Research Article

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Hotair mediates hepatocarcinogenesis through suppressing miRNA-218 expression and activating P14 and P16 signaling

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Background & Aims: Long non-coding RNA Hotair has been considered as a pro-oncogene in multiple cancers. Although there is emerging evidence that reveals its biological function and the association with clinical prognosis, the precise mechanism remains largely elusive.

Methods: We investigated the function and mechanism of Hotair in hepatocellular carcinoma (HCC) cell models and a xenograft mouse model. The regulatory network between miR-218 and Hotair was elucidated by RNA immunoprecipitation and luciferase reporter assays. Finally, the correlation between Hotair, miR-218 and the target gene Bmi-1 were evaluated in 52 paired HCC specimens.

Results: In this study, we reported that Hotair negatively regulated miR-218 expression in HCC, which might be mediated through an EZH2-targeting-miR-218-2 promoter regulatory axis. Further investigation revealed that Hotair knockdown dramatically inhibited cell viability and induced G1-phase arrest *in vitro* and suppressed tumorigenicity *in vivo* by promoting miR-218 expression. Oncogene Bmi-1 was shown to be a functional target of miR-218, and the main downstream targets signaling, $P16^{Ink4a}$ and $P14^{ARF}$, were activated in Hotair-suppressed tumorigenesis. In primary human HCC specimens, Hotair and Bmi-1 were concordantly upregulated whereas miR-218 was downregulated in these tissues. Furthermore, Hotair was inversely associated with miR-218 expression and positively correlated with Bmi-1 expression in these clinical tissues.

Conclusion: Hotair silence activates $P16^{Ink4a}$ and $P14^{ARF}$ signaling by enhancing miR-218 expression and suppressing Bmi-1 expression, resulting in the suppression of tumorigenesis in HCC.

Keywords: Hotair; microR-218; P14; P16; Hepatocellular carcinoma.
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Abbreviations: Bmi-1, B lymphoma mouse Moloney leukemia virus insertion region 1; CDS, the coding sequence; ceRNA, competitive endogenous RNAs; EZH2, Enhancer of zeste homolog 2; HCC, hepatocellular carcinoma; Hotair, Hox transcript antisense intergenic RNA; lncRNAs, Long non-coding RNAs; Lv-miR218, lentiviral pre-miR218 vector; miRNA, microRNAs; miR-218, microRNA-218; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control RNA duplex; PI, propidium iodide; PRC2, polycomb repressive complex 2; RIP, RNA immunoprecipitation; siBmi-1, specific siRNA of Bmi-1; siHotair, specific siRNA of Hotair; siRNA, small interfering RNA; shRNA, short-hairpin RNA; 3′UTR, 3′ untranslated region.

Introduction

Liver cancer is the fifth most prevalent cancer and the third leading cause of all cancer-related deaths worldwide. HCC is the most common primary malignant type in adults and is more frequent in men than in women [1,2]. Although advances in HCC diagnosis and treatment have increased the possibility of cure, HCC remains largely incurable because of poor prognosis and recurrence. Therefore, the development of innovative, targeted therapies is imperative and of high clinical significance. Recently, a variety of studies have proposed that non-coding RNAs contribute to hepatocarcinogenesis, indicating the potential of non-coding RNA as an effective molecular target for cancer diagnosis and therapeutics [3–5].

Long non-coding RNAs (lncRNAs), extensively transcribed from the mammalian genome, have gained widespread attention in recent years. They serve as important and powerful regulators of various biological activities and play critical roles in the progression of a variety of diseases including cancer [6–8]. Hotair (Hox transcript antisense intergenic RNA) is a 2158-bp lncRNA located in the Hoxc gene cluster but represses the transcription of Hoxd locus in foreskin fibroblasts [9]. As a novel regulator in

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tumorigenesis, Hotair was initially found to promote invasiveness and metastasis in breast cancer [8,9]. In addition, Hotair is associated with chromatin modifications and it exhibits pro-oncogenic activity in pancreatic cancer [10]. Moreover, its upregulation positively correlates with poor prognosis, tumor progression and recurrence in gastrointestinal cancers such as colorectal cancer, HCC and gastrointestinal stromal tumors [11–15]. Although an increasing number of studies have focused on its biological function and its association with clinical prognosis in cancers, the precise mechanism underlying its upregulation remains largely unknown.

As a broadly conserved microRNA, microRNA-218 (miR-218) is considered to be a tumor suppressor in multiple carcinomas, such as bladder cancer [16], nasopharyngeal cancer [17], non-small cell lung cancer [18], glioma [19], gastric cancer [20], and cervical carcinoma [21]. In the present study, miR-218 was found to be downregulated whereas Hotair was upregulated in HCC specimens and an inverse association was also observed in these samples. Further investigation revealed that the negative regulation of Hotair might be mediated through an EZH2-targeting-miR-218-2 promoter regulatory axis. Knockdown of Hotair was sufficient to inhibit tumorigenicity both *in vitro* and *in vivo* by promoting miR-218 expression. Furthermore, the downstream targets $P14^{ARF}$ and $P16^{Ink4a}$ signaling were activated in Hotair-miR-218-mediated tumorigenesis through directly suppressing oncogene Bmi-1 expression. Collectively, our findings dissected a novel mechanism of Hotair-mediated hepatocarcinogenesis and it might help to develop a promising molecular target for HCC therapy.

Materials and methods

Cell culture and tissue specimens

A panel of HCC cell lines including HepG2, Bel7404, PLC5, HuH7, and immortalized non-tumorigenic MIHA cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin.

Fifty-two paired primary HCC specimens, their non-tumor counterparts, and five normal liver tissues were collected by means of tumor resection at the Prince of Wales Hospital, The Chinese University of Hong Kong (CUHK). The information is further described in the Supplementary Table 1. All the human tissues were obtained with informed consent and this study was approved by Joint Chinese University of Hong Kong-New Territories Ease Cluster Clinical Research Ethics Committee.

RNA oligoribonucleotides and cell transfections

All RNA oligoribonucleotides were purchased from Genepharma (Shanghai, China). The small interfering RNAs (siRNAs) that specifically target human Bmi-1 mRNA (NCBI Reference No: NM_005180), Hotair (NCBI Reference No: NR_003716.3) and EZH2 (NCBI Reference Sequence: NM_001203247.1) were designated as siBmi-1, siHotair and siEZH2, respectively. The negative control RNA duplex (NC) for both miRNA mimics and siRNA, as well as the single-stranded negative control RNA for miRNA inhibitors (anti-NC), was non-homologous to any human genome sequences. Their sequences are listed in Table 1.

The transfection of RNA oligoribonucleotides was performed by using Lipofectamine 2000 (Invitrogen) [23]. The transfection of plasmid DNA was performed by using X-tremeGENE (Roche). Unless otherwise indicated, 100 nM of RNA duplex or 200 nM of miRNA inhibitor were used for each transfection and all the experiments were repeated in triplicate.

Lentiviral miR-218 expression plasmid construction and lentiviruses production

A 110 bp sequence of pre-miR218 encompassing the stem-loop was amplified and then cloned into a lentiviral vector (designated as Lv-miR218). The production and purification of the lentivirus were performed as mentioned previously [22,23]. Briefly, the pseudo-typed lentivirus was generated by co-transfecting 293T cells with Lv-miR218 vector and three packaging vectors (pRRE, pRSV-REV, and pCMV-VSVG). A lentiviral vector expressing a scramble RNA was used as the control (Lv-Sc).

Table 1. Sequences of RNA, siRNA and mRNA used.

NC:	5' UUCUCCGAACGUGUCACGUUU 3'
anti-NC:	5' GUGGAUAUUGUUGCCAUCA 3'
miR-218:	5' UUGUGCUUGAUCUAACCAUGU 3'
anti-miR218:	5' ACAUGGUUAGAUCAAGCACAA 3'
siBmi-1-1:	5' CGUGUAUUGUUCGUUACCUTT 3'
siBmi-1-2:	5' GCGGUAACCACCAAUCUUC 3'
siHotair-1:	5' CCACAUGAACGCCCAGAGAUUTT 3'
siHotair-2:	5' GAACGGGAGUACAGAGAGAUU 3'
siEZH2-1:	5' AAGAGGUUCAGACGAGCUGAUTT 3'
siEZH2-2:	5' GAAUGGAAACAGCGAAGGATT 3'

NC: control.

Hotair ShRNA and overexpression plasmids

Lv-ShHotair and Lv-ShNC (a small hairpin RNA acts as control) plasmids were kindly provided by Prof. Weidong Han of the First Affiliated Hospital to the Chinese PLA General Hospital. The Hotair overexpression plasmid (pHotair) was purchased from Addgene.

Bioinformatics analyses

The online bioinformatics programs, miRanda (http://www.microrna.org), Targetscan (http://www.targetscan.org), DINAN-LAB (http://diana.cslab.ece.ntua.gr), and Findtar (http://bio.sz.tsinghua.edu.cn) were applied to predict the target genes of miR-218.

Bmi-1 overexpressing plasmid construction

The full coding sequence (CDS) of Bmi-1 was amplified and then cloned into PCDNA3.1 vector. The Bmi-1 overexpressing vector was designated as PCDNA-Bmi-1, and the empty vector was used as control.

Cell viability and cell cycle analyses

Cell viability was analyzed by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylt etrazolium bromide (MTT, Sigma) assays as described previously [22]. Briefly, 5×10^3 cells per well were seeded into a 96-well plate. After microRNAs (miRNAs) transfection, the cells were maintained for 72 hours and cell viabilities were determined by using a Benchmark Plus™ microplate spectrometer (Bio-Rad). For cell cycle analysis, cells were plated in 6-well plates at 2×10^5 per well and transfected with miRNAs. After 72 hours, the cell cycle distribution was analyzed by propidium iodide (PI) staining by flow cytometry [24].

Colony formation assays

HepG2 and Bel7404 cells were infected with Lv-miR218 or Lv-Sc and cultured for 72 hours, and then they were re-plated in 6-well plates at the density of 5×10^2 per well and maintained for two weeks. The colonies were fixed and stained with 0.5% crystal violet for 15 minutes.

RNA extraction, reverse transcription and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA was extracted by Trizol reagent (Invitrogen). The reverse transcription was performed as described previously [22,23]. Primers are listed in the Supplementary Table 2. U6 or GAPDH were used as endogenous controls.