Molecular and Cellular Pathobiology

Upregulation of miR-196a and HOTAIR Drive Malignant Character in Gastrointestinal Stromal Tumors

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Abstract

Large intergenic noncoding RNAs (lincRNA) have been less studied than miRNAs in cancer, although both offer considerable theranostic potential. In this study, we identified frequent upregulation of miR-196a and lincRNA HOTAIR in high-risk gastrointestinal stromal tumors (GIST). Overexpression of miR-196a was associated with high-risk grade, metastasis and poor survival among GIST specimens. miR-196a genes are located within the HOX gene clusters and microarray expression analysis revealed that the HOXC and HOTAIR gene were also coordinately upregulated in GISTs which overexpress miR-196a. In like manner, overexpression of HOTAIR was also strongly associated with high-risk grade and metastasis among GIST specimens. RNA interference–mediated knockdown of HOTAIR altered the expression of reported HOTAIR target genes and suppressed GIST cell invasiveness. These findings reveal concurrent overexpression of HOX genes with noncoding RNAs in human cancer in this setting, revealing miR-196a and HOTAIR as potentially useful biomarkers and therapeutic targets in malignant GISTs.

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Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract (1–3). GISTs arise predominantly in the stomach (60%) and small intestine (25%) but also occur in colon and rectum (5%), esophagus (2%), and other organs (3). Immunohistochemically, GISTs are positive for KIT and CD34 and are negative or variably positive for other neural and smooth muscle cell markers. The expression of KIT and CD34 is a characteristic feature of the intestinal cells of Cajal (ICC), which are located in the intestinal wall and regulate gastrointestinal motility. GISTs are thus thought to originate from ICCs or ICC precursors. Activating KIT mutations have been identified in 80% to 90% of GISTs, and mutation of the platelet-derived growth factor receptor alpha gene (PDGFRA) is observed in approximately 5% of GISTs (1–3). In that context, imatinib mesylate (formerly STI571) was developed as a tyrosine kinase inhibitor and has been shown to inhibit the activities of BCR-ABL, KIT, and PDGFR. Imatinib mesylate is currently being used for the treatment of both chronic myeloid leukemia and metastatic GISTs.

Predicting the biologic potential of GISTs is often difficult, and considerable effort has been made to define the variables that could enable more accurate identification of tumors with malignant potential. In most classification systems, the key prognostic factors for estimating malignant potential are tumor size and mitotic rate, and to a more variable degree, the proliferation index or tumor site (4). Other potential and promising markers of GIST malignancy are molecular alterations. As mentioned, a large majority of GISTs exhibit activating KIT or PDGFRA mutations. By itself, however, mutation status does not fully explain the diverse biology of GISTs, and it is believed that additional molecular alterations are required for the progression of high-risk GISTs. For instance, expression of CD26 (encoded by DPP4) is strongly associated with poor survival among patients with gastric GISTs, suggesting its involvement in the malignant progression of the disease (5).
In addition, we recently showed that hypomethylation of repetitive DNA elements is predominantly observed in malignant GISTs, and that global hypomethylation correlates with increased chromosomal aberration (6).

miRNAs are a class of small noncoding RNAs that regulate gene expression by inducing translational inhibition or direct degradation of target mRNAs through base pairing to partially complementary sites (7). miRNAs are highly conserved among species and play critical roles in a variety of biologic processes, including development, differentiation, cell proliferation, and apoptosis. Consistent with their role in these processes, a number of studies have shown widespread alteration of miRNA expression patterns in cancer (8, 9). It has also been shown that in cancer global miRNA expression profiles, as well as expression of specific miRNAs, correlate with disease prognosis and clinical outcome (10). To date, however, only a few groups have studied miRNA expression in GISTs (11, 12), and no specific miRNAs that could serve as prognostic markers have yet been identified.

In this study, we investigated the global pattern of miRNA expression in GISTs. Our aim was to evaluate the contribution made by miRNAs to the malignant potential of GISTs and to identify predictive biomarkers. We determined that upregulation of miR-196a is strongly associated with high risk and poor prognosis in GIST patients. Furthermore, we provide evidence that overexpression of miR-196a is accompanied by upregulation of HOXC cluster genes and a metastasis-associated non-coding RNA in GISTs.

Materials and Methods

Tumor samples
A total of 56 fresh frozen GIST specimens were obtained from Sapporo Medical University Hospital, Keiyouki Sapporo Hospital, and Osaka University Hospital, as described (6). In addition, formalin-fixed paraffin-embedded (FFPE) tissue sections of 100 GIST specimens were obtained from Niigata University Hospital. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the respective Institutional Review Boards. Risk grade was assessed according to the risk definition system proposed by Fletcher and colleagues (4).

In this study, we investigated the global pattern of miRNA expression in GISTs. Our aim was to evaluate the contribution made by miRNAs to the malignant potential of GISTs and to identify predictive biomarkers. We determined that upregulation of miR-196a is strongly associated with high risk and poor prognosis in GIST patients. Furthermore, we provide evidence that overexpression of miR-196a is accompanied by upregulation of HOXC cluster genes and a metastasis-associated non-coding RNA in GISTs.

miRNA microarray analysis
One-color microarray-based miRNA expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies). Briefly, 100 ng of total RNA from fresh frozen GIST tissues was labeled using miRNA Labeling Reagent (Agilent Technologies), after which the labeled RNA was hybridized to a Human miRNA Microarray V3 (Rel 12.0, G4470C; Agilent Technologies), which covers 859 human miRNAs and 80 viral miRNAs. The microarray data were analyzed using GeneSpring GX version 11 (Agilent Technologies). The normalized microarray data were then compared with the TaqMan assay results using GraphPad PRISM version 5 (GraphPad Software Inc.). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE31741.

Quantitative RT-PCR of miRNA
miR-196a expression was analyzed using TaqMan microRNA Assays (Applied Biosystems). Briefly, 5 μg of total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems), and SDS v1.4 software (Applied Biosystems) was used for comparative ΔΔCt analysis. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control.

Gene expression microarray analysis
One-color microarray-based gene expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies). Briefly, 700 ng of total RNA were amplified and labeled using a Quick Amp Labeling Kit One-Color (Agilent Technologies), after which the synthesized cRNA was hybridized to the Whole Human Genome Oligo DNA microarray, which includes 41,000 probe sets covering 19,416 genes (G4112F; Agilent Technologies). The microarray data were analyzed using GeneSpring GX version 11 (Agilent Technologies). The Gene Expression Omnibus accession numbers for the microarray data are GSE31802 and GSE32064.

Quantitative RT-PCR of HOTAIR
Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative reverse transcriptase PCR (RT-PCR) of HOTAIR was carried out using a TaqMan Gene Expression Assay (Assay ID, Hs03296631_m1; Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems). GAPDH (Assay ID, Hs99999905_m1; Applied Biosystems) served as an endogenous control.

DNA copy number and chromatin signature analysis
DNA copy number was analyzed using array-based comparative genome hybridization (CGH) as described previously (6). Trimethylated Histone H3 lysine 4 (H3K4me3) was analyzed using chromatin immunoprecipitation (ChIP) as described previously (13, 14). Details of the experimental procedures are provided in the Supplementary Methods.
Transfection of miRNA inhibitors and siRNA molecules

GIST-T1 cells were described previously (15). For inhibition of miR-196a, cells (3 × 10^5 cells in 6-well plates) were transfected with 100 pmol of anti-miR miRNA Inhibitors (Ambion) or anti-miR miRNA Inhibitors Negative Control #1 (Ambion) using Lipofectamine2000 (Invitrogen). For RNA interference (RNAi)-mediated knockdown of HOTAIR, 3 different Stealth siRNAs against HOTAIR were generated by Invitrogen, after which a mixture of the 3 was used for transfection. Cells (3 × 10^5 cells in 6-well plates) were transfected with 100 pmol of siRNA or with Stealth RNAi Negative Control Medium GC (Invitrogen) using Lipofectamine2000 (Invitrogen). Total RNA extraction, cell viability assays, and Matrigel invasion assays were carried out 48 hours after transfection as described in the Supplementary Methods.

Statistical analysis

All gene expression levels were log transformed for subsequent statistical analysis because the distribution of expression data seemed to follow a log-normal distribution. Geometric means were therefore calculated as summary statistics for expression levels. Comparisons of continuous variables were made using t tests or one-way ANOVA with post hoc multiple comparisons (Games–Howell test). Pearson’s correlation coefficients were calculated to describe the strength of the correlation between 2 variables. Comparisons of categorical variables were made using Fisher exact test. To assess the association between prognostic factors and gene expression levels, logistic or Cox regression analyses were carried out. For these regression analyses, the most optimal cutoff points were employed to calculate ORs and HRs, with or without adjustment for clinical factors. Kaplan–Meier curves were plotted to compare 2 groups stratified by gene expression status. All statistical analyses were done using SPSS Statistics 18 (IBM Corporation).

Results

Detection of upregulated miR-196a expression in high-risk GISTs

To examine the miRNA expression signature in GISTs, we carried out miRNA microarray analysis with 32 fresh frozen GIST specimens (10 low-risk, 8 intermediate-risk, and 14 high-risk GISTs). The clinicopathologic features of the 32 patients are summarized in Table 1. The TaqMan assay results were highly consistent with the microarray data, though the TaqMan assay did reveal low levels of miR-196a expression in samples in which there was no detectable signal from the microarray (Supplementary Fig. S3).

Upregulation of miR-196a is associated with GIST malignancy

To assess the clinical importance of miR-196a upregulation in GISTs, we next carried out TaqMan assay with 56 fresh frozen GIST specimens (discovery cohort), including the 32 specimens initially analyzed by microarray. The clinicopathologic features of the patients are summarized in Table 1. The TaqMan assay results were highly consistent with the microarray data, though the TaqMan assay did reveal low levels of miR-196a expression in samples in which there was no detectable signal from the microarray (Supplementary Fig. S3).

Also consistent with the microarray results was the finding that miR-196a was markedly upregulated in high-risk GISTs, as compared with the other groups (P = 0.004, one-way ANOVA; Fig. 1C, Supplementary Table S2). In addition, logistic regression analysis revealed that the association between miR-196a upregulation and the high-risk category was highest when we employed a cutoff value of miR-196a/Up 0.4 or more (OR = 13.7; 95% CI: 3.4–546; P < 0.001; Supplementary Table S3). Survival data were obtained for 32 patients, and Cox hazard analysis revealed the highest HR for patients with elevated miR-196a expression when a cutoff value of 1.4 was employed (Table 2). Kaplan–Meier analysis showed poor overall survival among patients with GISTs expressing high levels of miR-196a, though the effect was not statistically significant (Fig. 1D).

We next used TaqMan assay to analyze an independent validation cohort consisting of 100 FFPE GIST specimens (Table 1). Consistent with the findings summarized above, we observed that upregulation of miR-196a was associated with high-risk GISTs (Fig. 1C, Supplementary Tables S2 and S3). By using the same cutoff value (miR-196a/Up ≥ 1.4), Cox hazard analysis revealed an elevated HR for patients exhibiting high levels of miR-196a expression (Table 2), and Kaplan–Meier analysis showed shorter survival times for the same patients (Fig. 1D). These results confirmed the prognostic value of miR-196a expression in both fresh frozen and FFPE GIST specimens.

Finally, we combined the GIST samples in the discovery and validation cohorts to examine the clinicopathologic significance of miR-196a. Expression of miR-196a correlated positively with high-risk grade (Fig. 1C, Supplementary Tables S2 and S3), poor clinical outcome (Fig. 1D, Table 2), tumor size, mitotic count, and metastasis (Table 3). Interestingly, although expression of miR-196a was not associated with age or gender, it was strongly associated with tumor location (Table 3). The median level of miR-196a expression was lowest in specimens from esophageal GISTS and then increased as the GIST site moved from the oral side toward the anal side of the gastrointestinal tract (P < 0.001; Table 3, Supplementary Fig. S4). Importantly, although the average level of miR-196a expression was higher in small intestine than in stomach, it was positively
correlated with high-risk grade in both organs (Supplementary Table S4).

**Concurrent upregulation of miR-196a and HOX cluster genes in GISTs**

To analyze the relationship between miR-196a upregulation and the global gene expression profiles in GISTs, we selected age-, gender-, and tumor location–matched GIST specimens showing either low ($n = 7$; average miR-196a/U6 = 0.1) or high miR-196a expression ($n = 7$; average miR-196a/U6 = 15.7) and subjected them to gene expression microarray analysis (Supplementary Table S5). We found that for 4,947 probe sets (corresponding to 3,206 unique genes), there was more than a 2-fold difference in expression between GISTs with miR-196a

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**Figure 1.** Upregulation of miR-196a expression in GISTs is associated with a high-risk grade and poor prognosis. A, scatter plots analyses: plotting low-risk ($n = 10$) versus high-risk GISTs ($n = 14$; left) and intermediate-risk ($n = 8$) versus high-risk GISTs ($n = 14$; right) revealed overexpression of miR-196a in high-risk GISTs. Microarray data are normalized and log transformed (base 2). Expression of miR-196a is highlighted by a circle. B, levels of miR-196a expression obtained from microarray analysis of 32 GIST specimens. Risk categories are indicated below. C, comparison of miR-196a expression using TaqMan assay in low- ($n = 14$), intermediate- ($n = 14$), and high-risk GISTs ($n = 23$) in a discovery cohort (left), very low- or low-risk ($n = 46$), intermediate-risk ($n = 25$), and high-risk GISTs ($n = 26$) in a validation cohort (middle) and all GIST specimens (right). Results are normalized to internal U6 snRNA. D, Kaplan-Meier curves showing the effect of miR-196a expression (high, miR-196a/U6 $> 1.4$; low, miR-196a/U6 $< 1.4$) on overall survival in the discovery cohort (left), validation cohort (middle), and all GIST patients (right).
overexpression and those without it. Hierarchical clustering analysis using the 4,947 probe sets clearly distinguished between tumors on the basis of their miR-196a expression status (Fig. 2A), and Gene Ontology analysis suggested that genes related to “immune system,” “plasma membrane,” and “cell communication” are strongly overrepresented among the differentially expressed genes (Supplementary Table S6).

To further characterize the differentially expressed genes, we carried out a gene set analysis and obtained the highest enrichment score with the HOX gene set (Supplementary Table S7). We found miR-196a to be encoded at 2 paralogous loci, miR-196a-1 and miR-196a-2, which are located within the HOXB and HOXC clusters, respectively (Fig. 2B; ref. 16). Hierarchical clustering analysis using the expression data for HOXC genes clearly differentiated the GIST samples into 2 groups, and we observed perfect correspondence between higher expression of multiple HOX genes and upregulation of miR-196a (Fig. 2C). By contrast, genes in other HOX clusters did not show such obvious correlations with miR-196a (Fig. 2C, Supplementary Fig. S5). We next compared the microarray signal for each HOX gene with the miR-196a expression and found strong positive correlations between the expression levels of a number of HOXC genes and those of miR-196a (Fig. 3D, Supplementary Fig. S6). Notably, we also found that expression of HOTAIR, which encodes a large intergenic non-coding RNA (lincRNA) and is located in an antisense orientation relative to the HOXC genes, is concurrently upregulated with miR-196a (Fig. 2C and D). Levels of miR-196a expression also correlated moderately with those of the HOXB genes neighboring miR-196a-1 (HOXB13 and HOXB9), but the correlations were less significant than those with HOXC genes (Supplementary Fig. S7).

The similarity between the expression patterns of HOXC genes and those of the noncoding RNAs encoded in the same locus is indicative of a common regulatory mechanism involved in the activation of these genes in GISTs. However, array CGH analysis of 27 GIST specimens failed to detect either gain or loss in any HOX loci, irrespective of miR-196a or HOX gene expression, which makes it unlikely that genomic amplification is the cause of their overexpression (Supplementary Fig. S8).

### Upregulation of HOTAIR is associated with GIST malignancy

A recent study showed that HOTAIR is overexpressed in primary breast cancer and is associated with metastasis (17). To examine its clinical significance in GISTs, we carried out TaqMan assays of HOTAIR with the discovery cohort samples. We found that the microarray signals for HOTAIR were highly correlated with those of miR-196a, and we next compared the microarray signals for each HOX gene with the miR-196a expression and found strong positive correlations between the expression levels of a number of HOXC genes and those of miR-196a (Fig. 3D, Supplementary Fig. S6). Notably, we also found that expression of HOTAIR, which encodes a large intergenic non-coding RNA (lincRNA) and is located in an antisense orientation relative to the HOXC genes, is concurrently upregulated with miR-196a (Fig. 2C and D). Levels of miR-196a expression also correlated moderately with those of the HOXB genes neighboring miR-196a-1 (HOXB13 and HOXB9), but the correlations were less significant than those with HOXC genes (Supplementary Fig. S7).

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### Table 2. miR-196a expression is associated with poor clinical outcome in GIST patients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>miR-196a/U6</th>
<th>Survival</th>
<th>Death</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>HR* (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discovery cohort</td>
<td>&lt;1.4</td>
<td>22</td>
<td>1</td>
<td>6.3 (0.7–57.5)</td>
<td>0.104</td>
<td>32.9 (2.0–551.3)</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>≥1.4</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Validation cohort</td>
<td>&lt;1.4</td>
<td>73</td>
<td>13</td>
<td>3.9 (1.4–11.1)</td>
<td>0.011</td>
<td>8.4 (2.6–26.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>≥1.4</td>
<td>9</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All samples</td>
<td>&lt;1.4</td>
<td>95</td>
<td>14</td>
<td>4.9 (2.1–11.7)</td>
<td>&lt;0.001</td>
<td>9.1 (3.5–23.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>≥1.4</td>
<td>14</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*Age and gender adjusted HR.
consistent with the TaqMan assay results (Supplementary Fig. S9). HOTAIR was upregulated exclusively in high-risk GISTs, as compared with low- or intermediate-risk GISTs ($P = 0.018$; Fig. 3A), and its expression correlated positively with the expression of miR-196a (Fig. 3B) and HOXC genes (Fig. 3C, Supplementary Fig. S10). In addition, logistic regression analysis revealed that high levels of HOTAIR expression in GISTs ($HOTAIR / GAPDH > 0.0002$) were strongly associated with metastasis (age and gender adjusted OR = 8.2; 95% CI: 1.4–48.4; $P = 0.021$). Cox hazard analysis suggested an elevated HR for patients with high HOTAIR expression (Table 4), and Kaplan–Meier analysis showed poor overall survival for the same patients, though the effect was not statistically significant (Fig. 3D). We also tried to analyze HOTAIR expression in the FFPE specimens; however, we failed to detect expression of either HOTAIR or GAPDH in these samples, most likely due to an inadequate quality of the RNA.

### Reduced expression of miR-196a and HOTAIR target genes in GISTs

To examine the functional role of miR-196a in GISTs, we interrogated our gene expression microarray data for miR-196a computationally predicted by TargetScan. Of the 2,248 genes whose expression was reduced in GISTs overexpressing miR-196a, 95 corresponded to predicted targets (Supplementary Fig. S11, Supplementary Table S8). This gene list included $ANXA1$ (Annexin A1), which is an experimentally validated miR-196a target gene (18). In addition, expression of several $HOX$ genes, including $HOXB8$, was reduced in GISTs overexpressing miR-196a, which is consistent with an earlier finding of miR-196a–directed cleavage of $HOXB8$ mRNA (Supplementary Fig. S11; ref. 19).

In normal human fibroblasts, HOTAIR represses HOXD gene expression by interacting with polycomb repressive complex 2 (PRC2; ref. 20). In breast cancer cells, overexpression of HOTAIR was shown to recruit PRC2 to more than 800 gene promoters, leading to histone H3K27 methylation and epigenetic silencing of the target genes (17). We therefore examined our microarray data for the reported HOTAIR-induced PRC2 target genes. Among 14 GISTs analyzed with the microarray, all 7 tumors strongly expressing miR-196a showed elevated HOTAIR expression (average $HOTAIR / GAPDH = 0.00254$), whereas all tumors only weakly expressing miR-196a showed little or no HOTAIR expression (average $HOTAIR / GAPDH = 0.00001$). We found that expression of 144 HOTAIR target genes was reduced in GISTs overexpressing HOTAIR (Supplementary Fig. S11, Supplementary Table S9). These results indicated that overexpression of miR-196a and HOTAIR may contribute to the malignant progression of GISTs by modulating expression of their target genes.

### Inhibition of miR-196a and HOTAIR suppresses GIST cell invasion

We next utilized a cultured GIST cell line to determine whether upregulation of miR-196a and HOTAIR is responsible for the malignant potential of GISTs. We found that both
miR-196a and *HOTAIR* are expressed in GIST-T1 cells (Supplementary Fig. S12). We then carried out cell viability and Matrigel invasion assays after transfecting GIST-T1 cells with an anti–miR-196a inhibitor molecule. Gene expression microarray analysis revealed that a number of predicted miR-196a target genes, including *ANXA1* and *HOXA5*, were upregulated by inhibition of miR-196a (Supplementary Table S10), and although we observed no effects on cell viability, inhibition of miR-196a moderately suppressed cell invasion (Supplementary Fig. S13). We next disrupted *HOTAIR* expression by transfecting the cells with siRNAs targeting it (Fig. 3E). Although knockdown of *HOTAIR* did not significantly affect cell viability, it suppressed the invasiveness of GIST-T1 cells (Fig. 3E and F). Moreover, gene expression microarray analysis revealed that a number of reported *HOTAIR* target genes, including *PCHD10*, *SEMA6A*, and *STK17B*, were upregulated upon knockdown of *HOTAIR* (Supplementary Table S11). In all, we found that 1,424 genes were upregulated by siHOT (>2-fold), and Gene Ontology analysis revealed enrichment of genes related to “nucleus,” “chromosome,” and “membrane-bounded...
organelle” (Supplementary Tables S12 and S13). These results suggested that HOTAIR may modulate transcription of a large number of genes and may have previously unidentified roles in GIST cells.

Finally, we sought to clarify the biologic relationship between miR-196a, HOTAIR, and HOXC genes. We first tested whether upregulation of miR-196a is a downstream effect of HOTAIR dysregulation, or vice versa. We found that inhibition
of miR-196a had no effect on HOTAIR expression in GIST-T1 cells, nor did knockdown of HOTAIR affect miR-196a expression. This suggested that overexpression of miR-196a or HOTAIR is not a simple downstream effect of their dysregulation (Supplementary Fig. S12). By contrast, analysis of the chromatin status in GIST-T1 cells using ChIP-PCR revealed enrichment of trimethylated histone H3 lysine 4 (H3K4me3), a hallmark of active gene transcription, at the transcription start sites of multiple HOX genes and HOTAIR (Supplementary Fig. S14). Moreover, we found concurrent overexpression of miR-196a, HOTAIR, and HOXC genes in other cancer cells, including the KatoIII gastric cancer cell line. By carrying out high-resolution ChIP-seq analysis with the KatoIII cells, we observed significant enrichment of H3K4me3 over a wide range (more than 50 kb) of the HOXC cluster, which suggested that an epigenetic mechanism is involved in the dysregulation of this genomic region (Supplementary Fig. S15).

Discussion

Although the results of recent studies suggest that the gene expression signatures of GISTs are predictive of malignancy and drug sensitivity of the tumors (5, 21), the clinical significance of the miRNA expression signature is not yet fully understood. In this study, we found that upregulation of miR-196a is strongly associated with a high-risk grade, metastasis, and poor prognosis in GIST patients. Furthermore, overexpression of miR-196a is accompanied by upregulation of multiple HOX genes and the metastasis-related lincRNA HOTAIR. To our knowledge, this is the first article to show concurrent overexpression of collinear HOX genes and non-coding RNAs in human malignancy.

A number of studies have implicated miR-196a in malignancy, but its role may differ among tumor types. Upregulation of miR-196a is observed in esophageal adenocarcinomas and their precancerous lesions, Barrett’s esophagus and dysplasia, which suggests miR-196a is a potential marker of the malignant progression of Barrett’s esophagus (22). Strong expression of miR-196a is also associated with a poor prognosis in pancreatic adenocarcinoma and glioblastoma patients (23, 24). In addition, functional analysis showed that expression of miR-196a in esophageal, breast, and endometrial cancer cells promotes proliferation and suppresses apoptosis through downregulation of ANXA1 (18). These results suggest that miR-196a contributes to oncogenesis in cancer. On the other hand, miR-196a is significantly downregulated in melanoma, and its reexpression inhibited the invasive behavior of melanoma cells by targeting HOXC8 (25). Similarly, miR-196a suppressed HOXC8 and inhibited invasion and metastasis by breast cancer cells (26). Thus miR-196a seems to exert opposite effects in tumors of different origins.

The HOX genes are a highly conserved subgroup of the homeobox superfamily, and they play essential roles in a variety of biologic processes, including development, differentiation, apoptosis, and angiogenesis (27). In humans, 4 HOX clusters containing 39 HOX genes have been identified, and dysregulation of their expression is observed in various malignancies. Although the role of HOXs in cancer is not fully understood, its aberrant expression is thought to affect pathways that promote tumorigenesis and metastasis (27). For instance, HOXC8 mRNA is overexpressed in prostate cancer cells and is associated with tumor cell proliferation and metastasis (28–30). In addition, HOXC5 and HOXC8 mRNAs are upregulated in cervical cancer cells (31), and one recent study suggested HOXC10 plays a key role of in the progression and invasion in cervical cancer (32).

An association between miR-196a and HOX expression in cancer has also been reported. Reduced expression of miR-196a in malignant melanoma cells leads to upregulation of HOXB7 and, in turn, activation of BMP4, a major modulator of migration (33). As mentioned above, miR-196a also inhibits invasion and metastasis by downregulating HOXC8 in melanoma and breast cancer cells (26, 34). Taken together, these results suggest that miR-196a acts as a tumor suppressor by targeting HOX genes in these tumor types. By contrast, we show in this study that both the miR-196a and HOTAIR genes are concurrently upregulated in malignant GISTs. Our findings are reminiscent of an earlier report showing that the expression patterns of miRNAs embedded in HOX clusters are very similar to those of HOX genes during mammalian embryogenesis (35). Global gene expression analysis revealed that expression of multiple putative miR-196a targets, including ANXA1, is diminished in GISTs overexpressing miR-196a, whereas their expression is enhanced upon inhibition of miR-196a in cultured GIST-T1 cells. In addition, inhibition of miR-196a in GIST cells overexpressing that miRNA moderately suppressed cell invasion. Taken together, our results indicate that upregulation of HOX genes along with miR-196a may contribute to the malignant potential of GIST.

HOTAIR is located within the HOXC cluster and encodes a lincRNA known to repress its target genes by directly interacting with histone modification complexes. Epigenetic gene

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Table 4. HOTAIR expression is associated with poor clinical outcome in GIST patients

<table>
<thead>
<tr>
<th>HOTAIR/GAPDH</th>
<th>Survival</th>
<th>Death</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>HR&lt;sup&gt;a&lt;/sup&gt; (95% CI)</th>
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<td>&lt;0.0002</td>
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<td>7</td>
<td>4</td>
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<sup>a</sup>Age and gender adjusted HR.

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HOTAIR that promote aggressiveness in GISTs. Interestingly, depletion of HOTAIR suppressed the HOXD locus by recruiting the PRC2 complex, which consists of the histone H3K27 methylase EZH2, SUZ12, and EED (20). It was also recently shown that HOTAIR serves as a scaffold for multiple repressor complexes, including PRC2 and LSD1/CoREST/REST (36). LSD1 is a demethylase that serves as a scaffold for multiple repressor complexes, including PRC2 and EED (20). It was also recently shown that HOTAIR is one of the mechanisms that promote aggressiveness in GISTs. Interestingly, depletion of HOTAIR induced a significant change in the gene expression profile in GIST cells, suggesting that HOTAIR may regulate a spectrum of genes other than the previously reported target genes. Further studies, including genome-wide histone modification analysis, may reveal as yet unidentified roles played by HOTAIR in the malignant progression of GISTs.

The mechanism underlying upregulation of HOX cluster genes and noncoding RNAs in GISTs is intriguing. Our array CGH analysis did not detect chromosomal aberrations in any HOX loci, making it unlikely that gene amplification is the cause of their overexpression. However, we found that the transcription start sites of multiple genes in the HOXC cluster are marked by an active histone mark, H3K4me3, in GIST-T1 cells. Moreover, high-resolution ChIP-seq analysis revealed that, in cancer cells, the entire region is significantly enriched with H3K4me3, leading to overexpression of the affected genes. Our results are reminiscent of the recent finding that rearrangement of MLL in leukemia induces active histone modifications at the promoters of HOX genes and miR-196b, resulting to their overexpression (38–40). Although such rearrangements are not known in GISTS, further study to clarify the involvement of epigenetic modifiers in malignant GISTS may lead to identification of new therapeutic targets.

Overall, our study has shown that noncoding RNAs encoded in the HOXC cluster could be useful predictive markers as well as novel therapeutic targets in malignant GISTS. As miRNAs are well preserved in FFPE specimens (41), miR-196a could be a reliable biomarker for risk assessment. We also provide evidence that HOTAIR is significantly upregulated in high-risk GISTS, indicating that this lncRNA also could be a useful biomarker, as well as a novel therapeutic target. Further study of the causes and functions of HOXC locus activation in GISTS may provide new strategies for the treatment of GIST patients.

Disclosure of Potential Conflicts of Interest
T. Nishida has received a research grant from Novartis Pharma K.K. The remaining authors disclose no conflicts of interest.

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Upregulation of miR-196a and HOTAIR Drive Malignant Character in Gastrointestinal Stromal Tumors

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