**microRNA-7 expression in colorectal cancer is associated with poor prognosis and regulates cetuximab sensitivity via EGFR regulation**

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| Complete List of Authors: | suto, toshinaga; Department of General Surgical Science, Gunma University, Graduate School of Medicine
Yokobori, Takehiko; Gunma University, General Surgical Science
Yajima, Reina; Gunma University, General Surgical Science
Morita, Hiroki; Gunma University, General Surgical Science
Fujii, Takaaki; Gunma University, General Surgical Science
Yamaguchi, Satoru; Gunma University, General Surgical Science
Bolag, Altan; Gunma University, General Surgical Science
Tsutsumi, Souichi; Gunma University, General Surgical Science
Asao, Takayuki; Gunma University, General Surgical Science
Kuwano, Hiroyuki; Gunma University Graduate School of Medicine, Department of General Surgical Science |
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microRNA-7 expression in colorectal cancer is associated with poor prognosis and regulates cetuximab sensitivity via EGFR regulation

Toshinaga Suto, MD, Takehiko Yokobori, MD, PhD, Reina Yajima, MD, Hiroki Morita, MD, PhD, Takaaki Fujii, MD, PhD, Satoru Yamaguchi, MD, PhD, Bolag Altan, Souichi Tsutsumi, MD, PhD, FACS, Takayuki Asao, MD, PhD, FACS and Hiroyuki Kuwano, MD, PhD, FACS

Departments of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22, Showamachi, Maebashi 371-8511, Japan.

Phone: +81-027-220-8224, Fax: +81-027-220-8230

Corresponding author: Takehiko Yokobori MD, PhD

Department of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22 Showamachi, Maebashi 371-8511 Japan.

Phone: +81-027-220-8224, Fax: +81-027-220-8230, E-mail: bori45@gunma-u.ac.jp

Running Head: Clinical significance of microRNA-7 in colorectal cancer
Abstract

MicroRNA 7 (miR-7) has been reported to be a tumor suppressor in all malignancies including CRC. However, its significance for CRC clinical outcomes has not yet been explored. The potential for miR-7 to act as a tumor suppressor by coordinately regulating the EGFR signaling pathway at several levels was examined. We investigated the tumor inhibitory effect of miR-7 in colorectal cancer, with particular focus on the relationship between miR-7 and the EGFR pathway. Quantitative RT-PCR was used to evaluate miR-7 expression in 105 colorectal cancer cases to determine the clinicopathologic significance of this miRNA. The regulation of EGFR by miR-7 was examined with miR-7 precursor-transfected cells. Furthermore, we investigated whether miR-7 suppresses proliferation of colorectal cancer cells in combination with cetuximab, a monoclonal antibody against EGFR. Multivariate analysis indicated that low miR-7 expression was an independent prognostic factor for poor survival (P=0.0430). In vitro assays showed that EGFR and RAF-1 are direct targets of miR-7, which potently suppressed the proliferation of colorectal cancer cells, and, interestingly, that the growth inhibitory effect of each of these was enhanced by cetuximab. miR-7 is a meaningful
prognostic marker. Furthermore, these data indicate that miR-7 precursor, alone or in combination with cetuximab, may be useful in therapy against colorectal cancer.

Summary: Our data suggest that microRNA 7 (miR-7) directly targets EGFR. miR-7 powerfully suppressed the proliferation of colorectal cancer cells, and the growth inhibitory effect was enhanced by cetuximab, a monoclonal antibody against EGF.
Introduction

Over 1.2 million new cases of colorectal cancer are diagnosed worldwide every year, and colorectal cancer accounts for 8% of cancer deaths (1). In recent years, the incidence of colorectal cancer and associated mortality have dramatically increased in Japan (2). According to the 2008 edition of Global Cancer Facts & Figures, colorectal cancer is globally the third and fourth most common cause of death from a malignant neoplasm among women and men, respectively. Surgery and administration of anticancer drugs, such as oxaliplatin, have been the conventional treatments for colorectal cancer. Recently, targeted molecular treatments using antibodies, such as anti-vascular endothelial growth factor (VEGF) antibody and anti-epidermal growth factor receptor (EGFR) antibody, have also been employed. Colon cancer chemotherapy can therefore involve traditional anti-cancer agents, which have nonspecific cytotoxic effects, and/or agents that target specific molecules and block specific intracellular signaling pathways, with both approaches playing an important role in improving prognosis and extending the lifespans of patients (3-6).

The EGF receptor, which is a member of the ErbB receptor family, regulates
important processes, such as cell proliferation, differentiation, and development (3-6).

It has been reported to be overexpressed in a variety of solid tumors, including colorectal tumors, and overexpression has been found to be associated with tumor progression, resistance to chemotherapy and radiation therapy, and poor prognosis (3-4).

Therefore, important therapeutics used in clinical practice include antibodies targeting EGFR and its downstream signaling effectors and low-molecular-weight compounds that inhibit signal transduction (7-9). Anti-EGFR antibodies have been used to treat colorectal cancer. However, tumors can develop resistance to these agents, limiting their clinical effectiveness (14). Resistance can be caused by mutations affecting EGFR downstream signaling and can be acquired during treatment (14). New therapeutic tools are currently being sought to help overcome this resistance.

A microRNAs (miRNAs) are noncoding RNAs of 21-23 nucleotides in length. miRNAs bind to complementary sequences in the 3'-untranslated regions (UTRs) of target mRNAs and inhibit translation. miRNAs are involved in cancer growth, differentiation, proliferation, and apoptosis (15). We focused on miR-7, which has been reported to target EGFR in vitro (16). Rai et al. determined that miR-7 targets not only
but also v-raf-1 murine leukemia viral oncogene homolog 1 (RAF-1), a gene downstream of. As such, miR-7 regulation might help overcome the resistance of tumors to EGFR inhibition therapies that are currently used in clinical practice (16).

The objectives of our study were to elucidate the clinical significance of miR-7 expression in clinical specimens of colorectal cancer and to perform functional analysis of miR-7 by using a colorectal cancer cell line. In our study, miR-7 expression in these clinical specimens was measured, and its relationships with clinicopathological features, prognosis, and EGFR protein expression were examined. Binding of miR-7 to the 3’-UTR of EGFR mRNA and the 3’-UTR of RAF-1 mRNA was analyzed by using a luciferase assay. Three colorectal cancer cell lines were used to determine the regulatory effects of miR-7 on cell proliferation, expression of genes downstream of, and cetuximab sensitivity.
Materials and Methods

Patients and sample collection

All clinical colorectal cancer samples (n = 105) in this study were used in accordance with institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants. All patients underwent resection of the primary tumor at the Department of General Surgical Science of Gunma University Hospital in Japan between 1999 and 2009. All patients had a clear histologic diagnosis of colorectal cancer; the diagnoses were based on the clinicopathological criteria described by the Japanese Society for Cancer of the Colon and Rectum (http://www.jsccr.jp/en/index.html). All patients were closely followed and were assessed every 3 months. The follow-up periods ranged from 0.7 months to 11 years, with a mean of 6 years. All sample data, including age, gender, histology, tumor size and depth, lymphatic invasion, vascular invasion, lymph node metastasis, liver metastasis, peritoneal dissemination, distant metastasis, and clinical stage were obtained from the clinical and pathologic records and are summarized in Table 1.
The resected cancer tissues and adjacent non-cancerous tissues were immediately cut, frozen in liquid nitrogen, and stored at -80°C until RNA and DNA were extracted. Total RNA was extracted by using the miRNeasy Mini kit (Qiagen) in accordance with the manufacturer’s instructions.

**Evaluation of miR-7 expression in clinical samples**

For quantitative real-time reverse transcriptase PCR (qRT-PCR) of miR-7, cDNA was synthesized from 10 ng of total RNA by using the TaqMan MicroRNA Reverse Transcription Kit and specific stem-loop reverse transcription primers (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol. PCR was performed in a LightCyclerTM 480 System (Roche, Basel, Switzerland). The 10 µL PCR reaction included 0.67 µL of RT products, 1× TaqMan Universal PCR master mix, and 1 µL of primers and probe mix included in the TaqMan miRNA assay kit. The reactions were incubated in 96-well optical plates at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 10 min. The expression levels of miR-7 were normalized to that of the small nuclear RNA RNU6B and analyzed by using the \(2^{-\Delta\Delta Ct}\) method.
Cell lines

The HCT116 and SW480 human colon cancer cell lines, which contain v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene (KRAS) mutations, were used. The HT29 human colon cancer cell line harbors a BRAF mutation. HCT116, SW480, and HT29 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO2 at 37°C.

Plasmid construction

The sequences in the 3'-UTR regions of EGFR mRNA and RAF1 mRNA that are targeted by miR-7 were predicted with Targetscan (release 5.1), and the 3'-UTRs from human EGFR mRNA and RAF1 mRNA were amplified from the genomic DNA of normal cells. The amplified fragments were inserted into the XhoI restriction site of the
pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) by using the In-Fusion® Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA, USA). The nucleotide sequences of the plasmids were confirmed by sequencing.

**Luciferase assay**

HCT116 cells were seeded in 96-well plates and then cotransfected with 0.2 µg Luc-EGFR and miR-7 precursor by using Lipofectamine RNAiMAX. Forty-eight hours following transfection, the activities of firefly and Renilla luciferase in cell lysates were measured by using the Dual-Glo® Luciferase Assay System (Promega) and the Fluoroskan Ascent FL (Thermo Fischer Scientific). The firefly luciferase activities produced by each vector were normalized to that of Renilla luciferase. All transfection experiments were conducted in triplicate.

**Immunohistochemistry**
Immunohistochemical studies of EGFR were conducted on formalin-fixed, paraffin-embedded (FFPE) surgical sections obtained from patients with colorectal cancer. The tissue sections were deparaffinized, soaked in 0.01 mol/L sodium citrate buffer, and boiled in a microwave oven for 5 minutes at 500 W to retrieve the cellular antigens. A rabbit monoclonal antibody against EGFR (Cell Signaling Technology, Danvers, MA, USA) diluted 1:100 was used as the primary antibody. All tissue sections were immunohistochemically stained with a streptavidin-biotin peroxidase complex solution (Nichirei Company, Tokyo, Japan) and counterstained with hematoxylin.

Transfection of the miR-7 precursor and miR-7 inhibitor

The pPre-miR™ miRNA Precursor hsa-miR-7-5p (miR-7 precursor; Applied Biosystems), Pre-miR™ miRNA Precursor Molecules Negative Control (miR-nc; Applied Biosystems), mirVana® miRNA inhibitor hsa-miR-7-5p (miR-7 inhibitor; Applied Biosystems), and mirVana® miRNA inhibitor Negative Control (miR inhibitor-nc; Applied Biosystems) were separately transfected at 20 nmol/L into
HCT116, SW480, and HT29 cells by using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer’s instructions.

**Protein expression analysis**

Western blotting was used to confirm the expression of the EGFR, RAF-1, ERK1/2, pAKT, and β-actin proteins in miR-7 precursor- and miR-7 inhibitor-transfected cells. Total protein (40 µg) was electrophoresed and then electrotransferred at 200 mA for 180 minutes at 4°C. These proteins were detected by using an anti-EGFR rabbit monoclonal antibody (1:1000; Cell Signaling Technology), an anti-ERK1/2 rabbit monoclonal antibody (1:1000; Cell Signaling Technology), a rabbit monoclonal antibody against Raf-1 (1:1000; Origene, Rockville, MD, USA), and an anti-pAKT rabbit monoclonal antibody (1:1000; Cell Signaling Technology); an anti-β-actin mouse monoclonal antibody (1:1000; Sigma-Aldrich, St. Louis, MO, USA) served as a control. Bands and band intensities were detected and calculated, respectively, by using ECL Prime.
Western Blotting Detection Reagent and an Image Quant LAS 4000 (GE Healthcare Life Sciences).

**Proliferation assay**

Analysis of proliferation was performed on cells that had been transfected with either the miR-7 precursor or the miR-7 inhibitor. The cells were plated in 96-well plates in 100 µL of medium at approximately 5000 cells per well. To quantitate cell viability with the cell-counting-kit-8 assay (CCK-8; Dojindo Laboratory, Tokyo, Japan), 10 µL of the cell counting solution was added to each well after 0, 24, 48, or 72 hours, and then the plates were incubated at 37°C for 2 hours. The cell proliferation rate was then determined by measuring the absorbance of the well at 450 nm with the reference wavelength set at 650 nm. The absorbances were measured with a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Regulation of the cetuximab sensitivity of colorectal cancer cells by miR-7**
HCT116, SW480, and HT29 cells, 5000 cells per well were seeded in 96-well plates and then treated with 0, 0.01, 0.1, 1, 10, 20, 50, or 100 µg/mL cetuximab for 96 hours. For each dose, cells from one plate were harvested to determine the absorbance value. Viable cells were counted 96 hours post-treatment with the CCK-8 assay by measuring the absorbances of the samples at 450 nm, with the reference wavelength set at 650 nm.

**Statistical analysis**

The differences between two groups were estimated by using the t test, the chi-square test, and the repeated measures ANOVA test. Kaplan–Meier curves were generated for overall survival, and statistical significance was determined by using the log-rank test. A probability value of < 0.05 was considered significant. In addition, univariate and multivariate survival analyses were performed using Cox’s proportional hazards model. All statistical analyses were performed with JMP5.0 software (SAS Institute Incorporated, Cary, NC, USA).
Results

The clinicopathological significance of miR-7 expression in colorectal cancer

The expression levels of miR-7 in cancerous tissues (T) were higher than those in adjacent, non-cancerous tissues (N) ($P<0.001$; Fig. 1A). In this study, the ROC (receiver operating characteristic) curve determined the cutoff point. Cancerous tissue below the cutoff point for miR-7 expression of 3.21, normalized to expression of the U6 small nuclear RNA RNU6B, was assigned to the low-expression group ($n=68$), whereas cancerous tissue with an expression level above the cutoff point was assigned to the high-expression group ($n=37$). Patients in the low-miR-7-expression group had a significantly poorer prognosis than those in the high-miR-7-expression group ($P=0.0489$; Fig. 1B). In addition, the clinicopathological factors of age, gender distribution, histology, tumor depth, lymphatic or venous invasion, lymph node, liver, or distant metastasis, peritoneal dissemination, or clinical staging were not significantly different between these two groups (Table 1). However, tumor size in the low-miR-7 expression group showed a non-significant increase ($P=0.083$) over that seen in the high-miR-7 expression group (Table 1). The results of univariate and multivariate Cox...
proportional hazards regression analyses for overall survival are shown in Supplementary Table 1. Multivariate analysis indicated that low expression of miR-7 was an independent and significant prognostic factor for survival (RR, 0.82; 95% CI, 0.54–0.94; \( P = 0.0430 \); Supplementary Table 1).

**miR-7 regulates EGFR and RAF-1 in colorectal cancer cells**

By using *in silico* miRNA target prediction tools, such as TargetScan, we identified miR-7 binding sites in the 3’-UTRs of transcripts encoding EGFR and RAF-1 (Fig. 2A, 2C). To investigate miRNA binding and repression, we performed a luciferase reporter assay with a vector in which the 3’-UTR sequences of EGFR mRNA and RAF-1 mRNA were inserted downstream of the luciferase reporter gene (Luc-EGFR, Luc-RAF-1). The luciferase activities of Luc-EGFR and Luc-RAF-1 were both significantly reduced when compared with that of the negative control in transient cotransfection of HCT116 cells with miR-7 precursor (\( P < 0.001 \), \( P < 0.001 \); Fig. 2B, 2D). These data suggest that the 3’-UTRs of both EGFR and RAF-1 are direct functional targets of miR-7.
**miR-7 and EGFR protein expression in clinical samples**

For each frozen tissue sample used to measure miR-7 levels in colorectal tumors and adjacent non-cancerous tissue, representing 105 patients with colorectal cancer, there were matching, adjacent FFPE surgical sections from the same tumor. The association between miR-7 and EGFR protein expression in clinical samples, we used immunohistochemistry sections of the FFPE samples, which were then divided into groups based on a score of EGFR protein expression. Samples were further classified according to the staining patterns in the tumor cell membranes as either incomplete staining, i.e., tumor cells were stained in only part of their membrane, and complete staining, i.e., tumor cells displayed a circumferential staining of the entire tumor cell membrane (17). The following scoring system for assessing EGFR immunostaining was used: score 0 = no staining or unspecific staining of tumor cells; score 1 = weak (intensity) and incomplete staining (quality) of more than 10% of tumor cells (quantity); score 2 = moderate and complete staining of more than 10% of tumor cells; score 3 = strong and complete staining of more than 10% of tumor cells. Representative examples
for the different scores are shown in Supplementary Fig. 1A. The expression of \textit{miR-7} was significantly increased in the \textit{EGFR} expression-negative group (score 0) compared with the \textit{EGFR} expression-positive group (score 1,2,3) (mean ±SEM: \textit{EGFR} expression-negative group, 6.01 ± 0.58; \textit{EGFR} expression-positive group, 2.19 ± 0.38; \textit{P} = 0.043; Supplementary Fig. 1B).

\textit{Expression of EGFR, RAF-1, ERK1/2, and pAKT is suppressed by \textit{miR-7} in vitro}

We used qRT-PCR to confirm that \textit{miR-7} expression in cells transfected with \textit{miR-7} precursor was significantly higher than that in both untreated cells (“Parent” in Figures 2-5) and cells transfected with \textit{miR-nc} (\textit{P} < 0.001, Supplementary Fig. 2). We also determined that \textit{miR-7} expression in cells transfected with \textit{miR-7} inhibitor was significantly lower than that in either untreated cells or cells transfected with the \textit{miR inhibitor-nc} (\textit{P} < 0.001, Supplementary Fig. 3). To determine whether \textit{miR-7} suppresses \textit{EGFR} expression and downstream signaling events in the colorectal cancer cell lines HCT116, SW480, and HT29, cell lysates of transfected cells were analyzed by Western
blotting. They were then compared with untreated cells and miR-nc-treated cells. In HCT116 and SW480 cell lines (KRAS mutation), the expression of EGFR, RAF-1, pAKT, and ERK1/2 was downregulated in cells transfected with miR-7 precursor (Fig. 3A, 3C), while the expression of EGFR, RAF-1, pAKT and ERK1/2 in cells transfected with miR-7 inhibitor was upregulated relative to the expression in untreated cells and cells transfected with the miR inhibitor-nc (Fig. 3B, 3D). However, in the HT29 cell line (BRAF mutation), the expression of EGFR, RAF-1, and pAKT was downregulated in cells transfected with the miR-7 precursor, while ERK1/2 expression was upregulated (Fig. 3E). These cells also upregulated the expression of EGFR, RAF-1, pAKT, and ERK1/2 upon transfection with miR-7 inhibitor relative to the expression in untreated cells and cells transfected with miR inhibitor-nc (Fig. 3F).

**miR-7 regulates proliferation in HCT116, SW480, and HT29 cells**

We analyzed the proliferation of HCT116, SW480, and HT29 cells that had been transfected with either miR-7 precursor or miR-7 inhibitor. In HCT116 and SW480
cells, the proliferation rate of miR-7 precursor–treated cells was significantly lower than that of untreated cells ($P < 0.001$; Fig. 4A, 4B). In contrast, the proliferation rate of miR-7 inhibitor–treated cells was significantly higher than that of untreated cells ($P < 0.001$; Fig. 4A, 4B). In HT29 cells, the proliferation rate of miR-7 precursor–treated cells was not significantly different from that of untreated cells ($P = 0.0636$; Fig. 4C). Similarly, there was no significant difference between the proliferation rates of miR-7 inhibitor–treated cells and untreated cells ($P = 0.2151$; Fig. 4C).

*miR-7 regulates cetuximab sensitivity in cetuximab-resistant HCT116 and SW480 cells with a KRAS mutation and HT29 cells with a BRAF mutation.*

To determine if cetuximab, an *EGFR*-targeted antibody, enhances the antitumor efficacy of miR-7 in colorectal cancer cells, we treated HCT116, SW480, and HT29 cells with cetuximab and analyzed proliferation in cells that received only cetuximab treatment and cells that were transfected with either miR-7 precursor or miR-nc after cetuximab treatment. HCT116 and SW480 colon cancer cells carry *KRAS* mutations and,
as such, are resistant to cetuximab. Whereas untreated cells and miR-nc-treated cells were highly resistant to cetuximab, the miR-7 precursor–treated cells responded to this drug ($P < 0.001$; Fig. 5A, 5B). However, in HT29 cells with a $BRAF$ mutation, there was no change in the sensitivity to cetuximab in cells treated with the miR-7 precursor ($P = 0.8584$; Fig. 5C).
**Discussion**

In this study, we determined that the expression of miR-7 in primary colorectal cancer is higher than in normal colorectal tissues; however, a low level of miR-7 expression is associated with cancer progression and poor prognosis. We also determined that miR-7 regulates proliferation and cetuximab sensitivity via EGFR suppression.

We focused on miR-7, which has been reported to target EGFR in vitro (16). Rai et al. demonstrated that miR-7 targets not only EGFR but also RAF1, a gene downstream of RAS. Mir-7 targets many other genes besides those analyzed in this study. Induction of miR-7 might overcome resistance of tumors to therapies that inhibit EGFR (16,18). Cetuximab is one of the targeted molecular drugs that have recently been used to treat patients with colorectal cancer. However, unresolved issues with this drug persist, such as acquired treatment resistance caused by mutations in KRAS and BRAF, both of which participate in the EGFR signaling pathway (19). Cetuximab is known to have low effectiveness in patients with either KRAS or BRAF mutations; this feature warrants limitations on its use (19). We examined how treatment with miR-7 changes cetuximab
sensitivity in cetuximab-resistant HCT 116 and SW480 cells, which harbor KRAS mutations, and HT29 cells, which harbor a BRAF mutation. Cetuximab treatment did not reduce proliferation of the parent HCT 116 and SW480 cells, but cetuximab sensitivity increased in miR-7 precursor–treated HCT 116 and SW480 cells. Since miR-7 does not target EGFR alone, it would be difficult to markedly increase cetuximab sensitivity by treatment with miR-7. miR-7 has been reported to bind to the 3’-UTR in the mRNA of not only EGFR, but also to that of RAF1, a gene downstream of KRAS, and inhibit translation (16). Similarly, in this study, RAF-1, which is downstream of EGFR, is also a potential target of miR-7 (Figure 2). In HT29 cells with a BRAF mutation, there was no change in the sensitivity to cetuximab in cells treated with the miR-7 precursor. When KRAS and BRAF are not mutated, KRAS signals to ERK1/2 through BRAF rather than RAF-1. When KRAS is mutated, however, KRAS signaling switches from BRAF to RAF-1, and the EGFR pathway is hyperactivated. When BRAF is mutated, the EGFR pathway is also hyperactivated, but instead by BRAF-dependent activation of ERK1/2 (20). In HT29 cells that do not have mutated
KRAS, EGFR signaling occurs through BRAF, and as such the combination effect of cetuximab with miR-7 that is targeted to RAF-1 would not be expected to occur.

miR-7 inhibits EGFR signaling that may regulate the growth capacity and cetuximab sensitivity of colon cancer cells. When there is a mutation in BRAF in colon cancer, a combined effect of an EGFR inhibitor and miR-7 does not occur, but in cancers that have both low miR-7 expression and KRAS mutations, miR-7 administration may be a strategy to overcome resistance to therapeutic agents.

We showed that expression of miR-7 was increased in colorectal cancer specimens. In lung cancer, EGFR mutations have also been reported to induce miR-7 expression (16).

When EGFR activation is caused by a driver mutation, the effects of changes in EGFR expression levels are small in colorectal cancer, which is in contrast to the effects of EGFR in lung cancer, suggesting that the signaling mechanisms may differ across cancer types (21). This finding suggests that activation of EGFR signaling and induction of miR-7 expression might be specific to the cancer type. Our study also showed that miR-7 expression was significantly lower in the clinical specimens of
patients with colorectal cancer who had positive $EGFR$ protein expression than in the clinical specimens of those with negative expression. An $\textit{in vitro}$ analysis revealed that $EGFR$ and $RAF-1$ translation were inhibited by the binding of $\textit{miR-7}$ to the 3’-UTR of $EGFR$ mRNA and $RAF-1$ mRNA in a colorectal cancer cell line. Our data suggest that $\textit{miR-7}$ might not be induced by $EGFR$ signaling and that $\textit{miR-7}$ inhibits $EGFR$ signaling. Thus, a low level of $\textit{miR-7}$ expression in colorectal cancer lesions is thought to be associated with the progression of cancer and a poor prognosis. From our $\textit{in vitro}$ data, it is expected that the cetuximab sensitivity in primary colorectal cancer with high $\textit{miR-7}$ expression is higher than in cases of low $\textit{miR-7}$ expression, even if they have a $KRAS$ mutation. $\textit{miR-7}$ might be a useful cetuximab sensitivity marker in colorectal cancer.

In conclusion, the low expression of $\textit{miR-7}$ correlated with cancer progression and poor prognosis. Low expression of $\textit{miR-7}$ could be a useful prognostic marker for colorectal cancer. In addition, $\textit{miR-7}$ regulates colorectal cancer cell proliferation and resistance to cetuximab in vitro. Finally, $\textit{miR-7}$ might be a promising candidate for
targeted therapy in patients with colorectal cancer whose tumors are resistant to

EGFR-directed antibodies.

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**Table and Figure Legends**

**Table 1. Relationship between *miR-7* expression and clinicopathological features**

**Fig. 1. Clinical significance of *miR-7* expression in colorectal cancer samples.**

(A) *miR-7* expression in cancerous (T) (n = 105) and adjacent non-cancerous (N) (n = 105) tissues from colorectal cancer patients assessed by TaqMan RT-PCR. All data were normalized to RNU6B. Horizontal lines indicate the means (P< 0.001).

(B) Kaplan-Meier curves according to *miR-7* expression levels in colorectal cancer patients (P=0.0489).
Fig. 2. *EGFR* and *RAF-1* expression is directly suppressed by *miR-7* in colorectal cancer.

(A) *miR-7* binding sites in the *EGFR* 3′-UTR. Putative conserved target sites in the 3′-UTR were identified using in silico miR target prediction tools.

(B) Luciferase assays of premiR-7 transfected HCT-116 cells. The error bars represent the SD from eight replicates. Left bar: *EGFR* 3′-UTR luciferase vector only. Middle bar: *EGFR* 3′-UTR luciferase vector + miR-nc. Right bar: *EGFR* 3′-UTR luciferase vector + *miR-7* precursor (P< 0.001).

(C) *miR-7* binding sites in the *RAF-1* 3′-UTR. Putative conserved target sites in 3′-UTR were identified using in silico miR target prediction tools.

(D) Luciferase assays of premiR-7 transfected HCT-116 cells. The error bars represent the SD from eight replicates. Left bar: *RAF-1* 3′-UTR luciferase vector only. Middle bar: *RAF-1* 3′-UTR luciferase vector + miR-nc. Right bar: *RAF-1* 3′-UTR luciferase vector + *miR-7* precursor (P< 0.001).
Fig. 3. Expression of EGFR, Raf-1, pAKT, and ERK1/2 is suppressed by miR-7 in colorectal cancer cells.

Western blotting of EGFR, Raf-1, pAKT, and ERK1/2 protein in miR-7 precursor-transfected HCT-116 cells (A), SW480 cells (C), and HT29 cells (E). Western blotting of EGFR, Raf-1, pAKT and ERK1/2 protein in miR-7 inhibitor-transfected HCT-116 cells (B), SW480 cells (D), and HT29 cells (F). Protein levels were normalized with respect to beta-actin.

Fig. 4. Proliferation potency is suppressed by miR-7 in HCT-116, SW480, and HT29 cells.

(A) (B) In HCT116 and SW480 cells, the proliferation rate of miR-7 precursor-treated cells was suppressed in comparison with that of untreated cells. In contrast, the proliferation rate of miR-7 inhibitor–treated cells was significantly higher than that of untreated cells.
(C) In HT29 cells, the proliferation rate of miR-7 precursor–treated cells was not significantly different from that of untreated cells. Similarly, there was no difference between the proliferation rates of miR-7 inhibitor–treated and untreated cells.

The data represent the means ± SD of 5 replicates.

Fig. 5. miR-7 inhibits the proliferation of the colorectal cancer cell line EGFR in combination with cetuximab.

To determine the cetuximab sensitivity of the cetuximab-resistant (A) HCT116 and (B) SW480 cells with KRAS mutations and (C) HT29 cells with a BRAF mutation, 5000 cells per well were seeded in 96-well plates and then treated with 0, 0.01, 0.1, 1, 10, 20, 50, or 100 µg/mL cetuximab for 96 hours. Viable cells were counted 96 hours post-treatment with the CCK-8 assay. The results are presented as means ±SD of 3 replicates.
Supplementary Table 1. Results of univariate and multivariate analysis of clinicopathological factors affecting overall survival rate following surgery

Supplementary Figure 1

(A) The staining pattern in tumor cell membranes was classified as score 0 when negative or unspecific, score 1 if the staining was weak and incomplete in more than 10% of tumor cells, score 2 if moderate and complete staining was detectable in more than 10% of the tumor cells, and score 3 if more than 10% of the tumor cells displayed strong and complete membrane staining. The EGFR expression-negative group is score 0. The EGFR expression-positive group is score 1, 2, or 3. (B) Expression of miR-7 was significantly increased in the EGFR expression-negative group compared with that in the EGFR expression-positive group.

Supplementary Figure 2
In HCT116, SW480 and HT29 cells, Bar graph shows miR-7 expression in the miR-7 precursor-treated group, miR-nc-treated group and control cell groups.

Supplementary Figure 3

In HCT116, SW480 and HT29 cells, The bar graph shows miR-7 expression in the miR-7 inhibitor-treated group, miR inhibitor-nc-treated group, and the control cell group using HCT116, SW480, and HT29 cells.
### Table 1: Relationship between miR-7 expression and clinicopathological features

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<td>III, IV</td>
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*p<0.05,*

Well: well differentiated, Moderate: moderately differentiated, Poor: poorly differentiated, Signet: signet ring cell.
Figure 1

A

- P < 0.001

miR-7 expression
(n=37)

miR-7 low expression
group (n=68)

B

Overall survival

Years after operation

P = 0.0489
Figure 2

A. EGFR 3' UTR 5’...GAGCACAAGCCACAAGUCUUCCA...
hsa-miR-7 3’ UGUUGUUUUAGUGAUCAGAAGGU

B. P < 0.001

C. RAF-1 3' UTR 5’...CCCUCUCAGGGAGCAGUCUUCCA...
hsa-miR-7 3’ UGUUGUUUUAGUGAUCAGAAGGU

D. P < 0.001
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<td>miR-nc</td>
<td>miR-7 precursor</td>
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<td>B</td>
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<td>miR-nc</td>
<td>miR-7 inhibitor</td>
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</table>
Figure 4

A) HCT116

B) SW480

C) HT29

Cell viability rate

Hours

* P<0.001

** P=0.0636

*** P=0.2151

Parent

miR-7 precurso

miR-7 inhibitor

Parent

miR-7 precurso

miR-7 inhibitor

Parent

miR-7 precurso

miR-7 inhibitor
Figure 5

A

HCT116

* P<0.001

B

SW480

* P<0.001

C

HT29

** P=0.8584

*** P=0.7659

parent

miR-nc

miR7 precurser

Cetuximab (µg/ml)

Cetuximab (µg/ml)

Cetuximab (µg/ml)
Supplementary Figure 1

A

EGFR negative

Score 0

×200

EGFR positive

Score 1

Score 2

Score 3

×200

B

miR-Expression (normalized to RNU6B)

P=0.043

EGFR immunohistochemistry

0

negative

positive
Supplementary Figure 2

miR-7 expression (normalized to RNU6B)

HCT116 (Parent)  premiR-nc  miR-7 precursor

P < 0.001

SW480 (Parent)  premiR-nc  miR-7 precursor

P < 0.001

HT29 (Parent)  premiR-nc  miR-7 precursor

P < 0.001
Supplementary Figure 3

For Peer Review

miR-7 expression (normalized to RNU6B)

HCT116 (Parent)  miR inhibitor-nc  miR-7 inhibitor

SW480 (Parent)  miR inhibitor-nc  miR-7 inhibitor

HT29 (Parent)  miR inhibitor-nc  miR-7 inhibitor

P < 0.001

Downloaded from http://carcin.oxfordjournals.org/ at Gunma University on February 25, 2015
### Supplementary Table 1

**Results of univariate and multivariate analysis of clinicopathological factors affecting overall survival rate following surgery**

<table>
<thead>
<tr>
<th>Clinicopathologic variable</th>
<th>Univariate analysis</th>
<th></th>
<th>Multivariate analysis</th>
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<td></td>
<td>RR</td>
<td>95%CI</td>
<td>p value</td>
<td>RR</td>
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<td>Depth (m, sm, mp vs ss, se, si)</td>
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<td>1.34-3.70</td>
<td>0.0057</td>
<td>1.48</td>
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<td>Lymphatic invasion (ly0 vs ly1, ly2, ly3)</td>
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<td>1.28-11.7</td>
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<td>2.51</td>
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<td>Venous invasion (v0 vs v1, v2, v3)</td>
<td>1.65</td>
<td>1.34-2.65</td>
<td>0.0267</td>
<td>1.49</td>
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<td>Lymph node metastasis (N0 vs N1, N2, N3)</td>
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<td>1.29-4.05</td>
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<td>Liver metastasis (H0 vs H1, H2, H3)</td>
<td>1.99</td>
<td>1.82-2.98</td>
<td>0.0001</td>
<td>3.24</td>
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<td>miR-7 expression (low expression vs high expression)</td>
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<td>0.56-1.48</td>
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<td>0.82</td>
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RR; Relative risk, CI; Confidence interval, *p<0.05