer as a prognostic marker for metastasis but is also associated with several other solid tumors. In hepatocellular carcinoma (HCC), MALAT1 is a novel biomarker for predicting tumor recurrence after liver transplantation. The mechanism of overexpression in tumor progression remains unclear. In the present study, we investigated the role of specificity protein 1/3 (Sp1/3) in regulation of MALAT1 transcription in HCC cells. The results showed a high expression of Sp1, Sp3 and MALAT1 in HCC vs. paired non-tumor liver tissues, which was associated with the AFP level (Sp1, r=7.44, P=0.0064; MALAT1, r=12.37, P=0.0004). Co-silencing of Sp1 and Sp3 synergistically repressed MALAT1 expression. Sp1 binding inhibitor, mithramycin A (MIT), also inhibited MALAT1 expression in HCC cells. In conclusion, the upstream of MALAT1 contains five Sp1/3 binding sites, which may be responsible for MALAT1 transcription. Inhibitors, such as MIT, provide a potential therapeutic strategy for HCC patients with MALAT1 overexpression.

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Abbreviations: HCC, hepatocellular carcinoma; lncRNAs, long non-coding RNAs; NEAT2, nuclear-enriched transcript 2; ncRNA, non-coding RNA; miRNA, micro-RNA; AFP, α-fetoprotein; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein; SRSF1, serine/arginine-rich splicing factor 1; YAP, Yes-associated protein; AMOT, angiomotin; LTBP3, latent-transforming growth factor β-binding protein-3; TGF-β, tumor growth factor-β; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; MIT, mithramycin A

Key words: hepatocellular carcinoma, transcriptional regulation, Sp1, Sp3, MALAT1, mithramycin A

Introduction

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts containing >200 nucleic acids (1). LncRNA functions to regulate gene transcription via post-transcriptional modification and interacts with microRNA (miRNA). LncRNA plays a crucial role in the regulation of human carcinogenesis (2-5). In hepatocellular carcinoma (HCC), an altered lncRNA expression is associated with tumor progression. Therefore, lncRNA is a potential target for HCC treatment and should be investigated in HCC development and progression (6-10).

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is highly conserved among mammals and primarily expressed in the nucleus (11-14). MALAT1 overexpression is associated with elevated levels of α-fetoprotein (AFP) and a number of tumor lesions, and has been identified in host reaction after liver transplantation (15). MALAT1 interacts with various target genes, such as serine/arginine-rich splicing factor 1 (SRSF1) and potential gene signaling pathways associated with human carcinogenesis (11-14). Nevertheless, the transcriptional regulation of MALAT1, per se, remains unknown.

The sequence database, ChIPBase, was established using the result of 543 ChIP-Seq experiments. Analysis of this database revealed the potential regulation between transcription factors and non-coding RNA (ncRNA), indicating a possible connection of a well-known transcription factor specificity protein 1 (Sp1) with MALAT1 expression (16). Moreover, Sp3 recognizes similar amino acid sequences of Sp1 DNA-binding domains (17-19). In the present study, we investigated the role of Sp1/3 in the regulation of MALAT1 transcription in HCC cells. Our findings provide mechanistic information for future investigations of the regulation of HCC progression mediated by Sp1/3 transcription of MALAT1 expression.

Materials and methods

Patient samples. Thirty-two fresh HCC and paired distant non-tumor liver tissues were randomly selected from histologically confirmed HCC patients from the First Affiliated Hospital of Guangxi Medical University (Guangxi, China) between March and November 2014. The paired non-tumor tissues were at least 2 cm away from the tumor lesion. The current study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from all the patients. Clinicopathological characteristics were collected and are provided in Table I. The mean of mRNA expression was set as the cut-off value to define low or high expression (>mean, high; <mean, low) (Table I). The relationship between Sp expression and MALAT1 expression are shown in Table II.

RNA isolation and RT-qPCR. Total cell RNA was isolated from fresh tissues or cells using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA using a Thermo Scientific RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. qPCR was performed in a Light Cycler 480 (Roche, Basel, Switzerland) with a SYBR-Green Premix Ex Taq (Roche). β-actin mRNA was used as an internal control. The primers used were: Sp1, 5'-TCCAGACCATTAACCTCAGTGC-3' and 5'-TGTATTCCATCACCACCAGCC-3'; Sp3, 5'-GCTTGCACCTGTCCCAACTGTA3' and 5'-CTCCAGAATGCCAACGCAGA-3'; MALAT1, 5'-CGCATTTACTAAACGCAGAC-3' and 5'-TCTCTATTCTTTTCTTCGCC-3'; β-actin, 5'-GCACCACACCTTCTACAATGAGC-3' and 5'-GGATAGCACAGCCTGGATAGCAAC-3'.

Cell lines and culture. Human embryonic kidney cells HEK293T, human normal liver cells L-02, and HCC cell lines Bel-7402, Huh7, and HepG2 were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Wisent, Nanjing, China) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China) in a humidified incubator with 5% CO_2 at 37˚C.

Protein extraction and western blotting. The total cell protein was extracted and western blotting was performed as previously described (20). The primary antibodies anti-Sp1 and anti-Sp3 IgG were obtained from Biolegend (San Diego, CA, USA). The horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and the Pierce ECL Western Blotting Substrate was purchased from Thermo Scientific. The western blot analysis results were quantified using Quantity One 4.62 (Bio-Rad, Hercules, CA, USA).

Small interfering RNA (siRNA) and gene silencing. SiRNAs targeting Sp1 and Sp3 were obtained from GeneGeme (Shanghai, China). HCC cell lines Bel-7402, Huh7, and HepG2 were seeded in 6-well plates at a density of $1x10^5$ cells/well, cultured overnight and transfected with lentivirus (siSp1 and siSp3) adding 5 ug/ml polybrene according to the manufacturer's instructions. The final MOI value of Sp1 and Sp3 lentivirus was 50 nM. After 72 h transfection, the cells were harvested and subjected to RT-qPCR and western blot analysis.

Cell viability assay. Cell viability was assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation/viability assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, the cells were treated with different concentrations (50-250 nm) of mithramycin A (MIT), (Sigma-Aldrich, St. Louis, MO, USA) and control medium (dimethyl sulfoxide). The 50% inhibition concentration (IC_{50}) at 96 h was calculated using Graphpad Prism 5.0. (GraphPad Software, La Jolla, CA, USA).

Chromatin immunoprecipitation (ChIP) assay. Approximately $5x10^6$ cells were harvested and cross-linked with 1% formaldehyde for 10 min, washed in cold PBS and resuspended in a lysis buffer. The cells were sonicated to obtain 200 and 1,000 bp chromatin fragments using the Sonics Sonication Instrument (Bioruptor, Diagenode, Liege, Belgium). The sonicated chromatin samples were then resuspended in an immunoprecipitation buffer and incubated overnight at 4˚C with magnetic beads conjugated to antibodies for Sp1 (17-601; Millipore) or Sp3 D-20 (SC-644; Santa Cruz Biotechnology, Inc.). On the following day, the samples were washed with a lysis buffer, LiCl buffer and TE buffer, respectively, and then eluted in an elution buffer. The DNA from these samples was recovered by reversing the crosslinks and purified by a Qiagen purification kit (Hilden, Germany). The immunoprecipitated DNA samples were amplified using qPCR with primers, specifically for MALAT1 promoter sequences (Table III). An unenriched DNA sample treated in a similar manner was used as the input control.

Electrophoretic mobility shift assay (EMSA). EMSA was performed using a ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas, Beijing, China) according to the manufacturer's instructions. The EMSA for Sp1 and Sp3 was performed using a non-radioactive gel shift assay system (Viagene Biotech, Beijing, China). Briefly, nuclear extracts were incubated with biotin-labeled or unlabeled Sp1 and Sp1/3 oligonucleotides (Sp1, 5'-ccagtggcgcccgcccac gagccag-3'; Sp1/3, 5'-tccctccccgcccccgctctcccct-3', produced by Viagene Biotech). Nuclear extracts and 80-fold excess of each competing probe were mixed together and incubated at room temperature for 20 min followed by the addition of 15 μl of each labeled probe for 20 min. The control sample was also incubated with unlabeled Sp1/3 probe. Protein/DNA complexes were then separated in a 6% non-denaturing polyacrylamide gel by electrophoresis. The gel was exposed to an X-ray film and digitized using a ChemiDoc XRS+ with Image Lab software (Bio-Rad).

Luciferase assay. LncRNA *MALAT1* promoter region covering -400 to -1 bp was amplified from the pcDNA3.1 (GV141) plasmid (Invitrogen) and then inserted into pGL3 plasmid (Promega, Madison, WI, USA). After DNA sequencing, the confirmed plasmid was transiently transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To detect luciferase reporter

Table I. Association of Sp1, Sp3 and MALAT1 expression with clinicopathological characteristics of HCC patients.

Characteristics	N	Sp1 expression			Sp3 expression			MALAT1 expression		
		Low	High	P-value[a]	Low	High	P-value[a]	Low	High	P-value[a]
Age (years)										
≤50	18	9	9	0.73	10	8	0.30	10	8	0.29
>50	14	8	6		5	9		4	10	
Gender										
Female	2	0	2	0.21	0	2	0.48	0	2	0.49
Male	30	17	13		15	15		14	16	
AFP (ng/ml)										
≤400	11	10	1	0.0027	5	6	1	10	1	1.42E-04
>400	21	7	14		10	11		4	17	
HBV										
-	3	0	3	0.091	1	2	1	0	3	0.23
+	29	17	12		14	15		14	15	
Tumor size (cm)										
≤5	12	5	7	0.46	5	7	0.72	4	8	0.47
>5	20	12	8		10	10		10	10	
No. of tumor										
Single	19	7	12	0.035	12	7	0.035	10	9	0.28
Multiple	13	10	3		3	10		4	9	
Histological grade										
Well + moderate	25	13	12	1	13	12	0.40	11	14	1
Poor	7	4	3		2	5		3	4	
PVTT										
Absent	24	13	11	1	12	12	0.69	10	14	0.70
Present	8	4	4		3	5		4	4	
Cirrhosis										
Absent	10	7	3	0.26	4	6	0.71	4	6	1
Present	22	10	12		11	11		10	12	

[a]Fisher's exact test. HCC, hepatocellular carcinoma; Sp, specificity protein; MALAT1, metastasis-associated lung adenocarcinoma transcript 1.

activities, HEK293T cells were harvested 48 h after transfection. Luciferase activity was measured using the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Statistical analysis. The experiments were performed independently at least three times and the data were statistically analyzed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as means ± standard deviations (SD) and analyzed by a Mann-Whitney-Wilcoxon test, Fisher's exact test, McNemar's test, Student's t-test, or one-way ANOVA. Spearman's correlation was applied to assess the relationship of gene expression with clinicopathological parameters. P≤0.05 was considered statistically significant.

Results

Upregulation of Sp1, Sp3 and MALAT1 expression in HCC tissues and cells. We first measured the expression level of Sp1, Sp3 and MALAT1 in 32 paired HCC and non-tumor liver tissues and found that the expression of Sp1, Sp3 and MALAT1 was significantly upregulated in cancer tissues compared to the non-tumor tissues (Fig. 1A). Expression of Sp1 and MALAT1 was significantly associated with the preoperative AFP level (Sp1, r=7.44, P=0.0064; MALAT1, r=12.37, P=0.0004) (Table I). The relationship between Sp and MALAT1 expression had no obvious statistical significance (Sp1 + MALAT1, r= 0.32, P=0.0709; Sp3 + MALAT1, r=0.31, P=0.0867) (Table II). Our results also showed that a high Sp3 positive immunocytochemical reaction and nuclear staining were observed (Fig. 1B and C). Their expression was also much higher in the three tested HCC cell lines compared to the normal hepatocellular epithelium (Fig. 1D and E).

Silencing Sp1 and Sp3 expression inhibits MALAT1 transcription in HCC cells. To investigate whether Sp1 and Sp3 silencing reduced the endogenous expression of the MALAT1 transcript, we transfected Sp1 and Sp3 siRNA into Bel-7402,

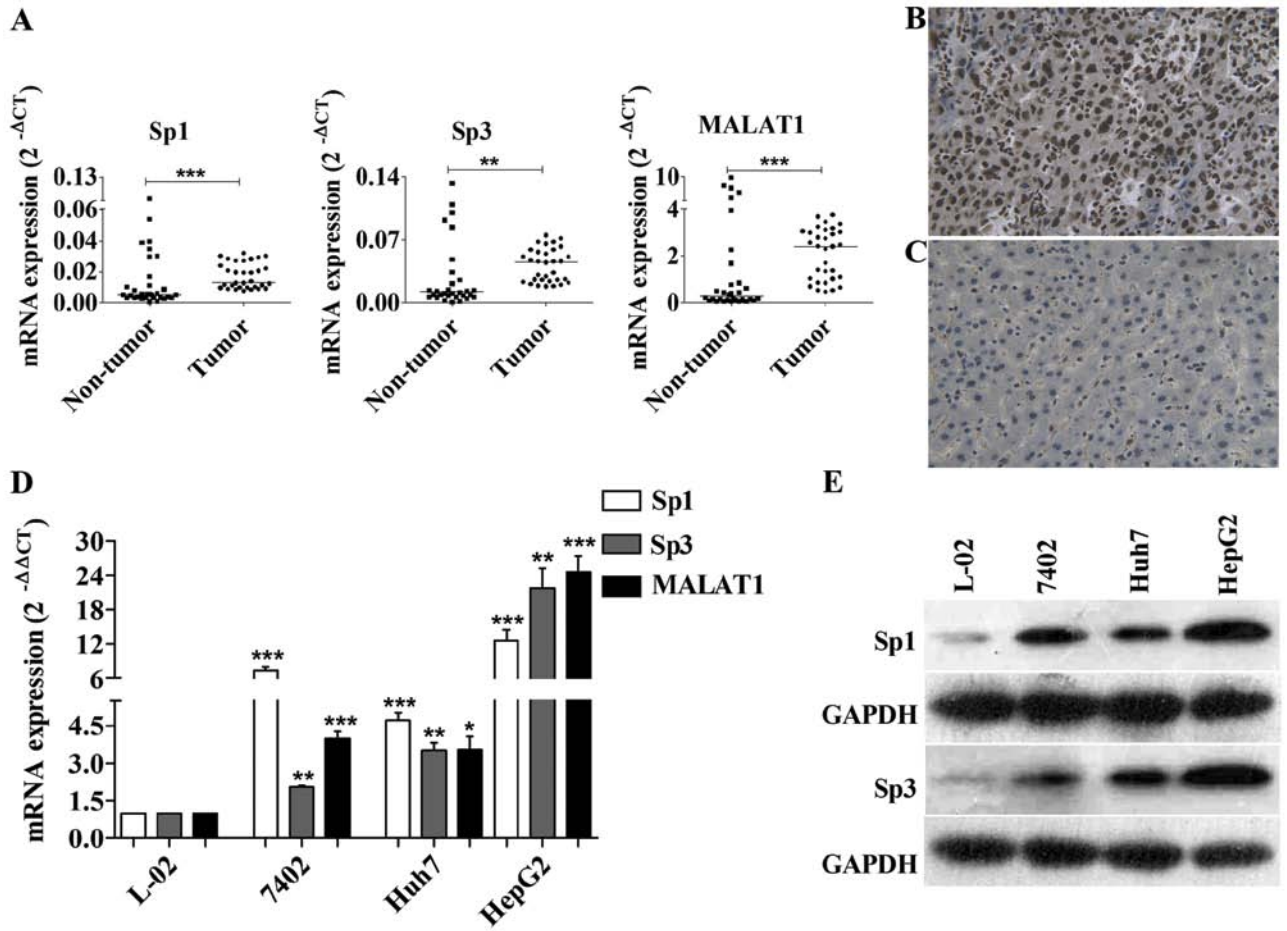

Figure 1. Differential expression of Sp1, Sp3 and MALAT1 in HCC tissues and cell lines. (A) HCC vs. non-tumor liver tissues analyzed by RT-qPCR. **P<0.01, ***P<0.001. (B) Immunocytochemistry of Sp3 in HCC tissues (x400 magnification, tumor tissue). (C) Immunocytochemistry of Sp3 in liver tissues (x400 magnification, paired non-tumor liver tissue). (D) HCC cells analyzed by RT-qPCR. **P<0.01, ***P<0.001. (E) Western blot analysis in cell lines. HCC, hepatocellular carcinoma; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein.

Table II. Assoication between Sp and MALAT1 expression.

	MALAT1 expression				
Factors	Low	High	P-value[a]	r	P-value[a]
Sp1					
Low	10	7	0.5488	0.32	0.0709
High	4	11			
Sp3					
Low	9	6	1.0000	0.31	0.0867
High	5	12			

[a]The data were analyzed by McNemar's test.

Table III. Primers for the ChIP assay.

Region name	Region (bp)	Primer
MALAT1-1	-894 to -811	5'-TTACAGGAGCCAAAGGAGTTT-3' 5'-CTGTATAGGTTAGGATGGCAAAA-3'
MALAT1-2	-1011 to -741	5'-TCAACAGGCCCTGCTTTATG-3' 5'-CCGGGTCTTTGGAACCTGT-3'
MALAT1-3	-772 to -543	5'-CCTCGGAGTTGACTGCCTA-3' 5'-AATATCTTCGTCGTTTGTATGTCA-3'
MALAT1-4	-285 to -4	5'-CAGGCACAGGCGTTAGGG-3' 5'-AGTCTCGGGCTGCAGGCT-3'
MALAT1-5	-85 to -4	5'-CGTTTGTCCCTGACGCAG-3' 5'-AGTCTCGGGCTGCAGGC-3'

ChIP, Chromatin immunoprecipitation; MALAT1, metastasis-associated lung adenocarcinoma transcript 1.

Huh7 and HepG2 cells and found that after 72 h, Sp1 and Sp3 mRNA and protein levels were significantly decreased. Notably, silencing of Sp1 or Sp3 alone did not significantly affect the level of MALAT1 in HCC cells, whereas silencing Sp1 and Sp3 significantly inhibited MALAT1 expression by 70-85% (Fig. 2A and B). Taken together, these data indicated that Sp1 cooperated with Sp3 to influence the basal endogenous expression of MALAT1.

Furthermore, we treated HepG2 HCC cells with different concentrations (0, 50, 100, 150, 200, and 250 nM) of MIT (21), an FDA-approved chemotherapeutic anticancer drug that inhibits the transcriptional activity of Sp1 by competitively binding to the Sp1-binding sites. Our data showed that

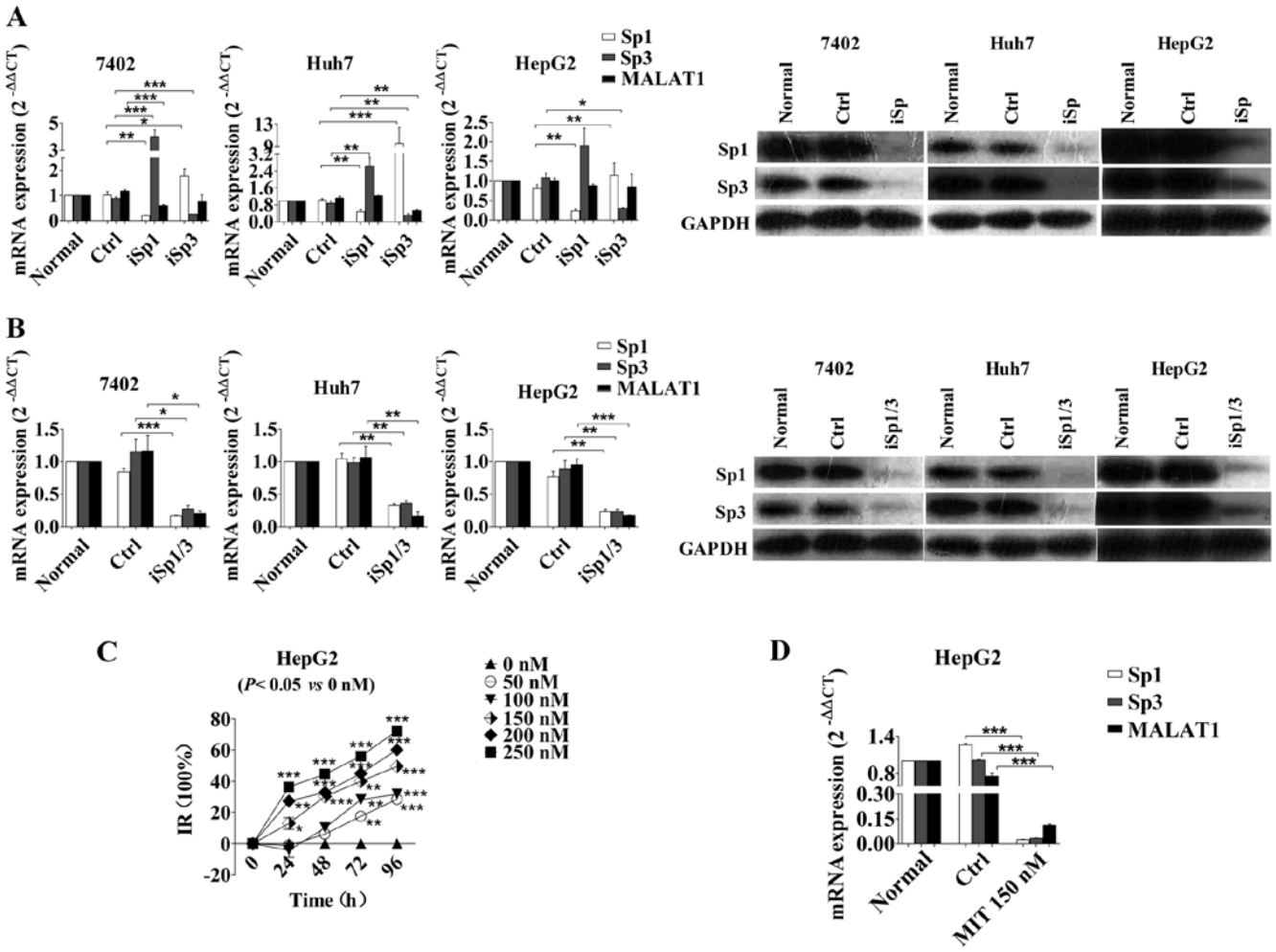

Figure 2. Effects of Sp1 and Sp3 knockdown on the regulation of MALAT1 level. (A) Bel-7402, Huh7 and HepG2 cells were transfected with Sp1- or Sp3-specific siRNA and subjected to RT-qPCR and western blot analysis. *P<0.05, **P<0.01, ***P<0.001. (B) Effect of combined Sp1 and Sp3 knockdown on the regulation of MALAT1 expression. *P<0.05, **P<0.01, ***P<0.001. (C) HepG2 cells were treated with different doses of MIT for up to 96 h and then subjected to cell viability assay. *P<0.05, **P<0.01, ***P<0.001. (D) HepG2 cells were treated with 150 nM MIT for 72 h and subjected to RT-qPCR analysis of Sp1, Sp3 and MALAT1 mRNA levels. *P<0.05, **P<0.01, ***P<0.001. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein; MIT, mithramycin A.

cell viability was significantly reduced (Fig. 2C) and the IC_{50} at 96 h was 146.7 nM. The transcription of MALAT1 decreased in HepG2 cells incubated with 150 nM MIT for 96 h (Fig. 2D).

Sp1/3 binding sites in the MALAT1 proximal promoter region. Online tools were used to predict Sp1/3 binding sites in the *MALAT1* proximal promoter region. These tools included the TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), MatInspector (http://www.genomatix.de/en/produkte/genomatix-sofware-suite.html), Tfsitescan (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) and CONSITE (http://asp.ii.uib.no:8090/cgibin/CONSITE/consite). We identified potential Sp1/3 binding sites upstream of MALAT1 between positions -1,000 bp and -1 bp, especially in the region -400 to -1 bp. This region contained four similarly high-scored sites from different online tools (Fig. 3A). From ChIPBase (http://deepbase.sysu.edu.cn/chipbase/), the first website for decoding the transcriptional regulation of lncRNA, we found the binding regions of MALAT1 to Sp1. The major predicted results are shown in Fig. 3B.

Identification of Sp1/3 binding site in the MALAT1 proximal promoter region using ChIP and EMSA . To confirm the Sp1 and Sp3 binding sites in the *MALAT1* promoter region, we first performed a ChIP assay to detect the interaction of Sp1/3 with endogenous *MALAT1* promoter. Chromatin fragments bound with transcription factor were prepared from HepG2 cells. Sp1 and Sp3 antibodies were separately used to immunoprecipitate Sp1- or Sp3-bound chromatin fragments followed by qPCR with primers corresponding to five different regions of the MALAT1 promoter. Promoter regions from MALAT1-1 to MALAT1-5 showed an extremely high affinity, especially the region -400 to -1 bp (MALAT1-4 and MALAT1-5) (Fig. 4B), which contained the predicted binding sites nos. 2, 3 and 4. The MALAT1-5 covered no. 4 site presented a high ratio of the input compared to the adjacent fragments (Sp1, F=144.9, P<0.0001; Sp3, F=68.54, P<0.0001). Additionally, Sp3 showed stronger binding than Sp1, which may be due to the expression disparity of endogenous Sp1 and Sp3 in HepG2 cells (Fig. 4A and B).

Furthermore, we performed EMSA and according to the consensus binding sequence of Sp1 (5'-(G/T)GGGCGG(G/A)

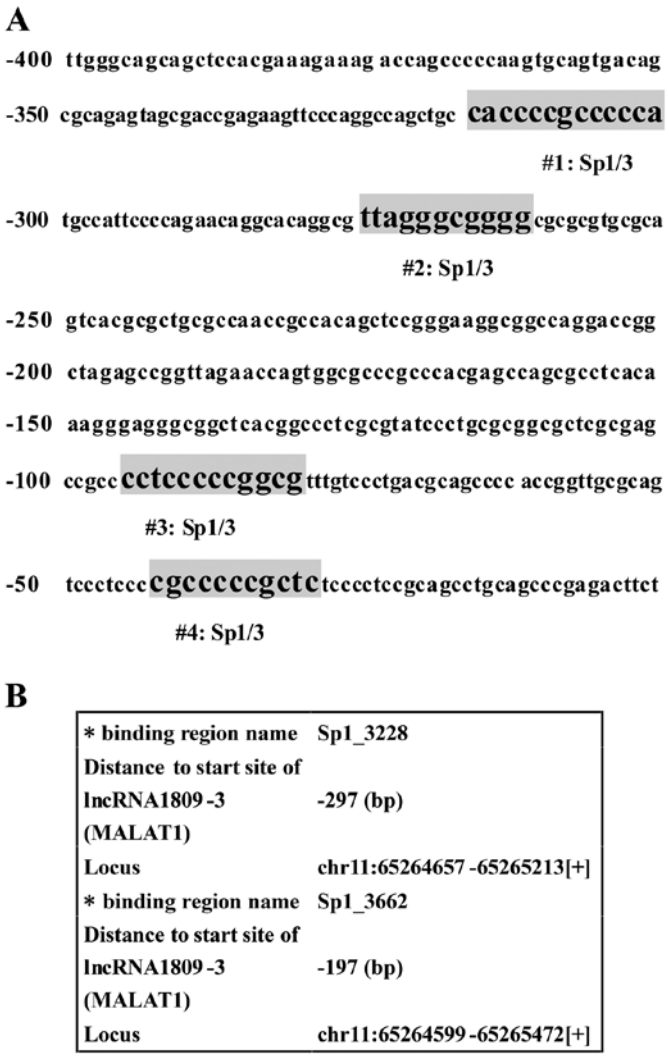

Figure 3. Prediction of potential binding sites in *MALAT1* promoter. (A) Position -1,000 to -1 bp of the *MALAT1* promoter was the predicted region for high scored transcriptional binding sites, especially -400 to -1 bp. Four putative sites involved in this region were confirmed by at least three online tools, designated as nos. 1, 2, 3, and 4. (B) ChIPBase database also predicted the binding sites of Sp1 to *MALAT1* gene promoter region. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein.

(G/A)(C/T)-3' (22), we designed Sp1 probes with or without biotin label, which also covers Sp3, according to a previous study (23). The biotin-labeled and unlabeled Sp1/3 oligonucleotides were prepared for EMSA. The NF-κB probes with or without related nuclear extract were used as a positive and negative control, respectively. The data showed that 80 excess cold probes successfully blocked the Sp1 and Sp1/3 binding activities (Fig. 4C). Thus, our data indicated that the MALAT1 promoter sites may not only bind to Sp1, but also to Sp3.

Sp1 and Sp3 enhance MALAT1 transcriptional activity. To confirm Sp1 and Sp3 MALAT1 transcriptional activity, we designed and subcloned the MALAT1 -400 to -1 promoter constructs with mutations in the potential binding sites according to data from ChIPBase. We co-transfected the clones with Sp1 or Sp3 expression plasmid into 293T cells and measured the transcriptional activity. As shown in Fig. 5A and B, the ratio between Sp1/3 plasmid and promoter construct indicated their positive role in regulating MALAT1 transcriptional activity. Sp1 and Sp3 wild-type constructs coincidentally showed the greatest activity in regulating MALAT1 transcription at a ratio of 25:1 (Sp1, $F=3.683$, $P=0.0172$; Sp3, $F=21.40$, $P<0.0001$). Separate transfection of Sp1 or Sp3 also enhanced MALAT1 promoter activity (Fig. 5A and B), whereas a combination of Sp1 and Sp3 showed much weaker action (Fig. 5C). Conversely, compared with the wild-type, mutation at no. 4 significantly abolished Sp1 and Sp3 transcriptional activity (Fig. 5D and E).

Discussion

MALAT1 is considered to be an oncogene as it is frequently upregulated in various human cancers (15,24-27). Previous findings demonstrated that an altered MALAT1 expression was an independent predictor for postoperative HCC recurrence (15). Our unpublished data also showed that knockdown of MALAT1 expression inhibited HCC cell proliferation and motility, and induced caspase-3/7 activity and HCC cell apoptosis. MALAT1 may prevent HCC cell apoptosis via inhibition of caspase-3/7 activity. However, the mechanism of prediction of postoperative HCC recurrence

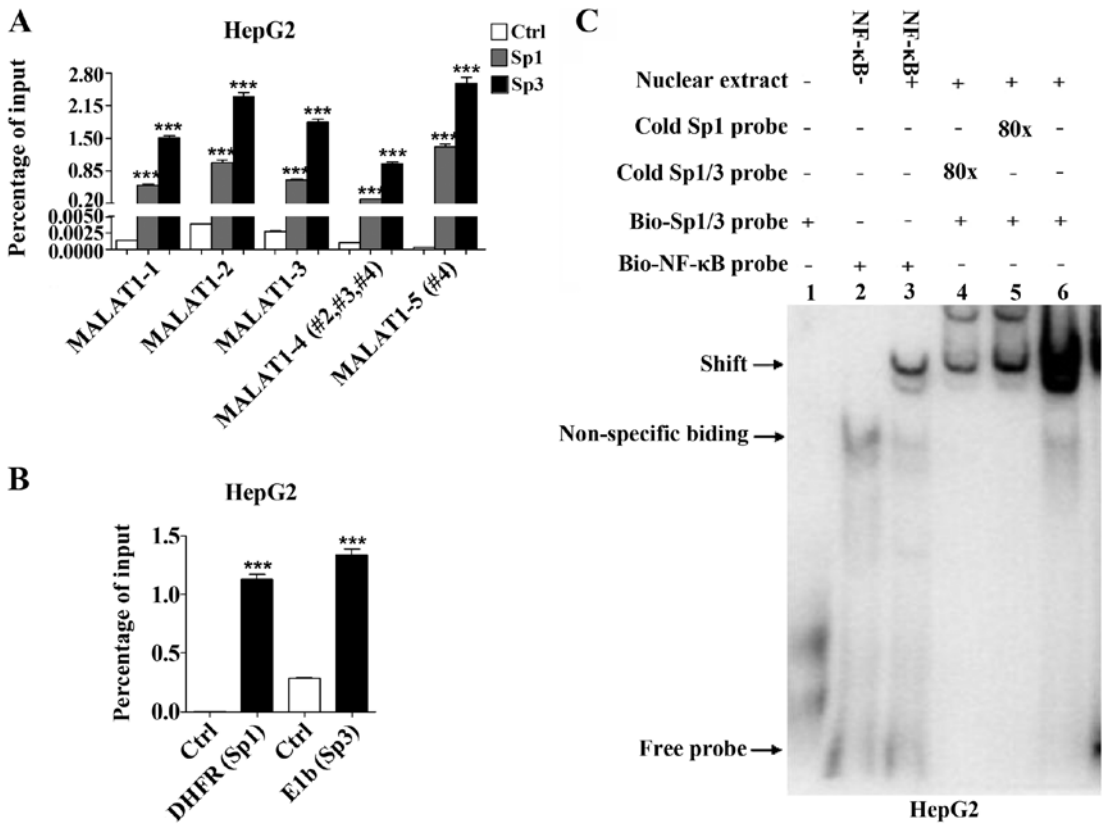

Figure 4. Detection of the binding activity between Sp1/3 and MALAT1 in HepG2 cells. (A) ChIP assay demonstrates the special binding activity between Sp1/3 and MALAT1. Anti-Sp1 and anti-Sp3 successfully enriched five DNA sequence from -1,000 to -1 bp of the *MALAT1* promoter. The normal rabbit IgG was used as a negative control. Pulled-down chromatin fragments were then analyzed by RT-qPCR. Percentage of the input suggested significant combination between transcription factors and the *MALAT1* promoter. The fragment from -85 to -1 bp of the *MALAT1* promoter, which covered predicted site no. 4, presented the highest ratio of input (Sp1, F=144.9, P<0.0001; Sp3, F=68.54, P<0.0001). *P<0.05, **P<0.01, ***P<0.001. (B) The qPCR primers included the Sp1 binding site in human DHFR promoter and the Sp3 binding site in human E1b promoter. (C) Two special probes were designed to determine DNA-binding activity using an electrophoretic mobility shift assay. Two biotin-labeled probes bound to the nuclear extract and were most competitively blocked by an unlabeled one (shift). MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein.

remains unclear. Thus, in the present study, we investigated the potential mechanism of MALAT1 transcriptional regulation. We primarily assessed the levels of MALAT1, Sp1 and Sp3 in HCC vs. non-tumor liver tissues and found a significant upregulation of their levels in HCC tissues. We performed a series of *in vitro* experiments to determine Sp1 and Sp3 regulation of MALAT1 expression in HCC cells. The present data provided information that may lead to the therapeutic targeting of Sp1 and Sp3 in MALAT1-ovexpressed HCCs.

MALAT1 is a large infrequently spliced non-coding RNA that is highly conserved among mammals (28). MALAT1 regulates alternative splicing by modulating SRSF1 phosphorylation to influence cancer-associated aberrant splicing (14,29) and positively regulates cell motility (30). Wang *et al* revealed a novel mechanism underlying the balance between SRSF1, Yes-associated protein (YAP) and MALAT1 and identified a new role of YAP in upregulating MALAT1. SRSF1 inhibited YAP activity by preventing its co-occupation with TCF/β-catenin on the MALAT1 promoter. By contrast, the overexpression of YAP interacted with Angiomotin (AMOT) to impair the nuclear retention of SRSF1 and itself. This effect resulted in removing the inhibitory role of SRSF1 on MALAT1 in the nucleus (29). Recent findings have shown that MALAT1 positively regulated latent transforming growth factor β-binding protein-3 (LTBP3) transcription in mesenchymal stem cells by recruiting Sp1 to the LTBP3 promoter. LTBP3 regulated the bioavailability of tumor growth factor-β (TGF-β) (31). However, the mechanism of MALAT1 overexpression in human cancers remains to be elucidated (32).

Sp family members have been reported to be associated with carcinogenesis (23,33). In the present study, Sp1 and Sp3 were upregulated in HCC. Similar results were obtained for pancreatic carcinoma (PC) (34). However, the factors that induced the high expression of Sp1 or Sp3 remain to be determined. More studies have concentrated on the target genes that are regulated by the two transcriptional factors and influence carcinogenesis. Sp1, a ubiquitous nuclear factor, was demonstrated to play a key role in the growth and metastasis of many human cancers (21,23,34-36). Previous microarray data showed that Sp1 was a potential regulator of MALAT1 transcription (16-19). In addition, Sp3 belongs to the family of Sp1-related genes that encode transcription factors to regulate transcription of GC-and GT-box containing genes (37). Previous results showed that Sp1 was responsible for many cancer-related genes including those associated with sustained proliferation (hTERT/hTERC, p53/MDM2, p16, and p21), apoptosis (survivin, Trail-R2, and Bcl-2), angiogenesis (VEGF, TSP-1, PDGF and uPA), DNA damage/stress response (Brca1, ATM, and MDC1), invasion and metastasis (MMP9, MT1-MMP, RECK, E-cadherin, Integrin

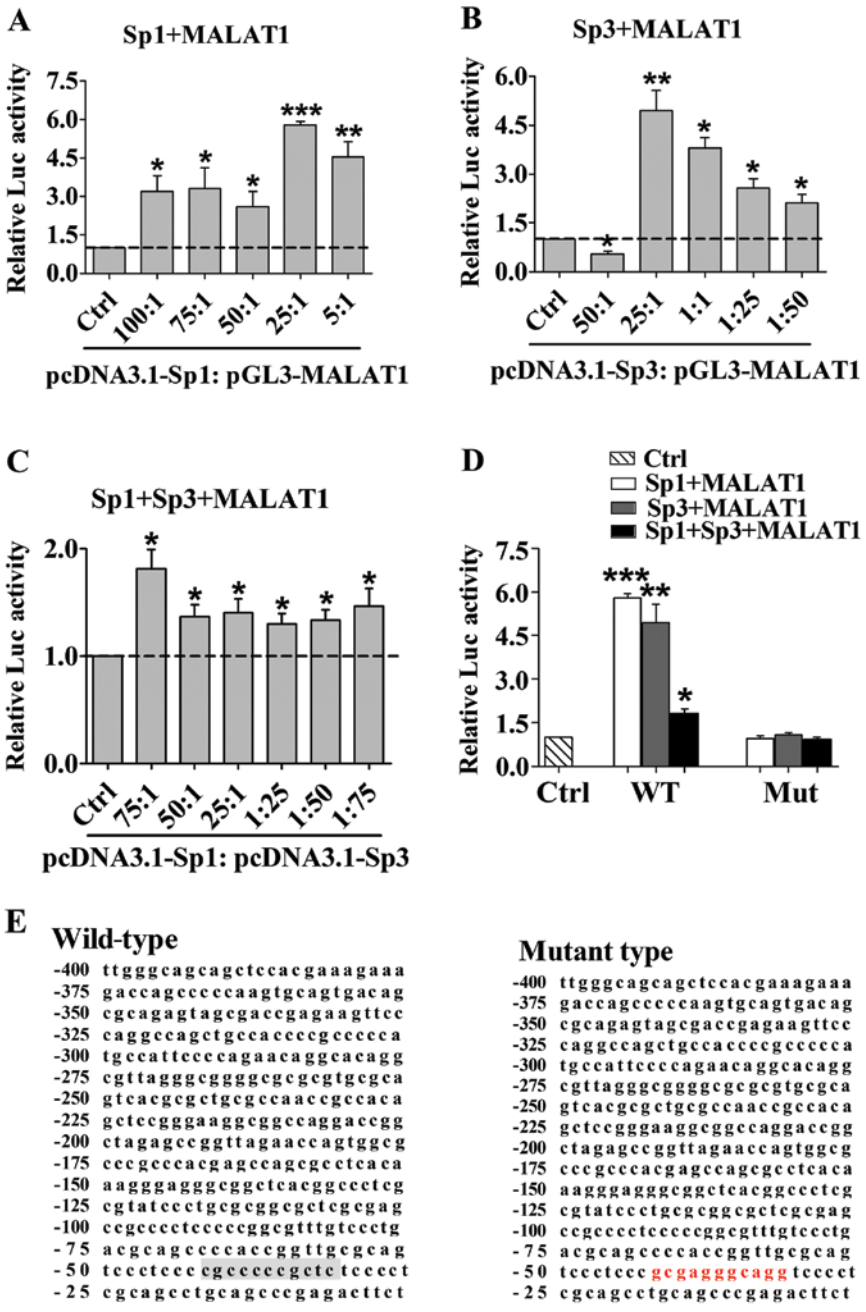

Figure 5. Luciferase assay detection of the transcriptional activity of MALAT1 promoter regulated by Sp1 and Sp3. (A) *MALAT1* -400/-1 promoter construct was transfected into 293T cells with (A) Sp1 and (B) Sp3 expression plasmid at different ratios to identify the one with the best effect. (C) Co-transfection of Sp1 and Sp3 vectors in different proportions (200 μg for transcription factors and 8 μg for MALAT1 promoter) to maintain the chosen ratio of 25:1. (D) Effects of wild-type promoter vs. mutated one. (E) The wild-type and the mutant type of MALAT1 report plasmid. The red section is the mutated sequence. *P<0.05, **P<0.01, ***P<0.001. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; Sp, specificity protein.

α5 and MMP2) (23,33). Similarly, Sp3 may bind to the same sites of cancer-related genes such as Sp1 and have different regulatory effects (23,33). For example, Sp1 and Sp3 physically interacted and co-operated with GABP to activate the utrophin promoter (37). Thus, although Sp1/Sp3 is upregulated in cancers such as HCC and PC, if Sp1/Sp3 were inhibited the cancer-related genes transcriptions would be affected. Thus, malignant behaviors, including proliferation, apoptosis, angiogenesis, DNA damage/stress response, invasion and metastasis, would be altered.

In the present study, we combined the two factors and analyzed their transcriptional regulation of MALAT1. The levels of Sp1, Sp3 and MALAT1 were markedly increased in the HCC tissues. The expression of Sp1 and MALAT1 was associated with AFP level. This result suggests that the co-analysis of serum AFP and MALAT1 levels may become a useful biomarker for HCC detection. In addition, Sp1 or Sp3 expression was not obviously associated with MALAT1 expression. Thus, MALAT1 may not be regulated by a single factor. Subsequent data have verified that Sp1 and Sp3 were

involved in the transcriptional regulation of MALAT1. In addition, Li *et al* (38) identified that only Sp1 can independently upregulate the expression of MALAT1 in A549 cells by binding to two specific transcriptional binding sites. Findings of the present study on HCC show that level of MALAT1 only decreased with the silencing of both Sp1 and Sp3, and not only of Sp1, which suggests a complementary effect between Sp1 and Sp3. The expression of MALAT1 is under the associated regulation of Sp1 and Sp3. The Sp1 inhibitor, MIT, showed a similar effect on MALAT1 inhibition. MIT, as a GC-rich region inhibitor, can suppress protein biosynthesis via transcription inhibition (39). It has been shown to inhibit cancer growth by blocking the Sp-family transcription factors from binding to GC-rich regions of gene regulatory elements and is used for the treatment of leukemia and testicular cancer in the US (21,40-45). By contrast, MIT has also been shown to exert neuroprotective effects in normal cells and potential as an agent for neurodegenerative diseases (41). Although MIT may produce some side effects, the data mentioned above has revealed that MALAT1 expression was downregulated in HepG2 cells treated with MIT. This result has demonstrated that MALAT1 expression is associated with Sp.

Furthermore, *MALAT1* promoter contained at least five biding regions of Sp1/3, particularly in the region -85 to -4 bp. The present results confirmed the prediction of Sp1 binding sites in the *MALAT1* promoter by using online prediction tools. Additionally, using the luciferase assay it was found that Sp1 contributed more to enhancing transcriptional activity than Sp3 in HCC cells. Sp1 and Sp3 affected transcriptional activity at a specific ratio, and alterations to this ratio induced suppression of MALAT1 transcriptional activation. However, additional studies are needed to investigate the mechanism of Sp1 and Sp3 overexpression in HCC tissues. The present study indicates that the specific Sp1 and Sp3 binding sites may be developed as novel therapeutic targets for treating HCC.

In summary, we have demonstrated the role of Sp1 and Sp3 in the regulation of MALAT1 expression in HCC cells. Future studies are to investigate targeting Sp1/3 and MALAT1 as a potential therapeutic strategy, including use of the Sp1 inhibitor MIT, to control HCC progression.

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