Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors

Takeshi Niinuma1*, Hiromu Suzuki1,2*, Masanori Nojima3, Katsuhiko Nosho1, Hiroyuki Yamamoto1, Hiroyuki Takamaru1, Eiichiro Yamamoto2, Reo Maruyama2, Takayuki Nobuoka4, Yasuaki Miyazaki5, Toshiro Nishida5,6, Takeo Bamba7, Tatsuo Kanda7, Yoichi Ajioka8, Takahiro Taguchi9, Satoshi Okahara10, Hiroaki Takahashi10, Yasunori Nishida11, Masao Hosokawa11, Tadashi Hasegawa12, Takashi Tokino13, Koichi Hirata4, Kohzoh Imai14, Minoru Toyota2, Yasuhisa Shinomura1

1First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
2Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo, Japan
3Department of Public Health, Sapporo Medical University School of Medicine, Sapporo, Japan
4First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan
5Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan
6Department of Surgery, Osaka Police Hospital, Osaka, Japan
7Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
8Division of Molecular and Diagnostic Pathology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
9Division of Human Health and Medical Science, Graduate School of Kuroshio Science, Kochi University, Nankoku, Japan
10Department of Gastroenterology, Keiyukai Sapporo Hospital, Sapporo, Japan
11Department of Surgery, Keiyukai Sapporo Hospital, Sapporo, Japan
12Department of Surgical Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan
13Medical Genome Science, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
14Division of Novel Therapy for Cancer, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

*These authors contributed equally to this work.
Running title: Upregulation of miR-196a and HOTAIR in GIST

Keywords: GIST, microRNA, lincRNA, clinical outcome, metastasis.

Grant support:
This study was supported in part by Grants-in-Aid for Scientific Research (B) from the Japan Society for Promotion of Science (Y. Shinomura), A3 foresight program from the Japan Society for Promotion of Science (H. Suzuki), a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control (M. Toyota, H. Suzuki), a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare, Japan (M. Toyota, H. Suzuki) and the Takeda Science Foundation (H. Suzuki).

Correspondence to:
Hiromu Suzuki, M.D, Ph.D.
Department of Molecular Biology, Sapporo Medical University
S1, W17, Chuo-Ku, Sapporo 060-8556, Japan
e-mail: hsuzuki@sapmed.ac.jp
Tel: +81-11-611-2111
Fax: +81-11-622-1918
or
Yasuhisa Shinomura, M.D, Ph.D.
First Department of Internal Medicine, Sapporo Medical University
S1, W16, Chuo-Ku, Sapporo 060-8543, Japan
e-mail: shinomura@sapmed.ac.jp
Tel: +81-11-611-2111
Fax: +81-11-611-2282

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

Abstract: 148 words
Main text: 4598 words
Total number of figures and tables: 7
Abstract

Large intergenic noncoding RNAs (lincRNAs) have been less studied than microRNAs (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 (GISTs). 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. RNAi-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.
Introduction

Gastrointestinal stromal tumors (GISTs) 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 (ICCs), 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 biological 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 \textit{KIT} or \textit{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 \textit{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).

MicroRNAs (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 biological processes, including development, differentiation, cell proliferation and apoptosis. Consistent with their role in these processes, a number of studies have demonstrated 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 the present 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 noncoding RNA in GISTs.
Materials and methods

Tumor samples

A total of 56 fresh frozen GIST specimens were obtained from Sapporo Medical University Hospital, Keiyukai 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 et al. (4). Tumors that were <2 cm in diameter with a mitotic count of <5/50 high-power fields (HPF) were categorized as very low risk. Tumors that were 2-5 cm in diameter with a mitotic count of <5/50 HPF were considered to be low risk. Tumors that were <5 cm in diameter with a mitotic count of 6-10/50 HPF, or were 5-10 cm with a mitotic count of <5/50 HPF were considered to be intermediate risk. Tumors that were >5 cm in diameter with a mitotic count of >5/50 HPF, >10 cm in diameter with any mitotic count, or any size with a mitotic count of >10/50 HPF were considered to be high risk. Total RNA was extracted from fresh frozen tissue specimens using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Total RNA was extracted from FFPE tissue specimens using a RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). Tumor
tissues were reviewed by pathologists and were macrodissected; laser capture microdissection was not carried out in this study.

**miRNA microarray analysis**

One-color microarray-based miRNA expression analysis was carried out according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA, USA). 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 ver. 11 (Agilent Technologies). The normalized microarray data were then compared with the TaqMan assay results using GraphPad PRISM ver. 5 (GraphPad Software Inc., La Jolla, CA, USA). 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, Foster City, CA, USA). Briefly, 5 ng 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 delta Ct 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, Santa Clara, CA). 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 ver. 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, Carlsbad, CA, USA). Quantitative 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 (3x10^5 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 RNAi-mediated knockdown of HOTAIR, three different Stealth siRNAs against HOTAIR were
generated by Invitrogen, after which a mixture of the three was used for transfection. Cells ($3 \times 10^5$ 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 h 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 appeared 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 two variables. Comparisons of categorical variables were made using Fisher’s exact test. To assess the association between prognostic factors and gene expression levels, logistic or Cox regression analyses were performed. For these regression analyses, the most optimal cut-off points were employed to calculate odds ratios and hazard ratios, with or without adjustment for clinical factors. Kaplan-Meier curves were plotted to compare two groups.
stratified by gene expression status. All statistical analyses were performed using SPSS Statistics 18 (IBM Corporation, Somers, NY, USA).
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 clinicopathological features of the 32 patients are listed in Supplementary Table S1. Of 939 probe sets, 470 were excluded because of the absence of a detectable signal in any of the samples tested. Unsupervised hierarchical clustering using the remaining 469 probe sets revealed that GISTs in which there was abundant expression of miRNAs encoded on chromosome 14q32.31 form a separate cluster (Supplementary Figs. S1 and S2). Moreover, by comparing the miRNA expression profiles with array CGH results, we found that this cluster is enriched in tumors without 14q loss. These results are consistent with recent reports showing an inverse relationship between 14q loss and expression of miRNAs located on 14q in GISTs (11, 12), which is indicative of the reliability of our microarray analysis. We next carried out a scatter plot analysis and found that miR-196a is markedly upregulated in high-risk GISTs, as compared to low- or intermediate-risk GISTs (Fig. 1A). As shown in Fig. 1B, miR-196a was undetectable in all but one of the low- and intermediate-risk GISTs tested, whereas it was upregulated in more than half of the high-risk
tumors. The elevated expression of miR-196a was observed in both gastric and small intestinal GISTs (Supplementary Table S1).

**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 clinicopathological 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 to 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/U6 $\geq 0.4$ (odds ratio, 13.7; 95% confidence interval, 3.4–54.6; $P < 0.001$) (Supplementary Table S3). Survival data were obtained for 32 patients, and Cox hazard analysis revealed the highest hazard ratio 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/U6 ≥ 1.4), Cox hazard analysis revealed an elevated hazard ratio 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 confirm 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 clinicopathological 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 4947 probe sets (corresponding to 3206 unique genes) there was more than a 2-fold difference in expression between GISTs with miR-196a overexpression and those without it. Hierarchical clustering analysis using the 4947 probe sets clearly distinguished between tumors based on 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 performed a gene set analysis (GSA) and obtained the highest enrichment score with the HOX gene set (Supplementary Table S7). We found miR-196a to be encoded at two paralogous loci, miR-196a-1 and miR-196a-2, which are located within the HOXB and HOXC clusters, respectively (Fig. 2B) (16). Hierarchical clustering analysis using the expression data for HOXC genes clearly differentiated the GIST samples into two groups, and we observed perfect correspondence between higher expression of multiple HOXC 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 noncoding 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 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 consistent with the TaqMan assay results (Supplementary Fig. S9). *HOTAIR* was upregulated exclusively in high-risk GISTs, as compared to 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 odd ratio, 8.2; 95%
confidence interval, 1.4–48.4; \( P = 0.021 \)). Cox hazard analysis suggested an elevated hazard ratio for patients with high \( \textit{HOTAIR} \) 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 \( \textit{HOTAIR} \) expression in the FFPE specimens; however, we failed to detect expression of either \( \textit{HOTAIR} \) or \( \textit{GAPDH} \) in these samples, most likely due to an inadequate quality of the RNA.

**Reduced expression of miR-196a and \( \textit{HOTAIR} \) target genes in GISTs**

To examine the functional role of miR-196a in GISTs, we interrogated our gene expression microarray data for miR-196a target genes computationally predicted by TargetScan. Of the 2248 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 \( \textit{ANXA1} \) (Annexin A1), which is an experimentally validated miR-196a target gene (18). In addition, expression of several \( \textit{HOX} \) genes, including \( \textit{HOXB8} \), was reduced in GISTs overexpressing miR-196a, which is consistent with an earlier finding of miR-196a-directed cleavage of \( \textit{HOXB8} \) mRNA (Supplementary Fig. S11) (19).
In normal human fibroblasts, *HOTAIR* represses *HOXD* gene expression by interacting with polycomb repressive complex 2 (PRC2) (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 indicate 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 1424 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 suggest 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 biological 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 suggests 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 HOXC 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 performing 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 suggests 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 the tumors’ malignancy and drug sensitivity (5, 21), the clinical significance of the miRNA expression signature is not yet fully understood. In the present 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 HOXC genes and the metastasis-related lincRNA HOTAIR. To our knowledge, this is the first to report to show concurrent overexpression of collinear HOX genes and noncoding 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 demonstrated 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 re-expression 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 appears to exert opposite effects in tumors of different origins.

The HOX genes are a highly conserved subgroup of the homeobox superfamily, and play essential roles in a variety of biological processes, including development, differentiation, apoptosis and angiogenesis (27). In humans, four 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 demonstrate in the present study that both the miR-196a and HOXC 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 HOXC 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 regulation is closely associated with histone modifications in which di- or trimethylation of histone H3 lysine 4 (H3K4me2 or H3K4me3) is enriched within active gene promoters. In addition, trimethylation of histone H3 lysine 27 (H3K27me3) is a marker of gene silencing. In normal adult
fibroblasts, *HOTAIR* suppresses 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 specifically mediates demethylation of H3K4, leading to repression of the target genes. *HOTAIR* is also strongly implicated in cancer metastasis. In breast cancer cells, *HOTAIR* induces re-targeting of the PRC2 complex throughout the genome, which leads to the silencing of multiple tumor suppressor and metastasis suppressor genes (17). Overexpression of *HOTAIR* is also predictive of recurrence in hepatocellular carcinoma patients after liver transplantation (37). We observed that upregulation of *HOTAIR* is closely associated with GIST aggressiveness and metastasis. In addition, functional analysis using GIST-T1 cells showed that RNAi-mediated knockdown of *HOTAIR* suppressed cell invasion. These results strongly suggest that upregulation of *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 HOXA 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 demonstrated 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 lincRNA 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.
Acknowledgements

The authors thank Dr. William F. Goldman for editing the manuscript, and M. Ashida for technical assistance.

Grant support

This study was supported in part by Grants-in-Aid for Scientific Research (B) from the Japan Society for Promotion of Science (Y. Shinomura), A3 foresight program from the Japan Society for Promotion of Science (H. Suzuki), a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control (M. Toyota, H. Suzuki), a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor, and Welfare, Japan (M. Toyota, H. Suzuki) and the Takeda Science Foundation (H. Suzuki).
References


Table 1. Clinical features of the GIST samples used in this study

<table>
<thead>
<tr>
<th>Table 1. Clinical features of the GIST samples used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Discovery cohort</strong></td>
</tr>
<tr>
<td>Age (years, median ± SD) 68.0 ± 15.2</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male 32 (57.1%)</td>
</tr>
<tr>
<td>Female 24 (42.9%)</td>
</tr>
<tr>
<td>Tumor location</td>
</tr>
<tr>
<td>Stomach 40 (71.4%)</td>
</tr>
<tr>
<td>Small intestine 14 (25.0%)</td>
</tr>
<tr>
<td>Omentum 1 (1.8%)</td>
</tr>
<tr>
<td>Colorectum 1 (1.8%)</td>
</tr>
<tr>
<td>Risk category (n = 51)</td>
</tr>
<tr>
<td>Low 14 (27.5%)</td>
</tr>
<tr>
<td>Intermediate 14 (27.5%)</td>
</tr>
<tr>
<td>High 23 (45.0%)</td>
</tr>
<tr>
<td><strong>Validation cohort</strong></td>
</tr>
<tr>
<td>Age (years, median ± SD) 64.0 ± 12.4</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male 44 (44.0%)</td>
</tr>
<tr>
<td>Female 56 (56.0%)</td>
</tr>
<tr>
<td>Tumor location</td>
</tr>
<tr>
<td>Esophagus 5 (5.2%)</td>
</tr>
<tr>
<td>Stomach 84 (84.0%)</td>
</tr>
<tr>
<td>Small intestine 8 (8.0%)</td>
</tr>
<tr>
<td>Colorectum 3 (3.0%)</td>
</tr>
<tr>
<td>Risk category (n = 97)</td>
</tr>
<tr>
<td>Very low 1 (1.0%)</td>
</tr>
<tr>
<td>Low 45 (46.4%)</td>
</tr>
<tr>
<td>Intermediate 25 (25.8%)</td>
</tr>
<tr>
<td>High 26 (26.8%)</td>
</tr>
</tbody>
</table>
### Table 2. miR-196a expression is associated with poor clinical outcome in GIST patients

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

HR, hazard ratio; CI, confidence interval. *Age and gender adjusted HR.
Table 3. Correlation between miR-196a expression and clinicopathological features of GISTs

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>miR-196a/U6</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Geometric mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 65</td>
<td>76</td>
<td>0.093</td>
<td>0.051 - 0.168</td>
<td></td>
</tr>
<tr>
<td>≥ 65</td>
<td>81</td>
<td>0.074</td>
<td>0.044 - 0.127</td>
<td>0.581</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>76</td>
<td>0.104</td>
<td>0.059 - 0.186</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>80</td>
<td>0.069</td>
<td>0.040 - 0.119</td>
<td>0.297</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td>5</td>
<td>0.019</td>
<td>0.001 - 0.417</td>
<td>Ref</td>
</tr>
<tr>
<td>Stomach</td>
<td>124</td>
<td>0.061</td>
<td>0.039 - 0.094</td>
<td>0.741</td>
</tr>
<tr>
<td>Small intestine</td>
<td>22</td>
<td>0.395</td>
<td>0.163 - 0.957</td>
<td>0.161</td>
</tr>
<tr>
<td>Colorectum</td>
<td>4</td>
<td>4.936</td>
<td>2.564 - 9.502</td>
<td>0.023 &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor size (cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5.0</td>
<td>70</td>
<td>0.045</td>
<td>0.026 - 0.075</td>
<td></td>
</tr>
<tr>
<td>&gt; 5.0</td>
<td>81</td>
<td>0.118</td>
<td>0.066 - 0.210</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Mitotic count (/50HPF)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>105</td>
<td>0.036</td>
<td>0.025 - 0.053</td>
<td></td>
</tr>
<tr>
<td>&gt; 5</td>
<td>35</td>
<td>0.539</td>
<td>0.215 - 1.353</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28</td>
<td>0.747</td>
<td>0.307 - 1.819</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>108</td>
<td>0.041</td>
<td>0.027 - 0.063</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

CI, confidence interval.
Table 4. HOTAIR expression is associated with poor clinical outcome in GIST patients

<table>
<thead>
<tr>
<th>Outcome</th>
<th>HOTAIR/GAPDH</th>
<th>Survival</th>
<th>Death</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
<th>HR*</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.0002</td>
<td>26</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 0.0002</td>
<td>7</td>
<td>4</td>
<td>3.8</td>
<td>0.7 - 21.2</td>
<td>0.123</td>
<td>9.0</td>
<td>1.2 - 68.9</td>
<td>0.034</td>
<td></td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval. *Age and gender adjusted HR.
Figure legends

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) vs. high-risk GISTs (n = 14) (left) and intermediate-risk (n = 8) vs. 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).

Figure 2
GIST gene expression signatures reveal a strong correlation between the miR-196a and HOXC genes. A, Heat map of the gene expression signatures correlated with miR-196a expression. Rows represent probe sets and columns represent patients. A total of 4947 probe sets differentially expressed (> 2-fold change) between GISTs with (n = 7) and without (n = 7) miR-196a overexpression were selected, after which hierarchical clustering was performed. The miR-196a expression status is indicated below. B, Schematic representations of the miR-196a family locations within the HOX gene clusters. C, Hierarchical clustering analysis using HOXB (left) and HOXC (right) expression data. miR-196a expression status is indicated below. D, Correlations between the expression levels of miR-196a and HOXC genes or HOTAIR. Expression of miR-196a was analyzed using TaqMan assay and was normalized to internal U6 snRNA. Microarray data for HOXC and HOTAIR were normalized and log transformed (base 2). The Pearson correlation coefficients and P-values are shown.
Figure 3
Upregulation of *HOTAIR* in malignant GISTs.  A, TaqMan assay of *HOTAIR* in a panel of GIST specimens (n = 52).  Results are normalized to internal *GAPDH* expression.  Risk categories are indicated below.  B, Correlation between levels of *HOTAIR* and miR-196a expression detected using TaqMan assay.  The Pearson correlation coefficient and *P*-value are shown.  C, Correlations between levels of *HOTAIR* expression detected using TaqMan assay and those of *HOXC* genes detected from microarrays.  The Pearson correlation coefficients and *P*-values are shown.  D, Kaplan-Meier curves showing the effect of *HOTAIR* expression (high, *HOTAIR*/*GAPDH* ≥ 0.0002; low, *HOTAIR*/*GAPDH* < 0.0002) on overall survival among GIST patients.  E, TaqMan assay for *HOTAIR* in GIST-T1 cells transfected with control siRNA (siCONT) or siRNA targeting *HOTAIR* (siHOT).  F, Cell viability assay using GIST-T1 cells transfected with siCONT or siHOT.  Cell viabilities were determined 48 h after transfection.  Shown are the means of eight replications; error bars represent standard deviations.  G, Matrigel invasion assay using GIST-T1 cells transfected with siCONT or siHOT.  Shown on the right are the means of three random microscopic fields per membrane; error bars represent standard deviations.
Figure 1

A

miR-196a

High risk

Low risk

Intermediate risk

B

B

miR-196a

Normalized microarray signal (log2)

Low

Intermediate

High

C

Discovery cohort

Validation cohort

All samples

P = 0.004

P = 0.080

P = 0.546

P = 0.001

P = 0.023

P = 0.806

P < 0.001

P = 0.001

P = 0.369

P = 0.001

D

Discovery cohort

Validation cohort

All samples

miR-196a low (n = 23)

miR-196a high (n = 9)

P = 0.065

miR-196a low (n = 86)

miR-196a high (n = 14)

P = 0.006

miR-196a low (n = 109)

miR-196a high (n = 23)

P < 0.001

Overall survival

Time (months)
Figure 2

A

B

HOXB

17q21

13

miR-196a-1

HOXC

12q13

miR-196a-2

C

D

HOXC9

HOX10

HOTAIR

miR-196a/U6

R = 0.927

P < 0.001

R = 0.969

P < 0.001

R = 0.860

P < 0.001
Figure 3

A

HOTAIR/GAPDH

0.000
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
0.010

Low-risk
Intermediate-risk
High-risk

B

miR-196a/U6

R = 0.622
P = 0.002

0.001 0.1 1 10 100

C

HOXC10

HOXC11

HOTAIR/GAPDH

0.000
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008

Normalized microarray signal (log2)

R = 0.939
P < 0.001
R = 0.850
P < 0.001

D

HOTAIR low

HOTAIR high

P = 0.097

E

Relative HOTAIR expression (%)

F

Cell viability (%)

G

Cell invasion (%)

siCONT

siHOT

siCONT

siHOT

EF
Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors

Takeshi Niinuma, Hiromu Suzuki, Masanori Nojima, et al.

Cancer Res Published OnlineFirst January 18, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1803

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/01/18/0008-5472.CAN-11-1803.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.