# miRNA-503 Promotes Tumor Progression and Is Associated with Early Recurrence and Poor Prognosis in Human Colorectal Cancer

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## Abstract

*Objectives:* MicroRNA (miR)-503 is downregulated in several cancers and plays a tumor-suppressive role in carcinogenesis. However, the miR-503 expression pattern, its clinical significance and its molecular mechanism in colorectal cancer (CRC) have not been investigated. *Methods:* We analyzed miR-503 expression in normal mucosa (n = 20), adenoma (n = 27) and CRC (n = 20). We quantified miR-503 expression in an independent cohort (n = 191) and investigated the clinical significance of miR-503 in CRC. CRC cell lines were transfected with anti-miR-503 to assess its function and target gene. *Results:* miR-503 expression increased according to the adenoma-carcinoma sequence. High miR-503 expression was significantly associated with large tumor size, serosal invasion, lymphatic and venous invasion as well as lymph node metastasis. CRC patients with high miR-503 expression had significantly earlier relapse and poorer prognosis than those with low expression. miR-503 was an independent recurrence marker in stage I/II CRC. In vitro, attenuated miR-503 expression resulted in inhibition of proliferation, invasion and migration and acquisition of anoikis of CRC cells. The putative target gene (calcium-sensing receptor) was significantly upregulated after miR-503 attenuation. *Conclusions:* miR-503 acts as an 'onco-miR' in CRC. High miR-503 expression is associated with early recurrence and poor prognosis in CRC.

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## Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide and a major cause of cancer-related deaths [1]. The survival rates of CRC patients have improved in recent years because of improved screening and/or treatment options. Nevertheless, among CRC pa-

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tients who have undergone potentially curative surgery, up to 30% eventually develop local recurrence or distant metastasis, which results in a worse overall prognosis [2, 3]. Therefore, an identification of CRC patients at a higher risk of developing metastatic disease might enable us to stratify and select patients that will truly benefit from treatment with cytotoxic chemotherapy and intensive posttreatment surveillance protocols. To move closer towards individualized therapeutic strategies, there clearly is an unmet need for developing promising biomarkers for the accurate identification of patients at a higher risk of developing recurrent disease.

MicroRNAs (miRNAs) are a class of small noncoding RNAs (17–25 ribonucleotides) that inhibit gene expression by binding to the 3' untranslated regions of mRNAs and inhibiting their translation [4, 5]. miRNAs are involved in essential biological processes such as development, cellular differentiation, proliferation and apoptosis. Therefore, dysregulation of miRNA expression contributes to development of cancer through posttranscriptional regulation of oncogenes and tumor suppressors in various types of cancer including CRC [6–8]. Accumulating data have shown that several consistently dysregulated miRNAs have been identified in CRC, and they are significantly associated with a variety of clinicopathological, diagnostic, recurrent and prognostic factors in CRC patients [9, 10].

miRNA (miR)-503 is a member of the miR-15/16 family, and recently there has been increasing interest in understanding the role of miR-503 in cancer [11]. miR-503 is downregulated in several types of cancer including oral, hepatocellular, gastric and endometrial [12–16] cancer, suggesting that it plays a tumor-suppressive role in carcinogenesis. In contrast, it is overexpressed in parathyroid carcinoma, retinoblastoma and adrenocortical carcinoma [17–19], and upregulation of miR-503 is associated with a shorter overall survival (OS) rate among patients with adrenocortical carcinoma [19]. These results suggest that the expression pattern of miR-503 is tissue or disease specific.

miR-503 expression according to the adenoma-carcinoma sequence in CRC and the correlation of miR-503 expression with clinical features, as well as the function of miR-503 in CRC, have not been fully studied. In the current study, we performed a functional investigation of miR-503 in human CRC cell lines. Then, we analyzed the expression pattern of miR-503 in human colorectal carcinogenesis, followed by evaluation of the clinical significance of miR-503 expression in CRC, using an independent, large cohort.

## Materials and Methods

### Study Design

This study included analysis of 260 tissue specimens obtained from healthy volunteers and consecutively enrolled patients with colorectal adenoma or cancer at the Mie University Medical Hospital, Japan, between January 1, 2005, and December 31, 2010. Exclusion criteria were inflammatory bowel disease, familial adenomatous polyposis, hereditary nonpolyposis colon cancer or other rare and complex types of tumors. This study undertook a functional analysis, including searching for target genes of miR-503 in CRC cells, followed by the association between miR-503 expression in CRC tissues and clinicopathological and survival outcomes.

This study was designed with a first and a second phase. In the first phase, miR-503 expression was measured in a subset of 67 tissue samples from normal colonic mucosa (n = 20), adenoma (n = 27) and CRC (n = 20) to analyze its expression according to colorectal carcinogenesis. In the second phase, the distribution of miR-503 expression was investigated in a large, independent cohort of 191 patients with CRC from whom surgical colorectal tissues were collected. The tumor-node-metastasis (TNM) staging system from the American Joint Committee on Cancer was used for classification of the pathological staging of CRC. All CRC patients who underwent surgery were followed up for tumor recurrence at regular intervals for up to 5 years. During each annual hospital visit, all patients underwent chest X-ray, colonoscopy and abdominal computed tomography. OS time was calculated from the date of diagnosis to the date of death or the last date of follow-up. Patients treated with radiotherapy or chemotherapy before surgery were excluded from the study. Patients with stage III/IV disease received 5-fluorouracil-based chemotherapy, whereas no adjuvant therapy was given to patients with stage I/II CRC.

The tissue specimen collection and studies were approved by the Institutional Review Boards of Mie University Hospital. All participants provided written informed consent and indicated their willingness to donate their medical data and tissue samples for research.

### RNA Isolation and qRT-PCR from Formalin-Fixed Paraffin-Embedded Tissues

Formalin-fixed paraffin-embedded samples were deparaffinized with deparaffinization solution (Qiagen, Tokyo, Japan) and digested with proteinase K solution (Qiagen). Total RNA isolation was performed using a miRNA Isolation Kit (Qiagen). Total RNA was eluted in the appropriate buffer, and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA). Reverse transcription reactions were carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif., USA) in a total reaction volume of 15 μl. miR-503 and miR-16 were quantified in duplicate by qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction), using MicroRNA Assay Kits (Applied Biosystems). qRT-PCR was performed on an Applied Biosystems 7000 Sequence Detection System with the following cycling conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were calculated with SDS version 1.4 software (Applied Biosystems). Expression levels of tissue miR-503 were normalized using miR-16 using the $2^{-\Delta Ct}$ method. Differences between the groups are presented as ΔCt, indicating differences between Ct values of miRNAs of interest and normalizer miRNAs.

## Cell Lines

The human CRC cell lines Caco2, DLD1, HT29, LoVo and SW480 were obtained from the Cell Resource Center of Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). These cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% humidified $CO_2$ atmosphere. The authenticity of various cell lines was routinely monitored by analyzing DNA (short tandem repeat) profiling specific for each cell line in an approved laboratory (last tested on July 15, 2014).

## Transfection of Oligonucleotides

The anti-miR-503 (mirVana miRNA inhibitors) and mirVana miRNA Inhibitor Negative Control were purchased from Ambion (Austin, Tex., USA). The mirVana miRNA Inhibitor Negative Control has a unique sequence designed such that it does not target any human, mouse or rat genes, and it has been validated not to produce any measurable effects on known miRNA functions. Cancer cell lines were seeded at $2 \times 10^5$ cells/well in a final volume of 2 ml in 6-well flat-bottom microtiter plates. The cells were cultured overnight for adherence. The anti-miR-503 and negative control oligonucleotides were diluted with Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, Calif., USA). The diluted oligonucleotides were mixed with diluted Lipofectamine RNAiMAX Reagent (Invitrogen) and incubated for 5 min at room temperature to allow for the formation of anti-miR-503/negative control-Lipofectamine RNAiMAX Reagent complexes. These complexes were added to each well at a final concentration of 10 nM. The cells were incubated at 37°C in a 5% humidified $CO_2$ atmosphere.

## qRT-PCR and miRNA Target Prediction

The doRiNA website (http://dorina.mdc-berlin.de/) was used to predict the miRNA targeting of miR-503 by combination search, using Web-based computational programs such as TargetScan and PicTar-Vert. We narrowed down the candidate target genes of miR-503 based on the definition that they act as tumor suppressor genes in CRC. For RT-PCR, RNA was reverse transcribed to cDNA from 1 µg total RNA, using random hexamers and an Advantage RT-for-PCR Kit (Clontech Laboratories, Mountain View, Calif., USA). qRT-PCR using Power SYBR Green (Applied Biosystems) was performed to investigate the expression of selected target genes in the CRC cell lines after treatment with anti-miR-503 and negative control oligonucleotides. The results were normalized to the expression of β-actin. All the experiments were performed in duplicate. The primer for the target gene was designed as follows: forward, 5'-CGAGGAGAAAATCCTGTGGA-3', and reverse, 5'-CACACTCAAAGCAGCAGGTG-3'. β-Actin was amplified as an internal control, for which the primer was designed as follows: forward, 5'-ACAGAGCCTCGCCTTTGC-3', and reverse, 5'-GC-GGCGATATCATCATCC-3'. We performed amplifications under the following conditions: 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Target gene expression was determined using the $2^{-\Delta Ct}$ method.

## Proliferation Assay

To assess the effect of knockdown of miR-503 on cell proliferation, migration and invasion, anti-miR-503 and negative control oligonucleotides were used. To evaluate changes in cell viability, a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay was performed. Cancer cells were seeded at $5 \times 10^3$ cells/well in 96-well plates in a final volume of 100 µl culture medium/well. After overnight preincubation, transfection was performed (at 0 h). At 0, 24, 48 and 72 h, the spectrophotometric absorbance of the samples was measured. At each time point, 15 µl WST-8 reagent solution (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) was added to the culture medium, followed by incubation for 2 h at 37°C. Cell viability was determined by colorimetric comparison by reading $OD_{450}$ values from a microplate reader (SoftMax Pro; Molecular Devices, Sunnyvale, Calif., USA). Each independent experiment was performed 3 times in triplicate.

## Anoikis Assay

Anoikis assays were performed in 6-well Costar Ultra-Low-Attachment microplates (Corning, N.Y., USA). Cancer cells transfected with anti-miR-503 or negative control oligonucleotides were seeded onto the microplates at $5 \times 10^5$ cells/ml and incubated for 24 h. MTT assays were performed to confirm anoikis induction. Floating cells from the anoikis assay were seeded at $5 \times 10^3$/well in 96-well flat-bottom microtiter plates in a final volume of 100 µl culture medium. Spectrophotometric absorbance of the samples was measured as described for the proliferation assay. Each independent experiment was performed 3 times.

## Invasion Assay

Transfected cells ($5 \times 10^4$ cells/well) were seeded in serum-free medium into 24-well (8-µm pore size) BD BioCoat Matrigel Invasion Chambers and 24-well (8-µm pore size) BD BioCoat Control Inserts (BD Biosciences Discovery Labware, Billerica, Mass., USA). Inserts were placed into BD Falcon TC Companion Plates containing 20% FBS medium and incubated for 48–72 h. The incubation medium plus cells were removed from the top chamber using cotton swabs and PBS. The membranes were fixed in methanol, stained with Diff-Quik stain (Sysmex, Kobe, Japan) and mounted on glass slides. The number of cells invading the underside of the membrane in 5 microscopic fields was then counted with a light microscope at ×40 magnification. Each experiment was performed 3 times in triplicate.

## Wound Healing Assay

Cancer cells transfected with anti-miR-503 or negative control oligonucleotides were seeded onto 24-well plates and cultured until confluent. Wounds were generated using a sterile 200-µl pipette tip. The cells were then cultured for an additional 48 h. Wound closure was assessed using an Olympus IX71 microscope (Center Valley, Pa., USA) at ×40 magnification. Cell migration distance was measured using Adobe Photoshop 9.0.2 software and compared with baseline measurements. Each independent experiment was performed 3 times in triplicate.

## Statistical Analysis

All data were analyzed using MedCalc version 12.3.0 (Mariakerke, Belgium). Quantitative variables were analyzed using Student's t test, the Wilcoxon test (nonparametric paired analysis) and the Mann-Whitney U test (nonpaired analysis). Qualitative variables were analyzed using the $\chi^2$ or Fisher's exact test. Receiver operating characteristic (ROC) curves were established for determining cutoff values for analyzing predictions of recurrence and poor prognosis by Youden's index. Survival analyses were per-
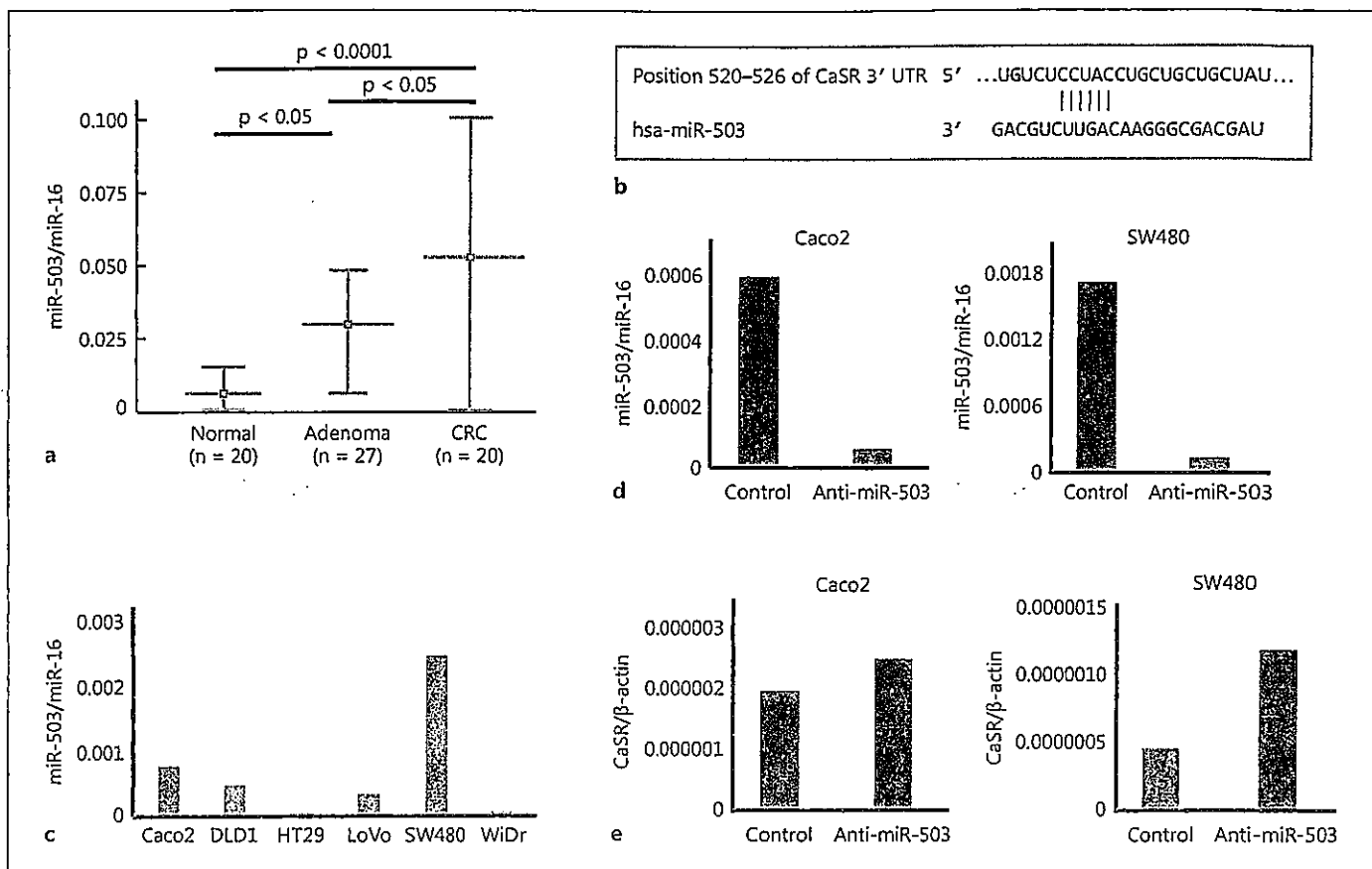
Fig. 1. Changes in miR-503 expression pattern throughout colonic carcinogenesis. miR-503 expression in CRC cell lines and the correlation between miR-503 and CaSR in CRC cell lines are displayed. a The y-axis represents miR-503 expression in tissues of normal colonic mucosa, adenoma and CRC. Data show means ± 95% CI. b The conserved predicted binding site of miR-503 with the 3′ UTR of CaSR mRNA is represented. c Columns illustrate the relative quantification of miR-503 expression in five CRC cell lines based on the ΔCt method. d Columns represent miR-503 expression levels in Caco2 and SW480 cells, which were suppressed by anti-miR-503 transfection. e Columns show CaSR expression levels in Caco2 and SW480 cells, which were overexpressed by anti-miR-503 transfection. The data for miR-503 and CaSR expression were normalized relative to miR-16 and β-actin expression, respectively. Statistically significant differences were determined using the Mann-Whitney test and Spearman's correlation test.

formed using the Kaplan-Meier method, and the differences in survival were examined using log-rank tests. Cox's proportional hazard regression analyses were used to estimate hazard ratios (HRs) of early recurrence and poor prognosis according to miR-503 expression levels. Two-sided p values <0.05 were considered to be statistically significant.

## Results

### miR-503 Expression Levels Are Attenuated in Normal Colonic Mucosa and Upregulated in Adenoma and CRC

We used TaqMan RT-PCR to assess the expression of miR-503 in 67 tissues of normal colonic mucosa (n = 20), adenoma (n = 27) and CRC (n = 20) and found substan-

tial upregulation in the adenoma and CRC tissues compared with the normal mucosa (p < 0.05, p < 0.0001; fig. 1a). Furthermore, miR-503 expression levels in CRC were significantly increased compared with those in adenoma (p < 0.05; fig. 1a). miR-503 is suggested to act as an onco-miRNA in CRC and is deregulated in the early phase of the adenoma-carcinoma sequence.

### Potential Gene Targets of miR-503

The function of miR-503 in tumor development and progression regarding CRC remains unresolved. Therefore, we searched predicted target genes associated with apoptosis and tumor suppression via Web-based programs such as TargetScan (http://www.targetscan.org) and PicTar-Vert (http://pictar.mdc-berlin.de) to predict the role

Noguchi et al.

miR-503 plays in tumor overgrowth. Calcium-sensing receptor (CaSR), a putative miR-503 target gene with a high target score (fig. 1b), participates in promoting the differentiation of colonic epithelial cells and functions as a tumor suppressor in colon cancer [20]. We determined whether CaSR could be restored by suppression of miR-503, using anti-miR-503 transfection of CRC cell lines.

We investigated the expression of miR-503 by real-time RT-PCR in human CRC cell lines. SW480 and Caco2 cells showed a higher expression of miR-503 compared with the other cell lines (fig. 1c). Thus, we selected SW480 and Caco2 cell lines on the basis of miR-503 expression levels for further experiments. After transfection of anti-miR-503 to both Caco2 and SW480 cells, CaSR expression was significantly upregulated compared with transfection with negative control oligonucleotides (fig. 1d, e). Thus, these data suggest that CaSR is a candidate target gene of miR-503 in CRC.

### Attenuated miR-503 Expression Inhibits Cell Proliferation, Migration and Invasion and Causes Acquisition of Anoikis

We performed functional studies to investigate whether miR-503 had oncogenic features in vitro following transfection of anti-miR-503 into CRC cells. An MTT assay showed that downregulation of miR-503 significantly reduced cell proliferation in both Caco2 and SW480 cells 24, 48 and 72 h after transfection of anti-miR-503 (fig. 2a, b). Anoikis resistance was evaluated by the proportion of viable tumor cells that proliferated nonadherently to low-attachment culture plates. Transfection with anti-miR-503 significantly reduced the proportions of anoikis-resistant tumor cells (floating viable tumor cells with nonadherent growth) in both cell lines (fig. 2c, d). In addition, a wound assay was performed to compare the migratory potential of CRC cells transfected with anti-miR-503 or negative control oligonucleotides. The number of migratory cells treated with anti-miR-503 was markedly decreased compared with cells treated with negative control oligonucleotides (fig. 2e, f). Finally, to determine whether attenuation of miR-503 levels affected cellular invasion, we performed invasion assays. Anti-miR-503 transfection of both Caco2 and SW480 cells showed a weakened invasive capacity compared with cells transfected with negative control oligonucleotides (fig. 2g, h).

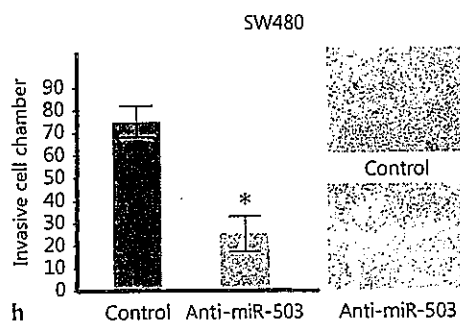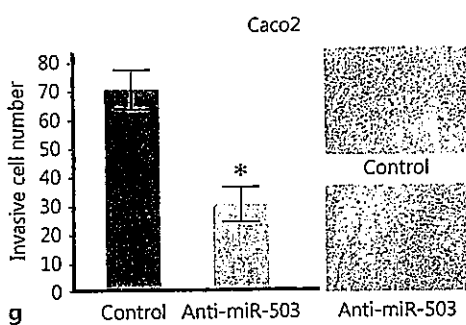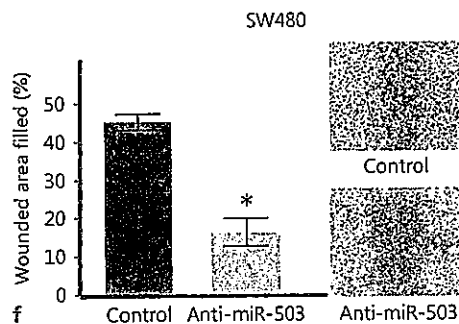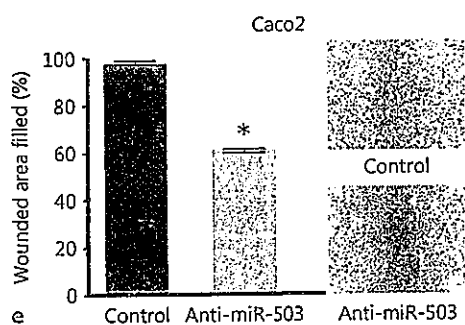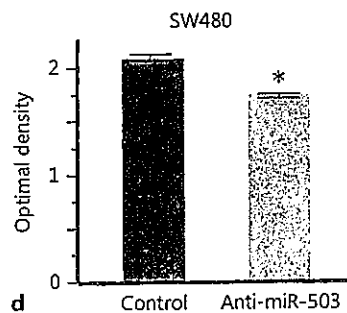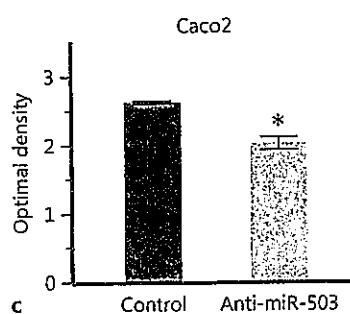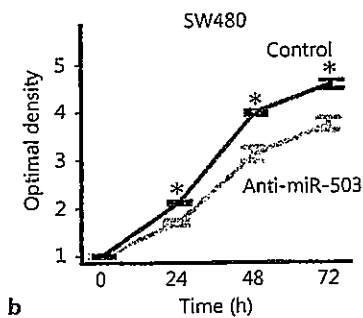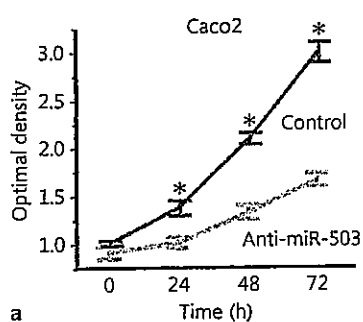### miR-503 Expression Is Significantly Associated with Tumor Growth in CRC

To assess the clinical significance of miR-503 expression, we quantified miR-503 expression in CRC using an independent, large cohort. In this cohort, expression of miR-503 increased in accordance with tumor TNM stage progression (p < 0.0001, Kruskal-Wallis test; fig. 3a). The relationships between miR-503 expression level in CRC and clinicopathological features are summarized in table 1. High miR-503 expression levels were significantly associated with large tumor size (p = 0.0006), serosal invasion (p < 0.0001), lymphatic invasion (p = 0.01), venous invasion (p = 0.01) and lymph node metastasis (p = 0.033). In contrast, no association was recognized between miR-503 expression and distant metastasis (p = 0.76). Collectively, these results revealed that miR-503 expression promotes local tumor growth, and this hypothesis is strengthened by the data showing a significant correlation between tumor size and miR-503 expression in CRC ($\rho$ = 0.24, p < 0.0001; fig. 3b).

### High miR-503 Expression Is Associated with Early Recurrence and Poor Prognosis of CRC Patients

To evaluate further whether miR-503 expression in CRC may serve as a predictor of patient outcome, we performed Kaplan-Meier survival analyses. We defined a cutoff value of >0.0049 as defining the high-expression group (n = 41) and ≤0.0049 as defining the low-expression group (n = 150), according to a maximum predictive value using OS based on ROC curve analysis. As anticipated, patients with higher levels of miR-503 expression had significantly worse OS (p = 0.0287; fig. 4a). Moreover, increased miR-503 expression was significantly associated with decreased disease-free survival (DFS; p = 0.0372; fig. 4b). The results of the Cox proportional hazard regression analyses for prognostic indicators are shown in table 2. Univariate analysis associated poor prognosis in CRC patients with high levels of miR-503 (p = 0.032), higher-grade pathological stage (T3/T4; p = 0.0009), lymph node metastasis (p < 0.0001) and distant metastasis (p < 0.0001). However, multivariate analysis revealed that high tumor miR-503 expression is not an independent prognostic marker for OS in CRC patients [HR: 1.362; 95% confidence interval (CI): 0.7143–2.597; p = 0.35].

In addition, to determine whether miR-503 could predict tumor recurrence in curative care patients (stages I–III), we used Cox's proportional hazard regression model (table 2). We defined a cutoff value of >0.0025 as defining the high-expression group (n = 56) and ≤0.0025 as defining the low-expression group (n = 92), according to a maximum predictive value using DFS based on ROC curve analysis. Univariate analysis showed that serosal invasion (p = 0.0138), lymph node metastasis (p = 0.0055)

and high miR-503 expression in CRC (p = 0.0428) were significantly associated with poor DFS. Multivariate analysis also showed that high miR-503 expression was not an independent predictor of tumor recurrence (HR: 1.7577; 95% CI: 0.7771–3.9755; p = 0.1778).

*High miR-503 Expression Is a Potential Independent Recurrence Marker in Curative Care CRC Patients without Lymph Node Metastasis (Stage I/II)*

High miR-503 expression in CRC may serve as a predictor of recurrence in stage I/II CRC patients who are not recommended to receive adjuvant chemotherapy postoperatively. A cutoff value for miR-503 level was determined as a maximum predictive value, using DFS based on ROC curve analysis (cutoff value: 0.0025). Kaplan-Meier sur-

vival analysis revealed that patients with stage I/II CRC with higher levels of miR-503 expression had significantly worse DFS (p = 0.0356; fig. 4c). We then used Cox's proportional hazard regression model to determine the risk factors for recurrence (table 3). Multivariate analysis indicated that miR-503 expression in CRC was an independent predictor of recurrence in patients with stage I/II CRC (HR: 4.4574; 95% CI: 1.56–13.01; p = 0.0395).

## Discussion

The current study is believed to be the first to have investigated the functional mechanism and the clinical significance of miR-503 in CRC. We demonstrated that miR-
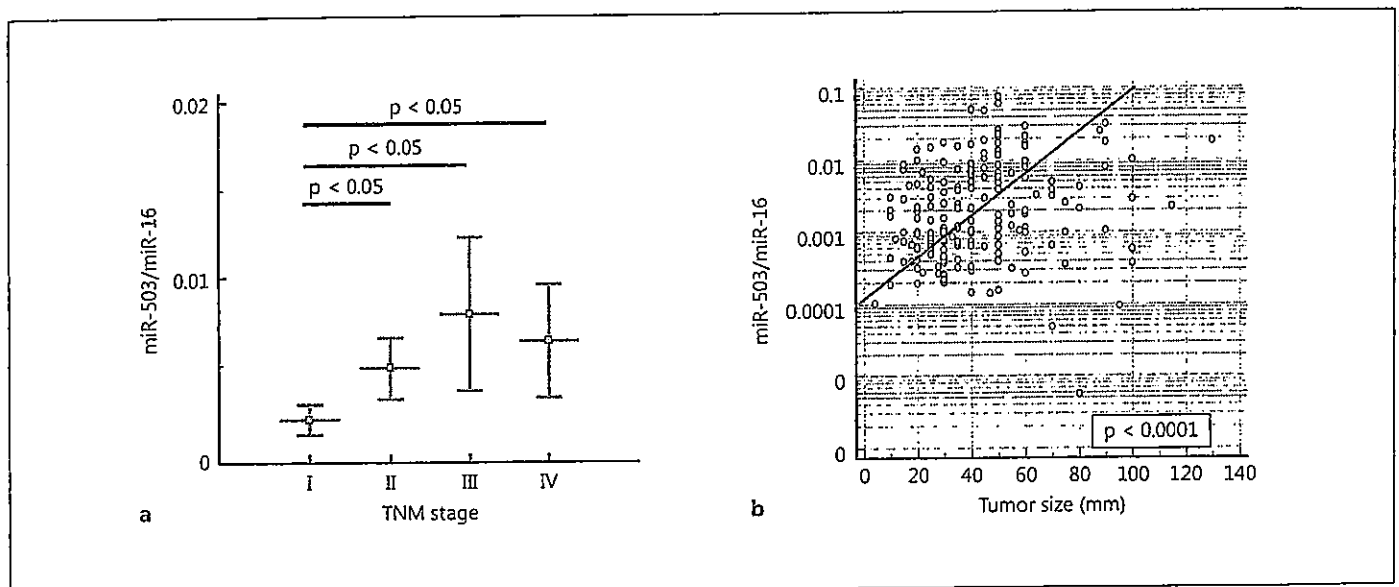


**Fig. 3.** miR-503 expression in CRC subdivided by TNM stage, and association between miR-503 expression and tumor size in CRC. **a** miR-503 was significantly overexpressed in stages II, III and IV compared with stage I. **b** There was a significant correlation between miR-503 expression in CRC and tumor size ($\rho$ = 0.385; 95% CI: 0.252–0.504; p < 0.0001).

**Fig. 2.** Knockdown of miR-503 expression results in suppression of CRC cell proliferation, invasion and migration and acquisition of anoikis function. Error bars represent SD. * p < 0.05; control: negative control oligonucleotide-treated cells. **a, b** Cell proliferation assays in Caco2 (**a**) and SW480 (**b**) cells 0–72 h after anti-miR-503 transfection. Growth of Caco2 and SW480 cells treated with anti-miR-503 (blue line) was significantly suppressed compared with that of cells treated with negative control (red line) at 24–72 h. Colors refer to the online version only. Anoikis assays in Caco2 (**a**) and SW480 (**b**) cells 24 h after anti-miR-503 transfection. **c, d** The proportion of viable floating Caco2 (**c**) and SW480

(**d**) cells in low-attachment plates treated with anti-miR-503 transfection was significantly decreased compared with the controls. **e, f** Wound healing assays for the evaluation of cancer cell migratory potential after miR-503 knockdown in Caco2 (**e**) and SW480 (**f**) cells. The y-axis represents migration rates relative to control cells. **g, h** The Transwell invasion system demonstrated significantly reduced invasive ability after miR-503 knockdown in Caco2 (**g**) and SW480 (**h**) cells. Images of invading cells were taken by phase contrast microscopy at ×100 magnification. The y-axis represents the number of invading cells.

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miR-503 in Tumorigenesis of CRC

**Table 1.** Association between miR-503 expression in CRC tissue and clinicopathological findings

| Category | Mean miR-503 expression | p value |
|---|---|---|
| Age | | |
| ≤67 years[a] | 0.0050858 | 0.57 |
| >67 years | 0.0054632 | |
| Gender | | |
| Male | 0.0049058 | 0.42 |
| Female | 0.0057779 | |
| Histology | | |
| Good and moderate | 0.0055495 | 0.50 |
| Poor and mucinous | 0.0026710 | |
| Tumor size | | |
| ≤40 mm[a] | 0.0030892 | 0.0006 |
| >40 mm | 0.0082458 | |
| Serosal invasion | | |
| Present | 0.0066725 | <0.0001 |
| Absent | 0.0019623 | |
| Lymph node metastasis | | |
| Present | 0.0075977 | 0.033 |
| Absent | 0.0035255 | |
| Lymphatic invasion | | |
| Present | 0.0061490 | 0.01 |
| Absent | 0.0022373 | |
| Venous invasion | | |
| Present | 0.0068571 | 0.01 |
| Absent | 0.0038315 | |
| Distant metastasis | | |
| Present | 0.0051879 | 0.76 |
| Absent | 0.0052789 | |

[a] Median age and tumor size.

503 promoted the malignant potential of CRC cells by decreasing the expression of CaSR, which functions as a tumor suppressor at the E2F-responsive promoter [21], resulting in cell overgrowth, invasion and migration as well as inhibition of anoikis. In order to support our in vitro findings, we used clinical samples to investigate the association between tissue expression of miR-503 and clinical parameters in CRC. Subsequently, we identified several intriguing associations between miR-503 and key clinical parameters. First, miR-503 expression was markedly increased in human colonic neoplasia, even in preneoplastic lesions (adenoma), compared with normal colonic mucosa. Second, overexpression of miR-503 in CRC tissues was significantly associated not only with tumor aggressiveness, including large tumor size as well as higher T stage, but also with local tumor invasion of the lymphatic and vessel ducts or regional lymph nodes. Finally, patients with high expression of miR-503 in CRC tissues

had significantly early recurrence and poor prognosis. Although high miR-503 expression in CRC was significantly associated with poor DFS and OS, multivariate analysis revealed that high tumor miR-503 expression is not an independent prognostic marker for OS and DFS. In fact, high miR-503 expression is mainly associated with tumor progression factors such as tumor size and pathological T stage but not with distant metastasis, all of which are highly important factors affecting recurrence and prognosis after surgery (fig. 3b; table 1). In addition, miR-503 expression in stage IV CRC is lower than that in stage III CRC (fig. 3a). In other words, miR-503 expression in CRC is significantly compromised by confounders such as tumor size and/or serosal invasion by multivariate analysis. Collectively, miR-503 expression in CRC does not work as an enhancer of cancer metastasis but in tumorigenesis.

Dysregulation of miRNA expression in human cancer appears to be tumor type specific. In addition, miRNAs act either as tumor suppressors or as oncogenes, depending on the downstream target genes or pathways that they regulate. Recent evidence has demonstrated reduced miR-503 expression levels induced by methylation associated with transcriptional silencing. Li et al. [22] revealed that miR-503 significantly decreases and acts as an anti-onco-miRNA in non-small-cell lung cancer (NSCLC) by regulating the resistance of NSCLC cells to cisplatin, in part by targeting Fanconi anemia, complementation group A protein. Yang et al. [23] also demonstrated miR-503 to be a tumor-suppressive miRNA in human NSCLC, the expression level of which correlates inversely with OS in NSCLC patients. In addition, downregulation of miR-503, which directly targets ARHGEF19, FGF2 and VEGFA and inhibits proliferation and angiogenesis, correlates with increased metastatic potential in vitro and in clinical specimens from hepatocellular carcinoma [13, 24]. Furthermore, miR-503 functions as a tumor suppressor by inhibition of several target genes associated with cell cycle transition in several cancers [14, 25].

Several studies have reported that miR-503 acts as an onco-miRNA in some cancer tissues. miR-503 is overexpressed in adrenocortical carcinoma compared with adjacent normal tissues. High expression of miR-503 is significantly associated with shorter OS [19], since miR-503 inhibits the G2/M DNA damage checkpoint by suppressing reprimo, which is a downstream protein of tumor suppressor p53 [26]. Recently, Li et al. [27] showed that miR-182 and miR-503 cooperatively targeted F-box/WD repeat-containing protein 7 (FBXW7), which normally functions as a cell cycle regulator in CRC cells. They also demonstrated that miR-503 contributed to the malignant
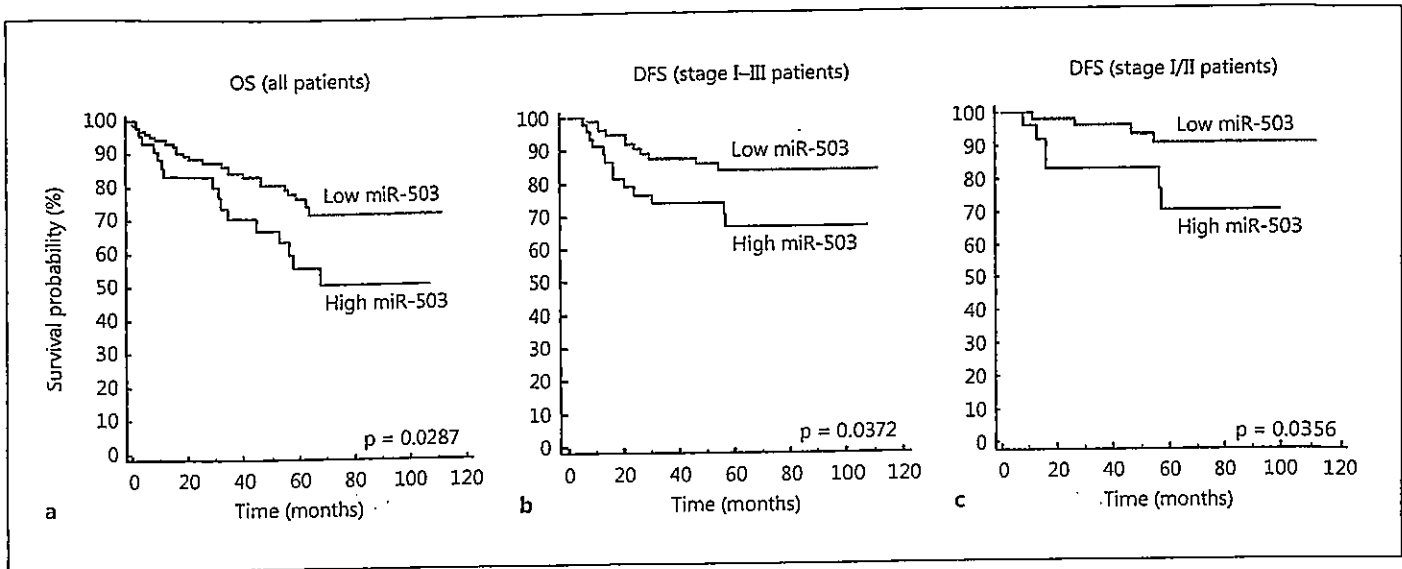
**Fig. 4.** Kaplan-Meier survival analysis in CRC patients. Cutoff values for miR-503 expression in CRC for OS and DFS were determined from the ROC curves with Youden's index. **a** The OS rate in CRC patients with high miR-503 expression levels (n = 51) in their tumor was significantly lower than in those with low expression (n = 140; p = 0.0287; log-rank test). **b** The DFS rate in CRC patients with high miR-503 expression levels (n = 56) was significantly lower than in those with low expression (n = 92; p = 0.0372; log-rank test). **c** The DFS rate in CRC patients with high miR-503 expression levels (n = 33) was significantly lower than in those with low expression (n = 66) in stage I/II CRC patients (p = 0.0356; log-rank test).

transformation of colonic adenoma to adenocarcinoma, and high expression of miR-503 in CRC tended to shorten OS [27]. The results of that study are consistent with those of the present study, in which miR-503 was gradually upregulated according to the adenoma-carcinoma sequence and high miR-503 expression was associated with malignant potential, in particular tumor growth (large and highly invasive tumor). Furthermore, we revealed the significant positive association between high miR-503 expression and early recurrence.

Surgical resection is highly effective for CRC patients without lymph node metastasis (stage I/II CRC), but some of these patients develop recurrence and succumb to the disease. However, whether postoperative adjuvant chemotherapy has any effect on patients with stage II colon cancer remains controversial. Therefore, identifying sensitive recurrence markers in this subgroup of patients would prompt the use of postoperative adjuvant therapy in patients with poor prognosis and thus improve survival. In the present study, we demonstrated for the first time that high miR-503 expression in CRC is an independent factor for recurrence in patients with stage I/II CRC. This ability to identify patients with stage I/II CRC with early relapse who would benefit from adjuvant therapy to prevent recurrence could improve cancer survival.

In addition, we demonstrated that CaSR is one of the miR-503 target genes. CaSR protein is a G protein-coupled receptor that regulates systemic calcium homeostasis. In the colon, CaSR plays important roles in nutrient sensing and fluid secretion/absorption [28]. CaSR expression is significantly reduced in colorectal tumors [29]. Indeed, the colons of mice lacking CaSR exhibit aberrant crypt foci, which are the earliest identifiable preneoplastic lesions [30], as well as enhanced intestinal inflammation [30], suggesting that CaSR is important for maintaining a normal colonic epithelium. Furthermore, activation of CaSR also inhibits invasion and anchorage-independent growth of human colon carcinoma cells [31]. Our in vitro proliferation demonstrated that CaSR expression in CRC cell lines was upregulated after reduction of miR-503 expression. Collectively, reduction of CaSR expression by miR-503 might be one of the mechanisms of colonic carcinogenesis and tumorigenesis. However, the effect of anti-miR-503 on the expression of CaSR in Caco2 cells does not appear to be significant (fig. 2a), even though the biological function in cell proliferation is obviously significant. These results might indicate that the other miR-503 target gene, FBXW7 [27], is also involved in the regulation of proliferation in CRC cells.

miR-503 in Tumorigenesis of CRC

**Table 2.** Univariate and multivariate analyses of OS and DFS (Cox's proportional hazard regression model)

| Factors | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | p value | HR | 95% CI | p value |
| **OS** | | | | | | |
| Age (>67/≤67 years)[a] | 1.5850 | 0.8602–2.9204 | 0.1417 | – | – | – |
| Gender (male/female) | 1.2263 | 0.6514–2.3083 | 0.5295 | – | – | – |
| Histology (poor and mucinous/good and moderate) | 1.6670 | 0.7039–3.9480 | 0.2478 | – | – | – |
| Tumor size (>40/≤40 mm)[a] | 1.7724 | 0.9620–3.2657 | 0.0678 | – | – | – |
| Serosal invasion (present/absent) | 11.0637 | 2.6874–45.5485 | 0.0009 | 5.9559 | 1.3971–25.3897 | 0.0164 |
| Lymph node metastasis (present/absent) | 4.2206 | 2.1576–8.2562 | <0.0001 | 1.8343 | 0.8956–3.7568 | 0.0989 |
| Distant metastasis (present/absent) | 9.7736 | 5.1905–18.4036 | <0.0001 | 6.7160 | 3.4793–12.9639 | <0.0001 |
| miR-503 expression (high/low)[b] | 1.9893 | 1.0642–3.7183 | 0.0320 | 1.3620 | 0.7143–2.5970 | 0.3506 |
| **DFS** | | | | | | |
| Age (>67/≤67 years)[a] | 1.2136 | 0.5474–2.6908 | 0.6355 | – | – | – |
| Gender (male/female) | 1.2074 | 0.5305–2.7481 | 0.6550 | – | – | – |
| Histology (poor and mucinous/good and moderate) | 0.8168 | 0.1935–3.4472 | 0.7840 | – | – | – |
| Tumor size (>40/≤40 mm)[a] | 1.2008 | 0.5275–2.7336 | 0.6644 | – | – | – |
| Serosal invasion (present/absent) | 4.5730 | 1.3716–15.2463 | 0.0138 | 3.0901 | 0.8715–10.9569 | 0.0822 |
| Lymph node metastasis (present/absent) | 3.1630 | 1.4099–7.0959 | 0.0055 | 2.0968 | 0.8930–4.9235 | 0.0908 |
| miR-503 expression (high/low)[b] | 2.2941 | 1.0313–5.1031 | 0.0428 | 1.7577 | 0.7771–3.9755 | 0.1778 |

[a] Median age and tumor size. [b] Cutoff: 0.0049.

**Table 3.** Multivariate analyses of DFS in stage I/II CRC patients (Cox's proportional hazard regression model)

| Factors | Univariate analysis | | |
|---|---|---|---|
| | HR | 95% CI | p value |
| Age (>67/≤67 years)[a] | 0.4505 | 0.0963–2.1064 | 0.3134 |
| Gender (male/female) | 1.2074 | 0.5305–2.7481 | 0.6550 |
| Histology (poor and mucinous/good and moderate) | 1.0531 | 0.1135–9.7706 | 0.9639 |
| Tumor size (>40/≤40 mm)[a] | 0.3531 | 0.0696–1.7908 | 0.2112 |
| Serosal invasion (present/absent) | 6.5335 | 1.3716–15.2463 | 0.0625 |
| Lymphatic duct invasion (present/absent) | 0.9466 | 0.1839–4.8738 | 0.9480 |
| Venous invasion (present/absent) | 1.0516 | 0.1934–5.7166 | 0.9538 |
| miR-503 expression (high/low) | 4.4574 | 1.0819–18.3638 | 0.0395 |

[a] Median age and tumor size.

In conclusion, we provided data for associations between miR-503 expression and progression, early recurrence and poor prognosis in CRC, suggesting that miR-530 acts as an onco-miRNA in this type of cancer. Our data showed that miR-503 appears to be an independent predictive marker for recurrence in stage I/II CRC, indicating that it can identify patients with high risk of recurrence who need to undergo adjuvant chemotherapy. Furthermore, our in vitro data demonstrate that miR-503 could be targeted therapeutically to attenuate cancer progression, which warrants further in vivo investigation. Moreover, in order to utilize the potential of miR-503 clinically, we must validate our findings in a larger cohort and/or established database containing relevant clinical parameters of CRC patients. In addition, we must compare the biomarker potential of miR-503 with that of other promising miRNA-based cancer biomarkers. The biomarker potential of miRNAs has to be rigorously evaluated to clarify whether these small noncoding RNAs can replace the conventional biomarkers in the future.

Noguchi et al.

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## Disclosure Statement

The authors have no conflicts of interest to disclose.

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