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Long non-coding RNA HOTAIR promotes carcinogenesis and invasion of gastric adenocarcinoma

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ABSTRACT

Gastric cancer is one of the major causes of cancer death worldwide; however, the mechanism of carcinogenesis is complex and poorly understood. Long non-coding RNA HOTAIR (HOX transcript antisense RNA) recently emerged as a promoter of metastasis in various cancers including gastric cancer. Here we investigated the impact of HOTAIR on apoptosis, cell proliferation and cell cycle to dissect the carcinogenesis of gastric cancer. We examined the mechanism of invasion and metastasis and analyzed the clinical significance of HOTAIR. Downregulation of HOTAIR was confirmed by two different siRNAs. The expression of HOTAIR was significantly elevated in various gastric cancer cell lines and tissues compared to normal control. si-HOTAIR significantly reduced viability in MKN 28, MKN 74, and KATO III cells but not in AGS cells. si-HOTAIR induced apoptosis in KATO III cells. Lymphovascular invasion and lymph node metastasis were more common in the high level of HOTAIR group. si-HOTAIR significantly decreased invasiveness and migration. si-HOTAIR led to differential expression of epithelial to mesenchymal transition markers. We found that HOTAIR was involved in inhibition of apoptosis and promoted invasiveness, supporting a role for HOTAIR in carcinogenesis and progression of gastric cancer.

1. Introduction

Gastric cancer is one of the major causes of cancer death worldwide, with almost 990,000 cases detected annually [1]. The incidence of gastric cancer varies with geographic location, and is highest in Eastern Asia including Korea, Japan and China. Despite its prevalence, there is still no curative modality for late-diagnosed gastric cancer. The mechanism of gastric carcinogenesis is complex and poorly understood, and is influenced by both infection with *Helicobacter pylori* and genetic factors. Gastric cancer appears to be caused by the accumulation of both genetic and epigenetic changes [2]. Non-coding RNAs (ncRNAs) like microRNA are major components of epigenetic regulatory networks, and have been shown to be deregulated in gastric cancer [3–6].

A new class of ncRNA that have been receiving increased attention are long non-coding RNA (lncRNA), with lengths ranging from 200 bp to 100 kbp [7]. HOX transcript antisense intergenic RNA (HOTAIR) is one of the well-studied lncRNAs that regulate gene expression by mediating the modulation of chromatin structure [7,8]. HOTAIR acts as a scaffold of histone modification complexes to coordinately interact with PRC2 complex and LSD1 histone modifiers [9]. HOTAIR promotes metastasis of breast cancer through the repression of multiple metastasis suppressor genes by interaction with PRC2 complex [10]. HOTAIR levels are also elevated in both primary and metastasized tumors of multiple other cancer types such as colorectal cancer, hepatocellular carcinoma, nasopharyngeal carcinoma and gastric carcinoma [11–14].

Altered apoptosis, cell proliferation and cell cycle regulation are key features of carcinogenesis of gastric cancer [2,15]. Although elevated HOTAIR is known to be related to invasiveness [11], metastasis, and poor prognosis, little is known about its relationship with carcinogenesis in gastric cancer.

In this study, we investigated the impact of HOTAIR on apoptosis, cell proliferation and cell cycle as indicators of the carcinogenesis of gastric cancer. We examined the mechanism of invasion and

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metastasis and analyzed the clinical significance of HOTAIR in patients with gastric cancer.

2. Materials and methods

2.1. Patients and tissue samples

Fifty fresh gastric cancer tissue and paired adjacent gastric tissue samples were obtained from 50 patients who underwent surgical resection for gastric cancer at Severance Hospital, Yonsei University College of Medicine. All samples were frozen in liquid nitrogen immediately after resection and stored at −80 °C until use. The mean age of patients was 60.7 (39–79) years and the male/female ratio was 2.2/1. This study was approved by the Ethics Committee of Yonsei University.

2.2. Cell lines and cell culture

A total of 22 gastric cancer cell lines were used. The Yonsei Cancer Center (YCC) series had been established by the Cancer Metastasis Research Center, Seoul, Korea, through isolation of ascites or peripheral blood from advanced gastric cancer patients. Other cell lines were obtained from the Korean Cell Line Bank (KCLB, SNU, Seoul, Korea) and the American Type Culture Collection (ATCC, Rockville, MD, USA). Human gastric cancer cells were cultured in RPMI-1640 medium (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The gastric cancer cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. Small interfering RNA (siRNA) transfection

MKN 28, MKN74 and AGS cells (2×10^5) were plated in 6-well culture plates and transfected after incubation for 24 h. KATO III cells (3×10^5) were plated in 6-well culture plates and immediately transfected with two si-HOTAIR or negative control siRNA (Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Target sequences for HOTAIR siRNAs: si-HOTAIR1, Sense: 5′-GAACGGGAGUACAGAGAG AUU-3′, Antisense: 5′-AAUCUCUCUGUACUCCCGUUC-3′; si-HOTAIR2, Sense: 5′-CCACAUGAACGCCCAGAGAUU-3′, Antisense: 5′-AAUCUCUGGGCGUUCAUGUGG-3′ [10].

2.4. Total RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted from gastric cancer tissues and cell lines using TRIzol reagent (Invitrogen). cDNA was synthesized from 2.0 μg of total RNA according to the manufacturer's instructions. The level of HOTAIR was measured by real-time PCR using iQ SYBR Green Supermix (Applied Biosystems Inc., Carlsbad, CA, USA). Normal gastric RNA was purchased from Agilent Technologies (Santa Clara, CA, USA) and the HOTAIR ct value was normalized to U6. Target sequences for HOTAIR and U6 are listed as: HOTAIR (Forward: GGTAGAAAAAGCAACCACGAAGC, Reverse: ACATAAACCTCTGTCTG TGAGTGCC); U6 (Forward: CTCGCTTCGGCAGCACA, Reverse: AACGCTTCAGGAATTTGCGT) [10,16].

2.5. Cell proliferation and luminescence assays

Gastric cancer cells were transfected with si-HOTAIR and incubated up to 72–96 h. Cell viability was detected by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, WI, USA) in 96-well culture plates at different time points. The number of viable cells was determined by reaction with MTS reagent for 1 h in the dark. The products of the reaction were measured by ELISA. For the caspase Glo 3/7 assay, the transfected cells (2×10^4) were re-plated in multiwell plates of a 96-luminometer using a 1:1 ratio of Caspase-Glo 3 and 7 Reagent (Promega) volume to sample volume. The plate was incubated in the dark for 1 h at 37 °C. Luminescence activity was measured using a Luminescence Microplate Reader (Molecular Devices Co., Sunnyvale, CA, USA).

2.6. Apoptosis analysis

The transfected KATO III cells were washed with PBS and resuspeded in 1× binding buffer (BD biosciences, San Jose, CA, USA). Fluorescein isothiocyanate (FITC) Annexin V and propidium iodide staining was performed using the FITC Annexin V detection kit (BD biosciences) according to the manufacturer's protocol. The ratio of percentage apoptosis was measured by flow cytometry (BD biosciences).

2.7. Cell cycle analysis

KATO III cells were transfected with 100 nM of si-HOTAIR, washed with PBS and fixed with 75% ethanol overnight at −20 °C. Cells were resuspended in PBS and treated with RNase for 30 min at room temperature. The cell nucleus was stained with propidium iodide (Sigma, Saint Louis, MO, USA) and incubated for 20 min in the dark. Cell cycle phases were determined by flow cytometry (BD biosciences) and analyzed with FlowJO 7.6 program.

2.8. Invasion assay

The matrigel invasion assay was carried out using BD biocoat trans-wells (BD biosciences) according to the manufacturer's protocol. MKN 28 and AGS cells were transfected with si-HOTAIR or si-CT. After 48 h, the transfected cells were re-plated in the upper chamber containing RPMI-1640 medium. The lower chamber was filled with RPMI-1640 medium containing 10% FBS. After 24 h, non-invading cells within the insert chamber were removed and the upper layer of the trans-well was wiped with a cotton swab. The membrane of the bottom part of the upper chamber was fixed and stained with Diff-Quik solution (Dade Behring Inc., Newark, DE). The invading cells on the membrane were counted under a bright-field microscope.

2.9. Scratch wound healing assay

MKN 28 and AGS cells transfected with si-HOTAIR, or control si-CT, were re-seeded in 24-well cultured plates. When cells formed an approximately 90% confluent monolayer, scratch wound healing was induced using a P-20 tip. The width of scratched cells was measured at 0 and 24 h under bright-field microscopy.

2.10. Western blot

The cells were lysed in 1× RIPA buffer (Cell Signaling Technology, MA, USA) containing protease inhibitor. Extracted proteins were separated by 8–10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked for 1 h at room temperature in tris–phosphate buffer containing 0.1% Tween 20 with 5% skim-milk (BD biosciences), and subsequently incubated at room temperature for 1–2 h with primary antibodies.

Primary antibodies were epithelial marker E-cadherin (1:1000, BD biosciences); mesenchymal marker N-cadherin (1:1000, BD biosciences), ZEB1 (1:1000, Cell Signaling Technology) and Snail (1:1000, Cell Signaling Technology); and cytochrome c (1:1000, BD biosciences) and poly(ADP-ribose) polymerase (1:1000, Cell Signaling Technology). For an internal loading control, the same membrane was stripped and reprobed with antibody against β-actin (1:5000, Bioworld Technology, Louis Park, MN, USA). The signal was developed in ECL solution (GenDEPOT, Barker, TX, USA) and exposed to an Image Quant LAS 4000 bio-molecular imager for 10 s–6 min.

2.11. Statistical analysis

All analyzing data for continuous and categorical variables are presented as the mean ± standard error and the number of lesions with the percentage. Statistical tests used to compare the measured results included the *t*-test, χ^2 test, and Fisher's exact test. The expression of HOTAIR in gastric cancer were categorized into low and high based on the median of HOTAIR expression. The Kaplan–Meier method and log-rank test were used for survival analysis. In addition, the Cox proportional hazard model was used for adjusting possible confounding variables including age, sex, differentiation, and stage. A value of $P < 0.05$ was regarded as a statistically significant difference for comparisons between groups. All statistical procedures were conducted using the statistical software SPSS for Windows (version 18.0; SPSS Inc., Chicago, IL, USA) with the exception of the survival analysis, which was performed in R (version 2.15.3; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Altered expression of HOTAIR in gastric cancer cell lines and gastric cancer tissues

HOTAIR was expressed at various levels in 22 gastric cancer cell lines compared to normal gastric RNA (Fig. 1A). Nineteen cell lines showed elevated HOTAIR expression compared to normal gastric RNA. Gastric cancer cell lines had an average of 43-fold increase in HOTAIR expression compared to normal gastric RNA. HOTAIR was detected in 48 of 50 gastric cancer tissues and the expression of HOTAIR was significantly higher in cancer tissues compared to adjacent normal tissues ($P = 0.007$) (Fig. 1B).

3.2. The expression of HOTAIR and clinicopathologic characteristics of gastric cancer

Clinicopathologic features were analyzed according to the level of HOTAIR expression (Table 1). Lymphovascular invasion and lymph node metastasis were more common in the high level of HOTAIR group (high vs. low; lymphovascular invasion, 68.8% vs. 31.3%, $P = 0.038$; lymph node metastasis, 72.0% vs. 28.0%, $P = 0.045$). In addition, tumors in the high level of HOTAIR group showed a more advanced TNM stage than those in the low level of HOTAIR group (high vs. low; stage III, 74.1% vs. 25.9%, $P = 0.012$). Age, sex and histologic differentiation were not related to HOTAIR expression. Kaplan–Meier plots were used to illustrate disease-free survival and overall survival according to the level of HOTAIR in patients who underwent surgery (Fig. 1C). The 2-year disease-free survival was 69.1% [95% confidence interval (C.I.), 53.2–89.6%] in the high level of HOTAIR group and 88.5% (95%

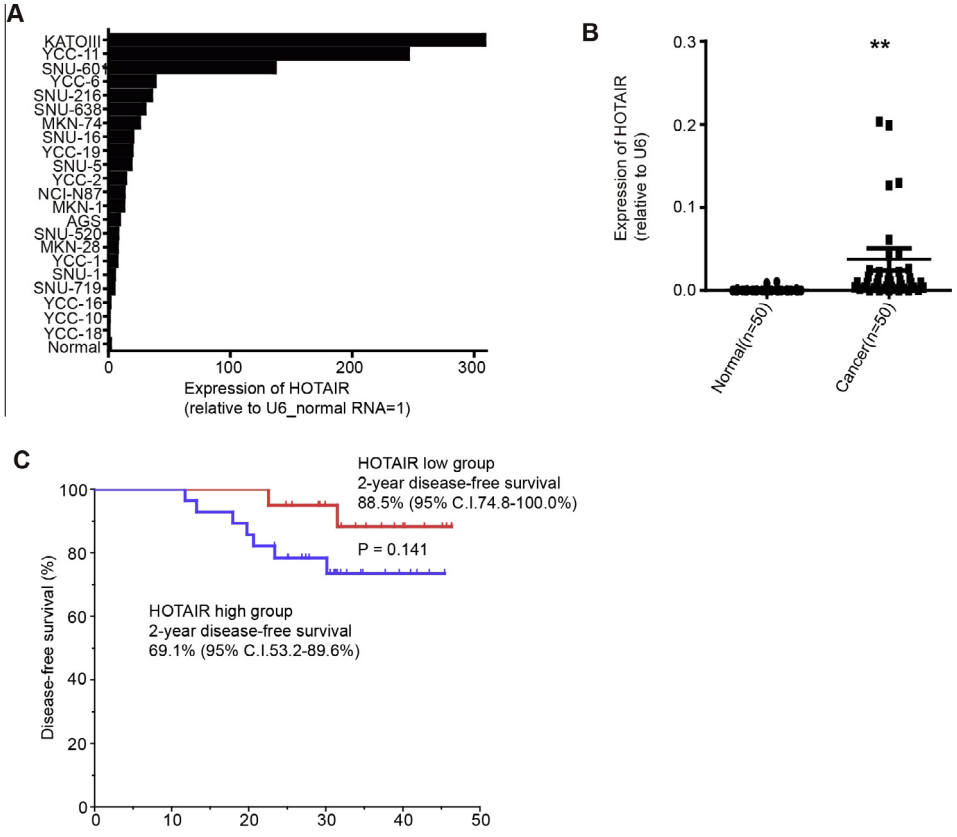

Fig. 1. Expression of HOTAIR was upregulated in gastric cell lines and tissues. The relative expression of HOTAIR was measured by quantitative real-time PCR in 22 gastric cancer cell lines (A) and 50 fresh gastric cancer tissues and paired adjacent gastric tissues (B). The expression of HOTAIR was calculated with the $2^{-\Delta\Delta CT}$ method using U6 levels for normalization. Kaplan–Meier plots show disease-free survival and overall survival according to the level of HOTAIR in patients who underwent surgery (C). Data shown represent the mean ± s.e.m. The asterisk represents a statistically significant difference compared with scrambled control (**$P \leqslant 0.01$).

Table 1
Increased expression of HOTAIR in 50 gastric cancer tissues compared to adjacent normal tissues.

Variables		Number	HOTAIR expression		*P*-Value
			Low	High	
Age (years)	<65	25	9 (36.0)	16 (64.0)	0.406
	⩾65	23	11 (47.8)	12 (52.2)	
Sex	Male	33	13 (39.4)	20 (60.6)	0.636
	Female	15	7 (46.7)	8 (53.3)	
CEA	⩽5	38	18 (47.4)	20 (52.6)	0.16
	>5	10	2 (20.0)	8 (80.0)	
Ca19-9	⩽37	42	19 (45.2)	23 (54.8)	0.379
	>37	6	1 (16.7)	5 (83.3)	
Lauren's classification	Intestinal	26	9 (34.6)	17 (65.4)	0.238
	Diffuse	20	11 (55.0)	9 (45.0)	
	Mixed	2	0 (0.0)	2 (100.0)	
Differentiation	Well to moderate	24	7 (29.2)	17 (70.8)	0.099
	Poorly	12	8 (66.7)	4 (33.3)	
	Signet ring	12	5 (41.7)	7 (58.3)	
Lymphovascular invasion	Absent	16	10 (62.5)	6 (37.5)	0.045
	Present	32	10 (31.3)	22 (68.8)	
Depth of tumor invasion	T1/T2	10	6 (60.0)	4 (40.0)	0.282
	T3/T4	38	14 (36.8)	24 (63.2)	
Lymph node metastasis	Absent	23	13 (56.5)	10 (43.5)	0.038
	Present	25	7 (28.0)	18 (72.0)	
Stage	I, II	21	13 (61.9)	8 (38.1)	0.012
	III	27	7 (25.9)	20 (74.1)	

C.I., 74.8–100.0%) in the low level of HOTAIR group. Disease-free survival in the low level of HOTAIR group tended to be superior to that in the high level of HOTAIR group; however, there was no significant statistical difference ($P = 0.141$). Cox proportional hazard models for recurrence and death revealed that the level of HOTAIR expression was not an independently associated factor for recurrence (hazard ratio (95% C.I.), 2.209 (0.533–9.163) (data not shown).

3.3. Knockdown of HOTAIR inhibited cell proliferation and induced apoptosis

We examined whether knockdown of HOTAIR in KATO III cells affected cell growth by determining the number of viable cells through an MTS assay. Treatment of gastric cancer cell lines with si-HOTAIR resulted in a significant reduction in HOTAIR expression compared to the siCT (Fig. 2A). Moreover, Inhibition of HOTAIR by

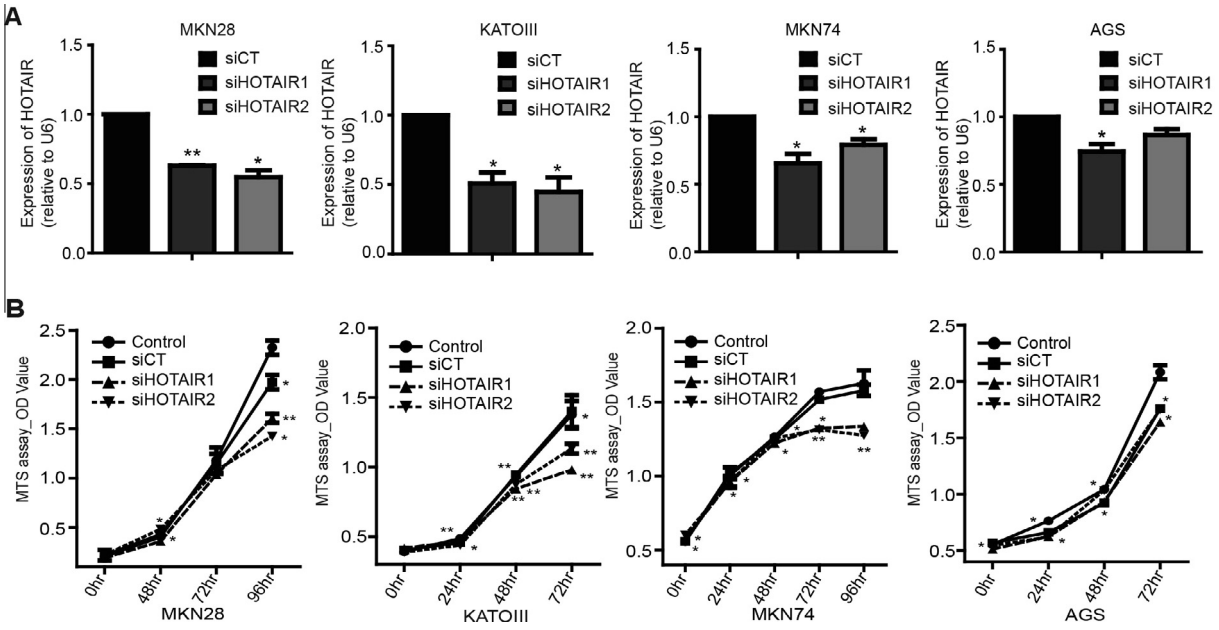

Fig. 2. siHOTAIR inhibited gastric cancer cell proliferation. KATO III, MKN 74, MKN 28 and AGS cells were transfected with siHOTAIR1, siHOTAIR2 or scrambled RNA (siCT), and expression of HOTAIR (A) and cell viability (B) was detected by MTS assay in gastric cancer cell lines. The data shown in panels are representative of three independent experiments. Data shown represent the mean ± s.e.m. The asterisk represents a statistically significant difference compared with scrambled control ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$).

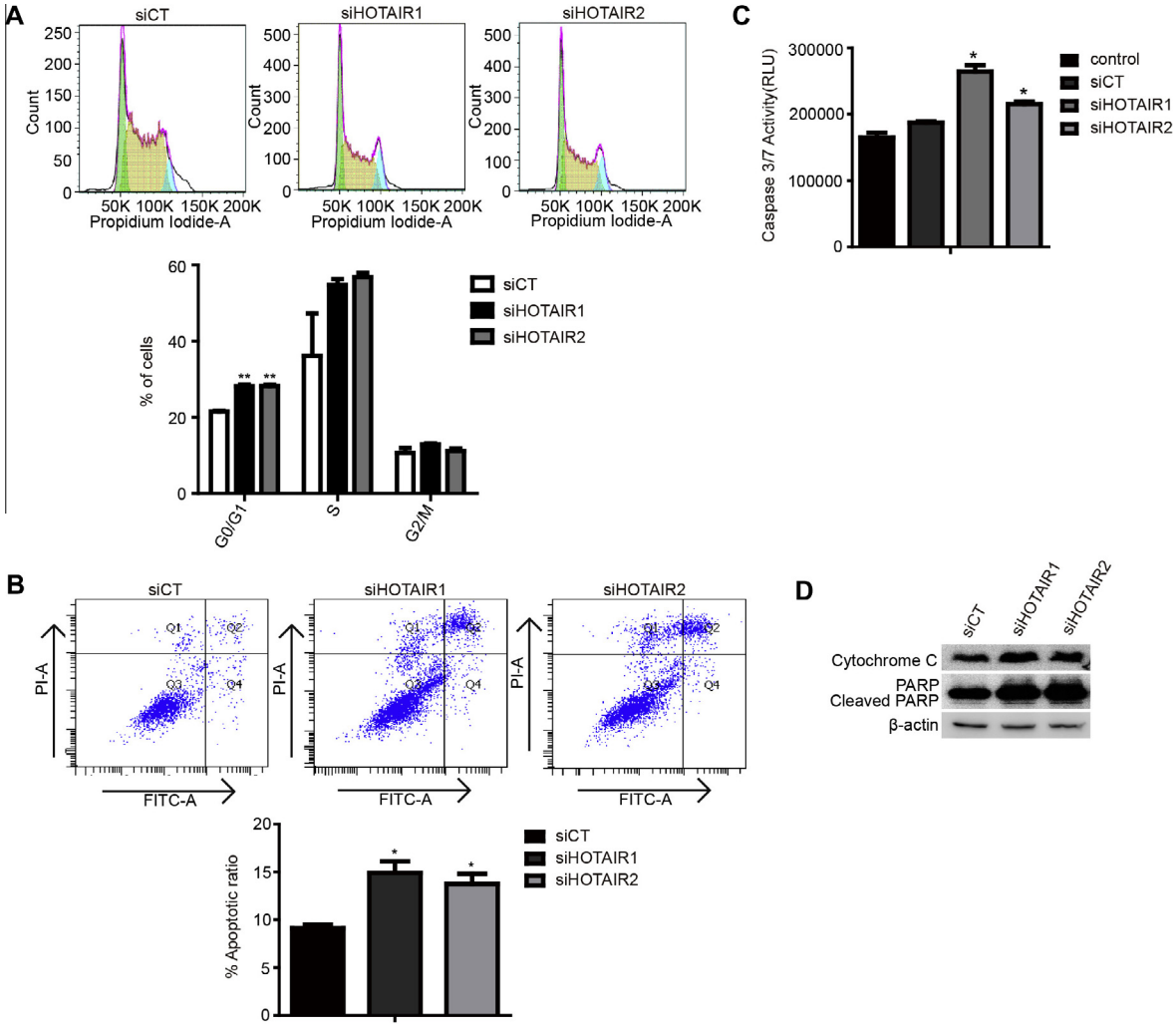

Fig. 3. siHOTAIR induced apoptosis. (A) KATO III was transfected with si-HOTAIR1 and 2, followed by cell-cycle analysis using propidium iodide staining. (B) PI/Annexin-V staining on flow cytometry was carried out in transfected KATO III cells. (C) Caspase Glo 3 and 7 assay of KATO III cells. Luminescence activity was measured using the Luminescence Microplate Reader. Data are the mean of three independent experiments ± s.e.m. (D) Western blot analysis of poly(ADP-ribose) polymerase (PARP) and cytochrome c after HOTAIR knockdown. The data shown in panels are representative of three independent experiments (*$P \leqslant 0.05$; **$P \leqslant 0.005$).

si-HOTAIR reduced cell proliferation of KATO III, MKN 74 and MKN 28 but not AGS cells (Fig. 2B). To analyze the effect of HOTAIR on cell proliferation, a cell-cycle analysis was carried out using propidium iodide staining. Knockdown of HOTAIR induced cell cycle arrest in G0/G1 (Fig. 3A). This finding suggested that HOTAIR silencing by siHOTAIR suppressed cell proliferation by arresting the cells in G0/G1 phase. To confirm the cell cycle analysis, PI/Annexin-V staining was carried out in KATO III cells after treatment with si-HOTAIR. The apoptotic ratio significantly increased by si-HOTAIR (Fig. 3B). Apoptotic profiling of KATO III by si-HOTAIR revealed that significantly increased expression of cleaved PARP-1, an enzyme with a role in repair of single stranded DNA breaks, as well as substrates Caspase-3 and Caspase-7 (Fig. 3C) and cytochrome c (Fig. 3D) which is released by mitochondria in response to pro-apoptotic stimuli. These data suggested that HOTAIR was a previously unrecognized member of the apoptosis pathway.

3.4. Knockdown of HOTAIR caused a significant decrease in cell invasiveness

To examine metastasis, we performed an invasion assay using a matrigel-based transwell. MKN 28 and AGS cells were transfected with si-HOTAIR and the invading cells on the membrane were counted on microscopy. We found that the number of invading cells was significantly decreased after treatment with si-HOTAIR compared to the siCT (Fig. 4A).

3.5. Knockdown of HOTAIR suppressed cell motility

The effects of HOTAIR silencing on migratory capacity in MKN 28 and AGS cells was confirmed. Compared to siCT, wound closure was repressed by si-HOTAIR (Fig. 4B). Knockdown of HOTAIR led to differential expression of EMT markers. Expression of the epithelial marker E-cadherin was increased. In contrast, expression of mesenchymal markers N-cadherin, Snail and ZEB1 was decreased after HOTAIR knockdown (Fig. 4C). A wound healing assay showed that knockdown of HOTAIR inhibited cell migration. Moreover, inhibition of HOTAIR disturbed the EMT in gastric cancer.

4. Discussion

In this study, HOTAIR was elevated in gastric cancer compared to normal tissue and this finding was supported by our results showing that HOTAIR could inhibit apoptosis in gastric cancer cell lines. We also clearly showed that higher expression of HOTAIR was associated with lymphovascular invasion, lymph node

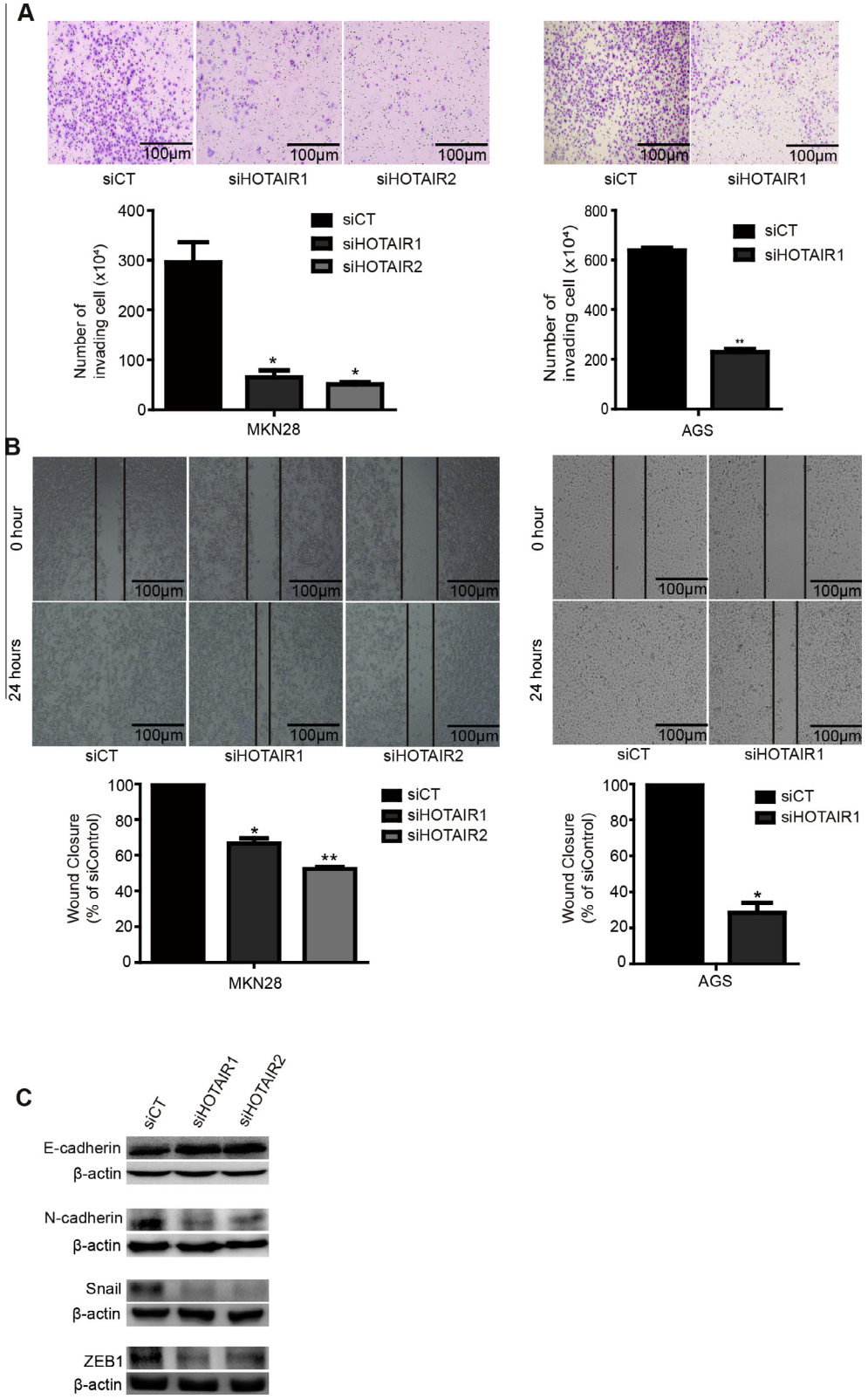

Fig. 4. HOTAIR silencing suppressed invasiveness and migratory capacity. (A) Matrigel invasion assay was carried out using an invasion chamber after treatment si-HOTAIR. (B) The width of the scratch wound was observed by microscopy at 0 and 24 h. (C) EMT markers were detected by Western blot in transfected MKN 28 cells. The figures are representative of three independent experiments Data shown represent the mean of three impendent experiments ± s.e.m. The asterisk represents a statistically significant difference compared with scrambled control ($^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.001$).

metastasis, advanced stage and poor clinical outcomes. Lastly, HOTAIR promoted the EMT through regulation of E-cadherin, N-cadherin, ZEB1 and Snail.

A considerable number of lncRNAs have been reported. While lncRNAs are known to be express in specific cell types and at specific times during development, it is not common to find one

lncRNA that is associated with a variety of cancers, like HOTAIR is. Based on the studies, HOTAIR is believed to play a role in proliferation and migration/invasion of cancer cells.

HOTAIR expression is upregulated in pancreatic tumors and si-HOTAIR decreases cell proliferation, alters cell cycle progression and induces apoptosis [17]. In breast, colon and liver cancer, HOTAIR has been shown to be unregulated, however, the involvement of HOTAIR in cell growth has not been demonstrated [10,13,18].

In our study, HOTAIR expression correlated with cell growth in some of the gastric cancer cell lines. The cell number by two different siRNAs for HOTAIR clearly decreased. This finding was reaffirmed by MTS assay. We found an increase in G0/G1 cells upon siRNA treatment of the KATO III cell line. This cell cycle regulatory checkpoint is important for preventing aberrant proliferation such as cancer, and non-proliferating cells stay in the G0/G1 phase. The inhibition of HOTAIR has previously been reported to be related to G0/G1 arrest [19]. We found that the apoptotic fraction was increased as inferred from propidium iodide staining and increased apoptotic upon knockdown of HOTAIR. Two previous reports did not detect an effect of HOTAIR on gastric cancer cells [11,20]. Xu et al. did not mention what types of cells were used, but we may assume that they used the AGS cell line. Endo et al. used MKN 74 and KATO III cell lines and shRNA to modulate HOTAIR. In our study, we extended the kinds of gastric cancer cell lines to include MKN 74, AGS, MKN 28 and KATO III to examine the effect of HOTAIR on cell proliferation. KATO III, MKN 74 and MKN 28 cells clearly showed impaired proliferation after HOTAIR silencing, whereas AGS cells showed no difference in proliferation by siRNA, similar to the findings of Xu et al. There are several possible explanations for this difference. First, the sequences and target motifs of siRNAs and shRNAs were not identical. Second, differences may exist in the biology and status of gastric cancer cells which have the same name, but were tested in different laboratories. Third, the degree of HOTAIR downregulation by siRNA was comparatively lower in AGS cells compared to other cell lines. This would lead to a different phenotypic effect.

We tried to address these concerns by using at least four different gastric cancer cell lines. Three of four gastric cancer cell lines showed reduced proliferation after si-HOTAIR and we therefore assumed that HOTAIR played a role in proliferation of gastric cancer. However, the mechanism of different responses to siRNA and the exact role of HOTAIR in the pathway of apoptosis and G0/G1 arrest should be determined in further studies.

In our study, patients with high HOTAIR expression showed more frequent lymph node metastasis and advanced stage cancer. These conditions will eventually cause poor patient prognosis. High levels of HOTAIR expression were found to correlate with both distant metastasis and lymph node metastasis in breast cancer, HCC, esophageal cancer and colorectal cancer [10,13,21,22]. Similarly, in our study, EMT associated with invasion and migration of gastric cancer cells was clearly enhanced in a HOTAIR-dependent manner. In fact, the HOTAIR-induced promotion of cancer invasion indicates that further studies may be able to identify the role of this lncRNA in the context of EMT [23]. Few previous studies have provided evidence to elucidate the actual contribution of HOTAIR to the EMT pathway. Recently Alves et al. reported that ablation of HOTAIR expression prevented EMT induction which had been activated by TGF-β1. This means that HOTAIR acts as a key regulator controlling different signaling mechanisms involved in the EMT [24]. Furthermore, the expression of snail, one of the major transcription factors regulating the EMT, can be induced by HOTAIR overexpression [10,20]. In our study, knockdown of HOTAIR decreased invasion and migration in gastric cancer cells with corresponding changes in snail, N-cadherin, ZEB1 and E-cadherin.

Although HOTAIR expression was reported with short overall survival in the diffuse type gastric cancer by previous study [11,20]. In our study, HOTAIR failed to show association with disease free survival. We think that number of patients were too few to show significant difference in this study. As survival rate is more valuable parameter than lymphovascular invasion and lymph node involvement in clinical setting, further study including more patients is needed to solve this limitation.

In conclusion, we found that HOTAIR was related with apoptosis in gastric cancer carcinogenesis, consistent with earlier reports of a correlation between elevated expression of HOTAIR and poor prognosis of patients. These results indicated HOTAIR as a likely therapeutic target in the treatment of gastric cancer.

Acknowledgments

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