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Circular RNA ciRS-7 – A promising prognostic biomarker and a potential therapeutic target in colorectal cancer

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Abstract

Purpose—Colorectal cancer (CRC) is one of the most common malignancies worldwide. Recently, a novel circular RNA, ciRS-7, was proposed to be a potential miR-7 sponge. Since miR-7 regulates the expression of several important drivers of CRC, we analyzed the clinical significance of ciRS-7 in CRC patients.

Experimental Design—Initially, we evaluated the expression levels of ciRS-7 in a training cohort comprising of 153 primary CRC 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 targets genes EGFR and RAF1. Functional analysis were performed in cell lines and an animal model to support clinical findings.

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Author contribution: Study concept and design: WWH, YM, AG; Specimen provider: KY, YM, QW, TN, TF; Acquisition of clinical data: KY, YM, QW, TN, TF; Analysis and interpretation of data and statistical analysis: YM, SC, QW, WWH and AG; Animal experiments: ST and WWH; Drafting of the manuscript: WWH and AG.

Results—Our data revealed that ciRS-7 was significantly up-regulated in CRC 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 blocking of tumor suppressive effects of miR-7 and resulted in a more aggressive oncogenic phenotype, and ciRS-7 overexpression permitted inhibition of miR-7 and subsequent activation of EGFR and RAF1 oncogenes.

Conclusions—CiRS-7 is a promising prognostic biomarker in CRC patients and may serve as a therapeutic target for reducingEGFR-RAF1 activity in CRC patients.

Keywords

Colorectal cancer; circular RNA; ciRS-7; miR-7; miRNA; EGFR; RAF1

INTRODUCTION

Colorectal cancer (CRC) 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 CRC pathogenesis and can be used as biomarkers for diagnosis, prognosis and metastasis-prediction in CRC 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 “cancer-driven” pathways (17, 18). In CRC, miR-7 was demonstrated as a tumor suppressor, and its down-regulation 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 mechanisms for its dysregulation in CRC, which still remains vastly unclear and unexplored.

It has become increasingly clear that RNA transcripts that 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 (ceRNAs). Accumulating evidence is now unveiling 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 soak up 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 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 present study, we have made first attempts to fill this gap in knowledge for the molecular contribution of ciRS-7 in CRC. 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 CRC patients. In addition, we performed a systematic and comprehensive functional analysis for the function of ciRS-7 in CRC, 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 CRC patients, and therapeutic targeting of ciRS-7 maybe a potential strategy for the management of CRC patients.

MATERIAL 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) CRC 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 (pCRC), and 40 specimens from matched adjacent normal mucosa (NM) tissues, obtained from CRC patient cohorts that were enrolled at the Shanghai Tenth People's Hospital in China and Okayama University Medical Hospital in Japan. The baseline characteristic of these patient cohorts is 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 CRC 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 Polymerase Reaction (qRT-PCR)

For the genes and cirRS-7 expression analysis, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) were used. The relative expression of target genes was determined by 2-Δct 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, Foster City, CA) and TaqMan® Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA) according to 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, Carlsbad, CA) and Opti-MEM (Gibco, Carlsbad, CA) were used according to the manufacturer's instructions. For the transfection studies, 30nM of miR-7 precursors were used for the functional assays. To investigate the suppressive effect of ciRS-7 on miR-7 with different concentrations, two different concentrations (30nM and 60nM) 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, Corning, NY) using 8 μm-size 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, Billerica, MA) 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 (Houston, TX) at 5 weeks of age and kept under controlled conditions (12 h light and dark cycles). The animal protocol was approved by the Institutional Animal Care and Use Committee of the Baylor Research Institute. The details were shown in Supplementary materials and methods.

Immunohistochemistry (IHC) and Western blotting (WB)

For IHC, the staining was performed using Dako envision+dual link system-HRP (DAB+) (Dako, Carpinteria, CA) according to the manufacturer's instructions. For western immunoblotting, the following primary antibodies were used: rabbit anti-EGFR (1:1000 dilutions; Cell Signaling, Boston, MA), rabbit anti-Phospho-Akt (1:1000 dilutions; Cell Signaling, Boston, MA), rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (1:1000 dilutions; Cell Signaling, Boston, MA), mouse anti-c-Raf (1:1000 dilutions; 12552, Cell Signaling, Boston, MA) and monoclonal mouse anti-β-actin (1:5000 dilutions; Sigma-Aldrich, St. Louis, MO). 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's 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's proportional

hazards models were used to estimate hazard ratios (HRs) for death. All *P* values were 2-sided, and those less than 0.05 were considered statistically significant.

RESULTS

CiRS-7 is overexpressed in colorectal cancer

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

High CiRS-7 expression correlates with advanced tumor stage, tumor depth and metastasis in CRC patients

We next examined the expression patterns of ciRS-7 in the training and validation cohorts of 318 CRC 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 cutoff threshold in all CRC patients. Interestingly, ciRS-7 expression was significantly higher in T4 stage patients (P=0.0179, Supplementary Table S1). Furthermore, ciRS-7 expression in CRC stage II~IV patients was significantly higher than stage I patients (P=0.0020, Figure 1C). To further confirm the clinical significance of ciRS-7 in CRC, we used the cutoff 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 clinicopathological 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, Figure 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, non-coding RNA that promotes the development of CRC.

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

We investigated the prognostic impact of ciRS-7 expression in two independent cohorts of CRC 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, Figure 1E), and this correlation was subsequently confirmed in the validation cohort (log-rank test: P=0.0061, Figure 1F). Univariate regression analyses revealed that HRs for death in patients with ciRS-7 high vs. 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 CRC patients.

CiRS-7 may serve as potential target through regulation of tumor suppressive miR-7 in CRC

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 Figure 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 (Figure 2, Supplementary Figure S2–3), highlighting the novel observation for the ability of ciRS-7 to inhibit tumor suppressive function of miR-7 in CRC.

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 Figure 3A–B, tumors in mice injected with miR-7 overexpressing cells grew significantly slower compared to 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 to miR-7 (Figure 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

Since our data showed that ciRS-7 effectively quenched normal function of miR-7 to suppress colorectal tumorigenesis, we 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 CRC patients. To prove our hypothesis, we first tested a panel of well-established miR-7 target genes (Reference#(15, 16, 20,30–37)) in HCT-116 and HT-29 cells. Interestingly, we noticed a significantly decreased expression of EGFR and RAF1 subsequent to overexpression of miR-7 (Supplementary Figure S4). Considering the important role of EGFR/RAF1/MAPK pathway in carcinogenesis, we deduced that ciRS-7 could be a major contributor for CRC 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 up-regulation 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 assumption, miR-7 over-expression 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 (Figure 4A). Likewise, inhibition of Erk phosphorylation by miR-7 was also attenuated by ciRS-7, confirming that suppression of miR-7 function by ciRS-7 activates EGFR1/RAF1/MAPK pathway (Figure 4B) in CRC, which has significant therapeutic implications for this malignancy.

To further validate our in vitro results that ciRS-7 regulated EGFR1/RAF1/MAPK pathway through miR-7 sponge 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 CRC (P=0.0003), suggesting that the loss of function of miR-7 may be not only due to its lowered expression, but also due to intimate association with upregulated ciRS-7 expression in this malignancy (Figure 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 could suppress EGFR and RAF1 expression in vivo (Figure 5B–C). Notably, we observed ciRS-7 overexpression was significantly associated with up-regulation of EGFR (P<0.0001) and RAF1 (P<0.0001) in CRC (Figure 5D–F), highlighting the clinical significance of these results, as these suggest that the up-regulation of ciRS-7 abrogates the tumor suppressive effect of miR-7 on its downstream targets, EGFR and RAF1, in CRC.

DISCUSSION

In this study, we first discovered that ciRS-7 is frequently upregulated in CRC, and this significantly correlated with several clinico-pathological 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 CRC. Third, from a biological perspective, we demonstrated that overexpression of ciRS-7 disrupted normal function of miR-7 and thus promoted aggressiveness of CRC 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 (circRNAs), which represent 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 up-regulated in colorectal cancer compared to normal colon mucosa, an observation, which was indirectly suggested in a previous study through RNA-seq analysis(38). Furthermore, our data provide convincing evidence that ciRS-7 contributes to the aggressive clinical CRC phenotype. Our data demonstrate that higher expression of ciRS-7 correlated with multiple clinic-pathological 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 pathological 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, the role of miR-7 in CRC should be considered. As expected, online pathway prediction (39, 40) revealed that majority of miR-7 targets are involved in cancer pathways (Supplementary Figure 5A). In line with our findings, data from publically available TCGA database also revealed that low miR-7 expression in CRC patients also correlated with poor prognosis (Supplementary Figure 5B). Consistent with this paradigm, our functional assays clearly showed that miR-7 exerts tumor suppressive effect in CRC. We therefore assumed that ciRS-7 could interfere with the tumor suppressive effect of miR-7 in CRC through its ceRNA activity. Accordingly, our results successfully proved our assumption, whereby overexpression of ciRS-7 in CRC 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 CRC. EGFR/RAF1/MAPK pathway is a well-known oncogenic pathway which correlates with metastasis and reduced survival rates in CRC. Indeed, anti-EGFR therapy has become clinically routine treatment, particularly for the treatment of advanced CRC. Our results suggest that miR-7 could effectively suppress this pathway in CRC 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 CRC(42), and recent study showed 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 CRC 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 (Figure 6). Therefore, dual targeting ciRS-7 and miR-7 could provide a new therapeutic strategy to suppress this oncogenic pathway for CRC patients.

In summary, ours 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 CRC. From a functional perspective, ciRS-7 impairs tumor suppressive effects of miR-7 in CRC 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 CRC patients; therapeutic targeting of ciRS-7 maybe a potential treatment option for patients with CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 CRC tissues, and its up-regulation 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 blocking of tumor suppressive effects 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 CRC patients, and provide novel evidence that therapeutic targeting of this circular RNA may be a potential treatment approach in CRC patients.

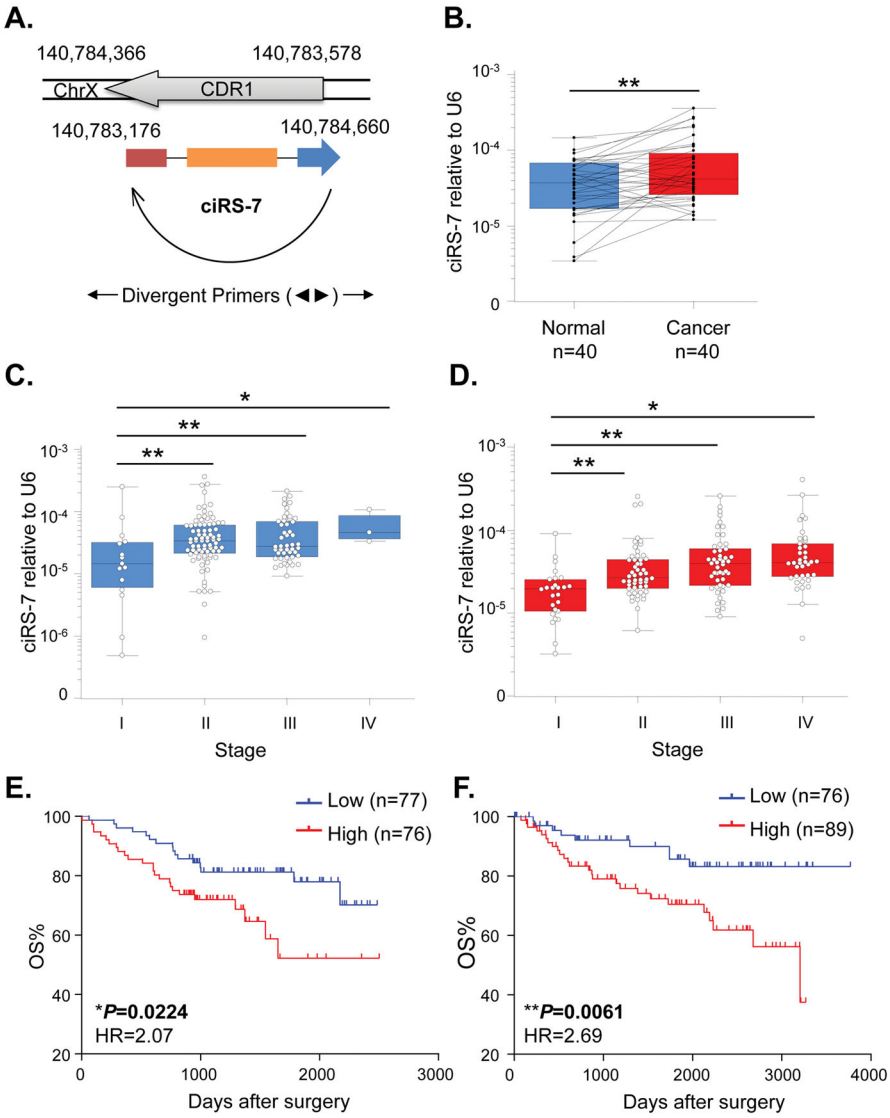

Figure 1.

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 to adjacent normal tissues (P=0.0018). The expression level of ciRS-7 was examined in cancer tissues from 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). CRC patients were divided into high- and low-expression groups based upon median cutoff 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; HR: Hazard Ratio)

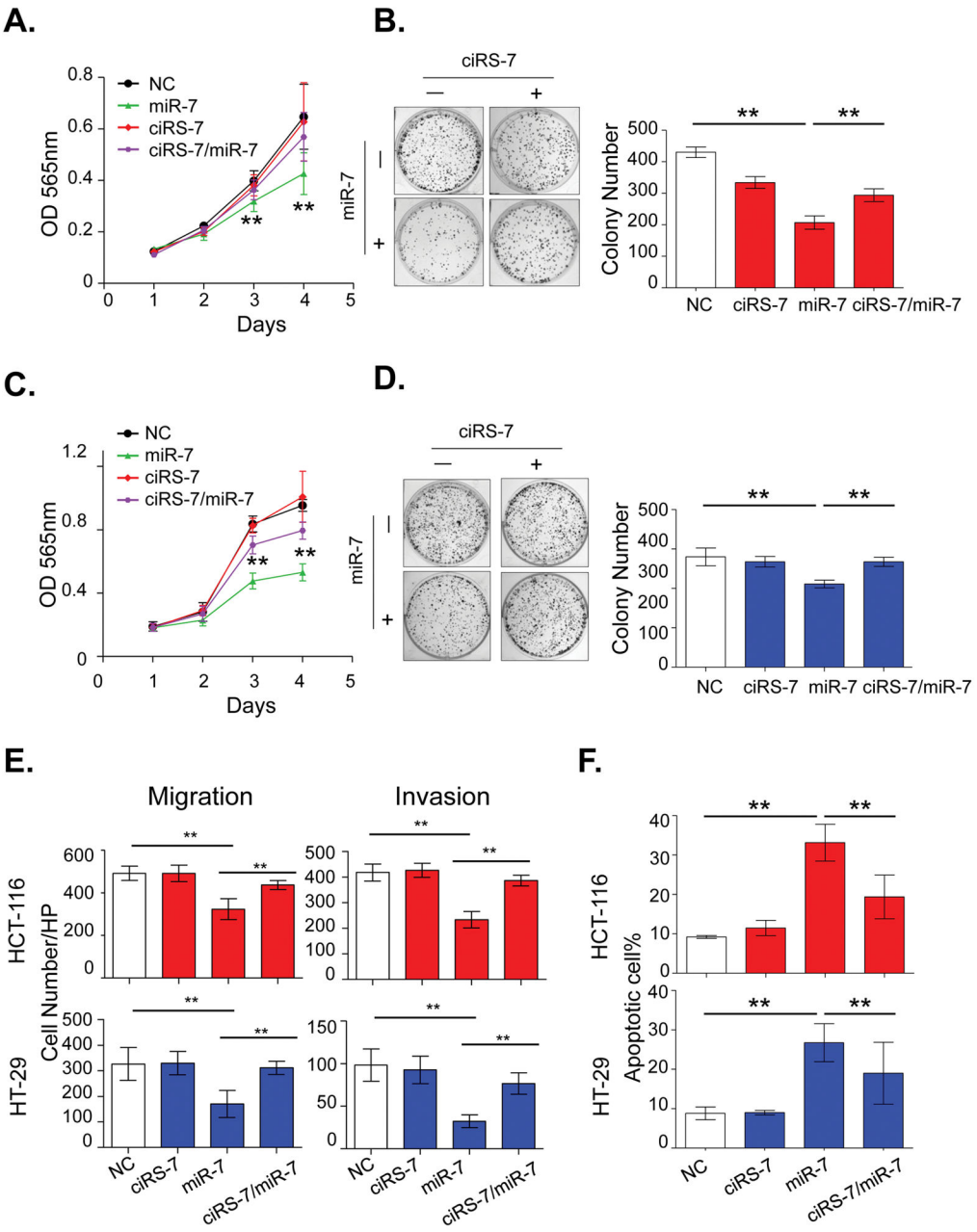

Figure 2.

CiRS-7 inhibits tumor suppressive effects of miR-7 in vitro. MTT assay and Colony formation assay was performed in HCT-116 (A–B) and HT-29 cells (C-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) Transwell and transwell-matrigel assay 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 CRC 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)

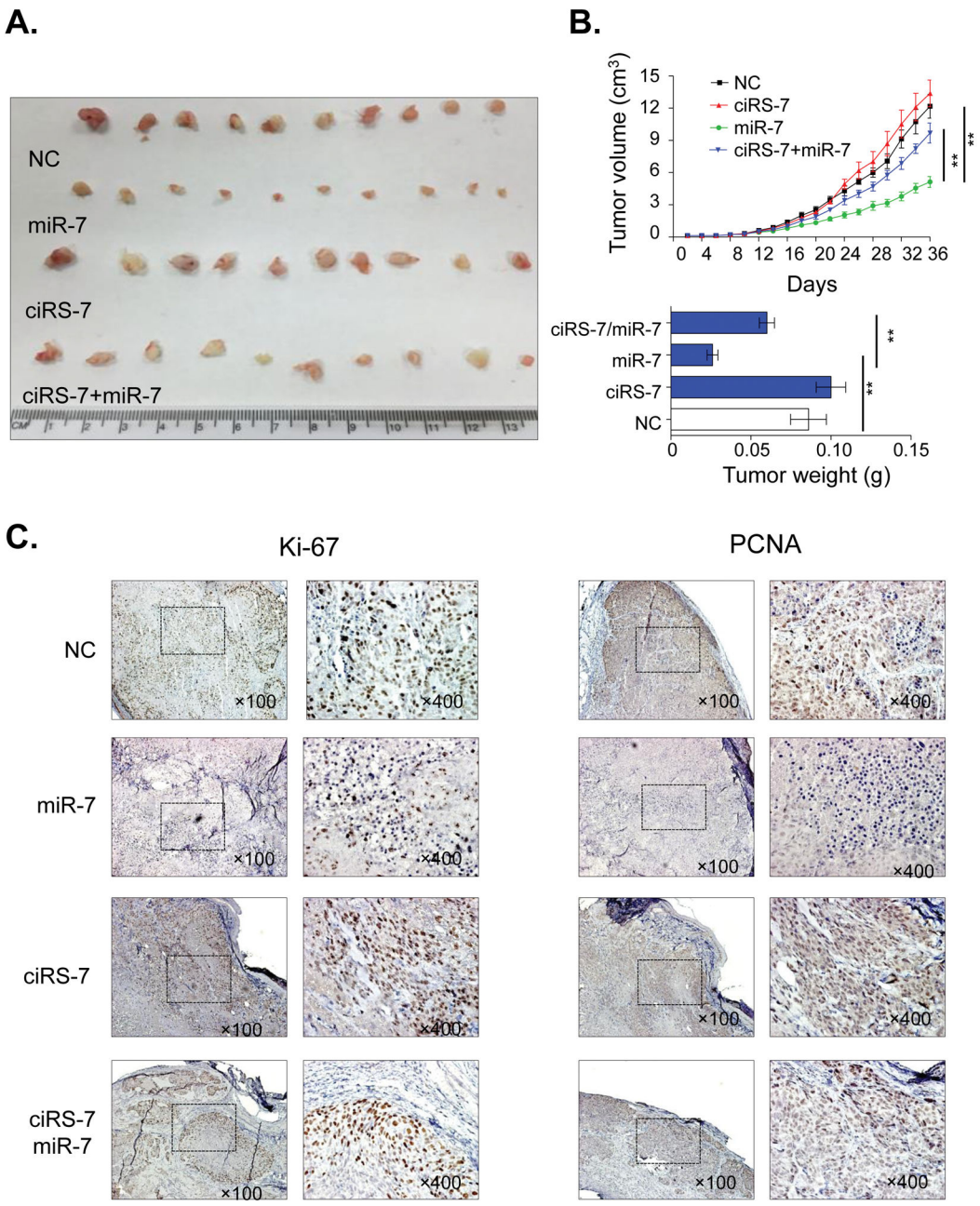

Figure 3.

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. Figure (A–B) depict 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). Figure (C) demonstrates expression level of Ki67 and PCNA in xenograft tissues from different treatment groups.

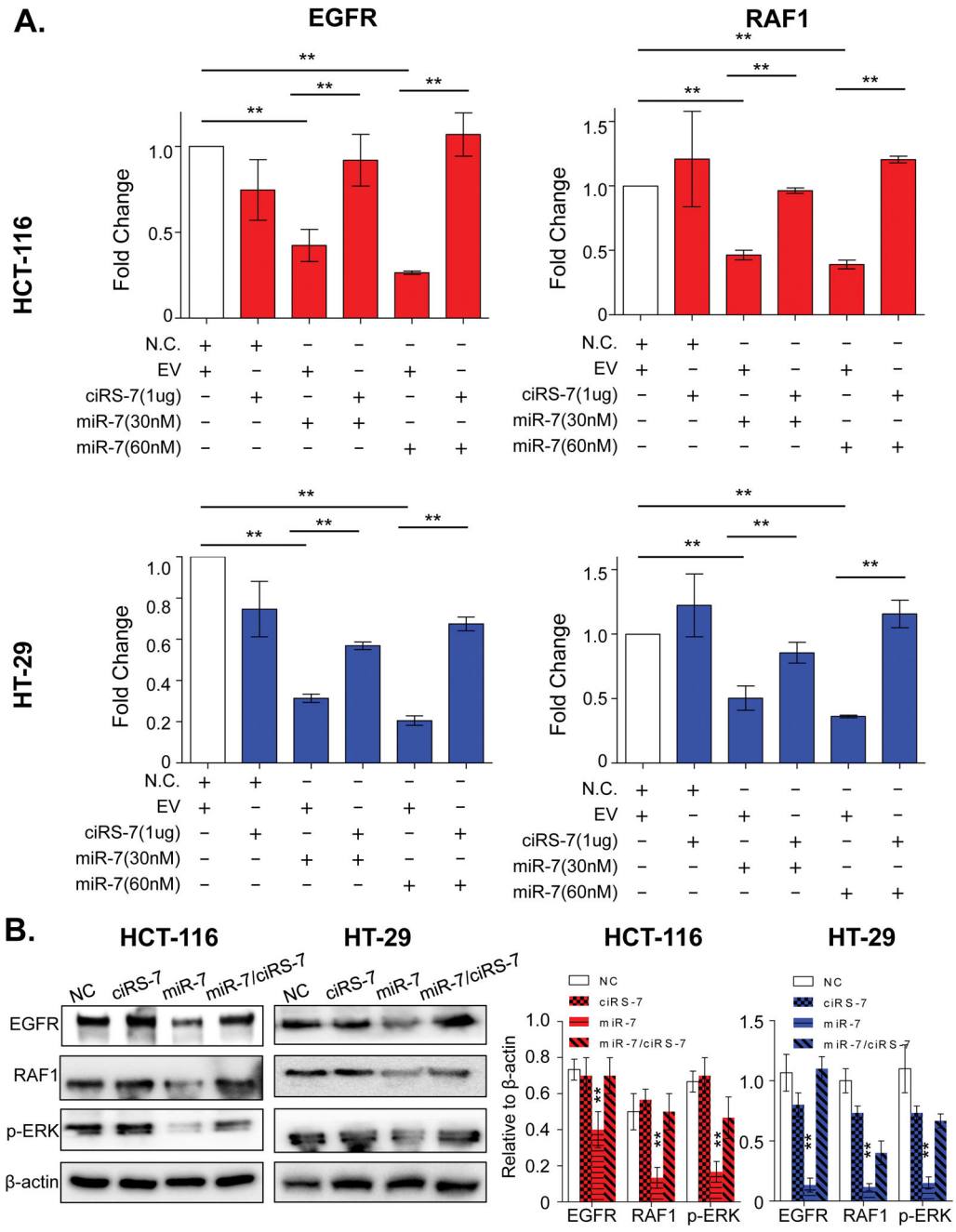

Figure 4.

CiRS-7 activated EGFR/RAF1 pathway via inhibition of miR-7 activity. HCT-116 and HT-29 cells were transfected with miR-7 precursor (30nM and 60nM) alone or combined with ciRS-7 (1μg). (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 was down-regulated in a dose-dependent manner. CiRS-7 overexpression endues the CRC 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 CRC 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)

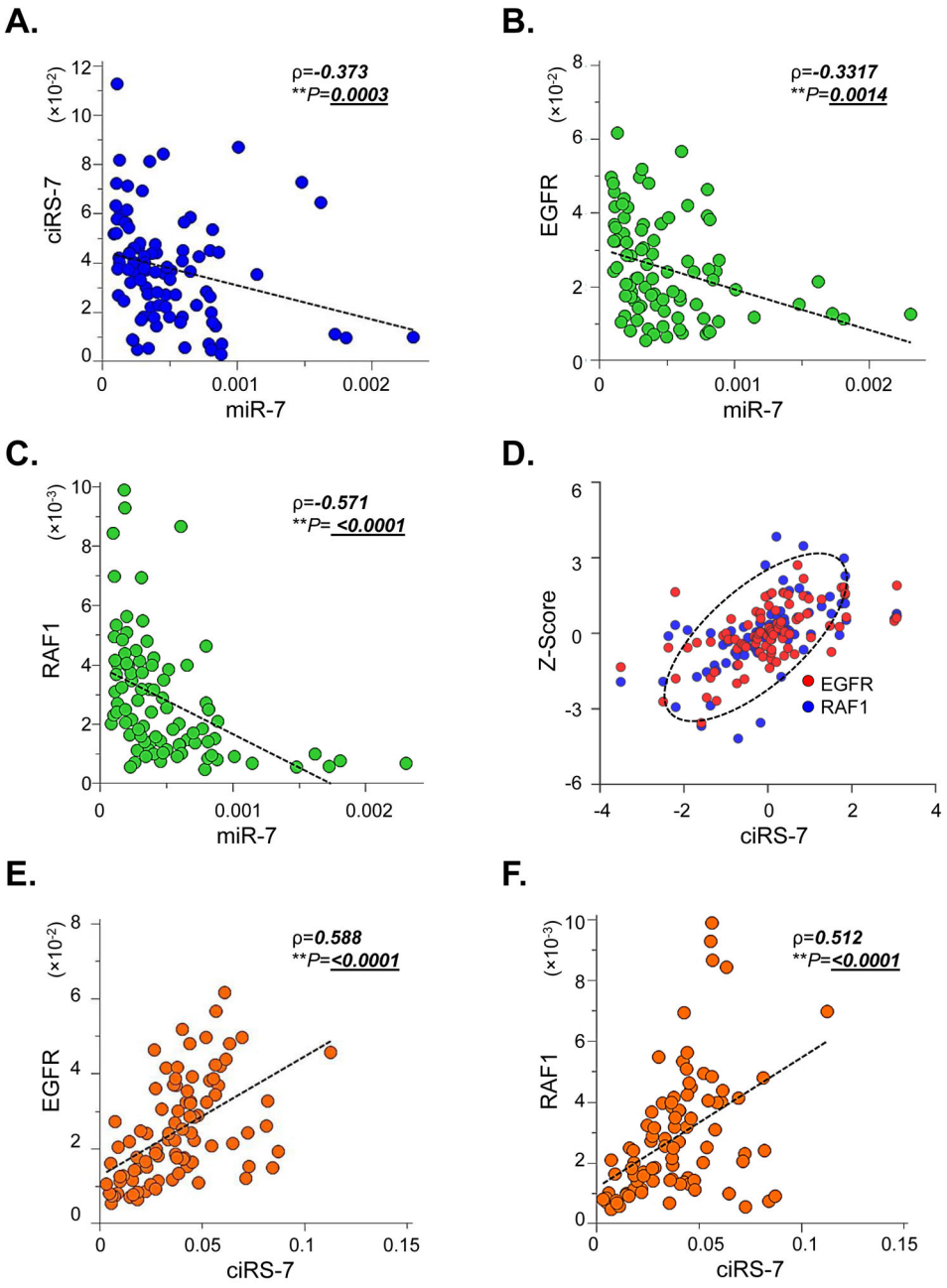

Figure 5.

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

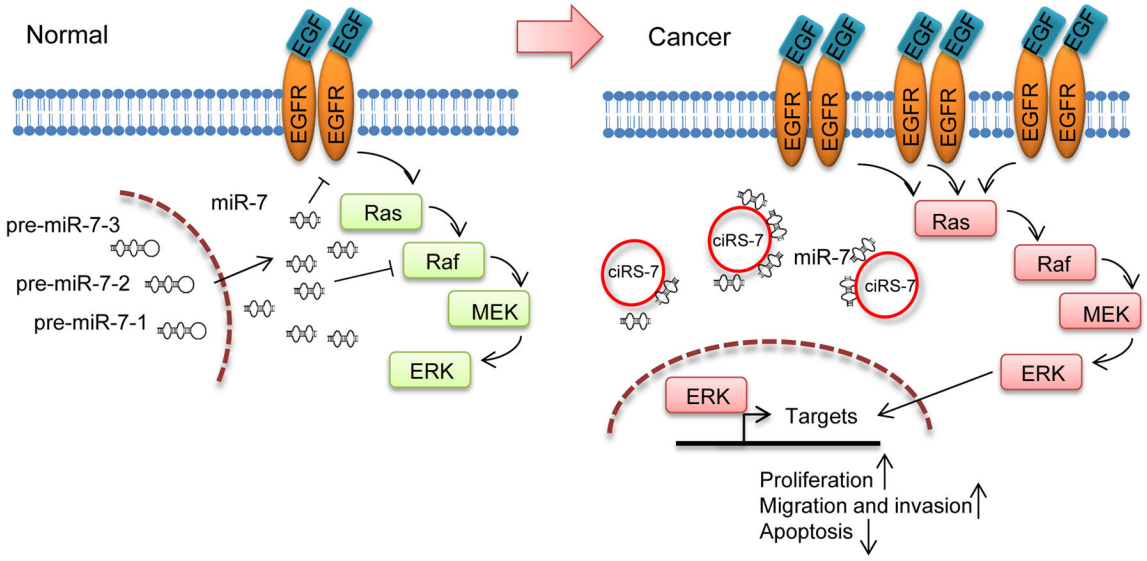

Figure 6.

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 up-regulation of ciRS-7 sequestered miR-7 and thereby lead to the accumulation of growth signaling to drive colorectal tumor growth.