Contrasting expression patterns of histone mRNA and microRNA 760 in patients with gastric cancer

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Running head: Histone/miR-760 expression in gastric cancer

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**Translational Relevance**

The presence of isolated tumor cells in the peripheral blood and bone marrow (BM) is an important factor contributing to the metastasis of solid cancers. Moreover, recent studies have demonstrated that various types of host cells are also involved in cancer development and metastasis. We performed RNA-seq analysis of the BM from patients with gastric cancer (GC) in order to identify candidate prognostic markers using next-generation sequencing and demonstrated that histone cluster genes were overexpressed in the BM and primary tumor samples from Stage IV GC patients compared to those from Stage I GC patients. Furthermore, we proposed the possibility that microRNA-760 (miR-760) was downregulated in order to degrade upregulated histone mRNA in response to an increase in S-phase cells in the BM and primary tumors of advanced GC patients. Our data also suggested that the histone mRNA/miR-760 axis had a crucial role in the development of GC.
Abstract

Purpose: Recent studies revealed that both disseminated tumor cells and noncancerous cells contributed to cancer progression cooperatively in the bone marrow (BM). Here, RNA-seq analysis of BM from gastric cancer (GC) patients was performed to identify prognostic markers for GC.

Experimental Design: BM samples from 8 GC patients (Stages I and IV: n = 4 each) were used for RNA-seq analysis. Results were validated through quantitative real-time PCR (qRT-PCR) analysis of HIST1H3D expression in 175 BM, 92 peripheral blood (PB), and 115 primary tumor (PT) samples from GC patients. miR-760 expression was assayed using qRT-PCR in 105 BM and 96 PT samples. Luciferase reporter assays were performed to confirm whether histone mRNAs were direct targets of miR-760. miR-760 expression was also evaluated in noncancerous cells from GC patients.

Results: RNA-seq analysis of BM samples from GC patients revealed higher expression of multiple histone mRNAs in Stage IV patients. HIST1H3D expression in the BM, PB, and PT of Stage IV patients was higher than that in Stage I patients (p = 0.0284, 0.0243, 0.0006, respectively). In contrast, miR-760 was downregulated in the BM and PT of Stage IV patients compared to Stage I patients (p = 0.0094 and 0.0018,
respectively). Histone mRNA and miR-760 interacted directly. Furthermore, miR-760 was downregulated in noncancerous mucosa in Stage IV GC patients.

**Conclusion:** Histone mRNA was upregulated, while miR-760 was downregulated in the BM and PT of advanced GC patients, suggesting that the histone mRNA/miR-760 axis had a crucial role in the development of GC.
Introduction

The occurrence of distant metastases is the main cause of death in cancer patients. The presence of isolated tumor cells (ITCs) is an important factor contributing to the metastasis of solid cancers. In clinical practice, the circulating tumor cell (CTC) detection system (CellSearch System) was first approved by the Food and Drug Administration (FDA) for metastatic breast cancer and has now been approved for the detection and monitoring of CTCs in the blood from patients with metastatic prostate and colorectal cancer. Moreover, recent studies have demonstrated that various types of host cells are also involved in cancer development and metastasis, including fibroblasts (carcinoma-associated fibroblasts or myofibroblasts), tumor-associated macrophages, mesenchymal stem cells, platelets, and hematopoietic progenitor cells (1-8).

We previously investigated the presence of ITCs in peripheral blood (PB) and bone marrow (BM) samples using quantitative real-time PCR (qRT-PCR) analysis of CEA, CK-7, and CK-19 in more than 800 cases of gastric cancer (GC) (9). We found that ITCs circulated in patients with a range of clinical stages of GC and demonstrated that the simultaneous expression of ITC-associated genes and VEGFR-1, which may originate from hematopoietic progenitor cells in PB and BM, was significantly associated with hematogeneous metastases (9). Therefore, multiple markers are
currently needed to predict distant metastasis and/or prognosis from BM or PB samples in GC patients by PCR analysis. Disseminated tumor cells (DTCs) in the BM have been detected in all solid tumor types, suggesting that the BM may be a preferred reservoir for blood-bone DTCs. The BM environment may allow these cells to persist for a prolonged period and to disseminate into other organs (10). Many cancer-associated host cells are derived from the BM. According to these findings, differences in the gene expression status of BM cells may reflect different cancer stages or the possibility of distant metastases. Moreover, the BM is a convenient organ to sample for analysis and is more easily accessible than other organs that are often sites of metastases, such as the lungs or liver. In this study, we performed RNA-seq analysis of the BM from patients with GC in order to identify candidate prognostic markers. We demonstrated that multiple histone cluster genes showed higher expression in the BM of Stage IV patients than in that of Stage I patients and evaluated the molecules that regulate these multiple histone mRNAs, revealing an interesting association between histone mRNA and microRNA.

Materials and Methods

Patients and sample collection
In our previous study, BM and PB samples were collected from Japanese GC patients who underwent surgery (9). BM samples from 8 patients with GC, including 4 Stage I and 4 Stage IV patients with liver metastasis, were used for RNA-seq analysis (Supplementary Table S1). Quantitative real-time PCR (qRT-PCR) was performed to analyze the expression of histone mRNA in 175 BM and 92 PB samples and expression of microRNA in 105 BM samples. For qRT-PCR analysis of primary tumors, another 127 GC and corresponding normal tissue samples were used. For microRNA microarray analysis of 3 fractions separated from the BM, BM samples were collected from another 4 GC patients. Detailed protocols for sample preparation are described in the Supplementary Data.

**RNA-seq analysis of BM samples from GC patients by massively parallel sequencing**

Briefly, a total of 1 μg of extracted RNA was used as a template to construct RNA-seq libraries. In this step of sample preparation, starting with total RNA, the mRNA was poly-A selected, fragmented, and converted into single-stranded cDNA using random hexamer priming. Detailed protocols are described in the Supplementary Data. Fold enrichment of the RNA-seq tags between the samples was calculated for each mRNA using the assigned tag counts and was normalized to read per kilobase
mRNA (RPKM) (11).

**Evaluation of HIST1H3D and miR-760 expression in clinical samples**

mRNA and microRNA levels were quantified using a LightCycler 480 Probes Master Kit (Roche Applied Science) following the manufacturer’s protocol. HIST1H3D mRNA expression levels were measured in 175 BM, 92 PB, and 115 primary tumor samples from patients with GC and corresponding noncancerous gastric mucosa samples. miR-760 expression was also assayed by qRT-PCR in 105 BM, 96 primary tumor, and 84 corresponding normal gastric mucosa samples from GC patients. Detailed protocols are described in the Supplementary Data.

**Cell lines and cell culture**

Seven human GC cell lines (NUGC3, NUGC4, MKN74, AGS, KATOIII, MKN45, and KE39) were provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University and the Riken Bioresource Center. Cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) or in serum-free conditions and cultured in a humidified 5% CO2 incubator at 37°C.

**Evaluation of HIST1H3D and miR-760 expression in GC cells**

For RNA analysis, cells were seeded at 1.0–4.0 × 10^5 cells per well in a volume
of 2 mL in 6-well flat-bottomed microtiter plates. Total RNA from these cell lines was isolated using a miRNeasy Mini Kit (Qiagen) following 3–72 h of incubation. Detailed protocols for qRT-PCR are described in the Supplementary Data.

**Protein expression analysis**

Western blotting was used to confirm HIST1H3D expression in GC cells. Primary antibodies targeting pan actin (NeoMarkers) and HIST1H3D (Abcam) were used. Detailed protocols are described in the Supplementary Data.

**Construction of reporter plasmids and the luciferase reporter assay**

To construct the luciferase reporter plasmid, full-length HSIT1H3D or HIST1H2AD was subcloned into the pmirGlo Dual-Luciferase miRNA Target Expression Vector (Promega, USA) at a location 5′ to the firefly luciferase. Furthermore, to confirm the direct interaction between miR-760 and its binding sites on HIST1H3D and HIST1H2AD 3′ UTRs, we constructed luciferase reporter plasmids in which the miR-760 binding sites were mutated. To construct these mutants, positions 45–52 of the HIST1H3D 3′ UTR and 56–63 of the HIST1H2AD 3′ UTR (the sequences were common: CAGAGCCA) were mutated to the sequence CTGTGTCA. A detailed protocol of the luciferase reporter assay is described in the Supplementary Data.

**Transfection of miR-760 precursor (Pre-miR-760)**
Either Pre-miR-760 or a Pre-miR negative control (Ambion miRNA Inhibitors, Applied Biosystems) was transfected into GC cells at 30 nmol/L (final concentration) using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer’s instructions.

**MicroRNA microarray of BM fractions from GC patients**

In 4 patients with Stage IV GC, BM cells were separated into 3 fractions using a 3-step automagnetic-activated cell separation system (MACS) by MACS Cell Separators. CD45+, CD14+, and CD45-/EpCAM+ cell fractions were collected using CD45, CD14, and EpCAM (CD326) microbeads according to the manufacturer’s instructions (Miltenyi Biotec, Germany). RNA was extracted from each BM fraction separated by the Auto MACS system, and microRNA microarrays were performed using the miRCURY LNA Array System. A detailed protocol is given in the Supplementary Data. The microRNA arrays have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession code GSE40325.

**Statistical analysis**

Data from qRT-PCR analyses and in vitro transfected cell assays were analyzed with JMP 5 software (SAS, Inc, USA). Overall survival rates were calculated.
actuarially according to the Kaplan-Meier method and were measured from the day of surgery. Differences between groups were estimated using the χ² test, Student’s t test, repeated-measures ANOVA, and log-rank test. Variables with a P-value of less than 0.05 in univariate analysis were used in subsequent multivariate analysis based on the Cox proportional hazards model for survival. All differences were considered statistically significant at the level of P < 0.05.

Results

RNA-seq analysis of BM from GC patients revealed increased histone mRNA expression in advanced cases.

Hierarchical clustering of genes evaluated by RNA-seq resulted in 2 main groups, one consisting of 2 cases from Stage I and the other consisting of 2 cases from Stage I and 4 cases from Stage IV (Supplementary Figure S1A, left). This result indicated that some cases of Stage I GC have characteristics similar to those of Stage IV GC in the BM microenvironment. Gene set enrichment analysis demonstrated that gene sets related to pathways of the immune response to cancer cells were significantly enriched in Stage IV GC patients (Supplementary Table S3). Upregulation of these gene sets appeared to occur in immune competent cells in the BM, and the immune response
to cancer was more activated in Stage IV GC patients than in Stage I GC patients.

RNA-seq analysis also revealed differential expression of many genes. Twenty-eight genes showed over a 5-fold significant increase in expression in the BM from Stage IV patients compared to that from Stage I patients and had an RPKM value of at least 2.0 (Supplementary Table S4). These genes included 4 histone genes, namely, *HIST1H1D*, *HIST1H3F*, *HIST1H2AD*, and *HIST1H2AL*. Interestingly, many other histone mRNAs also showed higher expression in the BM from Stage IV patients. Thirty-seven histone genes were highly expressed (at least a 3-fold increase in expression) in Stage IV patients compared to Stage I patients (schematized in Figure 1A). Hierarchical cluster analysis of histone cluster genes revealed that 3 of 4 cases of Stage IV GC were clustered in one group with histone upregulation, and all cases of Stage I and 1 of 4 cases of Stage IV (GC 57) were clustered in the other group without histone upregulation (Supplementary Figure S1A, right). This result indicated that some Stage IV GC patients had few S-phase cells in the BM and that BM cells in these patients may be maintained in a dormant state of cell growth.

*HIST1H3D* expression in GC patients

Intron spanning primers can prevent amplification of contaminating genomic DNA. The genes encoding canonical histones generally lack introns, whereas
HIST1H3D, corresponding to the 27th histone in Figure 1A, is unique in that it contains 1 intron; this gene showed elevated mRNA expression in the BM from Stage IV GC patients. We validated HIST1H3D mRNA expression in the BM and PB of GC patients (175 and 92 cases, respectively) by qRT-PCR using intron-spanning primers (Supplementary Figure S2A), and revealed that HIST1H3D expression was significantly higher in the BM and PB of Stage IV patients than in those of Stage I patients (Figure 1B). Furthermore, HIST1H3D expression in another set of 115 primary GC tissues was also evaluated. Primary tumor tissues exhibited higher HIST1H3D expression compared to corresponding normal tissues ($p = 0.0127$; Figure 1C, left), and the average expression of HIST1H3D mRNA in Stage I tumors was significantly lower than in other stages (Figure 1C, right). We then classified these 115 GC cases into 2 groups according to average HIST1H3D mRNA expression; patients with an average HIST1H3D expression below 1.069 (normalized to GAPDH) were assigned to the low expression group ($n = 77$), whereas those with an average expression above 1.069 were assigned to the high expression group ($n = 38$). These 2 groups were then used to analyze clinicopathological factors in relation to HIST1H3D levels. Patients with high expression of HIST1H3D exhibited significantly more frequent tumor invasion, lymph node metastasis, peritoneal dissemination, and advanced-stage cancers than those with
low HIST1H3D expression ($\chi^2$ test, Supplementary Table S2A). In terms of overall survival, patients in the HIST1H3D high expression group had a significantly poorer prognosis than those in the HIST1H3D low expression group (Figure 1D). Univariate analysis of overall survival revealed that the level of HIST1H3D expression was a prognostic predictor; however, multivariate analysis showed that HIST1H3D expression was not an independent prognostic predictor of prognosis in GC patients (Supplementary Table S2B).

Expression of specific genes involved in histone mRNA metabolism in the BM of GC patients

To indentify candidate prognostic/metastatic markers in GC patients, we compared the expression status of genes that regulate multiple histone mRNAs between Stage I and Stage IV GC patients. Many specific genes are involved in histone mRNA transcription, cleavage, translation, and degradation (12). Interestingly, most of these genes showed about a 2-fold increase in BM samples from Stage IV patients compared to those from Stage I patients by RNA-seq analysis (Supplementary Table S5). These results indicated enhanced histone mRNA metabolism in the BM of advanced GC patients and were consistent with our previous results demonstrating increased histone mRNA expression in the BM of patients with Stage IV GC. Some histone
metabolism-associated genes showed significantly higher expression in the BM from Stage IV patients, suggesting that these genes, which are involved in controlling the metabolism of dozens of histone mRNAs, could be prognostic markers for GC patients. However, these genes may regulate not only upregulated histones in Stage IV patients (schematized in Figure 1A) but also many other histones that showed no increased expression in Stage IV patients.

**In silico analysis predicted an association between histone mRNA and microRNA**

Because one or several microRNAs often regulate multiple target genes in a specific pathway of cellular function, we investigated the possibility that microRNAs regulate multiple histone mRNAs using an in-silico prediction algorithm (TargetScan v. 6.0). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA (13). Our analysis predicted that several microRNAs bind to the histone genes upregulated in BM samples from Stage IV GC patients (Table 1). Surprisingly, in this analysis, most of the histone genes upregulated in BM samples from Stage IV GC patients had predictive target sites for several common microRNAs in their 3’ untranslated regions (UTRs). In particular, 3 microRNA, namely, *miR-760*, *miR-1276*, and *miR-4766-5p*, were predicted to bind the 3’ UTRs of more than 20 histone genes (Table 1). Although histone H4
family genes had no binding sites for these 3 microRNAs, they had target sites for 2 other common microRNAs, *miR-1291* and *miR-4512* (Table 1). These results suggested that several microRNAs were involved in histone mRNA metabolism. Because the context score percentile of *miR-760* for each of the histone mRNAs was higher than those of *miR-1276* and *miR-4766-5p*, we examined *miR-760* expression and function in GC in subsequent experiments.

**microRNA 760 expression in GC cancers**

*miR-760* expression in BM samples from Stage IV patients (n = 53) was lower than that of Stage I patients (n = 52; \( p = 0.0094 \); Figure 2A). Likewise, *miR-760* expression in Stage IV primary GC tissues was significantly lower than in early-stage primary GC tissues (Figure 2B, *left*). These expression patterns for *miR-760* were opposite to those of *HIST1H3D* in both the BM and primary tumors from GC patients. We then classified 96 GC cases, which had been examined for *miR-760* expression in primary tumor tissues, into 2 groups according to the average *miR-760* mRNA expression. Patients with expression of *miR-760* that was below an average value of 42.256 (normalized to *RNU6B*) were assigned to the low expression group (n = 65), whereas those with expression values above an average of 42.256 were assigned to the high expression group (n = 31). Clinicopathological factors were then analyzed in
relation to miR-760 levels. Patients with low miR-760 expression exhibited significantly larger tumor sizes and more frequent tumor invasion, lymph node metastasis, peritoneal dissemination, and advanced stages than patients with high miR-760 expression (Table 2). In terms of overall survival, patients in the high miR-760 expression group had a significantly better prognosis than those in the low miR-760 expression group (Figure 2B, right). Multivariate analysis of overall survival showed that the level of miR-760 expression was an independent prognostic predictor (RR: 1.67, 95% CI: 1.03–3.11, \( p = 0.0374 \) by Cox proportional hazards model; Table 3).

**Direct interaction between miR-760 and histone mRNA in GC cells**

To confirm whether histone mRNAs were direct targets of miR-760, we generated HIST1H3D- and HIST1H2AD-luciferase constructs. Interestingly, among the histones upregulated in Stage IV BM in Figure 1A, HIST1H2AD shared a common genomic region with HIST1H3D in a region of the 5′ sides of transcripts (Supplementary Figure S2A). Therefore, we evaluated the differences in the binding abilities between these 2 histone variants. Cotransfectants expressing both miR-760 and HIST1H3D or HIST1H2AD showed significant reductions in luciferase activity compared with controls in the GC cell line, NUGC3 (\( p < 0.01 \) and \( p < 0.001 \), respectively; Figure 3A). This reduction in luciferase activity was more pronounced in
cells transfected with the *HIST1H2AD* construct than with the *HIST1H3D* construct.

The activities of both reporter constructs harboring point mutations in *miR-760* binding sites were unaffected by simultaneous transfection with *Pre-miR-760* (Figure 3A).

**HIST1H3D expression was downregulated in GC cell lines after Pre-miR-760 transfection**

In terms of mRNA expression, both *HIST1H3D* transcripts and *miR-760* were expressed at low levels in AGS cells, and KE39 cells showed high *HIST1H3D* and low *miR-760* expression among the 7 GC cell lines investigated (Supplementary Figure S2B). HIST1H3D protein levels did not necessarily correspond to *HIST1H3D* mRNA levels in each cell line (Supplementary Figure S2C), indicating that the proportions of S-phase cells differed between cell lines and that redundant untranslated histone mRNAs may be present in some types of GC cells. Three cell lines (NUGC3, KATOIII, and KE39) were used in the following *Pre-miR* transfection experiments because of their high transfection efficiency for microRNAs. In these cell lines, contrasting expression patterns of *HIST1H3D* and *miR-760* were observed in terms of mRNA levels (Figure 3B, upper panel). The expression of *HIST1H3D* mRNA was downregulated at 3 h after *Pre-miR-760* transfection (Figure 3C, middle panel), but upregulated at 24–48 h after transfection in these 3 cell lines. A time course of *HIST1H3D* expression data after
Pre-miR-760 transfection in NUGC3 cells is shown in Supplementary Figure S2D. Although HIST1H3D protein was upregulated at 3 h after Pre-miR-760 transfection in all 3 cell lines (Supplementary Figure S2E), downregulation of HIST1H3D protein was observed in NUGC3 and KE39 cells at 48 h (Figure 3C, bottom). In KATOIII cells, further upregulation of HIST1H3D was observed at 48 h (Figure 3C, bottom). Notably, only in NUGC3 cells, overexpression of miR-760 induced morphological changes at 24–48 h after transfection (Supplementary Figure S2F).

miR-760 expression was altered in response to histone mRNA expression in GC cells

It is possible that miR-760 expression changes in response to histone mRNA expression. We evaluated changes in miR-760 expression in GC cells under conditions of serum starvation and restimulation in which histone mRNA expression may be altered depending on cell cycle progression. GC cells were cultured in serum-free media for 72 h and then restimulated with serum for 24 h. The expression of HIST1H3D transcripts and miR-760 was measured at 24 and 72 h after starvation and at 48 h after restimulation. Although HIST1H3D and miR-760 behaviors differed in each cell line, contrasting expression patterns of these genes were observed under conditions of serum starvation and restimulation (Figure 3B, middle and bottom panels). These results
indicated that the expression levels of histone mRNA and miR-760 were altered in opposite directions in response to culture conditions in 3 GC cell lines.

**Contrasting expression of histone mRNA and miR-760 was also observed in noncancerous cells in GC patients**

Next, we examined which cells in the BM exhibited altered miR-760 expression in another set of 4 GC patients. Since all of these 4 patients had Stage IV GC, the expression of miR-760 was expected to be low. However, differential expression of miR-760 was observed in each fraction. In BM samples from all 4 patients, the CD45⁻/EpCAM⁺ fraction showed highest miR-760 expression among all fractions (Figure 2C). Tumor cells may be enriched in the CD45⁻/EpCAM⁺ fraction compared to other fractions. Interestingly, the CD14⁻ fraction exhibited the same degree of miR-760 expression (Figure 2C).

In corresponding noncancerous tissues from primary GC tumors, miR-760 expression was lower in Stage IV patients than in Stage I patients (p = 0.0243; Figure 2D, left). We then classified 84 GC patients, whose miR-760 expression could be evaluated in corresponding noncancerous gastric mucosa, into 2 groups based on the median expression of miR-760 (17.57 normalized to RNU6B). In terms of overall survival, patients in the low miR-760 expression group (n = 42) had a significantly
poorer prognosis than those in the high miR-760 expression group (n = 42; Figure 2D, right). These results suggested that changes in miR-760 expression in host noncancerous cells were also associated with GC progression.

Discussion

Histone protein synthesis is restricted to the S phase of the cell cycle, and regulation of histone protein synthesis is accomplished by regulation of histone mRNA levels. Histone mRNAs are tightly regulated during the cell cycle, allowing the synthesis of histone proteins to occur coordinately with the replication of DNA. In malignant tumors, upregulation of histone mRNA indicates proliferative activity of tumor cells, and many studies have demonstrated that histone mRNA accumulates in tumors (14-16). In most previous studies, histone levels have been measured by only considering H3 core histone levels. Histone gene clusters in mammals are heterogeneously organized and contain 1 or more copies of the 5 histone subtypes, i.e., core (H2A, H2B, H3, H4, and variants thereof) and linker (H1) histone genes (17, 18). The vast majority of the 74 human histone genes can be found within the major and minor clusters located on chromosomes 6p21 and 1q21, respectively. Recent studies have demonstrated that these histone clusters genes are overexpressed in several types
of malignancies. Sadikovic et al. performed integrative whole-genome analysis of DNA copy numbers, promoter methylation, and gene expression using 10 osteosarcomas and identified significant changes, including the hypomethylation, gain, and overexpression of histone cluster 2 genes on chromosome 1q21.1-q21.3 (19). Moreover, Perez-Margan et al. identified candidate genes related to meningioma recurrence by differential gene expression profiling analyses of 33 original and 7 recurrent meningiomas. They demonstrated that 16 histone cluster 1 genes, composed of 3 H1 linker and 13 H2 core histones, were overexpressed in recurrent meningiomas (20). Our current study presents the first evidence for overexpression of histone cluster genes, mainly histone cluster 1, in BM and primary tumor samples from Stage IV GC patients compared to those from Stage I GC patients. Because histone mRNAs are tightly regulated and increase or decrease simultaneously during the cell cycle, we speculate that genes regulating multiple histone mRNAs may be useful prognostic/metastatic markers for GC patients. Unlike most RNA polymerase II-transcribed mRNAs, histone mRNAs are not polyadenylated, but instead end in a conserved 30-bp stem-loop structure, which is recognized and cleaved by the stem-loop binding protein (SLBP) (12). SLBP binds to the 3′ end of histone mRNA and participates in many aspects of histone mRNA metabolism. SLBP is a cell cycle-regulated protein, accumulating just prior to entry into
S phase and then rapidly degraded by the proteasome at the end of S phase, similar to the timing of degradation of histone mRNAs (21). A sequence in the amino-terminal domain of SLBP is necessary for the rapid degradation of SLBP at the end of S phase. This region contains consensus cyclin phosphorylation and binding sites. Mutation of either of these sequences stabilizes SLBP. Despite the fact that SLBP is stabilized, histone mRNA is still degraded at the appropriate time (22). Thus, it has been suggested that histone mRNA is degraded at the end of S phase through a separate mechanism.

We proposed the possibility that several microRNAs were involved in histone mRNA degradation and uncovered contrasting expression patterns of miR-760 and histone mRNAs in GC patients and GC cells subjected to serum starvation or restimulation. Furthermore, Pre-miR-760 transfection experiments also indicated that the interaction between HIST1H3D and miR-760 occurred in living GC cells (Figure 3C). HIST1H3D mRNA and corresponding protein expression changes induced by miR-760 overexpression were somewhat complex. Just after Pre-miR-760 transfection, HIST1H3D mRNA was downregulated as expected, whereas HIST1H3D protein expression was upregulated in three cell lines (Figure 3C and Supplementary Figure S2E). At 48 h after Pre-miR-760 transfection, HIST1H3D protein was downregulated in NUGC3 and KE39 cells (Figure 3C, bottom panel); however, the expression of
HIST1H3D mRNA was significantly upregulated in all cell lines (Supplementary Figure S2D). Recently, 4 microRNAs, including miR-760, have been reported to cooperatively induce cellular senescence by targeting a subunit of protein kinase CKII in human colorectal cancer cells (23). Our in silico analysis also indicated that several microRNAs, including miR-760, targeted histone mRNAs cooperatively. Furthermore, an interaction between the histone 3’ UTR and microRNA is only one degradation pathway for histone mRNAs. Therefore, the expression of HIST1H3D mRNA and its corresponding protein is likely maintained by compensatory and/or feedback mechanisms, although exogenous miR-760 caused temporary destabilization of histone mRNA by targeting its 3’ UTR. Both mRNA and protein expression of HIST1H3D were continuously elevated only in KATOIII cells. Because KATOIII was a floating cell line, different from the other two cell lines, the effects of miR-760 may vary between cell types. Interestingly, morphological changes were observed only in NUGC3 cells by overexpression of miR-760. Pre-miR-760-transfected NUGC cells did not show spindle formation, which was observed in control cells (Supplementary Figure S2F). There were no differences in proliferation rates between Pre-miR-760-transfected cells and controls, whereas p21 expression was upregulated in transfected cells (Supplementary Figure S2G and S2H). These results suggested that miR-760 may prevent spindle
formation and direct senescence in specific cells. The predictive miR-760-binding site in histone 3’ UTRs was located in the stem-loop end (Figure 3D). All of the predicted binding sites for other microRNAs in histone 3’ UTRs shown in Table 1 were also in the stem loop regions. Under normal conditions, microRNAs are not likely to bind to these histone mRNA stem-loop structures. However, loss of normal histone pre-mRNA processing has been shown to result in the production of polyadenylated mRNAs from histone genes (24, 25). Levels of these polyadenylated histone mRNAs are very low in proliferating cells (24, 26), but may increase during terminal differentiation (27, 28) or tumorigenesis (29-31). The histone mRNAs detected in our RNA-seq analysis may also be polyadenylated since polyA-containing RNA was selected before first strand cDNA synthesis (see methods). Therefore, it is possible that upregulation of these polyadenylated histone mRNAs and downregulation of miR-760, which can bind to polyadenylated histone 3’ UTRs, are also involved in GC progression.

We also evaluated the association between HIST1H3D or miR-760 expression and the status of DTC markers in the BM. Significant differences were not observed between DTC-positive and -negative cases in both HIST1H3D and miR-760 expression (Supplementary Figure S1B), indicating that contrasting expression of HIST1H3D and miR760 did not occur only in DTCs of GC patients. To investigate whether host cells
were involved in cancer development and metastasis, we evaluated the miR-760 expression status of noncancerous cells in GC patients. Fractionated BM from GC patients was subjected to microRNA microarray; this analysis revealed that miR-760 was highly expressed in both the CD14+ and CD45-/EpCAM+ fractions (Figure 2C). Generally, it is thought that several types of host cells, such as macrophages and myofibroblasts, are enriched in CD14+ fractions, while tumor cells are enriched in CD45-/EpCAM+ fractions. Consistent with this, in corresponding noncancerous gastric mucosa, miR-760 expression was downregulated in tissues from Stage IV patients; moreover, low expression of miR-760 was associated with a poorer prognosis than high expression of this microRNA (Figure 2D). Kaplan et al. found that BM-derived hematopoietic progenitor cells expressing VEGFR-1 are attracted to tumor-specific, premetastatic sites and form cellular clusters before the arrival of tumor cells, functioning as a cancer niche to facilitate metastasis (7). Furthermore, BM-derived myofibroblasts have been reported to contribute to cancer-induced stromal reactions in the later stages of tumor development (32). From studies in mouse models of inflammation-induced GC, Quante et al. also demonstrated that carcinoma-associated fibroblasts were derived from mesenchymal stem cells in the BM and that myofibroblasts expressing alpha-smooth muscle actin increased markedly during cancer
progression (33). Therefore, our results indicate that downregulation of *miR-760* expression not only in GC cells, but also in specific host cells in the BM and gastric tissue may affect GC progression.

Area under the curve (AUC) values of both *miR-760* and *HIST1H3D* could not be used as sufficient predictors in receiver operating characteristic (ROC) curve analysis for discriminating patients with Stage I or Stage IV GC from BM samples (AUC = 0.680 for *miR-760*; AUC = 0.585 for *HIST1H3D*, Supplementary data and Figure S1C). Thus, further studies are warranted to develop prediction approaches for the prognosis of GC patients using these markers.

Histone mRNAs appeared to be upregulated in response to the increased proportion of S-phase cells in advanced GC tissue and the BM. Because redundant untranslated histone mRNAs are degraded rapidly at the end of S phase, a large amount of specific molecules involved in the degradation of histone mRNA, including *miR-760*, may be required at this period in advanced GC. Our present study revealed an interaction between *miR-760* and histone mRNA. Although it is unclear whether low levels of *miR-760* are the result or the cause of histone mRNA degradation in advanced GC, the histone mRNA/*miR-760* axis may have a crucial role in the development of GC and may become a new therapeutic target in the treatment of advanced GC.
References


23. Kim SY, Lee YH, Bae YS. MiR-186, miR-216b, miR-337-3p, and miR-760 cooperatively induce cellular senescence by targeting alpha subunit of protein kinase CKII in human colorectal cancer cells. Biochem Biophys Res Commun 2012;


**Figure Legends**

**Figure 1.** Histone genes expression in GC patients.

A, Expression of histone cluster genes in BM samples from GC patients. RPKM values
of histone cluster genes were schematized. RPKM, reads per kilobase per million mapped reads. B–D, HIST1H3D expression in GC patients. HIST1H3D expression was analyzed in the BM, PB (B), and primary tumors (C) of GC patients. D, Kaplan-Meier overall survival curves for GC patients based on the level of HIST1H3D expression from primary tumor samples.

Figure 2. miR-760 expression in GC patients.

miR-760 expression was analyzed in the BM (A) and primary tumors (B) of GC patients. Kaplan-Meier overall survival curves for GC patients based on the level of miR-760 expression from primary tumor samples (B, right). C, miR-760 expression in BM fractions separated by the AutoMACS system using CD45, EpCAM, and CD14 microbeads from 4 GC patients. D, Left, miR-760 expression in corresponding noncancerous gastric mucosa from GC patients. Right, Kaplan-Meier overall survival curves of GC patients based on the level of miR-760 expression in corresponding noncancerous gastric mucosa.

Figure 3. The association between histone mRNA and miR-760 in GC cells.

A, Luciferase analysis in NUGC3 cells. HIST1H3D (left) or HIST1H2AD (right) luciferase vector + miR-760 transfectants showed lower luciferase activities than control cells. The luciferase activities of both reporter constructs harboring point mutations in
miR-760 binding sites were unaffected by simultaneous transfection with Pre-miR-760.

Pre-miR n.c., Pre-miR negative control; WT, wild type. B, HIST1H3D and miR-760 mRNA expression in GC cell lines. Upper panel, HIST1H3D and miR-760 expression in normal culture conditions. Middle and lower panels, Changes in HIST1H3D and miR-760 expression in GC cells. Cells were cultured for 72 h in serum-free media and were restimulated with serum after 24 h of serum starvation. Expression of HIST1H3D and miR-760 was measured at 24 and 72 h after starvation and at 48 h after restimulation. C, miR-760 and HIST1H3D mRNA and protein expression after treatment with negative control or Pre-miR-760 for 3 h (mRNA) and 48 h (protein) in GC cell lines. Upper panel, miR-760; middle panel, HIST1H3D mRNA; bottom panel, western blotting analysis of HIST1H3D and pan actin. The lower panel shows a graphical representation of gel images in the upper panels. D, MicroRNA binding site in the 3′ UTR of histone mRNA. Gray region indicates the binding site for miR-760.

(* p < 0.05, ** p < 0.01, *** p < 0.001)
Figure 2

A  Bone marrow

B  Primary tumor

C  Bone marrow fractions

D  Corresponding normal gastric mucosa

* p < 0.05
** p < 0.01
*** p < 0.001

Overall survival rate

Years after operation

Low miR-760 (n = 65)
High miR-760 (n = 31)

0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

0
1
2
3
4
5
6

0
50
100
150
200
250

miR-760/RNU6

(miR-760/RNU6)

n = 52
n = 53

(n = 29)
(n = 22)
(n = 23)
(n = 22)

(p = 0.0094)**

(p = 0.0018**)

(p = 0.0490*)

(p = 0.0142*)

(p = 0.0105)

CD45 (+)
CD45 (-)/EpCAM (+)
CD14 (+)

miGC3, miGC15, miGC16, miGC18

(p = 0.0243*)

(p = 0.0265*)

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Figure 3
Table 1. The Association between Histone Cluster Genes and Conserved microRNAs predicted by in silico Analysis

<table>
<thead>
<tr>
<th>Histone genes</th>
<th>Conserved miR (Context score percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIST1H2AK</td>
<td>miR760 (98) miR4766-5p (54)</td>
</tr>
<tr>
<td>HIST1H2BM</td>
<td>miR1276 (75) miR4766-5p (39)</td>
</tr>
<tr>
<td>HIST1H1B</td>
<td>miR760 (98) miR1276 (78) miR4766-5p (43)</td>
</tr>
<tr>
<td>HIST1H3B</td>
<td>miR760 (99) miR1276 (84) miR4766-5p (51)</td>
</tr>
<tr>
<td>HIST1H1D</td>
<td>miR760 (99) miR1276 (74) miR4766-5p (48)</td>
</tr>
<tr>
<td>HIST1H1E</td>
<td>miR760 (98) miR1276 (80) miR4766-5p (44)</td>
</tr>
<tr>
<td>HIST1H3F</td>
<td>miR760 (98) miR1276 (82) miR4766-5p (47)</td>
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<tr>
<td>HIST1H2AD</td>
<td>miR760 (98) miR1276 (83) miR4766-5p (48)</td>
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<tr>
<td>HIST1H2AH</td>
<td>miR760 (99) miR1276 (83) miR4766-5p (55)</td>
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<tr>
<td>HIST1H3C</td>
<td>miR760 (98) miR1276 (81) miR4766-5p (46)</td>
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<tr>
<td>HIST1H4I</td>
<td>miR4512 (98)</td>
</tr>
<tr>
<td>HIST1H2BN</td>
<td>miR760 (99) miR1276 (76) miR4766-5p (43)</td>
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<tr>
<td>HIST1H4L</td>
<td>miR1291 (99) miR4512 (93)</td>
</tr>
<tr>
<td>HIST1H4K</td>
<td>miR1291 (99)</td>
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<td>HIST1H4E</td>
<td>miR1291 (89) miR4512 (95)</td>
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<td>HIST1H2BJ</td>
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<td>miR760 (98) miR1276 (82) miR4766-5p (45)</td>
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<td>HIST2H2AB</td>
<td>miR760 (98) miR1276 (78) miR4766-5p (47)</td>
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<td>HIST1H2BC</td>
<td>miR760 (67) miR1276 (87)</td>
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<tr>
<td>HIST1H2AL</td>
<td>miR760 (99) miR1276 (80) miR4766-5p (51)</td>
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<tr>
<td>HIST1H4B</td>
<td>miR1291 (89)</td>
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<tr>
<td>HIST1H2AG</td>
<td>miR1276 (70)</td>
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<td>HIST1H2BF</td>
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<td>HIST1H2BH</td>
<td>miR760 (99)</td>
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<td>HIST1H4C</td>
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<td>H3F3C</td>
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<td>miR760 (97) miR1276 (85) miR4766-5p (46)</td>
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<td>HIST1H4D</td>
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<tr>
<td>HIST1H2BO</td>
<td>miR760 (98) miR1276 (84)</td>
</tr>
<tr>
<td>HIST1H2BB</td>
<td>miR760 (99) miR1276 (82)</td>
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<tr>
<td>Average (%)</td>
<td>93.6 80.9 47.05 93.6 96.2</td>
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Table 2. miR-760 expression and clinicopathological factors

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<thead>
<tr>
<th>Factors</th>
<th>Low expression (n = 65)</th>
<th>High expression (n = 31)</th>
<th>p value</th>
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<tr>
<td></td>
<td>number (%)</td>
<td>number (%)</td>
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<td>Age (mean)</td>
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<tr>
<td>≥ 65</td>
<td>35 53.85</td>
<td>15 48.39</td>
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<tr>
<td>&lt; 65</td>
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<td>16 51.61</td>
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<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>44 67.69</td>
<td>17 54.84</td>
<td>0.2211</td>
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<tr>
<td>Female</td>
<td>21 32.31</td>
<td>14 45.16</td>
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<tr>
<td>Size</td>
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<td>(Large) &gt; 50mm</td>
<td>40 61.54</td>
<td>10 32.26</td>
<td>0.0056**</td>
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<tr>
<td>(Small) &lt; 50mm</td>
<td>24 36.92</td>
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<td>Histology a</td>
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<tr>
<td>well &amp; moderately</td>
<td>28 43.08</td>
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<tr>
<td>poorly &amp; others</td>
<td>36 55.38</td>
<td>17 54.84</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>T1</td>
<td>6 9.23</td>
<td>14 45.16</td>
<td>&lt;0.0001**</td>
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<td>T2-T4</td>
<td>59 90.77</td>
<td>17 54.84</td>
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<td>Lymph node metastasis</td>
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<td>17 26.15</td>
<td>15 48.39</td>
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<td>48 73.85</td>
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<td>Lymphatic invasion</td>
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<td>Venous invasion</td>
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<tr>
<td>Present</td>
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<td>7 22.58</td>
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<td>Liver metastasis</td>
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<tr>
<td>Absent</td>
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<td>Present</td>
<td>3 4.62</td>
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<tr>
<td>Peritoneal dissemination</td>
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<td>Absent</td>
<td>51 78.46</td>
<td>30 96.77</td>
<td>0.0209*</td>
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<td>Present</td>
<td>14 21.54</td>
<td>1 3.23</td>
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</tr>
<tr>
<td>Stage</td>
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<tr>
<td>I</td>
<td>14 21.54</td>
<td>15 48.39</td>
<td>0.0075**</td>
</tr>
<tr>
<td>II</td>
<td>14 21.54</td>
<td>8 25.81</td>
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<td>III</td>
<td>17 26.15</td>
<td>6 19.35</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>20 30.77</td>
<td>2 6.45</td>
<td></td>
</tr>
</tbody>
</table>

*a well, moderately and poorly: differentiated types of gastric adenocarcinoma

*p < 0.05, **p < 0.01
Table 3. Results of univariate and multivariate analysis of clinicopathological factor for 5-year overall survival (Cox proportional hazard model)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>RR  95%CI p value</td>
<td>RR  95%CI p value</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>1.12 0.81-1.60 0.5054</td>
<td>- - -</td>
</tr>
<tr>
<td>Tumor size (&lt; 5cm / &gt; 5cm)</td>
<td>1.63 1.19-2.27 0.0021**</td>
<td>0.95 0.62-1.48 0.8316</td>
</tr>
<tr>
<td>Histological differentiation (well*, moderatelyb/othersc)</td>
<td>1.32 0.97-1.83 0.0749</td>
<td>- - -</td>
</tr>
<tr>
<td>Tumor depth (T1/T2 - T4)</td>
<td>3.31 1.83-8.20 &lt;0.0001**</td>
<td>1.13 0.45-5.05 0.8306</td>
</tr>
<tr>
<td>Lymphnode metastasis (negative/positive)</td>
<td>6.41 2.98-27.02 &lt;0.0001**</td>
<td>3.3 1.45-14.24 0.0016**</td>
</tr>
<tr>
<td>Lymphatic invasion (negative/positive)</td>
<td>5.05 2.35-21.30 &lt;0.0001**</td>
<td>1.59 0.64-7.13 0.3623</td>
</tr>
<tr>
<td>Venous invasion (negative/positive)</td>
<td>2.17 1.58-2.98 &lt;0.0001**</td>
<td>1.62 1.04-2.56 0.032*</td>
</tr>
<tr>
<td>miR760 expression (high/low)</td>
<td>1.96 1.23-3.63 0.0035**</td>
<td>1.67 1.03-3.11 0.0374*</td>
</tr>
</tbody>
</table>

RR: Relative risk, CI: Confidence interval, *p< 0.05, **p < 0.01
*a: well: well differentiated type, b: moderately: moderately differentiated type, c: Others: poorly differentiated and schirrous type.
Clinical Cancer Research

Contrasting expression patterns of histone mRNA and microRNA 760 in patients with gastric cancer

Takeshi Iwaya, Takeo Fukagawa, Yutaka Suzuki, et al.

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