Amplification of Long Noncoding RNA ZFAS1 Promotes Metastasis in Hepatocellular Carcinoma

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Abstract

Despite progress in the diagnostics and treatment of hepatocellular carcinoma (HCC), its prognosis remains poor. In this study, we globally assessed long noncoding RNAs (lncRNA) for contributions to HCC using publicly available microarray data, in vitro and in vivo assays. Here, we report that ZFAS1, encoding a lncRNA that is frequently amplified in HCC, is associated with intrahepatic and extrahepatic metastasis and poor prognosis of HCC. ZFAS1 functions as an oncogene in HCC progression by binding miR-150 and abrogating its tumor-suppressive function in this setting. miR-150 repressed HCC cell invasion by inhibiting ZEB1 and the matrix metalloproteinases MMP14 and MMP16. Conversely, ZFAS1 activated ZEB1, MMP14, and MMP16 expression, inhibiting these effects of miR-150. Our results establish a function for ZFAS1 in metastatic progression and suggest its candidacy as a new prognostic biomarker and target for clinical management of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with nearly 600,000 deaths occurring each year worldwide (1–3). Although hepatic resection, liver transplantation, and chemotherapy are commonly used to improving outcomes of the HCC patients, the postoperative 5-year survival rate is low (4). HCC shows a propensity to metastasize and infiltrate adjacent and more distant tissues (5). The poor prognosis of HCC is largely the result of distant metastasis and high rate of tumor recurrence after surgery (6). However, the molecular mechanisms underlying these processes are not well understood.

The long noncoding RNAs (lncRNA) are a class of noncoding RNA over 200 nucleotides with no protein-coding potential (7). Through different mechanisms (epigenetic silencing, lncRNA–miRNA interaction and lncRNA–protein interaction), lncRNAs play critical roles in development, differentiation, and tumorigenesis (8, 9). LncRNA expression is frequently dysregulated in cancer, and specific lncRNAs are correlated with cancer recurrence, metastasis, and poor prognosis in different kinds of cancer (10). Examples include HOTAIR in breast cancer and HCC (11, 12), MALAT1 in lung cancer and bladder cancer (13, 14), HULC in HCC (15), and MEG3 in HCC (16), indicating that lncRNAs may be involved in tumorigenesis or tumor progression. HOTAIR, a well-known lncRNA, is increased in expression in various types of human cancers (11, 12). Overexpression of HOTAIR in epithelial cancer cells results in an increased cancer invasiveness and metastasis in a manner dependent on Polycomb repressive complex 2 (PRC2; ref. 11). LncRNAs also function as a competing endogenous RNA and sponge miRNAs, thus regulating the expression of target mRNAs (8). Wang and colleagues showed that HULC acts as an endogenous sponge, which downregulates miR-372 activities (17). The interaction results in an increased level of Prkacb (CAMP-dependent protein kinase catalytic subunit beta), a target of miR-372. lncRNA–CARL suppresses anoxia-induced mitochondrial fission and apoptosis in cardiomyocytes by impairing miR-539–dependent PHB2 downregulation (18).

ZFAS1, a newly identified lncRNA, was shown to be dysregulated in breast cancer (19). Askarian-Amiri and colleagues proposed a functional role for ZFAS1 in the regulation of alveolar development and epithelial cell differentiation in the mammary gland (19). However, the roles of ZFAS1 in tumor progression remain unclearly defined. It is also necessary to reveal the underlying molecular mechanisms by which ZFAS1 is involved in tumorigenesis and cancer progression. In the current study, we found that miR-150 inhibits hepatoma cell invasion by targeting ZEB1, MMP14, and MMP16 in HCC. ZFAS1 acts as an endogenous sponge of miR-150, which abrogates the role of miR-150 in suppressing cancer metastasis. ZFAS1 increases ZEB1, MMP14, and MMP16 expression and promotes HCC metastasis by sponging miR-150 and inhibiting its function.

Materials and Methods

Computational analysis

Clinical specimens and cell lines
HCC specimens and the corresponding adjacent noncancerous tissues were obtained from Ruijin Hospital with informed consent. The protocols used in the study were approved by the Hospital’s Protection of Human Subjects Committee. Recurrence-free survival was defined as the interval between treatment and first diagnosis of metastasis and recurrence. Overall survival was defined as the interval between resection and death or the last follow-up examination. Huh7, HepG2, Hep3B, SMMC7721, LM3 hepatoma cell lines and LO2, QSG7701 immortalized hepatic cells were directly obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Minimum Essential Medium (Gibco) with 10% FBS (Gibco). The cell lines were characterized at the bank by DNA fingerprinting analysis using short tandem repeat markers.

Quantitative real-time PCR
Total RNA was extracted from specimens or cell lines using TRIzol reagent (Invitrogen). The reverse transcription for mRNA and lncRNA was carried out using cDNA conversion kit and the Oligo(dT) primer (Invitrogen). Quantitative real-time PCR (qPCR) was carried out on Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems) using a standard protocol from the SYBR Green PCR Kit (Toyobo). The optimized primers used for qPCR were listed in Supplementary Table S1.

Overexpression and RNA interference
The plasmids pcDNA-ZFAS1, pcDNA-ZFAS1-mut (miR-150), and pcDNA-shZFAS1 were obtained from GenePharma (GenePharma) to overexpress or knockdown ZFAS1. The plasmids pPG-pri-miR-150 and pPG-miR-150-inhibitor (GenePharma) were used to overexpress or knockdown miR-150. Huh7 cells stably expressing ZFAS1, ZFAS1 plus miR-150, ZFAS1-mut (miR-150), shZFAS1, or shZFAS1 plus miR-152-inh were constructed in our lab. The siRNA or miRNA sequences were listed in Supplementary Table S1.

Transwell invasion assay
Cell invasion assay was carried out using Transwell chambers with inserts of 8-μm pore size (Corning Costar) as described previously (20). Briefly, Huh7 or HepG2 cells suspended in serum-free media were plated into the upper chamber for invasion assay after transfection, and media supplemented with 10% FBS was placed into the lower chamber. After 36 hours, the cells that had invaded through the membrane to the lower surface were fixed with methanol, stained with Crystal violet for 10 minutes, and counted.

RNA immunoprecipitation assay
The MS2bs-MS2bp–based RNA immunoprecipitation (RIP) assay was carried out according to previous reports (21). Huh7/HepG2 cells were transfected with pcDNA-ZFAS1-M2, pcDNA-ZFAS1-mut-M2, pcDNA-H19-M2, or pcDNA-M2. After 48 hours, cells were used to perform RIP using a GFP antibody (Abcam) and then the ZFAS1 level was analyzed by qPCR.

RNA pulldown assay
The RNA pulldown was performed as previously described (18). Huh7 or HepG2 cells were transfected with biotinylated miRNA (40 nmol/L). After 48 hours, the cells were harvested and the lysates were incubated with M-280 streptavidin magnetic beads (Sigma). The bound RNAs were purified using TRIzol and the ZFAS1 level was analyzed by qPCR.

The biotinylated DNA probe complementary to ZFAS1 was synthesized (GenePharm). Huh7 or HepG2 cells were overexpressed with ZFAS1, ZFAS1-mut or H19, and the cell lysates were prepared after 48 hours. Lysates were incubated with biotinylated probe and M-280 streptavidin magnetic beads (Sigma). miRNA level was analyzed by qPCR.

Luciferase reporter assay
pGL3 plasmid encoding a luciferase reporter gene was purchased from Promega. Recombinant plasmid of pGL3-MMP14-3’-UTR, pGL3-MMP16-3’-UTR, pGL3-ZEB1-3’-UTR (wild-type), or corresponding mutants was constructed in our laboratory. HepG2 cells (1–2×10^5 cells/well) were plated in a 24-well plate and cotransfected with 40 nmol/L of either miR-150 or miRNA control, 20 ng of either recombinant plasmids or corresponding mutants, and 2 ng of pRL-TK (Promega) by using Lipofectamine 2000. The pRL-TK vector was used as an internal control to correct the differences in both transfection and harvest efficiencies. HepG2 cells were collected 48 hours after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega).

In vivo experiments
The athymic BALB/C mice (4–6 weeks old) were purchased from the Chinese Academy of Sciences (Shanghai, China), and were maintained in a specific pathogen-free facility. Huh7 cells stably transfected with ZFAS1 (Huh7/ZFAS1 cell clone) or shZFAS1 (Huh7/shZFAS1 cell clone) were constructed in our lab and subcutaneously injected into the flanks of nude mice, and animal survival and lung metastasis were monitored. Intrasplenic injection model was used for liver colonization assays. For experimental metastasis assays, Huh7/shZFAS1 cell clones were labeled with firefly luciferase, and mice were then injected with 1–2×10^5/50 μL cell clones by lateral tail vein injection. Metastatic progression was monitored and quantified using a noninvasive bioluminescence In Vivo Imaging System (IVIS, Xenogen) as described previously (22, 23).

Statistical analysis
Data were presented as mean ± SD from at least three separate experiments. The Student t test, χ^2 test, log-rank test, nonparametric Mann–Whitney U test, or Fisher exact test was used for comparisons between groups. The Kaplan–Meier method was used to estimate overall survival. Differences were deemed statistically significant at \( P < 0.05 \).

Results
lncRNAs expression profile in HCC
To identify lncRNAs involved in HCC progression, we searched GEO database and two human lncRNA microarray datasets
ZFAS1 Promotes HCC Metastasis

(GSE58043 and GSE55191) were obtained to analyze differentially expressed lncRNAs between HCC and pair nontumor tissues. We developed a Venn analysis to screen the consistently upregulated or downregulated lncRNAs in GSE58043 and GSE55191. Analysis of these data showed that 3 lncRNAs were consistently downregulated and 58 lncRNAs were consistently upregulated in HCC tissues (fold change > 4.0, \( P < 0.05 \); Supplementary Fig. S1; Supplementary Table S2). We constructed gene coexpression networks to find the interactions among transcripts (Supplementary Fig. S2; Supplementary Table S3). Within the network analysis, degree centrality is the most simplest and important measures of the centrality of a gene within a network that determines the relative importance. Degree centrality is defined as the link numbers one node has to the other (24). Coexpression network analysis showed that FABP5P5, LOC100996735, LOC100996732, ZEB1-AS1, and ZFAS1 are core lncRNAs. To validate the microarray analysis findings, we analyzed their expression using qPCR in 20 pairs of HCC and pair nontumor tissues (Supplementary Fig. S3). Of these five lncRNAs, ZFAS1 is the most upregulated lncRNA in HCC tissues. Similar results were also found in HCC cell lines (Supplementary Fig. S3). Therefore, we focused on ZFAS1 and investigated its expression pattern, clinical significance, and biologic function in HCC.

Upregulation of ZFAS1 is correlated with poor prognosis in HCC

To further verify that ZFAS1 expression is upregulated in vivo, we assayed its expression in another panel of tumor tissues and pair nontumor tissues (Table 1). The ZFAS1 transcripts are expressed at higher levels in most tumor tissues compared with the nontumor tissues of the same donor (Fig. 1A). Portal vein tumor thrombus (PVTT) is the main route for intrahepatic recurrence and metastasis in HCC cells (25, 26). We next assayed ZFAS1 expression in 41 PVTT and pair primary HCC tissues, along with 20 normal liver tissues. Figure 1B shows a significantly higher ZFAS1 expression in the PVTT tissues than primary HCC and healthy liver tissues, indicating the correlation of ZFAS1 with HCC metastasis. ZFAS1 expression is also increased in HCC cell lines compared with normal liver cell in vitro (Fig. 1C). Upregulation of ZFAS1 is more frequently observed in HCC patients with microvascular invasion than in those with no microvascular invasion, though there is no significant correlation between ZFAS1 expression and gender, age, tumor number, tumor size, AFP level, or HCC stage (Table 1). HCC patients with high ZFAS1 expression have higher recurrence rates and shorter overall survival than those with low expression of ZFAS1 (Fig. 1D and E; Table 1). These results suggest that ZFAS1 is upregulated and correlated with tumor recurrence and metastasis in HCC.

Table 1. Correlation between ZFAS1 expression and clinicopathologic characteristics of HCC

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ZFAS1 promotes cell invasion and tumor metastasis in vitro and in vivo

The biologic consequences of ZFAS1 in regulating cancer cell proliferation and invasion were then examined using cell biology assays. To evaluate the role of ZFAS1 in the proliferation and invasion of HCC cells, we established two stable cell lines (Huh7/ZFAS1 and HepG2/ZFAS1) after transfection with the pcDNA-ZFAS1 (Fig. 2A). Meanwhile, three siRNAs (siRNA1#, siRNA2#, siRNA3#) were designed to silence ZFAS1 expression. The target site one (siRNA1#) is the most effective site and is chosen for further study (Fig. 2B). Cell proliferation assay did not show a significant effect of ZFAS1 on cell proliferation (data not shown). We then investigated the role of ZFAS1 in cancer cell invasion. As shown in Fig. 2C–E, forced expression of ZFAS1 in Huh7 or HepG2 cells markedly promotes cell invasion compared with control, whereas knockdown of ZFAS1 inhibits cell invasion.

On the basis of the above findings that ZFAS1 promotes cell migration and invasion, we next investigated the effects of ZFAS1...
Figure 1.
Upregulation of ZFAS1 is correlated with poor prognosis in HCC. A, qPCR analysis of ZFAS1 expression in 113 HCC tissues and pair nontumor tissues. The results were expressed as log_{2}(C_{0DD}^{CT})/C_{0DD}^{CT}. B, qPCR analysis of ZFAS1 expression level in 20 healthy liver samples, 41 PVTT, and pair primary HCC tissues. C, qPCR analysis of ZFAS1 expression level in normal liver cell (LO2 and 7701) and HCC cell lines (HepG2, Huh7, Hep3B, and LM3). D and E, Kaplan–Meier analysis of recurrence-free survival or overall survival based on ZFAS1 expression levels in 88 cases of patients with HCC. The median level of ZFAS1 is used as the cutoff. Patients with HCC were divided into ZFAS1-high expression group (whose expression is higher than the median) and low expression group. *, P < 0.05; **, P < 0.01.
on cancer metastatic ability in vivo. To establish a metastatic cancer model in vivo, Huh7 cells stably overexpressed with ZFAS1 (Huh7/ZFAS1) were labeled with firefly luciferase and injected into tail vein of nude mice. A bioluminescent signal was assayed at 10 weeks after tail vein injection. Figure 2F showed that the incidence of lung metastasis in the Huh7/ZFAS1 group is significantly increased, compared with the control group. Then, we inoculated the Huh7/ZFAS1 cells subcutaneously into mice and observed lung metastases after 6 weeks. Consistent with the above observations, ZFAS1 overexpression increases the number of visible lung metastases (Fig. 2G), whereas ZFAS1 knockdown in Huh7 cells inhibits lung metastases (Fig. 2H). The overexpression of ZFAS1 also results in a shorter overall survival time compared with control (Fig. 2I). These data show that ZFAS1 promotes cell invasion and tumor metastasis in vitro and in vivo.

ZFAS1 is physically associated with miR-150

Recent studies showed that many RNA transcripts may act as competing endogenous RNAs (ceRNA) by competitively binding miRNAs (27, 28). To explore the underlying mechanism responsible for ZFAS1 in tumor metastasis, we tested whether miRNAs are involved in the process. Bioinformatics analysis showed that ZFAS1 contains a binding site of miR-150 (Fig. 3A). miR-150, frequently downregulated in HCC (Supplementary Fig. S4), acts as a tumor suppressor (29, 30). To validate the direct binding between ZFAS1 and miR-150 at endogenous levels, we carried out a RIP with MS2-binding protein (MS2bp), which specifically binds RNA containing MS2-binding sequences (MS2bs). We generated a construct containing ZFAS1 transcripts combined with MS2bs elements (Fig. 3B) and cotransfected into hepatoma cells with a construct
containing MS2bp-GFP. The immunoprecipitation was then performed using GFP antibody (IgG was used as a negative control), and miR-150 level was analyzed using qPCR. Figure 3C showed that the ZFAS1 is able to significantly enrich miR-150 compared with the empty vector (MS2). ZFAS1 with mutations in miR-150 targeting sites [ZFAS1-mut(miR-150)], and lncRNA-H19 control. ZFAS1 cannot enrich other unrelated miRNAs (e.g., miR-155 and miR-146), which does not contain binding site in ZFAS1 (data not shown).

The specific combination between ZFAS1 and miR-150 was further validated by RNA pulldown assay using a biotin-labeled–specific ZFAS1 probe (Fig. 3D). We also used inverse pulldown assay to test whether miR-150 could pulldown ZFAS1. Hepatoma cells were transfected with biotinylated miR-150, then harvested

Figure 3.
ZFAS1 is physically associated with miR-150. A, schematic representation of the miR-150 site in ZFAS1. B, a construct containing ZFAS1 transcripts combined with MS2bs elements. C, MS2-RIP followed by miR-150 qPCR to assay miR-150 endogenously associated with ZFAS1. D, Huh7/HepG2 cell lysates were incubated with biotin-labeled ZFAS1 and the miR-150 expression level was assayed by qPCR after pulldown. E, Huh7/HepG2 cell lysates were incubated with biotin-labeled miR-150, and the ZFAS1 expression level was assayed by qPCR after pulldown. F, luciferase activity in Huh7 cells cotransfected with miR-150 and luciferase reporters containing ZFAS1 or mutant transcript. Data are presented as the relative ratio of firefly luciferase activity to Renilla luciferase activity. G, AGO2-RIP followed by ZFAS1 qPCR to assay ZFAS1 level after miR-150 overexpression. *, P < 0.05; **, P < 0.01.
for pulldown assay. ZFAS1 is pulled down, but several site-specific mutations that disrupt base pairing between ZFAS1 and miR-150 results in the inability of ZFAS1 to pull down ZFAS1 (Fig. 3E). We then produced a luciferase construct containing ZFAS1 or a mutated form [ZFAS1-mut (miR-150)]. Luciferase assay showed that miR-150 significantly inhibits the luciferase activity of ZFAS1, but it has less effect on the mutated form of ZFAS1 (Fig. 3F).

miR-150 inhibits cell invasion by targeting ZEB1, MMP14, and MMP16

The biologic consequences of miR-150 in regulating cancer cell invasion were then examined using cell biology assays. miR-150 mimic treatment increases miR-150 expression level, whereas miR-150 level is significantly decreased in Huh7 cells after treatment with miR-150 inhibitor (Supplementary Fig. S5). Functionally, miR-150 overexpression markedly inhibits cancer cell invasion, whereas miR-150 knockdown by specific inhibitor promotes cell invasion (Fig. 4A and B). miRNAs play a biologic function by targeting specific target gene. To identify potential target genes of miR-150, we searched for candidate genes using TargetScan6.2 and miRBase miRNA databases. Bioinformatics analysis showed that miR-150 directly targets ZEB1, MMP14, and MMP16, three important regulators involving in tumor metastasis (Fig. 4C, refs. 28, 29, 30). We therefore constructed luciferase reporter vectors containing 3′-UTR of ZEB1, MMP14, or MMP16. The reporter assay showed that miR-150 is able to markedly inhibit luciferase expression, whereas mutation of 4 nucleotides in miR-150–targeting sites results in complete abrogation of the suppressive effect (Fig. 4D and E). Expectedly, forced expression of miR-150 significantly suppresses the ZEB1, MMP14, and MMP16 protein levels in Huh7 cells (Fig. 4F). Furthermore, the negative association between miR-150 level and ZEB1, MMP14, or MMP16 mRNA level was measured in 21 HCC tissues. The significant negative
correlation is observed between the miR-150 level and the ZEB1, MMP14, or MMP16 level in vitro (Fig. 4G-I). These data suggest that miR-150 decreases cell invasion by suppressing endogenous ZEB1, MMP14, and MMP16 expression.

**ZFAS1 positively regulates ZEB1, MMP14, and MMP16 expression**

ZFAS1 directly binds miR-150 and inhibits its activity, and miR-150 targets ZEB1, MMP14, and MMP16. We therefore speculated whether ZFAS1 controls ZEB1, MMP14, and MMP16 expression in a miR-150–dependent manner. Ectopic expression of ZFAS1 results in the upregulation of ZEB1, MMP14, and MMP16 transcript and protein level, whereas the ZEB1, MMP14, and MMP16 expression level in ZFAS1-mut (miR-150) is similar to that of control (Fig. 5A and B). Furthermore, forced expression of miR-150 abrogates ZFAS1–inducing upregulation of ZEB1, MMP14, and MMP16 (Fig. 5A and B). Inversely, knockdown of ZFAS1 decreases ZEB1, MMP14, and MMP16 expression level in Huh7 cells (Fig. 5C and D). For the rescue experiment, we inhibited miR-150 in ZFAS1-downregulated Huh7 cells. Figure 5C and D showed that inhibition of miR-150 overcomes the decrease of ZEB1, MMP14, and MMP16 (Fig. 5C and D).

To further verify whether this observed effect depends on the role miR-150 in regulating the ZEB1, MMP14, and MMP16, 3′UTR, luciferase activity was assayed after cotransfection of luciferase plasmid (ZEB1-3′UTR-LUC, MMP14-3′UTR-LUC, or MMP16-3′UTR-LUC) with ZFAS1. Overexpression of ZFAS1, but not the mutant, increases the luciferase activity of ZEB1-3′UTR-LUC, MMP14-3′UTR-LUC, or MMP16-3′UTR-LUC, whereas forced expression of miR-150 abolishes this increase (Fig. 5E). Inversely, the depletion of ZFAS1 inhibits the luciferase activity of ZEB1-3′UTR-LUC, MMP14-3′UTR-LUC, or MMP16-3′UTR-LUC, whereas rescued by inhibition of miR-150 (Fig. 5F). We then assayed whether ZFAS1 is coexpressed with ZEB1, MMP14, and MMP16 in human HCC samples. We examined the expression levels of ZFAS1, ZEB1, MMP14, and MMP16 in 21 human HCC tissues. Fig. 5G–I showed that ZFAS1 transcript level is positively associated with ZEB1, MMP14, and MMP16 mRNA level. These results suggest that ZFAS1 positively regulates ZEB1, MMP14, and MMP16 expression by competitively binding miR-150.

**ZFAS1 promotes tumor metastasis in a miR-150–dependent manner**

We then tested whether ZFAS1 promotes HCC metastasis in a miR-150–dependent manner. Overexpression of ZFAS1, but not the mutant, increases Huh7 cells invasion, whereas forced expression of miR-150 abolishes this increase (Fig. 6A). Reciprocally, the depletion of ZFAS1 inhibits Huh7 cells invasion, which are rescued by inhibition of miR-150 (Fig. 6B). To explore the effect of ZFAS1 on liver metastasis, the different clones (Huh7/control, Huh7/ZFAS1, Huh7/ZFAS1 + miR-150 or Huh7/ZFAS1/mut) were injected intrasplenic into nude mice. As shown in Fig. 6C, overexpression of ZFAS1, but not the mutant, results in an increased liver metastases burden, whereas forced expression of miR-150 abolishes this increase. Inversely, the depletion of ZFAS1 inhibits tumor metastasis, which is partially rescued by inhibition of miR-150 (Fig. 6D). We then inoculated cancer cells subcutaneously into nude mice and lung metastasis was observed. Consistent with the above observations, overexpression of ZFAS1, but not the mutant, promotes lung metastasis, whereas miR-150 abolishes this increase (Fig. 6E). The depletion of ZFAS1 inhibits lung metastasis, which is rescued by miR-150 inhibition (Fig. 6F). We also used subcutaneous tumor tissues to establish orthotopic tumor models. In this model system, ZFAS1 overexpression in Huh7 significantly promotes intrahepatic and lung metastases, whereas mutating the miR-150–binding site in ZFAS1 or miR-150 overexpression partly abrogates the prometastatic role of ZFAS1 (Fig. 6G). Expectedly, ZFAS1 knockdown inhibits intrahepatic and lung metastases in a miR-150–dependent manner (Fig. 6H). Consistent with in vitro results, ZEB1, MMP14, and MMP16 are upregulated in the orthotopic tumor tissues with ZFAS1 overexpression (Supplementary Fig. S6). The mutation of the miR-150–binding sites or overexpression of miR-150 abolishes the effects (Supplementary Fig. S6). Taken together, these data confirm that ZFAS1 upregulates ZEB1, MMP14, and MMP16 expression, and promotes tumor metastasis in a miR-150–dependent manner.

**Discussion**

Large-scale complementary DNA cloning projects have identified that majority of the mammalian genome is transcribed, and only minority of these transcripts represent protein-coding genes (14, 31). In recent years, IncRNAs have gained massive attention as a potentially novel and crucial regulators of gene expression and cellular processes (32). In the study, we identified a set of IncRNAs differentially expressed in HCC using IncRNA microarrays. ZFAS1, a frequently amplified IncRNA, is correlated with poor prognosis in HCC. The ZFAS1 transcripts are expressed at higher levels in HCC tissues and cell lines. Especially, we found a significantly higher ZFAS1 expression in PVT1 than primary HCC, indicating the correlation of ZFAS1 with HCC metastasis. Clinical data analysis showed that HCC patients with high ZFAS1 expression have higher recurrence rates and shorter overall survival than those with low expression of ZFAS1. The biologic consequences of ZFAS1 overexpression in regulating cancer biology were then examined. Overexpression of ZFAS1 not only increases HCC cell migration, but also promotes the invasion activity of HCC cells compared with the control. In vivo, ZFAS1 increases the incidence of intrahepatic/extrahepatic metastasis in HCC.

Through regulating gene expression by different mechanisms, including gene transcription, genomic imprinting, and chromatin modification, IncRNAs play crucial roles in the regulation of multiple biologic processes (9). ZFAS1 is transcribed in antisense orientation of the protein-coding genes, znfx1. Emerging studies showed that upstream antisense transcription could function as enhancer for corresponding gene expression (33, 34). Therefore, we first assayed whether ZFAS1 regulates znfx1 expression. However, the results from qPCR and Western blot analysis did not show a significant effect of ZFAS1 in znfx1 expression (data not shown). Recently, a novel regulatory mechanism has been identified in which crosstalk between IncRNAs and mRNAs occurs by competing for shared miRNAs response elements. In the case, IncRNAs function as ceRNAs to sponge miRNAs, thereby derepressing the expression of miRNA targets (35). Cesana and colleagues showed that linc-MD1 regulates the time of muscle differentiation by acting as a ceRNA in myoblasts (27). Linc-MD1 sponges miR-133 and derepresses the expression of MAML1 and
MEF2C, two target genes of miR-133 (27). In HCC, HULC functions as an endogenous sponge that suppresses the activity of miR-372 (17). Meanwhile, emerging studies showed that ceRNAs cannot alter miRNA function in vivo (36, 37). They demonstrated that it is difficult to achieve sufficiently high expression of a ceRNA to significantly derepress the mRNAs regulated by that miRNA in vivo.

In the current study, bioinformatics analysis showed that ZFAS1 contains a binding site of miR-150. RIP, luciferase assay, and RNA pulldown demonstrated that ZFAS1 is able to significantly enrich miR-150, but ZFAS1 with mutations in miR-150–targeting sites abrogates the association of ZFAS1 with miR-150. Functionally, miR-150 overexpression markedly inhibits cancer cell invasion by directly targeting ZEB1, MMP14, and MMP16, three important regulators involving in tumor metastasis. We then investigated whether ZFAS1 regulates ZEB1, MMP14, and MMP16 in a miR-150–dependent manner. Overexpression of ZFAS1 results in the upregulation of ZEB1, MMP14, and MMP16 expression. Furthermore, forced

**Figure 5.** ZFAS1 positively regulates ZEB1, MMP14, and MMP16 expression. qPCR (A) or Western blot analysis (B) of ZEB1, MMP14, or MMP16 expression level after ZFAS1 overexpression. qPCR (C) or Western blot analysis (D) of ZEB1, MMP14, or MMP16 expression level after ZFAS1 knockdown. E and F, luciferase activity (ZEB1-3’UTR, MMP14-3’UTR, or MMP14-3’UTR) in Huh7 cells cotransfected with ZFAS1 (E) or siZFAS1 (F). Data are presented as the relative ratio of firefly luciferase activity to Renilla luciferase activity. G–I, the positive association between ZFAS1 transcript level and ZEB1 (G), MMP14 (H), or MMP16 (I) mRNA level was measured in 21 HCC tissues. *, P < 0.05.
expression of miR-150 abrogates ZFAS1-inducing upregulation of ZEB1, MMP14, and MMP16. In vivo, ZFAS1 transcript level is significantly positively associated with ZEB1, MMP14, and MMP16 mRNA level. Finally, we demonstrated that ZFAS1 promotes HCC metastasis in a miR-150–dependent manner. ZFAS1, but not the mutant on miR-150–binding site, increases cancer cells invasion, whereas forced expression of miR-150 abolishes this increase. The depletion of ZFAS1 inhibits Huh7 cells invasion, which are rescued by inhibition of miR-150. ZFAS1, but not the mutant, also results in intrahepatic/extrahepatic metastasis of HCC, whereas forced expression of miR-150 abolishes tumor metastasis.

A previous study showed that ZFAS1 functions as a putative tumor suppressor gene (19). They revealed that ZFAS1 is highly expressed in the mammary gland and is downregulated in breast tumors compared with normal tissue (19). ZFAS1 silencing in mammary epithelial cell line results in an increased cellular proliferation, indicating that ZFAS1 acts as a putative tumor suppressor gene in breast cancer (19). However, our results showed that the ZFAS1 transcripts are expressed at higher levels in most HCC tissues compared with the nontumor tissues of the same donor. Furthermore, the public NCBI web GEO profile indicates that ZFAS1 expression in HCC tissues is lower than that of normal control (GSE55191 and GSE58043). More importantly, we demonstrated that upregulated ZFAS1 promotes tumor metastasis, and is correlated with intrahepatic/extrahepatic metastasis and poor prognosis in HCC. Thus, it is possible that ZFAS1 plays different roles in different type of cancers, and the precise roles of ZFAS1 in cancer cells require further investigation.

**Conclusion**

Taken together, these results demonstrate that overexpression of ZFAS1 in HCC promotes tumor metastasis in a miR-150–dependent manner. Thus, ZFAS1 may be a novel target for the treatment of HCC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Li, D. Cheng, Y. Shi, Z. Wu, Q. Zhan, H. Chen.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Shen, D. Cheng, X. Deng, H. Li

References


# Amplification of Long Noncoding RNA ZFAS1 Promotes Metastasis in Hepatocellular Carcinoma

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doi:10.1158/0008-5472.CAN-14-3721

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