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Circulating microRNA-203 predicts prognosis and metastasis in human colorectal cancer

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Abstract

Background and Aims—Distant metastasis is a major cause of deaths in CRC patients, which is partly due to lack of robust metastasis-predictive biomarkers. In spite of the important function of miR-203 in cancer metastasis, its clinical significance in CRC metastasis remains unknown. Here, we evaluated the potential role of serum miR-203 as a non-invasive biomarker for CRC metastasis.

Methods—MiR-203 expression was quantified by qRT-PCR in 58 pairs of primary CRC (pCRC) and corresponding matched liver metastasis (LM), as well as 186 serum and 154 matched tissue specimens from CRC patients in *Cohort 1*. Next, we performed validation of miR-203 levels in serum from 144 CRC patients in an independent cohort (*Cohort 2*). A mouse model of CRC-associated metastases was established to identify the source of circulating miR-203. Expression patterns of miR-203 in tissues were determined by *in situ* hybridization.

Results—MiR-203 expression was significantly upregulated in LM compared to matched pCRC tissues. Serum miR-203 levels were significantly up-regulated in a stage-dependent manner, and high miR-203 expression was associated with poor survival in CRC patients in both patient cohorts. Increased miR-203 levels in serum indicated high risk for poor prognosis (HR=2.1), as well as metastasis to lymph nodes (OR=2.5), liver (OR=6.2), peritoneum (OR=7.2), and distant

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organs (OR=4.4). Serum miR-203 levels were significantly higher in animals with liver or systemic metastasis compared to controls.

Conclusions—High levels of serum miR-203 associate with poor survival and metastasis, suggesting it to be a promising non-invasive prognostic and metastasis-predictive biomarker in patients with metastatic CRC.

Keywords

Colorectal Cancer; Metastasis; Non-invasive Biomarker; miR-203

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers, and the second leading cause of cancer-related mortality worldwide.¹ Survival rates of patients with CRC have increased in the past few years, possibly as a result of earlier diagnosis and improved treatment regimens; nonetheless, approximately 30–50% of patients who undergo curative resection subsequently experience local and systemic recurrence.² Patients with tumor recurrence frequently receive cytotoxic chemotherapeutic regimens coupled with targeted monoclonal antibodies.³ However, the substantial financial costs associated with CRC treatment not only present an economic burden, but treatment of all patients with chemotherapy without *a priori* selection leads to overtreatment of patients with toxic agents that produce severe adverse effects.⁴ In addition, distant metastasis is the major causes of death in CRC patients, and the liver is the most common metastatic site for patients with CRC and approximately 15–25% of CRC patients present with liver metastasis at the time of diagnosis.⁵ Although resection of hepatic metastases improves 5-year overall survival rates ranging from 25% to 40%, less than one third of CRC patients with liver metastases are have a potentially resectable disease at the time of diagnosis.⁶ In order to overcome these clinical problems, there is a clear need for biomarkers that will facilitate the identification of patients with early recurrence or poor prognosis, and permit earlier diagnosis of the patients with systemic metastases.

Accumulating evidence indicates that circulating miRNAs reflect physiological and pathological alterations in cancer patients, and may serve as important surrogate minimally-invasive biomarkers. Indeed, several studies have identified differential expression of key miRNAs directly involved in the pathogenesis of CRC as circulating diagnostic biomarkers of CRC patients.^{7–14} Both miR-17-3p and miR-92 have been shown to be significantly elevated in pre-operative plasma from CRC patients compared to healthy controls, while their levels plummeted in post-surgery plasma specimens.¹¹ Moreover, plasma miR-29a and miR-92a expression successfully discriminated patients with advanced adenomas and CRCs from healthy controls.⁹ Recently, we and others have found that serum or plasma miR-21 expression is significantly upregulated in patients with adenomas and CRCs compared to healthy subjects, suggesting miR-21 to be a promising noninvasive biomarkers for the early detection of colorectal neoplasia.^{10,13} Although significant gains have been made in terms of developing non-invasive biomarkers for the detection of early-stage lesions, limited evidence exists for the potential significance of circulating miRNAs as biomarkers for prognosis and predicting metastasis in patients with advanced CRC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that post-transcriptionally regulate gene expression in multiple cancer-related signaling pathways, including metastasis.^{15–17} In cancer metastasis, the epithelial-mesenchymal transition (EMT) is a key process that converts polarized immotile epithelial cells into motile, invasive mesenchymal cells, enabling cancer cells to gain stem cell characteristics and an aggressive malignant phenotype.^{18,19} Several miRNAs have been reported to regulate transcription factors involved in the EMT process.^{20–22} Recently, we have demonstrated that miR-200c plays a pivotal role in regulating EMT and metastatic behavior in CRC.²³ Moreover, mir-203 has been shown to directly suppress EMT activators such as ZEB2 and SNAI1/2.^{24–27} However, in spite of the key functional role of miR-203 in cancer metastasis, no previous studies have investigated the clinical significance of miR-203 in CRC metastasis.

In current study, we focused on the clinical significance of serum miR-203 (official name: miR-203a-5p, Gene ID: 406986) and its origins in patients with CRC by analyzing matched pairs of primary CRC (pCRC) and corresponding liver metastasis (LM) tissues, followed by analysis of serum and matched tissue specimens from patients with CRC. In addition, we addressed the potential mechanism for the origin of circulating miR-203 levels in a series of *in vitro* and *in vivo* experiments. Our systematic approach demonstrated that serum levels of miR-203 are not only significantly associated with a metastatic phenotype in the colon, but also serve as a potential biomarker for predicting systemic metastases to lymph node, liver and peritoneal cavity and poor prognosis in CRC patients.

MATERIALS AND METHODS

Clinical specimens and cell lines

This study included examination of 762 serum and tissue specimens including 58 formalin-fixed, paraffin-embedded (FFPE) pCRC tissues and 58 matched corresponding LM tissues that were enrolled at Okayama University, Toho University, and Mie University Medical Hospital in Japan, as described in supplemental table 1. In addition, 24 healthy controls, 186 preoperative serum samples and 154 matched pCRC tissues (Cohort-1) and 144 preoperative serum samples from CRC patients (Cohort2) were analyzed from Mie University Medical Hospital (supplemental table 1). Furthermore, for evaluation of disease specificity, 162 preoperative serum samples from gastric cancer (GC, n = 113), esophageal cancer (EC, n = 19), ulcerative colitis (UC, n = 9) and another healthy controls (n = 21) were also analyzed. Careful microdissection was performed to collect and enrich tumor cells from the FFPE tissue specimens. Patients treated with radiotherapy or chemotherapy prior to surgery were not included in this study. Patients with stage III and IV disease received 5-fluorouracil-based chemotherapy, whereas no adjuvant chemotherapy was given to stage I and II CRC patients. CEA expression levels were also determined in the 186 serum samples by standard enzyme immunoassay. Written informed consent was obtained from all patients and the study was approved by the institutional review boards of all participating institutions.

Human CRC cell lines CaCO2, DLD1, HT29, LoVo, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) for *in vitro* and *in vivo* analysis. These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at

37 °C in a 5% humidified CO₂ atmosphere. The authenticity of various cell lines was routinely monitored by analyzing DNA profiles (short tandem repeats) specific for each cell line in an approved laboratory (last tested on July 15, 2014).

RNA isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

MiRNA extraction from serum and culture media samples was performed with miRNeasy RNA isolation Kits (Qiagen, Valencia, CA), whereas miRNA extraction from Formalin-fixed and paraffin- embedded (FFPE) samples was performed using RecoverAll Total Nucleic Acid Isolation Kits (Ambion Inc, Austin, TX). TaqMan miRNA qRT-PCR (Applied Biosystems, Foster City, CA) were used to detect and quantify miRNA expression using the 2⁻ Ct method (for details, refer to supplementary methods).

In situ hybridization

For in-situ hybridization (ISH) analysis, five micrometer thick FFPE tissue sections were hybridized with the miR-203 probe (LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon, Woburn, Massachusetts, USA), respectively, as described previously.²³ Positive controls (U6 snRNA, LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon) and negative controls (scrambled miRNA control, LNA-modified and 5'- and 3'-DIG-labeled oligonucleotide, Exiqon) were included in each hybridization reaction.²³

In vitro studies and In vivo mice model of liver and systemic metastasis

To determine the secretory potential of miR-203, CRC cell lines were cultured and a fraction of the culture medium was collected at 0, 24, and 48 hours after the initial seeding of cells in 10-cm dishes. Additionally, CRC cells ($2.0 \times 10^6/50 \mu\text{L}$ of PBS) were injected in the spleen of six-week-old male athymic nude mice (Balb/c nu; Harlan Laboratories, Inc.) during an open laparotomy to establish *in vivo* mice model of liver metastasis. After 10 weeks, mice were sacrificed. Liver and spleen tissues were resected, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm . With regard to systemic metastasis models, CRC cells ($1.0 \times 10^6/200 \mu\text{L}$ of PBS) were injected into the tail vein of six week old male athymic nude mice (Balb/c nu; Harlan Laboratories, Inc.). After 2 months, mice were sacrificed and the degree of systemic metastasis were checked and recorded. The control mice were the ones without mice without establishment of metastasis after injection of HT-29 in spleen and tail vein. Thereafter, the tissue sections were stained with hematoxylin and eosin (H&E). For analysis of serum miR-203 expression, whole blood was collected via cardiac puncture and serum was isolated using Vacutainer tubes with Serum Separator (BD Biosciences, San Jose, CA, USA).

All mice were group-housed in standard cages under a controlled temperature (25°C) and photoperiod (12 hour light/dark cycle). All experiments were approved by the Baylor Research Institute's Institutional Animal Care and Use Committee.

Statistical analysis

Paired *t*-test, Mann-Whitney test, Kruskal-Wallis test, and χ^2 tests were used to analyze miRNA expression data its relationship with various clinicopathological factors. Kaplan–Meier analysis and the log-rank test were used for survival analysis. Correlation between two continuous values was analyzed by Spearman’s correlation. The miRNA expression values were dichotomized into high- and low-expression groups based on receiver operating characteristic (ROC) curves with Youden’s Index correction.²⁸ We estimated that 154 patients were needed to achieve 80% power to substantiate more than 20% differences in prognostic outcomes (*n* = 184 in Cohort 1, and *n* = 144 in Cohort 2). Univariate and multivariate Cox’s proportional hazards models were used to identify independent prognostic factors dictating patient survival. Univariate and multivariate logistic regression models identified independent predictive factors for distant metastasis, liver metastasis, lymph node metastasis, and peritoneal metastasis. Data are presented as mean±S.D. (standard deviation), and all statistical analyses were conducted using the Medcalc version 12.3 (Broekstraat, Belgium) and the GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

MiR-203 expression is significantly up-regulated in LM compared to matched pCRC tissues

To determine the involvement of miR-203 in CRC metastasis, its expression was analyzed in 58 pairs of matched pCRC and corresponding LM tissue specimens (figure 1A). The qRT-PCR analysis revealed that miR-203 expression was significantly up-regulated in LM compared to pCRC tissues (mean=0.111 for pCRC vs. mean=0.153 for LM; *P*=0.0002, paired *t* test).

The pathological expression pattern of miR-203 was confirmed by *in situ* hybridization (ISH) staining in matched pairs of pCRC and LM tissues (figure 1B). ISH analysis showed that miR-203 expression was higher in the luminal regions compared to the invasive tumor front where metastasis originates in CRC. Of note, miR-203 was strongly expressed in the metastatic foci in liver, while normal hepatocytes barely expressed this miRNA.

Serum miR-203 is significantly up-regulated in CRC patients with liver metastasis

Since miR-203 was highly expressed in LM tissues, we hypothesized that this miRNA may be detectable in the circulation, which would provide a rationale for the development of a non-invasive biomarker for tumor metastasis. To determine whether miR-203 is a secretory miRNA, we examined if miR-203 is secreted by CRC cells cultured for various time periods. We first investigated the expression of miR-203 by real-time RT-PCR in human CRC cell lines (figure 1C). Of the 6 CRC cell lines, HT29 showed the highest expression level of miR-203. On the basis of these results, HT-29 CRC cell line was cultured and miR-203 expression was investigated in the culture medium by qRT-PCR assays (figure 1D). We observed that HT-29 cells secreted miR-203 into the culture medium and that its expression was directly proportional to cell number and cell culture duration. Moreover, treatment of

miR-203 inhibitor in HT-29 cells showed decreased level of miR-203 in the culture medium, suggesting culture medium miR-203 is directly secreted from CRC cells (figure 1E).

Next, the expression of miR-203 was analyzed in 186 serum samples as well as 154 matched tissue specimens from CRC patients in Cohort-1, 24 serum samples from healthy controls and 15 adjacent normal colonic mucosa. In comparison with healthy controls, the expression levels of serum miR-203 demonstrated significantly increase in patients with CRC ($P < .001$) (figure 1F). In addition, miR-203 expression was significantly up-regulated in a TNM stage-dependent manner in serum samples obtained from CRC patients (figure 1F; $P=0.0070$). On the other hand, although miR-203 expression in CRC was significantly higher than adjacent normal mucosa (figure 1G), tissue miR-203 expression did not change with CRC progression (figure 1G; $P=0.2748$). Collectively, these results suggest that miR-203 may be released by cancer cells, and high levels of circulating miR-203 could be a reflection of metastasized CRC cells in the systemic circulation, and indicate the specificity of serum miR-203 levels for identification of metastases in CRC patients. Furthermore, we quantified serum miR-203 expression levels in patients with other gastrointestinal cancers, including gastric and esophageal cancers, as well as ulcerative colitis patients, to investigate disease-specificity of serum miR-203. Interestingly, the expression levels of miR-203 were significantly lower in patients with other diseases vs. CRC (supplementary figure 1), highlighting that high serum miR-203 levels might be a specific feature of CRC patients.

High serum miR-203 expression is associated with poor prognosis and metastasis progression in CRC patients

To further demonstrate the clinical significance of miR-203 expression in CRC patients, we investigated the association between miR-203 expression and various clinicopathological variables in cohort-1 patients (table 1). In contrast to tissue expression, high serum miR-203 expression was significantly associated with TNM stage ($P=0.0071$), lymph node metastasis ($P=0.0139$), distant metastasis ($P=0.0008$), liver metastasis ($P=0.0004$), and peritoneal metastasis ($P=0.0030$). Moreover, Kaplan-Meier survival analysis revealed that high miR-203 serum expression was significantly associated with poor survival in CRC patients (figure 2A; log-rank $P<0.0001$), whereas tissue miR-203 expression did not associate with patient survival (figure 2B).

In subsequent analysis for determining associations between miR-203 expression and N classification for lymph node involvement (N0, absent lymph node metastasis; N1, metastasis in 1–3 regional lymph nodes; N2, metastasis in >4 regional lymph nodes; N3, lymph node metastasis to the aorta), H-classification for liver metastasis (H0, no liver metastasis; H1, liver metastasis with less than 5 nodules smaller than 5 cm; H2, metastasis that does not involve H1 and H3; H3, indicating liver metastasis with more than 5 metastases larger than 5 cm), and P-classification for peritoneal metastasis (P0, no peritoneal metastasis; P1, metastasis localized to the adjacent peritoneum; P2, limited metastasis to the distant peritoneum; P3, diffuse metastasis to the distant peritoneum), serum miR-203 expression levels were significantly associated with progression of lymph node (figure 2E; $P=0.0353$), liver (figure 2F; $P<0.0001$), and peritoneal metastases (figure 2G; $P=0.0090$), respectively. Our findings firstly demonstrate that serum miR-203 expression reflects tumor

progression and cancer cell dissemination to lymph nodes, distant organs, liver, and peritoneal cavity – all the features that directly influence survival rates of CRC patients. Next, to determine whether the effect of miR-203 on patient survival was affected by the presence of metastases, the prognostic potential of serum miR-203 was separately evaluated in curative (stage I-III) and non-curative (stage IV) CRC patients. Interestingly, high miR-203 serum expression was significantly associated with poor survival in CRC patients regardless of the presence of metastasis (figure 2C; log-rank $P=0.0038$, figure 2D; log-rank $P=0.0147$).

Serum miR-203 expression is an independent prognostic and metastasis-predictive biomarker in CRC

Since serum miR-203 expression was associated with specific clinicopathological factors that relate to cancer progression and metastases, we examined whether miR-203 also help to predict prognosis and metastasis in cohort-1 CRC patients. ROC analysis revealed that serum miR-203 yielded AUC values of 0.678, with a corresponding sensitivity of 47.50% and a specificity of 85.52% in discriminating patients with poor prognosis in Cohort 1 (supplementary figure 2A). Univariate Cox's proportional hazards analysis (table 2) revealed that high expression of miR-203 in serum (HR, 5.8; 95% CI, 3.1 to 10.8; $P<0.0001$), tumor size (HR, 2.3; 95% CI, 1.2 to 4.3; $P=0.0081$), lymph node metastasis (HR, 22.4; 95% CI, 7.0 to 72.1; $P<0.0001$), distant metastasis (HR, 37.0; 95% CI, 15.1 to 90.5; $P<0.0001$), and CEA expression (HR, 5.0; 95% CI, 2.2 to 11.3; $P=0.0001$) were associated with poor prognosis, while tissue miR-203 expression did not associate with any of these clinical factors. In multivariate analysis that included serum miR-203 expression, tumor size, lymph node metastasis, distant metastasis, and CEA expression, high serum miR-203 expression (HR, 2.1; 95% CI, 1.1 to 4.2; $P=0.0285$) was significantly associated with poor survival, and was independent of other clinical factors. Logistic regression analysis was used to examine the potential of miR-203 to predict lymph node, liver, peritoneal, and distant metastases, respectively (table 3). Univariate analyses revealed that serum miR-203 expression associated with lymph node metastasis (OR, 2.9; 95% CI, 1.4 to 6.1; $P=0.0035$), liver metastasis (OR, 7.3; 95% CI, 3.0 to 17.6; $P<0.0001$), peritoneal metastasis (OR, 7.4; 95% CI, 2.1 to 26.9; $P=0.0022$), and distant metastasis (OR, 5.3; 95% CI, 2.4 to 11.5; $P<0.0001$), while tissue miR-203 expression failed to associate with any of the metastasis-related factors. Our ROC analyses also revealed that serum miR-203 levels significantly discriminated CRC patients with lymph node metastasis, liver metastasis, peritoneal metastasis or distant metastasis, respectively (supplementary figure 2B–E). Each AUC value with associated sensitivity and specificity is as follows: Lymph node metastasis (AUC = 0.607, sensitivity = 35.06 %, Specificity = 85.05 %: supplementary figure 2B), liver metastasis (AUC = 0.719, sensitivity = 61.54 %, Specificity = 84.18 %: supplementary figure 2C), peritoneal metastasis (AUC = 0.767, sensitivity = 72.73 %, Specificity = 76.30 %: supplementary figure 2D) and distant metastasis (AUC = 0.674, sensitivity = 52.50 %, Specificity = 85.42 %: supplementary figure 2E). In multivariate analyses, serum miR-203 expression was an independent predictor of lymph node metastasis (OR, 2.5; 95% CI, 1.2 to 5.4; $P=0.0172$), liver metastasis (OR, 6.2; 95% CI, 2.1 to 18.3; $P=0.0009$), peritoneal metastasis (OR, 7.2; 95% CI, 2.0 to 26.5; $P=0.003$), and distant metastasis (OR, 4.4; 95% CI, 1.6 to 11.8; $P=0.0037$). Collectively, these results suggest that serum miR-203

expression status may be an important biomarker for predicting prognosis and metastases in CRC patients.

Validation of prognostic power and metastasis discriminating power of miR-203 in the validation cohort

To confirm the results from Cohort-1 which showed serum miR-203 in CRC as a promising prognostic and metastatic predictive biomarker, we further quantified serum miR-203 levels in another validation cohort (Cohort-2), which included 144 patients with CRC. Serum miR-203 levels increased significantly according to tumor stage (supplementary figure 3A; $P=0.00029$). In addition, we confirmed that serum miR-203 levels were significantly higher in CRC patients with multiple metastases including lymph node metastasis (supplementary figure 3B; $P=0.0054$), liver metastasis (supplementary figure 3C; $P=0.029$) or peritoneal dissemination (supplementary figure 3D; $P=0.029$), respectively. Kaplan-Meier survival curves showed that high miR-203 serum expression was significantly associated with poor overall survival in all patients with CRC (supplementary figure 3E; log-rank $P<0.0001$). Moreover, sub-analyses of overall survival demonstrated that the patients with high miR-203 expression had a significantly worse survival than those with low expression in both curative (supplementary figure 3F; log-rank $P=0.0001$) and non-curative patients (supplementary figure 3G; log-rank $P<0.0001$), respectively. To validate the prognostic power of serum miR-203, we conducted ROC analysis which showed that serum miR-203 yielded an AUC value of 0.748, with 62.5% sensitivity and 77.68% specificity in discriminating patients with poor prognosis in the validation cohort (supplementary figure 4A). On the other hand, to confirm the potential diagnostic applicability of serum miR-203 for predicting metastasis development, we also generated ROC curves. Each AUC value with sensitivity and specificity was as follows: Lymph node metastasis (AUC = 0.636, sensitivity = 49.15 %, specificity = 78.82 %: supplementary figure 4B), liver metastasis (AUC = 0.690, sensitivity = 91.67 %, specificity = 46.97 %: supplementary figure 4C), peritoneal metastasis: AUC = 0.690, sensitivity = 91.67 %, specificity = 46.97 %: supplementary figure 4D) and distant metastasis (AUC = 0.678, sensitivity = 95.83 %, specificity = 34.17 %: supplementary figure 4E).

Animal model for CRC-associated LM and systemic metastasis demonstrated increased serum miR-203 expression

In order to demonstrate that high levels of miR-203 expression observed in sera and CRC cells metastasized to the liver indeed originated from the primary tumor, we established an *in vivo* liver metastasis animal model. We injected HT29 cells in the spleens of animals, which provided unrestricted movement of these colon cancer cells through circulation and establishment in liver tissues as metastatic foci. As illustrated in figure 3, we analyzed the expression levels of miR-203 in serum samples as well as liver tissues from these animals. The animals with successful development of metastasis in the liver (figures 3A and 3B) revealed significantly higher expression of miR-203 in metastatic foci, compared to adjacent regions with normal hepatocytes and spleen (figure 3C; LM vs. H, $P=0.0006$; LM vs. S, $P<0.0001$). More interestingly, miR-203 expression was significantly elevated in serum specimens from animals with CRC-associated liver metastases compared to controls (figure 3D; $P=0.04$).

Next, to further confirm the pathological expression of miR-203, we performed ISH staining in tissue samples from the mouse liver tissues (figure 3B). Analogous to human CRC-related liver metastasis tissues, animals with liver metastases also displayed high miR-203 expression compared to adjacent normal hepatocytes.

In addition, to further demonstrate that high serum miR-203 levels were contributed by the metastatic sites, we injected HT29 cells in the tail vein of mice, which provided free movement of these colon cancer cells through circulation and establishment in distant organs as metastatic foci. As shown in figure 3E and 3F, systemic metastatic foci mainly occurred in subcutaneous tissues. We quantified the expression levels of miR-203 in serum samples from non-treated controls, and animals with established metastatic foci, and analyzed the correlation between serum miR-203 expression levels and tumor tissues. Consistent with the results of liver metastasis models, miR-203 expression was significantly elevated in serum specimens from animals with CRC-associated systemic metastases compared to controls (figure 3G; $P = 0.0064$). Furthermore, significant positive correlation was recognized between serum miR-203 levels and total volume of metastatic nodules (figure 3H; $\text{Rho} = 0.829$; $P = 0.0009$).

Taken together these results indicate that CRC cells, when metastasize to the liver and/or other organs express high levels of miR-203, which may be released into the circulation and serves as a substrate for the development of a non-invasive biomarker for determining prognosis and metastasis in CRC patients.

DISCUSSION

This study demonstrates the potential role of serum miR-203 as a non-invasive biomarker for CRC metastasis. In this report, we first highlight the clinical significance of serum miR-203 expression for determining patient prognosis and predicting CRC metastasis. Herein, we demonstrate that circulating miR-203 in serum could originate from metastasized cancer cells by directly analyzing miR-203 expression in primary CRC tissues and matched serum. In addition, we provide further credence to our findings by supporting these results *in vitro* and in an animal model of hepatic and/or systemic metastasis.

Accumulating data in recent years have convincingly demonstrated that the expression of various miRNAs is frequently dysregulated in CRC tissues compared to normal colonic mucosa. More importantly, recent studies have shown the promise that some of these can also be detected as circulating miRNAs in the systemic circulation, and their expression pattern in circulation can be directly related with physiological and pathological alterations in CRC patients.^{7–14} However, with regards to CRC metastasis, only a few circulating miRNAs have been reported to potentially play a role in advanced disease. Plasma miR-141 expression is elevated in metastatic stage IV compared to non-metastatic stage I-II CRC patients.⁸ More recently, Wang et al. identified elevated serum miR-29a expression in LM patients compared to CRC patients without distant metastasis.¹⁴ However, these previous studies did not address the origin of the circulating miRNAs, nor their clinical utility as possible biomarkers for determining prognosis and predicting distant metastasis.

MiR-203, which is known to be a putative tumor suppressor gene and a target of promoter hyper-methylation, has been shown to inhibit cell proliferation and invasion, migration and tumor angiogenesis in a variety of tumor cells^{29–31}. Although miR-203 expression has been reported in CRC tissues previously, none of the prior studies examined the expression pattern of this miRNA in detail, or explored its feasibility as a minimally-invasive biomarker in serum.^{32–38} This is the first study that explores the clinical relevance of miR-203 expression in CRC tissues and matched serum specimens from CRC patients. In the current study, we observed significantly increased expression of miR-203 in CRC tissues compared to normal colonic mucosa and a gradual decrease in miR-203 expression in CRC tissues in a stage-dependent manner. We observed a significant loss of methylation for miR-203 promoter in CRC, but we did not observe any correlation between expression and methylation levels of this miRNA (data not shown). In contrast, significant increase of miR-203 expression was observed in serum from CRC patients compared to healthy volunteers, and a simultaneous elevation in miR-203 expression levels in the serum of stage IV CRC patients. In addition, miR-203 expression was significantly upregulated in liver metastases compared to matched pCRCs, which was consistent with markedly high intensity of miR-203 in the liver metastasis in the *in situ* hybridization analysis. Furthermore, high levels of miR-203 in serum were significantly associated with metastases to lymph node, liver, peritoneum and other organs. Interestingly, the increase in serum miR-203 levels occurred in accordance with the N-category in the TNM classification (indicating involvement of lymph nodes) and the H- and P-system published from the Japanese Society for Cancer of the Colon and Rectum, in which metastases are classified according to the size of maximum metastasis diameter and the number of metastases in liver and peritoneum, respectively. In addition, high levels of serum miR-203 can predict curative CRC patients with poor prognosis, which indicates that serum miR-203 might be secreted from micro metastatic lesions that were not detected at the time of curative surgery. Furthermore, no correlation was observed between miR-203 expression in serum and matched primary CRC tissues, and serum miR-203 levels did not change after primary resection in CRC (data not shown). Collectively, our data from clinical specimens implies that systemic metastatic lesions excrete miR-203 in circulation of CRC patients.

Another unique strength of our study is that we were able to further substantiate our results from human clinical specimens with an *in vivo* metastasis animal model. We recognize that although our liver and/or systemic metastasis mouse model may not reproduce the complexity of metastatic CRC in humans, our animal model results corroborate the clinical data. Furthermore, ISH analysis of the CRC metastases in animal livers displayed a specifically high expression of miR-203 in the metastasized foci in the mouse livers, but not in regions of normal hepatocytes. It is known that miR-203 induces the reversal of the EMT process, i.e., mesenchymal-epithelial transition (MET), by directly targeting key EMT regulators such as ZEB2 and SNAIL1/2 in breast cancer cells and prostate cells, respectively.^{24–27} Recently, we found that miR-200c is overexpressed in liver metastasis samples compared to matched pCRC, and it is a key regulator of the EMT-MET process.²³ Furthermore, we observed high serum miR-200c expression correlated with distant and lymph-node metastasis, independently predicted tumor recurrence (especially with stage II CRC), and was an independent prognostic marker for CRC.³⁹ In the same context, high

miR-203 expression in localized metastasized CRC cells may promote metastatic colonization at secondary sites (ex. lymph node, liver, peritoneum and other distant organs) by inducing MET, and the origin of serum miR-203 from patients with metastasis might be from the metastatic site, from which the cancer cells are secreted abundantly into the systemic circulation in CRC patients. Future studies may address whether serum miR-203 levels may also be used as a disease monitoring marker for tumor recurrence after curative surgery.

In conclusion, current study demonstrates several novel pieces of evidence associated with serum levels of miR-203 in patients with CRC. First, miR-203 in serum was significantly associated with a metastatic phenotype in CRC and was an independent predictive marker for lymph node, liver and peritoneal metastasis in CRC, respectively. In addition, significant diagnostic potential in discrimination of metastatic CRC patients was evaluated by ROC analysis using independent two cohorts. Second, miR-203 in serum was an independent prognostic marker, whereas the prognostic value of CEA levels was statistically significantly compromised by other clinical factors by the multivariable Cox proportional hazards model. Finally, our data suggest that the source of miR-203 in serum might be contributed by foci of tumor metastasis within lymph nodes, liver, peritoneal space or other distant organs. Therefore, we propose that evaluation of serum miR-203 is a promising clinical tool for identification of CRC patients with metastasis who need early administration of intensive chemotherapy and/or resection of metastases to gain a survival much longer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CRC	colorectal cancer
miR-203	microRNA-203
qRT-PCR	quantitative real-time polymerase chain reaction
ISH	in situ hybridization

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SIGNIFICANCE OF THIS STUDY

What is already known about this subject?

- MiR-203 is involved in epithelial-to-mesenchymal transition in cancer cells.
- MiRNA biomarkers are emerging as important substrates for developing tissue-based and non-invasive biomarkers for cancer diagnosis and prognosis.

What are the new findings?

- In contrast to tissue expression, high serum miR-203 expression predicted prognosis as well as metastasis to lymph nodes, liver, peritoneum, and distant organs.
- MiR-203 expression is significantly upregulated in liver metastasis compared to primary CRC tissues.
- Culture media from CRC cell lines, and animal model of liver and systemic metastasis, demonstrated that miR-203 is a secretory miRNA, and hence may be exploited as a non-invasive biomarker for predicting metastasis in CRC patients.

How might it impact on clinical practice in the foreseeable future?

- Since high levels of serum miR-203 associate with poor survival and metastasis, these results suggest that this miRNA may serve as a promising non-invasive prognostic and metastasis-predictive biomarker in patients with metastatic CRC

Figure 1. Expression status of miR-203 in human clinical specimens and CRC cell culture medium

(A) Expression status of miR-203 in 58 pairs of primary CRC (pCRC) and corresponding matching liver metastasis (LM). The gray horizontal bars represent mean expression levels; *** $P < 0.001$, t test. (B) *In situ* hybridization analysis of miR-203 expression in pCRC and corresponding LMs. (Positive control, U6 snRNA; negative control, scrambled miRNA control) (C) Expression of miR-203 in CRC cell lines (CaCO2, HCT116, HT29, LoVo, SW480 and SW620). (D) The amount of miR-203 excreted in the HT-29 culture medium increased depending on cell number and duration of culture (E), whereas these levels decreased following treatment with miR-203 inhibitor in HT-29 cells (F). Serum miR-203 levels in healthy control subjects (N; $n = 24$) and different Tumor Node Metastasis (TNM) stages (I, II, III, and IV) of CRC ($n = 186$) in Cohort 1. *** $P < 0.001$, * $P < 0.05$ (G). Tissue miR-203 levels in different Tumor Node Metastasis (TNM) stages (I,II,III, and IV) of CRCs

(n=154) and adjacent normal mucosa (N; n = 20) in Cohort 1. NS: not significant, *** $P<0.001$.

Figure 2. Kaplan-Meier survival analysis for miR-203 expression in serum and matched tissue specimens from CRC patients, and miR-203 expression status in different classifications of tumor metastasis in Cohort 1

Overall survival analyses based on miR-203 expression of 186 serum specimens and 154 matched tissue specimens from CRC patients. (A) Overall survival of CRC patients (all stage) based on serum miR-203 expression. (B) Overall survival of all CRC patients (all stage) based on tissue miR-203 expression. (C) Overall survival of curative CRC patients (stage I-III) based on serum miR-203 expression. (D) Overall survival of non-curative CRC patients (stage IV) based on serum miR-203 expression. Serum miR-203 expression levels based on the Japanese Society for Cancer of the Colon and Rectum (JSCCR) classification system; (E) lymph node N-classification (N0, absent lymph node metastasis; N1, metastasis in 1–3 regional lymph nodes; N2, metastasis in 4 or more regional lymph nodes; N3, lymph node metastasis to the aorta), (F) liver metastasis H-classification (H0, no liver metastasis; H1, liver metastasis with less than 5 nodules smaller than 5 cm; H2, metastasis that does not

involve H1 and H3; H3, liver metastasis with more than 5 metastasis larger than 5 cm), and (G) Peritoneal metastasis P-classification (P0, no peritoneal metastasis; P1, metastasis localized to the adjacent peritoneum; P2, limited metastasis to the distant peritoneum; P3, diffuse metastasis to the distant peritoneum).

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Figure 3. MiR-203 expression in animal models of liver and systemic metastasis

Athymic nude mice for liver metastatic models were sacrificed 10 weeks after injection of HT-29 CRC cells ($2.0 \times 10^6/50 \mu\text{L}$ of PBS) in the spleen. (A) Representative images of non-treated control mouse. (B) Representative images of animals with established liver metastasis. Yellow arrows indicate metastatic foci. The upper-right panel illustrates representative results of H&E staining in mouse liver. The lower-left panel displays representative results for ISH staining for miR-203 in animal liver. (C) miR-203 expression status in spleen (S), adjacent hepatocytes (H), and liver metastasis (LM) tissues of liver metastasis established mouse group ($n=10$; *** $P<0.001$). (D) Serum miR-203 expression status between non-treated control animals (non-LM; $n=7$) and animals with established liver metastasis (LM; $n=10$; * $P<0.05$). Athymic nude mice for systemic metastatic models were sacrificed 8 weeks after injection of HT-29 CRC cells ($1.0 \times 10^6/200 \mu\text{L}$ of PBS) in the tail vein. Representative images of non-treated controls (E) and established systemic metastatic mouse (F). Red arrows indicate metastatic foci. (G) Serum miR-203 expression status between non-treated control animals (non-SM; $n=6$) and animals with established

systemic metastasis (SM; n=6; ** $P<0.01$). (H) Significant correlation between serum miR-203 expression and tumor volume in mice ($\text{Rho} = 0.829$, $P=0.0009$).

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Clinical Significance of Tissue and Serum miR-203 Expression in a Matched Tissues and Serum in *Cohort 1* CRC patients*

Table 1

	Tissue miR-203			Serum miR-203		
	<i>n</i>	<i>Mean±SD</i>	<i>P value</i>	<i>n</i>	<i>Mean±SD</i>	<i>P value</i>
Sex			0.8921			0.0894
Male	90	0.1139±0.09158		107	0.2220±0.4954	
Female	64	0.1061±0.07291		79	0.1817±0.1485	
Age (Years)			0.4294			0.2524
<Median (68)	74	0.1041±0.07688		96	0.1783±0.1848	
Median (68)	80	0.1167±0.09043		90	0.2327±0.5214	
T stage			0.3825			0.3693
T1/T2	45	0.1329±0.1134		59	0.1622±0.1657	
T3/T4	109	0.1008±0.06625		127	0.2257±0.4552	
N stage			0.3705			0.0139
N0	87	0.1211±0.09715		109	0.1456±0.1318	
N1/N2/N3	67	0.09818±0.06245		77	0.2896±0.5737	
M stage			0.6409			0.0008
M0	123	0.1110±0.08772		146	0.1657±0.2380	
M1	31	0.1112±0.06966		40	0.3505±0.6908	
TNM stage			0.2767			0.0071
I	37	0.1394±0.1146		45	0.1662±0.1796	
II	48	0.1106±0.08166		60	0.1282±0.08035	
III	39	0.08530±0.05411		41	0.2189±0.3919	
IV	30	0.1112±0.06966		40	0.3505±0.6908	
Liver Metastasis			0.3049			0.0004
Negative	132	0.1100±0.08678		160	0.1657±0.2292	
Positive	22	0.1177±0.06778		26	0.4500±0.8422	

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Tissue miR-203			Serum miR-203		
	<i>n</i>	<i>Mean</i> ± <i>SD</i>	<i>n</i>	<i>Mean</i> ± <i>SD</i>	<i>P value</i>
Peritoneal Metastasis					
Negative	145	0.1114±0.08488	173	0.1722±0.2253	0.003
Positive	9	0.1051±0.07718	13	0.7353±1.2561	

* by the Kruskal-Wallis Test

Table 2
Association Between miR-203 Expression and Prognosis in *Cohort 1* CRC patients

Characteristics	Association between miR-203 expression and CRC prognosis (Cox proportional hazards model)					
	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
Age (>Median vs. Median)	0.8271	0.4628 to 1.4783	0.5239			
Sex (Male vs. Female)	1.0627	0.5872 to 1.9233	0.8414			
Tumor size (>Median vs. Median)	2.3135	1.2472 to 4.2915	0.0081	1.1462	0.5574 to 2.3570	0.7121
Lymph node metastasis (Present vs. Absent)	22.4298	6.9746 to 72.1321	<0.0001	5.8503	1.6373 to 20.9038	0.0068
Distant metastasis (Present vs. Absent)	36.9655	15.0979 to 90.5059	<0.0001	15.9021	5.0977 to 49.6056	<0.0001
CEA (>5 vs. ≤5)	5.0092	2.2249 to 11.2777	0.0001	0.739	0.2594 to 2.1051	0.5732
Tissue miR-203 (High vs. Low)	1.5487	0.7964 to 3.0118	0.1996			
Serum miR-203 (High vs. Low)	5.7558	3.0629 to 10.8162	<0.0001	2.1404	1.0871 to 4.2142	0.0285

HR, hazard ratio; CI, confidence interval

Table 3
Association between miR-203 Expression and metastasis prediction in *Cohort 1* CRC patients

Association between miR-203 expression and CRC lymph node metastasis (Logistic regression model)						
Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Age (>Median vs. Median)	0.9462	0.5412 to 1.6545	0.8463			
Sex (Male vs. Female)	0.813	0.4615 to 1.4323	0.4736			
Tumor size (>Median vs. Median)	2.2631	1.2759 to 4.0139	0.0052	1.8544	0.9742 to 3.5299	0.06
Pathology (poor diff. vs. diff.)	2.1429	0.7945 to 5.7798	0.1322			
Venous Invasion (Positive vs. Negative)	3.9899	2.2057 to 7.2172	<0.0001	2.7534	1.4453 to 5.2453	0.0021
Tissue miR-203 (High vs. Low)	1.112	0.5823 to 2.1238	0.7477			
Serum miR-203 (High vs. Low)	2.9487	1.4284 to 6.0874	0.0035	2.5196	1.1778 to 5.3897	0.0172

Association between miR-203 expression and CRC liver metastasis (Logistic regression model)						
Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Age (>Median vs. Median)	0.604	0.2693 to 1.3547	0.2212			
Sex (Male vs. Female)	1.4153	0.6212 to 3.2248	0.4084			
Tumor size (>Median vs. Median)	3.4125	1.3783 to 8.4492	0.0047	2.3226	0.6995 to 7.7123	0.1687
Lymph node metastasis (Present vs. Absent)	51.8	6.8766 to 390.1987	0.0001	31.7013	3.9884 to 251.9716	0.0011
Pathology (poor diff. vs. diff.)	0.7176	0.156 to 3.3	0.6699			
Venous Invasion (Positive vs. Negative)	5	2.0236 to 12.3541	0.0005	2.5075	0.7849 to 8.0111	0.1208
Tissue miR-203 (High vs. Low)	2.0513	0.8076 to 5.2102	0.1309			
Serum miR-203 (High vs. Low)	7.2545	2.9862 to 17.624	<0.0001	6.245	2.1259 to 18.3454	0.0009

Association between miR-203 expression and CRC peritoneal metastasis (Logistic regression model)						
Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Age (>Median vs. Median)	0.6454	0.2036 to 2.0455	0.4568			

Association between miR-203 expression and CRC peritoneal metastasis (Logistic regression model)						
Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Sex (Male vs. Female)	0.5869	0.1898 to 1.8146	0.3548			
Tumor size (>Median vs. Median)	1.1418	0.3697 to 3.5266	0.8177			
Lymph node metastasis (Present vs. Absent)	NA	NA	0.9934			
Pathology (poor diff. vs. diff.)	5.4921	1.5003 to 20.1044	0.0101	3.9609	0.8568 to 18.3102	0.078
Venous Invasion (Positive vs. Negative)	3.0759	0.9145 to 10.3465	0.0694			
Tissue miR-203 (High vs. Low)	0.8262	0.1902 to 3.5893	0.7989			
Serum miR-203 (High vs. Low)	7.4242	2.0525 to 26.8553	0.0022	7.2047	1.9565 to 26.5312	0.003

Association between miR-203 expression and CRC distant metastasis (Logistic regression model)						
Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	OR	95% CI	P value
Age (>Median vs. Median)	0.6414	0.3267 to 1.2589	0.1968			
Sex (Male vs. Female)	1.0833	0.5508 to 2.1306	0.8166			
Tumor size (>Median vs. Median)	3.3237	1.5934 to 6.9329	0.0014	2.2714	0.8220 to 6.2764	0.1136
Lymph node metastasis (Present vs. Absent)	33.1739	9.7845 to 112.4748	<0.0001	21.8673	6.0454 to 79.0980	<0.0001
Pathology (poor diff. vs. diff.)	2.4115	0.8758 to 6.6397	0.0885			
Venous Invasion (Positive vs. Negative)	5.7811	2.7147 to 12.3112	<0.0001	3.0147	1.1289 to 8.0511	0.0277
Tissue miR-203 (High vs. Low)	1.5102	0.6775 to 3.3663	0.3134			
Serum miR-203 (High vs. Low)	5.2993	2.4435 to 11.4927	<0.0001	4.3626	1.6128 to 11.8003	0.0037

OR, odds ratio; CI, confidence interval