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Cell migration–inducing hyaluronan-binding protein is regulated by miR-140-3p and promotes the growth and invasion of colorectal cancer cells

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Abstract

Dysregulation of cell migration–inducing hyaluronan-binding protein (CEMIP) is associated with the growth and metastasis of multiple malignancies. But, the underlying mechanism by which CEMIP contributes to colorectal cancer (CRC) remains undocumented. The association of CEMIP or miR-140-3p expression with clinicopathological characteristics and prognosis in CRC patients was analyzed by the tissue microarray and TCGA dataset. MiR-140-3p-specific binding with CEMIP was confirmed by luciferase report assay. In vitro experiments were conducted to assess the effects of CEMIP on the growth and invasion of CRC cells. Consequently, we found that CEMIP expression was dramatically elevated in CRC tissues and associated with a poor prognosis in CRC patients. The upregulation of CEMIP was attributable to the dysregulation of miR-140-3p rather than its genetic and epigenetic alterations. Ectopic expression of CEMIP facilitated the cell viability, colony formation, and invasive potential, but silencing of CEMIP reversed these effects. Furthermore, CEMIP was identified as a direct target of miR-140-3p and attenuated miR-140-3p-induced anti-proliferation effects by regulating c-Myc, E-cadherin, and Twist-1 expression. MiR-140-3p indicated a negative correlation with CEMIP expression and was an independent prognostic factor of tumor recurrence in CRC patients. Taken together, CEMIP is regulated by miR-140-3p and promotes the growth and invasion of CRC cells. MiR-140-3p/CEMIP axis may represent the potential markers for CRC patients.

Keywords

cell migration–inducing hyaluronan-binding protein, colorectal cancer, growth, invasion, miR-140-3p

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Introduction

In spite of the declined incidence of colorectal cancer (CRC) due to the widespread popularization of colonoscopy, it is still one of the most common cancers and the third leading cause of cancer-related death worldwide.[1,2] Effective treatment strategies including chemotherapy, radiotherapy, and surgical

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and endoscopic resections have been considered as the primary options for CRC patients.[3] But, the long-term survival and prognosis of the patients remain unfavorable attributing to the tumor invasiveness and distant metastasis.[4] Therefore, identification of the potential biomarkers may provide the insights into the early detection of CRC.

Cell migration–inducing hyaluronan binding protein (CEMIP), also known as KIAA1199, has been reported to associate with the progression and prognosis in multiple malignancies. CEMIP expression can be induced by hypoxia in CRC and pancreatic ductal adenocarcinoma (PDAC)[5,6] and predicts a poor survival in CRC and PDAC.[6,7] CEMIP promotes Schwann dedifferentiation and selumetinib-resistant intestinal organoids via MEK1-ERK1/2 signaling[8,9] and accelerates the cell proliferation, apoptosis escape, and epithelial–mesenchymal transition (EMT) by regulating Wnt/β-catenin/Snail signaling.[10,11] CEMIP, induced by AMPK/GSK3β/β-catenin signaling, facilitates metabolic reprogramming to enhance migration and invasion in prostate cancer[12] and indicates a diagnostic marker in pancreatic cancer.[13]

Increasing data demonstrate that microRNA (miRNA)-target messenger RNA (mRNA) axis acts critical roles in cancer progression including CRC.[14–16] We found that the upregulation of CEMIP in CRC was attributable to the dysregulation of miRNAs rather than its genetic and epigenetic alterations. Individual studies showed that downregulation of CEMIP by miR-216a inhibits the invasion and metastasis of CRC cells,[17] uncovering the regulation of miRNA-CEMIP axis in CRC.

In this study, we found that upregulation of CEMIP was associated with poor survival and tumor recurrence in CRC patients. Furthermore, CEMIP was identified as a direct target of miR-140-3p to promote the growth and invasion of CRC cells and reversed miR-140-3p-caused anti-proliferation effects by regulating c-Myc, E-cadherin, and Twist-1 expression. The expression of miR-140-3p displayed a negative correlation with CEMIP expression and acted as an independent prognostic factor of tumor recurrence in CRC patients.

Materials and methods

Clinical samples

A total of 42 pair-matched CRC tissues were from the tissue microarray stored in our laboratory. The clinicopathological and prognostic data in another cohort for 453 cases of CRC samples and 41 adjacent normal tissues as well as the relative expression levels of CEMIP and 16 miRNAs (has-miR-24-3p, has-miR-27a-3p, has-miR-27a-5p, has-miR-27b-3p, has-miR-27b-5p, has-miR-29a-3p, has-miR-29b-3p, has-miR-29c-3p, has-miR-29c-5p, has-miR-128-3p, has-miR-140-3p, has-miR-181d-3p, has-miR-183-5p, has-miR-223-3p, has-miR-375, and has-miR-140-5p) were downloaded from The Cancer Genome Atlas 2015 RNA sequencing database (https://genome-cancer.ucsc.edu). The protocols used in our study were approved by the Ethics Committee of Minhang Branch of Zhongshan Hospital.

Immunohistochemistry analysis

The protein expression of CEMIP was examined by using immunohistochemistry (IHC) analysis in 42 pair-matched CRC tissues from the tissue microarray. These tissues were stained by using anti-CEMIP (DF12056, Rabbit polyclonal antibody; Affinity Biosciences, Cincinnati, OH, USA). The experimental process of IHC and the quantification of CEMIP protein expression were conducted as previously described.[15]

Identification of the miRNAs that target CEMIP 3′-UTR

A total of 16 miRNAs that target 3′-UTR of *CEMIP* gene were identified by using the TargetScan Human 7.1 (http://www.targetscan.org/vert_71/) according to the weighted context score.

Cell culture

RKO cell line was stored at Laboratory of Digestive Disease and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (HyClone, Los Angeles, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37°C.

Quantitative real-time PCR (qRT-PCR)

Total RNA of RKO cells was extracted by using TRIzol. Reverse transcription was performed by using Moloney Murine Leukemia Virus (M-MLV) and complementary DNA (cDNA) amplification by using the SYBR Green Master Mix kit (Takara, Otsu, Japan). Total RNA for miRNAs was isolated by using a High Pure miRNA isolation kit (Roche,

Basel, Switzerland) and RT-PCR using a TaqMan MicroRNA Reverse Transcription kit (Life Technologies, Shanghai, China). A miScript Primer Assay (QIAGEN, Dusseldorf, Germany) was used for the miR-140-3p and U6. Data were analyzed using the Ct method ($2^{-\triangle\triangle Ct}$). Three separate experiments were performed for each clone. The primers were listed in Supplemental Table S1.

Western blot analysis

RKO cells were harvested and their proteins were extracted by using lysis buffer. The primary antibodies against CEMIP (DF12056, Rabbit polyclonal antibody; Affinity Biosciences), GAPDH (ab128915; Abcam, Cambridge, MA, USA), c-Myc (ab39688; Abcam), E-cadherin (ab1416; Abcam), and Twist-1 (ab49254; Abcam) were diluted at a ratio of 1:1000, and the second antibody goat anti-Rabbit IgG H&L (HRP; ab6721; Abcam) was diluted at a ratio of 1:10,000 according to the instructions. The detailed process for Western blot analysis was conducted as previously described.[15]

Luciferase reporter assay

Luciferase reporter assay was performed as previously described.[15]

Plasmid, siRNAs, and miR-140-3p mimic and inhibitor

Plasmid-mediated pcDNA3.1-CEMIP vector, small interfering RNA (siRNA)-targeting CEMIP (si-CEMIP, 5′-CGAGAGAGAGAAGUUUGCU dTdT-3′), miR-140-3p mimic, and inhibitor were purchased from Genepharma (Shanghai, P.R. China), and the control vector was used as a control. RKO cells were planted in six-well plates 24 h prior to si-CEMIP, pcDNA3.1-CEMIP, miR-140-3p mimic, or inhibitor transfection with 50%–70% confluence and then were transfected with Lipofectamine 2000 according to the manufacturer instructions.

MTT, colony formation, Transwell assays, and statistical analysis

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), colony formation, Transwell assays, and statistical analysis were performed as previously described.[15]

Results

Upregulation of CEMIP expression was associated with poor survival and tumor recurrence in CRC patients

The protein expression levels of CEMIP were detected in 42 pair-matched CRC samples by using the IHC analysis, which indicated that the positive expression of CEMIP was predominantly localized in the cytoplasm and had the higher expression levels in CRC tissues as compared with the adjacent normal tissues (Figure 1(a)). TCGA cohort further validated that CEMIP expression levels were dramatically increased in paired and unpaired CRC samples (Figure 1(b) and (c)). According to the survival time, survival status, and CEMIP expression levels, the cutoff value of CEMIP was acquired by using the Cutoff Finder tool (http://molpath.charite.de/cutoff/load.jsp) in these 42 CRC samples (Figure 1(d)) and divided the patients into high and low CEMIP expression groups. We found that high expression of CEMIP was positively associated with lymph node infiltration in CRC patients ($P=0.009$), but had no association with other clinicopathological parameters (each $P>0.05$, Supplemental Table S2). Kaplan–Meier analysis showed that the patients with high CEMIP expression possessed a poorer survival as compared with those with low CEMIP expression (Figure 1(e)).

In another cohort from the TCGA dataset, we also obtained a cutoff value of CEMIP in 433 CRC samples (Figure 1(f)), but found no association between CEMIP expression and the clinicopathological factors (each $P>0.05$, Supplemental Table S3). Kaplan–Meier analysis indicated that the patients with high CEMIP expression had a poorer survival and higher recurrence as compared with those with low CEMIP expression (Figure 1(g)). Univariate and multivariate analyses revealed that high CEMIP expression was an independent prognostic factor of tumor recurrence (Supplemental Table S4) rather than poor survival (Supplemental Tables S5 and S6) in GC patients.

CEMIP promoted the viability, colony formation, and invasion of CRC cells

The transfection efficiency of CEMIP plasmid or si-CEMIP vector in RKO cell line was determined by qRT-PCR and Western blot analysis (Figure 2(a)), respectively. MTT, colony formation, and Transwell assays were conducted to assess the functional role of CEMIP in RKO cells. We found that the restored expression of CEMIP increased

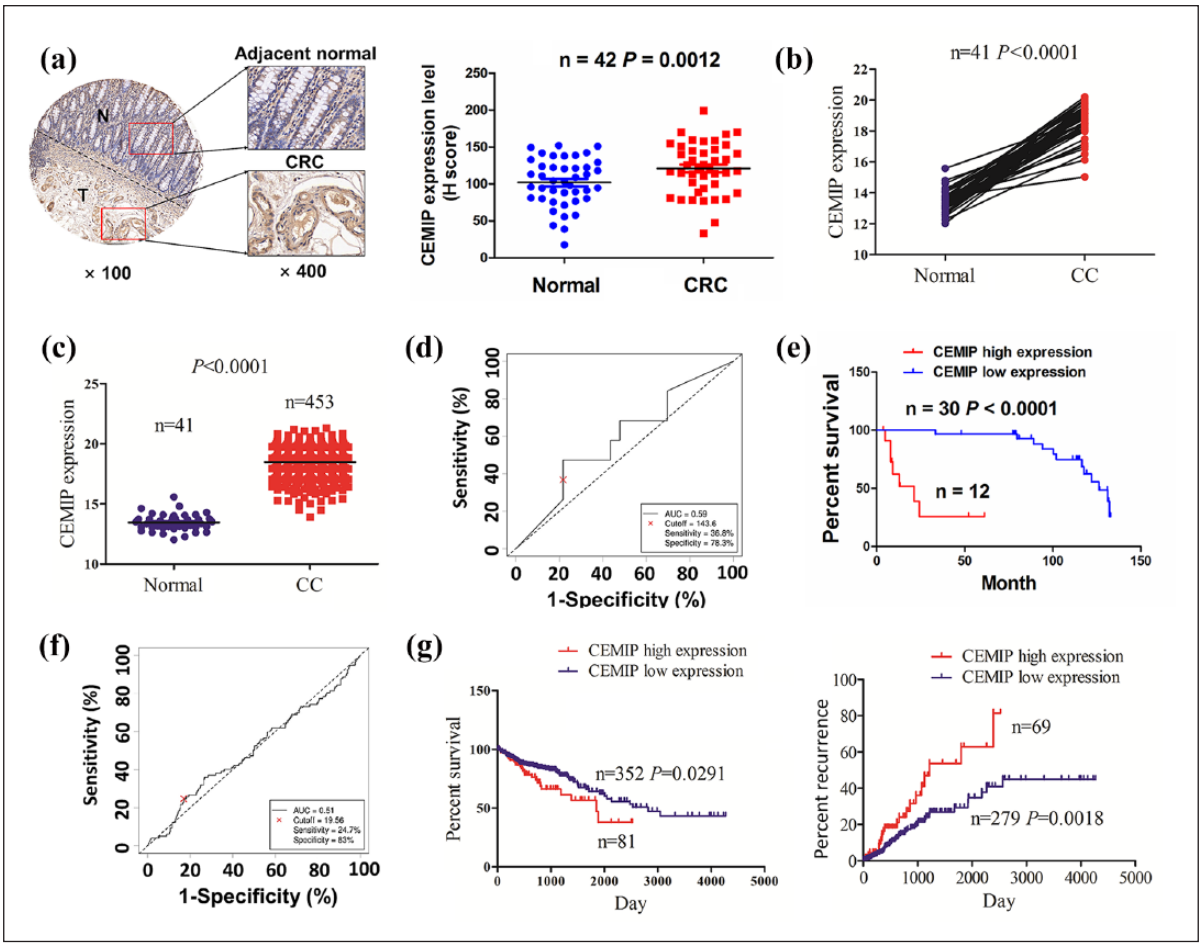

Figure 1. High expression of CEMIP was associated with poor survival and tumor recurrence in CRC patients. (a) Tissue microarray analysis of the protein expression of CEMIP in 42 pair-matched CRC tissues. (b and c) TCGA cohort analysis of the expression levels of CEMIP in paired and unpaired CRC tissues. (d) ROC curve analysis of the cutoff value of CEMIP in CRC samples (n = 42). (e) Kaplan–Meier analysis of the association of high or low CEMIP expression with the poor survival in 42 CRC patients. (f) TCGA cohort analysis of the cutoff value of CEMIP in CRC samples (n = 433). (g) TCGA analysis of the association of high or low CEMIP expression with the poor survival and tumor recurrence in 433 CRC patients.

cell viability (Figure 2(b)), colony formation number (Figure 2(c)), and invasive potential (Figure 2(d)), but knockdown of CEMIP produced the opposite effects (Figure 2(b)–(d)).

CEMIP was identified as a direct target of miR-140-3p in RKO cells

To unveil the cause of CEMIP upregulation in CRC samples, we estimated the genetic and epigenetic alterations of *CEMIP* gene in CRC (n = 333), indicating that CEMIP had no significant alterations at the genetic (Supplemental Figure S1A and B) and methylation levels (Supplemental Figure S1C), and DNA mutation, copy number, and methylation level could not account for the reason of CEMIP upregulation in CRC.

We hypothesized that CEMIP upregulation might be affected by miRNAs at post-transcriptional levels. To testify our hypothesis, we screened 16 miRNAs that may bind to CEMIP 3′-UTR by using TargetScanHuman7.1 and evaluated their expression levels in CRC samples. Among these miRNAs, miR-375, miR-140-3p, and miR-29c-5p showed a markedly decreased expression in CRC samples as compared with the adjacent normal tissues (Figure 3(a)). Pearson correlation analysis indicated that CEMIP had a negative correlation with miR-140-3p expression (Figure 2(b)) rather than miR-375 and miR-29c-5p (Supplemental Figure S2) in CRC samples.

The binding sites between miR-140-3p and wild-type (WT) or mutant (Mut) CEMIP 3′-UTR were

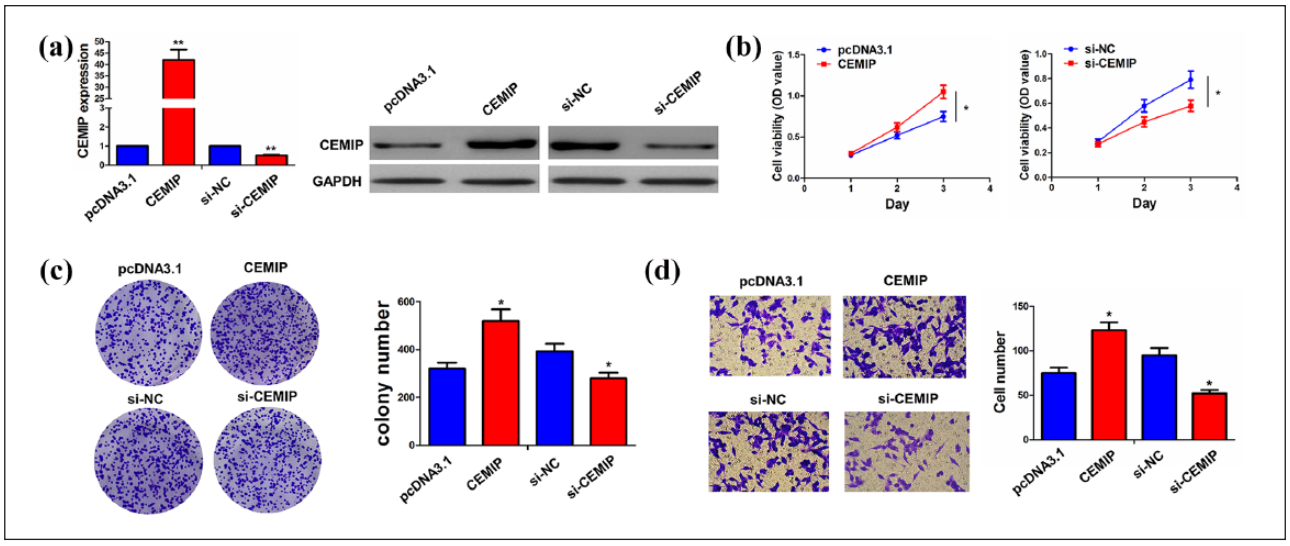

Figure 2. CEMIP promoted the cell proliferation, colony formation, and invasive potential. (a) qRT-PCR and Western blot analysis of the transfection efficiency of CEMIP plasmid or si-CEMIP in RKO cells. (b) MTT analysis of the cell viability after transfection with CEMIP plasmid or si-CEMIP in RKO cells. (c) Colony formation assay assessment of the cell colony number after transfection with CEMIP plasmid or si-CEMIP in RKO cells. (d) Transwell evaluation of the cell invasive capabilities after transfection with CEMIP plasmid or si-CEMIP in RKO cells. Data are the means ± SEM of three experiments (*$P < 0.05$, **$P < 0.01$).

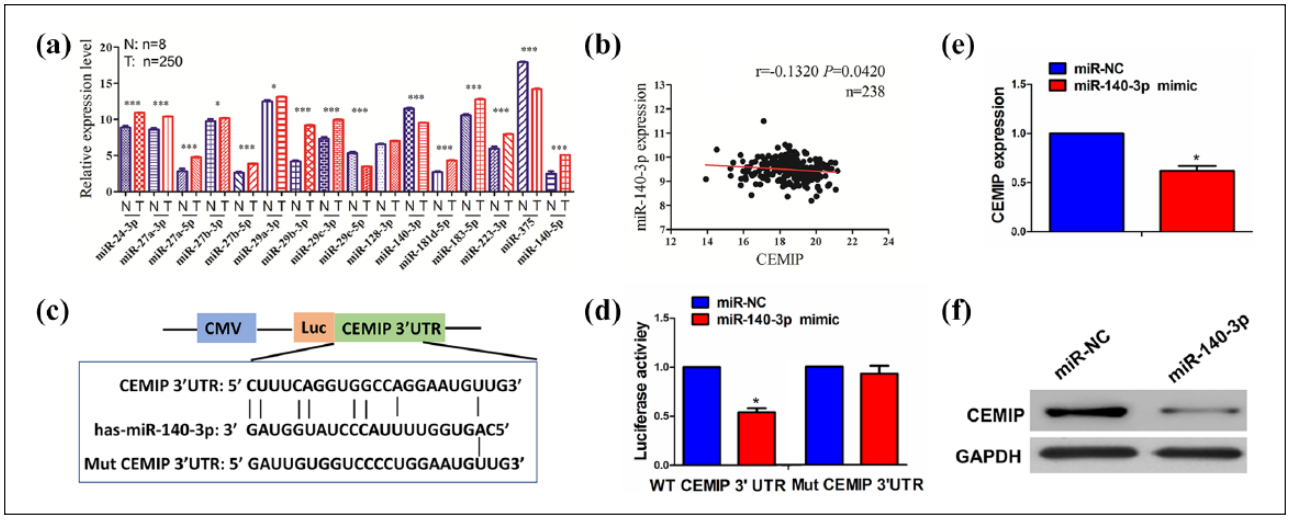

Figure 3. CEMIP was identified as a direct target of miR-140-3p in RKO cells. (a) TCGA analysis of the expression levels of 16 miRNAs in unpaired CRC tissues. (b) Pearson correlation analysis of the correlation of CEMIP with miR-140-3p expression in CRC tissues. (c) Schematic representation of the binding sites of miR-140-3p with WT or Mut 3′-UTR of CEMIP. (d) The luciferase activity of WT or Mut 3′-UTR of CEMIP was detected after transfection with miR-140-3p mimic or miR-NC in RKO cells. (e and f) qRT-PCR and Western blot analysis of the expression levels of CEMIP after transfection with miR-140-3p mimic or miR-NC in RKO cells. Data are the means ± SEM of three experiments (*$P < 0.05$, ***$P < 0.001$).

indicated in Figure 3(c). To validate miR-140-3p-specific binding with CEMIP 3′-UTR, we co-transfected miR-140-3p mimic and WT or Mut CEMIP 3′-UTR into RKO cells and found that miR-140-3p decreased the luciferase activity of WT CEMIP 3′-UTR, but had no effects on that of Mut CEMIP 3′-UTR, as compared with the miR-NC group (Figure 3(d)). qRT-PCR and Western blot analysis showed that miR-140-3p inhibited the expression of CEMIP in RKO cells (Figure 3(e) and (f)).

CEMIP reversed miR-140-3p-induced anti-proliferation effects

To further observe the functional association between miR-140-3p and CEMIP expression in

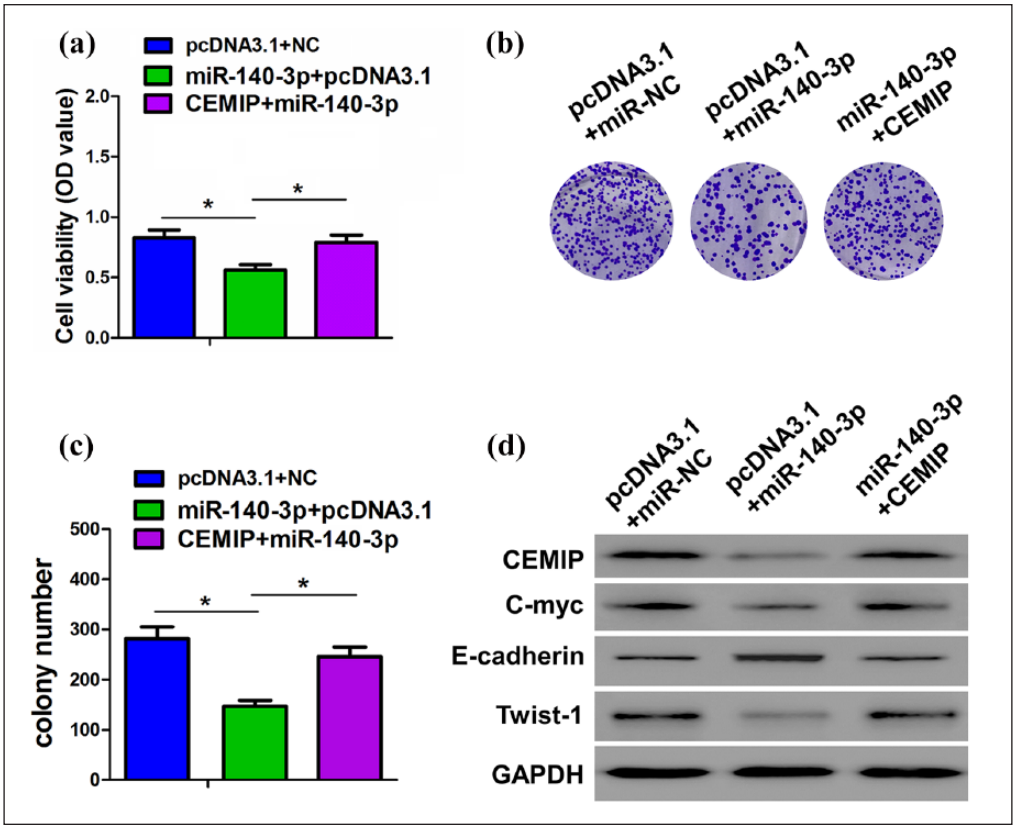

Figure 4. CEMIP-reversed miR-140-3p-caused anti-tumor effects in CRC cells. (a) MTT analysis of the cell viability after co-transfection with miR-140-3p mimic and CEMIP plasmid in RKO cells. (b and c) Colony assay assessment of the cell colony number after co-transfection with miR-140-3p mimic and CEMIP plasmid in RKO cells. (d) Western blot analysis of the protein expression levels of CEMIP, c-myc, E-cadherin, and Twist-1 after co-transfection with miR-140-3p mimic and CEMIP plasmid in RKO cells. Data are the mean ± SEM of three experiments (*$P < 0.05$).

RKO cells, we performed the MTT and colony formation assays. After co-transfection with CEMIP plasmid and miR-140-3p mimic into RKO cells, we found that miR-140-3p inhibited the cell viability (Figure 4(a)) and colony formation abilities (Figure 4(b) and (c)), but CEMIP overexpression reversed miR-140-3p-induced anti-tumor effects (Figure 4(a)–(c)). Western blot analysis further showed that miR-140-3p downregulated the expression of CEMIP, c-myc, and Twist-1 and upregulated E-cadherin expression, while CEMIP overexpression reversed these effects (Figure 4(d)).

Decreased expression of miR-140-3p was associated with poor survival and tumor recurrence in CRC patients

Having confirmed the decreased expression of miR-140-3p in CRC samples (Figure 3(a)), we analyzed the association between miR-140-3p expression and clinicopathological characteristics and prognosis in CRC patients. The cutoff value of miR-140-3p was acquired in CRC samples (n = 200; Figure 5(a)) and divided the patients into high and low miR-140-3p expression groups (Figure 5(b)). The area under the curve (AUC) value, sensitivity and specificity of miR-140-3p expression in CRC patients were respectively 0.55, 87.2% and 25.9% (Figure 5(a)), indicated that miR-140-3p might be a potential marker for CRC patients.

Furthermore, we found that low expression of miR-140-3p was associated with the gender, but had no association with the other factors in CRC patients (each $P > 0.05$, Supplemental Table S7). Kaplan–Meier analysis demonstrated that the patients with low miR-140-3p expression harbored a poorer survival (Figure 5(c)) and higher recurrence rate (Figure 5(d)), as compared with those with high miR-140-3p expression. Univariate and multivariate analyses revealed that low expression of miR-140-3p was an independent prognostic

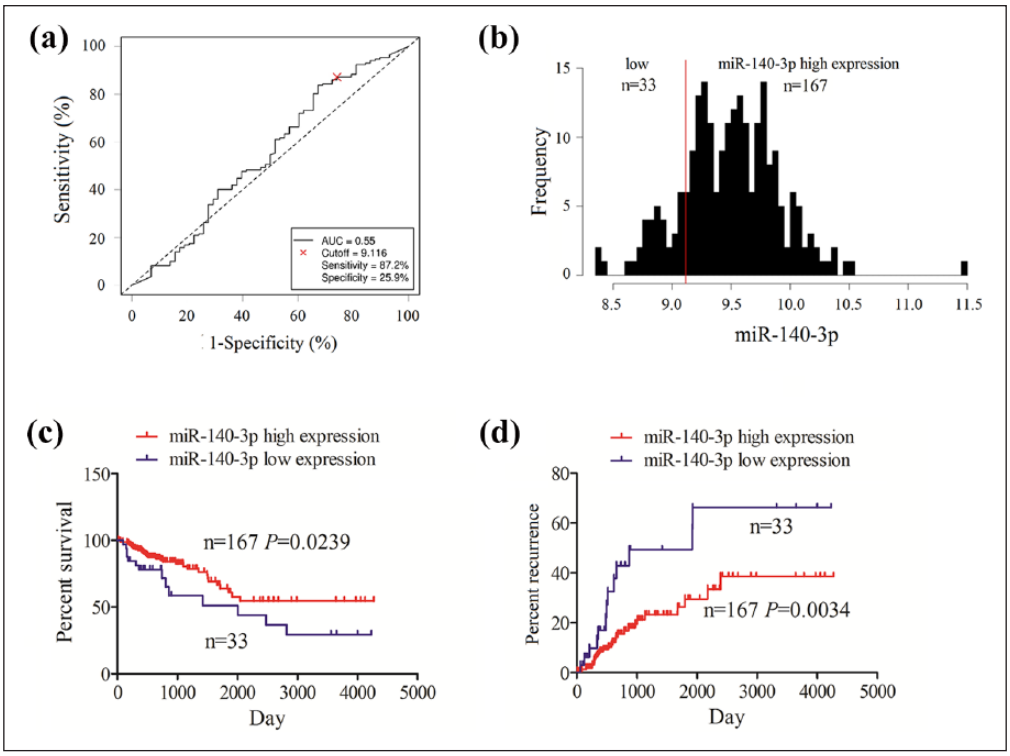

Figure 5. Low expression of miR-140-3p was associated with poor survival and tumor recurrence in CRC patients. (a) ROC curve analysis of the cutoff value and sensitivity and specificity of miR-140-3p in CRC samples (n = 200). (b) The cutoff value divided the CRC patients into high and low miR-140-3p expression groups. (c and d) Kaplan–Meier analysis of the association of high or low miR-140-3p expression with the poor survival and tumor recurrence in CRC patients.

factor of tumor recurrence (Supplemental Table S8) rather than poor survival (Supplemental Table S9) in CRC patients.

Discussion

Some studies showed that CEMIP expression is increased in CRC and PDAC and associated with a poor survival in these patients.[5–7] But, the association of CEMIP expression with the tumor recurrence in CRC is unclear. In this study, in coinciding with the previous reports,[7] a cohort from the tissue microarray indicated that CEMIP expression was upregulated and positively associated with lymph node infiltration in CRC patients. Due to the diversity of tissue sources and the size of CRC sample, another cohort from the TCGA dataset showed that high expression of CEMIP had no association with the clinicopathological factors, but was associated with poor survival and tumor recurrence. Multivariate analysis uncovered that CEMIP was an independent prognostic factor of tumor recurrence in CRC patients. These results indicated that CEMIP might be a potential marker for CRC.

We further found that the upregulation of CEMIP was attributable to the post-transcriptional modulation rather than the genetic and epigenetic alterations in CRC. A total of 16 miRNAs were screened to bind with CEMIP 3′-UTR, of which miR-140-3p displayed a negative correlation with CEMIP expression in CRC tissues. Luciferase gene report implied that CEMIP was a direct target of miR-140-3p in CRC cells. Previous studies showed that CEMIP is also a target of miR-216a involved in the growth and metastasis of CRC cells.[17] These results suggested that the dysregulation of miR-140-3p mediated CEMIP to participate in the pathogenesis of CRC.

Functional studies showed that CEMIP promotes the growth and metastasis of multiple malignancies.[10–12] In consistence with these results, we found that CEMIP encouraged the cell viability, colony formation, and invasion, but knockdown of CEMIP had the opposite effects in CRC cells. In addition, miR-140-3p acts as a tumor suppressor by inhibiting cell proliferation and invasion in lung and breast cancers.[18,19] Increased expression of miR-140-3p is associated with the tumor invasion

in spinal chordoma.[20] We found that miR-140-3p expression was downregulated in CRC tissues and had a negative association with the poor survival and tumor recurrence in CRC patients. MiR-140-3p repressed the cell proliferation and colony formation, while CEMIP attenuated miR-140-3p-caused anti-tumor effects in CRC cells. These results indicated that CEMIP was regulated by miR-140-3p to promote the growth of CRC.

c-Myc acts as an oncogenic transcription factor by promoting the tumor growth and metastasis.[21,22] E-cadherin and Twist-1 have been considered as the prognostic markers of aggressive tumors[23] and regulate the metastasis in pancreatic cancer.[24] Some studies showed that CEMIP acts as an oncogenic factor by regulating c-myc, E-cadherin, and Twist-1.[9,10] In our study, miR-140-3p downregulated the expression of CEMIP, c-myc, and Twist-1 and upregulated E-cadherin expression, while CEMIP overexpression reversed these effects. Our findings suggested that miR-140-3p might exhibit the anti-tumor effects by regulating CEMIP-mediated c-myc, E-cadherin, and Twist-1 expression.

In conclusion, high CEMIP expression or low miR-140-3p expression was associated with poor survival and tumor recurrence in CRC patients. CEMIP was regulated by miR-140-3p to promote the growth and invasion of CRC cells. MiR-140-3p/CEMIP axis might represent the potential markers for CRC patients.

Acknowledgements

Xiaodi Yang, Biwei Yang, and Sen Jiang contributed equally to this article. J.Z. and T.Y. designed this study, and J.Z. supervised the process of the experiments. X.-D.Y., B.-W.Y., and S.J. performed the experiments and contributed equally to this study. X.-Y.C. was responsible for downloading the data from the TCGA dataset and analyzing these data. X.-D.Y. wrote the manuscript and J.Z. checked the revised manuscript. All the authors read and approved this final manuscript. The authors thank Lidan Hou and Ming Jin for providing the experimental arrangement and data collection.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval and consent to participate

This study was approved by the Hospital's Protection of Human Subjects Committee.

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Supplemental material

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