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# HOTAIR, a long non-coding RNA driver of malignancy whose expression is activated by FOXC1, negatively regulates miRNA-1 in hepatocellular carcinoma

DONG-NA SU[1], SHI-PIN WU[1], HONG-TAO CHEN[1] and JIN-HUA HE[2]

[1]Department of Infectious Diseases, Shenzhen People's Hospital, The Second Affiliated Hospital of Jinan University, Shenzhen, Guangdong 518020; [2]Department of Laboratory, Panyu Central Hospital, Guangzhou, Guangdong 511400, P.R. China

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**Abstract.** Evidence is rapidly accumulating that long non-coding RNAs (lncRNAs) are involved in human tumorigenesis and are dysregulated in multiple cancers, including hepatocellular carcinoma (HCC). lncRNAs can regulate essential pathways that contribute to tumor initiation and progression with tissue specificity, which suggests that lncRNAs may be valuable biomarkers and therapeutic targets. HOX transcript antisense intergenic RNA (HOTAIR) has previously been demonstrated to be an oncogene and a negative prognostic factor in a variety of cancers; however, the factors that contribute to the upregulation of HOTAIR and the interaction between HOTAIR and microRNAs (miRNAs or miRs) are largely unknown. In the present study, the expression levels of HOTAIR, forkhead box C1 (FOXC1) and miRNA-1 were examined in 50 matched pairs of HCC and HCC cells. The effects of HOTAIR on HCC cell proliferation were tested using trypan blue exclusion assay. The effect of HOTAIR on HCC growth *in vivo* was determined in a (nu/nu) mouse model. A computational screening of HOTAIR promoter was conducted to search for transcription factor-binding sites. FOXC1 binding to the promoter region of HOTAIR was confirmed using a chromatin immunoprecipitation assay. A search for miRNAs that had complementary base paring with HOTAIR was performed utilizing an online software program. The interaction between miR-1 and HOTAIR was examined using a luciferase reporter assay. Gain and loss of function approaches were used to determine the changes of HOTAIR or miR-1 expression. The relative levels of FOXC1 and HOTAIR expression in HCC tissues and HepG2 cells were significantly higher than those in normal liver LO2 cells and adjacent carcinoma tissues; the relative expression of miR-1 exhibited the opposite pattern. Overexpression of HOTAIR promoted HCC cell proliferation and progression of tumor xenografts. The present authors have demonstrated that FOXC1 binds to the upstream region of HOTAIR in HCC cells and that FOXC1 activates lncRNA HOTAIR expression in HCC HepG2 cells, which suggests that HOTAIR harbors a miRNA-1 binding site. The present data revealed that this binding site is vital for the regulation of miRNA-1 by HOTAIR. Furthermore, HOTAIR negatively regulated the expression of miRNA-1 in HepG2 cells. Additionally, the present study demonstrated that the oncogenic activity of HOTAIR is in part based on the negative regulation of miR-1. Taken together, these results suggest that HOTAIR is a FOXC1-activated driver of malignancy, which acts in part through the repression of miR-1.

*Correspondence to:* Dr Shi-Pin Wu, Department of Infectious Diseases, Shenzhen People's Hospital, The Second Affiliated Hospital of Jinan University, 1017 Dong Men Bei Lu, Luo Hu, Shenzhen, Guangdong 518020, P.R. China
E-mail: sudona@qq.com

*Abbreviations:* lncRNA, long non-coding RNA; miR, microRNA; siRNA, small interfering RNA; TF, transcription factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HCC, hepatocellular carcinoma; HOTAIR, HOX transcript antisense intergenic RNA; FOXC1, forkhead box C1

*Key words:* long non-coding RNA, HOTAIR, miR-1, FOXC1, hepatocellular carcinoma

## Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortalities, with nearly 600,000 mortalities occurring worldwide each year (1). Although resection is considered a potentially curative treatment for HCC patients, the 1-year post-operative survival rate is only 30-40% (2). Thus, it is necessary to improve our understanding of the disease-causing mechanisms and to identify specific biomarkers for HCC progression to aid in the prediction and improvement in clinical outcomes.

Non-coding RNAs (ncRNAs) are subdivided into two groups: Small ncRNAs (<200 nt) and long ncRNAs (lncRNAs) (3,4). Small ncRNAs, including the well-documented microRNAs (miRNAs or miRs), have received great attention, since they are important in cancer (5). It has been proposed that lncRNAs are involved in the epigenetic regulation of coding genes, and thus, exert a powerful effect on a number of physiological and pathological processes, including the pathogenesis of numerous human cancers (6). It has been reported that

lncRNA HOX transcript antisense intergenic RNA (HOTAIR) expression is significantly higher in HCC tissues than in adjacent non-cancerous tissues (7). Patients with HCC tumors that overexpress HOTAIR have an increased risk of HCC recurrence following hepatectomy, and there is also a correlation between HOTAIR overexpression and increased risk of lymph node metastasis (8). The overexpression of HOTAIR is an independent prognostic factor for HCC recurrence in liver transplant patients (9). Furthermore, patients with high tumor expression of HOTAIR have a significantly shorter recurrence-free survival than patients with low expression of HOTAIR (10).

The mechanism by which HOTAIR exerts its oncogenic activity remains largely unknown. A regulatory mechanism has been proposed in which RNAs cross-talk via competing shared miRs (11). In addition, lncRNAs directly interact with RNA-binding proteins and localize to the gene promoter region to regulate gene expression (12). The proposed competitive endogenous RNAs (ceRNAs) mediate the bioavailability of miRs on their targets, thus imposing another level of post-transcriptional regulation (13). An example of this type of regulation is the lncRNA colon cancer associated transcript 1, (CCAT1), which binds miR-218-5p and forms a regulatory interaction (14). Using jaspar bioinformatics software (http://jaspar.genereg.net), forkhead box C1 (FOXC1) shared binding sites with the upstream promoter region of HOTAIR were predicted. Furthermore, miR-1 was identified as the miR that shares binding sites with HOTAIR using starBase 2.0 (http://starbase.sysu.edu.cn). Thus, the present authors hypothesized that transcription factors (TFs) activate the transcription of HOTAIR. Furthermore, HOTAIR overexpression inhibits the expression of miR-1. This hypothesis is discussed to better elucidate the pathogenesis of HCC.

## Materials and methods

*Tissue specimens*. Fresh-frozen and paraffin-embedded HCC tissues and corresponding adjacent non-tumorous gastric samples were obtained from Chinese patients at Shenzhen People's Hospital (Shenzhen, China) between January 2010 and December 2014. All cases were reviewed by a pathologist and histologically confirmed as HCC. Informed consent was obtained from all patients and the study was approved by the Institutional Ethics Committee of Shenzhen People's Hospital.

*Construction of recombinant HOTAIR lentiviral expression vectors*. The sequence of HOTAIR was obtained (https://www.ncbi.nlm.nih.gov/nuccore/383286742?report=fasta) and synthesized. Upon double digestion with *Bam*HI and *Eco*RI, HOTAIR was directionally-connected to the pGLV3/H1/GFP/Puro vector and then transformed into DH5α competent cells (Shanghai GenePharma Co., Ltd., Shanghai, China). Recombinant, packaging and envelope plasmids (Shanghai GenePharma Co., Ltd.) were co-transfected into 293T cells for 72 h. Viruses were then collected and the titers were determined using the dilution hole measurement method (15). The constructed recombinant HOTAIR lentiviral expression vector was designated LV3-HOTAIR (16).

*Design and construction of a eukaryotic expression vector for Homo sapiens (hsa)-miR-1 and FOXC1*. The mature hsa-miR-1 sequence (5'-UGGAAUGUAAAGAAGUAUGUAU-3') is available from the miR registry (MIMAT000416; http://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MIMAT0000416). To prevent the formation of a termination signal, TTGGCCACT GACT was selected as the region in a miR expression vector template (pcDNA3.1; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sequence TGCT was added to the 5' sense strand template of the miR expression vector, while GTCC was added to the 5' antisense strand template (17). In addition, a non-specific sequence was designed and synthesized by Shanghai GenePharma Co., Ltd. The eukaryotic expression vector plasmid targeting hsa-miR-1 was designated pmiR-1. The sequence of FOXC1 was synthesized and sub-cloned into pcDNA3.1 (Invitrogen; Thermo Fisher Scientific, Inc.) to generate pFOXC1. Empty pcDNA3.1 vector was used as a negative control (NC).

*Cell culture*. HCC HepG2 and LO2 cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified incubator at 37˚C in the presence of 5% $CO_2$. All cell lines were passaged for <6 months.

*Cell transfection*. All plasmid vectors (pmiR-1, pFOXC1 and empty vector) used for cell transfection were extracted using Hieff Trans™ Liposomal Transfection Reagent (Yeasen Biotechnology Co., Ltd., Shanghai, China). HepG2 cells cultured on 6-well plates were transfected with pmiR-1, pFOXC1 or empty vector using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were harvested after 48 h for quantitative polymerase chain reaction (qPCR). Methods for transfecting cells with LV3-HOTAIR were adopted, as previously described (17). Small interfering RNAs (siRNAs) were designed and synthesized by Shanghai GenePharma Co., Ltd. The siRNA sequence used in the present study was as follows: Sense, 5'-GGGAGATGTTCGAGT CACAGA-3' and antisense, 5'-GCGTAAAGCTCGGGTAAG TAG-3'. The siRNAs sequences targeting human HOTAIR were as follows: Sense, 5'-GGAGAACACUUAAAUAAGUTT-3' and antisense, 5'-ACUUAUUUAAGUGUUCUCCTA-3' (18).

*Trypan blue exclusion assay*. The density of the HepG2 cell line suspension was determined by counting on a hemacytometer. A 0.4% solution of trypan blue in phosphate-buffered saline (PBS) was prepared (pH 7.2-7.3). Trypan blue stock solution (0.1 ml) was added to 1 ml of cells. A hemacytometer was loaded and examined immediately under a microscope at low magnification. The number of blue-stained cells and the number of total cells were counted. Cell viability was considered to be ≥95% for healthy log-phase cultures. The percentage of viable cells was calculated as follows: Viable cells (%) = [1.00 - (number of blue-stained cells/number of total cells)] x 100.

*In vivo treatment*. A total of 15 BALB/c (nu/nu) male mice (200±2.6 g; age, 3 months) from the Animal Center of Guangzhou Province (Guangdong, China) received

Table I. Primers for quantitative polymerase chain reaction.

| Gene | Primers (5'-3') |
|---|---|
| U6 | F: CTCGCTTCGGCAGCACA |
| U6 | R: AACGCTTCACGAATTTGCGT |
| 18S | F: CCTGGATACCGCAGCTAGGA |
| 18S | R: AACGCTTCACGAATTTGCGT |
| HOTAIR | F: CAGTGGGGAACTCTGACTCG |
| HOTAIR | R: GTGCCTGGTGCTCTCTTACC |
| FOXC1 | F: CTCAACGAGTGCTTCGTCAA |
| FOXC1 | R: ACATGTTGTAGGAGTCCGGG |
| miR-1 | F: CACTCCAGCTGGGTGGAAT GTAAAGAAGTAT |

The level of expression was calculated using Cq and the $2^{-\Delta\Delta Cq}$ method (16). F, forward; R, reverse; HOTAIR, HOX transcript antisense intergenic RNA; FOXC1, forkhead box C1; miR, microRNA.

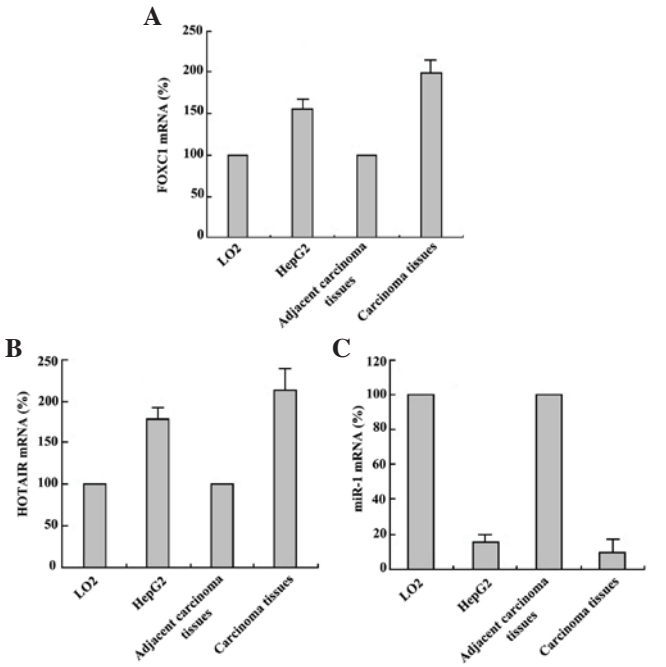


Figure 1. Relative levels of gene expression detected by reverse transcription-quantitative polymerase chain reaction. Relative expression of (A) FOXC1, (B) miR-1 and (C) HOTAIR. HOTAIR, HOX transcript antisense intergenic RNA; FOXC1, forkhead box C1; miR, microRNA; mRNA, messenger RNA.

subcutaneous injections of $2x10^6$ HepG2 cells into the axillae bilaterally. The mice were housed in a pathogen-free environment at a temperature of 20-26˚C and were exposed to 12 h light/dark cycles with free access to food and water. When xenograft tumors became palpable (~0.1 $mm^3$), mice were randomly divided (n=5 mice/group) into a control group receiving a PBS injection (100 $\mu$l), a transfection group receiving LV3-HOTAIR (200 nM) and a NC group receiving LV3 + scramble sequence (200 nM). There was no difference in the baseline tumor size between the groups. Tumor volume was calculated every 3 days according to the following formula: V=ab2π/6, where 'a' is the maximum tumor diameter and 'b' is the minimum tumor diameter. After treatment for 20 days, the mice were euthanized, and the tumors were extirpated and weighed.

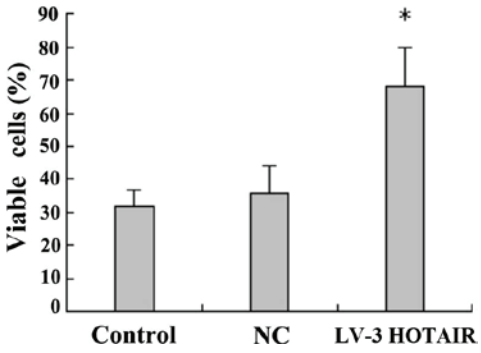


Figure 2. The proliferation of HepG2 cells was determined with trypan blue exclusion assay. Data are presented as the mean ± standard deviation; n=3; *P<0.05 vs. NC group. HOTAIR, HOX transcript antisense intergenic RNA; NC, negative control.

*Chromatin immunoprecipitation (ChIP) assay*. HepG2 cells were treated with formaldehyde and incubated for 10 min to generate DNA-protein cross-links. Cell lysates were then sonicated to generate chromatin fragments of 200-300 bp and immunoprecipitated with anti-FOXC1 (cat. no. 625905; 1:1,000; R&D Systems China Co., Ltd., Shanghai, China) for 1 h at room temperature or Alexa Fluor® 488 conjugated-immunoglobulin G (IgG) antibody (cat. no. 4408; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at room temperature as a control. Precipitated chromatin DNA was recovered and analyzed by qPCR.

*Reverse transcription (RT)-qPCR*. Total RNA samples were extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR analysis was performed using the Ultra SYBR Mixture with ROX (CWBio, Co., Ltd., Beijing, China) and an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). HOTAIR cDNA amplification was performed under the following conditions: Initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 30 sec and primer annealing at 55˚C for 30 sec, with a final extension step at 72˚C for 60 sec. FOXC1 cDNA amplification was performed under the following conditions: Initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 30 sec and primer annealing at 55˚C for 30 sec, with a final extension step at 72˚C for 60 sec. miR-1 cDNA was amplified under the following conditions: Initial denaturation at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 10 sec and primer annealing at 57˚C for 20 sec, with a final extension step at 72˚C for 10 sec. The level of 18S expression was used as an internal control for messenger RNAs, while the U6 level was used as an internal control for miRs. The primers used in RT-qPCR are indicated in Table I. The expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (19).

*Luciferase reporter assay*. The full sequence HOTAIR gene was obtained by qPCR amplification using the Ultra

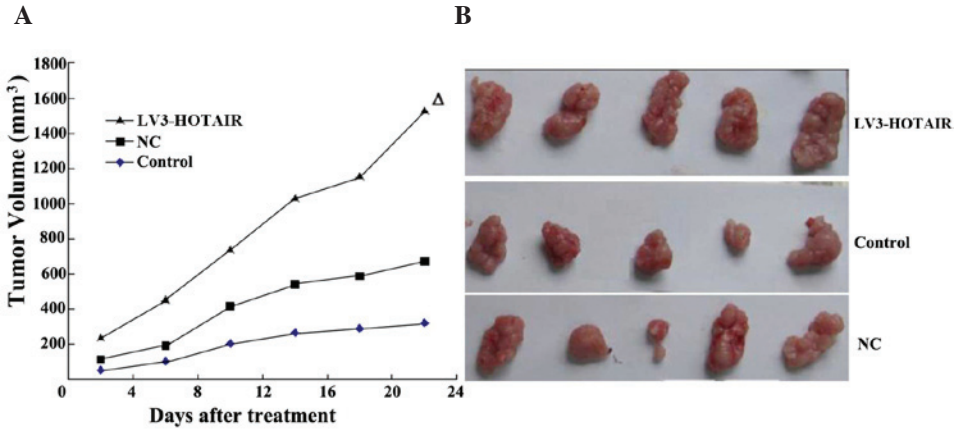


Figure 3. Effect of LV3-HOTAIR on hepatocellular carcinoma xenografts. HepG2 tumor xenografts were established in male athymic nude mice. Animals in the treatment group received LV3-HOTAIR (200 nM/kg once daily). Control mice received phosphate-buffered saline (100 $\mu$l/kg once daily). NC mice received scrambled sequence (200 nM/kg once daily). After 20 daily treatments, tumors injected with LV3-HOTAIR were significantly larger than those in the control and NC groups. (A) Growth curves of tumor xenografts, $^{\Delta}P<0.05$ compared with control and NC groups. (B) Mice were sacrificed and their xenografts were removed for weighing. HOTAIR, HOX transcript antisense intergenic RNA; NC, negative control.

SYBR Mixture with ROX and an Applied Biosystems 7500 Real-Time PCR System. HOTAIR cDNA was amplified under the following conditions: Initial denaturation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 30 sec and primer annealing at 55˚C for 30 sec, with a final extension step at 72˚C for 60 sec. The gene was cloned separately into the multiple cloning site of the psi-CHECK™-2 luciferase miR expression reporter vector. HepG2 cells were transfected with miR mimic, miR inhibitor, control miR, negative control (NC), negative control inhibitor (all purchased from Guangzhou Leader Bio-Technology Co., Ltd., Guangzhou, China) or empty plasmid using Lipofectamine 2000, according to the manufacturer's protocol. Nucleotide-substitution mutation analysis was conducted using direct oligomer synthesis of full sequences. All constructs were verified by sequencing. Luciferase activity was measured using the Dual Luciferase Reporter Assay System Kit (Promega Corporation, Madison, WI, USA) on an Infinite M200 luminescence reader (Tecan Group Ltd., Männedorf, Switzerland), according to the manufacturer's protocol.

*Data analysis*. All results are the average of ≥3 independent experiments from separately treated and transfected cultures. Data are expressed as the mean ± standard deviation. Statistical comparisons were performed by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*High expression of HOTAIR in HCC tissues and cells*. HCC HepG2 and LO2 cells were cultured conventionally. Total RNA was extracted from tissues and cells using TRIzol. The relative expression levels of FOXC1, HOTAIR and miR-1 were detected by RT-qPCR. The relative level of FOXC1 and HOTAIR expression in HCC tissues and HepG2 cells was significantly higher than that in normal liver LO2 cells and adjacent carcinoma tissues (Fig. 1A and B). The relative expression of miR-1 exhibited the opposite pattern (Fig. 1C). These results indicate that HOTAIR has a tumor-promoting role in HCC, while miR-1 has a tumor-suppressor role, and there may be negative regulation at the level of expression between HOTAIR and miR-1. The high expression of FOXC1 may activate the transcription of HOTAIR. These results provided a foundation to further explore the mechanism of action of the HOTAIR transcript.

*LV3-HOTAIR promotes HepG2 cell proliferation*. Following Lipofectamine 2000 transfection of HepG2 cells with the lentiviral expression vector LV3-HOTAIR, cells were stained 72 h later with 0.04% trypan blue, and the cell survival rate was calculated. In the experimental group (LV3-HOTAIR), the effects on cell proliferation activity decreased significantly compared with those in the blank control and NC groups ($P=0.01$), indicating that LV3-HOTAIR can promote the proliferation of HepG2 cells (Fig. 2).

*LV3-HOTAIR promotes the progression of tumor xenografts*. HepG2 tumor xenografts were established in athymic nude mice to evaluate the effects of LV3-HOTAIR on HCC growth *in vivo*. Compared with the untreated animals, application of LV3-HOTAIR significantly diminished the tumor volume, whereas no effect was observed in the NC group (Fig. 3). No body weight loss or diarrhea was observed, and all animals (treated and non-treated) survived. The results demonstrated that the overexpression of HOTAIR can effectively inhibit HCC cancer growth *in vivo*.

*FOXC1 binds to the promoter region of HOTAIR and activates lncRNA HOTAIR expression*. Recently, numerous important TFs have been demonstrated to be involved in regulating lncRNA transcription (20). To determine which TFs activate DD3 expression, the potential TF binding sites in the promoter region of HOTAIR were analyzed using the jaspar database (http://jaspar.genereg.net). The results revealed that there is one E-box element recognized by FOXC1. To further demonstrate that FOXC1 can directly bind to the HOTAIR promoter regions and activate the expression

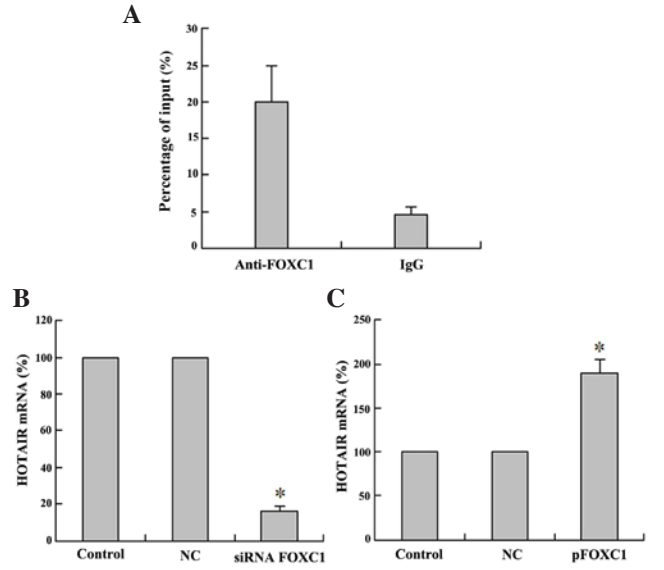


Figure 4. FOXC1 promotes HOTAIR expression via binding to its promoter regions. (A) ChIP assays were used to assess FOXC1 binding at the promoter region of HOTAIR containing the E-box element. The ChIP-derived DNA was amplified by quantitative polymerase chain reaction with specific primers and expressed as a percentage of input DNA. Data are presented as the mean ± standard error based on ≥3 independent experiments (*P<0.05 compared with the IgG group). (B) Levels of HOTAIR expression following the transfection of siRNA FOXC1 or scrambled siRNA into HepG2 cells (*P<0.05 compared with the NC group). (C) Levels of HOTAIR upon transfection of pFOXC1 or empty vector into HepG2 cells (*P<0.05 compared with the NC group). FOXC1, forkhead box C1; IgG, immunoglobulin G; ChIP, chromatin immunoprecipitation; HOTAIR, HOX transcript antisense intergenic RNA; NC, negative control; mRNA, messenger RNA; siRNA, small interfering RNA; p, plasmid.

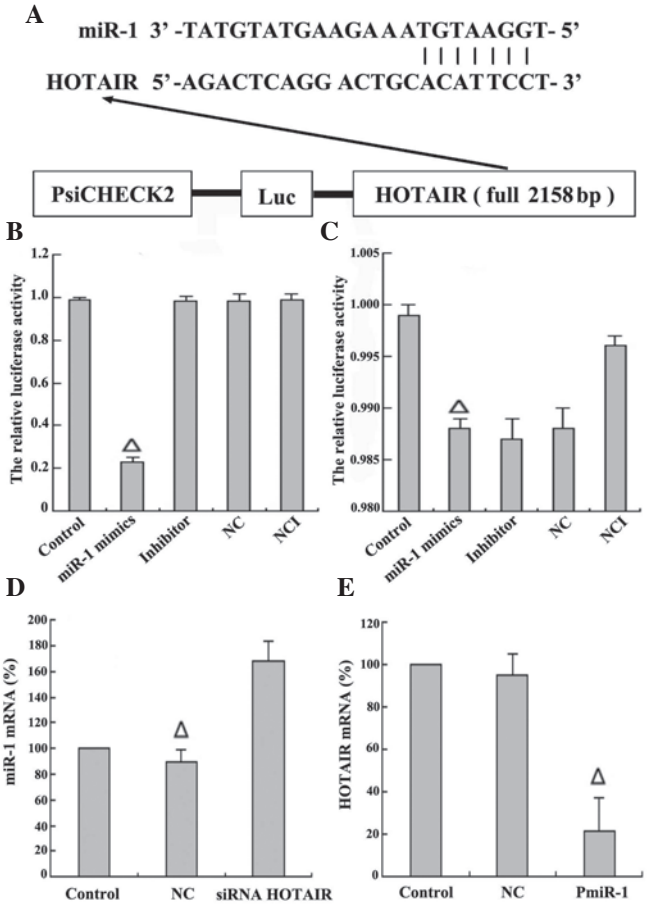


Figure 5. HOTAIR negatively regulates the expression of miR-1 in HepG2 cells. (A) Luciferase reporter plasmid containing the HOTAIR wild type sequence or a mutant transcript. (B) Comparison of luciferase activity of plasmid-transfected cloned HOTAIR full-length transcript (ΔP<0.05 vs. control and NC groups). (C) Comparison of the luciferase activity of the plasmid-transfected cloned HOTAIR mutant transcript (*P>0.05 vs. control and NC groups). (D) HepG2 cells were treated with siRNA HOTAIR for 48 h and the relative levels of miR-1 expression were analyzed by RT-qPCR (ΔP<0.05 vs. control and NC groups). (E) HepG2 cells were treated with pmiR-1 for 48 h and the relative levels of HOTAIR expression were analyzed by RT-qPCR (ΔP<0.05 vs. control and NC groups). miR, microRNA; HOTAIR, HOX transcript antisense intergenic RNA; Luc, luciferase; NC, negative control; NCI, negative control inhibitor; mRNA, messenger RNA; siRNA, small interfering RNA; p, plasmid; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

of HOTAIR, FOXC1 immunoprecipitates were observed to be highly enriched in DNA fragments compared with the NC IgG immunoprecipitates in a ChIP assay. The ChIP assays indicated that FOXC1 directly bound to HOTAIR promoter regions (Fig. 4A). These results suggest that HOTAIR interacts with the FOXC1 responsive element in the HOTAIR promoter to induce transcription. Furthermore, the qPCR results indicated that HOTAIR expression was increased upon pFOXC1 transfection (Fig. 4B), while HOTAIR expression was decreased following siRNA-FOXC1 transfection (Fig. 4C). These results indicated that there is a positive regulation between the TF FOXC1 and the lncRNA HOTAIR. Taken together, these data suggest that the TF FOXC1 activates the expression of the lncRNA HOTAIR in HCC HepG2 cells.

*HOTAIR negatively regulates the expression of miRNA-1.* The function of lncRNAs in human diseases may reflect their ability to regulate gene expression. Increasing evidence has suggested that ncRNAs may participate in the ceRNA regulatory network (21). For example, there is a negative correlation between CCAT1 and let-7 (22). To confirm the direct binding between HOTAIR and miR-1, luciferase reporter constructs were generated (Fig. 5A). As predicted, there was one putative binding site in HOTAIR (1,615-1,638 bp). It was observed that co-transfection of HepG2 cells with miR-1 mimics and wild type psi-CHECK™-2-HOTAIR significantly inhibited luciferase activity (P=0.01; Fig. 5B); however, co-transfection of HepG2 cells with miR-1 mimics and mutant psi-CHECK™-2-HOTAIR had little effect on the activity of luciferase (P=0.09; Fig. 5C). These data confirmed that miR-1 regulates the expression of HOTAIR by directly binding to target sites within the HOTAIR sequence. To further explore the regulatory association between HOTAIR and miR-1, the siRNA sequencing for HOTAIR was designed and transfected into HepG2 cells for 48 h to knock down the expression of HOTAIR. It was observed that the levels of miR-1 expression were significantly upregulated in contrast to those in the blank control (non-transfected siRNA HOTAIR) and NC (transfected with random sequences) groups (Fig. 5D). The eukaryotic expression vector plasmid of miR-1 was further constructed and transfected into HepG2 cells for 48 h to induce overexpression of miR-1. Upon overexpression of miR-1, the levels of HOTAIR expression were significantly downregulated in contrast to those in the blank

control (non-transfected miR-1) and NC (transfected with plasmid and random sequences) groups (Fig. 5E). Thus, there may be a regulatory effect between HOTAIR and miR-1. To summarize, HOTAIR negatively regulated the expression of miR-1 in HepG2 cells.

## Discussion

Recent evidence has suggested that ncRNAs serve an important role in cancer pathogenesis and can provide a novel insight into the biology of cancer (23). Over the past decade, research involving miRs has dominated the field of ncRNA regulation (24); however, the role of lncRNAs in the tumorigenesis of HCC remains largely unknown. Understanding the precise molecular mechanism by which lncRNAs function would facilitate the development of lncRNA-directed diagnostics and therapeutics against cancer. The present study provided evidence that FOXC1, a TF, activates the expression of HOTAIR, and that HOTAIR exhibits oncogenic activities in part through the modulation of miR-1. Therefore, HOTAIR promotes the tumorigenesis of HCC.

HOTAIR lncRNA was introduced by Kornienko *et al* (24) as a spliced and polyadenylated RNA with 2,158 nt and six exons (25). This RNA arises from the transcription of the antisense strand of the HOXC gene, which is specifically situated between HOXC11 and HOXC12 on chromosome 12q13.13 (25). HOTAIR is an oncogenic factor and can be used as a prognostic biomarker in different cancer types, since HOTAIR plays a key role in the initiation and progression of different types of cancer, including cervical cancer and nasopharyngeal carcinoma (26). HOTAIR, a lncRNA initially identified in breast cancer, was demonstrated to be upregulated in a variety of carcinomas (27,28). A large number of studies have focused on the biological role and association of HOTAIR with clinical prognosis (9,29,30), yet the precise factors regulating its expression remain largely unknown. HOTAIR is transcriptionally regulated by estradiol in breast cancer, which is tumor-specific (31). In the current study, a putative binding site of FOXC1 in the promoter region of HOTAIR was predicted. In addition, miR-1 was observed to have a binding site on HOTAIR. RT-qPCR results revealed that the relative level of FOXC1 and HOTAIR expression in HCC tissues and HepG2 cells was significantly higher than that in normal liver LO2 cells and adjacent carcinoma tissues. The expression of miR-1 exhibited the opposite pattern (Fig. 1). FOXC1 is a well-known TF. Knockdown of FOXC1 expression leads to cytoskeleton modification accompanied by a decreased ability of HCC cell proliferation, migration and invasion (32). miR-1 has been reported to be a tumor-suppressor gene that represses cancer cell proliferation and metastasis and promotes apoptosis by ectopic expression (33). The present results support the findings reported in the literature (33). Using technology in which LV3-HOTAIR can promote the expression of the HepG2 gene and tumor growth in animals (Figs. 2 and 3), HOTAIR was observed to promote the migration and invasion of HCC cells by inhibiting RNA binding motif protein 38 (RBM38), which indicates critical roles of HOTAIR and RBM38 in HCC progression (27). In contrast, knockdown of HOTAIR by siRNAs resulted in the reduction of motility and invasion of the human melanoma cell line A375 (34).

lncRNAs regulate gene expression through a variety of mechanisms, including transcription, post-transcriptional processing, chromatin modification, genomic imprinting and regulation of protein function (35,36). Recently, it has been reported that lncRNA transcription can be regulated by key TFs and epigenetic modification (23). For example, p53 can promote lncRNA-p21 transcription, and E2F1 regulates lncRNA E2F1-regulated inhibitor of cell death expression, while the core catalytic subunit of the polycomb repressive complex 2, enhancer of zeste homolog 2, represses lncRNA sprouty RTK signaling antagonist 4-intronic transcript 1 transcription via epigenetic maintenance of tri-methylation of lysine 27 on histone H3 (37). c-Myc directly binds to the CCAT1 promoter region and activates lncRNA CCAT1 expression in colon cancer cells (38). In the present study, ChIP assays determined that FOXC1 directly binds to the HOTAIR promoter region. Furthermore, overexpression of FOXC1 increased HOTAIR expression in HCC cells, and knockdown of FOXC1 expression decreased HOTAIR expression in HepG2 cells. FOXC1 also activated lncRNA HOTAIR expression in HCC cells. Taken together, these findings provide clues for exploring the mechanism underlying HOTAIR transcription.

Previous studies have established that lncRNAs can also regulate other non-coding RNAs, in particular miRs, and miRNAs may have an effect on the regulation of lncRNAs (39,40). In support of this notion, it was demonstrated in the current study that HOTAIR-mediated oncogenic activity occurs, at least in part, through the suppression of miR-1. Knockdown of HOTAIR induced the upregulation of miR-1. By contrast, overexpression of miR-1 could downregulate the HOTAIR level, while miR-1 overexpression repressed the HOTAIR level. Thus, HOTAIR and miR-1 may form a reciprocal repression feedback loop. In addition, the mechanism of such a feedback loop was explored in the present study, and it was observed that miR-1 regulates the expression of HOTAIR by directly binding to target sites within the HOTAIR sequence. lncRNA H19 has been demonstrated to inhibit muscle differentiation by antagonizing let-7 (41). It has been reported that long intergenic non-coding RNA muscle differentiation 1 'sponges' miR-133, and that miR-133 regulates muscle differentiation (42). A recently identified lncRNA, cardiac hypertrophy related factor, has been reported to regulate cardiac hypertrophy by targeting miR-489 (43).

It has been recently reported that HOTAIR is a c-Myc-activated driver of malignancy, which acts in part through repressing miR-130a (44). The current study demonstrated that LV3-HOTAIR effectively promotes HepG2 cell formation of clones, as well as growth of animal tumor xenografts. The underlying molecular mechanism may involve the TF FOXC1, which activates the transcription of the lncRNA HOTAIR. The current study suggested another layer of regulation involving ncRNAs (molecular and biological). A better understanding of the ncRNA interaction regulatory network will clearly advance the research in the tumorigenesis of HCC.

## Acknowledgements

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