Biology of Human Tumors

Clinical Cancer Research

Circular RNA ciRS-7—A Promising Prognostic Biomarker and a Potential Therapeutic Target in Colorectal Cancer

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Abstract

Purpose: Colorectal cancer is one of the most common malignancies worldwide. Recently, a novel circular RNA, ciRS-7, was proposed to be a potential miR-7 sponge. As miR-7, a putative tumor-suppressor, regulates the expression of several important drivers of colorectal cancer, we analyzed the clinical significance of ciRS-7 in colorectal cancer patients.

Experimental Design: Initially, we evaluated the expression levels of ciRS-7 in a training cohort comprising of 153 primary colorectal cancer tissues and 44 matched normal mucosae. We subsequently confirmed its clinical relevance in an independent validation cohort ($n = 165$), and evaluated the effect of ciRS-7 on miR-7, and its target genes EGFR and RAF1. Functional analyses were performed in cell lines and an animal model to support clinical findings.

Results: Our data revealed that ciRS-7 was significantly upregulated in colorectal cancer tissues compared with matched normal mucosae ($P = 0.0018$), and its overexpression was associated with poor patient survival ($P = 0.0224$ and 0.0061 in the training and validation cohorts, respectively). Multivariate survival analysis revealed that ciRS-7 emerged as an independent risk factor for overall survival ($P = 0.0656$ and 0.0324 in the training and validation cohorts, respectively). Overexpression of ciRS-7 in HCT116 and HT29 cells led to the blocking of miR-7 and resulted in a more aggressive oncogenic phenotype, and ciRS-7 overexpression permitted the inhibition of miR-7 and subsequent activation of EGFR and RAF1 oncogenes.

Conclusions: CiRS-7 is a promising prognostic biomarker in colorectal cancer patients and may serve as a therapeutic target for reducing EGFR-RAF1 activity in colorectal cancer patients. *Clin Cancer Res; 23(14); 3918–28. ©2017 AACR.*

Introduction

Colorectal cancer is a leading cause of tumor-associated morbidity and mortality worldwide, and its incidence continues to rise gradually (1). Although several critical events have been identified that play key roles during colorectal carcinogenesis (2, 3), only few of these molecular targets are clinically actionable. Wealth of published studies suggest that miRNAs play key roles in the development of various types of cancer, including colorectal cancer (4). Previous work from our group and others have highlighted that specific miRNAs contribute to colorectal cancer pathogenesis and can be used as biomarkers for diagnosis, prognosis, and metastasis prediction in colorectal cancer patients (5–9). One such miRNA that has aroused considerable interest recently is miR-7 (10–16). miR-7 is aberrantly expressed in several cancers and linked to many "oncogenic" pathways (17, 18). In colorectal cancer, miR-7 was demonstrated as a tumor suppressor, and its downregulation is correlated with poor prognosis (19, 20). Considering the critical biological role of miR-7 in colorectal tumorigenesis and its clinical relevance as a prognostic biomarker, it is essential to better understand the underlying mechanisms in colorectal cancer, which still remains vastly unclear and unexplored.

It has become increasingly clear that RNA transcripts share the same miRNA-binding sites can communicate with and regulate each other by competing for common pools of miRNA molecules (21), and thereby act as competing endogenous RNAs (ceRNA). Accumulating evidence has unveiled the importance of ceRNA-mediated regulatory mechanisms in cancer pathogenesis. Recently, circular RNA ciRS-7 was shown to act as a ceRNA of miR-7: ciRS-7 can bind up to 73 copies of miR-7, allowing genes repressed by miR-7 to be reactivated during the development of brain (22). This circular RNA has also been

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Translational Relevance

Recently, a novel circular RNA, ciRS-7, was proposed to be a potential miR-7 sponge, but the functional and clinical significance of this circular RNA in colorectal cancer remains unexplored. Herein, we found ciRS-7 was significantly overexpressed in colorectal cancer tissues, and its upregulation was associated with poor patient survival. We further confirmed its clinical relevance in another independent validation cohort. Functional assays identified overexpression of ciRS-7 in HCT116 and HT29 cells led to the blocking of miR-7 and resulted in a more aggressive oncogenic phenotype, which was subsequently validated in cell lines and a xenograft animal model. Collectively, we have firstly identified ciRS-7 as promising prognostic biomarkers in colorectal cancer patients, and provide novel evidence that therapeutic targeting of this circular RNA may be a potential treatment approach in colorectal cancer patients.

linked to human disease by affecting miR-7 activity (22, 23), suggesting its role as a miR-7 regulator. However, to the best of our knowledge, no studies have thus far interrogated the pathogenic role of ciRS-7 in cancer or its clinical relevance in colorectal cancer.

In the current study, we have made first attempts to fill this gap in knowledge to assess the molecular contribution of ciRS-7 in colorectal cancer. We specifically set out to investigate its relevance as a prognostic biomarker and potential therapeutic target. Accordingly, we analyzed the expression level of ciRS-7 in neoplastic tissues and matched normal tissues, followed by validation of our results in multiple, independent cohorts of colorectal cancer patients. In addition, we performed a systematic and comprehensive functional analysis of ciRS-7 in colorectal cancer, its regulatory effect on miR-7 activity in this disease, in a series of *in vitro* experiments followed by validation of these results on tumor growth in xenograft animal models. We conclude that ciRS-7 is a promising prognostic biomarker for colorectal cancer patients, and therapeutic targeting of ciRS-7 maybe a potential strategy for the management of colorectal cancer patients.

Materials and Methods

Patients and study design

This study included analysis of 448 specimens comprising of 90 fresh frozen and 358 formalin-fixed, paraffin-embedded (FFPE) colorectal cancer tissues. For the analysis of clinical significance of ciRS-7 expression, we measured expression levels of ciRS-7 in 358 FFPE samples which consisted of 318 FFPE tissues from primary colorectal cancers, and 40 specimens from matched adjacent normal mucosa (NM) tissues, obtained from colorectal cancer patient cohorts that were enrolled at the Shanghai Tenth People's Hospital in China and Okayama University Medical Hospital in Japan. The baseline characteristics of these patient cohorts are described in Supplementary Table S1. The study design consisted of an initial training cohort (Shanghai Tenth People's Hospital) and a subsequent validation cohort (Okayama University Medical Hospital). The details were shown in Supplementary Materials and Methods. Written informed consent was obtained from all patients and the study was approved by the institutional review boards of all participating institutions. The median follow-up time of colorectal cancer patients in the training cohort was 3.7 years and was 5.1 years for the validation cohort. Patients treated with radiotherapy or chemotherapy before surgery were excluded from the study.

Quantitative reverse transcription PCR

For the genes and cirRS-7 expression analysis, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Fast SYBR Green Master Mix (Applied Biosystems) were used. The relative expression of target genes was determined by $2-\Delta C_t$ method as described previously (24). Previously designed primer sequences for U6 and ciRS-7 were used for quantitation (22, 25, 26). Other primer sequences used are shown in Supplementary Table S2. For miRNA analysis, qRT-PCR was conducted using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan Universal PCR Master Mix kit (Applied Biosystems) according to the manufacturer's instructions. The details were shown in Supplementary Materials and Methods.

Cell lines, oligos, and plasmids

The cell lines, oligos, and plasmids were shown in Supplementary Materials and Methods.

Transient transfection and construction of stable cell lines

For transient transfections, Lipofectamine 2000 (Invitrogen) and Opti-MEM (Gibco) were used according to the manufacturer's instructions. For the transfection studies, 30 nmol/L of miR-7 precursors were used for the overexpression of miR-7. To investigate the suppressive effect of ciRS-7 on miR-7 with different concentrations, two different concentrations (30 nmol/L and 60 nmol/L) of miR-7 precursors were used. For stable transfections, we first established ciRS-7 and negative vector stable–expressing HCT-116 and HT-29 cells using G148 selection methods as described previously (27, 28). The stable cell lines were then infected by miR-7 and negative control virus according to the manufacturer's instructions. The details were shown in Supplementary Materials and Methods.

Cell proliferation assay and colony formation assay

The details were shown in Supplementary Materials and Methods.

Cell invasion, migration, and apoptosis assay

Migration and invasion assays were performed using Boyden chambers (Corning) using 8-μm pore membrane coated with Matrigel (for invasion assays) or without Matrigel (for migration assays). For apoptosis assays, Muse Annexin V and dead cell kit (Millipore) were used according to the manufacturer's instructions (the details were shown in Supplementary Materials and Methods).

Xenograft animal studies

Male athymic nude mice were obtained from Harlan Laboratories at 5 weeks of age and kept under controlled conditions (12-hour light and dark cycles). The animal protocol was approved by the Institutional Animal Care and Use Committee of the Baylor Research Institute (Dallas, TX). The details were shown in Supplementary Materials and Methods.

IHC and Western blotting

For IHC, the staining was performed using Dako envision+-dual link system-HRP (DAB+; Dako) according to the manufacturer's instructions. For Western immunoblotting, the following primary antibodies were used: rabbit anti-EGFR (1:1,000 dilutions; Cell Signaling Technology), rabbit anti-phospho-Akt (1:1,000 dilutions; Cell Signaling Technology), rabbit anti-phospho-p44/42 MAPK (Erk1/2; 1:1,000 dilutions; Cell Signaling Technology), mouse anti-c-Raf (1:1,000 dilutions; 12552, Cell Signaling Technology) and monoclonal mouse anti-β-actin (1:5,000 dilutions; Sigma-Aldrich). The details were shown in Supplementary Materials and Methods.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 or Medcalc version 12.3 programs. Data were expressed as mean ± SD. Statistical differences between groups were determined by Wilcoxon signed rank test or the χ^2 test. Kaplan–Meier analysis and log-rank test was used to estimate and compare survival, defined by the time from surgery until death (patients alive were censored at the time of their last follow-up), of patients with ciRS-7–positive and ciRS-7–negative primary tumors. The Cox proportional hazards models were used to estimate HRs for death. All *P* values were two-sided, and those less than 0.05 were considered statistically significant.

Results

CiRS-7 is overexpressed in colorectal cancer

As no previous studies have evaluated the expression of ciRS-7 in colorectal cancer, we first measured its expression level in a subset of 40 matched pairs of cancer and normal mucosa specimens from colorectal cancer patients by qPCR using ciRS-7 specific primers as described previously (ref. 22; Fig. 1A). We found that ciRS-7 expression was significantly higher (2.4-fold increase, $P = 0.0018$) in cancer versus normal tissues (Fig. 1B), suggesting its potential oncogenic role in colorectal cancer.

High ciRS-7 expression correlates with advanced tumor stage, tumor depth, and metastasis in colorectal cancer patients

We next examined the expression patterns of ciRS-7 in the training and validation cohorts of 318 colorectal cancer patients representing various clinical stages of the disease. In the training cohort, we categorized all patients into ciRS-7 high- and low-expression groups using the median ciRS-7 expression as the cut-off threshold in all colorectal cancer patients. Interestingly, ciRS-7 expression was significantly higher in T4 stage patients ($P = 0.0179$; Supplementary Table S1). Furthermore, ciRS-7 expression in colorectal cancer stage II–IV patients was significantly higher than stage I patients ($P = 0.0020$, Fig. 1C). To further confirm the clinical significance of ciRS-7 in colorectal cancer, we used the cut-off value derived from the training cohort, to categorize all patients into ciRS-7–high and -low expression groups, and analyzed the correlation between expression of ciRS-7 and clinicopathologic variables. Consistent with these findings, in the validation cohort, higher ciRS-7 expression was found in patients with T4 disease ($P = 0.0429$) and more advanced II–IV stages ($P = 0.0002$, Fig. 1D). In addition, high ciRS-7 expression was significantly frequent in patients with lymph node involvement ($P < 0.0001$) and distant metastasis ($P = 0.0162$). Taken together, our data highlight the potential role of ciRS-7 as a novel, oncogenic, noncoding RNA that promotes the development of colorectal cancer.

High ciRS-7 expression is an important prognostic biomarker in colorectal cancer patients

We investigated the prognostic impact of ciRS-7 expression in two independent cohorts of colorectal cancer patients by time-to-event analysis using Kaplan–Meier estimations. High ciRS-7 expression correlated with significantly poor overall survival in the training cohort (log-rank test: $P = 0.0224$, Fig. 1E), and this correlation was subsequently confirmed in the validation cohort (log-rank test: $P = 0.0061$, Fig. 1F). Univariate regression analyses revealed that HRs for death in patients with ciRS-7 high versus low were 2.07 and 2.69 along with corresponding, CI = 1.0977–3.9023 and 1.2570–5.7405, $P = 0.0253$ and 0.0108 in the training and validation cohorts, respectively. Multivariate survival analysis revealed that ciRS-7 emerged as an independent risk factor for overall survival (HRs for death = 1.8689 and 2.7262, CI =1.0977–3.9023 and 1.0879–6.8315, $P = 0.0656$ and 0.0324 in the training and validation cohorts, respectively; Supplementary Table S3).Collectively, our data demonstrate that overexpression of ciRS-7 has important clinical significance as a promising prognostic biomarker in colorectal cancer patients.

CiRS-7 may serve as a potential therapeutic target through regulation of tumor-suppressive miR-7 in colorectal cancer

Although the molecular roles of circular RNAs in cancer are still evolving, it has been suggested that ciRS-7 may function as a miR-7 sponge (22, 23, 29). Hence, we assumed that ciRS-7 may suppress miR-7 activity and promote development of colorectal cancer. We ectopically overexpressed miR-7 together with ciRS-7 in HCT-116 and HT-29 cells to evaluate the regulation of miR-7 function by ciRS-7 (Supplementary Fig. S1). Although miR-7 overexpression alone showed significant tumor-suppressive activity (suppression of cell proliferation, migration, invasion, and induction of apoptosis), ciRS-7 overexpression dramatically reduced miR-7 tumor-suppressive function (Fig. 2; Supplementary Figs. S2 and S3), highlighting the novel observation for the ability of ciRS-7 to inhibit tumor-suppressive function of miR-7 in colorectal cancer.

To further assess and confirm whether ciRS-7 promotes its oncogenic potential through inhibition of miR-7 activity, we inoculated stable clones of HCT-116 cells expressing miR-7 alone, ciRS-7, alone or both subcutaneously into nude mice. As illustrated in Fig. 3A and B, tumors in mice injected with miR-7–overexpressing cells grew significantly slower compared with the controls, ciRS-7–overexpressing or ciRS-7+miR-7 double overexpressing tumors. Also, the average weight of miR-7–expressing tumors at the time of sacrifice was approximately half that of the other 3 groups. Likewise, miR-7/ciRS-7 double overexpressing tissues showed higher level of Ki67 and PCNA compared with miR-7 (Fig. 3C), highlighting the ability of ciRS-7 to neutralize the tumor-suppressive effect of miR-7 and suggesting its potential role as therapeutic target.

CiRS-7 activated EGFR/RAF1/MAPK pathway via suppression of miR-7 activity

As our data showed that ciRS-7 effectively quenched normal function of miR-7 to suppress colorectal tumorigenesis, we

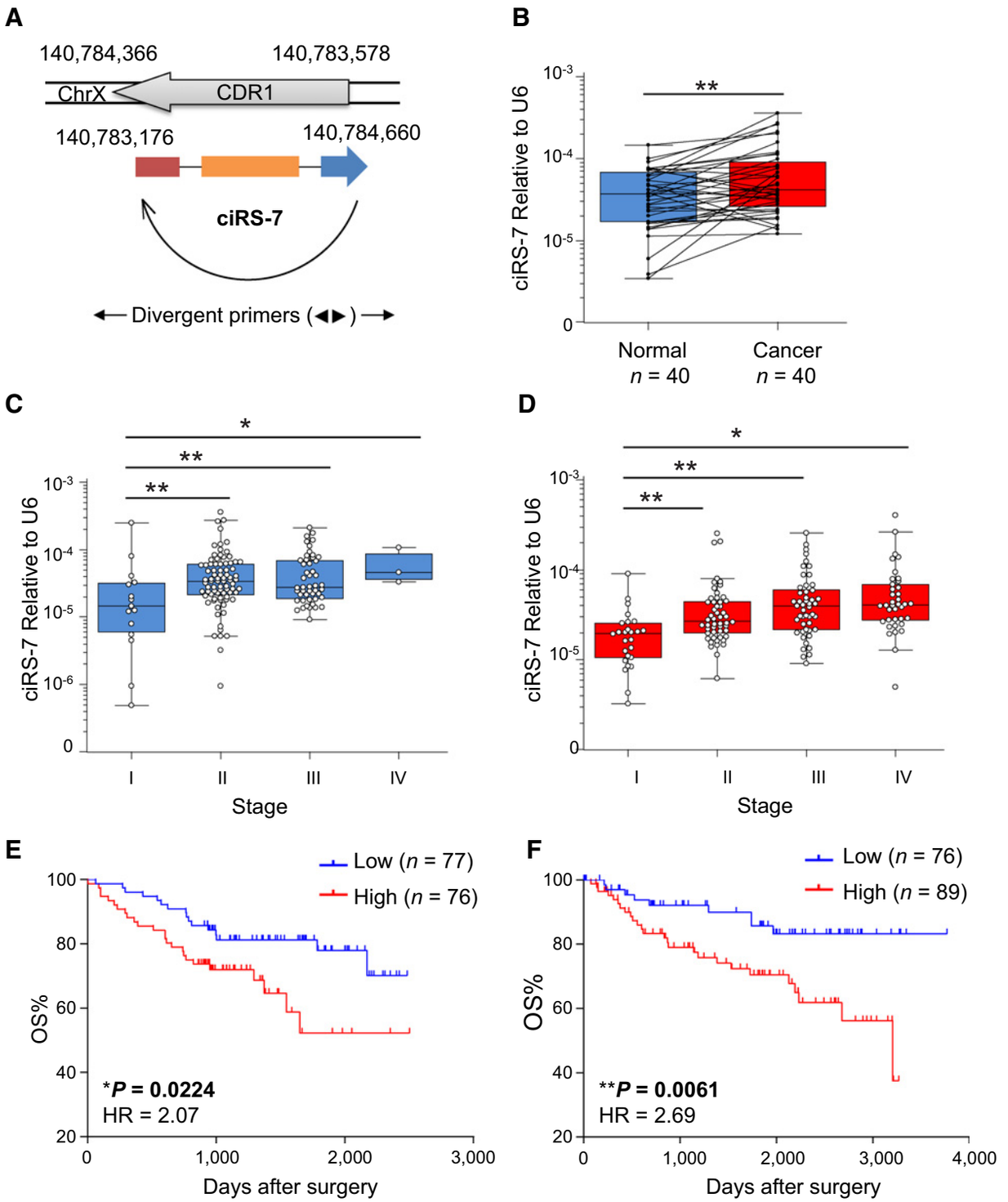

Figure 1.

CiRS-7 is overexpressed in colorectal cancer and correlates with poor prognosis. **A,** Schematic illustration of the ciRS-7 locus with specific divergent primers. **B,** Wilcoxon matched-pairs signed rank test showed ciRS-6 level is higher in colorectal cancer compared with adjacent normal tissues ($P = 0.0018$). The expression level of ciRS-7 was examined in cancer tissues from colorectal cancer (CRC) patients with I–IV stage from training cohort (**C**) and validation cohort (**D**). High level of ciRS-7 was found correlated with poor prognosis in training cohort (**E**) and validation cohort (**F**). Colorectal cancer patients were divided into high and low expression groups based upon median cut-off values established from training cohort. The overall survival (OS) analysis was performed by Kaplan–Meier analysis and log-rank method. (*, $P < 0.05$; **, $P < 0.01$).

hypothesized that ciRS-7 may be responsible for enhancing the expression levels of miR-7 targets by acting as a miR-7 sponge and facilitating a more aggressive phenotype in colorectal cancer patients. To prove our hypothesis, we first tested a panel of well-established miR-7 target genes (15, 16, 20, 30–37) in HCT-116 and HT-29 cells. Interestingly, we noticed a

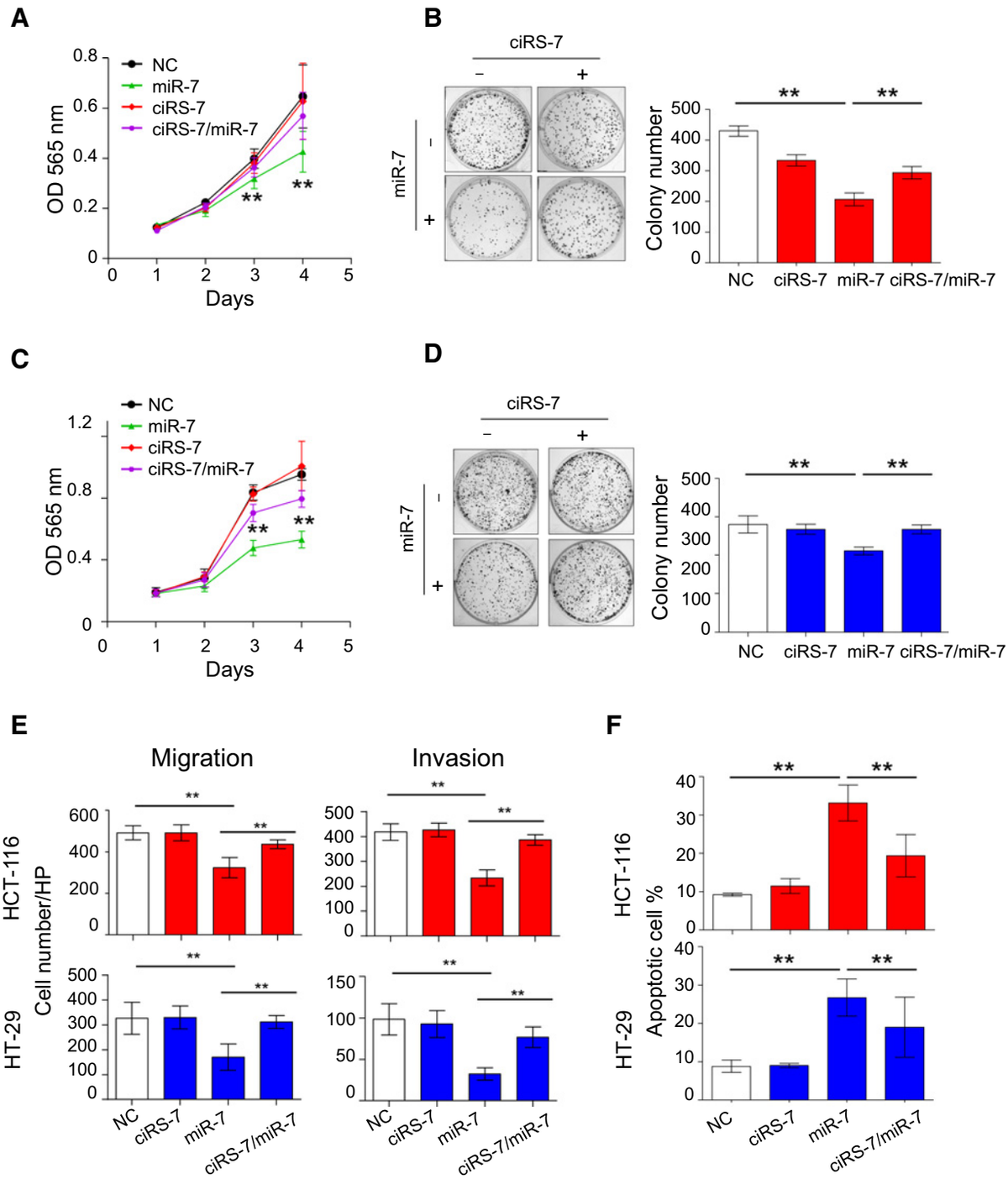

Figure 2.

CiRS-7 inhibits tumor-suppressive effects of miR-7 *in vitro*. MTT assay and colony formation assays were performed in HCT-116 (**A** and **B**) and HT-29 cells (**C** and **D**) with overexpression of miR-7 alone, ciRS-7 alone, or both ($n = 6$, *, $P < 0.05$; **, $P < 0.01$; independent t test was used to compare control and treated cells). **E,** Migration and Invasion assays showed miR-7 overexpression inhibited migration and ability of HCT-116 and HT-29 cells; such suppressive effect was neutralized by ciRS-7 overexpression in colorectal cancer cells. **F,** CiRS-7 overexpression reduced apoptotic cells, which was induced by miR-7 ($n = 3$, *, $P < 0.05$; **, $P < 0.01$; independent t test was used to compare control and treated cells).

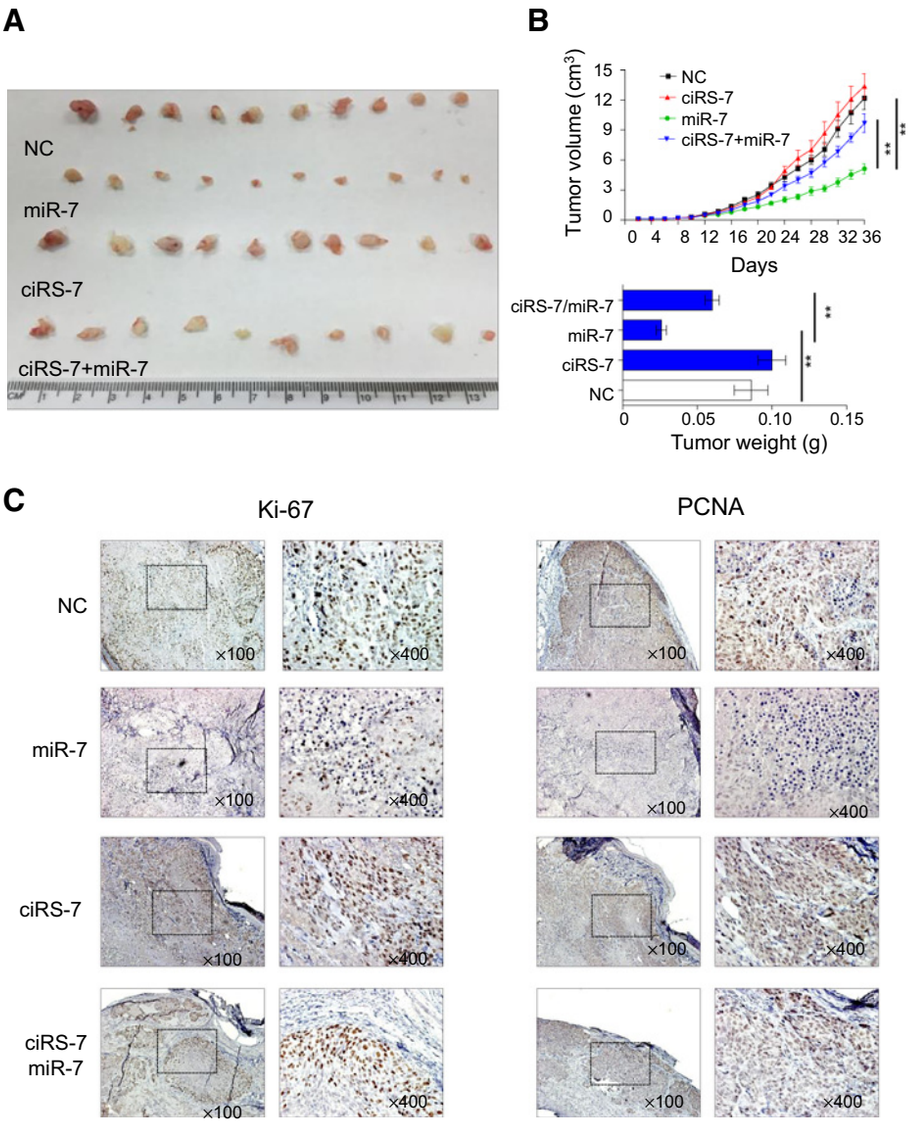

Figure 3.

CiRS-7 regulates miR-7 activity in xenograft animal models. Stably transfected HCT-116 cells were inoculated with miR-7 alone, ciRS-7 alone, or both subcutaneously into nude mice. **A** and **B**, The tumor growth curve and average weight of tumors at the time the animals were sacrificed in different treatment groups (*, $P < 0.05$; **, $P < 0.01$; paired *t* test was used to compare control and treated cells). **C**, Expression level of Ki67 and PCNA in xenograft tissues from different treatment groups.

significantly decreased expression of EGFR and RAF1 subsequent to overexpression of miR-7 (Supplementary Fig. S4). Considering the important role of EGFR/RAF1/MAPK pathway in carcinogenesis, we deduced that ciRS-7 could be a major contributor for colorectal cancer development via its ceRNA activity. The normal function of miR-7 is to prevent activation of EGFR/RAF1/MAPK pathway in intestinal epithelial cells. However, persistent upregulation of ciRS-7 sequestered miR-7 and thereby lead to the accumulation of growth signaling by driving colorectal tumor growth.

To test our hypothesis, we measured EGFR and RAF1 expression in HCT-116 and HT-29 cells transfected with pre-miR-7 alone or together with ciRS-7. In line with our hypothesis, miR-7 overexpression led to decreased mRNA and protein expression levels of EGFR and RAF1, while ciR-7 attenuated this inhibitory effect of miR-7 on EGFR and RAF1 expression (Fig. 4A). Likewise, inhibition of Erk phosphorylation by miR-7 was also attenuated by ciRS-7, confirming that ciRS 7 activates EGFR/RAF1/MAPK pathway through suppresion of miR-7 (Fig. 4B) in colorectal cancer, which has significant therapeutic implications for this malignancy.

To further validate our *in vitro* results that ciRS-7 regulated EGFR1/RAF1/MAPK pathway through suppression of miR-7 activity, we investigated the expression correlation between miR-7, ciRS-7, and EGFR/RAF1 in colorectal cancer tissues. We noticed that ciRS-7 expression was negatively correlated with miR-7 expression in colorectal cancer ($P = 0.0003$), suggesting that the loss of function of miR-7 is not only due to its lowered expression, but also due to intimate association with upregulated ciRS-7 expression in this malignancy (Fig. 5A). As expected, we also found miR-7 expression negatively correlated with EGFR ($P = 0.0014$) and RAF1 ($P < 0.0001$) in cancer tissues, indicating that miR-7 suppress EGFR and RAF1 expression in CRC (Fig. 5B and C). Notably, we observed ciRS-7 overexpression was significantly associated with upregulation of EGFR ($P < 0.0001$) and RAF1 ($P < 0.0001$) in colorectal cancer (Fig. 5D–F), highlighting the clinical significance of these results, as these suggest that the upregulation

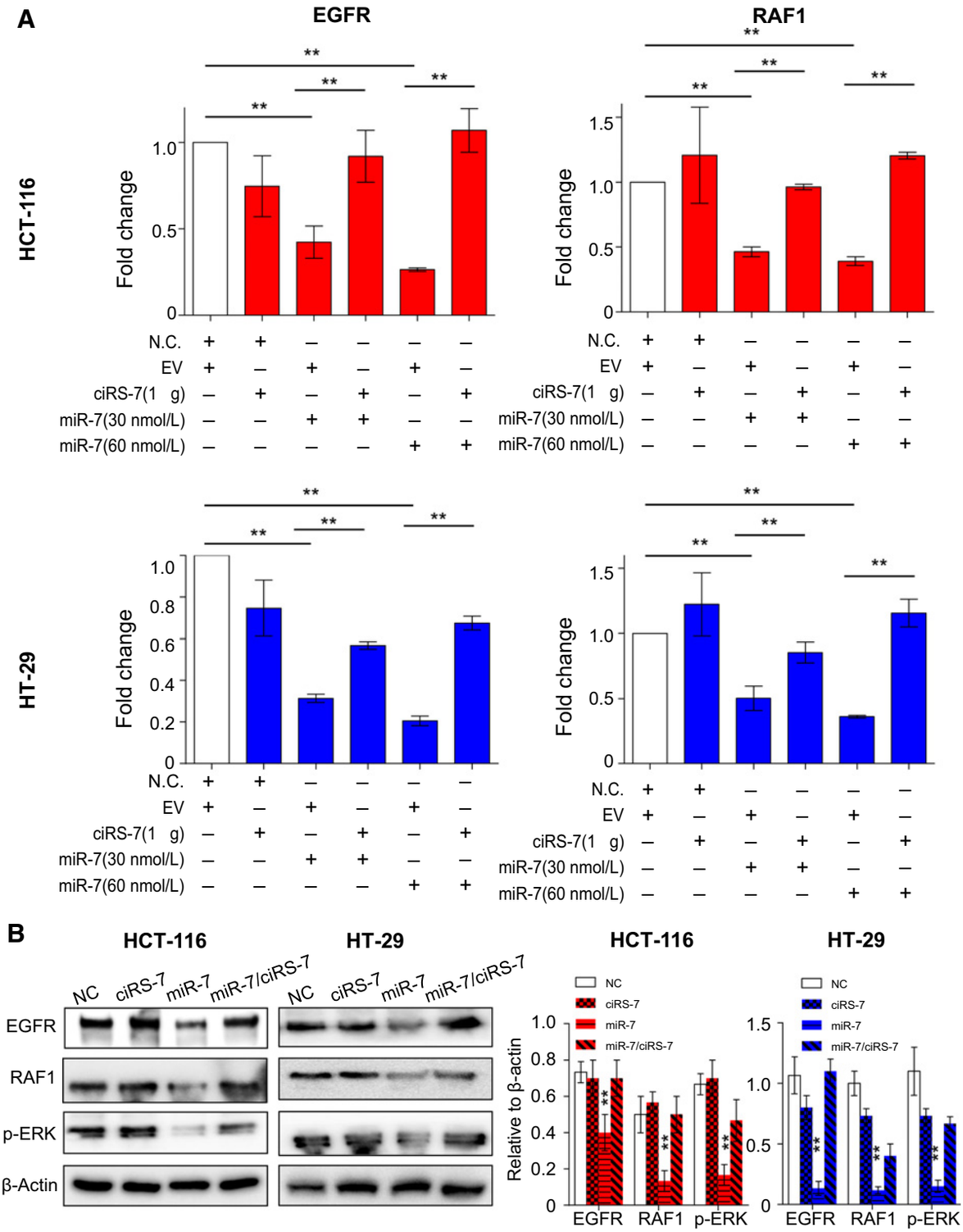

Figure 4.

CiRS-7 activated EGFR/RAF1 pathway via inhibition of miR-7 activity. HCT-116 and HT-29 cells were transfected with miR-7 precursor (30 nmol/L and 60 nmol/L) alone or combined with ciRS-7 (1 μg). **A,** The mRNA expression level of EGFR and RAF1 was examined by qPCR in different treatment groups. The results showed miR-7 targets EGFR and RAF1 were downregulated in a dose-dependent manner. CiRS-7 overexpression endues the colorectal cancer cells with resistance to miR-7 treatment. **B,** Western blotting assay, the expression level of EGFR/RAF1/MAPK pathway was suppressed by miR-7 overexpression in colorectal cancer cells, but such repressive effect was abolished by ciRS-7 overexpression ($n = 3$, *, $P < 0.05$; **, $P < 0.01$; independent t test was used to compare control and treated cells).

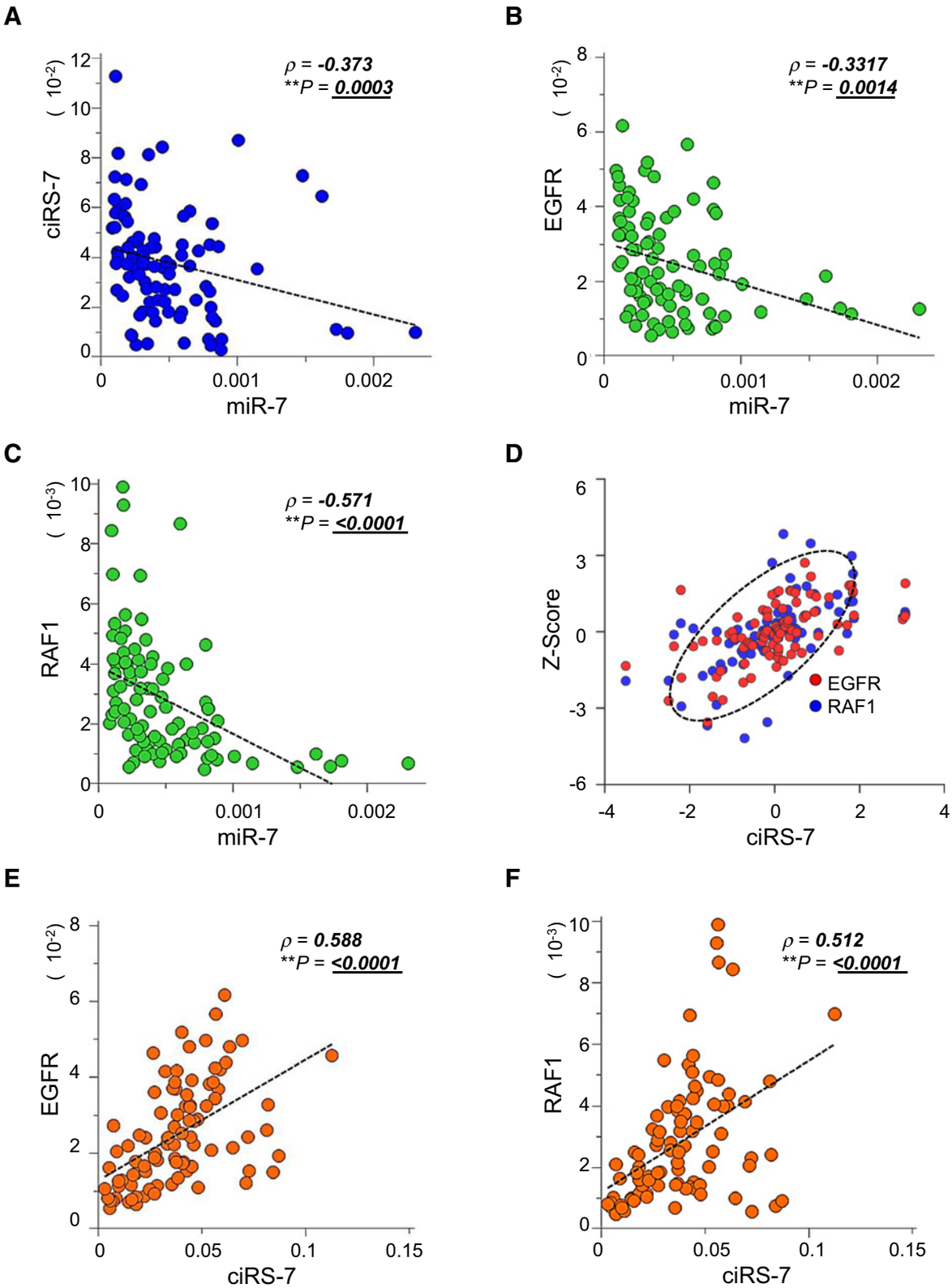

Figure 5.

The correlation between ciRS-7, miR-7, and EGFR/RAF1 in colorectal cancer (CRC) tissues. **A,** ciRS-7 expression was negatively correlated with miR-7 expression in colorectal cancer. **B** and **C,** miR-7 expression negatively correlated with EGFR and RAF1 in colorectal cancer tissues. **D–F,** ciRS-7 overexpression was significantly associated with upregulation of EGFR and RAF1 [$n = 90$, *, $P < 0.05$; **, $P < 0.01$; Spearman rank correlation (ρ) was used for the correlation analysis].

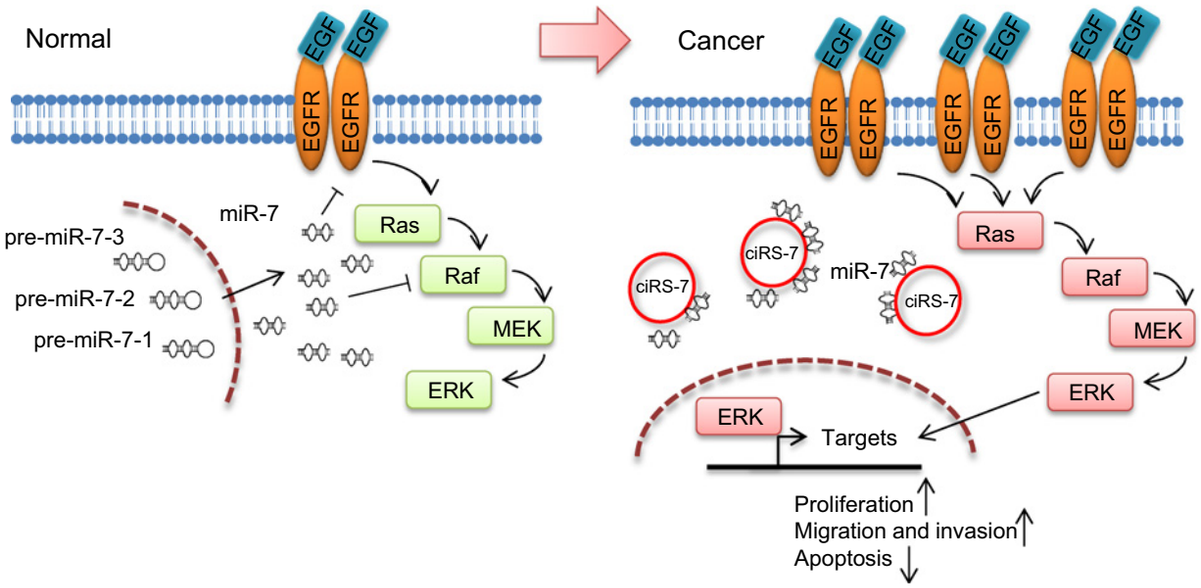

Figure 6.
A proposed mechanistic model as to how ciRS-7 functions as a miR-7 sponge and regulates EGFR/RAF1/MAPK pathway via inhibiting miR-7 activity. The normal function of miR-7 is to prevent excess activation of EGFR/RAF1/MAPK pathway in intestinal epithelial cells. Instead, persistent upregulation of ciRS-7 sequestered miR-7 and thereby led to the accumulation of growth signaling to drive colorectal tumor growth.

of ciRS-7 abrogates the tumor-suppressive effect of miR-7 on its downstream targets, EGFR and RAF1, in colorectal cancer.

Discussion

In this study, we first discovered that ciRS-7 is frequently upregulated in colorectal cancer, and its expression significantly correlated with several clinicopathologic variables. Second, our data showed that high expression of this noncoding RNA correlated with poor patient outcomes, highlighting its applicability as a promising prognostic biomarker in colorectal cancer. Third, from a biological perspective, we demonstrated that overexpression of ciRS-7 disrupted normal function of miR-7 and thus promoted aggressiveness of colorectal cancer cell lines. Fourth, we unraveled a novel mechanism that ciRS-7 acts as a ceRNA and regulates EGFR/RAF1 pathways by competing for miR-7. Considering the crucial role of EGFR/RAF1/MAPK signaling in development of colorectal cancer, our results for the first time revealed the therapeutic importance of ciRS-7 in this malignancy.

Circular RNAs (circRNA), a novel class of endogenous noncoding RNAs, are emerging as frontiers in cancer research. To date, ciRS-7 is one of few known circRNAs that has been proposed to inhibit tumor suppressor miR-7. Widespread expression of ciRS-7 in neuroblastomas, astrocytoma, renal cell, and lung carcinomas (29) implicates the role of ciRS-7 as a critical regulator in cancer. Our results for the first time clearly demonstrate that ciRS-7 is frequently upregulated in colorectal cancer compared with normal colon mucosa, an indirect observation in a previous study through RNA-seq analysis (38). Furthermore, our data provide convincing evidence that ciRS-7 contributes to the aggressive clinical colorectal cancer phenotype. Our data demonstrate that higher expression of ciRS-7 correlated with multiple clinicopathologic factors such as advanced T-stage, lymph node, and distant metastasis, and consequently, patients with high ciRS-7 expression had a worse prognosis than those with low expression. It is noteworthy to mention that patients in the training and validation cohorts had different pathologic T category, lymph node metastasis, and tumor stage. Nonetheless, in spite of such underlying tumor heterogeneity, the oncogenic role of ciR-7 was more significantly supported by our consistent clinical data.

To better appreciate the biological significance of ciRS-7 for its contribution to colorectal carcinogenesis, we investigated the role of miR-7 in colorectal cancer. As expected, online pathway prediction (39, 40) revealed that majority of miR-7 targets are involved in cancer pathways (Supplementary Fig. S5A). In line with our findings, data from publicly available TCGA database also revealed that low miR-7 expression in colorectal cancer patients also correlated with poor prognosis (Supplementary Fig. S5B). Consistent with this paradigm, our functional assays clearly showed that miR-7 exerts tumor-suppressive effect in colorectal cancer. We therefore hypothesized that ciRS-7 could interfere with the tumor-suppressive effect of miR-7 in colorectal cancer through its ceRNA activity. Accordingly, our results successfully proved our hypothesis, whereby overexpression of ciRS-7 in colorectal cancer cells completely abolished the tumor-suppressive function of miR-7.

More importantly, we found miR-7–mediated suppression of EGFR/RAF1/MAPK pathway could be alleviated by ciRS-7 overexpression in colorectal cancer. EGFR/RAF1/MAPK pathway is a well-known oncogenic pathway which correlates with metastasis and reduced survival rates in colorectal cancer. Indeed, anti-EGFR therapy has become clinically routine treatment, particularly for the treatment of advanced colorectal cancer. Our results suggest that miR-7 could effectively suppress this pathway in colorectal cancer cell lines despite the mutational status of the KRAS or BRAF

oncogenes. Notably, KRAS and BRAF mutations are present in HCT-116 and HT-29 cells, respectively (41); however, mir-7 still has a strong inhibitory effect on the EGFR/RAF1/MAPK pathway because miR-7 could successfully reduce expression levels of not only EGFR but also another important MAPK member RAF1. RAF1, similar to BRAF, contributes to activation of MAPK pathway, but is rarely mutated in colorectal cancer (42), and a recent study showed inhibition of RAF1 as a promising therapeutic strategy for BRAF- and KRAS-mutant cancers (43). These findings suggest miR-7 is a critical negative regulator of EGFR/RAF1/MAPK pathway. Interestingly, our results showed that ciRS-7 leads to persistent activation of EGFR/RAF1/MAPK pathway in colorectal cancer cells, regardless of treatment with low or high concentrations with miR-7 precursors supporting the hypothesis that ciRS-7 enhances this key oncogenic pathway through inhibition of miR-7 activity (Fig. 6). Therefore, dual targeting ciRS-7 and miR-7 could provide a new therapeutic strategy to suppress this oncogenic pathway for colorectal cancer patients.

In summary, this is the first study to systematically interrogate the functional and clinical significance of ciRS-7 in colorectal cancer, and we provide comprehensive evidence that it acts as a novel oncogenic circRNA, as well as a prognostic biomarker in colorectal cancer. From a functional perspective, ciRS-7 impairs the tumor-suppressive effects of miR-7 in colorectal cancer cells and in xenograft animal model. Mechanistically, overexpression of ciRS-7 enhanced EGFR/RAF1/MAPK pathway through inhibition of miR-7 activity. We conclude that ciRS-7 is a promising prognostic biomarker for colorectal cancer patients; therapeutic targeting of ciRS-7 maybe a potential treatment option for patients with colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Goel
Development of methodology: A. Goel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Toden, K. Yoshida, T. Nagasaka, T. Fujiwara, H. Qin, A. Goel
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Cai, H. Qin
Writing, review, and/or revision of the manuscript: A. Goel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Goel
Study supervision: A. Goel

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