Priority Report

Cancer Research

Long Noncoding RNA *HOTAIR* Regulates Polycomb-Dependent Chromatin Modification and Is Associated with Poor Prognosis in Colorectal Cancers

Ryunosuke Kogo[1,3], Teppei Shimamura[4], Koshi Mimori[1], Kohichi Kawahara[2], Seiya Imoto[4], Tomoya Sudo[1], Fumiaki Tanaka[1], Kohei Shibata[1], Akira Suzuki[2], Shizuo Komune[3], Satoru Miyano[4], and Masaki Mori[5]

Abstract

The functional impact of recently discovered long noncoding RNAs (ncRNAs) in human cancer remains to be clarified. One long ncRNA which has attracted attention is the *Hox transcript antisense intergenic RNA* termed *HOTAIR*, a long ncRNA expressed from the developmental *HOXC* locus located on chromosome 12q13.13. In cooperation with Polycomb complex PRC2, the *HOTAIR* long ncRNA is reported to reprogram chromatin organization and promote breast cancer metastasis. In this study, we examined the status and function of *HOTAIR* in patients with stage IV colorectal cancer (CRC) who have liver metastases and a poor prognosis. *HOTAIR* expression levels were higher in cancerous tissues than in corresponding noncancerous tissues and high *HOTAIR* expression correlated tightly with the presence of liver metastasis. Moreover, patients with high *HOTAIR* expression had a relatively poorer prognosis. In a subset of 32 CRC specimens, gene set enrichment analysis using cDNA array data revealed a close correlation between expression of *HOTAIR* and members of the PRC2 complex (SUZ12, EZH2, and H3K27me3). Our findings suggest that *HOTAIR* expression is associated with a genome-wide reprogramming of PRC2 function not only in breast cancer but also in CRC, where upregulation of this long ncRNA may be a critical element in metastatic progression. *Cancer Res; 71(20); 6320–6. ©2011 AACR.*

Introduction

Noncoding RNAs (ncRNA) are found throughout the genome. However, the functions of ncRNAs are only partially understood. The function and clinical significance of short ncRNAs, such as miRNA and siRNA were elucidated first and then, long ncRNAs were reported more recently. Most long ncRNAs work with DNA-binding proteins, such as chromatin-modifying complexes, and epigenetically regulate the expression of multiple genes (1–3). *Hox transcript antisense intergenic RNA* (*HOTAIR*) is a long ncRNA that was identified from a custom tilling array of the *HOXC* locus (12q13.13; ref. 2). *HOTAIR* trimethylates histone H3 lysine-27 (H3K27me3) of the *HOXD* locus with the polycomb-repressive complex 2 (PRC2), which is composed of EZH2, SUZ12, and EED, and inhibits *HOXD* gene expression (2). Thus, *HOTAIR* epigenetically regulates *HOXD* expression, located on a different chromosome.

Gupta and colleagues reported that *HOTAIR* induced genome-wide retargeting of PRC2, leading to H3K27me3, and promoted metastasis of breast cancer by silencing multiple metastasis suppressor genes (4). In particular, they concluded that *HOTAIR* suppressed tumor suppressor genes such as *HOXD10*, *PGR*, and the protocadherin gene family in breast cancer cells. *HOTAIR* expression was low in normal breast epithelia but high in primary breast cancer as well as metastatic lesions. Moreover, breast cancer patients with high *HOTAIR* expression had a poorer prognosis for overall survival and for metastasis-free survival than did those with low *HOTAIR* expression.

Colorectal cancer (CRC) is the one of the most common cancers in the world. However, the existence of multiple known carcinogens and varying genetic backgrounds makes it difficult to determine which factors are most important in the development of CRC. Therefore, the identification of a bona fide molecule involved in progression of CRC has been greatly sought after. In the current study, we clarified the clinical significance of *HOTAIR* expression in CRC. Moreover, to determine the function of *HOTAIR* in CRCs, we used cDNA microarray data from another subset of 32 CRC samples obtained by laser microdissection (LMD). We conducted gene

Authors' Affiliations: [1]Department of Surgery and [2]Division of Cancer Genetics, Medical Institute of Bioregulation, and [3]Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [4]Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo; and [5]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

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R. Kogo, T. Shimamura, and K. Mimori contributed equally to this work.

Corresponding Author: Masaki Mori, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-3251; Fax: 81-6-6879-3259; E-mail: mmori@gesurg.med.osaka-u.ac.jp

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set enrichment analysis (GSEA) and investigated whether *HOTAIR* expression was highly correlated with previously curated gene expression signatures of PRC2 (4).

Materials and Methods

Clinical samples and cell lines

One hundred CRC samples (bulk samples) were obtained from patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University, between 1993 and 2000. Another 32 CRC samples (LMD samples) were obtained from Medical Institute of Bioregulation Hospital, Kyushu University, Tokyo Medical and Dental University Hospital, Kitazato University Hospital, National Cancer center, and National Defense Medical College Hospital. All specimens were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction. Written informed consent was obtained from all patients. No patient received chemotherapy or radiotherapy before surgery. The follow-up periods ranged from 2 months to 11 years, with a mean of 3 years. HEK293T, HCT116, and SW480 cell lines were provided by the American Type Culture Collection and were maintained in Dulbecco's Modified Eagle's Media, McCoy 5A, or RPMI-1640, respectively, containing 10% FBS with 100 units/mL penicillin and 100 μg/mL streptomycin and cultured in a humidified 5% CO_2 incubator at 37°C.

RNA preparation, reverse transcription, and quantitative real-time PCR

One hundred bulk samples. Total RNAs from frozen CRC samples were extracted using ISOGEN (Nippon Gene) following the manufacturer's protocol.

Thirty-two LMD samples. Total RNAs were extracted using QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol.

As previously reported, cDNAs from all samples were synthesized from 8.0 μg of total RNA (5). *HOTAIR* levels were quantified using LightCycler 480 Probes Master kit (Roche Applied Science) following the manufacturer's protocol with the following specific *HOTAIR* primers (forward, 5′-CAGTGGGGAACTCTGACTCG-3′ and reverse, 5′-GTGCCTGGTGCTCTCTTACC-3′). *HOTAIR* levels were normalized to *GAPDH* (forward, 5′-GTCAACGGATTTGGTCTGTATT-3′ and reverse, 5′-AGTCTTCTGGGTGGCAGTGAT-3′).

Laser microdissection

RNAs from another 32 CRC tissues were collected for LMD. CRC tissues were microdissected using the LMD System (Leica Microsystems) as previously described (6).

GSEA of CRC with *HOTAIR* expression

HOTAIR/*GAPDH* levels in 32 CRC tissues (LMD samples) were measured by quantitative real-time PCR. Gene expression profiles of 32 CRC samples were measured by Agilent Whole Human Genome Microarray 4 × 44K G4112F and analyzed by GSEA (7). The expression profiles were quintile normalized. The batch effect in microarray experiments was also adjusted by an empirical Bayesian approach (8). To collapse each probe set on the array to a single gene, the probe with the highest variance among multiple probes that corresponded to the same gene was selected, which produced a 19,749 (genes) × 32 (CRCs) expression matrix. For GSEA, *HOTAIR* expression was treated as a binary variable divided into low or high *HOTAIR* expression by a criterion of whether or not its value was greater than 0.273. As a result, 9 CRC samples were categorized as high and 23 were labeled as low, of 32 tumors. For functional gene sets for GSEA, we used gene sets from global occupancy of H3K27me3, PRC2 subunits, SUZ12 or EZH2, and PRC2 (all of H3K27me3, SUZ12, and EZH2) induced by *HOTAIR* overexpression in MDA-MB-231 breast cancer cells (4). As a metric for ranking genes in GSEA, the difference between the means of samples with low and high *HOTAIR* expression was used, and the other parameters were set by their default values. Gene expression arrays have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession code GSE21815.

HOTAIR expression lentiviral vector

To generate *HOTAIR* expression lentiviral vector, we amplified insert (full-length human *HOTAIR*) by PCR from MCF7 cDNA. Lentiviruses were produced by transient transfection of HEK293T cells with pCMV-VSV-G-RSV-Rev, pCAG-HIVgp, and either CSII-CMV-*HOTAIR* or CSII-CMV-MCS (empty) plasmid DNAs (5′*Xho*I and 3′*Not*I site) plus Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Forty-eight hours after cotransfection, the lentivirus-containing supernatant was collected and passed through a 0.45-μm filter. The titer of the lentivirus vector in filtered supernatants was estimated by measuring the concentration of HIV p24 gag antigen with an ELISA kit (Perkin-Elmer Life Science).

Transfection of siRNA

Two individual siRNAs (siRNA HOTAIR1 and siRNA HOTAIR2) and negative control siRNA (silencer negative control siRNA) are purchased from Ambion. siRNA oligonucleotides (10 nmol/L) in Opti-MEM (Invitrogen) were transfected into SW480 cells using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol. Forty-eight hours posttransfection, *HOTAIR* expression levels were measured, and Matrigel invasion assays were conducted. Target sequences for *HOTAIR* siRNAs were as follows: siRNA1, 5′-UUUUCUACCAGGUCGGUAC-3′ and siRNA2, 5′-AAUUCUUAAAUUGGGCUGG-3′.

Matrigel invasion assay

The Matrigel invasion assay was done using the BD Biocoat Matrigel Invasion Chamber (pore size: 8 μm, 24-well; BD Biosciences) following the manufacturer's protocol. Cells (5×10^4) were plated in the upper chamber in serum-free medium. The bottom chamber contained medium with 10% FBS. After 48 hours, the bottom of the chamber insert was stained with Calcein AM (Invitrogen). The cells that had invaded through the membrane to the lower surface were evaluated in a fluorescence plate reader at excitation/emission

wavelengths of 485/530 nm. Each Matrigel invasion assay was conducted in at least 3 replicates.

Statistical analysis

The significance of differences between 2 groups was estimated with the Student *t* test and χ^2 test. Overall survival curves were plotted according to the Kaplan–Meier method, with the log-rank test applied for comparison. Variables with a value of $P < 0.05$ by univariate analysis were used in subsequent multivariate analysis on the basis of Cox proportional hazards model. All differences were considered statistically significant at the level of $P < 0.05$. Statistical analyses were done using JMP 5 (SAS Institute).

Results

HOTAIR expression and clinicopathologic factors in CRC

HOTAIR expression levels in 100 cancerous and noncancerous tissues were examined by quantitative real-time PCR. *HOTAIR* levels in cancerous tissues were significantly lower than those in the noncancerous tissues ($P = 0.002$; Fig. 1A). We divided the 100 patients with CRC into a high *HOTAIR* expression group ($n = 20$) and a low expression group ($n = 80$), according to a *HOTAIR*/*GAPDH* ratio of 0.273 in cancerous tissue (Fig. 1B). Clinicopathologic factors were analyzed in the high and low *HOTAIR* expression groups (Table 1). The high *HOTAIR* expression group ($n = 20$) showed a less differentiated histology, greater tumor depth, and liver metastasis than the low *HOTAIR* expression group ($n = 80$; $P < 0.05$). In particular, high *HOTAIR* expression was strongly associated with liver metastasis ($P = 0.006$). With regard to overall survival, patients with high *HOTAIR* expression had a significantly poorer prognosis than those with low *HOTAIR* expression ($P = 0.0046$; Fig. 1C). Univariate analysis of overall survival revealed that the relative level of *HOTAIR* expression, histologic grade, depth of tumor, lymph node metastasis, lymphatic invasion, and venous invasion were prognostic indicators (Table 2). Variables with a value of $P < 0.05$ were selected for multivariate analysis. Multivariate analysis showed that *HOTAIR* expression was an independent prognostic indicator for overall survival in patients with CRC (relative risk: 5.62, $P = 0.008$; Table 2). Moreover, we asked whether high *HOTAIR* expression contributed to poor prognosis in another independent subgroup. Using exon array data from 320 patients with CRC, we divided the cases according to the median, yielding a high *HOTAIR* expression group ($n = 160$) and a low expression group ($n = 160$). The patients in the high *HOTAIR* expression group had a significantly poorer prognosis than those in the low expression group ($P < 0.0001$; Supplementary Fig. S1).

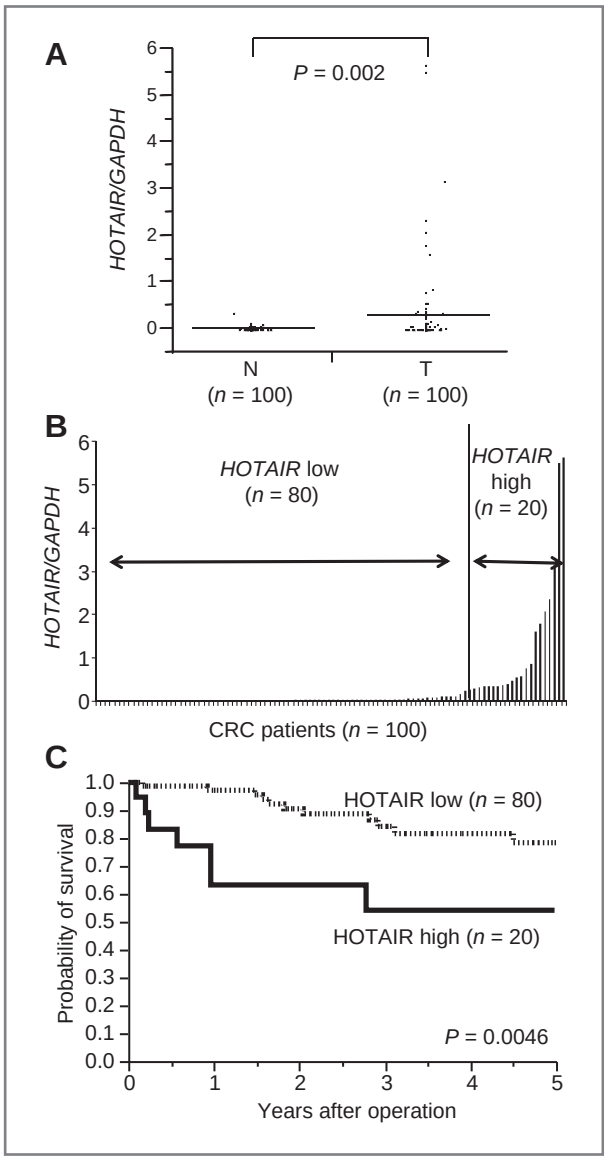

Figure 1. A, *HOTAIR* expression levels assessed by quantitative real-time PCR in cancerous (T) and noncancerous tissues (N) from 100 CRC samples. *HOTAIR* levels were normalized to *GAPDH*. *HOTAIR* levels in T were significantly lower than those in N ($P = 0.002$). Horizontal lines, mean value of each sample. B, quantitative real-time PCR analysis of *HOTAIR* in 100 CRC tissues and the classification based on *HOTAIR* level (*HOTAIR*/*GAPDH* = 0.0273). Vertical line, borderline of *HOTAIR* high ($n = 20$) or low ($n = 80$). C, Kaplan–Meier overall survival curves according to *HOTAIR* level. The overall survival of the high *HOTAIR* expression group ($n = 20$) was significantly higher than that of the low expression group ($n = 80$; log-rank test; $P = 0.0046$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

HOTAIR expression induced genome-wide retargeting of PRC2 in CRC

Next, we used a cDNA array on the basis of cancer tissue samples from 32 patients with CRC obtained by LMD and asked whether *HOTAIR* expression levels in the 32 CRC samples were highly correlated with previously curated gene expression signatures of PRC2 (4). First, we divided 32 patients with CRC into a high *HOTAIR* expression group ($n = 9$) and a low expression group ($n = 23$), according to the *HOTAIR*/*GAPDH* ratio of 0.273 as in Fig. 1B. We expected that CRC with high and low *HOTAIR* expression levels would be significantly enriched for these gene sets, as *HOTAIR* overexpression induced localization of H3K27me3 and PRC2

Table 1. *HOTAIR* expression and clinicopathologic factors

Factors	Tumor high expression ($n = 80$) *N* (%)	Tumor high expression ($n = 20$) *N* (%)	*P*
Age (mean ±SD)	68.3 ± 1.17	644 ± 2 33	0.136
Gender			
Male	51 (63.8)	12 (60)	0.757
Female	29 (36.2)	8 (40)	
Histologic grade			
Well and moderately	77 (96.3)	16 (80)	0.024[a]
Poorly and others	3 (3.7)	4 (20)	
Depth of tumor			
m, sm, mp	25 (31.2)	2 (10)	0.039[a]
ss, se, si	55 (68.8)	18 (90)	
Lymph node metastasis			
Absent	48 (60)	10 (50)	0.420
Present	32 (40)	10 (50)	
Lymphatic invasion			
Absent	54 (67.5)	10 (50)	0.151
Present	26 (32.5)	10 (50)	
Venous invasion			
Absent	67 (83.8)	15 (75)	0.378
Present	13 (16.2)	5 (25)	
Liver metastasis			
Absent	77 (96.3)	15 (75)	0.06[a]
Present	3 (3.7)	5 (25)	
Dukes' stage			
A and B	50 (62.5)	9 (45)	0.158
C and D	30 (37.5)	11 (55)	

Abbreviations: m, mucosa; mp, muscularis propria; se, penetration of serosa; si, invasion of adjacent structures; sm, submucosa; ss, subserosa.
[a]$P < 0$ 05.

subunits, SUZ12 and EZH2, in MDA-MB-231 breast cancer cells (4). Indeed, gene signatures with *HOTAIR*-induced SUZ12 occupancy [$P = 0.001$ and false discovery rate (FDR) = 0.004], EZH2 occupancy ($P = 0.029$ and FDR = 0.044), H3K27me3 occupancy ($P = 0.028$ and FDR = 0.032), and PRC2 occupancy (occupancy of SUZ12, EZH2, and H3K27me3; $P = 0.032$ and FDR = 0.024) were confirmed to be significantly enriched in CRC (Fig. 2A and B). More-

Table 2. Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

Factors	Univariate analysis		Multivariate analysis	
	Relative risk	*P*	Relative risk	*P*
Age (<67/<68)	0.73	0.497	—	—
Gender (male/female)	1.13	0.794	—	—
Histologic grade (well and moderately/poorly and others)	6.33	0.019[a]	2.28	0.272
Depth of tumor (m, sm, mp/ss, se, si)	8.78	0.004[a]	3.02	0.257
Lymph node metastasis (negative/positive)	5.72	<0.001[a]	5.30	0.010[a]
Lymphatic invasion (negative/positive)	3.76	0.006[a]	0.45	0.290
Venous invasion (negative/positive)	4.62	0.003[a]	6.96	0.003[a]
HOTAIR expression (low/high)	3.62	0.014[a]	5.62	0.008[a]

Abbreviations: m, mucosa; mp, muscularis propria; se, penetration of serosa; si, invasion of adjacent structures; sm, submucosa; ss, subserosa.
[a]$P < 0.05$.

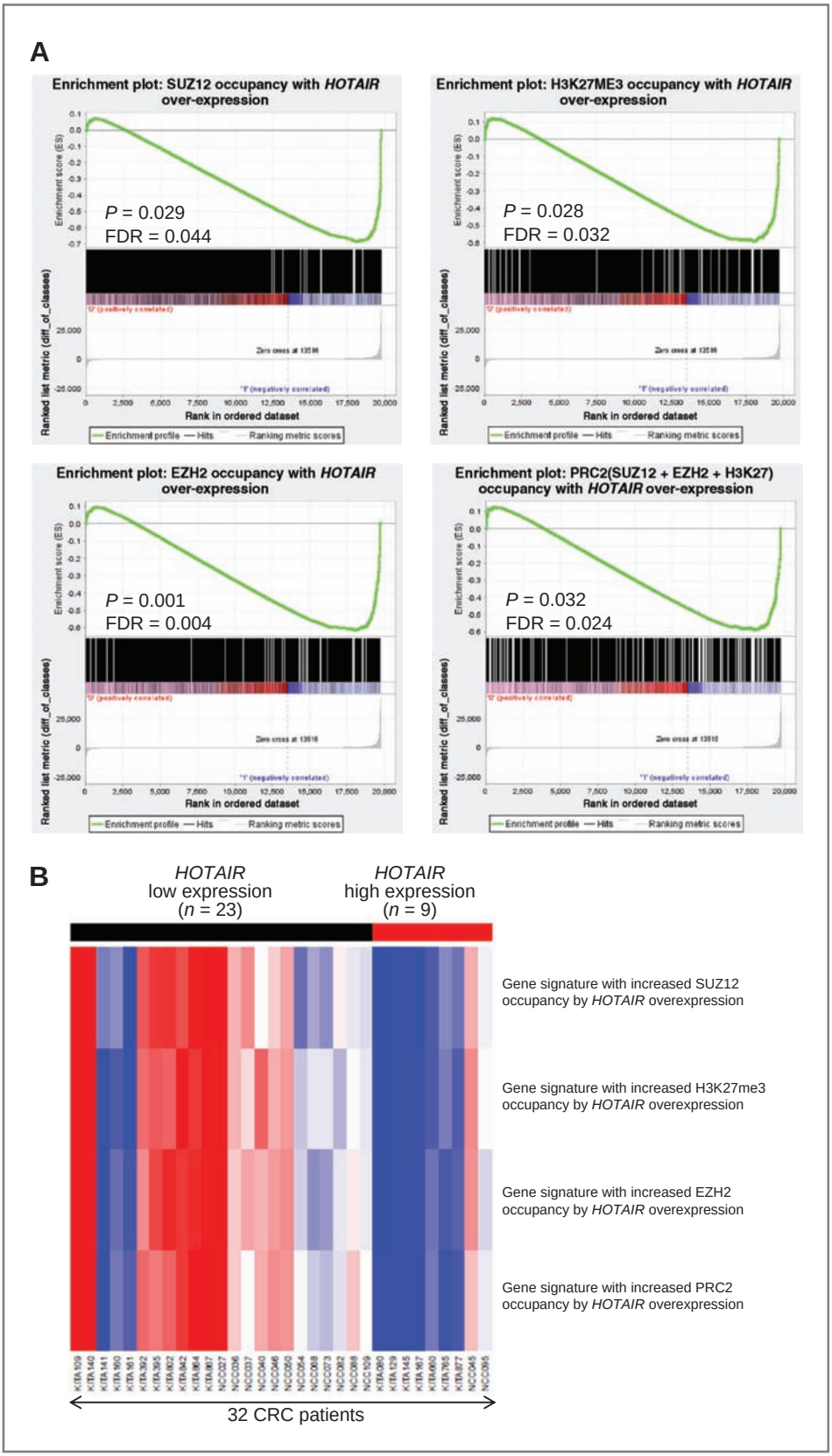

Figure 2. A, enrichment plots of gene expression signatures of *HOTAIR*-induced SUZ12, H3K27me3, EZH2, and PRC2 occupancy sorted according to the differences between the means of samples with high and low *HOTAIR* expression. The barcode plot indicates the position of the genes in each gene set; red and blue colors represent positive and negative Pearson correlation with *HOTAIR* expression, respectively. B, heat map of the gene expression averages for 32 CRC samples enriched in gene expression signatures of *HOTAIR*-induced SUZ12, H3K27me3, EZH2, and PRC2 occupancy. These gene expression signatures were obtained from Gupta and colleagues (4). The enriched gene expression averages were classified by high and low *HOTAIR* expression levels. The red and blue colors indicate high and low expression, respectively.

over, these enriched genes were expressed at lower levels in tumors with high *HOTAIR* expression than in those with low *HOTAIR* expression (Supplementary Fig. S2). These results suggest that *HOTAIR* expression induced genome-wide retargeting of PRC2 not only in breast cancer but also in CRC.

HOTAIR promotes invasion of CRC cells

Finally, we examined the effect of *HOTAIR* in CRC cells. We determined *HOTAIR* levels in CRC cell lines by quantitative real-time PCR (Supplementary Fig. S3A). We generated lentiviral *HOTAIR* expression vectors which were transduced to HCT116, a CRC cell line (Supplementary Fig. S3B). *HOTAIR* overexpression in HCT116 significantly promoted invasion in Matrigel ($P < 0.05$; Fig. 3A). Conversely, suppression of *HOTAIR* in SW480 CRC cells (that expressed endogenous *HOTAIR*) with specific siRNAs decreased invasion in Matrigel ($P < 0.05$; Fig. 3B and Supplementary Fig. S3C).

Discussion

In our current study, we found that *HOTAIR* expression levels in CRC tissues were higher than those in corresponding noncancerous tissues. Moreover, high *HOTAIR* expression in CRC tissues was associated with a poorer prognosis. As for clinicopathologic variables, *HOTAIR* expression levels were intimately linked to liver metastasis. Recently, Gupta and colleagues showed that *HOTAIR* expression was associated with metastasis of breast cancer (4). Therefore, we speculated that *HOTAIR* expression was also associated with metastasis in CRC. GSEA based on cDNA microarray data showed that *HOTAIR* expression was significantly associated with genome-wide retargeting of *PRC2* genes as shown in breast cancer by Gupta and colleagues (4). Using *in vitro* data, we showed that *HOTAIR* overexpression increased the invasiveness of CRC cells. These results indicate that *HOTAIR* might also play a role in promoting metastasis of CRC.

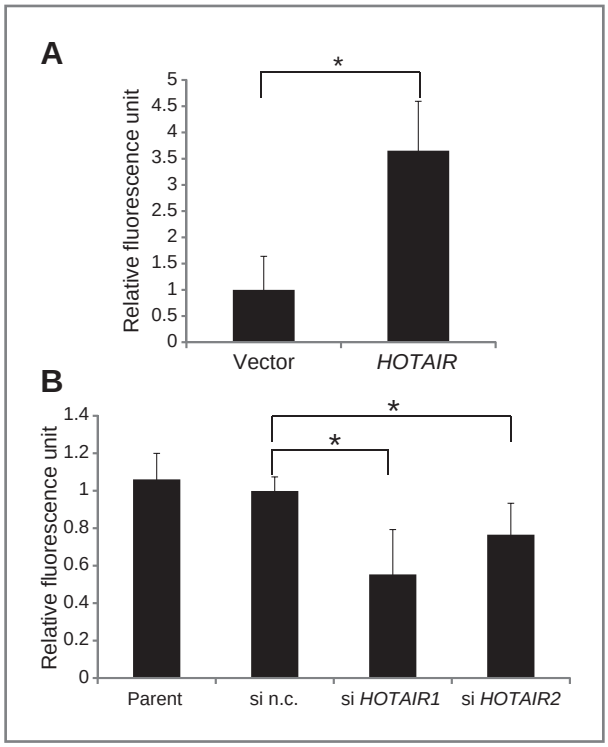

Figure 3. A, Matrigel invasion assay using HCT116 cells after enforced *HOTAIR* expression by lentiviral transduction. *HOTAIR* expression increased the invasiveness of HCT116 ($P < 0.05$). Error bars represent SD ($n = 3$). *, $P < 0.05$. B, Matrigel invasion assay using SW480 cells after transfection with siRNAs targeting *HOTAIR* (2 individual siRNAs: siRNA *HOTAIR1* and siRNA *HOTAIR2*) and negative control siRNA (si n.c.). Error bars represent SD ($n = 3$). *, $P < 0.05$.

In recent years, many long ncRNAs have been identified and their involvement in human disease has been reported. Long ncRNAs, such as *lincRNA-p21* (lung cancer; ref. 9), *uc.73* (CRC; ref. 10), and *uc.338* (hepatocellular carcinoma; ref. 11), have been associated with human malignancies. However, the detailed function and the clinical significance of the long ncRNAs have not yet been elucidated. This is the first report using clinical CRC samples to show that *HOTAIR* works in cooperation with the PRC2. It was previously shown that the PRC2 bound to the 5′ terminus of *HOTAIR* and trimethylated H3K27 (12). Also, the LSD1/CoREST/REST bound to the 3′ terminus of *HOTAIR*, which demethylated H3K4 (12). Thus, the modifications of those DNA-binding proteins by *HOTAIR* regulate global gene expression.

EZH2 and SUZ12, the components of PRC2, are overexpressed in several cancers (13, 14). In particular, SUZ12 is reportedly overexpressed in CRC (15–17). Thus, it is interesting that the current study identified an association between *HOTAIR* and SUZ12 in cancer-specific GSEA. Moreover, PRC2-targeted genes were identical to gene sets that were silenced by pluripotent stem cell–related transcription factors such as Oct4, Sox2, and Nanog (18, 19). Therefore, *HOTAIR* overexpression in CRC might be associated with multipotent differentiation of CRC cells. Gene pathway analysis also indicated that *HOTAIR*-regulated gene sets included CDH1 (E-cadherin) target genes (data not shown), whose expression is lost in metastatic cancer cells of the mesenchymal phenotype. Therefore, we suggest that *HOTAIR* might maintain mesenchymal and undifferentiated cancer cells in cooperation with the PRC2. The strong correlation between *HOTAIR* expression and liver metastasis might indicate an important role of *HOTAIR* in the proliferation of mesenchymal and undifferentiated cancer cells, which enhances the metastatic ability of CRC.

In conclusion, *HOTAIR* regulates expression of multiple genes in cooperation with PRC2 and is a novel molecule involved in the progression of CRC. *HOTAIR* might increase the number of undifferentiated cancer cells and contribute globally to cancer metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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