MicroRNA miR-125b is a prognostic marker in human colorectal cancer

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Abstract. MicroRNAs (miRNAs) are small, non-coding RNAs that can function as oncogenes or tumor suppressors in human cancer. Recent reports have highlighted the oncogenic aspects of microRNA miR-125b. However, the clinical significance of miR-125b in gastrointestinal cancers has not been sufficiently investigated. To this end, we analyzed miR-125b expression in colorectal cancer cases. Quantitative RT-PCR was used to evaluate miR-125b expression in 89 colorectal cancer cases to determine the clinicopathological significance of miR-125b expression. The high miR-125b expression group showed a greater incidence of advanced tumor size and tumor invasion compared to the low miR-125b expression group (P<0.05). In addition, the high miR-125b expression group had a significantly poorer prognosis compared to the low expression group (P<0.05). Multivariate analysis indicated that high miR-125b expression was an independent prognostic factor for survival. Our analysis of miR-125b focused on the miR-125b/p53 pathway. In vitro assays revealed that overexpression of miR-125b repressed the endogenous level of p53 protein in human colorectal cancer cells. These data show that miR-125b is directly involved in cancer progression and is associated with poor prognosis in human colorectal cancer. Our findings suggest that miR-125b could be an important prognostic indicator for colorectal cancer patients.

Introduction

Colorectal cancer is the third most common malignancy and the fourth most frequent cause of cancer mortality in the world (1). Over one million new cases of colorectal cancer are diagnosed each year and the incidence seems to increase with the progressive ‘westernization’ of lifestyles among Asian and African populations. For diagnosis and adequate treatment of colorectal cancer, identification and understanding of molecules responsible for cancer progression are critical.

Recent reports have demonstrated that post-transcriptional regulation is likely to be involved in the control of gene expression in addition to mutational status and transcriptional efficiency (2-3). MicroRNAs (miRNAs) constitute a class of small (19-25 nucleotides), non-coding RNAs that function as post-transcriptional gene regulators. Alterations in miRNA expression are involved in the initiation, progression, and metastasis of human cancer (4,5). In colorectal cancers, it is widely accepted that the accumulation of genetic alterations (to molecules such as APC, K-RAS, p53 and DCC) is critical for tumor development (6). Furthermore, those molecules are controlled by post-transcriptional regulation. Nagel and colleagues demonstrated that miR-135 regulates APC tumor suppressor gene and is associated with the progression of colorectal cancer cells (7). Recently, Le and colleagues demonstrated that miR-125b binds directly to the 3’ untranslated region (3’ UTR) of human p53 mRNAs, and represses the protein expression during development and during the stress response (8). These facts indicate that miR-125b is directly involved in cancer progression and is associated with poor prognosis in human colorectal cancer. Our findings suggest that miR-125b could be an important prognostic indicator for colorectal cancer patients.

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Materials and methods

Clinical samples. Primary colorectal cancer specimens were obtained from 89 patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University between 1993 and 2000. All specimens were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Written informed consent was obtained from all patients. Whenever possible, specimens were also prepared for immunohistochemical studies. All 89 patients were clearly identified as having colorectal cancer based on the clinicopathologic criteria described by the Japanese Society for Cancer of the Colon and Rectum. No patients received chemotherapy or radiotherapy before surgery. All patients were closely followed after surgery at regular three month intervals. The follow-up periods ranged from two months to 11 years, with a mean of three years. All data, including age, gender, tumor size and depth, lymphatic invasion, lymph node metastasis, vascular invasion, liver metastasis, peritoneal dissemination, distant metastasis and histological grade were obtained from clinical and pathologic records.

RNA preparation and reverse transcription. Frozen tissue specimens were homogenized in guanidium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion, as described previously (10). As previously reported, cDNA was synthesized from 8.0 μg of total RNA (11).

Evaluation of miR-125b in clinical samples. For miR-125b quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), cDNA was synthesized from 10 ng of total RNA using TaqMan™ MicroRNA hsa-miR-125b specific primers (Applied Biosystems) and a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The following temperature profile was used: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 65°C for 10 sec. PCR was performed in a LightCycler™ 480 System (Roche Applied Science) using the LightCycler 480 Probes Master kit (Roche Applied Science). Expression levels of target miRNAs were normalized to that of the small nuclear RNA RNU6B (Applied Biosystems) transcript.

Experimental studies

Cell lines and cell culture. The human colorectal cancer cell line HT29 was provided by the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. These cell lines were maintained in RPMI-1640 containing 1% fetal bovine serum with 100 units/ml penicillin and 100 units/ml streptomycin sulfate and cultured in a humidified 5% CO2 incubator at 37°C.

Transfection of Pre-miR™-125b. We used HT-29, a colorectal cancer cell line that expresses a high level of p53 mRNA, and which has a single mutational event in codon 273 (nucleotides: CGT→CAT, amino acid: Arg→His) (12). Either pre-miR-125b or pre-miR negative control (Ambion® Pre-miR miRNA Precursors, Applied Biosystems Japan Ltd.) was transfected at 30 nM (final concentration) using Lipofectamine™ RNAiMAX (Invitrogen Life Technologies) according to the manufacturer’s instruction. For RNA analysis, the HT-29 cell line was seeded at 2x10^5 cells per well in a volume of 2 ml in 6-well flat bottomed microtiter plates. Total RNAs were isolated using the miRvana™ miRNA Isolation kit (Ambion) 48 h after the transfection. Inhibition of p53 with Pre-miR-125b in HT-29 compared with parent and negative control was examined. Similarly, Western blot analysis was performed 72 h after transfection.

p53 sequences in clinical samples. Among 89 colorectal cancer samples in which miR-125b levels were measured, p53 was sequenced in 81 genomic DNA samples as we described previously (13). In short, DNA samples were used as templates to PCR amplify exons 4 to 9 of the p53 gene with primers derived from intronic sequences. The PCR was done with AmpliTaq Gold DNA Polymerase (Applied Biosystems). These PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and purified with ethanol precipitation. Purified PCR products were sequenced using a Big-Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI3100 sequencer (Applied Biosystems).

Evaluation of p53 and p21 mRNA expression in colorectal cancer cells. For RNA analysis, each cell line was seeded at 2x10^5 cells per well in a volume of 2 ml in 6-well flat bottomed microtiter plates. Total RNA from cell lines was isolated using the mirVana miRNA Isolation kit (Ambion) after 48 h incubation. Quantitative RT-PCR was performed to measure p53 and p21 mRNA expression with the Universal Probe Library probe (UPL) (Roche Diagnostics). Primer sequences corresponding to UPL were designed as follows:

- p53 forward: 5′-GTCAGTCTGTTCTGACGCT-3′
- p53 reverse: 5′-GAGCTGCCTCGCTCTT-3′
- p21 forward: 5′-AGTGGTGGTTTGCTGATCAC-3′
- p21 reverse: 5′-AACAGTGGTGGTTTGCTGATCAC-3′
Table I. miR-125b expression and clinicopathological factors.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Tumor low expression (n=44)</th>
<th>Tumor high expression (n=45)</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
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<tr>
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<td>25</td>
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</tr>
<tr>
<td>Female</td>
<td>19</td>
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<td>17</td>
</tr>
<tr>
<td>Histological grade</td>
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<tr>
<td>Well</td>
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<td>45.5</td>
<td>15</td>
</tr>
<tr>
<td>Moderately, poorly</td>
<td>24</td>
<td>54.5</td>
<td>30</td>
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<tr>
<td>Size</td>
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<tr>
<td>&gt;21 mm (large)</td>
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<td>Depth of tumor invasion</td>
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<td></td>
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<tr>
<td>m, sm, mp, ss</td>
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<td>45.5</td>
<td>11</td>
</tr>
<tr>
<td>se, si</td>
<td>24</td>
<td>54.5</td>
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<tr>
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<tr>
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<tr>
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<td>6</td>
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<tr>
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*pTumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent structures (si). *P<0.05.

**p53**: UPL no. 12, sense primer, 5'-agcaagtgaactcaaggat-3'; antisense primer, 5'-ccctttttggacttcaggtg-3'; p21: UPL no. 32, sense primer, 5'-tcactgtcttgtaccctgtgc-3; antisense primer, 5'-ggcgtttggagtggtagaaa-3'. The glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene served as an internal control: GAPDH: UPL no. 60, sense primer, 5'-agccacatcgctcagacac-3'; antisense primer, 5'-gcccaatacgaccaaatcc-3. The amplification conditions consisted of initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 65°C for 10 sec. PCR was performed in a LightCycler 480 System (Roche Applied Science) using the LightCycler 480 Probes Master kit (Roche Applied Science). All concentrations were calculated relative to the concentration of cDNA from Human Universal Reference total RNA (Clontech). The concentrations of p53 and p21 were then divided by the concentration of the endogenous reference (GAPDH) to obtain normalized expression values. Each assay was performed 3 times to verify the results, and the mean normalized value of mRNA expression was used for subsequent analyses.

**Protein expression analysis of p53.** Western blotting was used to confirm the expression of p53 in pre-miR-125b transfected cells. Total protein was extracted from samples using PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Inc.). Aliquots of total protein were applied to 10% acrylamide gradient gels. Following electrophoresis, the samples were electroblotted (0.2 A, 120 min, 4°C) onto a polyvinylidene
p53 protein was detected with p53 rabbit polyclonal antibody (Santa Cruz) at a 1:100 dilution. The levels of each protein were normalized to the level of ß-actin protein, which was detected by a 1:1000 dilution of mouse polyclonal anti-ß-actin antibody (Cyto-skeleton Inc.). The blots were developed using horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Promega, Inc.) at a dilution of 1:1000.

Statistical analysis. Data from RT-PCR analysis and in vitro transfected cell assays were analyzed with JMP 5. 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 test, and the log-rank test. Variables with a value of P<0.05 in univariate analyses were used in a subsequent multivariate analysis based on the Cox proportional hazards model. A probability level of 0.05 was chosen for statistical significance.

Results

The clinicopathologic significance of miR-125b mRNA expression in colorectal cancer. In this study, patients with values less than the median expression level of miR-125b were assigned to the low expression group (n=44), whereas those with expression values above the median were assigned to the high expression group (n=45). Patients in the high miR-125b expression group had a significantly poorer prognosis than those in the low miR-125b expression group (P=0.018; Fig. 1). Clinicopathologic factors were significantly different in the high miR-125b expression group. Tumor size (P=0.017), and tumor invasion (P=0.037) were greater than those in the low miR-125b expression group. However, no significant differences were observed regarding age, gender, histology, lymphatic invasion, venous invasion, lymph node metastasis, peritoneal dissemination and liver metastasis (Table I). The results of univariate and multivariate Cox proportional hazards regression analyses for overall survival are shown in Table II. Multivariate analysis indicated that the high expression level of miR-125b was an independent and significant prognostic factor for survival (OR, 1.84; CI, 1.14-3.15; P=0.011; Table II).

p53 and p21 were suppressed by miR-125b. Using in silico microRNA target prediction tools, such as miRanda (14), PicTar (15) and TargetScan (16), we identified the sequences of the miR-125b binding sites in the 3′ UTRs of transcripts encoding p53 (Fig. 2A). Using RT-PCR, we confirmed that miR-125b expression in Pre-miR-125b-treated cells was significantly higher than in untreated cells (parent) and in Pre-miR negative control-treated cells (p<0.05, Fig. 2B). To determine whether miR-125b suppressed p53 and its downstream target, p21, in the colorectal cancer cell line, HT-29,
cell lysates of transfected cells were analyzed by RT-PCR. Suppression of p53 and p21 was observed in Pre-miR-125b-treated cells, in comparison with untreated cells (parent) and Pre-miR negative control treated cells (p<0.05, Fig. 2C). While the inhibition of p53 mRNA level was quite modest, significant suppression in protein level was observed (Fig. 2D). These results support the fact that microRNA suppresses p53 in a post-transcriptional manner.

The association between p53 mutational status and miR-125b expression level. To explore the relationship between p53 mutation and miR-125b, we examined the expression level of miR-125b in 34 cases of colorectal cancer cases with p53 mutation and 41 cases without mutation. However, no significant difference was observed between the two groups (Fig. 3).

Discussion

Analysis of quantitative real-time PCR data demonstrated a concordant relationship between a number of clinicopathologic factors and the expression level of miR-125b in colorectal cancer. These results suggest that miR-125b plays a crucial role in cancer progression. High miR-125b expression was significantly associated with tumor invasiveness at the primary site and poor prognosis. Although some reports highlighted tumor suppressive aspect of miR-125b (17-19), many previous reports suggested that miR-125b works as an onco-genic microRNA in various kinds of cancers. Overexpression of miR-125b is associated with precursor B-cell acute lymphoblastic leukemia (pre-B ALL) (20) and myelodysplastic syndrome (MDS) (21). In addition, Klusmann and colleagues reported that miR-125b directly repressed Dicer1 and ST18,
and contributed to trisomy 21-associated megakaryoblastic leukemia (22). In prostate cancer, miR-125b targets Bak1, apoptosis related gene, and induces cancer progression (23). Among various pathways of miR-125b, Le and colleagues recently reported that miR-125b suppressed p53 and inhibited p53-induced apoptosis during development and during the stress response (8). These results suggest the possibility that overexpression of miR-125b contributes to the initiation and progression of human cancers through inhibition of p53, a key component of apoptosis. Consistent with this finding, our clinical data suggest that high expression of miR-125b is associated with poor prognosis in colorectal cancer. We confirmed that overexpression of miR-125b suppresses p53 and its downstream molecule, p21 in colorectal cancer cells. This suggests that miR-125b has anti-apoptotic effects and/or inhibits cell cycle arrest, permitting tumor progression, and worsening prognosis.

Reportedly, there is a complex regulatory network involving p53 and various microRNAs. MiR-29 and miR-122 activate the p53-dependent apoptosis pathway (24-26), while miR-192, miR-194, miR-215 are induced by p53 during stress responses (27,28). In addition, p53 promotes transcription of miR-34a, while miR-34a activates p53 by inhibiting the histone deacetylase, SIRT1. Thus, there exists a feed-forward loop between miR-34a and p53 (29,30). In this way, interaction between genes and microRNAs is frequently observed in the important signal transduction such as apoptosis inducing system (9). In our current study of miR-125b and p53, we investigated whether p53 mutational status influenced miR-125b expression in clinical samples. However, no significant difference was observed between samples with wild-type and mutant p53 (Fig. 3). At least in colorectal cancer, we could not demonstrate the existence of feedback loop between p53 and miR-125b.

There is growing evidence that suggests that accurate prediction of disease recurrence after complete resection and adequate induction of chemotherapy/radiation therapy reduces the mortality rate of colorectal cancer (31-33). Molecular based diagnostic tools and therapies are reaching clinical use, but their efficiency is still limited. MicroRNAs, which regulate various targets and play a crucial role in cancer initiation and progression, could be potential biomarkers that reflect tumor aggressiveness independent of TNM classification.

In conclusion, we demonstrated that high expression levels of miR-125b were associated with enhanced malignant potential and poor prognosis, and could be an independent prognostic factor. Although previous reports have demonstrated the oncogenic aspects of miR-125b, there has been little information regarding miR-125b expression and its clinical significance in gastrointestinal malignancy. MiR-125b is a meaningful prognostic indicator and potential therapeutic target in colorectal cancer.

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