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Prognostic Significance of miR-215 in Colon Cancer

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Abstract

The clinical utility of miR-215 as a potential biomarker in colon cancer was investigated. The levels of miR-215 were quantified by real-time qRT-PCR in 34 paired normal and tumor specimens. The expression levels of miR-215 were decreased in colon tumors, and were associated with patient survival. Thus, miR-215 is a potential prognostic biomarker in colon cancer.

Background—We have previously shown that miR-215 suppressed the expression of key targets such as thymidylate synthase (TS), dihydrofolate reductase, and denticleless protein homolog (DTL) in colon cancer. miR-215 is a tumor suppressor candidate due to the upregulation of p53 and p21 by targeting DTL. However, high levels of miR-215 conferred chemoresistance due to cell cycle arrest and reduced cell proliferation by suppressing DTL. In this study, the clinical significance of miR-215 was further investigated as a potential prognostic biomarker in colon cancer patients.

Methods—Total RNAs were extracted from 34 paired normal and colon (stage II and III) tumor specimens using the Trizol-based approach. The levels of miR-215 and a closely related miR-192 were quantified using quantitative real-time polymerase chain reaction (qRT-PCR) expression analysis. The expression of DTL mRNA and protein were quantified by real time qRT-PCR and immunohistochemistry.

Results—The expression levels of miR-192 ($P = .0008$) and miR-215 ($P < .0001$) were significantly decreased in colon tumors compared with normal tissues. DTL was significantly over-expressed and was inversely correlated with miR-215, further suggesting an in vivo physiologic relevance of miR-215 mediated DTL suppression. Kaplan-Meier survival analysis by Cox regression revealed that high levels of miR-215 expression (hazard ratio, 3.516; 95% confidence interval, 1.007–12.28, $P = .025$) are closely associated with poor patient's overall survival. Furthermore, an elevated expression of a miR-215 target protein DTL was detected in colon cancer tissues whereas no expression was present in normal tissues.

Conclusion—miR-215 has a unique potential as a prognostic biomarker in stage II and III colon cancer.

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Disclosure

All authors have no conflicts of interest.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.clcc.2011.06.002.

Keywords

Biomarker; Colon cancer; MicroRNA; Prognosis

Introduction

Cancer of the colon is a major cause of malignancy-related death worldwide and second only to lung cancer as a cause of cancer mortality.^{1,2} It is established that more than 80% of stage II and 65% of stage III colorectal cancer patients can be cured by surgery alone without adjuvant therapy.² Moreover, the 5-year survival rates for stage II (78.5%) and stage III (54%) colon cancer patients are much higher than stage IV patients (8.1%).³ Therefore, it is important to investigate the prognosis potential of miRNAs in stage II and III colon cancer both to spare patients who can be cured by surgery alone from unnecessary chemotherapy treatment, and to target a population who has a long-term survival with surgery and chemotherapy treatment.

Although improvements have been made in the treatment of colon cancer patients, the therapeutic outcome after chemotherapy is still suboptimal because of inherent or acquired anticancer drug resistance.⁴ Thymidylate synthase (TS) inhibitors such as 5-fluorouracil, tomudex (TDX, raltitrexed)–based chemotherapy are still the corner stones for treating colorectal cancer. The importance of post-transcriptional and translational mechanisms in chemoresistance has been well-documented,^{5–10} and one of the major regulators in this process is miRNAs.

miRNAs are 18- to 25-nucleotide, noncoding RNA molecules that cause translational arrest or mRNA cleavage, most likely, by binding to 3'UTRs of mRNAs.¹¹ miRNAs have been found to regulate many cellular processes including apoptosis,^{12–15} differentiation,^{16–18} and cell proliferation.^{12,17,19,20} Aberrant expression of miRNAs is observed in most tumor types, indicating a role in carcinogenesis and a potential as prognostic biomarkers in cancer.^{21,22} Approaches to modulating miRNA function may be an effective way to develop novel adjuvant therapeutics to increase chemoresponse and survival.

Previous studies have shown that the tumor suppressor gene p53 directly regulates some miRNAs,^{23,24} and this mechanism turns out to be a critical aspect of p53 function in regulating cell cycle and cell proliferation^{25–29}. Several miRNAs (miR-192, miR-215, miR-34) regulated by p53 have been shown to act on cellular mechanisms of anticancer drug resistance,^{30–34} through their effect on cell cycle and cell proliferation. Recently, a study from our laboratory has described a novel mechanism of chemoresistance mediated by miR-215. Despite the suppression of two of the most key anticancer targets, TS and dihydrofolate reductase (DHFR), cells with elevated miR-215 counter-intuitively became more resistant to TS inhibitor TDX and DHFR inhibitor methotrexate (MTX).³⁰ This raised a notion that miR-215 may act in a novel mechanism to mediate chemoresistance which is different from just inhibiting TS or DHFR. This also provides novel insights for the lack of consistency of using TS or DHFR as predictive and prognostic biomarkers.³⁵ Because miRNA regulates multiple targets, we reasoned that certain miRNAs may have better prognostic potential compared with single drug target such as TS or DHFR. Furthermore, we showed that colon cancer stem-like cells with elevated miR-215 were far more resistant to TDX and MTX. This resistance mechanism was mediated, at least in part, by reduced cell proliferation and increased G2-cell cycle arrest through the suppression of a critical target denticleless protein homolog (DTL) expression by miR-215.³⁰

DTL (RAMP, CDT2) is a protein that is thought to play an essential role in DNA synthesis, cell cycle progression, proliferation, and differentiation.³⁶ It has been reported that DTL

controls cell cycle through many distinct mechanisms. First, it is a component of the proliferating cell nuclear antigen (PCNA) – coupled CUL4-DDB1 E3 ubiquitin ligase complex and is required for degrading Cdt1 during S phase,³⁷ thereby preventing DNA rereplication.³⁸ Second, it is an important component of the early, radiation-induced G2/M checkpoint, possibly again through degrading key cell cycle regulators in G2 phase through its ubiquitin ligase function.³⁸ Moreover, PCNA-coupled CUL4/DDB1/DTL complex has been reported to ubiquitinate and degrade key cell cycle proteins such as p53, MDM2, p21, and E2F1, further emphasizing the significance of this ubiquitination system in cell cycle.^{39–41}

In this study, we analyzed the significance of miR-215 and DTL expression levels in stage II and III archival colon cancer specimens with clinical follow-up information to further investigate the potential clinical implications of miR-215 and its targets. To the best of our knowledge, this is the first report to show that DTL is elevated in colon cancer patients and a physiologic relevance of the miR-215 mediated DTL regulatory mechanism. We have shown that although miR-215 expression was decreased in tumor tissues compared with adjacent normal tissues, lower levels of miR-215 expression were associated with increased overall patient survival. As a result, our findings suggest that miR-215 holds a unique potential as a prognostic biomarker in colon cancer patients and as a therapeutic target for overcoming chemoresistance.

Materials and Methods

Patients and Samples

Two clinical sample cohorts were used for this study approved by the Institution Review Board. For the US cohort, tumor samples and the adjacent normal tissues were obtained from 34 patients with primary colon cancer who underwent surgery at the Stony Brook University Hospital, Stony Brook, NY. Formalin-fixed paraffin-embedded (FFPE) tissues were acquired from the archival collections of the Department of Pathology, and used for subsequent RNA extraction. The characteristics of these patients are shown in Table 1.

For the German cohort, snap fresh frozen tumor samples and the adjacent normal tissues were obtained from 24 patients with primary colorectal cancer who underwent surgery at the Department of General, Visceral and Transplantation Surgery, University of Ulm, Germany. Tissues were snap frozen in liquid nitrogen and stored at -80°C , and used for subsequent RNA extraction. The characteristics of these patients were described in detail in previous studies.⁴²

Tissue Microarrays

Tissue microarrays (TMAs) were prepared from FFPE blocks of colectomy specimens stored at the Stony Brook University Medical Center Pathology Department. The specimens were selected from 1998 to 2003 to allow that at least 5 years of clinical follow-up information was available and the miRNA in the tissue would remain viable.⁴³ Hematoxylin and eosin–stained sections from all cases were reviewed; for each case, areas of invasive colonic adenocarcinoma and benign colonic mucosa were designated. Using the Advanced Tissue Arrayer (Model: ATA-100, Millipore Corporate, MA), with a 1.5-mm diameter needle, three cores were extracted from the area of adenocarcinoma and three cores were removed from the benign area for each case. The cores were embedded into a paraffin tissue microarray block in predetermined positions, with up to 60 cores in each tissue microarray and two additional cores placed to designate the orientation of the block.

RNA Isolation

Using archival FFPE tissues, separate areas of solid tumor and normal colonic epithelium were identified using the corresponding hematoxylin and eosin–stained sections and cores measuring 1.5 mm in diameter and 2 mm in length (approximately 0.005 g) were extracted. Subsequently, the samples were treated with deparaffinization, hydration, proteinase K, and ultimately total RNAs were isolated by using the TRIZOL reagent (Invitrogen, Carlsbad, CA). The TRIZOL method was used to isolate total RNAs from snap frozen colorectal cancer tissues.

Quantitative Real-Time Polymerase Chain Reaction Analysis of miRNA Expression

The miR-192, miR-215 specific primers and the internal control RNU6B gene were purchased from Ambion (Applied Biosystems, Foster City, CA). cDNA synthesis was performed by the High Capacity cDNA Synthesis Kit (Applied Biosystems) with miRNA specific primers. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed on an Applied Biosystems 7500 Real-time system (ABI 7500HT instrument) with miRNA specific primers by TaqMan Gene Expression Assay. Expression of miR-215 was normalized according to the internal RNU6B control, and the relative expression values were plotted.

qRT-PCR Analysis of DTL mRNA

The PCR primers and probes for DTL and the internal control gene β -actin were purchased from Applied Biosystems. cDNA synthesis was performed by the High Capacity cDNA Synthesis Kit (Applied Biosystems) with random primers. qRT-PCR was performed on an ABI 7500HT instrument with mRNA specific primers by TaqMan Gene Expression Assay under the following conditions: 50°C, 2 minutes of reverse transcription; 95°C, 10 minutes; 95°C, 15 seconds; and 60°C, 1 minute for up to 40 cycles ($n = 3$). Expression of DTL mRNA was normalized according to the internal β -actin control, and the relative expression values were plotted.

Immunohistochemistry Analysis of DTL Expression

TMA's were sectioned at 5 μ m and heat immobilized at 60 °C overnight. After deparaffinization, antigen retrieval was performed in a citrate buffer (20 mmol/L [pH = 6]) at 120 °C for 10 minutes followed by 3% hydrogen peroxide for 5 minutes. Staining was applied to FFPE human colon cancer tissues by using an avidin-biotin (ABC) method (Vector Laboratories, Burlingame, CA). FFPE human testis tissues were used as positive controls. Sections were incubated for 1 hour with a rabbit polyclonal DTL (Atlas Antibodies AB, Stockholm, Sweden) antibody (1:100). Monoclonal mouse immunoglobulin G (IgG; BD Biosciences, Franklin Lakes, NJ) was used as an isotype–negative control. DTL staining was visualized using 3, 3'-diaminobenzidine (Dako, Carpinteria, CA), and sections were counterstained with dilute hematoxylin, dehydrated, and cover-slipped for bright-field microscopy. Staining scores for immunohistochemistry (range, 0–300) were calculated by multiplying the intensity score (0–3) by the percent of the cells staining (0–100) as described in another study,⁴⁴ and statistical analysis was performed using GraphPad Prism software 5.0.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism software 5.0. Gene expression Δ Ct values of miR-215 from each sample were calculated by normalizing according to internal control RNU6B expression, and relative quantification values were plotted. Gene expression Δ Ct values of DTL from each sample were calculated by normalizing according to internal control β -actin expression, and relative quantification values were plotted. The

differences between tumor and normal tissues were analyzed by Wilcoxon matched-pairs test. A Kaplan-Meier survival curve was generated by performing a Cox proportional hazards regression analysis to evaluate the expression level of miR-192 and miR-215 with survival rate. Statistical significance was set up to $P < .05$ in each test.

Results

Decreased Expression of miR-192 and miR-215 in Human Colon Cancer and Evaluation of Their Prognostic Values

To evaluate the clinical significance of the miR-215 and a closely related miR-192, we profiled their expression from 34 paired archival normal and tumor FFPE tissue specimens of stage II and stage III colon cancer patients by using real time qRT-PCR analysis. Of note, these naïve samples were obtained before chemotherapy treatment. The expression of both miR-192 ($P = .0008$) (Figure 1A) and miR-215 ($P < .0001$) (Figure 1B) were significantly decreased in colon cancer specimens compared with adjacent normal tissues. The fold changes of miR-192 and miR-215 between paired normal and tumor tissue specimens were presented in Figure 1C and 1D, respectively. The fold changes of normal versus low miR-215 tumor samples and normal versus high miR-215 tumor samples were shown in Supplementary Figure S1. This supports the notion that the putative function of miR-192 and miR-215 as tumor suppressors might be clinically relevant in the case of colon cancer.²³ We further analyzed the expression of miR-215 in colon cancer samples containing wild-type p53 or mutant/deletion p53. p53 status of these patients are described in detail in our previous study.⁴² The results revealed that the expression of miR-215 was significantly associated with p53 status (Supplementary Figure S2). To further analyze the significance of these miRNAs in terms of clinical prognosis, Kaplan-Meier survival analysis was performed using patient overall survival (Figure 2). Our results indicate that miR-215 expression was associated with patient survival (Figure 2B). Patients with low levels of miR-215 tended to have longer survival than patients with high levels of miR-215 ($P = .025$). However, miR-192 expression was not associated with patient survival (Figure 2A). These findings show that although miR-192 and miR-215 are both aberrantly expressed, only miR-215 shows a potential as a prognostic biomarker in colon cancer. Additionally, this evidence confirms that miR-192 and miR-215 are indeed two different miRNAs with different functions, consistent with their location on separate chromosomes. More importantly, miR-192 does not suppress the expression of DTL whereas miR-215 does.

Correlation of miR-215 and DTL Expression in Clinical Patients

Our previous studies in colon cancer cell lines showed that miR-215 inhibits G2 checkpoint regulator DTL, leading to upregulation of p53 and p21, and thereby causes cell cycle arrest.³⁰ To investigate the in vivo relationship between miR-215 and DTL, we profiled the expression of DTL mRNA from 24 paired fresh frozen tissue specimens of colorectal cancer patients by using real time qRT-PCR analysis as mRNAs were well-preserved in these tissue samples. Fresh frozen tissues were preferred because in FFPE tissues it is difficult to quantify mRNA due to different degrees of mRNA degradation while miRNAs remain stable.⁴³

The expression of DTL mRNA was significantly increased ($P < .0001$) in colorectal cancer specimens compared with adjacent normal tissues (Figure 3A). The DTL expression pattern is inversely correlated with miR-215 expression pattern (Figure 1B), suggesting that the in vitro results of miR-215 suppressing DTL expression is also valid in clinical specimens. In Figure 1B, miR-215 was quantified from archival FFPE tissues obtained from Stony Brook University Hospital; whereas in Figure 3A, DTL was quantified from fresh-frozen tissues obtained from University of Ulm, Department of General, Visceral and Transplantation

Surgery. Figure 3B shows the presence of a significant inverse correlation (Spearman correlation coefficient = -0.322) between miR-215 and DTL mRNA expression levels in clinical colorectal cancer patients.

To further investigate the *in vivo* relationship between miR-215 and DTL expression at the protein level, we performed immunohistochemistry with DTL antibody on sectioned archival FFPE colon cancer tissue specimens obtained from Stony Brook University Medical Center. Although DTL protein was not detected in normal colon tissues, an elevated protein expression was detected in colon cancer tissues (Figure 4). This pattern correlates similarly with DTL mRNA expression (Figure 3), and thus correlates oppositely with miR-215 expression (Figure 1B). Therefore, we suggest that an inverse correlation between miR-215 and DTL expression levels is present also at the protein level in clinical patients showing the physiologic relevance of this relationship.

Discussion

In this study, the prognostic potential of miR-215 in colon cancer was investigated using colon cancer specimens. The expression of miR-215 was significantly reduced in most of the colon cancer samples. This is consistent with our previous finding that high level of miR-215 suppresses cancer cell proliferation. However, the prognostic results were rather intriguing as patients with lower expression level of miR-215 tend to have a better survival. This seems to contradict with the “tumor suppressor” definition of miR-215. However, we reasoned that the function of miR-215 is a double-edge sword because miR-215 modulates multiple targets and pathways. A high level of miR-215 suppresses tumor cell proliferation and increases cell cycle arrest. However, with regard to chemosensitivity, it has an opposite effect. In general, the narrow chemotherapeutic window was achieved largely because of the differential proliferation rate of relatively rapid proliferation of cancer cells compared with normal cells. Chemotherapy in general is more effective in killing tumor cells with more rapid proliferation rate which requires lower level of miR-215, and we previously demonstrated that miR-215 is downregulated in fast-proliferating, chemotherapy-sensitive phenotype of differentiated cancer cells compared with slow-proliferating, chemotherapy-resistant phenotype of cancer stem cells.³⁰ In this translational study, we showed that patients with low expression level of miR-215 may have a more chemosensitive proliferative phenotype and they survived better than patients with elevated levels of miR-215. This brings up an important issue of developing future miRNA-mediated therapeutic strategy to sensitize slow-proliferating cancer cells and cancer stem cells to enhance anticancer chemotherapeutic efficacy.

Previous studies from our laboratory have shown several key targets of miR-215 as TS, DHFR, and DTL in colon cancer. The elevated expression of TS and DHFR in colon cancer has been extensively investigated in the past, which is consistent with the lower expression levels of miR-215 in colon tumor tissues. Despite extensive investigation from many laboratories, the prognostic value of TS in colon cancer is still debated.^{35,45} Despite the fact that TS and DHFR are the major anticancer targets in chemotherapy, their suppression by miR-215 resulted in increased chemoresistance.³⁰ However, reduced DTL expression mediated by miR-215 resulted in reduced chemosensitivity.³⁰ Therefore, we reasoned that DTL suppression by miR-215 could be the important mechanism controlling chemoresistance *in vivo*. DTL is a cell cycle G2/M checkpoint regulatory protein³⁸ and is a component of the CUL4/DDB1 E3 ubiquitin ligase complex which ubiquitinates and degrades target cell cycle proteins such as p53 and p21.^{39,40} Because miR-215 induces cell cycle G2-arrest by upregulation of p53 and p21, we hypothesize that DTL suppression by miR-215 is responsible for stabilizing and increasing p53 and p21 levels leading to G2-arrest.³⁰ When the cells are arrested and not proliferating, they become resistant to

chemotherapy agents and cannot be killed easily. In this study, evaluated the relationship between miR-215 and DTL expression levels in clinical patients to understand the physiologic relevance of this mechanism. This may also provide a novel molecular mechanism to explain for the inconsistent results of using TS or DHFR as prognostic biomarkers from previous reports and highlight the importance of this miR-215-mediated resistance mechanism.³⁵ It also further supports the notion of using miR-215 as a prognostic biomarker because of its broad effect on multiple targets and pathways.

Our results indicate that the expression levels of miR-215 and DTL were inversely correlated in clinical patients. Colon cancer specimens have reduced expression level of miR-215 to acquire increased proliferation phenotype with elevated DTL. This is also the first study to show over-expressed DTL in colon cancer. Thus, elevated DTL expression could be a potential hallmark of colon cancer. The expression level of miR-215 was associated with colon cancer patients' survival. Taken together with its critical function in regulating cell proliferation, cell cycle and chemoresistance, miR-215 has a unique potential as a prognostic biomarker in colon cancer. Future studies are required with multicenter large colon cancer patient cohorts to fully validate the clinical utility of miR-215.

Clinical Practice Points

Translational control mediated by non-coding microRNAs (miR-NAs) plays a key role in resistance to anti-cancer drug treatment. As a result, non-coding microRNAs hold great potential as new prognostic biomarkers. In this study, we show that the expression of miR-215 was significantly decreased in colon tumors compared with adjacent normal tissues. Kaplan-Meier survival analysis indicated that the expression levels of miR-215 were associated with patient overall survival such that patients with high levels of miR-215 tended to have a shorter survival time. Based on our previous findings, miR-215 induces chemoresistance through the suppression of a critical target, denticleless protein homolog (DTL). Thus, we further analyzed the physiologic relevance of this chemoresistance mechanism, and showed that the expression levels of miR-215 and DTL were inversely correlated in colon cancer patients. As a result, miR-215 holds a unique potential as a prognostic biomarker, and therapeutic approaches targeting miR-215 may enhance response rates in patients treated with chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. miR-192/miR-215 Expression in Normal and Colon Cancer Tissue Specimens. Gene Expression Values Were Expressed as Ratios Between miRNAs With an Internal Control RNU6B Gene. (A) miR-192 Expression ($P = .0008$). (B) miR-215 Expression ($P < .0001$). Statistical Significance Was Calculated by a Paired Wilcoxon Signed-Rank Test. (C) Fold Changes of miR-192 of Each Individual Paired Sample. (D) Fold Changes of miR-215 of Each Paired Sample

Figure 2. Relationship Between miR-192/miR-215 Expression and Survival in Colon Cancer Patients. Kaplan-Meier Overall Survival Curves by Cox Regression Analysis Were Plotted Based on miRNA Expression. (A) miR-192 Expression ($P = .6842$). (B) miR-215 Expression ($P = .025$)

Figure 3. Denticless Protein Homolog (DTL) mRNA Expression in Colorectal Cancer Patients. (A) DTL mRNA Expression Was Assayed by Quantitative Real-time Polymerase Chain Reaction in Normal and Colorectal Cancer Fresh Frozen Tissue Specimens ($P < .0001$). Gene Expression Values Were Expressed as Ratios Between DTL With an Internal Control β -actin Gene. (B) Correlation of DTL and miR-215 Expression Was Analyzed by Two-tailed Spearman Nonparametric Correlation Test ($P = .033$, Spearman Correlation Coefficient = -0.322)

Figure 4. Denticleless (DTL) Protein Expression in Colon Cancer Patients. (A) Scatter Plot of Immunostaining Scores for DTL (Range, 0–300) Calculated by Multiplying the Intensity Score (0–3) by the Percent of Cells Stained (0–100) for Normal Colon and Colon Cancer. Bars Represent the Median for Each Category (Normal Median = 0, Tumor Median = 83). DTL Protein Expression in Normal Colon (B, C) and Colon Cancer (D, E) as Detected by Immunohistochemistry

Table 1

Clinical Features of the 34 Colon Cancer Patients Used in This Study

Characteristics	Frequency	Percentage (%)
Age (Years)		
Mean (Range)	60 (28–81)	
Gender		
Male	20	58.8
Female	14	41.2
Histology		
Adenocarcinoma	34	100
TNM Stage		
I	0	0
II	15	44.1
III	19	55.9
IV	0	0
Survival (Months)		
Mean (range)	57 (5–126)	
0–40	10	29.4
40–80	14	41.2
> 80	10	29.4