Translational Oncology 14 (2021) 101036

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

LncRNA HOTAIR recruits SNAIL to inhibit the transcription of HNF4α and promote the viability, migration, invasion and EMT of colorectal cancer

Long Jin, Yu-Liang Pan, Jun Zhang, Pei-Guo Cao*

Department of Oncology, Third Xiangya Hospital, Central South University, Changsha 410013, Hunan, China

ARTICLE INFO

Keywords:
HOTAIR
SNAIL
HNF4α
Colorectal cancer
Tumorigenesis

ABSTRACT

Colorectal cancer causes severe burdensome on the health by its high fatality and poor prognosis. Hox transcript antisense intergenic RNA (HOTAIR) was believed closely related with the genesis and development of colorectal cancer, but the regulatory mechanism is still to be investigated. The expression of HOTAIR was analyzed in colorectal cancer using both qRT-PCR and ISH assay. The cell viability, migration, invasion and apoptosis rate were evaluated using MTT, BrdU,Transwell and flow cytometryexperiments. The interaction between HOTAIR and SNAIL was detected using RIP and RNA pull-down. The binding of SNAIL to HNF4α promoter was assessed by ChIP. The cell lines that knock down HOTAIR, SNAIL or overexpress HNF4α were constructed using retroviral vector system. The tumorigenic and metastatic capacity of colorectal cancer cells after knocking down HOTAIR were evaluated based on xenograft assay and liver metastases model. HOTAIR was highly expressed in both tissue and cell lines of colorectal cancer, indicated a regulatory function in colorectal cancer. Knock-down of HOTAIR suppressed cell viability, migration, invasion and epithelial-mesenchymal transition (EMT) of colorectal cancer cells *in vitro*, and inhibited the growth and metastasis of colorectal tumor in nude mice. We further found that HOTAIR suppressed HNF4α via recruiting SNAIL, and the overexpression of HNF4α inhibited cell viability, migration, invasion and EMT of colorectal cancer cells. We demonstrated that HOTAIR regulates the level of HNF4α via recruiting SNAIL, knocking down HOTAIR repressed the cell viability and metestasis of colorectal cancer cell line *in vitro*, and suppressed the tomorgenesis and migration/invasion of colorectal cancer *in vivo*.

Introduction

Colorectal cancer (CRC) causes severe burdensome on the health and economies of countries worldwide by its high fatality and poor prognosis. In 2018, 18.1 million of new cancer cases were reported, of which 10.2% were colorectal cancer. Besides, in 2018, colorectal cancer caused death accounted for 9.2% of cancer death in the year, was the second leading cause [1]. Moreover, most colorectal cancer was diagnosed in advanced stages and often with migration, leading to more difficulties for treatment. Therefore, to further investigate the pathogenesis and mechanism of colorectal cancer is essential and significant to explore novel biomarkers for colorectal cancer diagnosis and metastasis prevention. So far, several factors related with colorectal cancer were reported, such as miRNAs [2], E-CADHERIN/T-cadherin [3,4], CD164 [5], Cytokines [6], Forkhead transcription factors (FOX) [7], and oncogenes such as *KRAS, PIK3CA* and *BRAF* [8], etc.

Long non-coding RNAs (LncRNAs) are RNA transcripts which are non-protein coding and longer than 200 bp [9,10]. Hox transcript antisense intergenic RNA (HOTAIR) was first reported in 2007, and found to be closely related with the genesis and progression of several cancers such as colorectal cancer, lung cancer, breast cancer and liver cancer [11–20]. Moreover, Kogo et al. reported that in stage IV colorectal cancer with liver metastasis and unfavorable prognosis, the level of HOTAIR in tumor tissue was much higher than the level of HOTAIR in adjacent tissue [13]. It could be speculated that HOTAIR plays a part in colorectal cancer progression, whereas the regulatory mechanism remains unclear.

SNAIL, a transcription factor was reported to give rise to cell metastasis, including epithelial-mesenchymal transition (EMT) [21]. Huber et al. reported that SNAIL bound with E-box in the promoter of *E-CADHERIN* and inhibited its expression, which played important role in epithelial differentiation and cell polarization [22]. HOTAIR was found related with the expression of SNAIL in several cancers, such as kidney cancer and esophageal cancer [23,24]. Moreover, in the report of non-tumorigenic hepatocytes, Battistelli et al. found that HOTAIR recruited SNAIL, suppressed the expression of downstream genes and then regulated cell viability and EMT [25]. However, whether similar regulatory mechanisms exist in colorectal cancer needs further exploration.

Hepatocyte nuclear factor 4 alpha (HNF4α) is a member of nuclear hormone receptor superfamily. HNF4α is enriched in liver, but low expressed in pancreatic cancer, gastric cancer and renal cell carcinoma

* Corresponding author.
E-mail address: xy3caopg@csu.edu.cn (P.-G. Cao).

https://doi.org/10.1016/j.tranon.2021.101036
Received 23 September 2020; Received in revised form 7 January 2021; Accepted 1 February 2021

[26–29]. As a downstream gene of SNAIL,HNF4α was reported acting as a cancer suppressor gene in liver cancer [30], and P1 promoter-driven HNF4α isoforms are specifically repressed in colorectal cancer cells [31]. However, the specific regulatory mechanism of HNF4α in colorectal cancer still requires to be confirm.

Accordingly, in this study, via both *in vitro* and *in vivo* experiments, we investigated the regulatory effect of HOTAIR on colorectal cancer, and further explored the interaction among HOTAIR, SNAIL and HNF4α and the molecular mechanism of regulatory process.

Methods and materials

Cell culture

FHC, HCT116 and HT29 cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM; HyClone); SW480 cells were cultured using Leibovitz's L-15 Medium; P53R cells were cultured using Minimum Eagle's Medium (MEM, HyClone). All the mediums were supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% Penicillin-Streptomycin (Gibco). All the cell lines were obtained from the Chinese Academy of Medical Sciences and grown in a humidified incubator with 5% CO_2 at 37 °C.

Construction of recombinant retroviruses and retroviral infections

Target of sh-HOTAIR or sh-SNAIL were picked using BLOCK-iT^{TM} RNAi Designer (http://rnaidesigner.thermofisher.com/rnaiexpress/). Sh-NC, sh-HOTAIR, sh-SNAIL (ordered from Invitrogen, Thermo Fisher Scientific) and HNF4α cDNA (amplified by PCR) were inserted in retroviral vector pLPCX (Addgene) to obtain pLPCX-shNC, pLPCX-shHOTAIR, pLPCX-shSNAIL and pLPCX- HNF4α. Green Fluorescent Protein (GFP) sequence (amplified by PCR) was also inserted into pLPCX as a control retroviral vector pLPCX-GFP.

Each constructed plasmid was co-transfected with the packaging plasmids into HEK293T cells using Lipofectamine^{TM} 3000 (Thermo Fisher Scientific), according to the standard procedures. The supernatants were collected after 48 h, and filtered using 0.45 mm filter.

P53R and HT29 cells were infected using recombinant retrovirus expressing sh-HOTAIR, sh-Snail and HNF4α and controls with polybrene (8 mg/mL). The infection efficiency was around 90% (data not shown).

In situ hybridization (ISH) assay

The ISH assay was performed following previous study [32]. In brief, dewaxed and rehydrated sections were digested using proteinase K, and then fixed using paraformaldehyde (4%). Then, sections were hybridized with 5′ digoxin-labeled LNA-modified probe (Exiqon) at 56 °C for 12 h, and incubated with anti-digoxin antibody (Roche) for another 12 h at 4 °C. The samples were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP), avoiding light. Sections were then observed and images were captured.

Methyl thiazolyl tetrazolium (MTT) assay

Cells were grown in 96-well culture plates (2000/well) and incubated with . MTT reagent (5 mg/mL; Sigma-Aldrich) (20 μL/well) at 37 °C for 4 h. After incubation, the supernatant was removed. Dimethylsulfoxide (DMSO, Sigma-Aldrich) (150 μL/well) was added to dissolve the formazan crystals in each well, and the absorbance at 490 nm was detected by Microplate Spectrophotometer. The MTT assay was conducted in triplicate.

Bromodeoxyuridine / 5-bromo-2′-deoxyuridine (BrdU) assay

Cells were fixed with 4% paraformaldehyde for 30 mins at 4 °C, washed in 0.1 M PBS (pH 7.4) with 1% TritonX100 for 3 times. Samples were then then incubated in HCl (2 M) for 10 mins at 37 °C. Immediately after the acid washes, Borate buffer (0.1 M, pH 8.3) was added to buffer the cells for 10 min. Samples were then washed and added 0.2% TritonX100, incubated for 10 min. After washed with PBS, samples were blocked in 3% BSA for 1 h, prior to incubating overnight (at 4 °C) with anti-BrdU. Following the incubation overnight, samples were washed and then incubated with secondary antibody (AlexaFlour 488 conjugated). After washed, DAPI (0.5 μg/mL) were added to stain the nucleus. Samples were observed with fluorescence microscope.

Transwell migration assay

Cells were grown in Transwell inserts (Corning Life Sciences) at a concentration of 1×10^5 cells per insert using serum free medium containing 0.1% BSA in a 200 μL final volume for each insert. Transwell inserts were then put in 24-well plates containing complete medium and incubated for 12 h. After incubation, remove cells in the inner side of membrane. The migrated cells were fixed using 4% paraformaldehyde and stained using 0.1% crystal violet. Images of migrating cells were captured for five different areas in the membrane. The numbers of invading cells for each sample were calculated as the average number.

Transwell invasion assay

Transwell inserts were coated with 100 μL Matrigel (1:5) per insert, and incubated in 37 °C over night. Cells were then added in the inserts at a concentration of 1×10^5 cells per insert using serum free medium containing 0.1% BSA in a 100 μL final volume for each insert. Transwell inserts were then put in 24-well plates containing complete medium and incubated for 24 h. After incubation, remove medium and wash twice using PBS. Cells were fixed using 4% paraformaldehyde and stained using 0.1% crystal violet. The cells in the upper side were removed. Images of invased cells were captured for five different areas in the membrane. The numbers of invading cells for each sample were calculated as the average number.

Western blot

Cells were harvested and rinsed using phosphate buffered saline (PBS), and then lysed using NP-40 Lysis Buffer (Sigma-Aldrich). Cell lysate was then gathered and sampled to determine the concentration using the Bicinchoninic acid protein assay (NEB). Equal amount of total protein (50 μg) for each sample was quantified using SDS-PAGE electrophoresis. The resolved protein was transferred to polyvinylidene difluoride membrane and blocked in 2.5% skim milk for 1 h. The membranes were incubated with the primary antibodies against SNAIL, HNF4α, E-CADHERIN, VIMENTIN, SLUG, N–CADHERIN (Cell Signaling; Danvers, MA, USA, 1:1000 dilution), β-actin and GAPDH (Cell Signaling; Danvers, MA, USA, 1:4500 dilution). After rinsing, blots were probed with second antibody (Abcam; Cambridge, UK, 1:1000 dilution) for 1 h. The blots were visualized using chemiluminescence detection system (CWBIO; Beijing, China).

Annexin V staining to detect cell apoptosis

Cells were stained and analyzed following the manual (Becton, Dickinson and Company). Cells were washed twice with cold PBS and then resuspend around 1×10^5cells in 100 μL of binding buffer for each sample. FITC (5μL, cat. no. 556,420,) and PI (3μL, cat. no. 556,463) were added in each sample, gently mixed and incubated for 15 min at RT in the dark. Binding buffer (400 μL) was added to each tube, and the samples were analyzed by flow cytometry within 1 h.

RNA isolation and qRT-PCR assay

Total RNA was isolated using TRI reagent (Sigma-Aldrich) from tissues or cell lines. Complementary DNA (cDNA) was reversely-

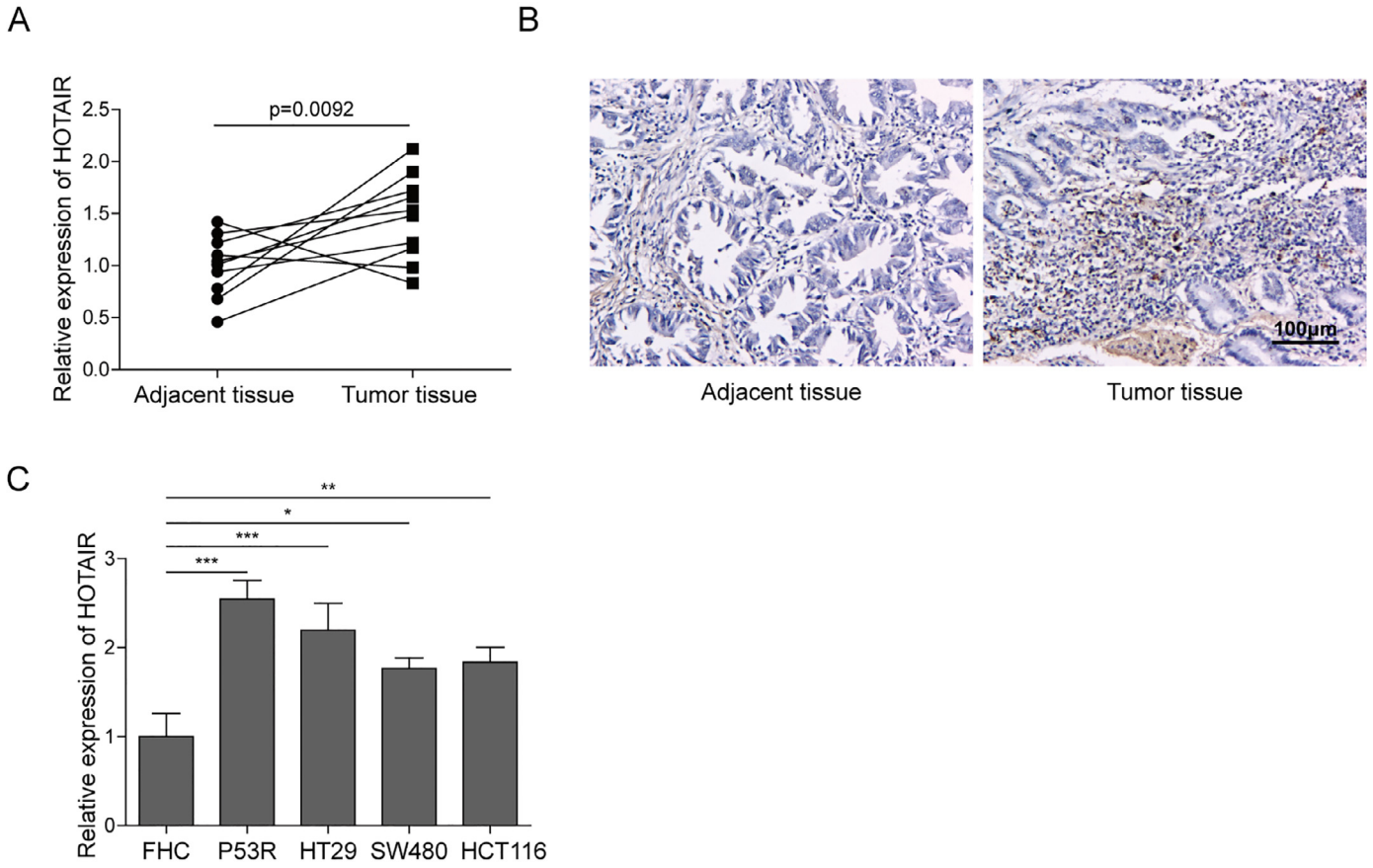

Fig. 1. HOTAIR expression in colorectal cancer tissue and cell lines.
(A) Plots of the relative expression of HOTAIR in colorectal cancer tissue and adjacent tissue. $n = 10$. (B) Representative images of expression and distribution of HOTAIR in colorectal cancer tissue and adjacent tissue detected by ISH assay. (C) Plots of the relative expression of HOTAIR in colorectal cancer cell lines. $n = 3$. HOTAIR expression was assessed by qRT-PCR. β-actin was taken as internal control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) with random primer. Primers for qRT-PCR were picked using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), ordered from Invitrogen, Thermo Fisher Scientific, and checked by gel, melting curve analysis and sequencing for specificity. The qRT-PCR cycling was performed at 98 °C for 2 min, then following 40 cycles of 98 °C/10 s, 60 °C/15 s and 65 °C/15 s. The relative expression of each target gene was analyzed using the formula $2^{-\triangle\triangle Ct}$. The expression of β-actin gene and sample without cDNA were used as internal control and negative control, respectively.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted generally following previous reports [33,34]. In brief, cells were washed and then cross-linked with 1% formaldehyde solution for 10 min at room temperature. Cross-linking was stopped by glycine with a final concentration of 0.125 M, then shake for 5 min at room temperature, and then rinsed twice. Cells were scraped off in cold PBS supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors cocktail (Takara), centrifuged for 5 min at 1000 RPM in 4 °C. Supernatant was removed gently and the pellet was resuspended in lysis buffer supplemented with protease inhibitors cocktail, and incubated for 10 min on ice, disrupted using homogenizer, and the nuclei were collected by centrifugation for 5 min at 1000 RPM in 4 °C.

Nuclei pellet was resuspended in nuclear lysis buffer containing protease inhibitors mixture, and sonicated for 10 times (1 min on/off) on ice. Supernatant were collected by centrifugation for 20 min at 13,000 RPM in 4 °C. Twenty microliters of each sample was took as input and stored at −20 °C. The rest supernatant was diluted in 1:10 ratio with IP dilution buffer supplemented with protease inhibitors cocktail, divided into two parts and mixed with Pierce™ ChIP-grade Protein A Magnetic Beads (Thermo Fisher Scientific) which pre-blocked using Salmon Sperm DNA and bovine serum albumin (BSA) in advance. After shaking in 4 °C for 1 h, antibodies (anti-SNAIL or IgG) were added respectively, shake gently in 4 °C overnight. Beads were then washed once use low salt wash buffer and twice with IP wash buffer. Samples was then extracted using elution buffer, incubated with RNase A at 65 °C overnight, incubated with proteinase K for 1 h, extracted using phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in 30 μL of water at last. The follow-up quantification was conducted using qRT-PCR.

RNA immunoprecipitation (RIP) assay

RIP assay was conducted generally following previous reports [35]. In brief, cell lysate was incubated with anti-SNAIL or IgG for 4 h at 4 °C. Prepared protein A magnetic beads were mixed with samples, shaken gently for 2 h, then eluted and treated with DNase and Proteinase K. Samples were extracted using phenol/chloroform/isoamyl alcohol and precipitated with ethanol, then the precipitated RNAs were resuspended with RNase-free water. Complementary DNA was reverse transcript using random primer. The follow-up quantification was conducted using qRT-PCR amplifications.

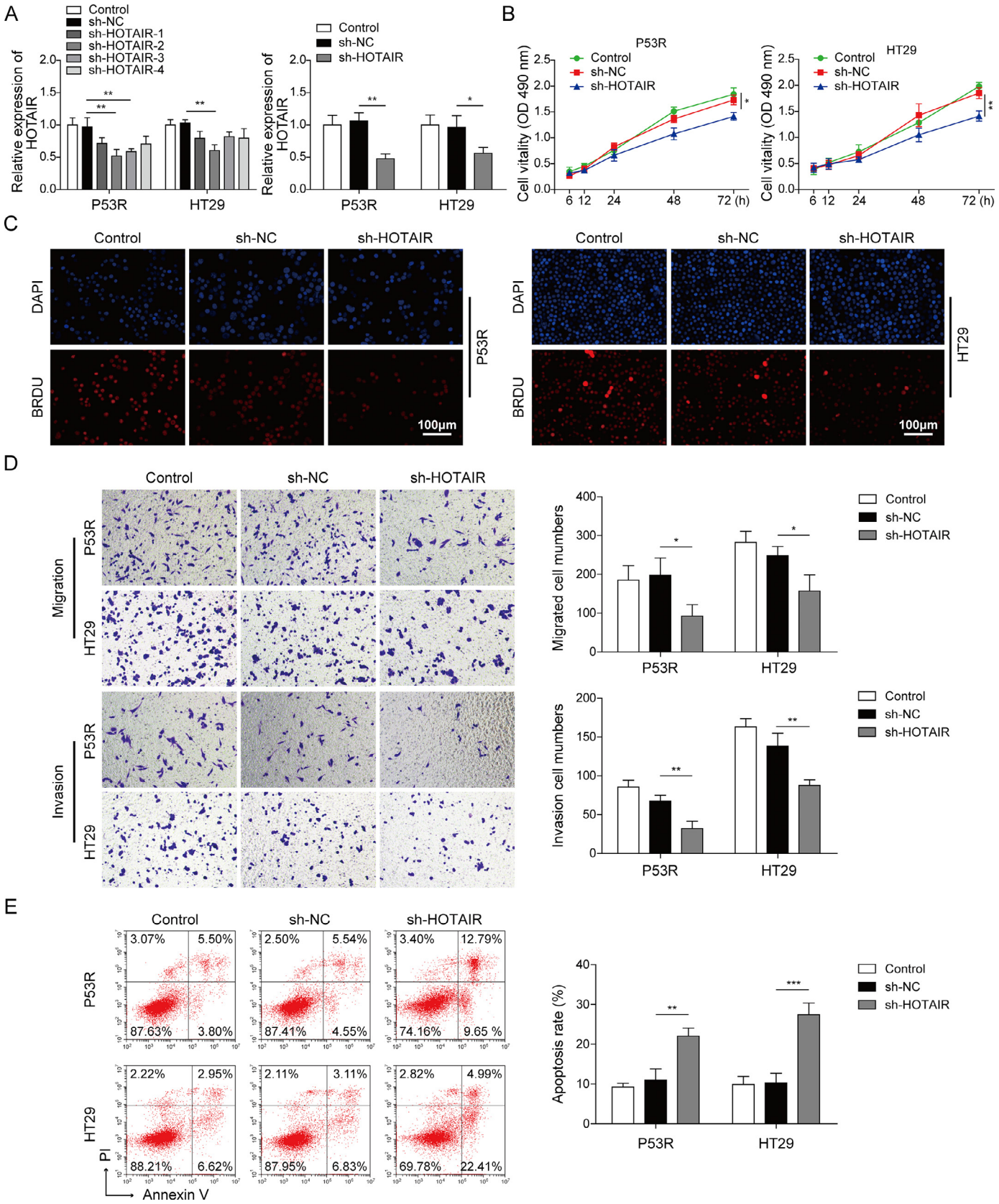

Fig. 2. The effect of low level of HOTAIR on the viability, migration, invasion and EMT of colorectal cancer cells

(A) Plots of the relative expression of HOTAIR in colorectal cancer cells expressing 4 shRNAs against HOTAIR detected by qRT-PCR. (B-C) Plots of cell proliferation of colorectal cancer cells expressing sh-HOTAIR or sh-NC assessed by MTT and BrdU assay. (D) Representative images and plots of the metastasis and invasion ability of colorectal cancer cells expressing sh-HOTAIR or sh-NC assessed by Transwell assay. (E) Representative images and plots of the apoptosis of colorectal cancer cells expressing sh-HOTAIR or sh-NC by Annexin V assay. (F) Representative images of protein levels of E-CADHERIN, VIMENTIN, SLUG and N–CADHERIN assessed by western blot. (G) Representative images of E-CADHERIN and N–CADHERIN expression in colorectal cancer cells expressing sh-HOTAIR or sh-NC, assessed by immunofluorescence assay. Wild-type of P53R and HT29 cells were shown as control group. β-actin was taken as internal control in qRT-PCR and western blot assay. $n = 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

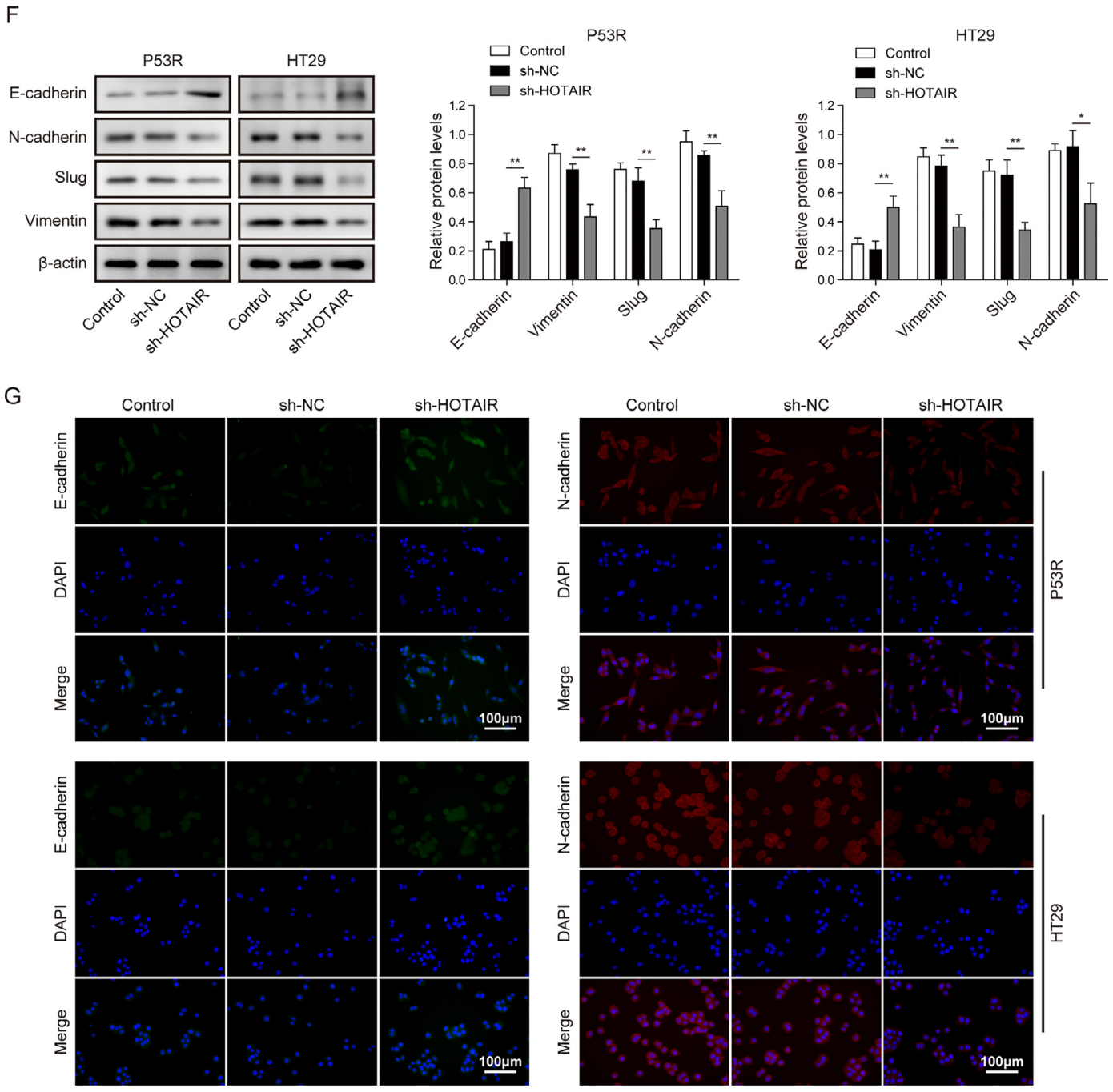

Fig. 2. Continued

Immunofluorescence assay

Cells were fixed in 3.7% paraformaldehyde, washed twice with PBS after fixation, and then blocked in 2% FBS/2% BSA in PBS with 0.1% NP40 for 45 min. Cells were then treated with antibodies of E-CADHERIN (Cell Signaling Technology, 14,472) and N–CADHERIN (Cell Signaling Technology, 13,116) for 1 hour. After washed, samples were incubated with secondary antibodies in the same blocking buffer (Goat Anti-house IgG, Cy3, A22210, Abbkine, and Goat Anti-rabbit IgG, Cy5, 111–175–144, Jackson). After washed, DAPI solution was added to each sample and incubate for 15 min, wash twice. Mounting solution and coverslip were added. Samples were observed with fluorescence microscope.

RNA pull-down

RNA pull-down was performed using Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) following the specification. Cells were harvested, washed and lysed using Pierce IP Lysis Buffer (Thermo Scientific) supplemented with proteinase inhibitors. After incubation and homogenate, samples were centrifuged for 20 min at 13,000 RPM in 4 ˚C, and supernatant were collected for further study. HOTAIR RNA transcripts were obtained using T7 *in vitro* transcription kit (Biomics Biotech) and labeled using Pierce RNA 3'Desthiobiotinylation Kit. Labeled HOTAIR RNA (100 pmol) was used to mix with 50 μL pre-washed beads, incubated at room temperature. After 30 min, the supernatant was removed, and the beads were washed twice for

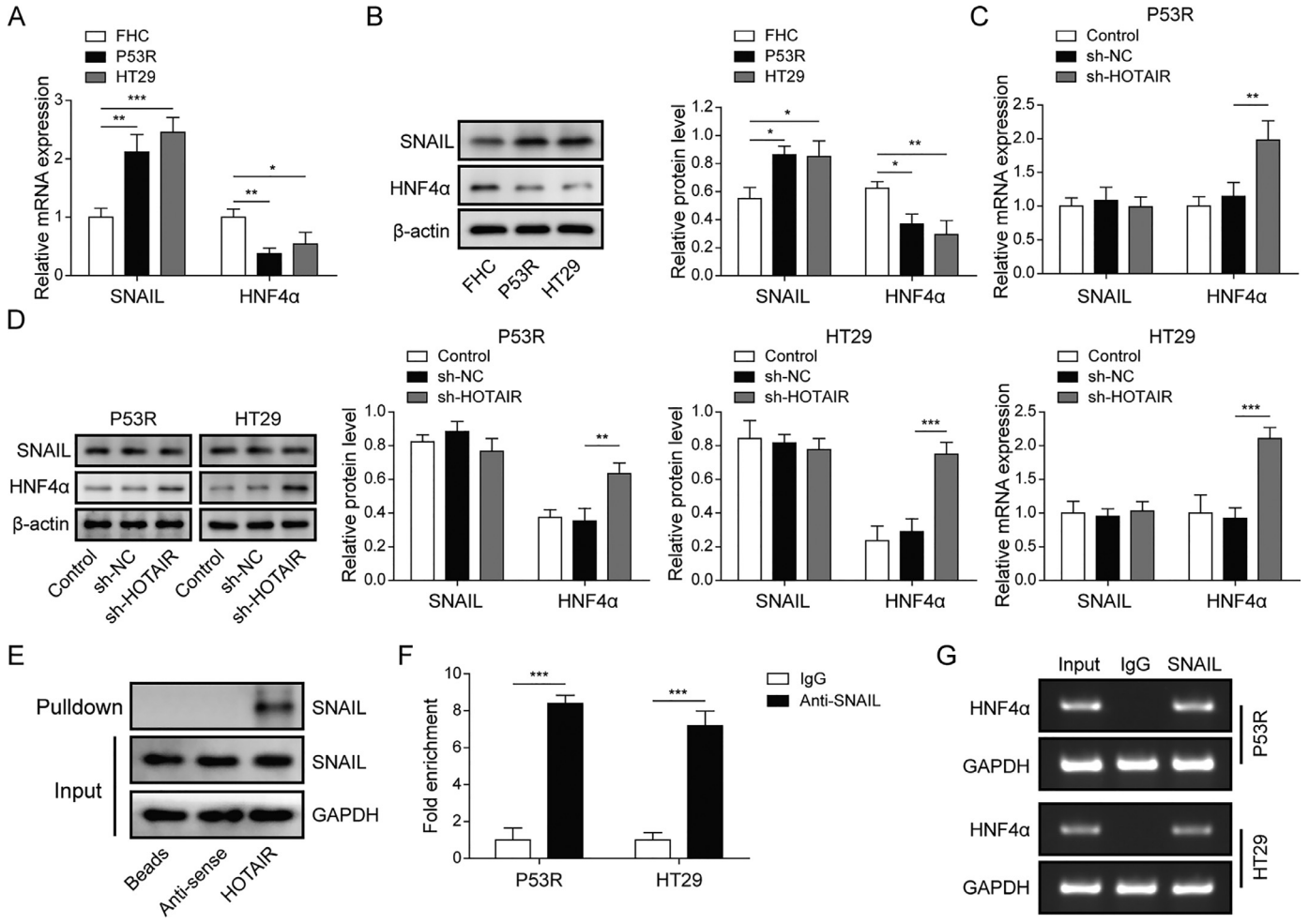

Fig. 3. The interaction among HOTAIR, SNAIL and HNF4α in colorectal cancer cells
(A) Plots of the relative expression of SNAIL and HNF4α mRNA in FHC, P53R and HT29 assessed by qRT-PCR. (B) Representative images of protein levels of SNAIL and HNF4α in FHC, P53R and HT29 cells assessed by western blot. (C) Plots of the relative expression of SNAIL and HNF4α mRNA in colorectal cancer cells expressing sh-HOTAIR or sh-NC assessed by qRT-PCR. (D) Representative images of protein levels of SNAIL and HNF4α in colorectal cancer cells expressing sh-HOTAIR or sh-NC assessed by western blot. (E) Protein levels of SNAIL precipitated with biotin labeled HOTAIR were assessed by western blot in RNA pull-down assay. GAPDH was taken as the internal control. Biotin-labeled antisense transcript was taken as negative control. (F) The RNA levels of HOTAIR precipitated with SNAIL by anti-SNAIL/SNAIL complex were assessed by qRT-PCR in RIP assay. IgG was taken as negative control. β-actin was taken as internal control. (G) Representative images of levels of HNF4α promoter precipitated with anti-SNAIL/SNAIL complex were assessed by semi-quantitative PCR assessed by CHIP assay. GAPDH was taken as the internal control. IgG was taken as negative control in qRT-PCR and western blot assay. $n = 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

protein-binding. Cell lysate was prepared following the manual, added into RNA-bound beads and incubated 1 h at 4 ˚C with rotation. After washing, binding proteins were eluted and assessed using western blot.

Xenograft assay and liver metastases model

Xenograft assay was used to analyze the function of HOTAIR *in vivo*. Animal experiment in this study was approved by the ethics committee of Medical ethics committee, The Third Xiangya Hospital, Central South University. In brief, 1×10^7 sh-NC or sh-HOTAIR expressing cells were injected to nude mice in flanks subcutaneously. The growth of tumors was observed for a total of 35 days. The tumor volumes were analyzed for each time point. Xenograft experiments were conducted with 5 biological duplications.

Liver metastases model in nude mice were preformed following Kawaguchi et al. reported previously [36]. In brief, after washed with serum-free medium, 5×10^5 sh-NC or sh-HOTAIR expressing cells (re-suspended in serum-free medium with 50% matrigel) were injected into spleen at the superior and inferior pole. Four weeks later, all experimental nude mice were euthanized using CO_2 asphyxiation and decapitated. Liver tissues were sampled, fixed in 10% formalin and then embedded using paraffin, stored for further analyses.

Hematoxylin-Eosin (HE) staining

Paraffin sections (5-μm thick) were treated for deparaffinization and rehydration, stained with hematoxylin solution for 5 min, washed with water and dipped in 1% hydrochloric acid ethanol (1% HCl in 70% ethanol) for 5 s, and then washed in deionized water. Next, eosin solution was used to stain the sections. After 2 min of staining, the sections were washed with deionized water, 80% ethanol, 95% ethanol, 100% ethanol in turn to be dehydrated. After dehydration, the sections were cleared in xylene and mounted. Following observation and photographing were conducted using fluorescence microscope (Olympus, Tokyo, Japan).

Immunohistochemistry (IHC) assay

In brief, blocked tissues were sectioned at a 5-μm thickness. Slides were deparaffinized and hydrated in a series washes using xylenes and

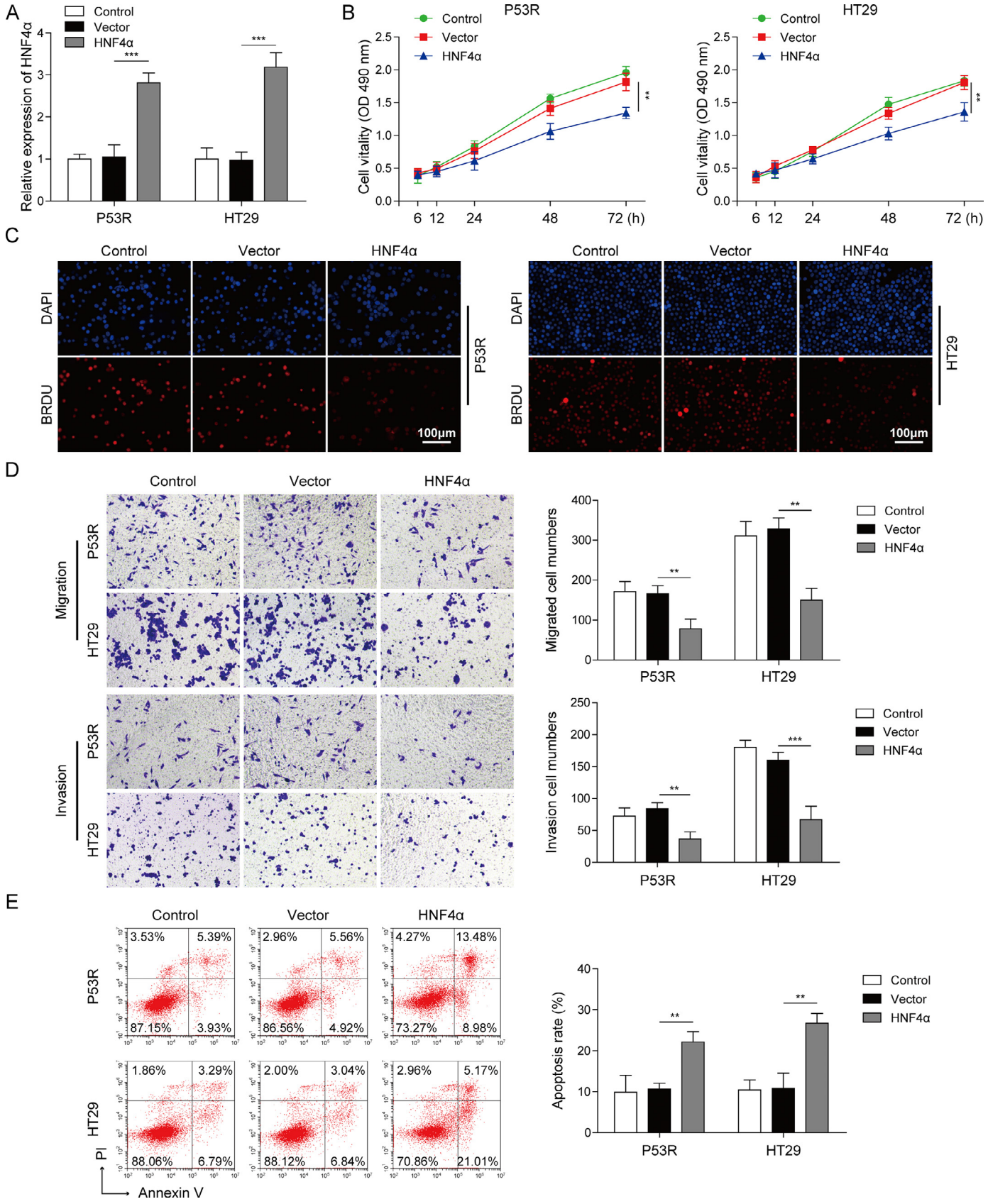

Fig. 4. The effect of high level of HNF4α on the viability, migration, invasion and EMT of colorectal cancer cells

(A) Plots of the relative expression of HNF4α in colorectal cancer cells overexpressing HNF4α detected by qRT-PCR. (B-C) Plots of cell proliferation of colorectal cancer cells overexpressing HNF4α assessed by MTT and BrdU assay. (D) Representative images and plots of the metastasis and invasion ability of colorectal cancer cells overexpressing HNF4α by Transwell assay. (E) Representative images and plots of the apoptosis of colorectal cancer cells overexpressing HNF4α by Annexin V assay. (F) Representative images of protein levels of E-CADHERIN, VIMENTIN, SLUG and N-CADHERIN in colorectal cancer cells overexpressing HNF4α assessed by western blot. (G) Representative images of E-CADHERIN and N–CADHERIN expression in colorectal cancer cells overexpressing HNF4α assessed by immunofluorescence assay. Wild-type of P53R and HT29 cells were shown as control group. P53R and HT29 cells transfected with mock-vector were taken as negative control. β-actin was taken as internal control in qRT-PCR and western blot assay. $n = 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

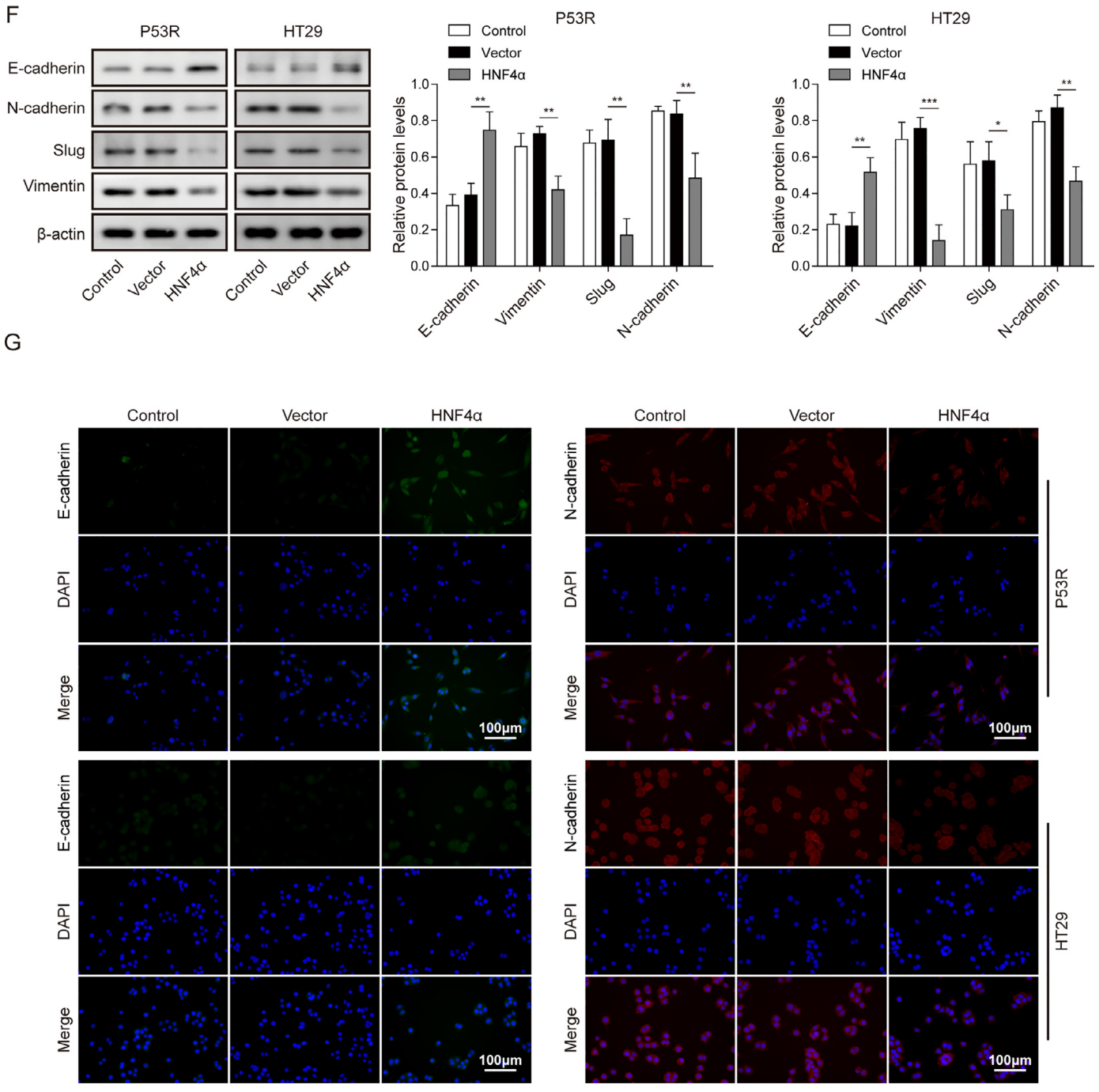

Fig. 4. Continued

progressively diluted alcohols to water. After washed, the slides were boiled in 10 mM sodium citrate buffer (pH 6.0) and maintain a sub-boiling temperature for 10 min. Sections were then washed and incubated in 3% hydrogen peroxide for 10 min. After washed, sections were blocked with 200 μl blocking solution for 1 hour at room temperature. After removing blocking buffer. primary antibodies were then added to sections and incubated for 30 min. Sections were then washed, incubated with biotin-conjugated secondary antibodies, horseradish peroxidase enzyme–conjugated streptavidin, and diaminobenzidine, respectively. Sections were dehydrated in a series of alcohols and xylenes, and observed using microscopic evaluation.

Statistical analysis

Data were shown as mean ± SD of at least three biological replicates. For comparisons between 2 groups, unpaired Student *t*-test was conducted. $P<0.05$ was considered as statistically significant for *t*-test. For the comparison among multiple groups, one-way ANOVA and Bonferroni post-hoc test were conducted. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

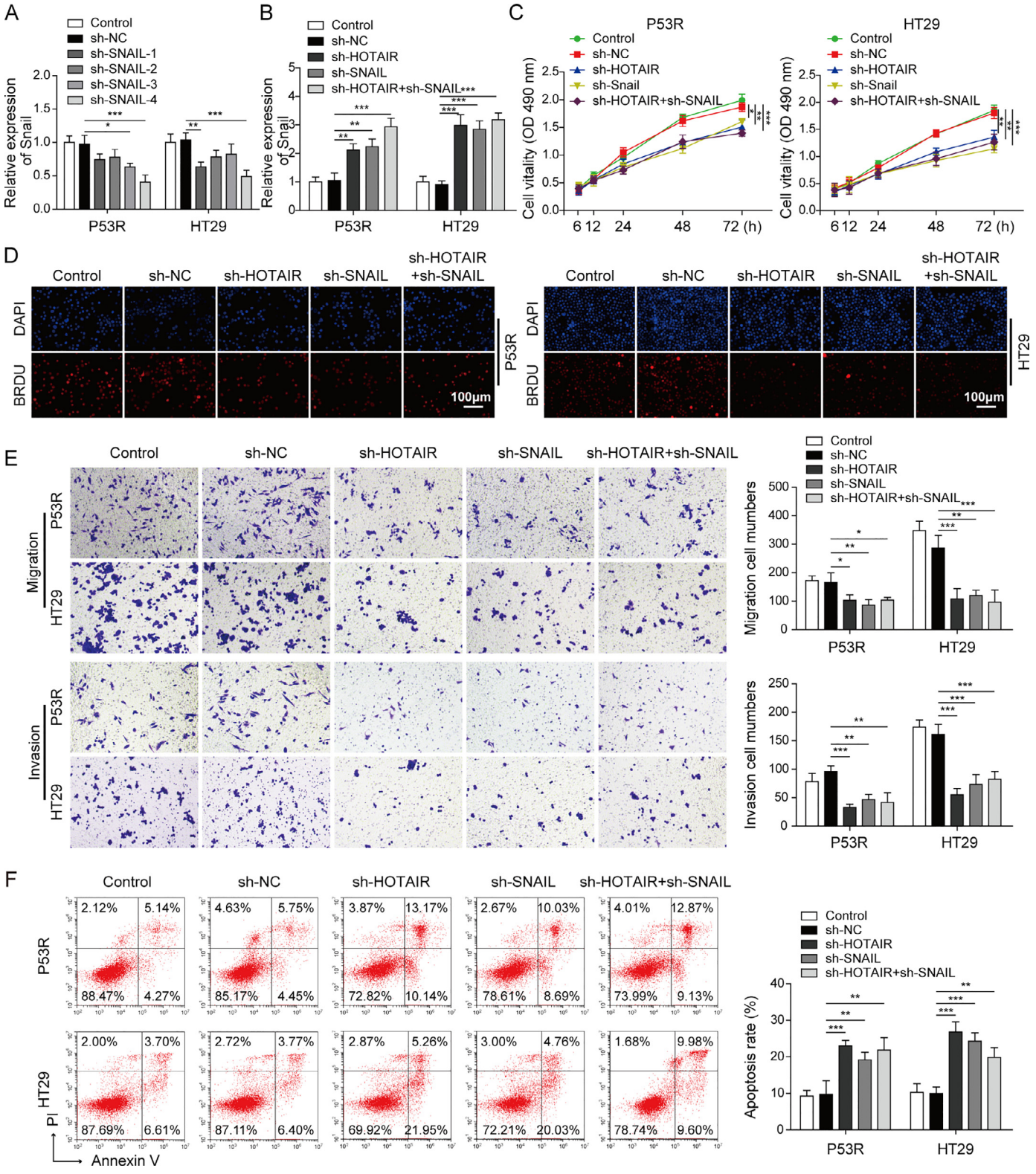

Fig. 5. The effect of double-knockdown of HOTAIR and SNAIL on the viability, migration, invasion and EMT of colorectal cancer cells
(A) Plots of the relative expression of SNAIL mRNA in colorectal cancer cells expressing 4 shRNAs against SNAIL. (B) HNF4α mRNA expression was detected by qRT-PCR. (C-D) Plots of cell proliferation of colorectal cancer cells expressing sh-HOTAIR, sh-Snail or both, assessed by MTT and BrdU assay. (E) Representative images and plots of the metastasis and invasion ability of colorectal cancer cells expressing sh-HOTAIR, sh-Snail or both, assessed by Transwell assay. (F) Representative images and plots of the apoptosis of colorectal cancer cells expressing sh-HOTAIR, sh-Snail or both, by Annexin V assay. (G) Representative images of protein levels of E-CADHERIN, VIMENTIN, SLUG and N–CADHERIN in colorectal cancer cells expressing sh-HOTAIR, sh-Snail or both, assessed by western blot. (H) Representative images of E-CADHERIN and N–CADHERIN expression in colorectal cancer cells expressing sh-HOTAIR, sh-Snail or both, assessed by immunofluorescence assay. Wild-type of P53R and HT29 cells were shown as control group. P53R and HT29 cells transfected with sh-NC were taken as negative control. β-actin was taken as internal control in qRT-PCR and western blot assay. $n = 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

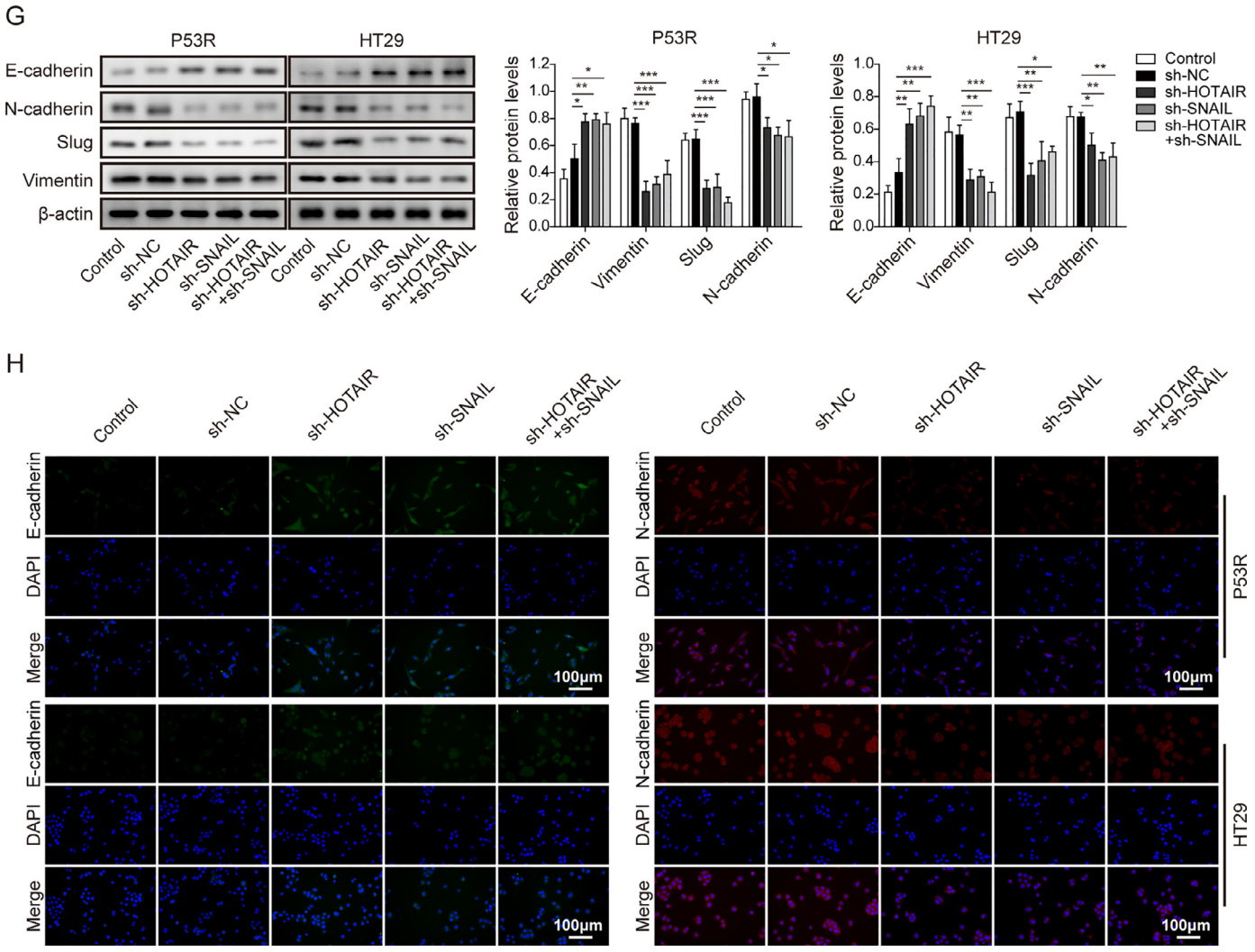

Fig. 5. Continued

Results

HOTAIR is highly expressed in colorectal cancer tissue and cell lines

To explore the correlation between HOTAIR and colorectal cancer, first, we analyzed the expression level of HOTAIR in colorectal cancer tissue and the adjacent tissue based on 10 pairs of colorectal cancer tissue and the adjacent tissue samples. Results showed that the expression of HOTAIR was significantly higher in colorectal tumor tissue than that in adjacent tissue (Fig. 1A). In addition, ISH assay was conducted to present the expression and distribution of HOTAIR in colorectal cancer tissue and the adjacent tissue. Result also showed higher expression of HOTAIR, which distributed in both nucleus and cytoplasm, in colorectal cancer tissue (Fig. 1B).We further assessed the expression of HOTAIR in colorectal cancer cell lines, including P53R, HT29, SW480 andHCT116. FHC cells, human normal colorectal mucosal cells, were used as control cell line. Consistent result was shown in colorectal cancer cell lines that comparing with FHC cells, the expression of HOTAIR was significantly higher in each colorectal cancer cell lines (Fig. 1C). Thus, from aspects of both patient tissues and database, a higher expression of HOTAIR was demonstrated in colorectal cancer.

Knock-down of HOTAIR inhibits the viability, migration, invasion and EMT of colorectal cancer cells

First, we constructed stable P53R and HT29 cell strains with stable expression of shRNA targeting HOTAIR. The efficacy of sh-RNAs targeting HOTAIR were detected by qRT-PCR (Fig. 2A). The expression of sh-RNA-2 (sh-HOTAIR) effectively suppressed the level of HOTAIR to less than 50% in both P53R and HT29 cells and was used in the following study. MTT and BrdU assays were performed to investigate the influence of HOTAIR on colorectal cancer cell viability and proliferation (Fig. 2B&C). Compare with sh-NC group, the proliferation of P53R cells after transfected with sh-HOTAIR was significantly reduced. Consistent result was found in HT29 cells, showed that low level of HOTAIR inhibits the proliferation of colorectal cancer cells. Transwell assay also showed that the migration and invasion capacity of colorectal cancer cells with low HOTAIR level was weaker than the control groups for both P53R and HT29 cells (Fig. 2D). Using Annexin V assay, we found that sh-HOTAIR induced the cell apoptosis in colorectal cancer cells as well (Fig. 2E). In addition, we assessed the expression of several biomarkers of EMT to evaluate the EMT of colorectal cancer cells with low HOTAIR level. E-CADHERIN, VIMENTIN, SLUG and N–CADHERIN were

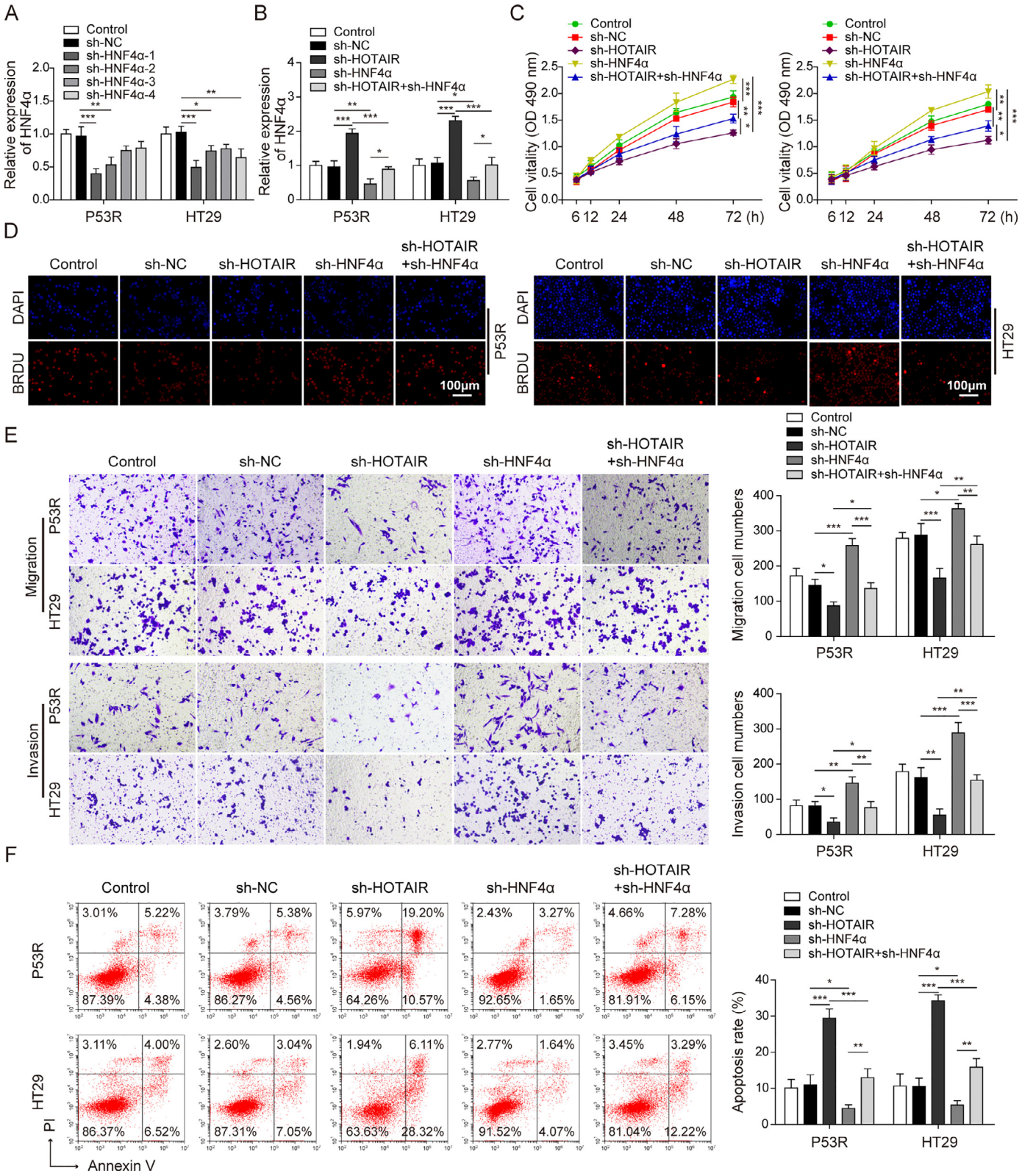

Fig. 6. The effect of double-knockdown of HOTAIR and HNF4*α* on colorectal cancer cells

(A) Plots of the relative expression of HNF4*α* in colorectal cancer cells expressing 4 shRNAs against HNF4*α*. HNF4*α* expression was detected by qRT-PCR. (B) Plots of the relative expression of HNF4*α* in colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both. (C-D) Plots of cell viability and representative images of proliferation of colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both, assessed by MTT assay and BrdU assay. (E) Representative images and plots of the metastasis and invasion ability of colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both, assessed by Transwell assay. (F) Representative images and plots of the apoptosis of colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both, by Annexin V assay. (G) Representative images of protein levels of E-CADHERIN, VIMENTIN, SLUG and N–CADHERIN in colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both, assessed by western blot. (H) Representative images of E-CADHERIN and N–CADHERIN expression in colorectal cancer cells expressing sh-HOTAIR, sh-HNF4*α* or both, assessed by immunofluorescence assay. Wild-type of P53R and HT29 cells were shown as control group. P53R and HT29 cells transfected with sh-NC were taken as negative control. *β*-actin was taken as internal control in qRT-PCR and western blot assay. $n = 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

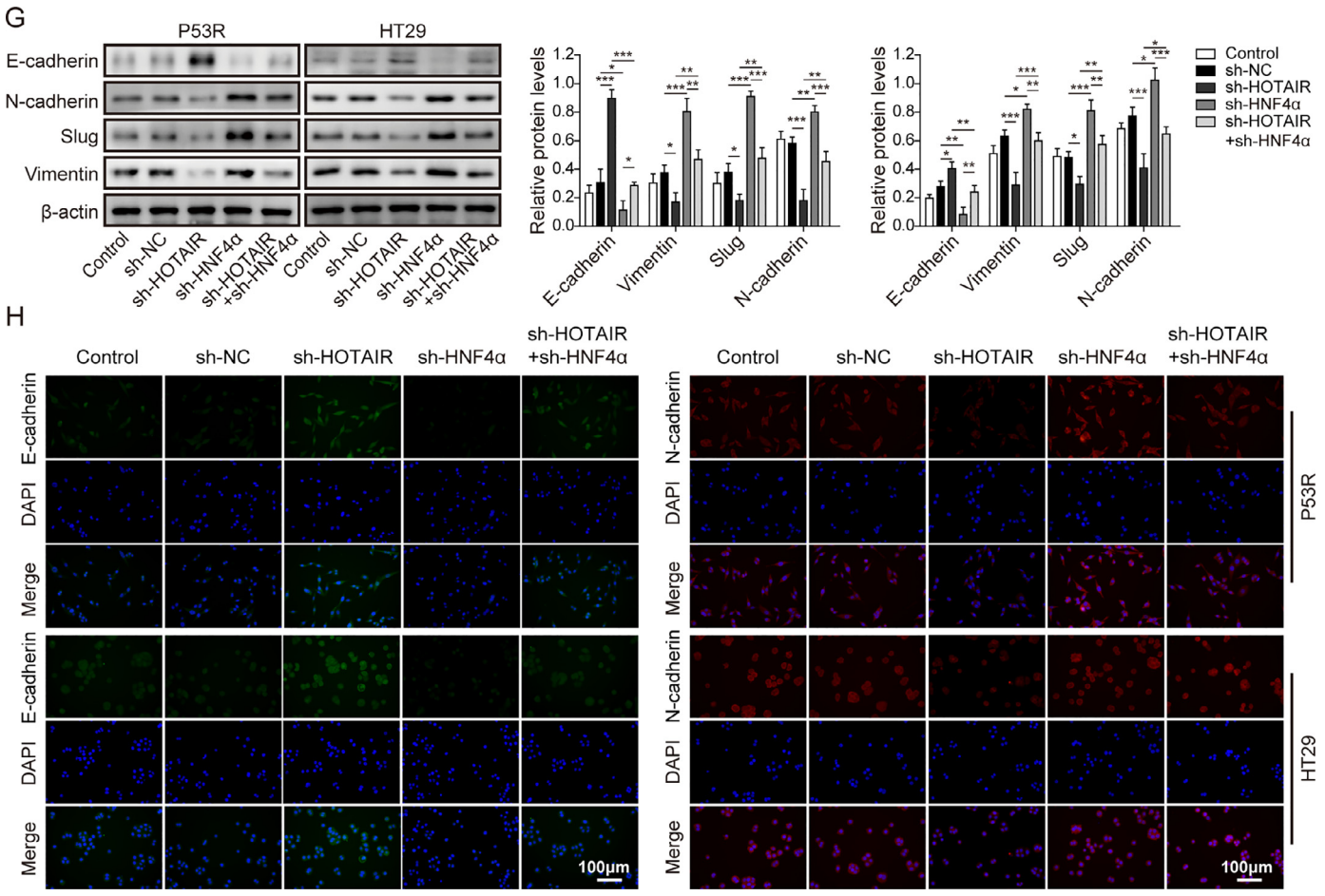

Fig. 6. Continued

detected by western blot and immunofluorescence assay (Fig. 2F&G), and found that, for both P53R and HT29, the decrease of HOTAIR induced E-CADHERIN expression, reduced the level of VIMENTIN, SLUG and N–CADHERIN in protein level, indicating a reversion of EMT was triggered by sh-HOTAIR in colorectal cancer cells.

HOTAIR suppresses HNF4α via recruiting SNAIL

To clarify the interactive relationship among HOTAIR, SNAIL and HNF4*α*, the differential expression of *SNAIL* and *HOTAIR* in colorectal cancer cells and normal colorectal mucosal cells were assessed by qRT-PCR and western blot assay. In both RNA level and protein level, the expression of *SNAIL* was higher in P53R and HT29 cells than that in FHC cells. Whereas HNF4*α* showed a reverse trend that expressed lower in P53R and HT29 cells than FHC cells (Fig. 3A&B). The expression of *SNAIL* and *HNF4α* under low HOTAIR condition was further assessed in the HOTAIR knockdown cell lines. From both RNA and protein aspect, comparing with wild-type control cells, HNF4*α* expression was significantly higher in both sh-HOTAIR-P53R and sh-HOTAIR-HT29, while the expression of *SNAIL* was unchanged in the two cell lines. (Fig. 3C&D), indicating that HOTAIR could have inhibitory effect on HNF4*α*, but not on SNAIL.

The effect of HOTAIR on Snail-mediated repression suggests the possibility that HOTAIR may interact with SNAIL [25]. Thus, we explored the interaction of HOTAIR and SNAIL by RNA pull-down and RIP assay. Biotin labeled HOTAIR RNA transcript was used to pull-down interacting proteins with the antisense transcript as control. As shown in Fig. 3E, SNAIL was found to bind with HOTAIR in P53R cells. This specific HOTAIR-SNAIL binding was verified by RIP assay in both P53R and HT29 cells (Fig. 3F). Furthermore, to confirm the function of SNAIL in the regulatory process, ChIP assay was conducted to analysis the interaction between SNAIL and HNF4*α*. In both P53R and HT29 cells, specific binding of SNAIL with HNF4*α* promoter was found, indicating a direct binding of SNAIL to the promoter of HNF4*α* in colorectal cancer cells (Fig. 3G). Taken together, these results supported the hypothesis that HOTAIR recruited SNAIL and suppressed the expression of HNF4*α* subsequently in colorectal cancer cells.

Overexpression of HNF4α inhibits the viability, migration, invasion and EMT of colorectal cancer cells

Given that the down-regulation of HOTAIR induced HNF4*α* expression, and repressed the viability, migration, invasion and EMT of colorectal cancer cells, the effect of HNF4*α* was further investigated based on colorectal cancer cell lines stable overexpressing HNF4*α*. Data in Fig. 4A showed the efficiency of overexpression. The levels of HNF4*α* increased to around 3-fold in overexpressing P53R and HT29, comparing with those of mock-vector groups. According to the results of MTT, Transwell and Annexin V assay, overexpression of HNF4*α* reduced the cell viability, migration, invasion capacity, and induced the apoptosis of P53R and HT29 cells (Fig. 4B-E). In addition, western blot and immunofluorescence results showed that high level of HNF4*α* induced E-CADHERIN expression, and reduced the level of VIMENTIN, SLUG and N–CADHERIN (Fig. 4F&G), suggested a reversion of EMT. Therefore, overexpressing of HNF4*α* induced the apoptosis, inhibited the viability, migration, invasion and EMT of P53R and HT29.

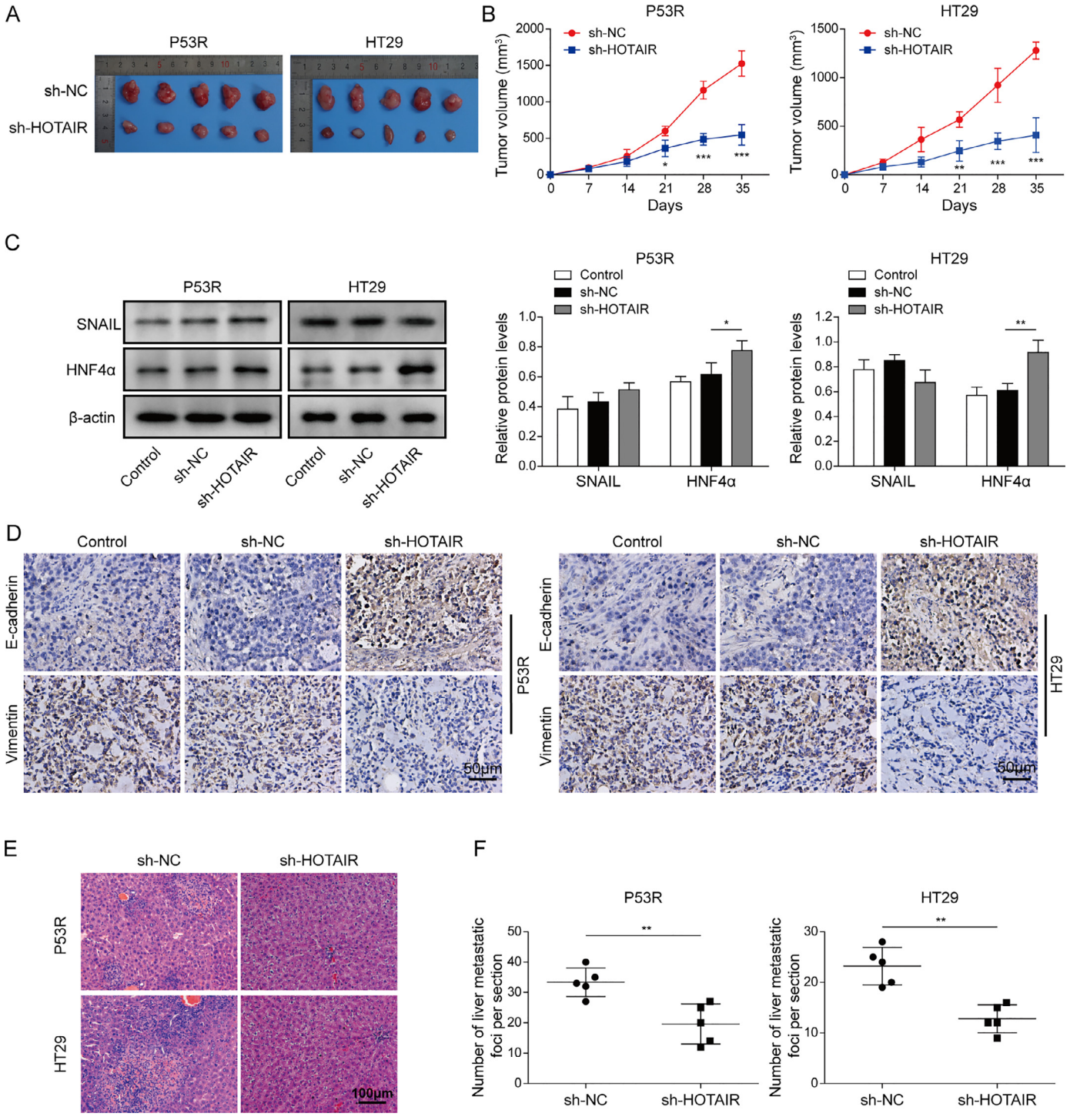

Fig. 7. The effect of HOTAIR on the viability, migration, invasion and EMT of colorectal cancer *in vivo*

Xenograft assay was preformed and sh-HOTAIR expressing P53R and HT29 were injected to nude mice in flanks subcutaneously. (A) Picture of the tumors formed in nude mice injected P53R and HT29 that expressing sh-HOTAIR or shNC. (B) Plots of the tumor volumes in 35 days. The tumor volumes were assessed at day 7, 14, 21, 28 and 35. (C) Representative images of protein levels of SNAIL and HNF4α in colorectal cancer cells expressing sh-HOTAIR or shNC, assessed by western blot. Wild-type of P53R and HT29 cells were shown as control group. P53R and HT29 cells transfected with sh-NC were taken as negative control. β-actin was taken as internal control. (D) Representative images of E-CADHERIN and VIMENTIN expression in tumors generated from colorectal cancer cells expressing sh-HOTAIR or sh-NC, assessed by IHC assay. (E) Representative images of the liver metastasis status of colorectal cancer cells expressing sh-HOTAIR or shNC, based on colorectal cancer liver metastases model in nude mice. (F) Plots of the number of liver metastatic foci per section in the colorectal cancer liver metastases model in nude mice. $n = 5$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

The promotive effect of HOTAIR on the viability, migration, invasion and EMT of colorectal cancer cells depends on the inhibition of SNAIL on HNF4α

To further address the hypothesis that HOTAIR inhibits HNF4α via recruiting SNAIL, sh-SNAIL was stable transfected to P53R and HT29 cells with or without sh-HOTAIR to investigate the regulatory function of HOTAIR on HNF4α in a low-Snail condition. Four shRNAs of SNAIL were tested for the knock-down efficacy in P53R and HT29 cells and sh-SNAIL-4 was selected to use in the following experiments (Fig. 5A). qRT-PCR result showed that both sh-HOTAIR and sh-SNAIL led to the increase of *HNF4α* level in both P53R and HT29 cells (Fig. 5B). In the following analysis of MTT assay, Annexin V assay, Transwell assay and detection of EMT biomarkers by both western blot and immunofluorescence assay, the inhibition of cell viability, migration and invasion, the promotion of apoptosis, and the reversion of EMT were found to be triggered by either sh-HOTAIR or sh-SNAIL (Fig. 5C–H). More importantly, the double knockdown of HOTAIR and SNAIL didn't trigger stronger responses comparing with those indued by single knockdown of HOTAIR or SNAIL, indicated that the regulatory function of HOTAIR on the viability, migration, invasion and EMT of colorectal cancer cells depends on the inhibition on HNF4α.

The effect of double-knockdown of HOTAIR and HNF4α on colorectal cancer cells

To further investigate the correlation between HOTAIR and HNF4α, we invastigated whether the loss of HNF4α could rescue the knock-down of HOTAIR, and the effect of low levels of both HOTAIR and HNF4α on colorectal cancer cells. Four shRNAs of HNF4α were tested for the efficacy and sh-HNF4α−1 (sh-HNF4α) was selected for the following experiments (Fig. 6A). It was shown that comparing with sh-HNF4α group, knock-down of both HOTAIR and HNF4α resulted in a higher level of HNF4α, indicating a potential up-regulation of HNF4α induced by HOTAIR (Fig. 6B). In the following analysis of MTT assay and BrdU assay, knockdown of HNF4α reversed the inhibitory effect of HOTAIR knockdown on cell viability and proliferation (Fig. 6C&D). According to the results of Transwell assay, Annexin V assay and the detection of EMT biomarkers by both western blot and immunofluorescence assay, in a certain extent, knock-down of HNF4α reversed the effect of sh-HOTAIR on the migration and invasion capaticy, on cell apoptosis, and on the EMT of colorectal cancer cells (Fig. 6E-H). These results showed knockdown of HNF4αrescued the phonotypes found in sh-HOTAIR cells, suggested a regulatory effect of HOTAIR on HNF4α.

Konckdown of HOTAIR suppressed the growth, migration and invasion of colorectal tumor in nude mice

Xenograft assay was proformed to invistigate the effect of HOTAIR on the tumorigenesis ability of colorectal cancer cells. sh-HOTAIR expressing P53R and HT29 were injected to nude mice in flanks subcutaneously, the tumor volume was observed every 7 days from day 7 to day 35. Fig. 7A and B showed that for both P53R and HT29, the volume of tumors in sh-HOTAIR group was significantly smaller than that in control groups, indicating an inhibited tumorigenesis ability in sh-HOTAIR expressing colorectal cancer cells. We further assessed the expression of SNAIL and HNF4α in the tumores generated in sh-NC and sh-HOTAIR groups by western blot, and found that the level of HNF4α protein increased in sh-HOTAIR expressing P53R and HT29, consistent with what we found *in vitro* experiments (Fig. 7C). IHC assay showed the higher level of E-CADHERIN and the lower level of VIMENTIN triggered by sh-HOTAIR *in vivo*. Moreover, nude mice model of colorectal cancer liver metastases was established to investigate the effect of HOTAIR on colorectal cancer liver metastasis *in vivo*. HE staining showed that the number of liver metastatic foci per section in sh-HOTAIR group decreased to about 30% comparing with that in control group (Fig. 7E). In summary, low level of HOTAIR induced the expression of HNF4α, and suppressed the tumorigenesis and liver metastasis capacity of colorectal cancer *in vivo*.

Discussion

Current conventional therapy of colorectal cancer is surgery combined with chemotherapy and radiotherapy. However, the lack of effective early diagnostic approach make the treatment harder, the occurrence of recurrence and metastases further Increased the mortality [37,38]. Therefore, it is essential to explore novel target of colorectal cancer for early diagnosis and precision therapy. In current study, we demonstrated that HOTAIR regulates the level of a cancer suppressor gene HNF4α, via recruiting the transfection factor SNAIL in colorectal cancer cells, and further inhibits the EMT, viability, migration and invasion of colorectal cancer cells both invitro and *in vivo*.

LncRNAs have been revealed to play vital roles in cancers, especially HOTAIR [12,39]. The elevated level of HOTAIR in colorectal tumor tissue and colorectal cancer cell lines implied the correlation between HOTAIR and the development of colorectal cancer. Besides, HOTAIR was reported to induce the EMT in several cancers [40–44], and EMT is closely related with the tumorigenesis and progression of epithelial malignancy, and highly regarded by investigators [45]. Other studies also shown that HOTAIR binds to PRC2 & LSD1 (lysine-specific demethylase 1), and act as a scaffold of histone modification complexes [46] and the genetic variants in HOTAIR were associated with the risk of colorectal cancer [47]. Taken together, the regulatory function and the molecular mechani of HOTAIR in colorectal cancer is worth to clarify. In hepatocytes, HOTAIR was reported to recruit SNAIL, suppress downstream genes expression, and impact cell viability and EMT suppress the expression of downstream genes and then regulate hepatocyte viability and EMT [15]. Moreover, SNAIL was reported to regulate the differentiation of hepatocytes via repressing HNF4α [34], which also has been mentioned with the regulatory function on the epithelial phenotype in other studies [48,49]. Thus, we discussed the correlation among HOTAIR, SNAIL and HNF4α, and their regulatory function in the progression of colorectal cancer.

First, we determined that suppressing either HOTAIR or SNAIL individually would repress the EMT of colorectal cancer cells, which is consistent with previous studies. It's worth noting that the double-knockdown of HOTAIR and SNAIL didn't show a better inhibition of colorectal cancer cells, suggesting that HOTAIR and SNAIL could play their role in the same pathway. Then, RNA pull-down and RIP experiments were performed to demonstrate the direct binding between HOTAIR and SNAIL. Additional *in vivo* experiments were conducted using sh-HOTAIR expressing cell lines, the lower tumorigenesis and metastasis capacity of sh-HOTAIR expressing cell lines further confirmed that the decrease of HOTAIR regulates the EMT, viability, migration and invasion of colorectal cancer cells. Identifying that HNF4α works as a downstream gene of HOTAIR in colorectal cancer further supplemented the regulatory network of HNF4α.

Conclusions

In summary, current study revealed that HOTAIR is an oncogene of colorectal cancer. The inhibition of HOTAIR repressed the viability and metastasis of colorectal cancer cell line *in vitro*, and suppressed the tumorigenesis, migration and invasion of colorectal cancer *in vivo*. Furthermore, we demonstrated that the molecular mechanism of the regulatory process is that HOTAIR recruits SNAIL, reduce the expression of HNF4α, and then inhibit the EMT of colorectal cancer cells. Our study provides further insight into the regulatory mechanism of HOTAIR regulation in colorectal cancer, and potential novel lncRNA-directed early diagnosis and therapy of colorectal cancer.

Declaration of Competing Interest

The authors declare that there are no competing interests.

Acknowledgments

We would like to give our sincere gratitude to the reviewers for their constructive comments.

Funding

This work was supported by the National Natural Science Foundation of China (Grant number:81872473), the Hunan Province Science and Technology Plan (Grant number:2017SK2052) and the New Xiangya Talent Project of the Third xiangya hospital of Central South University (Grant number:20180301).

Consent for Publication

The informed consent obtained from study participants

Availability of Data and Material

All data generated or analysed during this study are included in this article.

Ethical Approval

Animal experiment in this study was approved by the ethics committee of Medical ethics committee, The Third Xiangya Hospital, Central South University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101036.

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (6) (2018) 394–424.
[2] D.G. Sur, M. Colceriu, G. Sur, C. Aldea, C. Silaghi, G. Samasca, et al., MiRNAs roles in the diagnosis, prognosis and treatment of colorectal cancer, Expert Rev. Proteom. 16 (10) (2019) 851–856.
[3] J-z Ren, J-r. Huo, Correlation between T-cadherin gene expression and aberrant methylation of T-cadherin promoter in human colon carcinoma cells, Med. Oncol. 29 (2) (2012) 915–918.
[4] I. Druzhkova, N. Ignatova, N. Prodanets, N. Kiselev, I. Zhukov, M. Shirmanova, et al., E-cadherin in colorectal cancer: relation to chemosensitivity, Clin. Colorectal Cancer 18 (1) (2019) e74–e86.
[5] J. Tang, L. Zhang, X. She, G. Zhou, F. Yu, J. Xiang, et al., Inhibiting CD164 expression in colon cancer cell line HCT116 leads to reduced cancer cell proliferation, mobility, and metastasis *in vitro* and *in vivo*, Cancer Invest. 30 (5) (2012) 380–389.
[6] R.S. Olsen, J. Nijm, R.E. Andersson, J. Dimberg, D. Wågsäter, Circulating inflammatory factors associated with worse long-term prognosis in colorectal cancer, World J. Gastroenterol. 23 (34) (2017) 6212–6219.
[7] P. Laissue, The forkhead-box family of transcription factors: key molecular players in colorectal cancer pathogenesis, Mol. Cancer 18 (1) (2019) 5.
[8] K.M. Marks, N.P. West, E. Morris, P. Quirke, Clinicopathological, genomic and immunological factors in colorectal cancer prognosis, Br. J. Surg. 105 (2) (2018) e99–e109.
[9] J.L. Rinn, M. Kertesz, J.K. Wang, S.L. Squazzo, X. Xu, S.A. Brugmann, et al., Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs, Cell 129 (7) (2007) 1311–1323.
[10] C.A. Brosnan, O. Voinnet, The long and the short of noncoding RNAs, Curr. Opin. Cell Biol. 21 (3) (2009) 416–425.
[11] A. Bhan, S.S. Mandal, LncRNA HOTAIR: a master regulator of chromatin dynamics and cancer, Biochim. Biophys. Acta 1856 (1) (2015) 151–164.
[12] M. Hajjari, A. Salavaty, HOTAIR: an oncogenic long non-coding RNA in different cancers, Cancer Biol. Med. 12 (1) (2015) 1–9.
[13] R. Kogo, T. Shimamura, K. Mimori, K. Kawahara, S. Imoto, T. Sudo, et al., Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers, Cancer Res. 71 (20) (2011) 6320–6326.
[14] J. Heubach, J. Monsior, R. Deenen, G. Niegisch, T. Szarvas, C. Niedworok, et al., The long noncoding RNA HOTAIR has tissue and cell type-dependent effects on HOX gene expression and phenotype of urothelial cancer cells, Mol. Cancer 14 (2015) 108.
[15] R.A. Gupta, N. Shah, K.C. Wang, J. Kim, H.M. Horlings, D.J. Wong, et al., Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis, Nature 464 (7291) (2010) 1071–1076.
[16] K. Kim, I. Jutooru, G. Chadalapaka, G. Johnson, J. Frank, R. Burghardt, et al., HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer, Oncogene 32 (13) (2013) 1616–1625.
[17] M. Ishibashi, R. Kogo, K. Shibata, G. Sawada, Y. Takahashi, J. Kurashige, et al., Clinical significance of the expression of long non-coding RNA HOTAIR in primary hepatocellular carcinoma, Oncol. Rep. 29 (3) (2013) 946–950.
[18] Y. Zhuang, X. Wang, H.T. Nguyen, Y. Zhuo, X. Cui, C. Fewell, et al., Induction of long intergenic non-coding RNA HOTAIR in lung cancer cells by type I collagen, J. Hematol. Oncol. 6 (2013) 35.
[19] B. Liu, E.D. Shen, M.M. Liao, Y.B. Hu, K. Wu, P. Yang, et al., Expression and mechanisms of long non-coding RNA genes MEG3 and ANRIL in gallbladder cancer, Tumour Biol. 37 (7) (2016) 9875–9886.
[20] N. Zhou, Z. Si, T. Li, G. Chen, Z. Zhang, H. Qi, Long non-coding RNA CCAT2 functions as an oncogene in hepatocellular carcinoma, regulating cellular proliferation, migration and apoptosis, Oncol. Lett. 12 (1) (2016) 132–138.
[21] M.A. Nieto, The snail superfamily of zinc-finger transcription factors, Nat. Rev. Mol. Cell Biol. 3 (3) (2002) 155–166.
[22] M.A. Huber, N. Kraut, H. Beug, Molecular requirements for epithelial-mesenchymal transition during tumor progression, Curr. Opin. Cell Biol. 17 (5) (2005) 548–558.
[23] M. Imai-Sumida, P. Dasgupta, P. Kulkarni, M. Shiina, Y. Hashimoto, V. Shahryari, et al., Genistein Represses HOTAIR/Chromatin Remodeling Pathways to Suppress Kidney Cancer, Cell. Physiol. Biochem. 54 (1) (2020) 53–70.
[24] F. Xu, J. Zhang, Long non-coding RNA HOTAIR functions as miRNA sponge to promote the epithelial to mesenchymal transition in esophageal cancer, Biomed. Pharmacother. 90 (2017) 888–896.
[25] C. Battistelli, C. Cicchini, L. Santangelo, A. Tramontano, L. Grassi, F.J. Gonzalez, et al., The Snail repressor recruits EZH2 to specific genomic sites through the enrollment of the lncRNA HOTAIR in epithelial-to-mesenchymal transition, Oncogene 36 (7) (2017) 942–955.
[26] D.T. Odom, N. Zizlsperger, D.B. Gordon, G.W. Bell, N.J. Rinaldi, H.L. Murray, et al., Control of pancreas and liver gene expression by HNF transcription factors, Science 303 (5662) (2004) 1378–1381.
[27] Q. Sun, W. Xu, S. Ji, Y. Qin, W. Liu, Q. Hu, et al., Role of hepatocyte nuclear factor 4 alpha in cell proliferation and gemcitabine resistance in pancreatic adenocarcinoma, Cancer Cell Int. 19 (2019) 49.
[28] H.R. Chang, S. Nam, M.C. Kook, K.T. Kim, X. Liu, H. Yao, et al., HNF4alpha is a therapeutic target that links AMPK to WNT signalling in early-stage gastric cancer, Gut 65 (1) (2016) 19–32.
[29] B. Lucas, K. Grigo, S. Erdmann, J. Lausen, L. Klein-Hitpass, G.U. Ryffel, HNF4alpha reduces proliferation of kidney cells and affects genes deregulated in renal cell carcinoma, Oncogene 24 (42) (2005) 6418–6431.
[30] B. Fekry, A. Ribas-Latre, C. Baumgartner, J.R. Deans, C. Kwok, P. Patel, et al., Incompatibility of the circadian protein BMAL1 and HNF4alpha in hepatocellular carcinoma, Nat. Commun. 9 (1) (2018) 4349.
[31] J.P. Babeu, C. Jones, S. Geha, J.C. Carrier, F. Boudreau, P1 promoter-driven HNF4alpha isoforms are specifically repressed by beta-catenin signaling in colorectal cancer cells, J. Cell Sci. 131 (13) (2018).
[32] X.B. Lv, G.Y. Lian, H.R. Wang, E. Song, H. Yao, M.H. Wang, Long noncoding RNA HOTAIR is a prognostic marker for esophageal squamous cell carcinoma progression and survival, PLoS One 8 (5) (2013) e63516.
[33] P.M. Das, K. Ramachandran, J. vanWert, R. Singal, Chromatin immunoprecipitation assay, BioTechniques 37 (6) (2004) 961–969.
[34] C. Cicchini, D. Filippini, S. Coen, A. Marchetti, C. Cavallari, I. Laudadio, et al., Snail controls differentiation of hepatocytes by repressing HNF4alpha expression, J. Cell. Physiol. 209 (1) (2006) 230–238.
[35] G.M. Dahm, M.M. Gubin, J.D. Magee, P. Techasintana, R. Calaluce, U. Atasoy, Method for the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts using RIP-Chip, J. Vis. Exp. (67) (2012).
[36] K. Kawaguchi, T. Murakami, A. Suetsugu, T. Kiyuna, K. Igarashi, Y. Hiroshima, et al., High-efficacy targeting of colon-cancer liver metastasis with Salmonella typhimurium A1-R via intra-portal-vein injection in orthotopic nude-mouse models, Oncotarget 8 (12) (2017) 19065–19073.
[37] H. Tanaka, S. Hazama, M. Iida, R. Tsunedomi, H. Takenouchi, M. Nakajima, et al., miR-125b-1 and miR-378a are predictive biomarkers for the efficacy of vaccine treatment against colorectal cancer, Cancer Sci. 108 (11) (2017) 2229–2238.
[38] C. Rat, C. Pogu, D. Le Donne, C. Latour, G. Bianco, F. Nanin, et al., Effect of Physician Notification Regarding Nonadherence to Colorectal Cancer Screening on Patient Participation in Fecal Immunochemical Test Cancer Screening: a Randomized Clinical Trial, JAMA 318 (9) (2017) 816–824.
[39] W.X. Peng, P. Koirala, Y.Y. Mo, LncRNA-mediated regulation of cell signaling in cancer, Oncogene 36 (41) (2017) 5661–5667.
[40] Q. Hong, O. Li, W. Zheng, W.Z. Xiao, L. Zhang, D. Wu, et al., LncRNA HOTAIR regulates HIF-1alpha/AXL signaling through inhibition of miR-217 in renal cell carcinoma, Cell Death Dis. 8 (5) (2017) e2772.
[41] T. Rajagopal, S. Talluri, R.L. Akshaya, N.R. Dunna, HOTAIR LncRNA: a novel oncogenic propellant in human cancer, Clin. Chim. Acta 503 (2019) 1–18.

[42] Y. Song, R. Wang, L.W. Li, X. Liu, Y.F. Wang, Q.X. Wang, et al., Long non-coding RNA HOTAIR mediates the switching of histone H3 lysine 27 acetylation to methylation to promote epithelial-to-mesenchymal transition in gastric cancer, Int. J. Oncol. 54 (1) (2019) 77–86.
[43] G. Yang, Y. Fu, X. Lu, M. Wang, H. Dong, Q. Li, LncRNA HOTAIR/miR-613/c-met axis modulated epithelial-mesenchymal transition of retinoblastoma cells, J. Cell. Mol. Med. 22 (10) (2018) 5083–5096.
[44] Y. Ren, H.H. Jia, Y.Q. Xu, X. Zhou, X.H. Zhao, Y.F. Wang, et al., Paracrine and epigenetic control of CAF-induced metastasis: the role of HOTAIR stimulated by TGF-ss1 secretion, Mol. Cancer 17 (1) (2018) 5.
[45] H. Hugo, M.L. Ackland, T. Blick, M.G. Lawrence, J.A. Clements, E.D. Williams, et al., Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression, J. Cell. Physiol. 213 (2) (2007) 374–383.
[46] M.C. Tsai, O. Manor, Y. Wan, N. Mosammaparast, J.K. Wang, F. Lan, et al., Long noncoding RNA as modular scaffold of histone modification complexes, Science 329 (5992) (2010) 689–693.
[47] Y. Xue, D. Gu, G. Ma, L. Zhu, Q. Hua, H. Chu, et al., Genetic variants in lncRNA HOTAIR are associated with risk of colorectal cancer, Mutagenesis 30 (2) (2015) 303–310.
[48] F. Parviz, C. Matullo, W.D. Garrison, L. Savatski, J.W. Adamson, G. Ning, et al., Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis, Nat. Genet. 34 (3) (2003) 292–296.
[49] S. Satohisa, H. Chiba, M. Osanai, S. Ohno, T. Kojima, T. Saito, et al., Behavior of tight-junction, adherens-junction and cell polarity proteins during HNF-4alpha-induced epithelial polarization, Exp. Cell Res. 310 (1) (2005) 66–78.