Long non-coding RNA *HOTAIR* regulates Polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers.

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Abstract

The functional impact of recently discovered long non-coding RNAs (ncRNAs) in human cancer remains to be clarified. One long ncRNA which has attracted attention is the Hox 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 stage IV colorectal cancer (CRC) patients who have liver metastases and a poor prognosis. HOTAIR expression levels were higher in cancerous tissues than 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.
Introduction

Non-coding RNAs (ncRNAs) 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, 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 tiling array of the *HOXC* locus (12q13.13) (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 *et al.* reported that *HOTAIR* induced genome-wide re-targeting 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 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 CRC, we used cDNA microarray data from another subset of 32 CRC samples obtained by laser micro dissection (LMD). We performed Gene 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 two months to 11 years, with a mean of three years. HEK293T, HCT116 and SW480 cell lines were provided by the American Type Culture Collection (ATCC) and were maintained in DMEM, McCoy 5A, or RPMI1640, respectively, containing 10% fetal bovine serum with 100 units / mL penicillin and 100 μg / mL streptomycin and cultured in a humidified 5% CO₂ 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 (Nippongene) following the manufacturer’s protocol.

**32 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 \textit{HOTAIR} primers (forward, 5’-CAGTGGGGAACTCTGACTCG-3’; reverse, 5’-GTGCCTGGTGCTCTCTTACC-3’). \textit{HOTAIR} levels were normalized to \textit{GAPDH} (forward, 5’-GTCAACGGATTTGGTCTGTATT-3’; reverse, 5’-AGTCTTCTGGGTGCCAGTGAT-3’).

\textbf{Laser microdissection}

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

\textbf{Gene set enrichment analysis of CRC with HOTAIR expression}

\textit{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 4x44K G4112F and analyzed by gene set enrichment analysis (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, out 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’Xhol and
3’NotI site) plus Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Forty-eight hr after co-transfection, 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, siRNA HOTAIR2) and negative control siRNA (Silencer negative Control siRNA) are purchased from Ambion. siRNA oligonucleotides (10nM) in Opti-MEM (Invitrogen) were transfected into SW480 cells using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s protocol. Forty-eight hr post-transfection, HOTAIR expression levels were measured and Matrigel invasion assays were performed. Target sequences for HOTAIR siRNAs were as follows: siRNA1, 5’-UUUCUACCAGGUCGUAC-3’ and siRNA2 5’-AAUUCUAAAUUGGCGUUG-3’.

Matrigel invasion assay

The Matrigel invasion assay was done using the BD Biocoat Matrigel Invasion
Chamber (pore size 8μm, 24 well, BD Biosciences) following the manufacturer’s protocol. Cells (5 x 10^4) were plated in the upper chamber in serum-free medium. The bottom chamber contained medium with 10% FBS. After 48 hr, 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 performed in at least three replicates.

**Statistical analysis** The significance of differences between two groups was estimated with Student's *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 based on 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 (T) and noncancerous (N) tissues were examined by quantitative real time PCR. HOTAIR levels in cancerous tissues (T) were significantly lower than those in the noncancerous tissues (N) (P = 0.002, Fig. 1A). We divided the 100 colorectal cancer patients 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 (T) (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 did those with low HOTAIR expression (P = 0.0046, Fig. 1C). Univariate analysis of overall survival revealed that the relative level of HOTAIR expression, histological grade, depth of tumor, lymph node metastasis, lymphatic invasion and venous invasion were prognostic indicators (Table 2). Variables with a P value < 0.05 were selected for multivariate analysis. Multivariate analysis showed that HOTAIR expression was an independent prognostic indicator for overall survival in CRC patients (RR: 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 CRC patients, we divided the cases according to the median, yielding a high \textit{HOTAIR} expression group (n = 160) and a low expression group (n = 160). The patients in the high \textit{HOTAIR} expression group had a significantly poorer prognosis than did those in the low expression group (P < 0.0001, Supplementary Fig. S1).

\textit{HOTAIR} expression induced genome-wide re-targeting of \textit{PRC2} in CRC

Next, we used a cDNA array based on cancer tissue samples from 32 CRC patients obtained by LMD and asked whether \textit{HOTAIR} expression levels in the 32 CRC samples were highly correlated with previously curated gene expression signatures of \textit{PRC2} (4). First, we divided 32 CRC patients into a high \textit{HOTAIR} expression group (n = 9) and a low expression group (n = 23), according to the \textit{HOTAIR}/\textit{GAPDH} ratio of 0.273 as in Fig. 1B. We expected that CRC with high and low \textit{HOTAIR} expression levels would be significantly enriched for these gene sets, since \textit{HOTAIR} overexpression induced localization of H3K27me3 and \textit{PRC2} subunits, SUZ12 and EZH2, in MDA-MB-231 breast cancer cells (4). Indeed, gene signatures with \textit{HOTAIR}-induced SUZ12 occupancy (P = 0.001 and FDR = 0.004), EZH2 occupancy (P = 0.029 and FDR = 0.044), H3K27me3 occupancy (P = 0.028 and FDR = 0.032), and \textit{PRC2} occupancy
(occupancy of SUZ12, EZH2, and H3K27me3) \( (P = 0.032 \text{ and } \text{FDR} = 0.024) \) were confirmed to be significantly enriched in CRC (Fig. 2A, Fig. 2B). Moreover, these enriched genes were expressed at lower levels in tumors with high \( \text{HOTAIR} \) expression than in those with low \( \text{HOTAIR} \) expression (Supplementary Fig. S2). These results suggest that \( \text{HOTAIR} \) expression induced genome-wide re-targeting of PRC2, not only in breast cancer but also in CRC.

**\( \text{HOTAIR} \) promotes invasion of CRC cells**

Finally, we examined the effect of \( \text{HOTAIR} \) in CRC cells. We determined \( \text{HOTAIR} \) levels in CRC cell lines by quantitative real time PCR (Supplementary Fig. S3A). We generated lentiviral \( \text{HOTAIR} \) expression vectors which were transduced to HCT116, a CRC cell line (Supplementary Fig. S3B). \( \text{HOTAIR} \) overexpression in HCT116 significantly promoted invasion in Matrigel \( (P < 0.05, \text{Fig. 3A}) \). Conversely, suppression of \( \text{HOTAIR} \) in SW480 CRC cells (that expressed endogenous \( \text{HOTAIR} \)) with specific siRNAs decreased invasion in Matrigel \( (P < 0.05, \text{Fig. 3B, Supplementary Fig. S3C}) \).
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 *et al.* demonstrated 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 re-targeting of PRC2 genes as shown in breast cancer by Gupta *et al.* (4). Using *in vitro* data, we demonstrated that *HOTAIR* overexpression increased the invasiveness of CRC cells. These results indicate that *HOTAIR* might also play a role in promoting metastasis of CRC.

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) (9), *uc.73* (CRC) (10), and *uc.338* (hepatocellular carcinoma) (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.

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Technological Development for Chromosome Analysis.

References


17. Kirmizis A, Bartley SM, Farnham PJ. Identification of the polycomb group protein SU(Z)12 as a potential molecular target for human cancer


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Table 1: HOTAIR expression and clinicopathological factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>Tumor low expression (n = 80)</th>
<th>Tumor high expression (n = 20)</th>
<th>P value</th>
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<td>Age</td>
<td></td>
<td></td>
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<tr>
<td>Gender</td>
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<td></td>
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<tr>
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<td>12</td>
<td>0.757</td>
</tr>
<tr>
<td>Female</td>
<td>29</td>
<td>8</td>
<td></td>
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<td>Histological grade</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Well &amp; Moderately</td>
<td>17</td>
<td>16</td>
<td>0.024*</td>
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<tr>
<td>Poorly &amp; others</td>
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<td>3</td>
<td></td>
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<td>Depth of tumor</td>
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<td></td>
<td></td>
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<tr>
<td>vs. w, m, mp</td>
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<td>2</td>
<td>0.029*</td>
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<tr>
<td>vs. no, s, si</td>
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<td>16</td>
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<tr>
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<td>Present</td>
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<td>Liver metastasis</td>
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<td>15</td>
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<td>Dukes stage</td>
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<td>0.158</td>
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<tr>
<td>C, D</td>
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<td>11</td>
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SD; Standard deviation, *P < 0.05, †Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent structures (si)

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

<table>
<thead>
<tr>
<th>Factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<td></td>
<td>RR</td>
<td>P value</td>
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<tr>
<td>Age (&lt; 67 / 68 +)</td>
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<td>0.497</td>
</tr>
<tr>
<td>Gender (male / female)</td>
<td>1.13</td>
<td>0.794</td>
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<tr>
<td>Histological grade (well &amp; moderately / poorly &amp; others)</td>
<td>6.33</td>
<td>0.019*</td>
</tr>
<tr>
<td>Depth of tumor (m, sm, mp / ss, se, si)†</td>
<td>8.78</td>
<td>0.004*</td>
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<td>Lymph node metastasis (negative / positive)</td>
<td>5.72</td>
<td>&lt; 0.001*</td>
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<td>Lymphatic invasion (negative / positive)</td>
<td>3.76</td>
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<tr>
<td>Venous invasion (negative / positive)</td>
<td>4.62</td>
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<tr>
<td>HOTAIR expression (low / high)</td>
<td>3.62</td>
<td>0.014*</td>
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RR; Relative risk, *P < 0.05, †Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent structures (si)
Figure Legends

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).

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 \textit{HOTAIR} expression, respectively.

B. Heat map of the gene expression averages for 32 CRC samples enriched in gene expression signatures of \textit{HOTAIR}-induced SUZ12, H3K27me3, EZH2, and PRC2 occupancy. These gene expression signatures were obtained from Gupta \textit{et al.} (4). The enriched gene expression averages were classified by high and low \textit{HOTAIR} expression levels. The red and blue colors indicate high and low expression, respectively.

Figure 3

A. Matrigel invasion assay using HCT116 cells after enforced \textit{HOTAIR} expression by lentiviral transduction. \textit{HOTAIR} expression increased the invasiveness of HCT116 (P < 0.05). Error bars represent S.D. (n = 3) *P < 0.05

B. Matrigel invasion assay using SW480 cells after transfection with siRNAs targeting \textit{HOTAIR} (two individual siRNAs; siRNA HOTAIR1, siRNA HOTAIR2) and negative control siRNA (si n.c.). Error bars represent S.D. (n = 3) *P < 0.05
Figure 1

A

B

C

HOTAIR low (n = 80)  
HOTAIR high (n = 20)

CRC patients (n = 100)

HOTAIR/GAPDH

HOTAIR/GAPDH

HOTAIR/GAPDH

N  (n = 100)  
T  (n = 100)

0.0  0.5  1.0  1.5  2.0  
0.6  0.7  0.8  0.9  1.0  
0.0  0.1  0.2  0.3  0.4  0.5  0.6  0.7  0.8  0.9  1.0

Probability of survival

Years after operation

P = 0.002

P = 0.0046
Figure 2

A

![Enrichment plot: SUZ12 OCCUPANCY WITH HOTAIR OVER-EXPRESSION](image1)

\[ P = 0.029 \]
\[ FDR = 0.044 \]

![Enrichment plot: H3K27ME3 OCCUPANCY WITH HOTAIR OVER-EXPRESSION](image2)

\[ P = 0.028 \]
\[ FDR = 0.032 \]

![Enrichment plot: EZH2 OCCUPANCY WITH HOTAIR OVER-EXPRESSION](image3)

\[ P = 0.001 \]
\[ FDR = 0.004 \]

![Enrichment plot: PRC2(SUZ12+EZH2+H3K27)](image4)

\[ P = 0.032 \]
\[ FDR = 0.024 \]

B

**HOTAIR**

low expression
(n = 23)

high expression
(n = 9)

- **Gene Signature with Increased SUZ12 Occupancy by HOTAIR Overexpression**
- **Gene Signature with Increased H3K27me3 Occupancy by HOTAIR Overexpression**
- **Gene Signature with Increased EZH2 Occupancy by HOTAIR Overexpression**
- **Gene Signature with Increased PRC2 Occupancy by HOTAIR Overexpression**

32 CRC Patients
Long non-coding RNA HOTAIR regulates Polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers

Ryunosuke Kogo, Teppei Shimamura, Koshi Mimori, et al.

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