Investigation of mechanistic role of HOX transcript antisense intergenic RNA and Protocadherin10 in gastric carcinogenesis

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Investigation of mechanistic role of HOX transcript antisense intergenic RNA and Protocadherin10 in gastric carcinogenesis

Directed by Professor Sang Kil Lee

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

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December 2020

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The Graduate School
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December 2020

ACKNOWLEDGEMENTS

I'm truly happy for submitting my doctoral thesis.
Firstly, I've been able to get a degree thanks to my supervisor professor, Sang Kil Lee. He provided me with various supports in my doctor's course. And I'd like to thank to Dr. Jung-Ho Yoon and Hyo Joo Byun. I was very happy to have opportunities of research with them. I've learned many things from Professor Lee and Dr. Yoon. I also appreciate to Professor Yong Chan Lee, Joon Seok Lim, Hyunki Kim and Woon Geon Shin. They gave me some important advice, and it was great help to me. In addition, I'd like to send messages to my family. My parents have supported my doctor's degree for a long time. They always believed me and it was great help to me. I also thank to my two sons and my husband. I love all of them, and I'll always try to be a good mother and wife.

It was very honorable to participate in the doctor's course in graduate school of Yonsei University. I think that I've been changed better in terms of academic research during the period. Thank you for kind support.

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ABSTRACT

Investigation of mechanistic role of HOX transcript antisense intergenic RNA and Protocadherin10 in gastric carcinogenesis

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HOX transcript antisense intergenic RNA (HOTAIR), as a long non-coding RNA (lncRNA) has been reported to regulate the carcinogenesis by epigenetic mechanism in many cancers. Protocadherin 10 (PCDH10) is one of well-known tumor suppressor genes and it is frequently methylated in gastric cancers (GC). Although both HOTAIR and PCDH10 play a major role in gastric carcinogenesis, there are no reports on the interactions between the two in the

carcinogenic mechanism. Therefore, we tried to find out the function of HOTAIR related to the gastric carcinogenesis of PCDH10 in this thesis. For this purpose, we investigated mechanism of HOTAIR on apoptosis, cell proliferation, and invasiveness as indicators of carcinogenesis and metastasis of GC. Quantitative real-time reverse transcription polymerase chain reaction (PCR) and western blotting were used to examine the expression of HOTAIR and its related genes. The effect of HOTAIR on the promoter methylation of PCDH10 was analyzed by Methylation-specific PCR(MS-PCR) method. In addition, we investigated the interaction between miR-148b and HOTAIR by dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay. The expression of HOTAIR was significantly up-regulated in GC tissues ($P<0.05$) and GC cell lines ($P<0.01$), while PCDH10 was down-regulated in GC tissues ($P<0.05$). Knockdown of HOTAIR by siRNA of HOTAIR (si-HOTAIR1 and 2) significantly upregulated the mRNA/protein expression of PCDH10 and reduced the methylation of PCDH10 compared to scramble in MKN 28 and MKN 74 cells. Si-HOTAIR1 and 2 significantly reduced DNA methyltransferase 1

(DNMT1) expression, and over-expression of HOTAIR enhanced DNMT1 expression. In RIP, we found that miR-148b interacted with HOTAIR. Si-HOTAIRs increased miR-148b expression, and miR-148b mimic inversely reduced HOTAIR expression. Si-HOTAIRs and miR-148b mimic reduced DNMT1 expression and increased PCDH10 expression compared to control. Finally, we found that DNMT1 was downregulated after si-HOTAIRs and it was restored after treatment with miR-148b inhibitor. We demonstrate that HOTAIR interacts with miR-148b and DNMT1, eventually leading to PCDH10 methylation, which contributes to the progression of GC. The current data provide a better understanding for the key roles of lncRNA HOTAIR in epigenetic mechanism of GC.

Key words: HOX transcript antisense intergenic RNA (HOTAIR); protocadherin 10; microRNA 148b; gastric cancer; methylation

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Ⅰ. INTRODUCTION

Gastric cancer (GC) incidence has declined worldwide over the past half-century, however, GC remains a global health problem as the fifth leading cancer and third most common cause of cancer-related deaths worldwide.[1,2] The incidence of GC is disproportionately high in East

Asia. Overall, declining *Helicobacter pylori* (*H. pylori*) prevalence and active GC screening and surveillance have resulted in reduced GC incidence and mortality, however, the mechanism of gastric carcinogenesis is still poorly understood.

The concept of "field cancerization", first introduced by Slaughter et al. in 1953, proposes that normal tissue adjacent to the tumor harbors specific molecular characteristics that can eventually lead to the development of local recurrence or second primary tumors.[3] A field for cancerization is formed by the accumulation of genetic and/or epigenetic alterations in normal-appearing tissues and can correlate with risk of cancer development.[4] The most widely studied epigenetic modification is DNA methylation.[4] Inactivation of tumor-suppressor gene through promoter methylation is known to play an important role in the development of GC.[5,6] Previous studies showed that PCDH10 is an important tumor suppressor gene with key roles of suppressing cell proliferation, inducing apoptosis, and inhibiting cell invasion in the development of multiple cancer including GC.[7,8]

Recent studies have indicated that long non-coding RNA (lncRNAs) are extensively associated with diverse chromatin remodeling complexes and target them to specific genomic loci to alter DNA methylation or histone status.[9,10] Over the last decade, remarkable progresses about GC-associated lncRNAs have been achieved.[11] Although thousands of lncRNAs have been identified, the exact function of most lncRNAs remains unknown, and there is still requirementss for characterization to reveal the mechanisms by which they regulate biological processes.

HOX transcript antisense intergenic RNA (HOTAIR) is one of the well-studied lncRNAs that regulate gene expression by mediating the modulation of chromatin structure.[12-19] HOTAIR acts as a scaffold of histone modification complexes to coordinately interact with polycomb repressive complex (PRC) 2 and LSD1 histone modifiers. In our previous study, we showed HOTAIR promotes carcinogenesis and invasion of gastric adenocarcinoma.[20] Previous genome-wide and microarray studies reported changes in the expression of tumor-associated genes following HOTAIR overexpression.[13] In breast cancer,

PCDH10 was transcriptionally repressed upon HOTAIR expression and de-repressed upon concomitant PRC2 depletion.[13] PCDH10 is a member of the protocadherin gene family, which is a direct p53 transcriptional target and regulate tumor cell motility and further cell migration.[21]

We hypothesized that HOTAIR may regulate PCDH10 expression by promoter methylation and influence the early-stage gastric carcinogenesis. Until now, several studies have documented the important prognostic role of HOTAIR on GC, however, studies on how exactly HOTAIR contributes to gastric carcinogenesis are needed. The epigenetic alteration of tumor suppressor genes such as PCDH10 is an important step in the early phase of gastric carcinogenesis, and the research to find out whether HOTAIR is involved in this process and its mechanism will be a very important study to find a way to prevent GC. This study aimed to investigate the mechanistic role of HOTAIR and PCDH10 in GC from the epigenetic perspectives.

Ⅱ. MATERIALS AND METHODS

1. Cell lines and cell culture

A total of 5 GC cell lines (Kato III, MKN-28, MKN-45, MKN-74, AGS) and normal gastric GES-1 cell were used. Human GC cells and GES-1 cell were cultured in RPMI-1640 medium (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. The GC cells and GES-1 cell were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37℃.

2. Patients and tissue samples

Forty-nine fresh GC tissue and paired adjacent non-tumor gastric tissue samples were obtained from patients who underwent surgical resection or endoscopic resection for gastric cancer at Kangdong Sacred Heart Hospital, Hallym University College of Medicine. All samples were frozen in liquid nitrogen immediately after resection and

stored at -80℃ until use. The mean age of patients was 62.2±1.7 years and the male/female ratio was 65.3%. This study was approved by the Ethics Committee of Kangdong Sacred Heart Hospital and written informed consent was obtained from all patients (IRB no. 2017-11-007).

3. RNA extraction, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from GC tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2.0 μg of total RNA with Superscript II (Invitrogen). The level of HOTAIR was measured by real-time PCR using iQ SYBR Green Supermix (Applied Biosystems Inc., Carlsbad, CA, USA). The Ct value of the sample was normalized to the U6 or GAPDH expression, and the $2^{-\triangle\triangle Ct}$ value was calculated. Primers used for qRT-PCR are shown in Table 1.

Table 1. Primer sequences used for qRT-PCR

Primer name	Direction	Sequences (5' to 3')
HOTAIR	Forward	TGGGAGTGTGTTTTGTTGGA
	Reverse	CTACACAACCCCTTCGCTTC
PCDH10	Forward	AACGGTGGAGATGAGGACAG
	Reverse	TCTCCGGATGGATGTTCTTC
DNMT1	Forward	GCCTCTCTCCGTTTGGTACA
	Reverse	TCGGAGGCTTCAGCAGAC
EZH2	Forward	CCTCGAGTACTGTGGGCAAT
	Reverse	CACTTTGCAGCTGGTGAGAA
SUZ12	Forward	AGTTACTCGGCCTCCTCCTC
	Reverse	AGGAAAAGCTCGTGGTCAGC
p53	Forward	CACATGACGGAGGTTGTGAG
	Reverse	ACACGCAAATTTCCTTCCAC

U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCAGGAATTTGCGT
GAPDH	Forward	CCGGGAAACTGTGGCGTGATGG
	Reverse	AGGTGGAGGAGTGGGTGTCGCTGTT
miR-148b		UCAGUGCAUCACAGAACUUUGU

HOTAIR, HOX transcript antisense intergenic RNA; PCDH10 Protocadherin 10; DNMT1, DNA-methyltransferase 1; EZH2, Enhancer of zeste 2; SUZ12, Suppressor of zeste 12; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; mir148b, microRNA 148b

4. Small interfering RNA (siRNA) transfection

MKN 28 and MKN74 cells (2 x 10^5 cells) were plated in 6-well culture plates and transfected after incubation for 24 h. Cells were transfected with two si-HOTAIRs or negative control siRNA (Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Target sequences for HOTAIR siRNAs are shown in Table 2.

Table 2. SiRNAs targeting lncRNA HOTAIR

Gene	Direction	Sequence (5' to 3')
Si-HOTAIR 1	Sense	50-GAACGGGAGUACAGAGAGAUU-30
	Antisense	50-AAUCUCUCUGUACUCCCGUUC-30
Si-HOTAIR 2	Sense	50-CACAUGAACGCCCAGAGAUU-30
	Antisense	50-AAUCUCUGGGCGUUCAUGUGG-30

5. HOTAIR overexpression plasmid construction

To overexpress HOTAIR in GC cell, we used human HOTAIR cDNA (Addgene, Plasmid #26110, Cambridge, MA) as reported previously.[22] The HOTAIR cDNA was amplified by PCR and the resulting product was inserted in the pcDNA3.1 vector (Addgene, Plasmid #47388) using TOPO cloning (Invitrogen) following the manufacturer's protocol. AGS cell was transfected with 1ug

pcDNA3.1HOTAIR (pcDNA-HOTAIR) using Lipofectamine 2000 (Invitrogen) for 24 h.

6. Cell proliferation analysis

MKN 28 and MKN 74 cells were transfected with 50nM si-HOTAIRs and AGS cell was transfected with pcDNA-HOTAIR from 0 to 72 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. Luminescence activity was measured using a Luminescence Microplate Reader (Molecular Devices Co., Sunnyvale, CA, USA).

7. Apoptosis analysis and ELISA

MKN28 and MKN74 cells were transfected with siHOTAIR1, siHOTAIR2 or si-Control (siCT) and washed with 1X phosphate buffered saline (PBS; Thermo Scientific). After 48 h, the cells were

pelleted and washed with PBS. After washing with 1X binding buffer (BD Bioscience, San Jose, CA, USA), the cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and PI, using a FITC-Annexin V kit (BD Bioscience, San Jose, CA, USA). The apoptotic ratio was measured using flow cytometry (BD Bioscience, San Jose, CA, USA). To determine the quantitative relationship, caspase 3/7 protein levels were measured by ELISA after treatment with si-HOTAIRs.

8. Cell cycle analysis

MKN28 and MKN74 cells were transfected with 100 nM of siHOTAIR1, siHOTAIR2 or siCT, then 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 FlowJO7.6 program.

9. Invasion assay and migration assay

To perform invasion assay, the matrigel invasion assay was carried out using BD biocoat trans-wells (BD bioscience, San Jose, CA, USA). MKN 28 and MKN 74 cells were transfected with si-HOTAIRs or si-CT. After 48 hours, 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 48 hours, 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). Invading cells were visualized using a virtual microscope (BX51; Olympus, Tokyo, Japan) in five random fields, counted, and averaged. For migration analysis, GC cells were transfected with si-HOTAIR and si-CT. The cells were grown for 48 hours, and a wound was then generated using a P-20 tip. The width of scratched cells was measured at 0 and 48 hours under bright-field microscopy.

10. Western blot

The cells were lysed in 1 X 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. The following primary antibodies were used for Western blot analysis: PARP (Cell Signaling Technology, #9542), bcl-2 (Cell Signaling Technology, #2870), bcl-xl (Cell Signaling Technology, #2764), p53 (Santa Cruz Biotechnology, sc-126), cleaved caspase-9 (Cell Signaling Technology; 9504), SUZ12 (Abcam, Cambridge, MA, ab12073), PCDH10 (Thermo Scientific, Cell Signaling Technology, #7237), PA5-31042), β-actin (Bioworld Technology, Louis Park, MN, USA, AP0060). EZH2 (Abcam; ab186006) and DNMT1(Abcam; ab13537).

11. RNA immunoprecipitation (RIP)

For immunoprecipitation of endogenous RNA-protein complexes, cells were lysed with IP buffer (Thermo Fisher Scientific, USA) and resuspended in RNA immunoprecipitation **(**RIP) buffer (Abcam) with RNase inhibitor (GenDEPOT) and protease inhibitor (GenDEPOT). For shearing of chromatin, we used 20 cycles of shearing under cooling conditions, with 15s on and 30s off for each cycle (170-190W) and water bath sonication. After sonication, the antibodies were added to the supernatant obtained by centrifugation and then incubated overnight at 4℃ with a rotator. After incubation, 20μL of MagnaChip protein magnetic beads (Millipore) was added, and samples were reacted on a rotator at 4℃ for 1 hour. After washing twice with RIP buffer, samples were dissolved with TRIzol reagent or RIPA.

12. Methylation specific PCR (MS-PCR)

Genomic DNA was extracted from MKN 28 and MKN 74 cells using a DNeasy

Blood & Tissue kit (Qiagen, Valencia, CA, USA). An EZ DNA methylation-gold kit (ZymoResearch, Irvine, CA, USA) was used for DNA bisulfate transformation. The following methylation-specific PCR (MS-PCR) primers for PCDH10 were used as below: Methylation (forward: GTTAGGGAGGATGGATGTAAGTATC, reverse: GCGAAATAAAAACAATAAAACGAC), and un-methylation (forward: GTTAGGGAGGATGGATGTAAGTATT, reverse: CCCACAAAATAA AAACAATAAAA AA).

13. Luciferase reporter assay

For the luciferase reporter assay, MKN 28 and MKN 74 cells were co-transfected with miRNAs (miR148b mimics or miR148b inhibitors; Biomics Biotechnologies Co., Ltd., Nantong, China) and reporter vectors (pmirGLOWT or pmirGLOMUT) using Lipofectamine 2000. Luciferase activity was assayed 48 h after transfection using a Dual Luciferase Reporter Assay system (Beyotime Institute of

Biotechnology, Haimen, China). The values were normalized to those obtained for miRNA negative control transfection.

14. 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, chi-square test, and Fisher's exact test. The expression of HOTAIR in GC were categorized into low and high based on the median value of HOTAIR expression. 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).

Ⅲ. RESULTS

1. HOTAIR expression in GC cell lines and GC tissues

The relative expression of HOTAIR was higher in MKN 74, MKN 28, MKN 45 and KATO III cells compared to normal gastric cell GES-1 ($P<0.01$), however, the level of HOTAIR expression in AGS cell was lower than GES-1 cell ($P<0.05$) (Figure 1A). The expression of HOTAIR in 49 GC tissues were significantly higher than adjacent non-tumor tissue ($P<0.05$) (Figure 1B). The Cancer Genome Atlas (TCGA) data showed that the expression of HOTAIR in GC tissues were significantly higher than normal gastric tissue (Figure 1C).

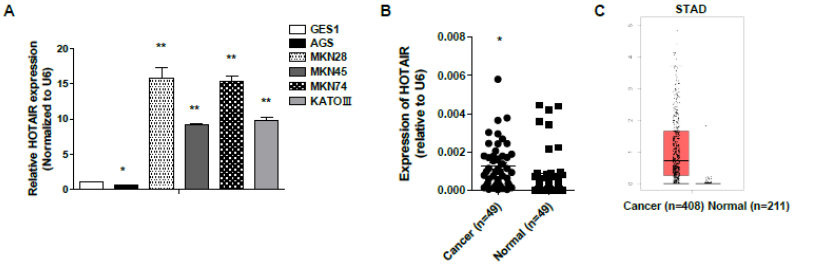


Figure 1. HOTAIR expression in GC cells and tissues. (A) The relative expression of HOTAIR by qRT-PCR in 5 GC cell lines (B) The relative expression of HOTAIR by qRT-PCR in 49 GC tissues and

paired adjacent gastric tissues (C) The relative expression of HOTAIR in 408 GC tissues and 211 normal gastric tissues from TCGA data. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

After transfection with si-HOTAIRs, HOTAIR was significantly downregulated in MKN 28 and MKN 74 cells (Figure 2, left and middle). In AGS cell, which showed low expression of HOTAIR than other cancer cell lines, we over-overexpressed HOTAIR by transfection with pc-DNA-HOTAIR (Figure 2, right).

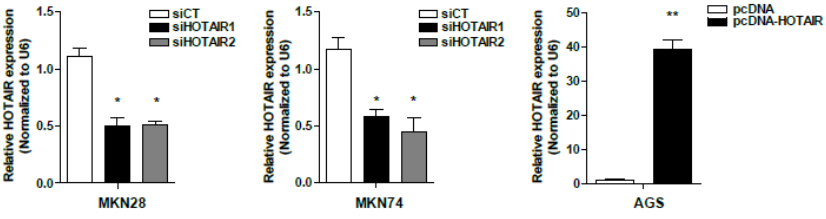


Figure 2. Generation of loss of function HOTAIR using siRNA in MKN 28 and MN 74 cell and gain of function HOTAIR using overexpression vector in AGS cell. MKN 28 and MKN 74 cells were

transfected with siHOTAIR1, siHOTIAR2 and or scrambled RNA (si-CT), and AGS cell was transfected with pcDNA and pcDNA-HOTAIR. The relative HOTAIR expression by qRT-PCR assay is shown. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

2. The effect of HOTAIR on proliferation and apoptosis of GC cells

We examined whether knockdown of HOTAIR in MKN 28 and MKN 74 cells affect cell growth by MTS assay. From 48hr after treatment with si-HOTAIR1 and si-HOTAIR2, the proliferation of MKN 28 and MKN 74 cells were significantly reduced, respectively compared to the si-CT (Figure 3). In the contrary, over-expression of HOTAIR upregulated the proliferation in AGS cell (Figure 3).

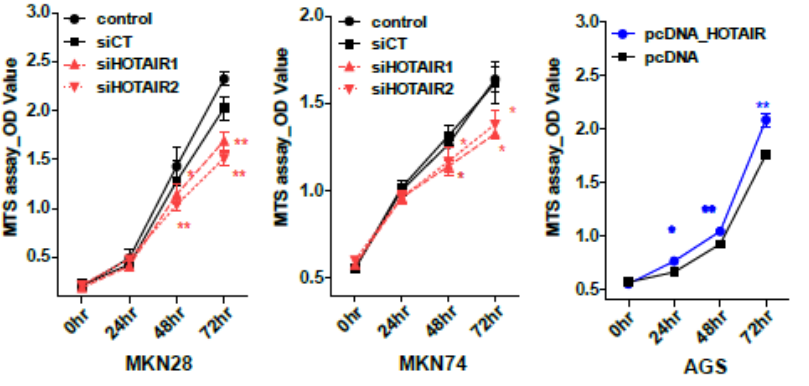
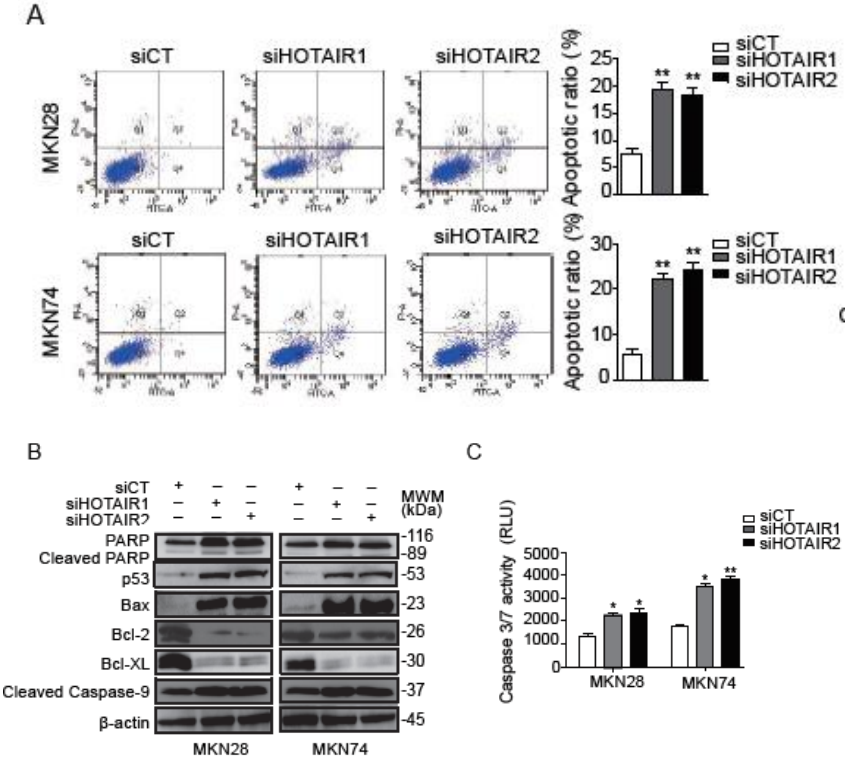


Figure 3. Knockdown of HOTAIR inhibits cell proliferation of GC cells, while over-expression promotes proliferation. Cell viability was detected by MTS assay in MKN 28, MKN 74 and AGS cells. Data are presented as the mean ± standard deviation of three independent experiments. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

We next analyzed the effect of HOTAIR on apoptosis of MKN 28 and MKN 74 cells using PI/Annexin-V. Si-HOTAIRs significant

increased in the early-to-late apoptotic ratio compared to control in both cell lines (Figure 4A). Protein levels of two anti-apoptotic markers such as Bcl-2 and Bcl-xL were decreased by si-HOTAIRs, while an induction of apoptotic factors such as p53, Bax, cleaved caspase-9 and cleaved PARP were increased compared to the control (Figure 4B). Si-HOTAIRs significantly increased caspase 3/7 activity in MKN 28 and 74 cells (Figure 4C). These data suggested that HOTAIR was a member of the apoptosis pathway. Flow cytometry analysis revealed a significant increase in the proportion of cells in the sub-G1 phase in the si-HOTAIRs compared with siCT (Figure 4D). Si-HOTAIRs subsequently reduced the proportion of cell in the S and G2 compared to siCT (Figure 4D). These data suggested that HOTAIR consistently inhibits apoptosis in GC cells.



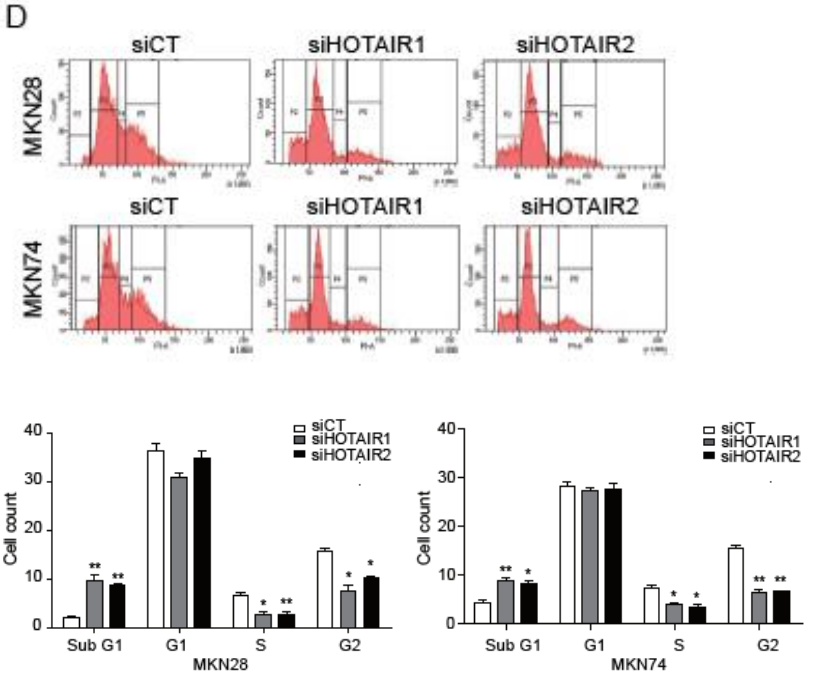


Figure 4. Knockdown of HOTAIR induces apoptosis of GC cells. (A) PI/annexin-V staining on flow cytometry was carried out in si-RNAs transfected MKN 28 and MKN 74 cells (A.; left panel), and the apoptotic ratio was measured by flow cytometry (A.; right panel). (B) Western blot analysis of apoptotic markers after treatment with si-

HOTAIRs (C) Caspase Glo 3 and 7 assays after treatment with si-HOTAIRs. (D) Cell cycle analysis by flow cytometry. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

3. The effect of HOTAIR on invasion and migration of GC cells

To investigate whether HOTAIR was involved in the invasion of GC, we performed an invasion assay. MKN 28 and MKN 74 cells were transfected with si-HOTAIRs and the invading cells on the membrane were counted on microscopy. We found that the number of invading cells were significantly decreased after treatment with si-HOTAIRs compared to the si-CT (Figure 5A). Next, the effects of HOTAIR knockdown on migratory capacity was confirmed by scratch wound healing assay. Compared to si-CT, wound closure was significantly repressed by both si-HOTAIRs (Figure 5B). This result shows that knockdown of HOTAIR inhibited GC cell migration.

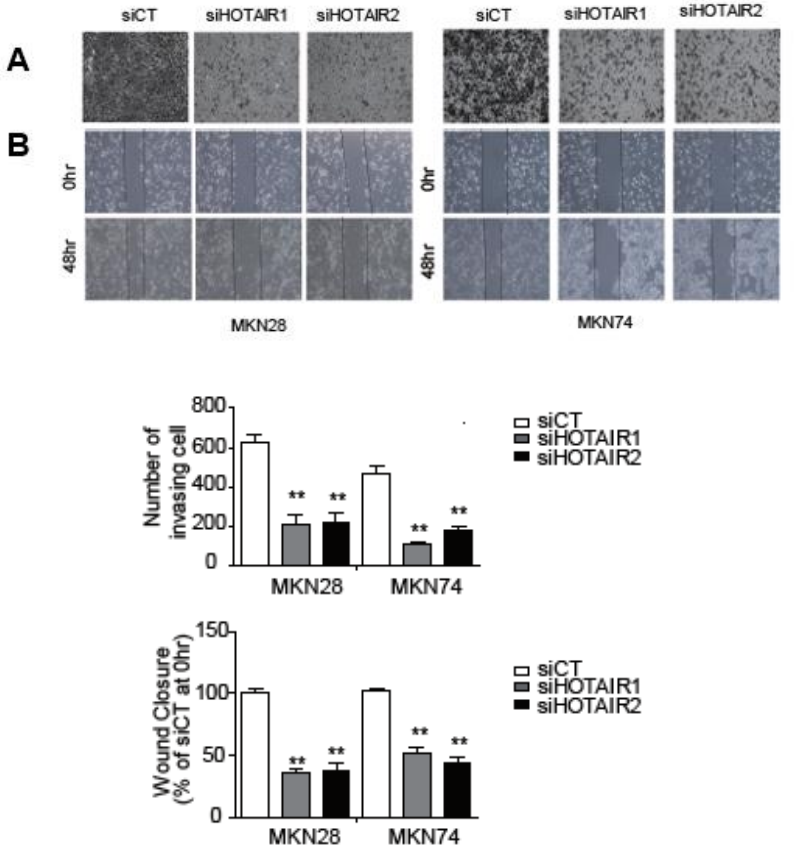


Figure 5. Inhibition of HOTAIR expression inhibits invasion and migration of GC cells. Matrigel invasion assay (A) and wound healing assay (B) after transfection with si-HOTAIRs.

4. The relation between the HOTAIR expression and clinicopathologic characteristics of GC

The HOTAIR expression in GC tissues was dichotomized by median value of HOTAIR of total patients. Clinicopathologic features of GC were compared between two groups (Table 3). Diffuse and mixed type cancer by Lauren classification were more prevalent in high HOTAIR group (high vs. low; diffuse and mixed type, 64.7% vs. 37.5%, P = 0.04). Moreover, tumors with the high level of HOTAIR group showed a more advanced TNM stage than those with the low level of HOTAIR group (TNM stage II, III; high HOTAIR vs. low HOTAIR group; 82.4% vs. 56.3%, P = 0.04). High HOTAIR group was significantly associated with advanced depth of invasion. Age, sex, proportion of *H. pylori* infection, CEA level and histologic differentiation were not related to HOTAIR expression. In addition, lymphovascular invasion and lymph node metastasis were not statistically different according to HOTAIR level.

Table 3. Clinicopathological analysis of 49 patients with gastric cancer according to HOTAIR expression

	High HOTAIR (n=17)	Low HOTAIR (n=32)	P-value
Age	62.1±13.5	62.1±11.5	0.99
Sex, male (%)	12 (70.6%)	20 (62.5%)	0.75
H. pylori infection	8/17	16/32	0.30
CEA	3.2±3.2	2.5±2.5	0.50
PCDH10	0.0014±0.0016	0.01±0.012	<0.01
Lauren's classification			
Intestinal	6 (35.3%)	20 (62.5%)	0.04
Diffuse, mixed	11 (64.7%)	12 (37.5%)	
Differentiation			
WD,MD	6 (35.3%)	15 (46.9%)	0.54
PD, signet ring cell	11 (64.7%)	17 (53.1%)	
Lymphovascular	12 (70.6%)	21 (65.6%)	0.49

invasion			
Depth of tumor			
invasion	4 (23.5%)	18 (56.3%)	
T1/T2	13 (76.5%)	14 (43.8%)	0.03
T3/T4			
Lymph node metastasis	13 (76.5%)	20 (62.5%)	0.36
TNM stage			
I	3 (17.6%)	14 (43.8%)	0.04
II, III	14 (82.4%)	18 (56.3%)	

Data are presented as mean ± S.D. *H. pylori*, *Helicobacter pylori*; CEA, carcinoembryonic antigen; WD, well differentiated; MD, moderate differentiated; PD, poorly differentiated.

5. The effect of HOTAIR on tumor suppressor gene, PCDH10 in GC

We noticed that knockdown of HOTAIR reduced PCDH10 in breast cancer cell in microarray,[13] therefore, we need to validate PCDH10 as a new target of HOTAIR in GC. PCDH10 is known as a transcriptional

target of p53,[21] therefore, we investigated the expression of PCDH10 and p53 after overexpression of HOTAIR. The over-expression of HOTAIR significantly reduced the transcription and translation of p53 in normal gastric cell line GES-1(Figure 6A). This result suggests that p53 reduction by HOTAIR may be major cause of PCDH10 transcription reduction. Next, we conducted an experiment to compare the effects of p53 and HOTAIR on PCDH10 transcription. P53 significantly increased PCDH10 transcription, however, co-infection of HOTAIR, which is expected to induce the promoter methylation of PCDH10 and reduce the PCDH10 transcription, reduced only a part of PCDH10 transcription by p53 (Figure 6B).

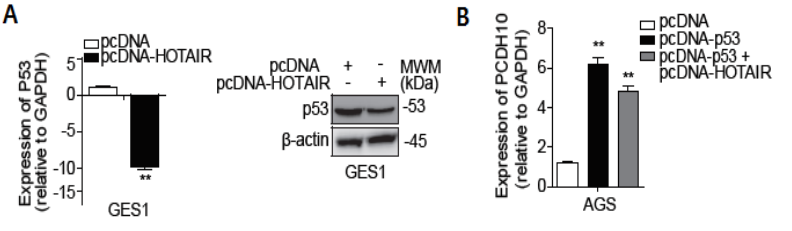


Figure 6. Upregulation of HOTAIR reduced p53 and PCDH10 expression. (A)The relative expression of p53 after transfection with pcDNA-HOTAIR in GES1 cell line by qRT-PCR and Western blot analysis. (B) The relative expression of PCDH10 after transfection with pcDNA-p53 and pcDNA-HOTAIR in AGS cell by qRT-PCR. The asterisk represents a statistically significant difference compared with scrambled control. **$P \leq 0.01$.

We performed qRT-PCR to analyze mRNA level of PCDH10 in 49 GC tissues and the adjacent non-tumor tissues. The mRNA of PCDH10 was significantly higher in adjacent non-tumor tissue compared with GC tissues ($P<0.05$) (Figure 7A). We evaluated the protein level of PCDH10 by Western blot in 4 GC tissues and the adjacent non-tumor tissues. The expression of PCDH10 was downregulated in GC tissue compared with adjacent non-tumor tissue,

and oppositely the expression of HOTAIR was upregulated in GC tissue compared to adjacent non-tumor tissue (Figure 7B).

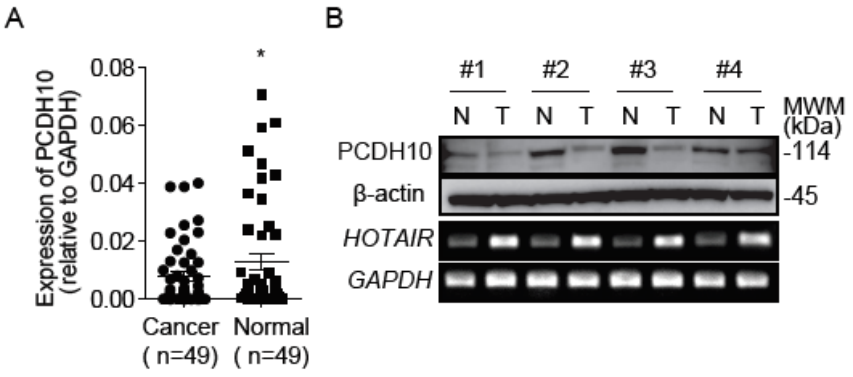


Figure 7. PCDH10 was downregulated in GC tissues. (A) The relative expression of PCDH10 by qRT-PCR in GC tissues and paired adjacent gastric tissues. (B) Western blot analysis of PCDH10 and RT-PCR analysis of HOTIAR in 4 GC tissues and paired adjacent gastric tissues. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05.

Next, we investigated PCDH10 level in GC cells after knockdown of HOTAIR. Both si-HOTAIR1 and 2 significantly upregulated mRNA of PCDH10 (Figure 8A) and protein (Figure 8B) in MKN 28 and MKN 74. In MS-PCR, si-HOTAIR1 and 2 significantly reduced the methylation of PCDH10 compared to control in in MKN 28 and MKN 74 (Figure 8C).

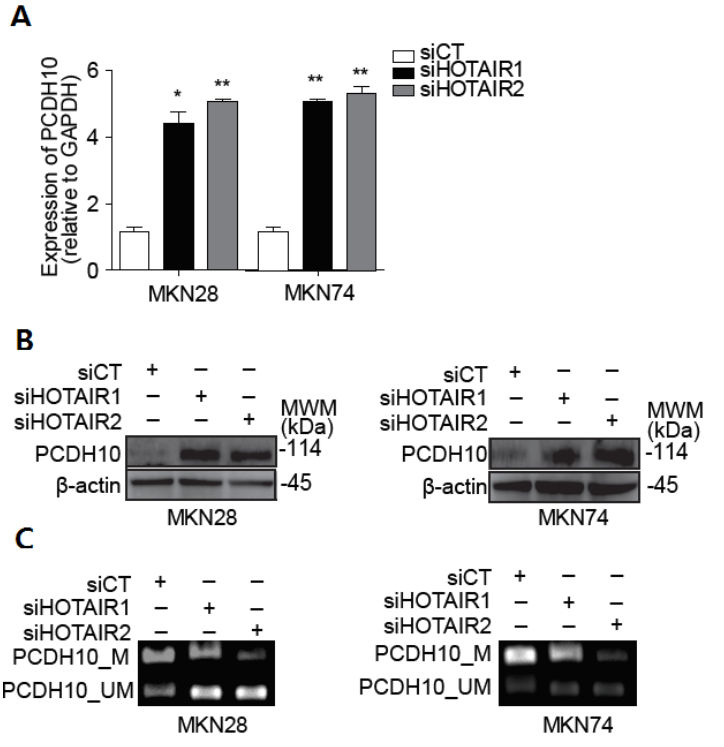


Figure 8. Knockdown of HOTAIR increased PCDH10 expression and reduced the methylation of PCDH10. (A) The relative expression of PCDH10 by qRT-PCR after transfection with si-HOTAIRs in MKN 28 and MKN 74 cells. (B) Western blot analysis of

PCDH10 after treatment with si-HOTAIRs. (C) MS-PCR analysis of PCDH10 after treatment with si-HOTAIRs. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

To investigate the mechanism of HOTAIR to control methylation of PCDH10, we performed RIP with DNMT1, EZH2 and SUZ12. HOTAIR was significantly interacted with DNMT1 and EZH2, not with SUZ12 (Figure 9A). DNMT1 and EZH2 were downregulated by si-HOTAIRs in MKN 28 and MKN 74 cells, while overexpression of HOTAIR induced upregulation of DNMT1 and EZH2 in AGS cell (Figure 9B). The result suggested that HOTAIR enhanced DNMT1 and EZH2 expression.

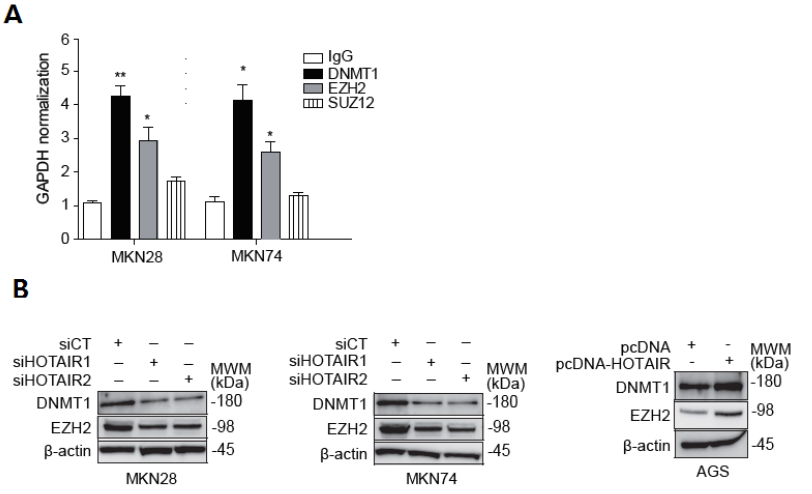


Figure 9. HOTAIR interacts with DNMT1 and EZH2. (A) The interaction between DNMT1, EZH2, SUZ12 and HOTAIR was identified by RIP analysis. The bars present relative enrichment. (B) Western blot analysis of DNMT1 and EZH2 after treatment with si-HOTAIRs in MKN 28 and MKN 74 cells and pcDNA-HOTAIR in AGS cell. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

6. HOTAIR regulates methylation of PCDH 10 as a competitive endogenous RNA by sponging miR-148b

In previous study, several miRNAs interacting with lncRNA were identified in GC by computer-aided algorithm.[23-26]. In this study, we further investigated the underlying mechanism of HOTAIR as competitive endogenous RNA (ceRNA) related with miR-148b in reference to previous study.[24] Among 5 GC cells, the expression level of miR-148b was significantly lower in 4 GC cells including MKN 28 cell and MKN 74 cell compared with GES-1 cell (Figure 10A). In RIP, miR-148b interacted with HOTAIR in both MKN 28 and MKN 74 (Figure 10B). Both si-HOTAIR1 and 2 increased miR-148b expression (Figure 10C), while the treatment of miR-148b mimic reduced HOTAIR expression (Figure 10D), indicating inverse correlation between HOTAIR and miR-148b in GC cells.

To verify whether miR-148b is regulated by HOTAIR as a ceRNA, we predicted a predicted binding site for miR-148b using the online software program,[27] and then an wild-type or mutant miR-148b target

binding sequences were cloned into the pmirGLO luciferase vector. Following co-transfection with pmirGLO luciferase construct (pmirGLO-mutant or pmirGLO-wild-type) and miR-148b mimic or inhibitor, a dual-luciferase assay was performed to determine the luciferase activity.

The MKN 28 and MKN 74 cells co-transfected with the constructs containing the pmirGLO-Mut and miR-148b mimic had significantly higher luciferase activity compared with that of those transfected with pmirGLO-WT and miR-148b mimic ($P<0.05$; Figure 10E). In addition, the relative luciferase activity showed significantly lower in MKN 28 and MKN 74 cells co-transfected with the constructs containing the pmirGLO-Mut and inhibitor of miR-148b compared with that of those transfected with pmirGLO-WT and inhibitor of miR-148b ($P<0.01$; Figure 10E).

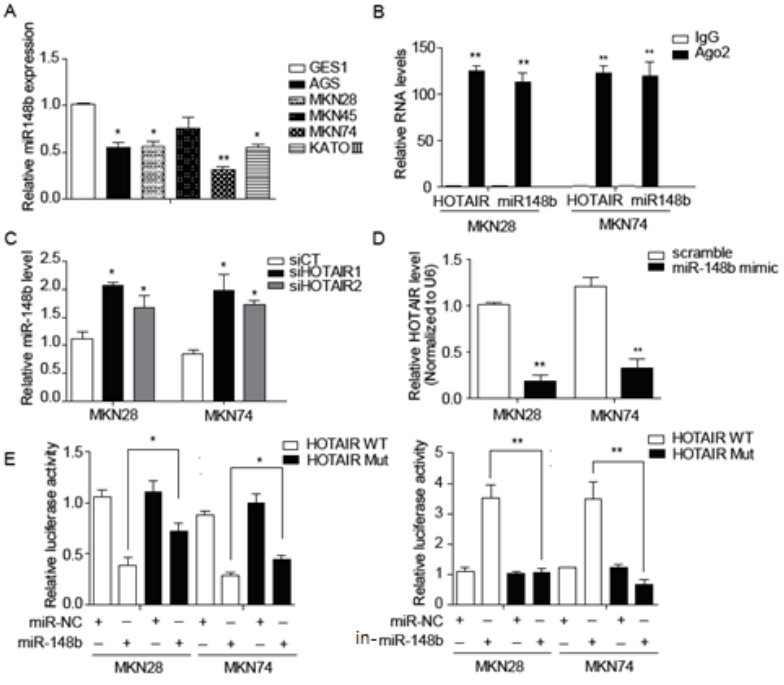


Figure 10. Reciprocal negative regulation of miR-148b and HOTAIR. (A) The relative miR-148b expression by qRT-PCR in 5 GC cell lines. (B) RIP with anti-Ago2 was performed in MKN 28 and MKN 74 cells transfected with HOTAIR and miR-148b. HOTAIR and miR-148b expression level were detected using qRT-PCR. (C) The

relative miR-148b level after transfection with si-HOTAIRs by qRT-PCR. (D) The relative HOTAIR level after transfection with miR-148 mimic by qRT-PCR. (E) The relative luciferase activity of HOTAIR after treatment with miR-148b mimic/inhibitor in MKN 28 and 74 cells transfected with wild type/mutant type HOTAIR. The asterisk represents a statistically significant difference compared with scrambled control. *P≤0.05; **P≤0.01.

In Western blot analysis, treatment with si-HOTAIRs and miR-148b mimic reduced DNMT1 expression and increased PCDH10 expression compared to control in MKN 28 cell and MKN 74 cell (Figure 11A). HOTAIR overexpression increased DNMT1 expression and reduced PCDH10 expression in AGS cell, however treatment with miR-148b mimic showed opposite result (Figure 11A). Finally, we found that DNMT1 was downregulated after si-HOTAIRs and it was restored after treatment with miR-148b inhibitor in MKN 28 and MKN 74 cells (Figure 11B).

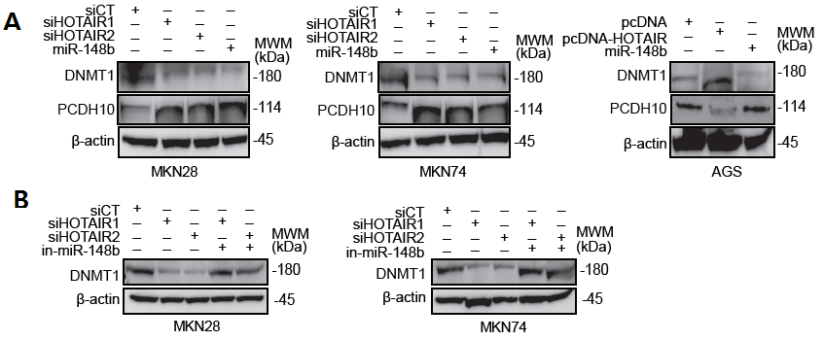


Figure 11. HOTAIR and miR-148b regulates DNMT1 and control PCDH10 expression reciprocally. (A) Western blot analysis of DNMT1 and PCDH10 after treatment with si-HOTAIRs and miR-148b mimic. (B) Western blot analysis of DNMT1 after treatment with si-HOTAIRs and miR-148b inhibitor.

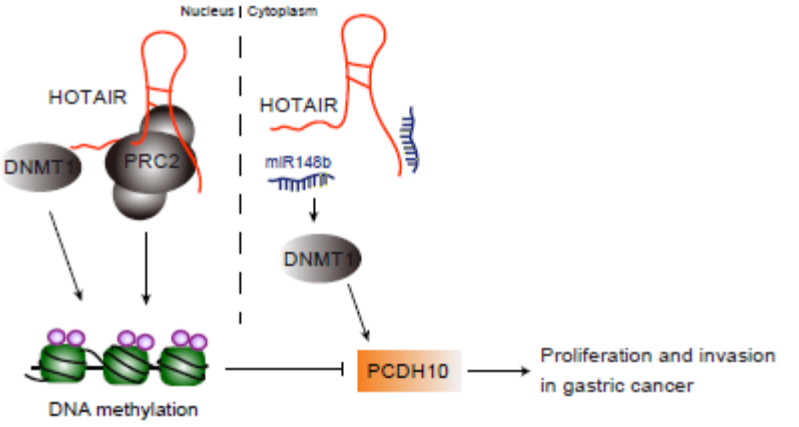


Figure 12. A schematic diagram for the epigenetic mechanism of HOTAIR in GC.

HOTAIR promotes proliferation and invasion by methylation of PCDH10 through regulating DNMT1 competitively with miR-148b in GC.

Ⅳ. DISCUSSION

In the current study, we found that lncRNA HOTAIR upregulated the expression of DNMT1, thereby promoting aberrant methylation of PCDH10, which has known to be tumor suppressor gene in GC. Moreover, we demonstrated that reciprocal negative regulation of HOTAIR and miR-148b in regulation of PCDH10 methylation. The mechanism of lncRNA on cancer progression is complex and there have been numerous reports investigating the role of lncRNA and miRNA in the carcinogenesis.[9,11,28-30] However, to our knowledge, there have been few studies that investigated complex mechanism involving methylation of tumor suppressor genes related with HOTAIR and miRNAs in GC.

HOTAIR is one of the well-studied lncRNA that regulate gene expression by mediating the modulation of chromatin structure and widely studied in various cancers.[13-15,17,19,20,31-33] Several studies found that HOTAIR plays an important role in the progression and metastasis of GC, and serve as a novel biomarker for GC.[14,20,34] Our result also showed that HOTAIR was involved in inhibition of apoptosis, proliferation, invasiveness and metastasis in GC cell lines as similar in

previous our study.[20] Further, clinicopathologic analysis in GC tissues indicated that high level of HOTAIR group showed more advanced depth of invasion and TNM staging, which was consistent with previous studies.[14,16,17,20,35]

Previous genome-wide and microarray studies reported global gene expressions that were induced or repressed as a consequence of HOTAIR overexpression, and PCDH10 was transcriptionally repressed upon HOTAIR expression in breast cancer and gastrointestinal stromal tumor (GIST).[13,22] PCDH10 belongs to a protocadherin protein family and is a potential tumor suppressor protein as the dysregulation of PCDH10 gene frequently existed in multiple human tumors.[8,21] Inactivation of PCDH10 through promoter methylation has been studied in multi-kind of human cancers including GC. [7,8,21] Consistent with previous result, the mRNA level of PCDH10 in GC tissues was significantly lower than non-tumor tissue in our study.

In this study, we investigated epigenetic mechanism of HOTAIR in GC and found that HOTAIR promoted the methylation of PCDH10 by up-regulating DNMT1. DNA methylation is mediated by DNMTs,

including DNMT1, DNMT3a and DNMT3b, which catalyze the methylation at cytosine-C5 mainly in a CpG dinucleotide context[36]. DNMT1 is the most-widely known to be responsible for maintenance of the DNA methylation.[36] Overexpression of DNMT1 has been shown in several cancers and targeting DNMT1 could be a potential target in the treatment of cancers.[36] In the previous study, we reported lncRNA LUCAT1, which modulates the stability of DNMT1, resulting in DNA methylation of tumor suppressor genes.[37] Aberrant DNA methylation in the promoter regions of tumor suppressor genes, which plays a key role in the initiation and progression of tumor, is the most well-defined epigenetic mechanism in GC.[5,38,39] Additionally, aberrant methylation of a number of genes is significantly associated with clinicopathological characteristics and clinical outcomes in GC[5]. PCDH10 promoter methylation appears to be a relatively early event during gastric carcinogenesis, and causes PCDH10 repression.[7] Thus, our findings provide a better understanding for early carcinogenesis of GC.

Furthermore, we found that miR-148b could be involved in reciprocal regulation process of PCDH10 methylation with HOTAIR. To date, researchers have found the role of lncRNAs and miRNAs in gene regulation, and bioinformatic analysis revealed the interaction between lncRNAs and miRNAs in many cancers.[23,25,31,32,40,41] Role of miR-148a was widely studied by previous researchers and Song et al reported miR-148b is frequently down-regulated in GC and acts as a tumor suppressor.[24,26] The expression of miR-148b was found to be associated with tumor size in GC patients, and the data also suggested that miR-148b can inhibit cell proliferation in vitro and in vivo.[24]

In the current study, we found inverse correlation between HOTAIR and miR-148b by qRT-PCR and luciferase reporter assay indicated that miR-148b suppresses HOTAIR by binding to HOTAIR in a sequence-specific manner. In Western blot analysis, the expression of DNMT1 and PCDH10 was regulated by HOTAIR and miR-148b subsequently. These results are similar with the previous findings that HOTAIR may act as an endogenous sponge of miR-148b, which regulates expression of the DNMT1/MEG3/p53 pathways in hepatic stellate cells and

fibrogenesis in liver.[32] Therefore, our results demonstrated the interaction between miRNA and lncRNA in the methylation of tumor suppressor gene from the perspectives of epigenetic mechanism in GC.

Finally, as seen in our and other researchers' studies,[21,22] the p53 was important for the transcription of PCDH10, and HOTAIR also reduced the expression of p53 in our study. Since our results showed that HOTAIR's effects on PCDH10 may be less than that of p53, further studies on how HOTAIR regulates p53 and how p53 regulates PCDH10 are needed to investigate the overall role of PCDH10 in GC.

Our study has some limitations. Firstly, we could not include *in vivo* model to complement our results. Even though, we collected gastric and non-cancer tissues from 49 patients with gastric cancer as a means of representative of in vivo findings to overcome this limitation, therefore, the attention is needed in the interpretation of our research results. Secondly, AGS cell showed relatively lower expression of HOTAIR level compared with other GC cell lines, similarly with previous study, the differences may reflect cancer cell variability.[20] Thirdly, we could not differentiate between cancer cell and stromal cell

to analyze HOTAIR expression in GC tissues. The localization of HOTAIR might be detected by RNA fluorescence in situ hybridization. Lastly, quite a few papers already have reported the similar HOTAIR action like this paper in cancer. Although it may be unique and clear that PCDH10 is HOTAIR's down-stream target in our results, we have not studied the relationship or differentiation with other pathways such as Wnt/beta-catenin signaling pathway.

Ⅴ. CONCLUSION

Taken together, we found novel epigenetic mechanism of HOTAIR which was involved in methylation of PCDH10 by interacting with miR-148b in GC. HOTAIR enhanced the methylation of PCDH10 by up-regulating DNMT1, and acts as a ceRNA of miR-148b, which regulated DNMT1 reciprocally. This mechanism may be a potential biomarker and therapeutic target against GC, although it is needed to be further investigated.

REFERENCES

1. Balakrishnan M, George R, Sharma A, Graham DY. Changing Trends in Stomach Cancer Throughout the World. Curr Gastroenterol Rep 2017;19:36.

2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87-108.

3. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6:963-968.

4. Baba Y, Ishimoto T, Kurashige J, Iwatsuki M, Sakamoto Y, Yoshida N, Watanabe M, et al. Epigenetic field cancerization in gastrointestinal cancers. Cancer Lett 2016;375:360-366.

5. Qu Y, Dang S, Hou P. Gene methylation in gastric cancer. Clin Chim Acta 2013;424:53-65.

6. Leung WK, Yu J, Ng EK, To KF, Ma PK, Lee TL, Go MY, et al. Concurrent hypermethylation of multiple tumor-related genes in

gastric carcinoma and adjacent normal tissues. Cancer 2001;91:2294-2301.

7. Yu J, Cheng YY, Tao Q, Cheung KF, Lam CN, Geng H, Tian LW, et al. Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric cancer. Gastroenterology 2009;136:640-651.e641.

8. Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW, Putti T, et al. Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Oncogene 2006;25:1070-1080.

9. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, Barrette TR, et al. The landscape of long noncoding RNAs in the human transcriptome. Nat Genet 2015;47:199-208.

10. St Laurent G, Wahlestedt C, Kapranov P. The Landscape of long noncoding RNA classification. Trends Genet 2015;31:239-251.

11. Li T, Mo X, Fu L, Xiao B, Guo J. Molecular mechanisms of long noncoding RNAs on gastric cancer. Oncotarget 2016;7:8601-8612.

12. Geng YJ, Xie SL, Li Q, Ma J, Wang GY. Large intervening non-coding RNA HOTAIR is associated with hepatocellular carcinoma progression. J Int Med Res 2011;39:2119-2128.

13. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010;464:1071-1076.

14. Hajjari M, Behmanesh M, Sadeghizadeh M, Zeinoddini M. Up-regulation of HOTAIR long non-coding RNA in human gastric adenocarcinoma tissues. Med Oncol 2013;30:670.

15. Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, et al. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 2013;32:1616-1625.

16. Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, et al. Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. Cancer Res 2011;71:6320-6326.

17. Li X, Wu Z, Mei Q, Li X, Guo M, Fu X, Han W. Long non-coding RNA HOTAIR, a driver of malignancy, predicts negative prognosis and exhibits oncogenic activity in oesophageal squamous cell carcinoma. Br J Cancer 2013;109:2266-2278.

18. Xu ZY, Yu QM, Du YA, Yang LT, Dong RZ, Huang L, Yu PF, et al. Knockdown of long non-coding RNA HOTAIR suppresses tumor invasion and reverses epithelial-mesenchymal transition in gastric cancer. Int J Biol Sci 2013;9:587-597.

19. Yu X, Li Z. Long non-coding RNA HOTAIR: A novel oncogene (Review). Mol Med Rep 2015;12:5611-5618.

20. Lee NK, Lee JH, Park CH, Yu D, Lee YC, Cheong JH, Noh SH, et al. Long non-coding RNA HOTAIR promotes carcinogenesis and invasion of gastric adenocarcinoma. Biochem Biophys Res Commun 2014;451:171-178.

21. Shi D, Murty VV, Gu W. PCDH10, a novel p53 transcriptional target in regulating cell migration. Cell Cycle 2015;14:857-866.

22. Lee NK, Lee JH, Kim WK, Yun S, Youn YH, Park CH, Choi YY, et al. Promoter methylation of PCDH10 by HOTAIR regulates the

progression of gastrointestinal stromal tumors. Oncotarget 2016;7:75307-75318.

23. Dong X, He X, Guan A, Huang W, Jia H, Huang Y, Chen S, et al. Long non-coding RNA Hotair promotes gastric cancer progression via miR-217-GPC5 axis. Life Sci 2019;217:271-282.

24. Song YX, Yue ZY, Wang ZN, Xu YY, Luo Y, Xu HM, Zhang X, et al. MicroRNA-148b is frequently down-regulated in gastric cancer and acts as a tumor suppressor by inhibiting cell proliferation. Mol Cancer 2011;10:1.

25. Wang G, Li Z, Tian N, Han L, Fu Y, Guo Z, Tian Y. miR-148b-3p inhibits malignant biological behaviors of human glioma cells induced by high HOTAIR expression. Oncol Lett 2016;12:879-886.

26. Xia J, Guo X, Yan J, Deng K. The role of miR-148a in gastric cancer. J Cancer Res Clin Oncol 2014;140:1451-1456.

27. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res 2014;42:D92-97.

28. Sun W, Yang Y, Xu C, Xie Y, Guo J. Roles of long noncoding RNAs in gastric cancer and their clinical applications. J Cancer Res Clin Oncol 2016;142:2231-2237.

29. Chen J, Xue Y. Emerging roles of non-coding RNAs in epigenetic regulation. Sci China Life Sci 2016;59:227-235.

30. Lin XC, Zhu Y, Chen WB, Lin LW, Chen DH, Huang JR, Pan K, et al. Integrated analysis of long non-coding RNAs and mRNA expression profiles reveals the potential role of lncRNAs in gastric cancer pathogenesis. Int J Oncol 2014;45:619-628.

31. Yu F, Chen B, Dong P, Zheng J. HOTAIR Epigenetically Modulates PTEN Expression via MicroRNA-29b: A Novel Mechanism in Regulation of Liver Fibrosis. Mol Ther 2017;25:205-217.

32. Bian EB, Wang YY, Yang Y, Wu BM, Xu T, Meng XM, Huang C, et al. Hotair facilitates hepatic stellate cells activation and fibrogenesis in the liver. Biochim Biophys Acta Mol Basis Dis 2017;1863:674-686.

33. Abdeahad H, Avan A, Pashirzad M, Khazaei M, Soleimanpour S, Ferns GA, Fiuji H, et al. The prognostic potential of long noncoding

RNA HOTAIR expression in human digestive system carcinomas: A meta-analysis. J Cell Physiol 2019;234:10926-10933.

34. Xu Z, Chen H, Yang B, Liu X, Zhou X, Kong H. The Association of HOTAIR with the Diagnosis and Prognosis of Gastric Cancer and Its Effect on the Proliferation of Gastric Cancer Cells. Can J Gastroenterol Hepatol 2019;2019:3076345.

35. Liu FT, Qiu C, Luo HL, Zhang Y, Xia GF, Hao TF, Zhu PQ. The association of HOTAIR expression with clinicopathological features and prognosis in gastric cancer patients. Panminerva Med 2016;58:167-174.

36. Xiang S, Zou P, Tang Q, Zheng F, Wu J, Chen Z, Hann SS. HOTAIR-mediated reciprocal regulation of EZH2 and DNMT1 contribute to polyphyllin I-inhibited growth of castration-resistant prostate cancer cells in vitro and in vivo. Biochim Biophys Acta Gen Subj 2018;1862:589-599.

37. Yoon JH, You BH, Park CH, Kim YJ, Nam JW, Lee SK. The long noncoding RNA LUCAT1 promotes tumorigenesis by controlling

ubiquitination and stability of DNA methyltransferase 1 in esophageal squamous cell carcinoma. Cancer Lett 2018;417:47-57.

38. Maeda M, Moro H, Ushijima T. Mechanisms for the induction of gastric cancer by Helicobacter pylori infection: aberrant DNA methylation pathway. Gastric Cancer 2017;20:8-15.

39. Tan P, Yeoh KG. Genetics and Molecular Pathogenesis of Gastric Adenocarcinoma. Gastroenterology 2015;149:1153-1162 e1153.

40. Sa L, Li Y, Zhao L, Liu Y, Wang P, Liu L, Li Z, et al. The Role of HOTAIR/miR-148b-3p/USF1 on Regulating the Permeability of BTB. Front Mol Neurosci 2017;10:194.

41. Zhang JG, Shi Y, Hong DF, Song M, Huang D, Wang CY, Zhao G. MiR-148b suppresses cell proliferation and invasion in hepatocellular carcinoma by targeting WNT1/beta-catenin pathway. Sci Rep 2015;5:8087.

ABSTRACT (IN KOREAN)

위암 발생에 관여하는 HOTAIR 와 PCDH10 의 기전 탐구

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서 승 인

HOX transcript antisense intergenic RNA (HOTAIR)는 long non-coding RNA (lncRNA)로 여러 암종에서 발암과정에 관여하는 유전자로 알려져 있다. 여러 lncRNA 의 발암기전 중에 후성유전학적 기전이 중요하며 이는 주로 종양억제유전자의 발현을 조절함으로써 암 발생에 관여할 수 있다. Protocadherin 10 (PCDH10)은 잘 알려진

종양억제유전자로 위암에서 메틸화 되어있으며 이로 인하여 발현이 억제되어 위암 발생에 관여하는 것으로 알려져 있다. 본 연구에서는 lncRNA HOTAIR 가 암 발생에 관여하는 기전에 대하여 실험을 통해 증명하며 HOTAIR 와 PCDH10 의 발암과정에 관여하는 후성유전학적 기전에 관하여 탐구하는 것을 목적으로 하였다. 또한 lncRNA 의 기전 중 microRNA 와 연관하여 경쟁적인 상호작용을 통해 표적 유전자를 조절하는 것이 알려져 있는 상태로 HOTAIR 와 연관이 있는 것으로 알려진 miR-148b 를 이용하여 추가적인 실험을 진행하였다.

49 명의 위암 조직에서 주변 정상조직에 비해 HOTAIR 발현이 높았으며 ($P<0.05$), 4 개의 위암 세포주에서도 HOTAIR 의 발현이 높았다 ($P<0.01$). 반면 PCDH10 은 위암 조직에서 주변 정상조직에 비해 낮은 발현을 보였다 ($P<0.05$). MKN 28 과 MKN 74 위암 세포에서 HOTAIR 가 apoptosis 를 차단하고 이동과 침습을 유도함을 확인하였으며 위암 조직에서 HOTAIR 과발현이 불량한 예후와 연관됨을

확인하였다. siRNA 를 이용해서 HOTAIR 의 발현을 억제하였을 때 PCDH10 의 mRNA 와 단백질이 감소함을 MKN 28 과 MKN 74 위암세포에서 확인하였고 methylation-specific PCR 을 통해 PCDH10 의 메틸화가 감소함을 확인하였다. 메틸화에 관여하는 기전을 확인하기 위하여 RNA immunoprecipitation (RIP)를 통해 HOTAIR 와 DNA 메틸 전이 효소 1 (DNMT1)의 상호작용을 확인하였으며 HOTAIR 발현억제후 DNMT1 발현이 감소함을 Western blot 에서 확인하였다. miR-148b 는 위암세포주에서 감소되어 있었으며 HOTAIR 발현을 감소시키면 miR-148b 발현이 증가하고 반대로 miR-148b 과발현시 HOTAIR 발현이 감소하는 상호 경쟁적인 관계를 보여주었다. 마지막으로 HOTAIR 억제와 miR-148b 과발현시 DNMT1 발현이 억제되며 PCDH10 발현이 증가함을 확인하였다.

결론적으로 본 연구는 lncRNA HOTAIR 가 miR-148b 와의 경쟁적 상호작용을 통해 DNMT1 발현을 조절하고 이를 통한 PCDH10 의 메틸화를 유도하여 종양억제유전자 발현을

억제함으로써 위암 발생에 관여함을 시사한다. 이번 연구를 통해 위암에서 후성유전학적 기전에 관여하는 lncRNA HOTAIR 의 역할에 대한 이해와 향후 위암에서의 바이오마커로서의 가능성을 발견할 수 있었다.

핵심되는말: Homeobox (HOX) transcript antisense intergenic RNA, protocadherin 10, 위암