Overexpression of miR-19a inhibits colorectal cancer angiogenesis by suppressing KRAS expression

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Abstract. The microRNA miR-19a is closely related to tumor formation and development and is a key oncogene. Previous studies have demonstrated that miR-19a is upregulated in multiple cancer types, including colorectal cancer (CRC). However, most of these experiments were performed in vitro, and consequently, the mechanisms underlying the effects of miR-19a on CRC are still unclear. Therefore, in the present study, we investigated the role of miR-19a in the development of solid CRC tumors. We generated KRAS 3’UTR-Mut by deleting the predicted binding site for miR-19a in KRAS, and observed that the expression of a reporter gene containing the KRAS 3’UTR in HCT116 cells was suppressed by miR-19a, while that of a reporter gene with mutant KRAS 3’UTR was unaffected by miR-19a. We observed that high miR-19a levels reduced KRAS expression. In the tube formation assay, overexpression of miR-19a exhibited anti-angiogenesis effects, which were rescued by KRAS expression. We established a nude mouse xenograft model to investigate the specific role of miR-19a in solid tumors. The results revealed that the sizes of xenograft tumors and density of blood vessels developed from HCT116 cells expressing miR-19a were smaller/lower compared with those of the control. KRAS and VEGFA levels were also reduced. In conclusion, our results revealed that miR-19a overexpression suppressed KRAS expression and angiogenesis in CRC, indicating possibilities of using miR-19a in future therapeutic applications.

Introduction

Colorectal cancer (CRC) is one of the most common cancers in China, and its incidence rate has increased rapidly in both men and women in recent years (1). Various factors affect the expression of colorectum-specific genes, which contribute to the development and progression of CRC. Mutations and microRNAs (miRNAs) play important roles in the development and progression of CRC. KRAS mutations, particularly the activated KRAS mutation, induces cell invasion and maintains metastases in CRC, which is considered a landmark event in CRC (2), and patients with KRAS mutations have lower survival rates (3). Therefore, targeting KRAS is a promising treatment strategy for CRC.

miRNAs play critical roles in CRC tumorigenesis (4). In particular, the following miRNA genes are important: the miR-17-92 cluster consisting of 6 miRNA genes (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1), the miR-106b-25 and the miR-106a-363 cluster (5). Previous studies have shown that the miR-17-92 cluster is upregulated in various human cancers, including CRC (6), and is often thought to play important roles in the development and progression of CRC (7,8).

Among the miRNAs within the miR-17-92 cluster, miR-19 functions as a key oncogenic miRNA (9). miR-19 is comprised of miR-19a and miR-19b, which share a 96% identity, differing by a single nucleotide at position 11, and are likely to regulate the same mRNA targets. Indeed, miR-19a expression has been revealed to be closely related to the development and progression of several types of tumors (10,11). For example, miR-19a contributed to the proliferation and invasion of CRC cells (12). Additionally, hsa-miR-19a is associated with lymph metastasis and mediates tumor necrosis factor (TNF)-α-induced epithelial-to-mesenchymal transition in CRC (13). miR-19 mediated inhibition of transglutaminase-2 led to enhanced invasion and metastasis in CRC (14). However, several studies have revealed that miR-19a may negatively affect CRC development. For example, Yu et al demonstrated that miR-19a can suppress tissue factor expression in vitro and inhibit colon cancer cell migration and invasion (15). Moreover, most of the experiments reported have been based on in vitro cell culture, and few experiments have ascertained the actual role of miR-19a in colorectal solid tumors in vivo. Thus, the specific role of miR-19a in CRC remains unclear.

We identified KRAS as one of the target genes of miR-19a, using a site-prediction web site, and were interested in investigating the effect of miR-19a overexpression in CRC.

In the present study, we aimed to elucidate the precise role and underlying mechanisms of miR-19a in CRC, using both
CHEN et al: miR-19a OVEREXPRESSION INHIBITS KRAS EXPRESSION AND CRC ANGIogenesis

620

Table I. Sequences of primers used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>miR-19a Probe</td>
<td>5'-UCAGUUUUGCAUAGAUVUGCACA-3'</td>
</tr>
<tr>
<td>KRAS qRT-PCR</td>
<td>5'-ACAGAGAGTGAGGATGCTTT-3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5'-TTTCACACACCAGGAGTCTT-3' (reverse)</td>
</tr>
<tr>
<td>hKRAS-3'UTR</td>
<td>5'-CCGTGTAATCTAGGCTTATTTAAAAATGACAGTGGAAGT-3' (forward, F1)</td>
</tr>
<tr>
<td></td>
<td>5'-CGCCCCCAGTCTAGGATAGGGTTCTGCTATTCATACC-3' (reverse, R1)</td>
</tr>
<tr>
<td>hKRAS-3'UTR-M</td>
<td>5'-TTGGATAGCCTCAAAAGATAAATCTCACTGTGG-3' (forward, F2)</td>
</tr>
<tr>
<td></td>
<td>5'-TGTTGAGCTATCCCAAGTCCCTCCATTTGGACTA-3' (reverse, R2)</td>
</tr>
<tr>
<td>sh-KRAS-1#</td>
<td>5'-AAAAAGGTTCCTACAGGAGCAAGTTGGATCCAAACTTGTCCCTGAGGATACCC-3'</td>
</tr>
<tr>
<td>sh-KRAS-2#</td>
<td>5'-AAAAAGGTTCCTACAGGAGCAAGTTGGATCCAAACTTGTCCCTGAGGATACCC-3'</td>
</tr>
<tr>
<td>sh-control</td>
<td>5'-AAAATACAACACCGCAAACCTATATTTGGATCCAAATAGACGTGGCTTGGTA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AGAAAGGCTGTTGGCTATTTG-3' (forward)</td>
</tr>
<tr>
<td></td>
<td>5'-AGGGGCAACATCAGTCTTTC-3' (reverse)</td>
</tr>
<tr>
<td>U6 probe</td>
<td>5'-CTCGCTTCCAGCAGCACA-3'</td>
</tr>
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in vitro assays and an in vivo mouse tumor model. We believe that our findings provide important insights into CRC biology and highlight the possibility of using miR-19a as a therapeutic agent for CRC in future.

Materials and methods

Study approval. Animal experiments were performed in compliance with the rules of the Animal Use Committee of Fujian Medical University. The present study was approved by the Institutional Ethics Board of Fujian Medical University Union Hospital.

Northern blotting. Northern blotting was performed to detect miRNAs as previously described (16). Briefly, we used RNAiso Plus to extract 10-20 µg total RNA from cells. The RNA was then separated on 10% denaturing polyacrylamide gels and electrotransferred to nylon membranes. DNA oligonucleotides that were antisense to mature miRNAs and end-labeled with 32P were used as probes. U6 snRNA was used as an internal control. A miR-19a probe (Pr009177336, 5'-UCAGUUUUGCAUAGAUVUGCACA-3') was used to detect miR-19a.

Cell culture and transfection. The human CRC cell line HCT116 (a gift from Professor Pan Jinshui) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Gaithersburg, MD, USA) and was maintained at 37°C in a humidified 5% CO2 atmosphere. The miR-19a mimic (5'-UGUGCAAAACUAUGCAAAACUGA-3'), inhibitor and negative control were purchased from Ribobio (Guangzhou, China). For transfection, cells (3x10^6/well) were cultured in a six-well plate until they reached 40-50% confluency, and the mimics were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Histology and immunohistochemistry. Hematoxylin and eosin (H&E) staining was performed using standard protocols. Briefly, deparaffinized rehydrated sections were stained with hematoxylin for 5 min, incubated in 0.1% acid alcohol, and then counterstained in 0.5% eosin Y solution. Sections were finally dehydrated and examined by a skilled pathologist. For immunohistochemistry, sections were deparaffinized and quenched with 3% H2O2. After the antigen was retrieved in 10 mM sodium citrate buffer and blocked with goat serum (ZLI-9022; ZSGB-BIO, Xicheng, Beijing, China), the sections were incubated with antibodies recognizing CD31 (ab28364; Abcam, Cambridge, UK) or KRAS (12063-1-AP; Proteintech, Chicago, IL, USA) and then with secondary antibodies conjugated with horseradish peroxidase (PV-6101; ZSGB-BIO). Sections were processed according to the manufacturer's instructions and counterstained with hematoxylin.

Western blotting and antibodies. We performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate total cell lysates, and then transferred the proteins to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Western blotting was performed with the appropriate antibodies, which were visualized by enhanced chemiluminescence in accordance with the manufacturer's instructions (ECL; Millipore).

Quantitative real-time polymerase chain reaction (qRT-PCR). Reverse transcription of total RNA was performed using 4 µg total RNA from HCT116 cells. M-MLV reverse transcriptase (BGI, Shenzhen, China) was used to generate cDNAs. The mRNA level of KRAS was assessed by qRT-PCR using SYBR-Green I on a CFX96 real-time RT-PCR detection system (Bio-Rad, Hercules, CA, USA). The sequences of the primers used in the present study are listed in Table I. PCR was performed for 45 cycles using the following conditions: denaturation at 95°C for 20 sec, annealing at 58°C for
20 sec, and elongation at 72°C for 20 sec. All values were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and the relative expression was calculated according to the ΔΔCT method (17), where ΔΔCT = ΔCTsample - ΔCTGAPDH.

In vitro tube formation assay. The tube formation efficiency of human umbilical vein endothelial cells (HUVECs) was assessed by an angiogenesis assay on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). First, we prepared the GFP-labeled HUVECs, as described by Guo et al (18), and then 36 h after transfection with miR-19a, KRAS/sh-KRAS-2# or VEGFA expression constructs, the HUVECs (1x10^5 cells) were added to a 24-well plate coated with 100 µl Matrigel basement membrane matrix (BD Biosciences), which was derived from an Engelbreth-Holm-Swarm tumor. After culturing for 12 h, we recorded tube formation with an IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan) at a low magnification of (x10). Four wells for each treatment were photographed. The images were saved as JPEG files and analyzed using TCS Cellworks AngioSys 1.0 software (Botolph Claydon, Buckingham, UK) to quantify the effects on angiogenesis. Data were analyzed using unpaired Student’s t-test of GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Tumorigenesis assay. HCT116 cells were infected using the appropriate amount of lentivirus, and then subjected to amplification. Infected cells were trypsinized and suspended in FBS-free DMEM-F12 medium at a density of 6x10^6 cells/0.1 ml. The suspended cells were then implanted into the backs of 27 five-week-old BALB/c female nude mice divided into 3 groups, with each group containing nine mice for initiating tumor xenografts. Tumor diameters were measured every 3 days. Data were analyzed using non-parametric tests such as Kruskal-Wallis tests were performed when comparing >2 groups. For qualitative data, non-parametric tests such as Kruskal-Wallis tests were performed.

DNA constructs. The plv vector carrying a copy of miR-19a or VEGFA was a kind gift from Dr Lixin Hong (School of Life Science, Xiamen University, Fujian, China). The pGL3 control vector carrying a 1,923-bp fragment of the 3′ UTR of human VEGFA mRNA was directly purchased from RiBoBio. The KRAS 3′ UTR harboring predicted target sites for miR-19a (Fig. 1A) was PCR-amplified using primers containing XbaI sites and cloned into the XbaI-digested pGL3 control vector. The recombinant plasmids were constructed using ligation-independent combination (LIC). F1 and R1 primers were used to generate the KRAS wild-type 3′ UTR. To construct 3′UTRs with deletion mutations, two sets of PCR were performed using the primer pairs F1/R2 and F2/R1 to generate two fragments. Then, the two fragments were cloned into the XbaI-digested pGL3 control vector using LIC.

The DNA oligos encoding shRNA sequences were designed and cloned into the expression vector pLV-H1-EF1a-puro using single oligonucleotide RNAi technology developed by Biosettia (San Diego, CA, USA). All lentiviral-shRNA vectors were constructed following the manufacturer’s protocol. The primers used in the present study are listed in Table I.

Dual-luciferase reporter assay. HCT116 cells were plated in six-well culture plates 24 h before transfection. The cells were then transfected with the recombinant pGL3 vector (1.6 µg), plv-miR-19a (0.2 µg) and the pRLTK vector (0.2 µg) for normalization of transfection efficiency. Cell lysates were collected and assayed 48 h after transfection. Firefly and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activities were calculated as the mean ± standard deviation (SD) after normalization to Renilla luciferase activity. Three independent experiments were performed.

Statistical analysis. Mann-Whitney tests were performed to compare the differences between two groups when vari-ances were unequal. Analysis of variance with post hoc tests were performed when comparing >2 groups. For qualitative data, non-parametric tests such as Kruskal-Wallis tests were performed.

Results

miR-19a suppresses KRAS expression in HCT116 cells. We identified the conserved miR-19a-binding site in the 3′ UTR region of human KRAS using the microRNA.org website (Fig. 1A). The KRAS 3′UTR-Mut was generated by deleting the predicted binding site. We observed that a reporter gene containing the KRAS 3′ UTR was suppressed by miR-19a,
CHEN et al: miR-19a OVEREXPRESSION INHIBITS KRAS EXPRESSION AND CRC ANGIogenesis

while removal of the predicted miR-19a-binding site resulted in insensitivity of the reporter gene to miR-19a in HCT116 cells (Fig. 1B).

To ascertain the effect of miR-19a on KRAS expression in HCT116 cells, the mature miR-19a mimic/inhibitor was transfected in HCT116 cells. The results revealed that the endogenous KRAS mRNA and protein levels were significantly downregulated in the miR-19a mimic cells and upregulated in the miR-19a inhibitor cells compared with those in the untransfected cells (Fig. 2A and B). Accordingly, the VEGFA protein level was also downregulated in the miR-19a mimic cells and upregulated in the miR-19a inhibitor cells (Fig. 2B).

Next, in order to construct stable miR-19a-overexpressing HCT116 cell lines for subsequent studies, we transfected HCT116 cells with a lentivirus expressing miR-19a. Then, via northern blotting, we confirmed miR-19a overexpression in HCT116 cells (Fig. 2C and D). Next, we compared the levels of KRAS expressed in HCT116 cells with and without
lentiviral transfection for overexpression of miR-19a. The resulting in vitro data revealed that overexpression of miR-19a decreased KRAS mRNA and protein levels in HCT116 cells compared with that in the control (miR-Con; Fig. 2E and F).

TargetScan analysis revealed that VEGFA was not a miR-19a target. Since the sites that match the miRNA seed sequence are located in 3’UTRs in most mammalian mRNAs, we performed the dual-luciferase reporter assay to detect whether miR-19a bound to the 3’UTR of VEGFA. The results revealed that the reporter gene containing the VEGFA 3’UTR was not suppressed by miR-19a, which confirmed that VEGFA was not directly regulated by miR-19a in HCT116 cells (Fig. 2G).

miR-19a suppresses tube formation by targeting KRAS in vitro. Since high levels of miR-19a downregulates KRAS, we hypothesized that miR-19a may affect angiogenesis. Hence, we performed tube formation assays using HUVECs, which

Figure 3. miR-19a targets KRAS inhibiting tube formation in vitro. (A) GFP-labeled HUVECs were transfected with the expression vector miR-con or miR-19a as indicated, with or without co-transfection with a KRAS expression vector. Representative images are shown; scale bar, 100 µm. (B) Quantitative tube formation data from A. Four wells were photographed for each treatment, and the cells were analyzed to quantify angiogenesis; *P<0.01 vs. miR-con, †P<0.01 vs. miR-19a. (C) RT-PCR analysis confirmed that KRAS was knocked down by shRNA in HCT116 cells. The results are presented as the mean ± SD (n=3); **P<0.01 vs. sh-control. (D) Western blotting revealed that KRAS was knocked down by shRNA in HCT116 cells. Data from 3 experiments and loading controls are listed below. (E) GFP-labeled HUVECs were transfected with the expression vector sh-KRAS-2# or sh-control as indicated, with or without co-transfection with the VEGFA expression vector. Representative images are shown; scale bar, 100 µm. (F) Quantitative tube formation data from E. Four wells were photographed for each treatment, and the cells were analyzed to quantify angiogenesis; *P<0.01 vs. sh-control, †P<0.01 vs. sh-KRAS-2#.
differentiate and form capillary-like structures on Matrigel, to mimic the process via which endothelial cells form capillaries in vivo (19). The results revealed that overexpression of miR-19a significantly (P<0.01) inhibited angiogenesis in the Matrigel assay, and that rescuing KRAS expression could restore angiogenesis inhibited by miR-19a (Fig. 3A and B). This confirmed our hypothesis that miR-19a suppressed angiogenesis by targeting KRAS.

KRAS is a key regulator in VEGF-related angiogenesis. A previous study revealed that the vascular endothelial growth factor receptor (VEGFR) signaling pathway plays a major role in angiogenesis (20) and that KRAS plays a key role in VEGF signaling (21). To ascertain these results, we knocked down KRAS using shRNA and determined the expression level of VEGFA in HCT116 cells. The results revealed that two KRAS shRNAs reduced KRAS mRNA and protein levels significantly, and consequently, VEGFA was also downregulated (Fig. 3C and D). Furthermore, we knocked down KRAS using shRNA in HUVECs and performed the tube formation assay. As shown in Fig. 3E, KRAS knockdown disrupted tube formation, whereas re-expression of VEGFA rescued the defect. Thus, the results demonstrated that KRAS was a key regulator of VEGFA expression and angiogenesis.

miR-19a suppresses CRC tumor growth in nude mice. The above data indicated that miR-19a overexpression could target KRAS in HCT116 cells and inhibit tube formation. Therefore, we next examined whether overexpression of miR-19a could suppress CRC progression by blocking angiogenesis via targeting of KRAS using a xenograft model.

We transplanted the transfected cells into the flanks of nude mice. The results revealed that the sizes of xenograft tumors that developed from HCT116 cells expressing miR-19a were much smaller than those from HCT116 cells transfected with the control lentivirus (Fig. 4A and B). In addition, tumors that
developed from miR-19a-expressing lentivirus-transfected cells tended to be paler in color than xenograft tumors developed from control lentivirus-transfected cells, indicating decreased blood flow in miR-19a-expressing tumors (Fig. 4A).

We performed H&E staining to examine the blood vessels in these tumors. We observed that tumors that developed from miR-19a-expressing cells had fewer blood vessels than the control tumors. Immunohistochemical staining for CD31, a marker of vascular endotheliocytes, also revealed that the blood vessels in tumors that developed from miR-19a-expressing cells were smaller than those in the control group (Fig. 4D). Moreover, immunohistochemical staining for KRAS and western blotting analysis confirmed that the tumors that developed from miR-19a-expressing cells exhibited reduced KRAS expression compared with those in the control group (Fig. 4C and D). Moreover, western blotting analysis revealed that the expression level of VEGFA in tumors that developed from miR-19a-expressing cells was also reduced (Fig. 4C). These results indicated that overexpression of miR-19a inhibited KRAS expression in CRC, which may reduce the production of VEGFA and suppress tumor angiogenesis, thereby delaying tumor growth.

Discussion

In the present study, we evaluated the role of miR-19a in colorectal cancer (CRC) development and progression, and observed that overexpression of miR-19a resulted in the inhibition of CRC angiogenesis owing to reduced expression of KRAS, a known oncogene that has been demonstrated to be a target of miR-19a in chronic myeloid leukemia (22).

A study in 2010 revealed that overexpression of miR-17, miR-18a, miR-19a and miR-20a considerably inhibited three-dimensional spheroid sprouting in vitro (23). Additionally, Landskroner-Eiger et al suggested that miR-19 of the miR-17-92 family may negatively affect the formation of arterial blood vessels (13). Hence, we hypothesized that miR-19a inhibited angiogenesis. In the present study, we revealed that overexpression of miR-19a reduced KRAS expression in the HCT116 cell line and inhibited angiogenesis, as determined by an in vitro tube formation assay. This inhibition, which could be rescued by KRAS, consequently slowed down xenograft tumor growth. Hence, we believe that miR-19a may be closely related to CRC angiogenesis, and that the anti-angiogenesis effect results from the targeting of KRAS by a microRNA. To the best of our knowledge, this is the first study to investigate the primary function of miR-19a in solid CRC tumors and to provide a theoretical foundation for the application of miRNA treatment to CRC.

Vascular supply is key for tumor development (24) in various types of cancers, including CRC. Vascular endothelial growth factor (VEGF) and the VEGF receptor pathway play key roles in tumor angiogenesis (25). VEGF/VEGFR receptor activation can initiate a signaling cascade that promotes endothelial cell proliferation, migration and differentiation (26). VEGF can also be secreted by CRC cells (27), and it induces angiogenesis. KRAS and VEGF have a close relationship. KRAS activation significantly enhances the production of angiogenic factors, including CXC chemokines and VEGF (28), and KRAS mutations significantly increase VEGF production (29). Therefore, KRAS and VEGF may be involved in creating tumor vascular networks.

Moreover, previous studies have shown a pivotal role of KRAS in human cancer development (30) and demonstrated that KRAS mutations were indicative of poor prognosis in CRC (31). Therefore, targeting KRAS is a promising therapeutic strategy for CRC treatment, particularly for patients with mutations in KRAS, which are common in CRC (32). Use of miRNAs for degrading KRAS holds promise. For most mammalian miRNAs, the sites that match the miRNA seed sequence (nucleotides 2-7), particularly those in 3'UTRs, are preferentially conserved (33). Therefore, miRNA therapy is unlikely to be affected by gene variations.

Our findings in the present study provided important insights into the role of miR-19a in cancer and laid the foundation for future research in this area. High miR-19a levels reduced KRAS expression and affected angiogenesis in CRC. Thus, miR-19a may have applications as a therapeutic agent. However, the present study has one limitation. The in vitro analyses were only performed in HCT116 cells, and additional studies using other CRC cell lines are required. With greater and improved understanding of the role of miR-19a in CRC, the usefulness of this microRNA in the diagnosis and treatment of CRC may be maximized.

Acknowledgements

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References


