Molecular Cancer Therapeutics

Decreased miR-340 Expression in Bone Marrow Is Associated with Liver Metastasis of Colorectal Cancer

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Abstract

Studies have shown the prognostic significance of disseminated tumor cells (DTC) in bone marrow of patients with colorectal cancer. However, the molecular characteristics of DTCs, including their miRNA expression profiles, remain mostly unknown. In this study, we analyzed the miRNA expression of DTCs in bone marrow. $EpCAM^+$ bone marrow cells were collected using immunomagnetic beads after exclusion of $CD14^+$ and $CD45^+$ cells, then subjected to miRNA microarray analysis. Cluster analysis (7 colorectal cancer patients with liver metastasis and 12 colorectal cancer patients without liver metastasis) indicated that miR-340 and miR-542-3p expressions were significantly decreased in $EpCAM^+$ bone marrow cells of patients with liver metastasis ($P = 0.019$ and 0.037, respectively). We demonstrated that pre-miR-340 administration inhibited growth of colon cancer cells and suppressed c-Met expression *in vitro*. In clinical samples of colorectal cancer, miR-340 was expressed at significantly lower levels in tumor tissues compared with normal mucosa. Survival analysis in 136 patients with colorectal cancer indicated that low miR-340 expression was correlated with shorter 5-year disease-free survival ($P = 0.023$) and poor 5-year overall survival ($P = 0.046$). It was of note that the colorectal cancer group with low miR-340 and high *c-Met* expression had the worst prognosis. We further demonstrated that systemic pre-miR-340 administration suppressed growth of pre-established HCT116 tumors in animal therapeutic models. These findings indicate that miR-340 may be useful as a novel prognostic factor and as a therapeutic tool against colorectal cancer. Our data suggest that miR-340 in bone marrow may play an important role in regulating the metastasis cascade of colorectal cancer. *Mol Cancer Ther; 13(4); 976–85.* ©*2014 AACR.*

Introduction

Disseminated tumor cells (DTC) are thought to represent the beginning of systemic disease from localized human cancer. In patients with various types of tumors, DTCs are detectable in regional lymph nodes, peripheral blood, and bone marrow at early stages of tumor progression (1, 2). Cumulative evidence indicates that metastatic relapse is significantly correlated with the presence of DTCs in bone marrow in several tumor types, including carcinomas of the colorectum (3, 4), breast (5, 6), lung (7, 8), and prostate (9, 10). These findings suggest that DTCs in bone marrow can be sensitive marker for cancer spread from the primary tumor, although they are usually present at a microscopic level. Two methods are commonly employed to detect DTCs in bone marrow: immunologic assays using antibodies directed against specific epithelial cell proteins, such as cytokeratins, EpCAM, and Ber-EP4 (11); and PCR-based molecular assays targeting *CK19*, *CK20*, *EpCAM*, and *CEA* transcripts (12).

Many studies have reported that DTCs in bone marrow are associated with prognosis; however, little is known about how DTCs acquire metastatic properties. Previous reports indicate that all patients with DTCs do not necessarily develop distant metastasis (3, 5, 10). Therefore, it is likely that the DTCs of different individual case have varying characteristics that influence their ability to develop metastasis. It was recently reported that the *VEGFR-1* or *miR-144-ZFX* axis plays a role in the bone marrow-associated metastasis cascade of gastric cancer (13, 14).

miRNAs, small noncoding RNA gene products ranging in size from 19 to 25 nucleotides, have been identified as

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factors involved in cancer development and metastasis through negative regulation of oncogenic or antioncogenic genes (15). miRNAs can play roles in regulating mRNA translation and degradation through base pairing to partially complementary sites, predominantly in the untranslated region of the mRNA (15). Through this negative regulation of gene expression, miRNAs can influence various biologic processes, including cell proliferation, cell death, and stress resistance (16).

To test the hypothesis that DTCs in bone marrow might have a specific miRNA expression profile when they acquire the metastatic potential, here, we collected bone marrow samples from patients with colorectal cancer with liver metastasis and nonmetastatic patients, and analyzed the miRNA profile differences between the two groups. Our data provide novel information and help further our understanding of the bone marrow-mediated metastasis cascade of colorectal cancer.

Material and Methods

Clinical samples

Bone marrow samples were collected from 19 patients with colorectal cancer (7 with liver metastasis and 12 without metastasis) during surgery at Osaka University Hospital between 2010 and 2012. Supplementary Table S1 shows the characteristics of these 19 patients. Among the cases with liver metastasis, 5 cases were synchronous (including 3 patients with multiple liver metastases and 2 with a single liver metastasis) and 2 were metachronous (1 with multiple liver metastases and 1 patient with a single liver metastasis).

In addition, 136 primary colorectal cancer samples were collected from patients who had curative surgery between 1999 and 2010 at Osaka University Hospital and its two related hospitals. The tumor samples were stored at −80°C with RNAlater until RNA extraction. These 136 patients included 4 stage 0, 15 stage I, 38 stage II, 52 stage IIIA/B, and 27 stage IIIC colorectal cancer diseases. The mean follow-up period for these patients was 4.2 years. All patients gave written informed consent, in accordance with the guidelines approved by the Institutional Research Board of each institute. This study was conducted under the supervision of the ethical board of Osaka University Hospital.

Bone marrow aspiration and MACS sorting

Patients with colorectal cancer were administered anesthesia and 20 mL bone marrow was taken from the right and left anterior iliac crests before surgery. Mononucleated cells were collected using a standard Ficoll-Hypaque gradient technique. To enrich for $EpCAM^+$ cells, $CD14^+$ cells were removed (designated as BM2) from the whole bone marrow (BM1, Fig. 1A) using auto MACS pro (Miltenyi Biotec) with anti-CD14 immunomagnetic beads (clone; TÜK4, Miltenyi Biotec). Next, $CD45^+$ cells were removed by treatment with anti-CD45 immunomagnetic beads (clone; 5B1; Miltenyi Biotec), and the $CD14^-CD45^+$

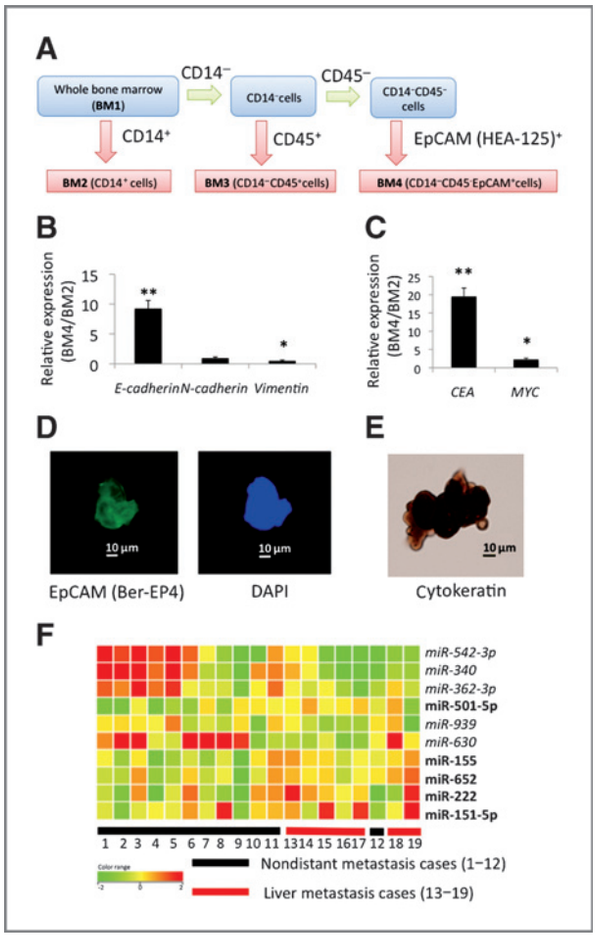

Figure 1. Characteristics of $CD14^-CD45^-EpCAM^+$ cells. A, schematic of the immunomagnetic beads-based separation of bone marrow cells using auto MACS pro. To obtain the $EpCAM^+$ fraction, $CD14^+$ cells and $CD45^+$ cells were excluded from the whole bone marrow (BM1), then $EpCAM^+$ cells were collected using the anti-EpCAM (HEA-125) antibody; this fraction was named BM4 ($CD14^-CD45^-EpCAM^+$). B, transcript expressions in BM4 (epithelial fraction) relative to BM2 (monocyte or macrophage lineage). Compared with BM2, BM4 showed 9.25-fold higher levels in the epithelial marker *E-cadherin* mRNA, a 0.48-fold change in the mesenchymal marker *Vimentin* mRNA, and no change in *N-cadherin* level. *, $P < 0.005$, Mann–Whitney *U* test. C, relative expression of *CEA* and *MYC* transcripts. Compared with BM2, BM4 showed significantly higher levels of *CEA* and *MYC* mRNA (19.5-fold and 2.26-fold, respectively). **, $P < 0.0001$; *, $P < 0.005$; Mann–Whitney *U* test. D and E, immunostaining of BM4 cells using the epithelial cell–specific antibodies Ber-EP4 antibody (D) and cytokeratin antibody (E). Tumor cell clusters comprised of individual cells of >10 μm in diameter were clearly visualized. Magnification, ×40. F, heatmap of the miRNA profile in BM4 cells from colorectal cancer (CRC) patients with liver metastasis and those without metastasis. Using miRNA microarray analysis, we determined the miRNA levels in BM4 cells obtained from 12 nonmetastasis patients and 7 liver metastasis patients. Cluster analysis showed 10 miRNAs that were significantly differentially expressed between the two groups with a >1.50-fold change (range, 1.50- to 3.39-fold). The heatmap summarizes the expressions of these 10 miRNAs from BM4 in bone marrow cells of 19 patients. Colors range from green to red, corresponding to low to high expression, respectively. Patients no. 1–12 had no distant metastasis and patients no. 13–19 had liver metastasis. miRNAs in italic were decreased and miRNAs in bold were increased in patients with liver metastasis. *P* values < 0.05, unpaired *t* test.

cells were designated as BM3. The residual $CD14^-CD45^-$ cells were then incubated with FcR blocking reagent (Miltenyi Biotec), followed by incubation with anti-EpCAM immunomagnetic beads (clone; HEA-125, Miltenyi Biotec), and the $CD14^-CD45^-EpCAM^+$ cells were taken up and designated BM4 (Fig. 1A).

Immunocytochemistry

Cells were attached onto glass slides and quickly air-dried. After permeabilization with 70% ethanol for 30 minutes, immunocytochemistry with anti-cytokeratin-large spectrum antibody (clone; KL1, Beckman Coulter) at a 1:1,000 dilution was performed using a standard ABC method as previously described (17, 18). Fluorescent immunochemistry was performed using fluorescence isothiocynate-conjugated anti-human epithelial antigen antibodies (clone; Ber-EP4, DAKO) at a 1:100 dilution and observed with a BZ-9000 fluorescence microscope (Keyence).

RNA isolation and miRNA microarray analysis

Total RNA was isolated using the miRNeasy kit (Applied Biosystems) following the manufacturer's protocol. Total RNA concentration and purity were assessed with a spectrophotometer and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (100 ng) was directly labeled with cyanine 3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise and accurate measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. Each 100 ng total RNA sample was competitively hybridized to a miRNA array (Agilent Microarray Kit; G4470C) containing 866 miRNAs (version 12.0 of the Sanger miRNA database; http://www. mirbase.org/; ref. 19). The intensity of each hybridization signal was evaluated using Feature Extraction Software Version 10.7.3.1 (Agilent Technologies), which used the global normalization method (90 percentile shift) using GeneSpring GX Software Version 11.5.1 (20). *P* values were calculated using an unpaired *t* test. All data from the miRNA array have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession code GSE51716.

cDNA microarray

Total RNA (500 ng) was converted into labeled cRNA with Cy3-coupled nucleotides (PerkinElmer) using the Quick Amp Labeling Kit, one-color (Agilent Technologies). Cy3-labeled cRNA (1.65 mg) was hybridized to an oligonucleotide microarray (Whole Human Genome 4 × 44 K Agilent G4112F) for 17 hours at 65°C. The intensity of each hybridization signal was evaluated using Feature Extraction Software Version 10.7.3.1 (Agilent Technologies). *P* values were calculated using an unpaired *t* test.

Reverse transcription PCR and TaqMan miRNA assay

The TaqMan miRNA Assay (Applied Biosystems) was used to measure miRNA levels. First, 5 ng RNA was reverse transcribed, and the resulting cDNA was amplified using the following specific TaqMan miRNA assays: hsa-miR-340 ID 002258, hsa-miR-542-3p ID 001284, and RNU6B ID 001093. The quantitative reverse transcription (qRT)-PCR reactions were performed using the 7900HT Sequence Detection System (Applied Biosystems), following the manufacturer's protocol. Amplification data were normalized to RNU6B expression. Relative expression was quantified using the $2^{-\Delta\Delta C_t}$ method.

Reverse transcription mRNA PCR and quantitative real-time PCR

The LightCycler TaqMan Master (Roche Diagnostic) was used to measure mRNA levels. First, 500 ng total RNA was reverse transcribed using High Capacity RNA-to-DNA Kit (Applied Biosystems), and the resulting cDNA was amplified using the following specific primers: *c-Met*: forward primer, 5′-AAATGTGCATGAAGCAGGAA-3′, reverse primer, 5′-TCTCTGAATTAGAGCGATGTTGA-3′, *MITF*: forward primer, 5′-CAGCGTGTATTTTTCCCACA-3′, reverse primer, 5′-TGCGGTCATTTATGTTAAATCTTC, *Survivin*: forward primer, 5′-GCCCAGTGTTTCTTCTG-CTT-3′, reverse primer, 5′-AACCGGACGAATGCTTT-TTA-3′, *ACTB*: 5′-AGAGCTACGAGCTGCCTGAC-3′, reverse primer, 5′-CGTGGATGCCACAGGACT-3′.

The qRT-PCR reactions were performed using the LightCycler 2.0 System (Roche Diagnostics), following the manufacturer's protocol. Amplification data were normalized to *β-actin* (*ACTB*) expression.

Cell lines and culture

Human colorectal cancer cell lines HCT116 and SW480 were obtained from the American Type Culture Collection in 2001. Stocks were prepared after passage 2 and stored in liquid nitrogen. All experiments were performed with cells of passage of <8. These cell lines were authenticated by morphologic inspection, short tandem repeat profiling, and Mycoplasma testing by ATCC. Mycoplasma testing was done also by the authors. They were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS and 1% antibiotic/antimycotic solution (Sigma) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Transient miRNA transfection

Cells were transfected with 30 nmol/L pre-miR miRNA precursor molecules of hsa-miR-340 and has-miR-542-3p (Applied Biosystems) using Lipofectamine iMax (Invitrogen) in 6- or 12-well plates following the manufacturer's protocol. Pre-miR negative control (Applied Biosystems) was used as a control.

Proliferation assays

Cells were seeded at a density of 3–4 × 10^4 cells/well in 12-well dishes, and cultured for 72 hours to determine proliferation. Cell counting was performed using a NucleoCounter kit (Chemometec).

In vivo tumor growth

HCT116 cells were mixed with Matrigel (BD Biosciences) and medium in a 1:1 ratio by volume. Cells (1×10^6) in 100 μL of medium/Matrigel solution were injected subcutaneously into the bilateral lower back region of female nude mice (NIHON CLEA). Formulated miRNA (40% conjugation; ref. 21; 30 μg per injection) was administered intravenously with carbonate apatite as vehicle (22, 23) via tail vein injections when tumor volume reached approximately 75 to 80 mm^3. Mice were treated four times with formulated miR-340 ($n = 7$) or miR-NC ($n = 9$), every 3 days. Mature hsa-miR-340 sense (5′-UUAUAAAGCAAUGAGACUGAUU-3′) and antisense (5′- AAUCAGUCUCAUUGCUUUAUAA-3′), and negative control sense (5′- UAAAUGUACUGCGCGUGGAGAGGAA-3′) and antisense (5′- UUCCUCUCCACGCGCAGUACAUUUA-3′) were purchased from Gene Design. Tumor volumes were determined as previously described (22). All animal experiments were performed in accordance with the currently prescribed guidelines and using a protocol approved by Osaka University.

Statistical analysis

Statistical analysis was performed using the JMP9 program (SAS Institute). Clinicopathologic factors were compared using the χ^2 test and continuous variables were compared using the Student *t* test. Survival curves were computed using the Kaplan–Meier method and Cox hazard model, and statistically significant differences between survival curves were determined using the log-rank test and Cox hazard ratio. In *in vivo* and *in vitro* analysis, data are expressed as mean ± SE and were analyzed with the Mann–Whitney *U* test. *P* values < 0.05 were considered statistically significant.

Results

Characteristics of $CD14^-CD45^-EpCAM^+$ cells

To obtain the $EpCAM^+$ fraction, $CD14^+$ cells and $CD45^+$ cells were excluded from the whole bone marrow (BM1), then $EpCAM^+$ cells were collected using the anti-EpCAM (HEA-125) antibody and the $CD14^-CD45^-EpCAM^+$ fraction was named BM4 (Fig. 1A). We first compared gene expression between BM4 cells and the $CD14^+$ BM2 cells, which were expected to be of monocyte or macrophage lineage and to thus not include epithelial cells. Compared with BM2, BM4 showed 9.25-fold higher levels of the epithelial marker *E-cadherin* mRNA, a 0.48-fold change in the mesenchymal marker *Vimentin* mRNA, and no change in *N-cadherin* level (Fig. 1B). Furthermore, BM4 exhibited significantly increased levels of the putative tumor marker *CEA* mRNA and the oncogene *MYC* mRNA (19.5-fold and 2.26-fold, respectively; Fig. 1C). Immunostaining of BM4 cells using the epithelial cell–specific antibodies Ber-EP4 antibody (Fig. 1D) and cytokeratin antibody (Fig. 1E) revealed the presence of tumor cell clusters, comprising individual cells of >10 μm in diameter.

Distinct miRNA profiles in BM4 cells between liver metastasis and nonmetastasis

Using the miRNA microarray, we analyzed the miRNA levels of BM4 cells obtained from nonmetastasis patients ($n = 12$) and liver metastasis patients ($n = 7$). Cluster analysis revealed 10 miRNAs that showed a >1.50-fold change (range, 1.50- to 3.39-fold) with significant between-group differences ($P < 0.05$; Fig. 1F). Five miRNAs were decreased and the other five were increased in BM4 cells from the patients with colorectal cancer with liver metastasis compared with in those without metastasis. Table 1 presents information about each miRNA, including the target molecule and whether it is an anti-oncomir or oncomir according to the available literature (24–37). All data from the miRNA array are shown in Supplementary microarray results.

Validation of miRNA expression by qRT-PCR

We performed qRT-PCR assays to verify the significant differences for the two miRNAs that were most downregulated in patients with liver metastasis: miR-340 and miR-542-3p. Our results confirmed that compared with in nonmetastasis patients, liver metastasis patients showed significantly lower BM4 cell expressions of miR-340 and miR-542-3p ($P = 0.008$ and 0.015, respectively; Supplementary Fig. S1).

miR-340 and miR-542-3p inhibited colorectal cancer cell proliferation *in vitro*

In HCT116 and SW480, pre-miR-340 and pre-miR-542-3p treatment significantly inhibited cell growth compared with that in parental cells ($P < 0.001$) or in negative control miR-treated cultures ($P < 0.01$; Fig. 2A and B). These *in vitro* proliferation assays were repeated three times and similar results were obtained. When we examined the levels of miRNAs in HCT116 and SW480 cells, both miR-340 and miR-542-3p levels were significantly higher at 24 hours as compared with those treated with pre-miR-negative control, and those in normal colonic mucosa ($P < 0.05$; Fig. 2A and B).

Expression of miR-340 and miR-542-3p in normal colonic mucosa and colorectal cancer tissue samples

Normal mucosa showed significantly higher miR-340 expression compared with in colorectal cancer tissue ($P = 0.010$; Fig. 2C). No significant difference was found in miR-542-3p expression ($P = 0.973$; Fig. 2C).

Association between miR-340 and miR-542-3 expressions and colorectal cancer patient prognosis

We analyzed miR-340 expression in tissue samples from 136 colorectal cancer patients who underwent curative surgery. Patients were divided into two groups: lower than average miR-340 expression ($n = 73$) and higher than average miR-340 expression ($n = 63$). Kaplan–Meier estimation indicated that high miR-340 expression was significantly associated with better 5-year disease-free survival (DFS) and better 5-year overall

Table 1. Top 10 miRNAs expressed in bone marrow between patients with liver metastasis and non-metastasis

Decreased miRNAs (liver metastasis patients)						
Rank	**miRNAs**	**Fold change**	***P***	**Anti-oncomir or Oncomir**	**Target**	**Ref.**
1	miR-340	3.39	0.019	Anti-oncomir	c-Met, MITF	24, 25
2	miR-542-3p	2.91	0.037	Anti-oncomir	Survivin, ILK	26, 27
4	miR-630	2.37	0.035	Anti-oncomir	N.R.	28, 29
6	miR-362-3p	1.78	0.025	Anti-oncomir	E2F1, USF2, PTPN1	30
9	miR-939	1.57	0.049	N.R.	N.R.	
Increased miRNAs (liver metastasis patients)						
Rank	**miRNAs**	**Fold change**	***P***	**Anti-oncomir or Oncomir**	**Target**	**Ref.**
3	miR-222	2.43	0.036	Oncomir	p27, p53 PTEN, TIMP3, TRPS1	31–33
5	miR-151-5p	1.90	0.028	Oncomir	RhoGDIA	34
7	miR-155	1.77	0.040	Oncomir	CDC73, TP53BP1, SOCS1	35–37
8	miR-501-5p	1.60	0.028	N.R.	N.R.	
10	miR-652	1.50	0.049	N.R.	N.R.	

Abbreviations: CDC73, cell division cycle 73; ILK, integrin-linked linase; MITF, microphthalmia-associated transcription factor; N.R., not reported; PTPN1, protein tyrosine phosphatase non receptor type1; PTEN, phosphatase and tensin homolog; RhoGDIA, Rho GDP dissociation inhibitor (GDI) alpha; SOCS1, suppressor of cytokine signaling 1; TIMP3, tissue inhibitors of metalloproteinases; TRPS1, trichorhinophalangeal syndrome type 1; TP53BP1, tumor protein p53 binding protein 1; USF2, upstream transcription factor.

survival (OS; $P = 0.023$ and 0.046, respectively; Fig. 3A). Similar survival analysis showed that miR-542-3p expression had no effect on either 5-year DFS or 5-year OS ($P = 0.429$ and 0.577, respectively; data not shown). Clinicopathologic survey indicated that miR-340 expression correlated with smaller tumor size ($P < 0.001$) but not with any other factors (Supplementary Table S2).

Table 2 shows the results of univariate and multivariate analyses of factors related to 5-year DFS. Univariate analysis indicated that lymph node metastasis ($P = 0.003$), serosal invasion ($P = 0.001$), and low miR-340 expression ($P = 0.020$) were significantly related to DFS. Multivariate analysis indicated that low miR-340 expression was an independent predictor of DFS [relative risk (RR), 2.499; 95% confidence interval (CI), 1.031–6.962; $P = 0.042$], as were serosal invasion (RR, 3.053; 95% CI, 1.311–7.041; $P = 0.011$) and lymph node metastasis (RR, 2.918; 95% CI, 1.081–10.16; $P = 0.033$; Table 2).

Systemic delivery of formulated miR-340 inhibited the growth of established colorectal cancer tumors *in vivo*

In the therapeutic animal models of HCT116 tumors, tumor growth was significantly inhibited by systemic administration of formulated miR-340 compared with in the pre-miR–negative control group ($P < 0.05$; Fig. 3B and C). This experiment was repeated twice and reproducible results were obtained (data not shown). To evaluate the miRNAs delivery to the subcutaneous tumors, we measured the miR-340 level of the subcutaneous tumors at 1, 8, and 12 hours after administration via tail vein. As shown in Fig. 3B, pre-miR-340–treated mice had significantly higher miR-340 levels at 1, 8, and 12 hours as compared with the control mice treated with pre-miR–negative control ($P < 0.01$ for each).

Association between miR-340 and c-Met expressions and colorectal cancer patient prognosis

We transiently transfected HCT116 and SW480 cells with pre-miR-340 and miR-542-3p, and then analyzed target gene expression, *c-Met* and *MITF* for miR-340, and Survivin for miR-542-3p. We observed that *c-Met* levels in pre-miR–340-treated HCT116 and SW480 cells were suppressed to 24.6% and 35.9% of that in negative control miR–treated cells, respectively (Fig. 4A). Similar results were obtained in terms of *MITF* and *Survivin* when HCT116 and SW480 cells were treated with pre-miR-340 and pre-miR-542-3p, respectively (Supplementary Fig. S2A and S2B).

We also confirmed *c-Met* expression level of the subcutaneous tumors at 8-, 12-, and 24-hour *in vivo* models. The expression level of *c-Met* significantly decreased after systemic administration of pre-miR-340 compared with the pre-miR–negative control group at each time point ($P < 0.01$; Fig. 4B).

Furthermore, we evaluated the association between miR-340 and *c-Met* expression levels in 135 clinical samples, which was the part of the initial 136 clinical samples. There was not significant association between miR-340 and c-Met expression levels in the whole (data not shown). However, subgroup analysis revealed that miR-340-low/c-Met-high cases had a significantly worse

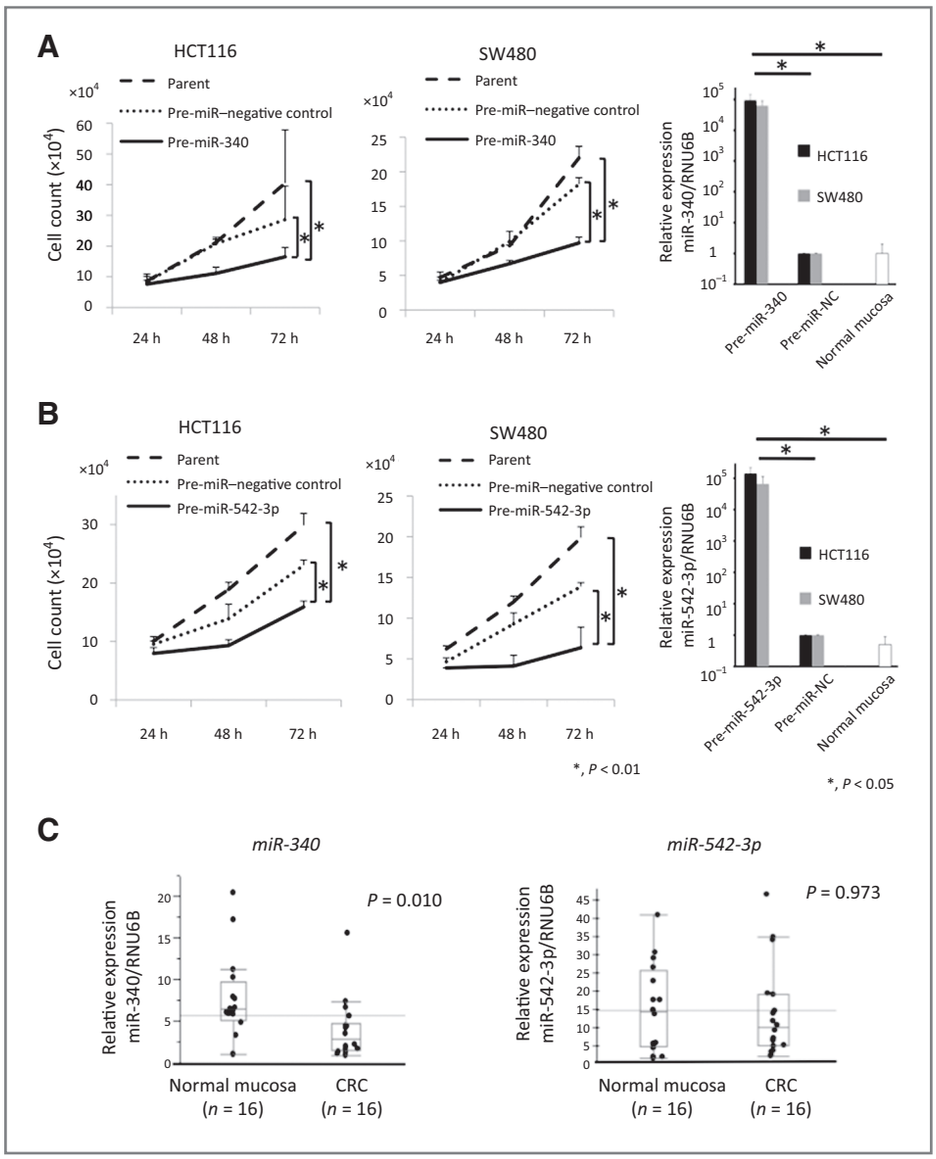

Figure 2. *In vitro* inhibitory effects of miR-340 and miR-542-3p on proliferation of colorectal cancer cells and miR-340 and miR-542-3p expression in clinical colorectal cancer samples. A, pre-miR-340 treatment led to significant growth inhibition in HCT116 and SW480, compared with that of parental cells or cultures treated with pre-miR–negative control ($P < 0.01$), $n = 4$ for each group, Mann–Whitney *U* test. The results are the mean ± SE of three replicates. The miR-340 expression in pre-miR-340–treated colorectal cancer cells was significantly higher than that of pre-miR–negative control-treated cells and clinical normal mucosa samples. B, similar results were obtained in miR-542-3p. C, expression of miR-340 and miR-542-3p in normal colonic mucosa and colorectal cancer tissue samples. miR-340 expression was significantly higher in normal mucosa than in colorectal cancer tissue ($P = 0.010$). No significant difference was found in miR-542-3p expression ($P = 0.973$). Analyzed samples of 16 colorectal cancer patients included 3 stage I, 4 stage II, 5 stage IIIA/B, 1 stage IIIC, and 3 stage IV colorectal cancer diseases.

prognosis compared with miR-340-high/c-Met-low cases ($P = 0.015$; Fig. 4C)

Discussion

In 2002, a metastasis model was proposed that metastatic capacity was gained early during tumor development (38). In this model, bone marrow is considered to be an important reservoir that allows DTCs to adapt, escape the host immune defenses in a dormant state, and develop into overt metastases in different organs (2, 39); however, the precise mechanism is largely unknown. In the present study, we attempted to uncover the nature of DTCs in bone marrow of patients with colorectal cancer who developed liver metastasis, from a view of miRNA expression.

Flatmark and colleagues (3) previously described the use of immunomagnetic selection with an anti-EpCAM antibody to determine the presence of DTCs in bone marrow of patients with colorectal cancer. EpCAM is a useful epithelial marker for detecting colorectal cancer cells, with reported detection rates ranging from 89.6% to 100% (40, 41). In the present study, we also employed an immunomagnetic capture method to encircle DTCs from bone marrow aspirates using anti-EpCAM antibody (clone HEA-125), with some modifications. To facilitate DTC capture, $CD14^+$ and $CD45^+$ cells were removed beforehand. The resultant $CD14^-CD45^-EpCAM^+$ fraction displayed typical DTC characteristics, i.e., high expressions of the epithelial marker *E-cadherin*, tumor-associated *CEA*, and oncogene *MYC*. DTC identity was further confirmed by microscopic observation with the BM4 fraction stained with EpCAM antibody (Ber-EP4 clone) and anti-cytokeratin antibody. Thus, we verified that the employed method successfully enriched the BM4 fraction with DTCs from bone marrow aspirates.

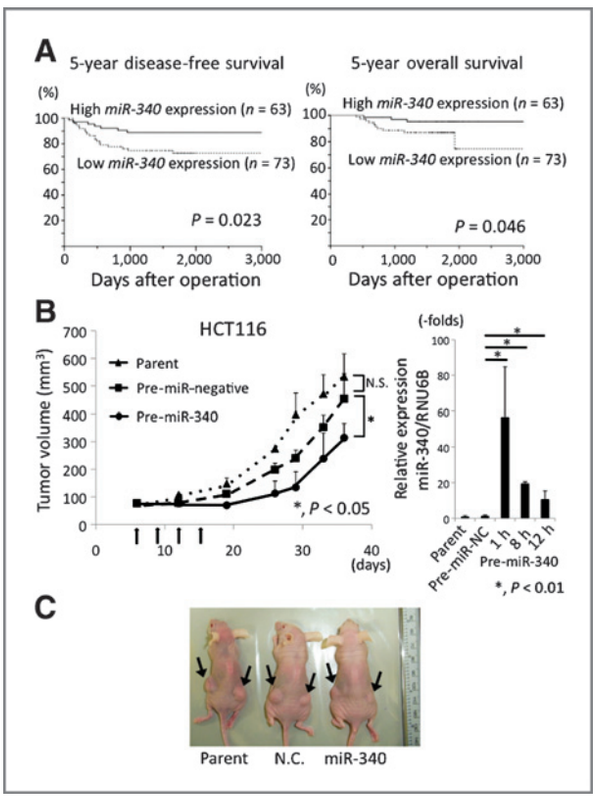

Figure 3. Impact of miR-340 expression on prognosis of patients with colorectal cancer and systemic delivery of formulated miR-340 to established colorectal cancer tumors *in vivo*. A, impact of miR-340 expression on prognosis of patients with colorectal cancer. Seventy-three patients had less than average miR-340 expression levels, and 63 patients had above average miR-340 expression. Kaplan–Meier estimation indicated that high miR-340 expression was significantly associated with better 5-year disease-free survival (DFS) and better 5-year overall survival (OS; $P = 0.023$ and 0.046, respectively). miR-542-3p expression had no effect on 5-year DFS or 5-year OS ($P = 0.429$, 0.577; data not shown). B, in therapeutic animal models with HCT116 tumors, tumor growth was significantly inhibited by systemic administration of formulated miR-340 compared with the pre-miR–negative control group ($P < 0.05$). Mice were treated on days 6, 9, 12, and 15 with carbonate apatite-formulated pre-miR-340 or pre-miR–negative control via tail vein injection. Each shot contained 30 μg of formulated oligo, with a 40% conjugation rate. miR-340–treated ($n = 7$), miR-NC–treated ($n = 9$), and parent ($n = 8$) for each group. *, $P < 0.05$. The miR-340 expression in tumors at 1, 8, and 12 hours after systemic administration of pre-miR-340 into mice tail vein was significantly higher than that of mice treated with pre-miR–negative control ($P < 0.01$). The results are the mean ± SE. $n = 5$ for each group. C, representative image of tumor growth of posterior bilateral flanks in each group after treatment. The arrows indicate tumor location. Left, nontreated mouse; middle, pre-miR–negative control–treated mouse; right, miR-340–treated mouse.

miRNA analysis of BM4 cells identified 10 miRNAs that were significantly differentially expressed between the nonmetastasis and liver metastasis groups. It is notable that the putative oncomirs miR-222 (32, 42) and miR-155 (35–37) were increased in the BM4 cells of the liver metastasis group. Increased miR-155 expression has been reported in oral cell and pancreatic carcinomas (35, 37), and represents oncomir functions, including downregulation of anti-oncogenes, such as *tumor protein 53-induced nuclear protein 1 (TP53INP1)*, *suppressor of cytokine signaling 1 (SOCS1)*, and *cell division cycle 73 (CDC73)*, which negatively regulates *β-catenin*, *cyclin D1*, and *c-MYC* (35–37). On the other hand, miR-222 is increased in non–small lung cell and hepatocellular carcinoma, and it suppresses *p27*, *PTEN*, and *tissue inhibitor of metalloproteinases-3 (TIMP3)*, all of which are usually linked to tumor-suppressive effects (32, 42). Literature survey revealed that 7 miRNAs included 4 anti-oncomirs, all of which were decreased in the liver metastasis group and 3 oncomirs, which exclusively increased in the metastasis group. The consistency between reported function of miRNAs and clinical outcome would justify collection method for DTCs and performance of employed miRNA array. These results also suggest that DTCs in bone marrow may play a role in the establishment of liver metastasis through regulation of miRNA expression, i.e., downregulation of anti-oncomirs or upregulation of oncomirs.

Among the 10 miRNAs, we focused further experiments on those that were downregulated, as we expected they might have simple and physiologic effects that could be useful in replacement therapy. Using qRT-PCR, we validated the results of miRNA array analysis for the two most strongly downregulated miRNAs: miR-340 and miR-542-3p. Although another cohort of bone marrow of colorectal cancer is expected to be investigated, further collection of 7 to 10 cases with liver metastasis and 10 cases without metastasis is practically difficult. For this reason, we attempted to investigate the malignant features of these miRNAs in colorectal cancer cell lines and clinical sample of the primary colorectal cancers.

In vitro studies showed that addition of pre-miR-340 or pre-miR-542-3p led to inhibition of cell growth of colon cancer cells, suggesting these miRNAs to be anti-oncomirs, as has been reported in other cancer types: miR-340 in breast cancer (24) and colorectal (43), and miR-542-3p in colon (27) and lung cancer (26). Furthermore, pre-miR-340 and pre-miR-542-3p suppressed the target genes: *c-Met* (24) and *MITF* (25) for miR-340, and *Survivin* (26) for miR-542-3p.

To further investigate the potential essential role of these anti-oncomirs, we examined their expression levels in clinical colorectal cancer samples, particularly in terms of normal versus tumor tissue, and the impact on patient survival. We found that expression of miR-340, but not miR-542-3p, was significantly decreased in tumor tissues compared with in normal mucosa. This finding supports the idea that miR-340 functions as an anti-oncomir in colorectal cancer, and may be of clinical importance in replacement therapy against colorectal cancer. Moreover, multivariate analysis indicated that low expression of miR-340, but not miR-542-3p, was a significant predictor of poor 5-year DFS, and high miR-340 expression was significantly associated with smaller tumor size. These findings suggest that although both miR-340 and miR-542-3p act as anti-oncomirs in colorectal cancer cell lines *in vitro*, only miR-340 may have clinical relevance.

Table 2. Univariate and multivariate analysis for 5-year disease-free survival (Cox proportional regression model)

	Univariate			Multivariate		
Factors	**RR**	**95% CI**	***P***	**RR**	**95% CI**	***P***
Gender (Male/Female)	1.621	0.739–3.805	0.232			
Histologic grade (others/well/mod)	1.920	0.455–5.544	0.329			
Size (in cm; >3 cm/≤3 cm)	2.464	0.854–10.41	0.102			
Lymph node metastasis (present/absent)	4.145	1.570–14.26	0.003[a]	2.918	1.081–10.16	0.033[a]
Venous invasion (present/absent)	1.490	0.677–3.427	0.324			
miR-340 expression (low/high)	2.637	1.157–6.755	0.020[a]	2.499	1.031–6.962	0.042[a]
Serosal invasion (SE-SI/Tis-SS)	4.008	1.817–8.917	0.001[a]	3.053	1.311–7.041	0.011[a]
Tumor type (3-4/0-2)	1.656	0.574–6.996	0.383			
Tumor site (rectum/colon)	1.235	0.556–2.724	0.599			

Abbreviations: Histologic grade; well, well-differentiated adenocaricinoma; Mod, moderately differentiated adenocarcinoma; others, poorly differentiated and mucinous adenocarcinoma.
[a]$P < 0.05$.

Our finding was consistent with the previous report that the low expression of miR-340 was associated with poor prognosis in colorectal cancer (43). However, this study performed only the array analysis that included very small sample size (each group, $n = 6$) without further confirmation study using RT-PCR. In this regard, our data substantially extended the previous small study and have established a clinical value of miR-340 as a strong indicator for prognosis of patients with colorectal cancer. In relation to disease relapse, liver metastasis is the most major recurrent mode of colorectal cancer. When we consider the link between miR-340 and liver metastasis, it is postulated that *c-Met*, proto-oncogene encoding a receptor tyrosine kinase might be a key molecule. A previous study demonstrated that *c-Met* gene amplification was linked to metastatic progression of colorectal cancer, especially in liver metastasis of colorectal cancer (44). On the other hand, a direct target of miR-340 is

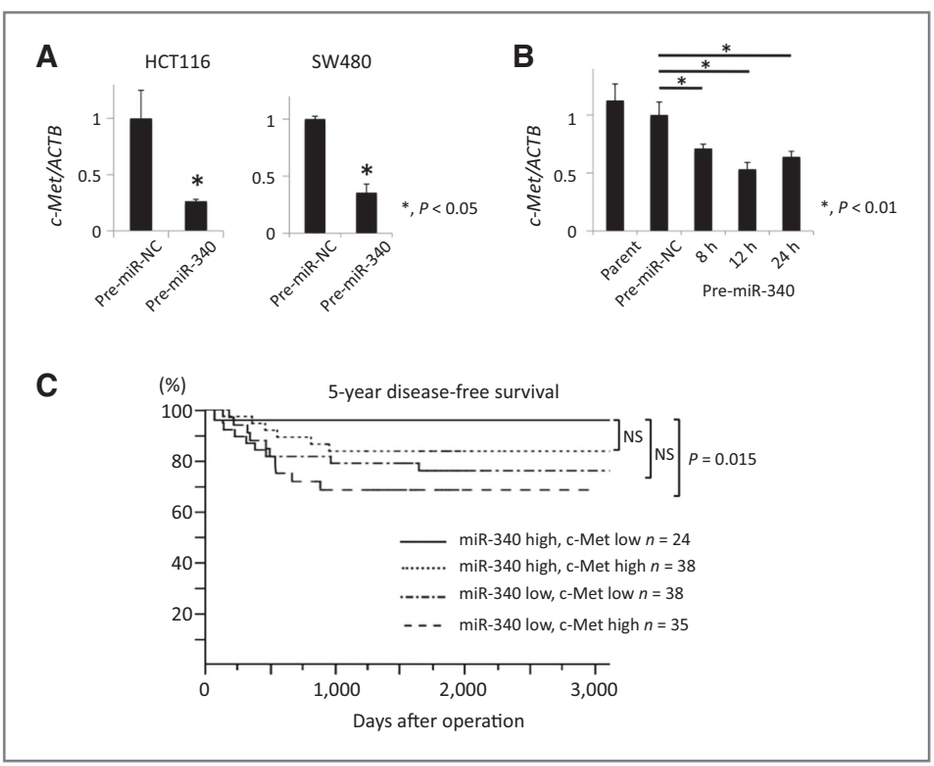

Figure 4. *c-Met* inhibition by miR-340 *in vitro* and combination survival analysis of *c-Met* and miR-340. A, the expression level of *c-Met* of pre-miR-340–treated HCT116 and SW480 were suppressed to 24.6% and 35.9% of that of negative control miR-treated cells ($P < 0.05$, $n = 3$ for each group). The results are the mean ± SE of two replicates. B, *c-Met* expression in the subcutaneous tumors was significantly suppressed at 8, 12, and 24 hours after systemic administration of miR-340 ($P < 0.01$, $n = 5$ for each group). C, combination survival analysis of *c-Met* and miR-340 expression in 135 patients with colorectal cancer. One of the initial 136 samples was excluded. *c-Met*-high and *c-Met*-low groups were divided at the median value. Kaplan–Meier curves indicated that miR-340-low/*c-Met*-high group alone showed a significantly worse DFS compared with miR-340-high/c-Met-low cases ($P = 0.015$).

reportedly shown to be *c-Met* in breast cancer (24). It was of interest that combination analyses with miR-340 and *c-Met* showed that miR-340-low/*c-Met*-high group was solely a significant worse prognostic marker as compared with miR-340-high/*c-Met*-low group. These results suggest that miR-340/*c-Met* axis may play an important role in metastasis cascade of colorectal cancer.

In animal models, it has been demonstrated that several anti-oncomir miRNA replacement therapies using either virus-mediated transduction or non-virus vehicle have inhibitory effects on cancer metastasis and tumor growth. Several examples include reduced tumor growth by adenoviral transfer of miR-let-7 in mouse models of lung cancer (45) and blockade of prostate cancer cell metastasis by miR-34a and miR-16 using RNA-Lancer II and atelocollagen, respectively (46, 47). Furthermore, anti-oncomir uptake is believed to confer no adverse effects to normal cells, because the pathway regulated by miRNA administration is already activated by the endogenous miRNA (48). In the present study, we found that administration of miR-340 synthetic nucleotides via tail vein injection into nude mice significantly inhibited growth of pre-established HCT116 tumors. To our knowledge, this is the first evidence that systemic delivery of miR-340 exhibits antitumor effects *in vivo* in an animal therapeutic model. Since administration of synthesized miRNA agonist is considered clinically safer than virus-mediated techniques, miR-340 could be a promising therapeutic agent.

There is some unresolved debate regarding the exact contents of the BM4 fraction. It is obvious from our data that this EpCAM-positive fraction contains an enriched population of DTCs, as confirmed by immunostaining with the anti–Ber-EP4 antibody or the anti-CK antibody. However, we could not rule out a possibility that bone marrow cells other than DTC might express specific miRNAs. It has recently been proposed that niche cells surrounding tumor cells in the cancer microenvironment could cherish cancer cells and endow their metastatic ability (49, 50). This possibility is currently under investigation in our laboratory.

In conclusion, the results of the present study demonstrated that DTCs in bone marrow of patients with colorectal cancer with or without liver metastasis displayed distinct miRNA expression profiles, suggesting that the characteristics of DTCs, as defined by miRNA expression, may be essential for allowing tumor metastasis. Our data also imply that miR-340 is a novel prognostic factor, and could be a useful small nucleic acid for replacement therapy against colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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