MiR-205 suppresses tumor growth, invasion, and epithelial–mesenchymal transition by targeting SEMA4C in hepatocellular carcinoma

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ABSTRACT: Growing evidence indicates that microRNAs are involved in tumorigenesis and progression of hepatocellular carcinoma (HCC). However, the functional mechanisms of miR-205 in HCC remain largely unknown. Here, we demonstrate that miR-205 expression was significantly down-regulated in HCC tissues and cell lines and was correlated with metastatic pathologic features and shorter disease-free and overall survival. Overexpression of miR-205 dramatically inhibited HCC cell proliferation, apoptosis, migration, invasion, epithelial–mesenchymal transition (EMT) in vitro, and tumor growth in vivo. We subsequently identified semaphorin 4C (SEMA4C) as a novel target of miR-205. Furthermore, high expression levels of SEMA4C were frequently found in HCC tissues and were associated with poor prognosis. Ectopic expression of SEMA4C restored the suppressive effect of overexpressed miR-205 on migration, invasion, and EMT. Taken together, our findings provide new insight into the critical role of miR-205 in regulating tumor growth, invasion, and EMT of HCC, suggesting miR-205 may serve as a promising therapeutic target and novel prognostic indicator for patients with HCC.

KEY WORDS: microRNA · EMT · SEMA4C · HCC

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer-related mortality worldwide (1). Despite numerous therapeutic strategies used to improve the prognosis of HCC, the long-term survival rate is still unsatisfactory because of high rates of recurrence and metastasis (2, 3). Several factors are involved in the pathogenesis of HCC; however, the roles and underlying molecular mechanisms of those factors remain poorly understood. Consequently, there is an urgent need to explore the molecular mechanisms responsible for HCC metastasis, which may contribute to identifying novel therapeutic targets and improving the prognosis of patients with HCC.

MicroRNAs (miRNAs) are a class of highly conserved, small, noncoding RNAs that negatively control gene expression at the posttranscriptional level by resulting in mRNA degradation or repressing protein translation (4). Increasing evidence indicates that miRNAs are aberrantly expressed in various types of cancer, and the dysfunction of miRNAs has a crucial role in tumor initiation, invasion, and metastasis (5, 6). Recent studies have demonstrated that miRNAs are correlated with the proliferation, angiogenesis, drug resistance, and prognosis of HCC (7–10).

The roles of miR-205 in human malignancies remain controversial (11). Several reports have indicated that miR-205 is frequently down-regulated in several types of cancer and serves as a tumor suppressor by inhibiting tumor proliferation, invasion, and metastasis (12–15). However, miR-205 also exhibits oncogenic properties in endometrial carcinoma and cervical cancer (16, 17). Therefore, miR-205 appears to carry out various functions depending on tumor types or its target genes. Nevertheless, the function of miR-205 and its molecular mechanisms in HCC are largely unknown.

Epithelial–mesenchymal transition (EMT)—a series of events during which epithelial cells acquire...
phenotypic characteristics of mesenchymal cells—has been recognized as the key process facilitating invasion and metastasis of tumors (18, 19). Numerous studies reveal that EMT is involved in HCC invasion and metastasis (20, 21). The molecular mechanisms of the EMT process are complex, and multiple molecules have been shown to be capable of orchestrating EMT programs, including miRNAs (22). However, the functional roles of miR-205 in the regulation of EMT in HCC remain to be elucidated.

In this study, we found that miR-205 was significantly down-regulated in HCC, which was associated with poor prognosis. Overexpression of miR-205 inhibited malignant behaviors and EMT of HCC cells in vitro as well as tumor growth in vivo. Notably, SEMA4C was identified as a direct target of miR-205 and mediated the biologic functions of miR-205 in HCC cells. Furthermore, we revealed that miR-205 may inhibit the EMT process to influence migration and invasion of HCC cells by regulating SEMA4C expression.

MATERIALS AND METHODS

Patients and specimens

Fifty paired human HCC and adjacent nontumor liver tissues were collected from the West China Hospital of Sichuan University between January 2010 and December 2011. Histopathologic diagnosis was performed according to the TNM Staging Guide [based on the extent of the tumor (T), the extent of spread of the lymph nodes (N), and the presence of metastasis (M); American Joint Committee on Cancer, Chicago, IL, USA]. The tissues were stored at −80°C or embedded in paraffin. None of the patients received any preoperative chemotherapy or radiotherapy. Informed consent was obtained from each patient, and all protocols of this study were approved by the Ethics Committee of West China Hospital. The demographic and clinicopathologic features of the patients are described in the Table 1.

TABLE 1. Correlations between miR-205 and clinicopathologic features of patients with HCC

| Factor                          | miR-205 expression | n   | Low (n = 25) | High (n = 25) | P     | χ²   |
|--------------------------------|-------------------|-----|-------------|-------------|-------|------|
| Age (yr)                       |                   |     |             |             |       |      |
| <50                            |                   | 22  | 14          | 8           | 0.382 | 0.764|
| ≥50                            |                   | 28  | 11          | 17          |       |      |
| Gender                         |                   |     |             |             |       |      |
| Male                           |                   | 39  | 22          | 17          | 0.088 | 2.914|
| Female                         |                   | 11  | 3           | 8           |       |      |
| Tumor size (cm)                |                   |     |             |             |       |      |
| ≤5                             |                   | 21  | 10          | 11          | 0.774 | 0.082|
| >5                             |                   | 29  | 15          | 14          |       |      |
| Lesion number                  |                   |     |             |             |       |      |
| Single                         |                   | 32  | 10          | 22          | <0.001| 12.5 |
| Multiple                       |                   | 18  | 15          | 3           |       |      |
| Capsule formation              |                   |     |             |             |       |      |
| No                             |                   | 31  | 16          | 15          | 0.771 | 0.085|
| Yes                            |                   | 19  | 9           | 10          |       |      |
| Macrovascular invasion         |                   |     |             |             |       |      |
| No                             |                   | 38  | 17          | 21          | 0.185 | 1.754|
| Yes                            |                   | 12  | 8           | 4           |       |      |
| Lymphovascular invasion        |                   |     |             |             |       |      |
| No                             |                   | 30  | 11          | 19          | 0.021 | 5.333|
| Yes                            |                   | 20  | 14          | 6           |       |      |
| TNM staging                    |                   |     |             |             |       |      |
| I/II                           |                   | 28  | 9           | 19          | 0.004 | 8.117|
| III/IV                         |                   | 22  | 16          | 6           |       |      |
| Edmondson-Steiner grade        |                   |     |             |             |       |      |
| I–II                           |                   | 22  | 6           | 16          | 0.002 | 9.859|
| III–V                          |                   | 28  | 19          | 9           |       |      |

Underlined values signify P < 0.05.

Cell culture and miRNA transfection

Human HCC cell lines (MHCC97-L, HepG2, Huh-7, Bel-7402, MHCC97-H, and HCCLM3) and the human normal hepatocyte cell line (L02) were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). All cells were cultured in complete DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific) and incubated at 37°C in a humidified incubator with 5% CO₂.

MiR-205 overexpression and its negative control (NC) lentiviral vectors were designed and synthesized by Shanghai R&S Biotechnology Co. (Shanghai, China). The SEMA4C overexpression vector and its NC vector were obtained from GeneCopoeia (Rockville, MD, USA). Cells were transiently transfected with above vectors using Polo Deliverer 3000 Transfection Reagent (Shanghai R&S Biotechnology Co.) in accordance with the manufacturer's protocol.
RNA extraction and real-time, quantitative RT-PCR analysis

Total RNA was extracted from fresh-frozen HCC tissues or cell lines with Trizol reagent (Thermo Fisher Scientific). cDNA synthesis was performed with 2 μg total RNA using a reverse transcription kit (Takara Bio, Kusatsu, Japan). Real-time, quantitative RT-PCR (qRT-PCR) was performed with All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia) on an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific). Real-time qRT-PCR was performed with SYBR green 2× master mixture. Hsa-miR-205 primer, U6 small nuclear RNA qPCR primer, and SEMA4C were purchased from GeneCopoeia. U6 was used as an internal control for miRNA, and β-actin was used for mRNA. The ΔΔCt and 2−ΔΔCt methods were used to analyze gene expression relative to the endogenous control. The specific primer sequences used are listed in Supplemental Table S1.

Transfection and vector construction

The miR-205–mimic and NC miRNA sequences (GeneCopoeia) were amplified and cloned into a pcDNA6.2-GW/EmGFP vector (Shanghai R&S Biotechnology). The SEMA4C expression lentivirus was constructed by inserting open reading frame–encoded SEMA4C into the pLenti6.3 vector (Thermo Fisher Scientific). For lentivirus production, 293T cells were transfected with the above-mentioned plasmids and the virus-packaging plasmid mix using the Poly Deliverer 3000 transfection reagent. After transfection for 48 h, the viral supernatant was collected and filtered. The HCC cells were then cocultured with lentivirus and polybrene. To obtain cells that stably overexpressed miR-205, the lentiviral transduced cells were selected with blasticidin.

Western blot

HCC cells (HCCLM3 and HepG2) were lysed with RIPA buffer (Beyotime, Shanghai, China) and put on ice for 30 min. The cell lysates were centrifuged at 10,625 g for 15 min; then, the supernatant was collected as whole proteins. Protein concentration was quantified with the BCA Protein Assay Kit (Thermo Fisher Scientific). For Western blot analysis, 250 μg proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (MilliporeSigma, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline and Tween for 1 h at room temperature and incubated with specific primary antibodies against E-cadherin, vimentin, and SEMA4C (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). Afterward, the membranes were incubated with horseradish peroxidase–labeled secondary antibody (1:5000; Santa Cruz Biotechnology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500; Santa Cruz Biotechnology) was used as the loading control. The protein bands on the membranes were detected by an Enhanced Chemiluminescence Kit (Thermo Fisher Scientific). Images were photographed through Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA, USA) and analyzed by the Quantity One software program (Bio-Rad). The Western blot experiment was performed in triplicate, and images shown in the figures are representative results.

Immunohistochemical analysis

All the paraffin-embedded tissues were sliced into 5-μm-thick consecutive sections. Tissue sections were subsequently deparaffinized, rehydrated, and quenched in 3% hydrogen peroxide. After blocking with goat serum, the slides were incubated with anti-SEMA4C and, then, incubated with secondary horseradish peroxidase–labeled rabbit anti-goat IgG antibodies. The sections were visualized with a 3,3′-diaminobenzidine kit (Beyotime) and counterstained with hematoxylin. Immunoreactive scoring was assessed according to the percentage of positively staining cells and the staining intensity. To determine the average scores, each section was identified for 5 independent high-magnifications (×400) fields.

Cell proliferation, colony formation, and cell apoptosis assays

Cell proliferation was measured with the cell counting kit 8 (CCK-8) and colony-formation assays. For the CCK-8 assay, cells were seeded in 96-well plates at 4000 cells/well. After 24 h, 10 μl of CCK-8 was added into each well. Subsequently, the optical density was measured at a wavelength of 450 nm with a microplate reader (Bio-Rad). For the colony-formation assay, 500 cells were seeded into each well of a 6-well plate. Colonies were fixed with 20% methanol and stained with 0.1% crystal violet, and the colonies were counted. For the apoptosis assay, cells were stained with 0.5% propidium iodide (Shanghai R&S Biotechnology) and annexin V (Shanghai R&S Biotechnology). The percentage of apoptotic cells was determined by flow cytometry.

Cell migration and invasion assays

Cell migration and invasion assays were performed in 24-well Transwell chambers (Corning, Corning, NY, USA) with or without being coated Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). Briefly, a 200-μl transfected-cell suspension, with serum-free medium (1 × 10^5/ml), was added to the upper chamber, and the lower chamber was filled with a culture medium containing 10% fetal bovine serum as the chemoattractant. After 24 h, the cells that had migrated into or invaded the lower surface were fixed and stained with 0.1% crystal violet. Photographs of 5 randomly selected fields of the fixed cells were taken, and cell numbers were calculated under a microscope.

MiRNA target prediction and dual luciferase reporter assay

The analysis of miR-205–predicted targets was performed with TargetScan, miRDB, and miRanda algorithms. The 3′-UTR sequence of SEMA4C, which was predicted to interact with miR-205, was combined with a corresponding mutated sequence of SEMA4C that contained the miR-205 binding sites, and was synthesized and cloned into the psiCheck2 dual-luciferase reporter vector (GeneCopoeia) called wild-type (wt)-SEMA4C
3’-UTR (wt) and mutant (mt)-SEMA4C 3’-UTR (mutated). Subsequently, the cells were cultured in 24-well plates and cotransfected with the wt or mt 3’-UTR of the SEMA4C vector, together with the miR-205 mimics or NC miRNA with Lipofectamine 2000 Reagent (Thermo Fisher Scientific). Forty-eight hours after cotransfection, firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to the manufacturer’s protocol. The ratio of firefly to Renilla was used to normalize the firefly luciferase activity.

Animal experiments

All animal studies were approved by the Institutional Animal Care and Use Committee of Sichuan University, China. Male immune-deficient nude mice (BALB/C-nu) at 4–6 wk of age were purchased from the Sichuan Laboratory Animal Center (Chengdu, China) and were randomized into 2 groups (n = 5 each). The mice were raised in cages under specific pathogen-free conditions. A total of 1 × 10⁷ HCCLM3 cells, which stably transfected with either miR-205 or the NC vector, were subcutaneously injected into the right flank of each mouse. Mice were monitored daily for general condition, and tumor size was measured every 3 d. Tumor volume (V) was calculated with the following formula: V = 0.5 × L × W² (mm³), where L is the length, and W is the width of each tumor. Forty days later, all mice were euthanized, and the tumors were removed, weighed, and photographed.

Statistical analysis

All data are expressed as means ± SD. Statistical analyses were conducted using SPSS software (v.21.0; IBM, Armonk, NY, USA). Statistical differences between the 2 groups were determined by independent Student’s t test. The χ² test or Fisher’s exact test was used to analyze the relationship between miR-205 expression and clinicopathologic characteristics. Overall survival (OS) and disease-free survival (DFS) were evaluated WITH Kaplan-Meier survival curves and were compared by the log-rank test. Univariate and multivariate analyses (Cox proportional hazard-regression model) were performed to determine potential prognostic factors for OS and DFS. A value of P < 0.05 was considered statistically significant.

RESULTS

MiR-205 is down-regulated in HCC and is correlated with a poor prognosis

To investigate the expression and significance of miR-205 in HCC, we first examined the expression level of miR-205 in 50 matched pairs of HCC and adjacent nontumor tissues. We found that miR-205 expression was significantly down-regulated in 76% (36 of 50) HCC tissues when compared with corresponding adjacent nontumor tissues (P < 0.001, Fig. 1A). Moreover, miR-205 expression was obviously lower in aggressive HCC tissues than in nonaggressive HCC tissues (P < 0.01, Fig. 1B). Aggressive HCC tissues were defined as HCC tissues with intrahepatic metastasis and vascular and bile duct invasion. To further confirm the relationship between miR-205 expression and clinicopathologic features, 50 patients with HCC were divided into 2 subgroups based on the cutoff values, which were determined as the median level of miR-205 in HCC tissues. Interestingly, clinicopathologic analysis showed that miR-205 expression was strongly correlated with the number of tumor nodules, microvascular invasion, TNM staging, and Edmondson-Steiner grading (Table 1). In addition, Kaplan-Meier survival analysis showed that patients with HCC who had low miR-205 expression levels had significantly shorter OS (Fig. 1C) and DFS (Supplemental Fig. S1) than those with high miR-205 expression levels. The 1-, 3-, and 5-y OS rates of patients with low miR-205 expression were 56, 16, and

Figure 1. MiR-205 was down-regulated in HCC and was significantly associated with a poor prognosis for patients with HCC. A) qRT-PCR detection of miR-205 expression levels in 50 pairs of HCC tissues and their matched tumor-adjacent tissues. B) qRT-PCR detection of miR-205 expression levels in aggressive and nonaggressive HCC tissues. C) Kaplan-Meier analysis of overall survival between high (n = 25) and low (n = 25) miR-205 expression levels in patients with HCC. D) qRT-PCR detection of miR-205 expression in 6 HCC cell lines, with normal human hepatocyte cell line LO2 as the control. **P < 0.01, ***P < 0.001.
8%, respectively, which were significantly lower than those with high miR-205 expression (84, 52, and 20%, respectively; \( P = 0.001 \)). A multivariate Cox regression analysis revealed that low miR-205 expression was the independent risk factor for OS (\( P = 0.001 \); Table 2) and DFS (\( P = 0.015 \); Supplemental Table S2).

Consistent with miR-205 expression in HCC tissues, miR-205 expression was also decreased in HCC cell lines with different metastatic potentials as compared with the normal human hepatocyte line LO2 (\( P < 0.001 \), Fig. 1D). Importantly, the expression level of miR-205 decreased with an increase in metastatic potentials in the HCC cell lines. These results indicated that down-regulation of miR-205 is correlated with poor prognosis in HCC and might be involved in HCC progression.

**MiR-205 not only inhibits cell proliferation, migration, and invasion of HCC cells but also induces HCC cells apoptosis in vitro**

To further investigate the biologic role of miR-205 in the development and progression of HCC, gain-of-function experiments were performed in HCCLM3 and HepG2 cells that showed different expression levels of endogenous miR-205. Those 2 HCC cell lines were transfected with the miR-205 expression plasmid or the NC miRNA vector. As detected by qRT-PCR, we confirmed that miR-205 was effectively overexpressed in both cell lines (\( P < 0.001 \), Fig. 2A). The CCK-8 assay showed that up-regulation of miR-205 in HCCLM3 and HepG2 cells resulted in significant suppression of cell proliferation compared with those in the NC groups (\( P < 0.01 \), Fig. 2B). The colony-formation assay also demonstrated that HCCLM3 and HepG2 cells overexpressing miR-205 showed remarkably decreased colony-formation abilities (Fig. 2C). We also investigated whether miR-205 modulated cell apoptosis and found that miR-205 overexpression induced apoptosis of HepG2 cells (15 vs. 5% in the control group; \( P < 0.001 \)) (Fig. 2D). Similar effects of miR-205 overexpression were found in HCCLM3 cells (\( P < 0.001 \), Fig. 2D). To determine the influence of miR-205 on migration and invasion of HCC cells, we performed transwell assays with HCCLM3 and HepG2 cells. Overexpression of miR-205 was accompanied by decreased migration and invasion of HCCLM3 cells in comparison to those in the NC group (\( P < 0.001 \), Fig. 2E). Similar effects of the inhibition of miR-205 were observed in HepG2 cells (\( P < 0.001 \), Supplemental Fig. S2). Collectively, these results indicate that miR-205 acts as a tumor suppressor and attenuates cell growth, apoptosis, invasion, and migration of HCC cells *in vitro*.

**TABLE 2. Univariate and multivariate analysis of prognostic factors influencing overall survival of patients with HCC**

| Variable                        | n  | Univariate analysis |     | Multivariate analysis         |     |
|---------------------------------|----|---------------------|-----|-------------------------------|-----|
|                                 |    | Survival (mo)       |     | HR (95% CI)                   |
|                                 |    |                     |     |                              |
| Age (yr)                        |    |                     |     |                              |
| <50                             | 22 | 15.4                | 0.164 |                              |
| \( \geq 50 \)                   | 28 | 32.3                | 0.293 |                              |
| Gender                          |    |                     |     |                              |
| Male                            | 39 | 15.4                | 0.293 |                              |
| Female                          | 11 | 36.1                | 0.293 |                              |
| Tumor size (cm)                 |    |                     |     |                              |
| \( \leq 5 \)                    | 21 | 33.5                | 0.097 |                              |
| >5                              | 29 | 13.9                | 0.097 |                              |
| Lesion number                   |    |                     |     |                              |
| Single                          | 32 | 37.1                | \(<0.001^{*}\) | 2.097 (0.773–5.686) | 0.146 |
| Multiple                        | 18 | 8.8                 |     |                              |
| Capsule formation               |    |                     |     |                              |
| No                              | 31 | 15.3                | 0.010* | 0.603 (0.263–1.383) | 0.232 |
| Yes                             | 19 | 45.1                | 0.010* | 0.603 (0.263–1.383) | 0.232 |
| Macrvascular invasion           |    |                     |     |                              |
| No                              | 38 | 28.1                | 0.001* | 1.481 (0.526–4.169) | 0.457 |
| Yes                             | 12 | 7.7                 | 0.001* | 1.481 (0.526–4.169) | 0.457 |
| Lymphovascular invasion         |    |                     |     |                              |
| No                              | 30 | 28.1                | 0.196 |                              |
| Yes                             | 20 | 15.3                | 0.196 |                              |
| TNM staging                     |    |                     |     |                              |
| I/II                            | 28 | 40.8                | \(<0.001^{*}\) | 2.281 (0.789–6.598) | 0.128 |
| III/IV                          | 22 | 8.8                 |     |                              |
| Edmondson-Steiner grade         |    |                     |     |                              |
| I–II                            | 22 | 36.1                | 0.004* | 1.289 (0.601–2.763) | 0.515 |
| III–V                           | 28 | 12.8                | 0.004* | 1.289 (0.601–2.763) | 0.515 |
| miR-205 expression              |    |                     |     |                              |
| Low                             | 25 | 13.5                | 0.001* | 2.497 (1.118–5.577) | 0.026* |
| High                            | 25 | 40.8                | 0.001* | 2.497 (1.118–5.577) | 0.026* |

CI, confidence interval. \*\( P < 0.05 \).
Figure 2. MiR-205 not only inhibits cell proliferation, migration, and invasion of HCC cells but also induces HCC cells apoptosis in vitro. A) HepG2 and HCCLM3 cells, which were transfected with corresponding miRNA vectors, were measured by qRT-PCR for miR-205 expression. B) Effect of miR-205 on HCC cell proliferation was determined with a CCK-8 assay. C) Colony formation assays in HCC cells transfected with miR-205 or NC. D) Flow cytometer was used to analyze the changes in HCC cell apoptosis when transfected with miR-205 or NC. E) Transwell assays in HCCLM3 cells transfected with miR-205 or NC. **P < 0.01, ***P < 0.001.
Figure 3. MiR-205 suppresses the EMT process of HCC cells in vitro and inhibits the growth of HCCLM3 xenograft tumors. A–C) qRT-PCR and Western blot results of the expression of EMT markers E-cadherin and vimentin in HepG2 and HCCLM3 cells with miR-205 or NC vector transfection. D) Immunofluorescence staining of EMT markers E-Cadherin and vimentin in HepG2 and HCCLM3 cells with miR-205 overexpression. E) Xenograft tumors harvested from the nude mice at 40 d. F) Tumor growth curves of subcutaneous injection of HCCLM3 cells that stably overexpressed miR-205 or NC vector. *P < 0.05.
MiR-205 inhibits the EMT process of HCC cells

Considering that EMT has a critical role in the invasion and metastasis of HCC and that miRNAs have been implicated in regulating EMT, we performed experiments to assess whether miR-205 inhibits EMT in HCC cells. qRT-PCR and Western blot results revealed that miR-205 overexpression remarkably increased the mRNA and protein levels of the epithelial marker E-cadherin, whereas the mRNA and protein levels of mesenchymal marker vimentin were significantly reduced (P < 0.05, respectively; Fig. 3A–C). Immunofluorescence analysis also confirmed this result by showing that overexpression of miR-205 was associated with enhanced expression of E-cadherin and decreased expression of vimentin (Fig. 3D). Taken together, these data suggest that miR-205 suppresses the EMT process in HCC cells.

MiR-205 inhibits tumor growth of HCC in vivo

Based on our results in vitro, we further identified the biologic function of miR-205 in HCC growth in vivo. HCCLM3 cells that stably overexpressed miR-205 or the NC vector were injected subcutaneously into nude mice. Although tumors successfully formed in all mice, the tumor growth rate of HCCLM3-miR-205 group was significantly slower than that of the HCCLM3-NC group (Fig. 3E). In addition, the sizes of tumors from mice injected with miR-205 were significantly smaller than those of mice injected with the NC vector (Fig. 3F). Therefore, these results indicate that overexpression of miR-205 suppresses tumor growth of HCC in vivo.

SEMA4C is directly targeted by miR-205 and up-regulated in HCC

To reveal the underlying molecular mechanisms by which miR-205 exerts its functional effects on HCC cells, we predicted and identified the potential target genes of miR-205 by searching 3 databases, including TargetScan, miRDB, and miRNAAda. SEMA4C was screened and selected as the candidate target gene of miR-205 because the complementary sequence of miR-205 was found in the 3'-UTR of SEMA4C (Fig. 4A). Furthermore, previous studies have demonstrated that SEMA4C is highly expressed in breast cancer and lung cancer cells and can prompt the invasion and metastasis of tumors by inducing EMT (23, 24).

To further verify that SEMA4C was directly targeted by miR-205 in HCC, we investigated whether miR-205 directly interacted with the predicted 3'-UTR of SEMA4C mRNA by a dual-luciferase reporter system. Results showed that miR-205 overexpression significantly inhibited the luciferase activity of wt-SEMA4C-3'-UTR compared with that of the NC miRNAs (P < 0.05, Fig. 4B). In contrast, the luciferase activity of mt-SEMA4C-3'-UTR was not influenced by miR-205 or the NC miRNA, indicating that SEMA4C was directly targeted by miR-205. Consistent with the above findings, qRT-PCR and Western blot also demonstrated that overexpression of miR-205 markedly suppressed the mRNA and protein levels of SEMA4C (Fig. 4C, D).

The mRNA levels of SEMA4C were also validated by qRT-PCR in the previous cohort of 25 paired HCC clinical specimens. Compared with adjacent, healthy liver tissues, SEMA4C mRNA was significantly up-regulated in 16 (64%) HCC tissues (P < 0.05, Fig. 5A). Additionally, SEMA4C protein expression in HCC tissues detected by immunohistochemical staining analysis was dramatically increased when compared with adjacent, noncancerous tissues (P < 0.05, Fig. 5B). We also performed a Kaplan-Meier survival analysis to evaluate the prognostic value of SEMA4C in patients with HCC. As shown in Fig. 5C, patients with high SEMA4C expression levels had shorter OS and DFS (Supplemental Fig. S3) than those with low expression levels of SEMA4C (P < 0.05). These data suggest that SEMA4C is up-regulated in HCC and correlated with poor prognosis in patients with HCC.

SEMA4C mediates the functional effects of miR-205 and EMT on HCC cells

To ascertain whether miR-205 elicits inhibitory effects on HCC cells through SEMA4C, SEMA4C was...
Figure 5. SEMA4C is up-regulated in HCC and overexpression of SEMA4C restores miR-205–mediated migration, invasion, and EMT of HCC cells in vitro. A) qRT-PCR detection of SEMA4C mRNA expression levels in 25 pairs HCC tissues and their matched tumor-adjacent tissues. B) Immunohistochemical staining analysis of SEMA4C protein expression levels in 25 pairs HCC tissues (continued on next page)
restored by overexpression plasmids in HCCLM3 cells stably overexpressing miR-205. Subsequent Western blotting results confirmed the overexpression of SEMA4C in miR-205-overexpressing HCCLM3 cells (Fig. 5D). As expected, SEMA4C restoration abrogated the inhibitory effects on migration and invasion of HCCLM3 cells induced by miR-205 (P < 0.05, Fig. 5E). Likewise, the repressive effects of miR-205 on EMT were rescued by overexpression of SEMA4C, leading to molecular changes associated with up-regulation of E-cadherin and reduced expression of vimentin (Fig. 5D). Thus, these data provided evidence that reexpression of SEMA4C could rescue miR-205-mediated migration, invasion, and EMT of HCC cells.

**DISCUSSION**

Emerging evidence has demonstrated that dysregulation of miRNAs has a crucial role in the carcinogenesis and progression of HCC (25–27). A previous study (28) showed that miR-205 expression levels decrease in HCC tissues as compared with those in matched healthy tissues, and down-regulation of miR-205 promotes stemness of HCC by targeting PLCβ1 and increasing CD24 expression. However, the relationship between miR-205 expression and prognosis in patients with HCC, as well as its functional roles in HCC are still largely unknown. In this study, we found that miR-205 was significantly down-regulated in HCC, especially in aggressive HCC phenotypes. In addition, reduced miR-205 expression was correlated with poor clinicopathologic features and shorter DFS and OS. These data strongly suggest that miR-205 may serve as a tumor suppressor in HCC and is a promising predictor for prognosis in patients with HCC.

MiRNAs have been recognized as key regulators in the invasion and metastasis of human cancers (29, 30). Thus, we explored whether miR-205 was involved in the progression of HCC through modulating the metastatic behaviors of HCC cells. Our results showed that overexpression of miR-205 inhibited the metastatic ability of HCC cells in vitro. Additionally, to confirm the similar biologic function of miR-205 in a complex microenvironment, we evaluated the suppressive effect of miR-205 in vivo. In agreement with our in vitro findings, miR-205 also inhibited tumor growth of HCC grafted in mice.

EMT, a key process characterized by loss of epithelial marker E-cadherin, acquisition of mesenchymal marker vimentin, and enhanced migratory and invasive behaviors, is attracting increasing attention as a crucial mechanism that facilitates progression and metastasis of cancer (18, 31). Previous studies have revealed that up-regulation of miR-205 promoted EMT process in cervical cancer and lung cancer cells (32), whereas other reports showed the opposite results, that down-regulation of miR-205 resulted in acceleration of EMT in prostate cancer and breast cancer cells (13, 33). In the present study, we found that ectopic expression of miR-205 significantly increased E-cadherin expression and concomitantly decreased vimentin expression. These findings suggest miR-205 may inhibit invasion and metastasis of HCC by hindering the EMT process.

SEMA4C, a member of the semaphorin family, has been reported to be highly expressed in several human cancers and encourages proliferation, migration, and EMT of cancer cells (23, 34). Moreover, miR-25-3p can reverse the EMT process in cisplatin-resistant cervical cancer cells via targeting SEMA4C (35). However, the functional role of SEMA4C in HCC remains unclear. Here, SEMA4C was confirmed as a direct downstream target of miR-205 and mediated the biologic function of miR-205 in HCC. Interestingly, this interaction between miR-205 and SEMA4C has not, to our knowledge, been previously reported. Our conclusion was based on the following evidence. First, miR-205 overexpression remarkably decreased the expression of SEMA4C at both the mRNA and protein levels in HCC cells. Second, the complementary sequence of miR-205 was identified in the 3’UTR of SEMA4C mRNA, and, importantly, the luciferase reporter activity of wt-3’UTR instead of mt-3’UTR was specifically attenuated by miR-205 overexpression. Third, SEMA4C expression was up-regulated in HCC tissues, and that up-regulation was inversely correlated with down-regulation of miR-205, and elevated SEMA4C expression was closely related to poor prognosis in patients with HCC. Fourth, we discovered that recovery of SEMA4C expression reversed the inhibitory effects of miR-205 on migration, invasion, and EMT process of HCC cells. These results indicate that SEMA4C is directly targeted by miR-205 and functions as an oncogene in HCC.

However, the molecular mechanisms by which miR-205/SEMA4C contributes to the metastasis and EMT of HCC are still entirely unknown. Previous studies have demonstrated that SEMA4C promoted the proliferation and migration of cancer cells by activating PlexinB2-MET signaling. Interestingly, aberrant activation of Met signaling was confirmed to be closely associated with tumor growth, angiogenesis, metastasis, and poor survival in HCC (36, 37). Furthermore, Met can also activate an EMT program in human HCC cells (38). Consequently, miR-205/SEMA4C probably promotes the metastasis and EMT of HCC by activating Met signaling. Further studies and their matched tumor-adjacent tissues. C) Kaplan-Meier analysis of overall survival between high (n = 13) and low (n = 12) SEMA4C expression levels of patients with HCC. D) Western blot analysis of SEMA4C and EMT markers E-Cadherin and vimentin in HCCLM3 cells that stably express miR-205 or NC vector after restoration of SEMA4C expression. E) Transwell assays in HCCLM3 cells that stably expressing miR-205 or NC vector after restoration of SEMA4C expression. *P < 0.05, **P < 0.01.
should identify the pathways that miR-205/SEMA4C mediates for its inhibitory effects on metastasis and EMT in HCC.

In summary, we discovered that miR-205 is frequently down-regulated in HCC and is significantly correlated with poor prognosis in patients. Our results indicate that miR-205 inhibits the metastatic behaviors and EMT of HCC cells by directly targeting SEMA4C. Therefore, this study provides new insight into the mechanism involved in HCC progression and suggests that miR-205 may act as a novel biomarker and promising therapeutic target in HCC.

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AUTHOR CONTRIBUTIONS

X. Xiong and N. Cheng conceived and designed the study: J. Lu, Y. Lin, R. Zhou, Y. Jin, and B. Li performed the experiments; J. Lu, Y. Lin, and N. Cheng analyzed the data and drafted the manuscript; F. Li and H. Ye interpreted the data and revised the manuscript; J. Lu, Y. Lin, R. Zhou, Y. Jin, and D. Li.

REFERENCES

1. El-Serag, H. B., and Rudolph, K. L. (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132, 2557–2576
2. Forner, A., Llovet, J. M., and Bruix, J. (2012) Hepatocellular carcinoma. Lancet 379, 1245–1255
3. Maluccio, M., and Covey, A. (2012) Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. CA Cancer J. Clin. 62, 394–399
4. Zanone, P. D., and Haley, B. (2005) Ribo-gnome: the big world of noncoding RNA. Science 309, 1510–1524
5. Calin, G. A., and Croce, C. M. (2006) MicroRNA signatures in human cancers. Nat. Rev. Cancer 6, 857–866
6. Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., Downing, J. R., Jacks, T., Horvitz, H. R., and Golub, T. R. (2005) MicroRNA expression profiles classify human cancers. Nature 435, 834–838
7. Yang, X., Zhang, X. F., Lu, X., Jia, H. L., Liang, L., Dong, Q. Z., Ye, Q. H., and Qin, L. X. (2014) MicroRNA-20a inhibits angiogenesis in human hepatocellular carcinoma by targeting hepatocyte growth factor-CMet pathway. Hepatology 59, 1874–1885
8. Dou, C., Liu, Z., Xu, M., Jia, Y., Wang, Y., Li, Q., Yang, W., Zheng, X., Tu, K., and Liu, Q. (2016) miR-187-3p inhibits the metastasis and epithelial–mesenchymal transition of hepatocellular carcinoma by targeting S100A4. Cancer Lett. 381, 380–389
9. Kapur, T. D., Ganda, C., Brown, R. M., Beveridge, D. J., Richardson, K. L., Chaturvedi, V., Candy, P., Epis, M., Winkle, L., Kalnowski, F., Kopf, C., Smart, L. M., Yeoh, G. C., George, J., and Leedman, P. J. (2017) A miR-7/GAS5/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma. Hepatology 67, 216–231
10. He, J., Zhao, K., Zheng, L., Xu, Z., Gong, W., Chen, S., Shen, X., Huang, G., Gao, M., Zeng, Y., Zhang, Y., and He, F. (2015) Upregulation of microRNA-122 by farnesoid X receptor suppresses the growth of hepatocellular carcinoma cells. Mol. Cancer 14, 163
11. Qin, A. Y., Zhang, X. W., Liu, L., Yu, J. P., Li, H., Wang, Z. Z., Ren, X. B., and Gao, S. (2013) MiR-205 in cancer: an angel or a devil? Eur. J. Cell Biol. 92, 54–60
12. Chaudhary, A. K., Mondal, G., Kumar, V., Kattel, K., and Mahato, R. I. (2017) Chemosensitization and inhibition of pancreatic cancer stem cell proliferation by overexpression of microRNA-205. Cancer Lett. 402, 1–8
13. Tucci, P., Agostini, M., Grespi, F., Markert, E. K., Terrinoni, A., Vosuden, K. H., Muller, P. A., Dötsch, V., Kehrlhoesser, S., Sayan, B. S., Giaccone, G., Lowe, S. W., Takahashi, N., Vandenabeele, P., Knight, R. A., Levine, A. J., and Melino, G. (2012) Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer; erratum: 111, 2855. Proc. Natl. Acad. Sci. USA 109, 15312–15317
14. Zhang, P., Wang, L., Rodriguez-Aguayo, C., Yuan, Y., Debeb, B. G., Chen, D., Sun, Y., You, M. J., Liu, Y., Dean, D. C., Woodward, W. A., Liang, H., Yang, X., Lopez-Berestein, G., Sood, A. K., Hu, Y., Ang, K. K., Chen, J., and Ma, L. (2014) miR-205 acts as a tumour radiosensitizer by targeting ZEB1 and Ubc13. Nat. Commun. 5, 5671
15. Hirata, H., Hinoda, Y., Shahryari, V., Deng, G., Nakajima, K., Tabatabai, Z. L., Ishii, N., and Dahiya, R. (2015) Long non-coding RNA MALAT1 promotes aggressive renal cell carcinoma through EzH2 and interacts with miR-205. Cancer Res. 75, 1322–1331
16. Su, N., Qu, H., Chen, Y., Yang, T., Yan, Q., and Wan, X. (2013) miR-205 promotes tumor proliferation and invasion through targeting ESRG in endometrial carcinoma. Oncol. Rep. 29, 2297–2302
17. Xie, H., Zhao, Y., Caramuta, S., Larsson, C., and Lui, W. O. (2012) miR-205 expression promotes cell proliferation and migration of human cervical cancer cells. PLoS One 7, e46990
18. Lamouille, S., Xu, J., and Derynck, R. (2014) Molecular mechanisms of epithelial–mesenchymal transition. Nat. Rev. Mol. Cell Biol. 15, 178–196
19. Zhao, J., Ou, B., Han, D., Wang, P., Zong, Y., Zhu, C., Liu, D., Zheng, M., Sun, J., Feng, H., and Lu, A. (2017) Tumor-derived CXL5 promotes human colorectal cancer metastasis through activation of the ERK/Erk-1/2-Snail and AKT/GSK3β/b-catenin pathways. Mol. Cancer 16, 70
20. Ye, L. Y., Chen, W., Bai, X. L., Xu, X. Y., Zhang, Q., Xia, X. F., Sun, X., Li, G. G., Hu, Q. D., Fu, Q. H., and Liang, T. B. (2016) Hypoxia-induced epithelial-to-mesenchymal transition in hepatocellular carcinoma induces an immunosuppressive tumor microenvironment to promote metastasis. Cancer Res. 76, 818–830
21. Xiao, S., Chang, R. M., Yang, M. Y., Lei, X., Liu, X., Gao, W. B., Xiao, J. L., and Yang, L. Y. (2016) Actin-like 6A predicts poor prognosis of hepatocellular carcinoma and promotes metastasis and epithelial–mesenchymal transition. Hepatology 63, 1256–1271
22. Zhao, X., Lu, Y., Nie, Y., and Fan, D. (2013) MicroRNAs as critical regulators involved in regulating epithelial–mesenchymal transition. Curr. Cancer Drug Targets 13, 935–944
23. Li, J., Wang, Q., Wen, R., Liang, J., Zhong, X., Yang, W., Su, D., and Tang, J. (2015) MiR-138 inhibits cell proliferation and reverses epithelial–mesenchymal transition in non-small cell lung cancer cells by targeting GT1 and SEMA4C. J. Cell. Mol. Med. 19, 2797–2805
24. Yang, Q., Wang, Y., Lu, X., Zhao, Z., Zhu, L., Chen, S., Wu, Q., Chen, C., and Wang, Z. (2015) MiR-125b regulates epithelial–mesenchymal transition via targeting Sema4C in paclitaxel-resistant breast cancer cells. Oncotarget 6, 3268–3279
25. Wu, H., Tao, J., Li, X., Zhang, T., Zhao, L., Wang, Y., Zhang, L., Xiong, J., Zeng, Z., Zhan, N., Steer, C. J., Che, L., Dong, M., Wang, X., Niu, J., Li, Z., Yan, G., Chen, X., and Song, G. (2017) MicroRNA-296 prevents the pathogenesis of hepatocellular carcinoma via modulating expression of Cmet and CdK6. Hepatology 66, 1952–1967
26. Fang, F., Chang, R. M., Yu, L., Lei, X., Xiao, S., Yang, H., and Yang, L. Y. (2015) MicroRNA-185-5p suppresses tumor cell proliferation and metastasis by directly targeting FGFR5 in hepatocellular carcinoma. J. Hepatol. 63, 874–885
27. Fu, X., Wen, H., Jing, L., Yang, Y., Wang, W., Liang, X., Nan, K., Yao, Y., and Tian, T. (2017) MicroRNA-155-5p promotes hepatocellular carcinoma progression by suppressing PTEN through the PI3K/Akt pathway. Cancer Sci. 108, 620–631
28. Zhao, J., Xu, G., Qian, Y. W., and Li, Y. W. (2015) Down-regulation of miR-205 promotes stemness of hepatocellular carcinoma cells by targeting PLCβ1 and increasing CD24 expression. Neoplasma 62, 567–573.

29. Song, S., and Ajani, J. A. (2013) The role of microRNAs in cancers of the upper gastrointestinal tract. Nat. Rev. Gastroenterol. Hepatol. 10, 109–118.

30. Su, Y., Li, X., Ji, W., Sun, B., Xu, C., Li, Z., Qian, G., and Su, C. (2014) Small molecule with big role: microRNAs in cancer metastatic microenvironments. Cancer Lett. 344, 147–156.

31. De Craene, B., and Berx, G. (2013) Regulatory networks defining EMT during cancer initiation and progression. Nat. Rev. Cancer 13, 97–110.

32. Wang, X., Yu, M., Zhao, K., He, M., Ge, W., Sun, Y., Wang, Y., Sun, H., and Hu, Y. (2016) Upregulation of MiR-205 under hypoxia promotes epithelial–mesenchymal transition by targeting ASPP2. Cell Death Dis. 7, e2517.

33. Lee, J. Y., Park, M. K., Park, J. H., Lee, H. J., Shin, D. H., Kang, Y., Lee, C. H., and Kong, G. (2014) Loss of the polycomb protein Mel-18 enhances the epithelial-mesenchymal transition by ZEB1 and ZEB2 expression through the downregulation of miR-205 in breast cancer. Oncogene 33, 1325–1335.

34. Wei, J. C., Yang, J., Liu, D., Wu, M. F., Qiao, L., Wang, J. N., Ma, Q. F., Zeng, Z., Ye, S. M., Guo, E. S., Jiang, X. F., You, L. Y., Chen, Y., Zhou, L., Huang, X. Y., Zhu, T., Meng, L., Zhou, J. F., Feng, Z. H., Ma, D., and Gao, Q. L. (2017) Tumor-associated lymphatic endothelial cells promote lymphatic metastasis by highly expressing and secreting SEMA4C. Clin. Cancer Res. 23, 214–224.

35. Song, J., and Li, Y. (2017) miR-25-3p reverses epithelial-mesenchymal transition via targeting Sema4C in cisplatin-resistant cervical cancer cells. Cancer Sci. 108, 23–31.

36. Requinsou, S., La Bella, T., Rekik, S., Imbeaud, S., Calatayud, A. L., Rohr-Udilova, N., Martin, Y., Couchy, G., Bioulac-Sage, P., Grasl-Kraupp, B., de Koning, L., Ganne-Carrie, N., Nault, J. C., Ziol, M., and Zucman-Rossi, J. (2017) Proliferation markers are associated with MET expression in hepatocellular carcinoma and predict Tivantinib sensitivity in vitro. Clin. Cancer Res. 23, 4364–4375.

37. Goyal, L., Muzumdar, M. D., and Zhu, A. X. (2013) Targeting the HGF/c-MET pathway in hepatocellular carcinoma. Clin. Cancer Res. 19, 2310–2318.

38. Kodama, T., Newberg, J. Y., Kodama, M., Rangel, R., Yoshihara, K., Tien, J. C., Parsons, P. H., Wu, H., Finegold, M. J., Copeland, N. G., and Jenkins, N. A. (2016) Transposon mutagenesis identifies genes and cellular processes driving epithelial-mesenchymal transition in hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 113, E3384–E3393.

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