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.

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

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.

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).

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.

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Western blotting

Protein lysates were separated by SDS-PAGE (10%) and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% skimmed milk for 1 hour and incubated with primary antibodies including rabbit polyclonal anti-Bmi-1 (Cell Signaling Technology), anti-P14 (Santa Cruz), anti-pRb (Santa Cruz), anti-E2F1 (Santa Cruz), anti-P16 (Santa Cruz), anti-Mdm2 (Santa Cruz), anti-P53 (Cell Signaling Technology) at 4 °C overnight. They were then followed by the HRP-labeled corresponding secondary antibodies for 1 hour and the chemiluminescence (ECL, USA) was used to detect the results. GAPDH was used as the internal control.

Luciferase activity assay

Bmi-1 3′-UTR fragment (nt 1337–1800) was inserted into the pMIR vector (Promega) to generate Bmi-1-3′UTR-Wt luciferase reporter. The binding sites mutant vector was then generated by using a Site-Directed Mutagenesis Kit (Invitrogen). The Wt and Mu constructs were verified by DNA sequencing. The Wt or Mu luciferase reporter and miRNAs were co-transfected into HepG2 cells, and the luciferase activity was measured at 28–30 hours by using the luciferase reporter assay system (Promega).

Xenograft mouse model

Female athymic nude mice (4–6 weeks old) were purchased from the Laboratory Animal Services Centre of CUHK. The usage and treatment of nude mice were approved by the Animal Experimental Ethics Committee of CUHK. HepG2 cells infected with Lv-miR218 or Lv-Sc or Lv-ShHotair or Lv-ShNC and 1×10^6 infected cells were injected subcutaneously into the dorsal flank of nude mice. Tumor size was measured twice a week and tumor volumes (V) were calculated as the formula: $V = (D \times d^2)/2$, in which D means the longest diameter and d means the shortest diameter [22].

Immunohistofluorescence

The specimens were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (5 μm) were used to analyze Ki-67 (Calbiochem) and Bmi-1 expression. After being counterstained with DAPI (Invitrogen), the images were captured using a Zeiss Axiophot 2 microscope.

Statistical analysis

Data are expressed as means ± SD. The two-tailed Student's *t* test was used to compare cellular proliferation, cell cycle distribution, colony formation, gene expression, and tumorigenicity between the two selected groups. The correlation between two factors in HCC specimens was performed by using Pearson's correlation in GraphPad Prism 5.0. The difference was considered as statistically significant when the *p* value is less than 0.05.

Results

Hotair negatively regulates miR-218 expression in hepatoma cells

Previous studies have demonstrated that Hotair expression was increased in multiple cancers [12–15], and our results corroborated that it was significantly upregulated in most HCC cells (Fig. 1A). We also found that Hotair was increased in HCC tissues compared to their adjacent non-tumor tissues and the normal liver tissues (Fig. 1B). Additionally, miR-218 was downregulated in HCC cells (Fig. 1C) and specimens (Fig. 1D). Therefore, Hotair upregulation while miR-218 downregulation is a frequent event in human HCC, and this may be involved in malignant tumor development and progression. To further validate the negative regulation of Hotair on miR-218, we silenced Hotair by using its specific siRNAs (siHotair) and the results showed that Hotair expression was downregulated by siHotair-1 and siHotair-2

(Supplementary Fig. 1A). The siHotair-1 was selected to be used in the following experiments and a dramatically enhanced miR-218 expression was observed in HepG2 and Bel7404 cells (Fig. 1E). On the other hand, miR-218 was downregulated in the two HCC cells with Hotair overexpression (Fig. 1F; Supplementary Fig. 1B). These data suggest that Hotair negatively regulates miR-218 expression in HCC cells.

Hotair modulated miR-218 expression through an EZH2-miR-218-2 promoter regulatory axis

Hotair mediated invasion and metastasis by acting as a bridge to recruit the polycomb repressive complex 2 (PRC2), thereby leading to chromatin modifications [8]. Enhancer of zeste homolog 2 (EZH2), a key component of PRC2, is frequently upregulated in HCC, and it possesses oncogenic properties in tumorigenesis [25–27]. In our study, EZH2 was upregulated in HCC specimens (Supplementary Fig. 2A). To assess whether Hotair associates with EZH2, RNA immunoprecipitation (RIP) assay was performed and the results showed that Hotair was preferentially enriched in the EZH2-recruited complex (Fig. 2A). The siRNAs against EZH2 were designed and its expression was decreased by siEZH2-1 and siEZH2-2 (Supplementary Fig. 2B and C). Moreover, the cell viability was suppressed by siEZH2-1 in HCC cells (Supplementary Fig. 2D).

The mature miR-218 is produced from two separate loci, namely, miR-218-1 and miR-218-2, which are co-expressed with their host genes *Slit2* and *Slit3*, respectively [20]. The previous study reported that *Slit3*, consistent with miR-218-2 precursor, was silenced in pancreatic cancer cells [28]. Our results also displayed the similar effect in HepG2 and Bel7404 cells (Supplementary Fig. 3A and B). *Slit3* was found to be downregulated whereas *Slit2* remained unchangeable in HCC specimens (Supplementary Fig. 3C and D). In addition, miR-218-2 precursor and *Slit3* were upregulated by siEZH2-1 whereas miR-218-1 precursor and *Slit2* did not show any obvious change in HepG2 cells (Fig. 2B and C). Moreover, the expression of miR-218 was promoted by siEZH2-1 in HepG2 and Bel7404 cells (Fig. 2D). To verify the direct regulatory role of EZH2 on miR-218 expression, a luciferase assay was performed in HepG2 cells. A 2 k bp sequence of miR-218-2 promoter (miR-218-2-Luc) was cloned into PGL3 luciferase reporter. As expected, EZH2 knockdown generated a higher luciferase activity of miR-218-2-Luc reporter (Fig. 2E).

Hotair mediated cell cycle arrest through upregulating miR-218 expression

To explore the functional significance of Hotair in tumorigenesis, the HepG2 and Bel7404 cells were transfected with siHotair-1 or pHotair, and cell viabilities were detected. As shown in Fig. 3A, siHotair-1 suppressed cell viability (left panel) while pHotair promoted cell survival (right panel) in HCC cells. Furthermore, the HuH7 and PLC5 cells with a lower expression of Hotair were transfected with pHotair, and the cells viability were monitored. As shown in Supplementary Fig. 4, pHotair significantly enhanced the cells viability in the two HCC lines. Next, we investigated the proliferative effect of miR-218 on HCC cells and the results showed that miR-218 induced a suppressive effect whereas anti-miR-218 induced a proliferative effect on cell viability (Fig. 3B).
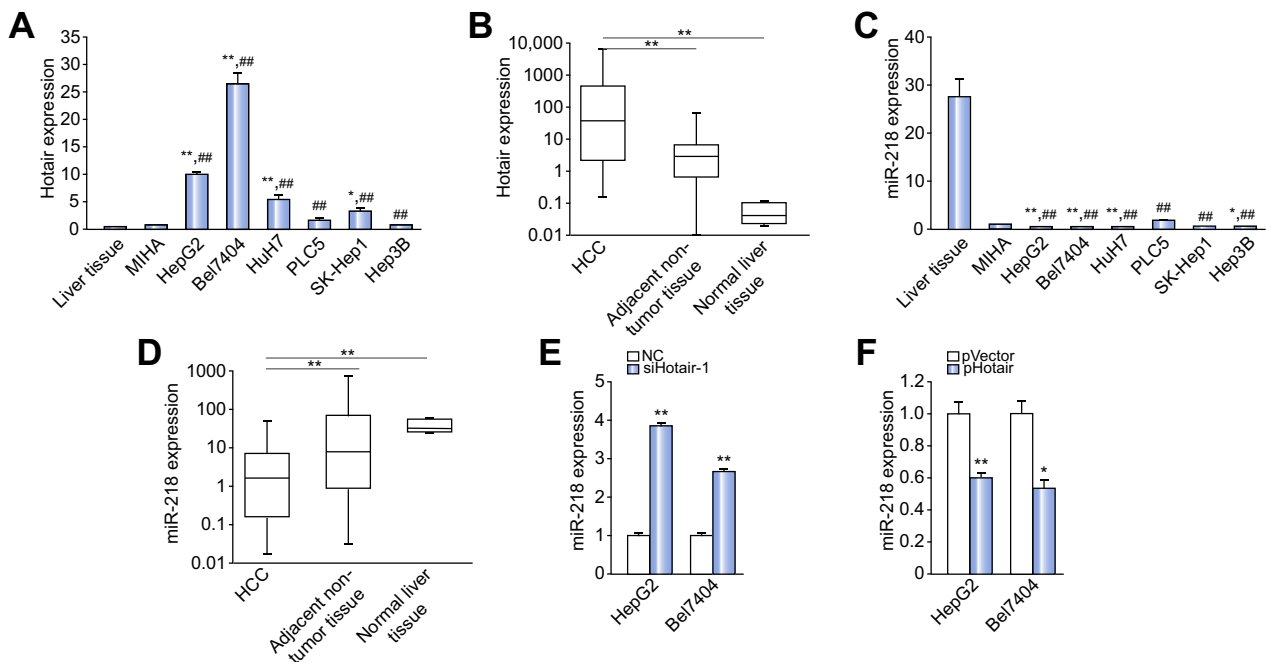
Fig. 1. Hotair negatively regulated miR-218 expression in HCC cells. (A & B) Hotair was upregulated in a panel of HCC cells (A) and specimens (B). (C & D) miR-218 was downregulated in a panel of HCC cells (C) and specimens (D). $^*p$ <0.05 *vs.* MIHA; $^{**}p$ <0.01 *vs.* MIHA; $^{##}p$ <0.01 *vs.* liver tissue. (E & F) miR-218 was upregulated by siHotair-1 (E) whereas it was downregulated by pHotair in HepG2 and Bel7404 cells (F). $^*p$ <0.05; $^{**}p$ <0.01.
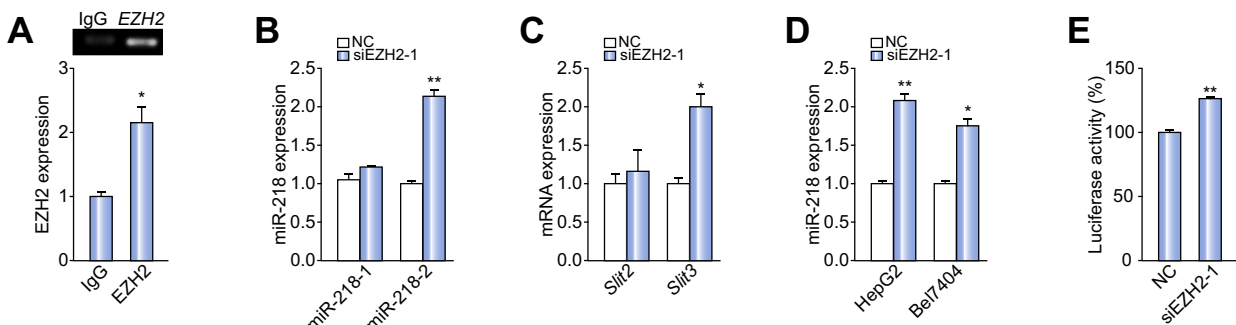
Fig. 2. Hotair modulated miR-218 expression through an EZH2-miR-218-2 promoter regulatory axis. (A) Hotair expression was enhanced in the immunoprecipitates. (B & C) miR-218-2 (B) and its host gene *Slit3* (C) were upregulated by siEZH2-1 in HepG2 cells. (D) The total miR-218 expression was also upregulated by siEZH2-1 in HCC cells. (E) siEZH2-1 promoted the luciferase activity of the miR-218-2-Luc in HepG2 cells. $^*p$ <0.05; $^{**}p$ <0.01.

In addition, the cell cycle distribution demonstrated that siHotair-1 or ectopic miR-218 induced an increased percentage of HepG2 cells in G1-phase and fewer cells in S-phase (Fig. 3C and D), indicating that the growth-suppressive effect resulted from G1-phase arrest. We further evaluated the proliferative effect of stable miR-218 overexpression. A lentiviral vector was used to stably restore miR-218 expression, and mature miR-218 was strongly enhanced in the Lv-miR218-infected HepG2 and Bel7404 cells (Supplementary Fig. 5A). Moreover, the growth inhibition induced by Lv-miR218 was similar to that induced by miR-218 mimics in HepG2 and Bel7404 cells (Supplementary Fig. 5B). Next, the capacity of colony formation was assessed and results showed that Lv-miR218-infected HCC cells displayed much fewer and smaller colonies compared with those obtained with Lv-Sc-infected cells (Fig. 3E).

To evaluate the antagonistic effects of Hotair and miR-218 on cell viability, miR-218 was transfected into the pHotair stably infected HepG2 cells and the proliferation was assayed. As shown in Fig. 3F, miR-218 reversed the proliferation induced by Hotair overexpression (left panel). On the other hand, anti-miR-218 was transfected into Lv-ShHotair infected HepG2 cells and it significantly abrogated growth inhibition induced by Hotair knockdown (right panel).

Bmi-1 was identified as a functional target of miR-218 in HCC cells

Bmi-1 was predicted to be a functional target of miR-218 by several bioinformatics programs including Targetscan, miRanda, Findtar and DINAN-microT. The sequence of 1459-1477 in Bmi-1 3′UTR perfectly matches with miR-218 seed

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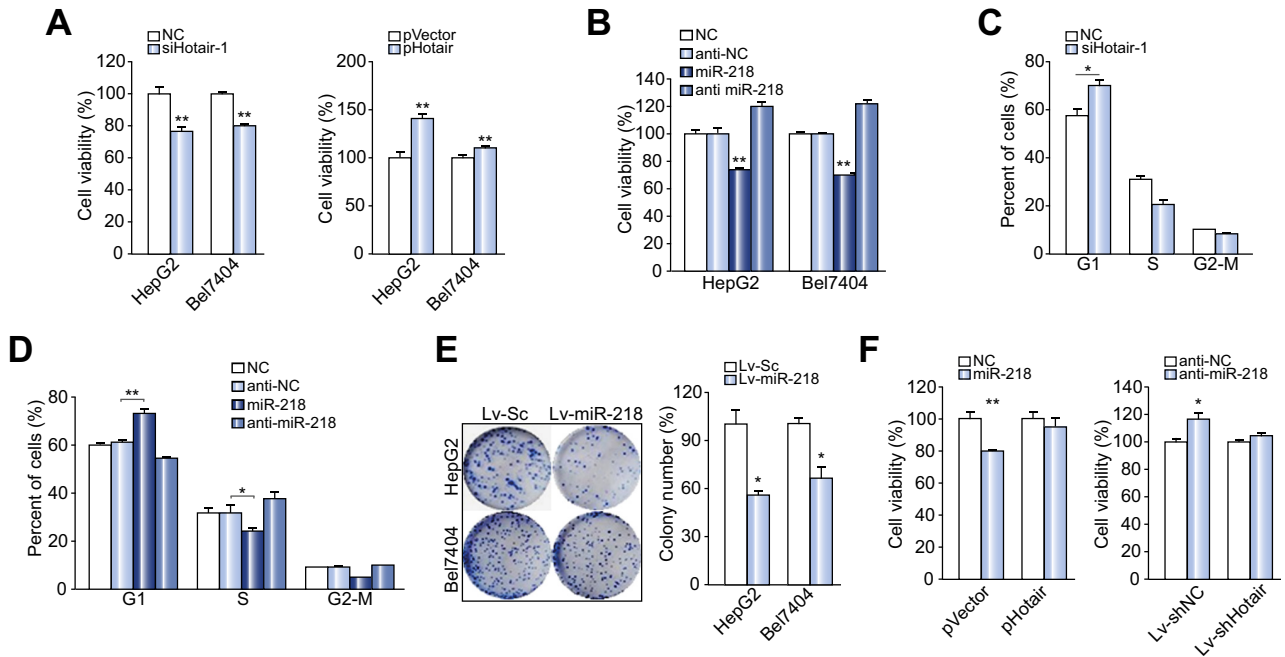
Fig. 3. Hotair mediated cell growth through upregulating miR-218 expression. (A) Hotair knockdown suppressed (left panel) whereas Hotair overexpression promoted cell viability (right panel) in HCC cells. (B) miR-218 suppressed whereas anti-miR-218 promoted cell proliferation in HCC cells. (C & D) siHotair-1 or miR-218 induced the G1-phase arrest in HepG2 cells. (E) The representative pictures and statistical analyses of colony formation assay of Lv-miR218 infected HCC cells. (F) miR-218 reversed the enhanced proliferative effect of Hotair overexpression (left panel) whereas anti-miR-218 rescued the suppressive effect of ShHotair on cell growth (right panel). $^*p$ <0.05; $^{**}p$ <0.01. (This figure appears in colour on the web.)
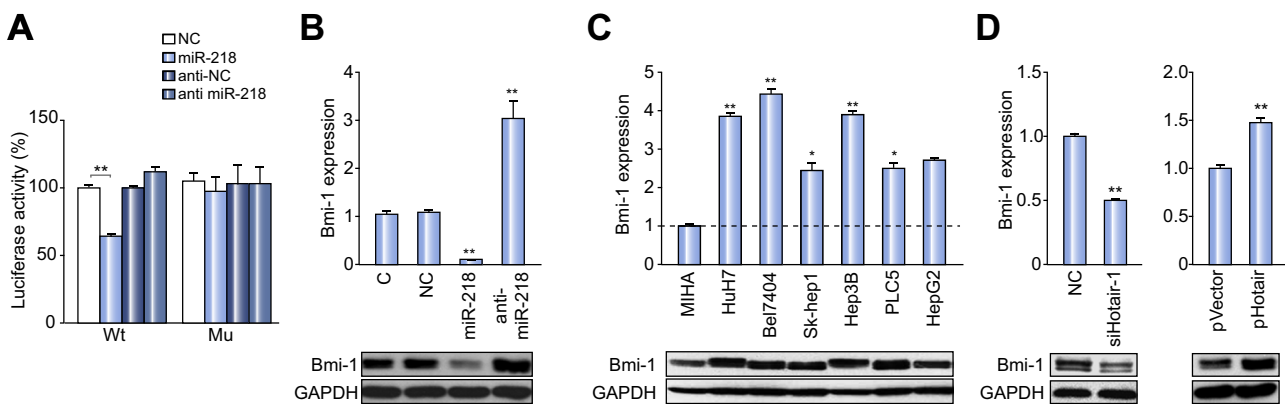

Fig. 4. Bmi-1 was a functional target of miR-218 in HCC cells. (A) miR-218 inhibited the luciferase activity of the Wt reporter. (B) Bmi-1 was downregulated by miR-218 whereas it was upregulated by anti-miR-218 at mRNA and protein levels. (C) Bmi-1 was upregulated in a panel of HCC cells at mRNA and protein levels. (D) Bmi-1 expression was suppressed by siHotair-1 whereas the ectopic expression of Hotair by pHotair could upregulate Bmi-1 expression. $^*p$ <0.05; $^{**}p$ <0.01.

(Supplementary Fig. 6). According to the predicted sequence, Bmi-1-3′UTR-Wt and Bmi-1-3′UTR-Mu reporters were generated. The luciferase activity assays showed that miR-218 dramatically suppressed the firefly luciferase activity of the Bmi-1-3′UTR-Wt reporter but not that of the Bmi-1-3′UTR-Mu reporter in HepG2 cells (Fig. 4A). Further investigation displayed a significant decrease in the endogenous expression of Bmi-1 at mRNA and protein levels in response to miR-218 transfection (Fig. 4B; Supplementary Fig. 7A). As an oncogene [29], Bmi-1 was upregulated in HCC cells (Fig. 4C; Supplementary Fig. 7B) and specimens (Supplementary Fig. 8). In addition, Bmi-1 was suppressed by

siHotair-1 and promoted by pHotair at mRNA and protein levels (Fig. 4D; Supplementary Fig. 7C). Collectively, all these data indicated that Hotair regulated carcinogenesis in HCC, at least partially through suppressing miR-218 and activating Bmi-1 expression.

P14^ARF and P16^Ink4a signaling were activated by siHotair through promoting miR-218 expression

It is well-established that tumor suppressors P16[Ink4a] and P14[ARF] are the main targets of Bmi-1 that contribute to repressing cell
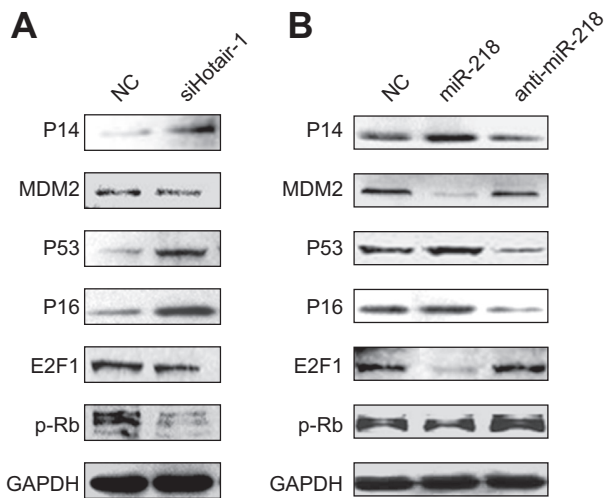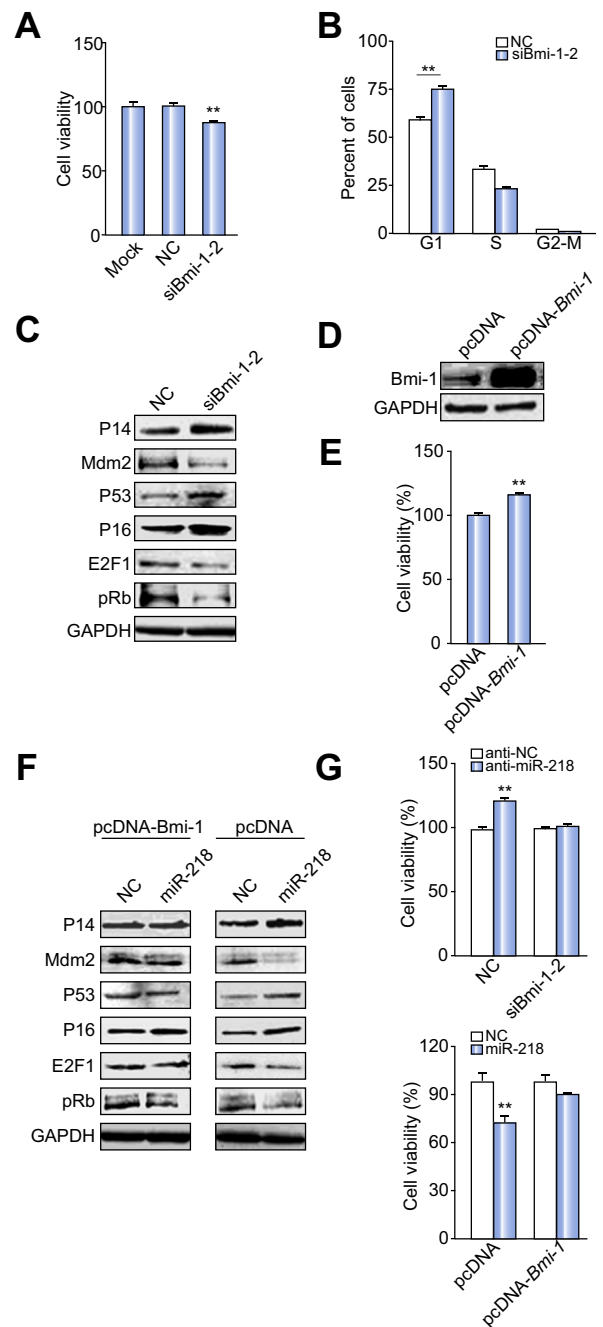
Fig. 5. P14^{ARF} and P16^{Ink4a} signaling were activated by either siHotair or upregulation of miR-218 overexpression. (A) P14^{ARF} signaling was stimulated by siHotair-1 including P14^{ARF} and P53 were upregulated, and Mdm2 was downregulated in HepG2 cells. Moreover, P16^{Ink4a} was upregulated and E2F1 and pRb were downregulated by siHotair-1, leading to activation of P16^{Ink4a} signaling. (B) miR-218 induced the activation of P14^{ARF} and P16^{Ink4a} signaling whereas anti-miR218 induced the inactivation of the two signaling pathways.

proliferation and senescence [30,31]. Our results have demonstrated that Bmi-1 expression was suppressed by siHotair-1, therefore, we wondered whether P14^{ARF} and P16^{Ink4a} signaling were involved in this event. As expected, the expression of P14^{ARF} and P53 were upregulated, whereas Mdm2 was downregulated by siHotair-1, indicating the activation of P14^{ARF}-P53 signaling (Fig. 5A; Supplementary Fig. 9A). Moreover, P16^{Ink4a}-Rb signaling was stimulated by siHotair-1 including the upregulation of P16^{Ink4a} and the downregulation of pRb and E2F1 (Fig. 5A; Supplementary Fig. 9A). Similarly, P14^{ARF}-P53 and P16^{Ink4a}-Rb signaling were also activated by ectopic miR-218 (Fig. 5B; Supplementary Fig. 9B). Conversely, inhibition of endogenous miR-218 led to inactivation of the two signaling pathways (Fig. 5B; Supplementary Fig. 9B).

To elucidate whether miR-218 suppressed the carcinogenesis by reducing Bmi-1 expression, we performed loss- and gain-of-function studies. We firstly silenced Bmi-1 by its specific siRNAs (siBmi-1) and the result revealed that it was suppressed at the mRNA and protein levels (Supplementary Fig. 10A and B). Then the cell growth and cell cycle distribution were further investigated by using siBmi-1-2. Notably, Bmi-1 knockdown significantly attenuated cell growth (Fig. 6A) and induced G1-phase arrest (Fig. 6B). Moreover, the P14^{ARF}-P53 and P16^{Ink4a}-Rb pathways were also activated by siBmi-1-2 (Fig. 6C; Supplementary Fig. 11A).

We next determined whether Bmi-1 overexpression could reverse the suppressive effect of miR-218 in HCC cells. An expression vector PCDNA-Bmi-1, which encoded the full-length coding sequence of Bmi-1, was transfected into HepG2 cells (Fig. 6D; Supplementary Fig. 11B). Intriguingly, reinforced expression of Bmi-1 dramatically abrogated the miR-218-induced cell viability inhibition (Fig. 6E) and reversed the activation of P14^{ARF}-P53 and P16^{Ink4a}-Rb signaling induced by miR-218 (Fig. 6F; Supplementary Fig. 11C). Furthermore, we also found that the anti-miR-218 partially rescued siBmi-1-2 induced cell growth

Fig. 6. Bmi-1 mediated miR-218-induced tumorigenesis in HCC cells. (A) Bmi-1 silence suppressed the cell viability in HepG2 cells. (B & C) siBmi-1-2 induced G1-phase arrest (B) and activated P14^{ARF} and P16^{Ink4a} signaling (C). (D) Bmi-1 expression was upregulated with PCDNA3-Bmi-1 transfection. (E) PCDNA3-Bmi-1 abrogated the miR-218-induced cell viability inhibition. (F) Activation of P14^{ARF} and P16^{Ink4a} signaling by miR-218 were suppressed by Bmi-1 overexpression. (G) Bmi-1 knockdown suppressed the proliferation induced by anti-miR-218 whereas its overexpression reversed the growth inhibition induced by miR-218. $^{*}p < 0.05$; $^{**}p < 0.01$.

inhibition (Fig. 6G, top panel) and the reinforced expression of Bmi-1 dramatically abrogated the miR-218-induced cell viability inhibition (Fig. 6G, bottom panel).

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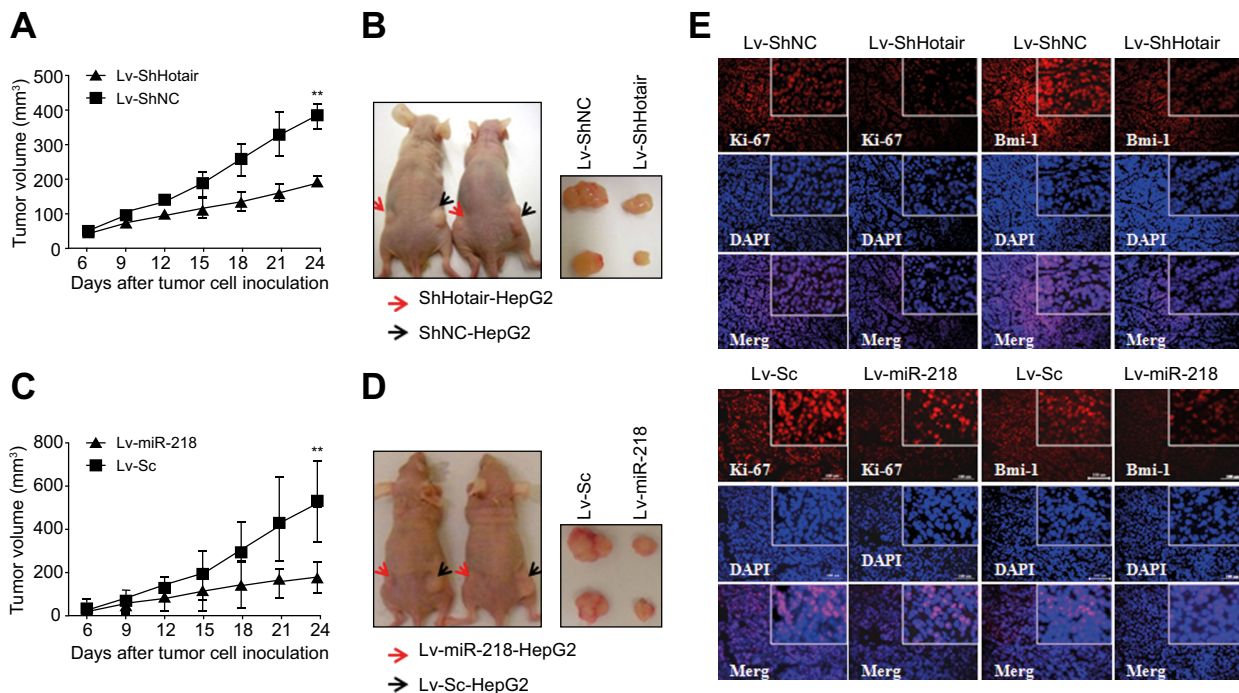
Fig. 7. Hotair mediated tumorigenicity through miR-218 upregulation and Bmi-1 downregulation in vivo. HepG2 cells were infected with Lv-ShHotair or Lv-miR-218 injected subcutaneously into nude mice. (A & C) The growth curves of tumor volumes were measured. Each data point represented the mean ± SD of five mice. (B & D) Lv-ShHotair and Lv-miR-218-infected cells generated smaller tumors than their control cells. (E) The immunofluorescence of Ki-67 and Bmi-1 stained sections followed by counterstaining with DAPI. $^*p$ <0.05; $^{**}p$ <0.01. (This figure appears in colour on the web.)

Hotair mediated tumorigenicity through miR-218 upregulation and Bmi-1 downregulation in vivo

Following the above observation, we further verified these *in vitro* findings by using an *in vivo* xenograft model. The HepG2 cells stably infected with Lv-ShHotair or Lv-miR218 were subcutaneously injected into the dorsal flank of nude mice. Compared with the ShNC group, the ShHotair group revealed a significant reduction in tumor volume (Fig. 7A) and size (Fig. 7B). The similar anti-cancer effect of miR-218 on HCC cells *in vivo* are displayed in Fig. 7C and D and Supplementary Fig. 12. Furthermore, the cell proliferation marker Ki-67 and Bmi-1 were detected by immunohistofluorescence analyses. Decreased Ki-67 and Bmi-1 expression were observed in xenografts of mice treated with ShHotair or Lv-miR218 cells (Fig. 7E).

Hotair expression negatively correlates with miR-218 and positively correlates with Bmi-1 in primary HCCs

miR-218 was frequently decreased in hepatoma and 67.3% of the HCC specimens displayed the decreased miR-218 expression in our study (Fig. 8A). Hotair and Bmi-1 expression were increased in 76.9% and 78.8% of HCC patients, respectively (Fig. 8A). Furthermore, the association analyses showed that a significant inverse association between the expression of Hotair and miR-218 was observed in these HCC specimens (Fig. 8B). More importantly, a negative correlation between Bmi-1 and miR-218 (Fig. 8C) and a positive association between Hotair and Bmi-1 were identified in these specimens (Fig. 8D). Taken together, we pinpoint the following regulatory axis: miR-218,

negatively regulated by Hotair, may inhibit cell growth and induce G1-phase arrest by directly suppressing Bmi-1 expression, thereby activating P14^{ARF}-P53 and P16^{Ink4a}-Rb signaling pathways in HCC (Fig. 8E).

Discussion

Elucidating the function of transcriptional factors in HCC has been considered as a key for revealing the critical pathways in hepatocarcinogenesis. LncRNAs have recently been identified as novel regulators of the transcriptional and epigenetic networks. LncRNA Hotair has been documented to play a part in the epigenetic regulation of gene transcription in a number of epidemiological studies [10,32]. The aberrantly upregulated Hotair was detected in several tumors, including breast cancers, colorectal cancers, pancreatic cancers, cervical cancers, bladder cancers, HCC and gastrointestinal stromal tumors [8,10,33–35]. Our data validated that Hotair was overexpressed in HCC specimens, and for the first time, we determined that Hotair knockdown could inhibit cell growth, induce cell cycle arrest and suppress tumorigenicity.

Recent studies have established that Hotair negatively regulates miRNA-130a in gallbladder cancer [36]. Our study identified that Hotair negatively regulated miR-218 expression in which Hotair overexpression decreased miR-218 expression while its silence promoted miR-218 expression. The antagonistic effects of Hotair and miR-218 on cell proliferation further validated this negative regulation. Furthermore, the negative association between Hotair and miR-218 was also determined in HCC

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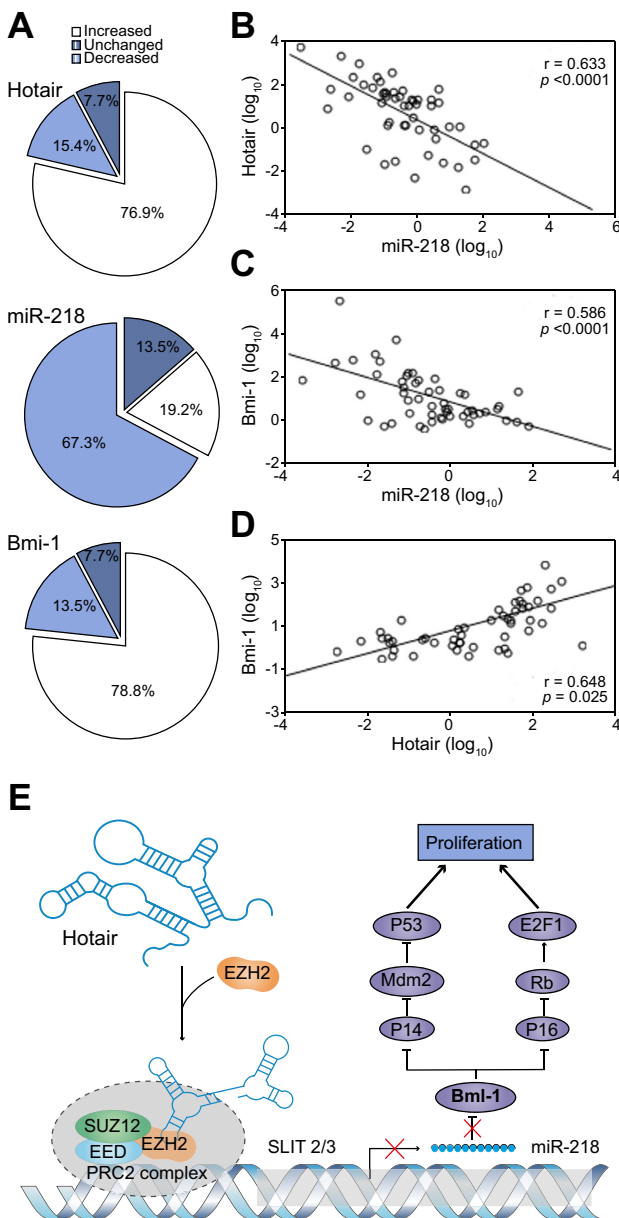
Fig. 8. Hotair expression negatively correlated with miR-218 and positively correlated with Bmi-1 in primary HCCs. (A) The expressions of Hotair, miR-218 and Bmi-1 in 52 paired HCC specimens. Hotair and Bmi-1 abundance were normalized to GAPDH, and the miR-218 expression was normalized to U6. (B–D) The statistically significant association between Hotair, miR-218 and Bmi-1 expression in HCC specimens. (E) Schematic overview of Hotair-miR-218 mediated tumorigenesis. (This figure appears in colour on the web.)

specimens. Distinguished from the function as a "competitive endogenous RNAs (ceRNA)" in gallbladder cancer [36], Hotair serves as a bridge to interact with PRC2, which leads to chromatin remodeling and H3K27 trimethylation [8,37]. Our results also suggest that Hotair acts as a scaffold to recruit EZH2 into a complex, and this has been confirmed by RIP assays. EZH2, a subunit of PRC2, negatively regulated miR-218-2 expression through

directly targeting its promoter, of which the knockdown of EZH2 increased miR-218 expression and its promoter activity. This negative regulatory pattern is consistent with previous studies of pancreatic cancer [28].

As a well-known tumor suppressor, miR-218 was found to suppress cell viability and reduce tumorigenesis in HCC *in vitro* and *in vivo*. Generally, miRNAs negatively regulate their mRNA targets in a sequence-specific binding manner. In this study, the polycomb group transcriptional repressor Bmi-1 was predicted to be a promising target of miR-218 by bioinformatics analyses. Further investigation confirmed that it was a *bona fide* target of miR-218 in HCC, which was reported in colorectal cancer [38]. As a member of the polycomb group family, Bmi-1 has an essential role in embryogenesis and regulation of cell cycle. Moreover, it serves as a pro-oncogene in tumorigenicity [39] and also contributes to the maintenance of the cancer stem cells [29,40].

P16^{Ink4a} and P14^{ARF} are the main targets of Bmi-1 in mediating cell proliferation and senescence [30,31]. The two tumor suppressors play important roles in regulating cell cycle arrest and carcinogenesis. P14^{ARF} binds and inhibits the P53 antagonist Mdm2, leading to the accumulation of P53 [29,40]. Furthermore, P16^{Ink4a} is frequently inactivated in tumors and activation of P16^{Ink4a}-Rb signaling results in senescence [41]. In the present study, P14^{ARF} and P16^{Ink4a} signaling pathways were activated by Hotair knockdown and ectopic miR-218. As a target gene of miR-218, Bmi-1 knockdown induced cell growth inhibition, cell cycle arrest and activation of P14^{ARF} and P16^{Ink4a} signaling pathways. On the other hand, exogenous introduction of Bmi-1 could rescue the suppressive effect and the inactivation of P14^{ARF} and P16^{Ink4a} signaling induced by miR-218. These data strongly support the notion that Bmi-1 serves as a critical mediator in the Hotair-miR-218-mediated hepatocarcinogenesis.

Emerging evidence have demonstrated the clinical significance of Hotair in gastrointestinal cancer [11–15], and suggested the potential role of Hotair in diagnosis and therapeutics [42,43]. We further addressed the association between Hotair, miR-218 and Bmi-1 in 52 pairs of HCC specimens. Hotair was upregulated in approximately 76.9% and Bmi-1 was increased in approximately 78.8% whereas miR-218 was suppressed in nearly 67.3% HCCs. More importantly, an inverse correlation between Hotair and miR-218 was observed, demonstrating the clinical significance of Hotair combined with miR-218 as a diagnostic or therapeutic target for HCC. In conclusion, our integrated approach shows for the first time that Hotair plays a critical role in hepatocarcinogenesis through the downregulation of miR-218 and inactivation of P14^{ARF} and P16^{Ink4a} signaling. This lncRNA directly recruits EZH2 and silences miR-218 expression through binding its promoter which provides a mechanistic basis for the aberrant Bmi-1 activation in HCC. Thus, the disruption of the Hotair-EZH2-miR-218 negative regulatory axis is highly promising to the design of therapeutic interventions in HCC.

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Research Article

Conflict of interest

Authors' contributions

Zhang J.F. spearheaded and supervised all the experiments. Fu W.M., Wang W.M., Zhu X., Hu B.G., Liang W.C., Wang S.S., Wang H., Lu Y.F., and Zhang J.F. designed and conducted experiments. Ko C.H., Waye M.M.Y, Li G., and Kung H.F. provided technical and material support. Fu W.M., Wang W.M., and Zhang J.F. analyzed the data and prepared the manuscript.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.05.016.

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