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Large noncoding RNA HOTAIR enhances aggressive biological behavior and is associated with short disease-free survival in human non-small cell lung cancer

Takayuki Nakagawa [a,c], Hiroyuki Endo [a], Misa Yokoyama [a], Jiro Abe [c], Keiichi Tamai [b], Nobuyuki Tanaka [b], Ikuro Sato [d], Satomi Takahashi [c], Takashi Kondo [e], Kennichi Satoh [a,*]

[a] Division of Cancer Stem Cell, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi 981-1293, Japan
[b] Cancer Biology & Therapeutic, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi 981-1293, Japan
[c] Department of Thoracic Surgery, Miyagi Cancer Center, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi 981-1293, Japan
[d] Department of Pathology, Miyagi Cancer Center, 47-1 Nodayama, Medeshima-Shiode, Natori, Miyagi 981-1293, Japan
[e] Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo Aobaku, Sendai, Miyagi 980-8575, Japan

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ABSTRACT

HOTAIR is one of long non-coding RNAs and its expression correlates with the prognosis and metastasis in various cancers. We showed that HOTAIR expression has an important role in the development of non-small cell lung cancer (NSCLC). In this study, we examined the expression of HOTAIR in 77 NSCLCs, their corresponding normal lung tissues and 6 brain metastases by quantitative real-time RT-PCR. High expression of HOTAIR (tumor/normal ratio $\geqslant 2$) was detected in 17 patients (22.1%) and was frequently found in patients with advanced stage, lymph node metastasis or lymph-vascular invasion and short disease free interval. Furthermore, brain metastases show significantly higher HOTAIR expression compared to primary cancer tissues. HOTAIR-expressing A549 cells showed induced cell migration and anchorage-independent cell growth *in vitro*. These results indicate the expression of HOTAIR enhanced the aggressive behavior of NSCLC cells.

1. Introduction

Lung cancer is the most common cause of cancer death worldwide [1]. Non-small cell lung cancer (NSCLC) occupies more than 80% of all lung cancer. The prognosis of NSCLC patients depends on the stage, overall health, pathological type, and other factors. Overall, less than 10% of patients with unresectable NSCLC survive five years after the diagnosis [2,3]. Multidisciplinary management of NSCLC which contains surgery, chemotherapy and radiotherapy does not satisfactorily improve the patients' prognosis [2,3]. Recently, a novel type of medication, "Targeted therapy", is changing the treatment strategy for NSCLC. The epigenetic molecular targeted drugs, such as Gefitinib [4,5], Erlotinib [6] and crizotinib [7], are likely to extend overall survival or progression-free survival in a portion of NSCLC patients, when compared to traditional chemotherapy alone. Despite the use of these new drugs, mortality in patients with advanced stage and recurrence after surgery are still high. The pathogenic mechanisms contributing to the resistance to various therapeutic approaches in this cancer still remain to be clarified.

The genome sequencing projects have shown the human genome is comprised of less than 2% protein coding genes and that more than 90% of the genome is transcribed as non-coding RNAs (ncRNA) [8–10]. These ncRNAs are classified into two groups depending on the nucleotide size. Micro RNAs (miRNAs) are approximately 18–25 nucleotides in length and have been reported to regulate many biological processes including oncogenesis [11–13]. Several miRNAs are known to be associated with malignant behaviors in lung cancer [14–20]. On the other hand, large intervening non-coding RNA (lincRNA) consists of more than 200 nucleotides. Although more than 3000 lincRNAs have been found, 99% of their functions are still unknown [21–23].

Abbreviations: cDNA, complementary DNA; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; DMEM, Dulbecco's modified Eagle's medium; EV cells, empty vector transfected cells; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3K27, histone H3 methylated Lys 27; HE, hematoxylin eosin; lincRNA, large intervening non-coding RNA; miRNA, micro RNA; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ncRNA, non-coding RNA; NSCLC, non-small cell carcinoma; PBS, phosphate buffered saline; PRC2, polycomb repressive complex 2; RT-PCR, reverse transcription polymerase chain reaction; SCLC, small cell lung cancer.

* Corresponding author. Fax: +81 22 381 1195.
E-mail address: ksatoh-gi@umin.ac.jp (K. Satoh).

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Hox transcript antisense intergenic RNA (HOTAIR) is one of the lincRNAs identified by custom tilling array of the HOXC locus. HOTAIR was shown to interact with polycomb repressive complex 2 (PRC2), which consists of the histone H3 lysine-27 (H3K27) methylase EZH2, SUZ12 and EED [24], to decrease the expression of multiple genes. Recently, the enhanced expression of this molecule has been shown to be associated with metastasis and/or poor prognosis in several human carcinomas such as breast [25], liver [26], colon [27] and pancreas [28]. However, little is known about the roles of this gene in lung cancer.

In this study, we examined the expression of HOTAIR in 77 NSCLCs and analyzed the correlation between the expression of this gene and the clinical symptom. Moreover, we evaluated the role of this gene in the development of NSCLC using retrovirally transduced HOTAIR expressing lung cancer cells.

2. Materials and methods

2.1. Clinical samples

77 NSCLC samples and their corresponding normal lung tissues were obtained from patients who underwent complete resection at the Miyagi Cancer Center between 2008 and 2011. In addition, 6 metastatic lesions to the brain were employed. All specimens were immediately frozen and stored at −80 °C after surgery. Written informed consent was obtained from all patients. No patient received chemotherapy or radiotherapy before lung surgery, while 2 of 6 patients received chemotherapy before the resection of brain metastases.

2.2. RNA preparation, cDNA synthesis and quantitative real-time RT-PCR

Total RNA was extracted from frozen samples and lung cancer cell lines using ISOGENE (NIPPON GENE, Tokyo, Japan) and an mirVana miRNA isolation Kit (Ambion, Life Technologies, CA), respectively. The quality assessment of RNA was evaluated in the 28S and 18S bands using agarose gel electrophoresis. cDNAs were synthesized from 1ug of total RNA using PrimeScript® 1st strand cDNA Synthesis Kit (TaKaRa Bio, Siga, Japan) according to the manufacturer's protocol.

The expression of HOTAIR and other genes was quantified using LightCyler™ 480 Probes Master kit (Roche Applied Science, IN) with the specific primer sets according to a previous study [25]. All reactions were performed according to the manufacturer's protocol. The annealing temperature for these primer sets was 60 °C. The specificity of each PCR reaction was confirmed by melting curve analyses. The level of target gene expression in each sample was normalized to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level [29]. The relative HOTAIR expression in 77 NSCLCs was evaluated in comparison with the corresponding normal lung tissues. The patients with equal to or more than 2-fold and less than 2-fold the HOTAIR level in the tumor compared to the corresponding normal tissue were defined as high and low expression, respectively.

2.3. Cell lines and cell culture

Lung cancer cell line A549 was obtained from Cell Resource Center For Biological Research, Institute of Development Aging and Cancer, Tohoku University (Sendai, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/Life technologies Co., CA) supplemented with 10% Fetal Bovine Serum (EuroClone, Milano, Italy) and with 1% penicillin–streptomycin (Gibco/Life technologies Co.). Cells were incubated in a humidified incubator at 37 °C, 5% CO_2.

2.4. Retroviral transduction

Human HOTAIR cDNA (addgene, Cambridge, MA) was amplified by PCR and was inserted into the pBabepuro vector (pBabepuroHOTAIR). The recombinant retrovirus were produced with Platinum-A (Plat-A, Provided by Prof. Kitamura) packaging cell lines as described previously [30]. Briefly, Plat-A cells were transfected with pBabepuro-HOTAIR or pBabe-puro Vector (Empty Vector). Fugene-6 (Roche Applied Science) and Opti-MEM I (Gibco/Life technologies Co.) were added following the manufacturer's protocol. Forty-eight hours after transfection, the retrovirus-containing supernatant was collected and passed through a 0.45 μm filter. Cells were infected with the recombinant retroviruses and then selected with puromycin.

2.5. siRNA transfection

HOTAIR-overexpressing A549 cells were transfected with 10 nM of control siRNA (siRNA Universal Negative Control, Sigma–Aldrich Co., MO), HOTAIRsiRNA1 or HOTAIRsiRNA2 purchased from Sigma Aldrich Co (MO) employing Lipofectamine RNAiMAX (Invitrogen) with Opti-MEM I (Gibco/Life technologies Co.) following the manufacturer's protocol. The HOTAIR expression levels were measured using quantitative real-time RT-PCR and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT) assays, migration assays and wound healing scratch assays.

2.6. Cell proliferation assay

1×10^4 cells were seeded per well in 96 well plates in normal cell growth media. MTT assay was performed using Cell proliferation Kit I (GE HealthcareLife Sciences, NJ) according to the manufacturer's protocol. Measurements of absorbance at 570 nm by VersaMax (Molecular Devices, CA) were made to estimate MTT-formazan production after 24 h and 72 h incubation. The index at 72 h was normalized to that at 24 h.

2.7. Soft agar assay

1×10^4 cells were suspended in 0.3% Bacto™ agar (BD Bioscience, NJ) supplemented with DMEM containing 10% FBS and layered over 1 ml of an 0.8% agar–medium base layer in 6 well plates. After 4 weeks, the number of colonies was evaluated using a microscope.

2.8. Migration assay

The cell migration was assessed by two-chamber assay using the Cell Culture Insert (8 μm pore size, BD Biosciences) in 24 well plates. 5×10^4 cells were plated in each insert in serum-free medium. The bottom well contained medium with 10% FBS. After 48 h, the bottom of the insert was stained with hematoxylin. The number of cells invaded through the membrane to the lower surface was counted.

2.9. Wound healing scratch assay

Cells were seeded in 6 well plates in normal cell growth media and incubated to confluence. A yellow pipette tip was used to make a straight scratch, simulating a wound. The medium was changed to DMEM containing 2% FBS. After 48 h incubation, the area occupied by migration cells in the straight scratch was estimated.

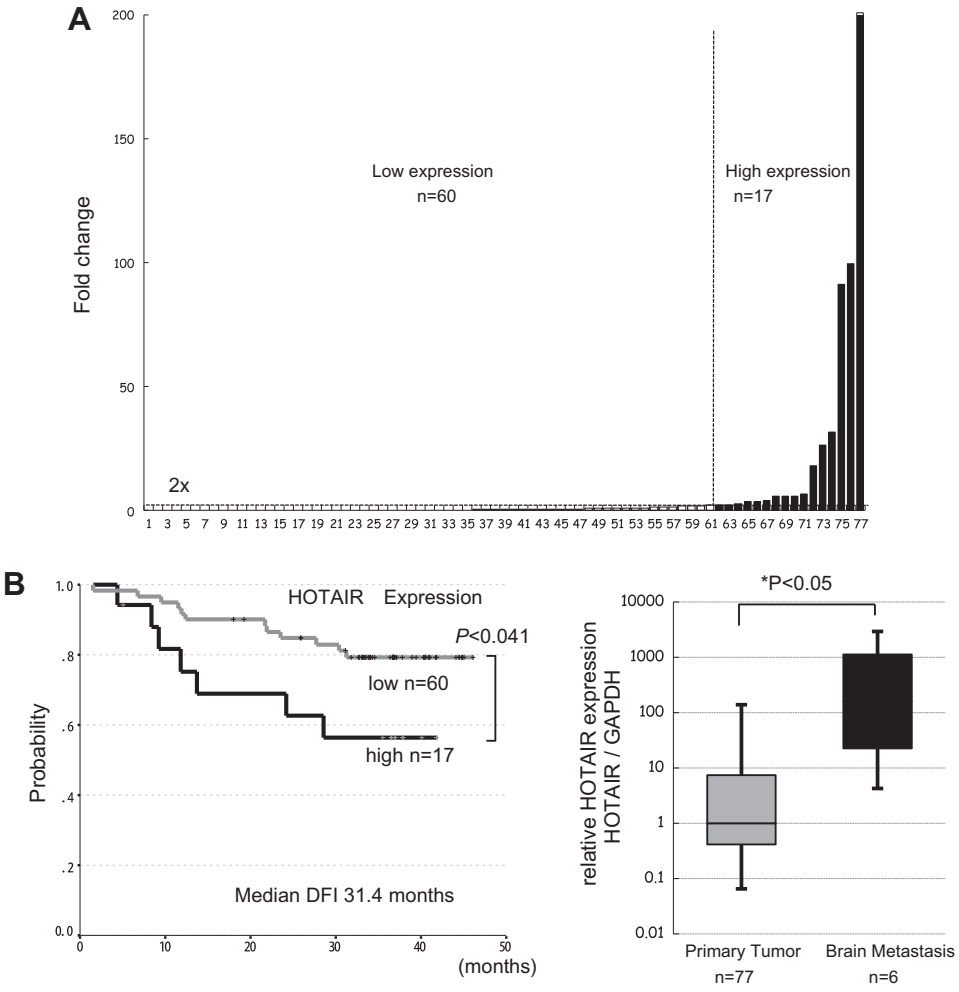

Fig. 1. HOTAIR expression in human non-small lung cell carcinomas (NSCLCs). (A) HOTAIR expression was investigated by quantitative real-time RT-PCR in 77 NSCLCs and their corresponding normal lung tissues. 17 of 77 in NSCLC patients showed high HOTAIR expression (>2-fold their normal counter parts). (B) HOTAIR expression was also examined in 6 brain metastatic lesions. The HOTAIR expression was significantly higher in the brain metastases than in primary NSCLCs ($p < 0.05$). (C) Kaplan–Meier curves for disease free interval (DFI) ratio after surgery according to expression of HOTAIR in 77 NSCLC patients. The patients with high HOTAIR expression showed significantly shorter DFI than those with low expression ($p < 0.05$).

Table 1
The association between HOTAIR expression and clinicopathological features in human non-small lung cell carcinoma patients.

HOT AIR expression		Low ($n = 60$)	High ($n = 17$)	P value
Age	<70	23	8	.517
	⩾70	37	9	
Gender	Male	37	12	.578
	Female	23	5	
Smoking	0–400	29	5	
BI status[a]	>400	31	12	.268
Histology	Adenocarinoma	43	13	.768
	Squamous	17	4	
Tumour size	(mean ± SD)	28.1 ± 13.8	42.5 ± 22.5	.005[b]
T	T1	36	5	.031[b]
	T2–4	24	12	
N	N0	50	10	.047[b]
	N1–2	10	7	
Stage	I	49	8	.010[b]
	II–IV	11	9	
Lymph and/or vascular infiltration	−	54	10	.006[b]
	+	6	7	
Pulmonary metastatis	−	59	16	.395
	+	1	1	

[a] BI, Brinckman index.
[b] Statistically significant.

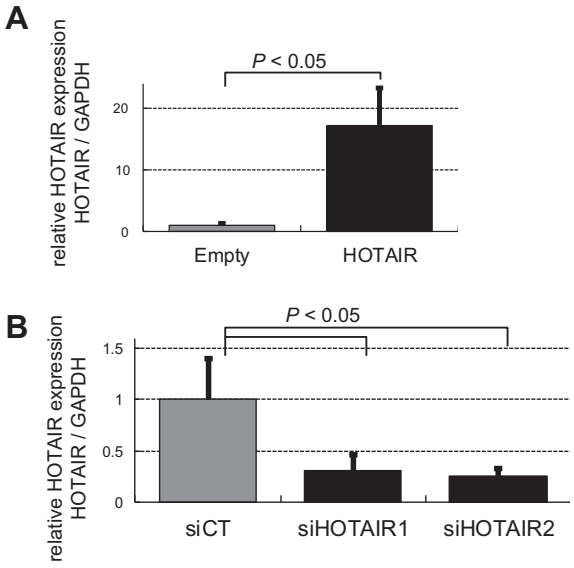

Fig. 2. Generation of HOTAIR expressing A549 cells. The HOTAIR expression level was 17-fold higher in HOTAIR-transduced cells than in EV cells (A). The expression of HOTAIR was significantly reduced compared to control cells (siCT) when the ectopic HOTAIR-expressing cells were transfected with siRNAs [control siRNA (siCT), siHOTAIR1 and siHOTAIR2] (B).

2.10. Statistical analysis

Statistical significance of difference between 2 groups was determined using *t*-test, Pearson's chi-square test and Mann–Whitney *u*-test. Disease- free interval probability was analyzed by the Kaplan–Meier methods and evaluated by log-rank test. All statistical analyses were performed using SPSS for Windows v.11.0 (SPSS, Chicago, IL). A *P* value of <0.05 was regarded as statistically significant.

3. Result

3.1. HOTAIR expression and clinicopathological characteristics in NSCLC

We examined the HOTAIR expression levels among 77 NSCLC (56 adenocarcinomas and 21 squamous cell carcinomas) and their corresponding normal lung tissues using quantitative real-time RT-PCR. There were 17 patients (22.1%) whose HOTAIR expression levels in their tumor tissues were equal to or more than 2-fold that of corresponding normal tissues (Fig. 1A). Clinicopathological features such as gender, age, smoking, histological type, tumor size, T stage, N stage, pathological stage and lymph-vascular invasion were analyzed between the high and low HOTAIR expression groups. The high expression group showed greater tumor size, advanced T and N stage, P stage and more frequent lymphatic and/or

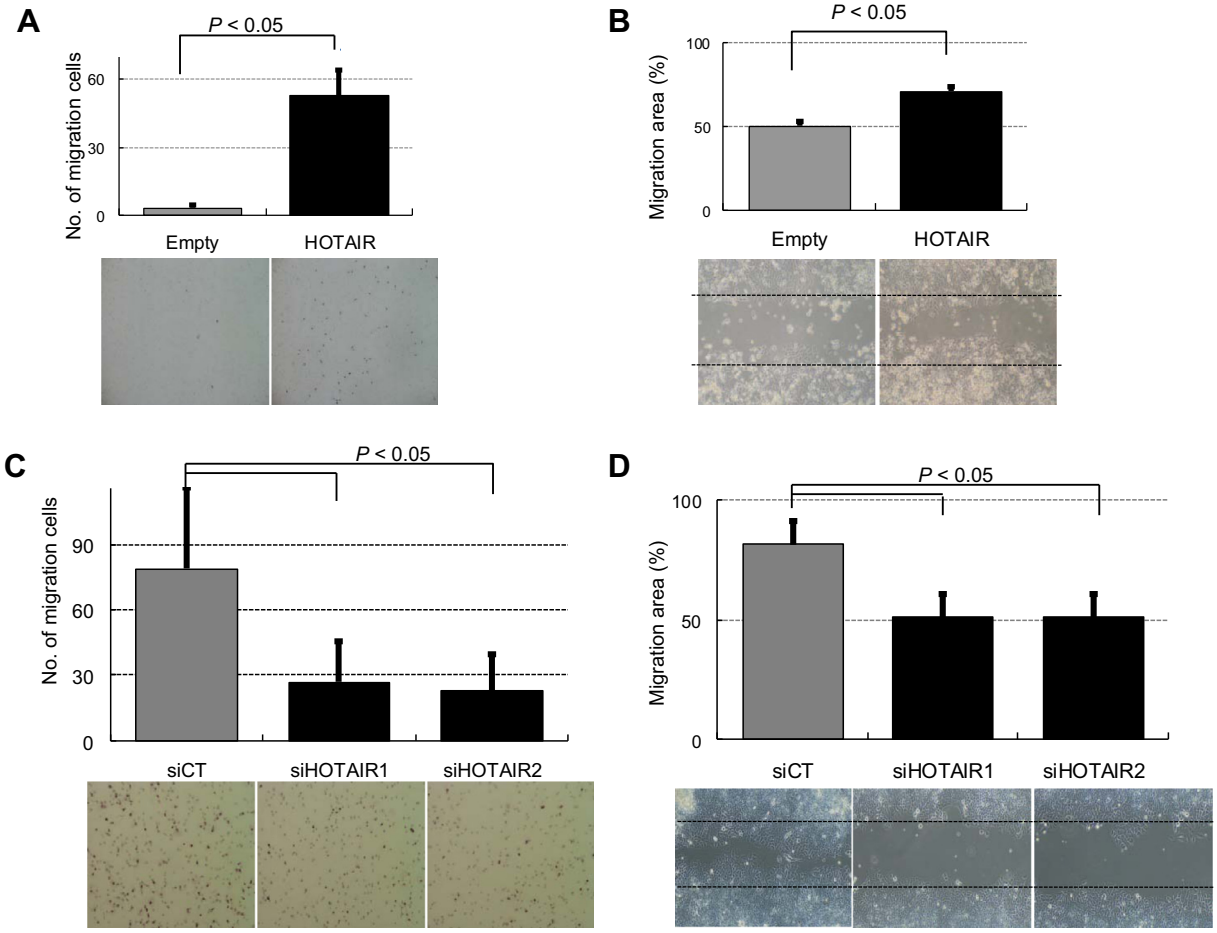

Fig. 3. Modulation of HOTAIR expression alters the migration of A549 cells. (A) Two-chamber assay reveals that forced expression of HOTAIR markedly increased the cellular migration of A549 cells. The columns indicate number of migrated cells per field ($p < 0.05$). (B) The effect of HOTAIR on cell migration was also confirmed by scratch wound healing assay. HOTAIR expressing A549 cells show enhanced cell migration. The columns denote occupied area (%) by migrated cells in scratched interval ($p < 0.05$). (C) Reduced cell migration is observed in HOTAIR knockdown cells compared to control cells by two chamber assay ($P < 0.05$). (D) Wound healing assay also shows the disappearance of the enhanced cell migration by siRNA transfection in HOTAIR expressing cells.

venous invasion than the low expression group did (Table 1). In addition, we also revealed that the disease-free interval after surgery was significantly shorter in the patients with high HOTAIR expression compared to those with low expression (Fig. 1B). These results strongly suggest that HOTAIR expression is involved in the development of NSCLC. Furthermore, we measured the HOTAIR expression levels in 6 metastatic lesions to the brain and compared them with those in primary lung cancer. The expression levels of HOTAIR in distant metastases were significantly higher than those in the primary tumors (Fig. 1C).

3.2. HOTAIR enhanced cell migration and anchorage-independent cell growth

To clarify the role of HOTAIR in NSCLC cells, we generated stably HOTAIR-expressing lung cancer cells. We transduced the retroviral HOTAIR expression vector to A549 cells, which expressed relatively low levels of endogenous HOTAIR in lung cancer cell lines. The HOTAIR level was 17-fold higher in the HOTAIR transduced cells than in EV cells (Fig. 2A). To confirm the effect of ectopic expression of HOTAIR, we also performed knockdown of overexpressed HOTAIR with specific siRNA to stably HOTAIR-expressing A549 cells. The transfection of siRNA resulted in a 70% decrease of the gene expression level enough to abolish the effects of forced HOTAIR expression (Fig. 2B). Both the two-chamber assay and wound healing scratch assay showed that HOTAIR overexpression promoted cell migration (Figs. 3A and B), and that this was caused to disappear in siRNA transfected-cells (Figs. 3C and D). MTT assay revealed that HOTAIR overexpression decreases cell proliferation (Fig. 4A), while knockdown of this gene restored this effect (Fig. 4C). In contrast, HOTAIR overexpression in A549 cells promoted colony formation in soft agar, suggesting that HOTAIR enhances the anchorage-independent but not –dependent cell growth of lung cancer (Fig. 4B).

4. Discussion

This is the first study to demonstrate the expression of HOTAIR in lung cancer and reveal that HOTAIR expression was correlated with the aggressive biological behavior of NSCLC. The patients with high HOTAIR expression had more advanced stage, more-frequent lymph-vascular infiltration and shorter disease-free interval than those with low expression. The expression of HOTAIR is enhanced in various types of cancer and is associated with metastasis and/or short overall survival [25–28]. Thus, the current results are consistent with those of previous studies, indicating that the expression of HOTAIR is involved in the enhancement of aggressive biological behavior of cancer cells of various origins. On the other hand, greater time would be required to conclude about the association between HOTAIR expression and overall survival of NSCLC patients since our study was limited to the patients who underwent complete resection without prior treatment and the follow-up period after surgery was short.

In breast cancer, HOTAIR expression was increased in metastatic lesions compared to primary tissues and a high HOTAIR level was a predictor of subsequent metastasis in early stage patients (stage I and II) [25], indicating the important relevance of HOTAIR expression to cancer cell metastasis. Similarly, the metastatic tissues to the brain from NSCLC demonstrated significantly higher levels of HOTAIR than primary NSCLCs. In addition, HOTAIR expressing A549 cells induced cell migration and colony formation in the anchorage independent environment, suggesting that HOTAIR plays a pivotal role in the development of NSCLC cells.

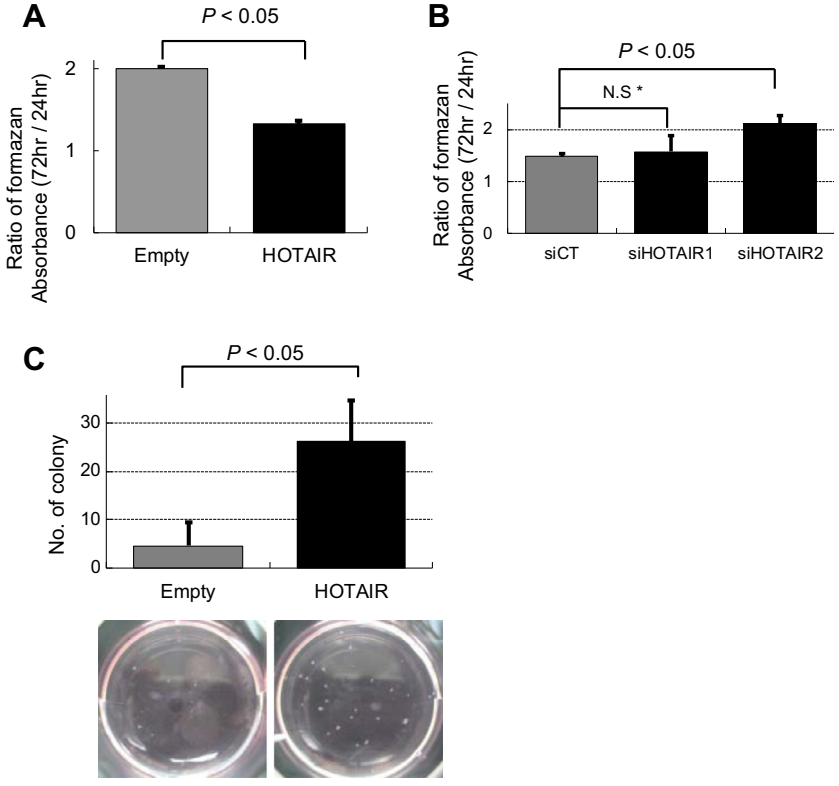

Fig. 4. Effect of HOTAIR expression on the growth of NSCLC cells. MTT assay shows that the overexpression of HOTAIR significantly suppressed lung cancer cell growth compared to control cells (A), while the knockdown of HOTAIR in overexpressing cells restores this effect (B). *N.S., not significance (B). The anchorage-independent cell growth was investigated by soft agar assay. The columns show number of colonies after 4 weeks of cell culture in soft agar. HOTAIR overexpression significantly enhanced the anchorage-independent cell growth of A549 cells (C).

Interestingly, HOTAIR overexpression was shown to induce both migration and cell growth in pancreatic cancer [28]. The authors postulated that HOTAIR must exert pro-oncogenic activities in addition to promoting invasion and metastasis. However, HOTAIR did not facilitate lung cancer cell proliferation. This conflicting characteristic of cancer cells was demonstrated in the process of the epithelial-mesenchymal transition (EMT), which is a well-known phenomenon that promotes carcinoma cell invasion and metastasis [31–33]. For example, TGF-β enhances cell migration and/or invasion via the induction of EMT [34], but it suppresses cell growth. Alternatively, it is likely that the link between HOTAIR expression and cell proliferation is cancer species- dependent because it was seen only in pancreatic cancer. In this context, HOTAIR might facilitate the tumor development but not the carcinogenesis of NSCLC.

In conclusion, enhanced HOTAIR expression in NSCLC was associated with advanced stage and short disease-free survival. Forced expression of this gene induced cell migration and anchorage-independent-cell growth *in vitro*, indicating that the evaluation of HOTAIR in NSCLC samples could be a useful tool to predict the biological behavior of tumors, and potentially a therapeutic target in advanced NSCLC.

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