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.

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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* targets *p53* activity, reducing excessive apoptosis and ensuring homeostasis (9). However, these also suggest the possibility that overexpression of *miR-125b* leads to the inhibition of p53, and contribute to the initiation and progression of human cancers.

In the current study, we focused on *miR-125b* as a negative regulator of *p53*. We analyzed the *miR-125b*/*p53* pathway in colorectal cancer, and show its clinical significance and importance as a prognostic indicator of *miR-125b* in human colorectal cancer.

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 10% fetal bovine serum with 100 units/ml penicillin and 100 units/ml streptomycin sulfate and cultured in a humidified 5% CO_2 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.

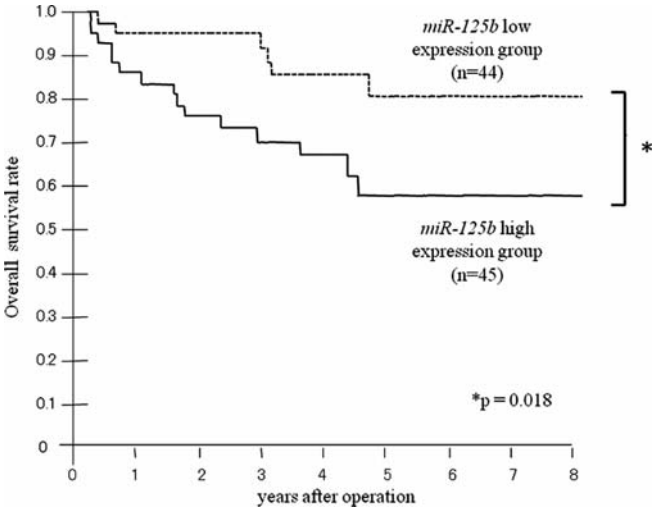

Figure 1. Five-year Kaplan-Meier survival curves for colorectal cancer patients according to the level of *miR-125b* expression. Colorectal cancer cases (n=89) were classified into two groups according to the median *miR-125b* expression level as determined by quantitative RT-PCR. *MiR-125b* expression was normalized to RNU6B, an internal control. The survival rate for patients in the high expression group was significantly lower than that for patients in the low expression group (p=0.018).

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:

Table I. miR-125b expression and clinicopathological factors.

Factors	Tumor low expression (n=44)		Tumor high expression (n=45)		P-value
	Number	%	Number	%	
Age (mean ± SD)	65.0±1.87		64.07±1.85		0.724
Sex					
Male	25	56.8	28	62.2	0.603
Female	19	43.2	17	37.8	
Histological grade					
Well	20	45.5	15	33.3	0.24
Moderately, poorly	24	54.5	30	66.7	
Size					
<20 mm (small)	7	15.9	1	2.2	0.017[b]
>21 mm (large)	37	84.1	44	97.8	
Depth of tumor invasion					
[a]m, sm, mp, ss	20	45.5	11	24.4	0.037[b]
se, si	24	54.5	34	75.6	
Lymph node metastasis					
Absent	30	68.2	24	55.3	0.15
Present	14	31.8	21	46.7	
Lymphatic invasion					
Absent	32	72.7	28	62.2	0.29
Present	12	27.3	17	37.8	
Venous invasion					
Absent	39	88.6	37	82.2	0.39
Present	5	11.4	8	17.8	
Liver metastasis					
Absent	39	88.6	39	86.7	0.78
Present	5	11.4	6	13.3	
Peritoneal dissemination					
Absent	42	95.5	43	95.6	0.95
Present	2	4.5	2	14.4	

[a]Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures (si). [b]P<0.05.

p53: UPL no. 12, sense primer, 5'-aggccttggaactcaaggat-3'; antisense primer, 5'-ccctttttggacttcaggtg-3; *p21*: UPL no. 32, sense primer, 5'-tcactgtcttgtaccttgtgc-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 PROPREP™ 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

Table II. Univariate and multivariate analysis of overall survival (Cox regression model).

Factors	Univariate analysis			Multivariate analysis		
	RR	95% CI	P-value	RR	95% CI	P-value
Age (<65/>66)	1.80	0.78-4.47	0.17	-	-	-
Sex (male/female)	0.86	0.54-1.31	0.48	-	-	-
Histology grade (well/others)	1.3	0.87-2.15	0.48	-	-	-
Depth of tumor invasion ([a]m, sm, mp/ss, se, si)	2.50	1.35-6.23	0.0017[b]	1.40	0.71-3.61	0.37
Lymph node metastasis (negative/positive)	2.32	1.49-3.85	0.0001[b]	1.73	1.06-2.98	0.0295[b]
Lymphatic invasion (negative/positive)	1.66	1.1-2.52	0.017[b]	1.41	0.88-2.27	0.155
Venous invasion (negative/positive)	1.59	0.95-2.47	0.0729	-	-	-
Liver metastasis (negative/positive)	2.36	1.48-3.61	0.0006[b]	1.69	1.01-2.82	0.048[b]
Peritoneal dissemination (negative/positive)	2.36	1.48-3.61	0.0006[b]	4.77	2.09-11.4	0.0004[b]
miR-125b expression (low/high)	3.02	1.37-7.59	0.0055[b]	1.84	1.14-3.15	0.011[b]

RR, relative risk; CI, confidence interval. [a]Tumor invasion of mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), penetration of serosa (se), and invasion of adjacent strucures. [b]P<0.05.

membrane (Immobilon; Millipore, Inc.). 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 (Cytoskeleton 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 χ^2 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,

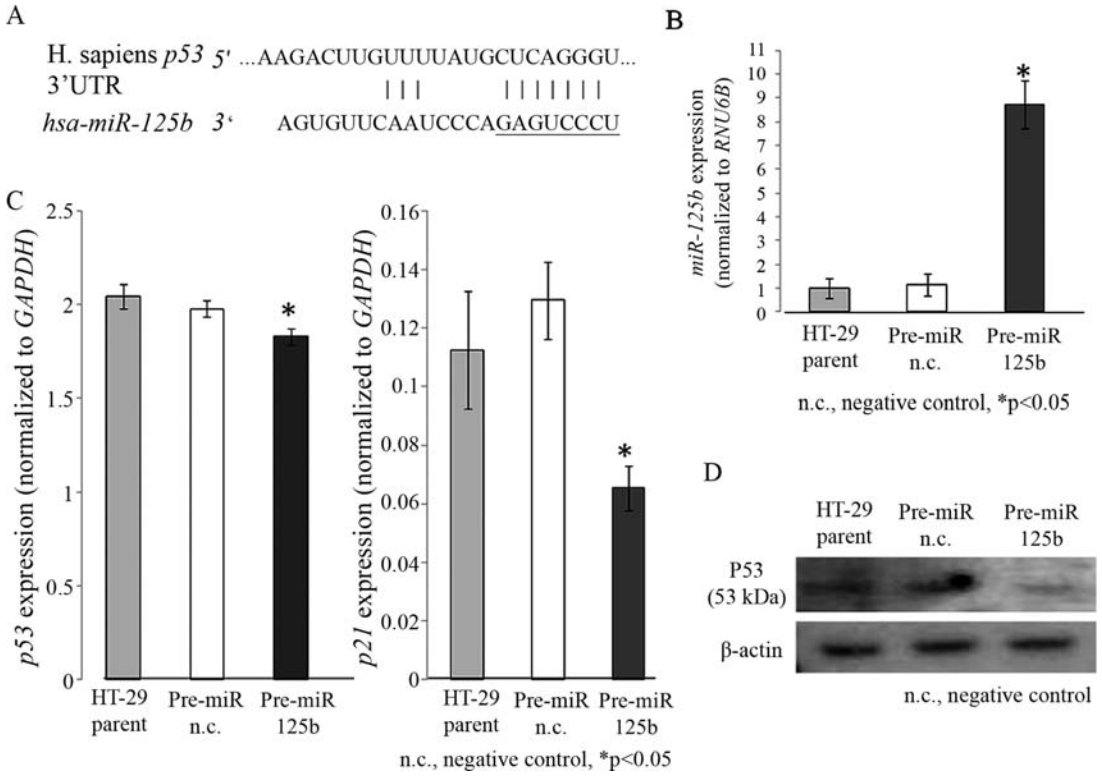

Figure 2. *MiR-125b* targets *p53* in colorectal cancer cells. (A) Sequence of the *miR-125b* binding sites in the 3'UTRs of transcripts encoding p53. Seed sequence of microRNA is underlined. (B) *MiR-125b* expression after treatment with negative control or Pre-*miR-125b* in HT-29 (quantitative RT-PCR). *MiR-125b* expression in Pre *miR-125b*-treated cells is significantly higher than in untreated cells (parent) and in Pre-miR-treated (negative control) cells. The results are the means ± SD of triplicates. (C) *p53* (left) and *p21* (right) mRNA expression after treatment with negative control or Pre-*miR-125b* in HT-29 cells. Expression of *p53* and *p21* in Pre-*miR-125b*-treated cells was significantly lower than in untreated cells (parent) and in Pre-miR-treated (negative control) cells. The results are the means ± SD of triplicates. mRNA levels were normalized to *GAPDH*. (D) Western blotting analysis of p53 in HT-29 cells transfected with Pre-*miR-125b* or negative control. These proteins were normalized to the level of ß-actin.

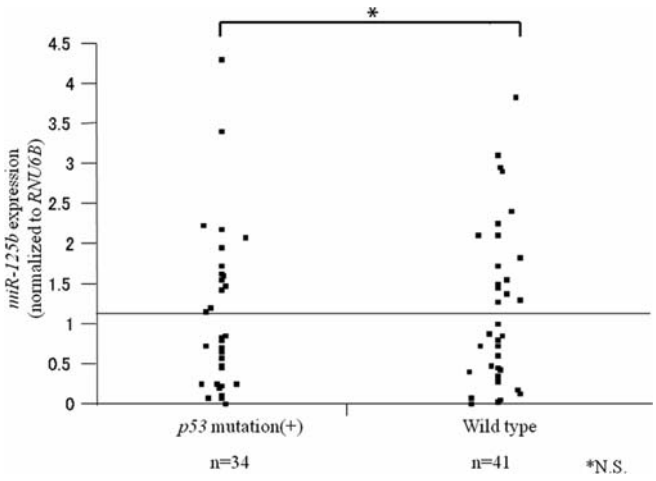

Figure 3. *miR-125b* expression according to the mutational status of *p53*. There were no significant differences between the mutant *p53* group (n=34) and the wild-type *p53* group (n=41). Horizontal lines indicate the mean value of each group. *MiR-125b* expression was normalized to *RNU6B*.

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 oncogenic 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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