Regulation of UHRF1 by microRNA-9 modulates colorectal cancer cell proliferation and apoptosis

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second most common in females worldwide.1(1) Despite substantial progress being made in CRC diagnosis and treatment, the clinical outcome and prognosis of patients with metastatic colorectal cancer still remains poor. Different genetic and epigenetic abnormalities are common in CRC, such as KRAS mutation, MLH1 methylation, and microsatellite instability.2–5 Epigenetic regulation could lead to the activation of oncogenes and/or the inactivation of tumor suppressor genes. Thus, exploring the underlying epigenetic mechanisms of CRC could benefit the understanding of tumorigenesis and improve the current therapeutic strategies of CRC.

Ubiquitin-like with plant homeodomain and ring finger domains 1 (UHRF1) also known as ICBP90, was characterized as a multidomain protein, which is emerging as a major player in DNA methylation and cell proliferation.5,6 Its multiple domains comprise five structural elements: an N-terminal ubiquitin-like domain, followed by a tandem Tudor domain, a plant homeodomain, a SET- and RING-associated (SRA) domain, and a C-terminal RING domain.7 The plant homeodomain could bind to trimethylated histone H3 lysine 9 (H3K9me3) and the SRA domain recognizes hemimethylated DNA.5 By binding to recruiting DNA (cytosine-5)-methyltransferase 1, UHRF1 could maintain genomic DNA methylation.8 UHRF1 is also linked with heterochromatin formation by binding to histone deacetylase 1 and H3K9me3.9,10 Recent research has elucidated that UHRF1 is upregulated in various cancers, including CRC, hepatocellular cancer, and prostate cancer,16,11,12 and knockdown of UHRF1 expression in cancer cells inhibits cell growth.13 Therefore, UHRF1 inhibition may provide a promising route to cancer therapy.

MicroRNAs (miRNAs), a class of approximately 19–25-nt non-coding RNA molecules, also exert a critical role in tumorigenesis. They could regulate protein-coding genes through direct interaction with the 3’-UTR of their target mRNA and leading to translational inhibition.14,15 A growing number of studies have shown that aberrant expression of miRNAs is tightly linked to the development of CRC, such as miR-9, miR-143, miR-145, miR-103-1, miR-191, miR-21, miR-221, miR-222, miR-135b, and miR-34a,16–20 and plays pivotal roles in CRC pathophysiology such as cell proliferation, differentiation, apoptosis, and metastasis.19–24 In CRC, miR-9 serves as a tumor suppressor.25 The expression of miR-9 in CRC tissue was downregulated compared with normal colonic mucosa.18,26 Ectopic expression of miR-9 could enhance the motility of RKO cells and change cells’ morphological appearance, resulting in promoting the metastatic process of CRC.27 By binding to the 3’-UTR, miR-9 has the potential to suppress Cdx2 and E-cadherin transcript.28,29 Overall, miR-9 is associated with the development of CRC and could be used for CRC molecular-targeted therapies.

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Playing an important role in connecting DNA methylation and histone modifications, UHRF1 is implicated in CRC development and progression. However, little is known of the factors that modulate UHRF1 expression. In this study, we examined the expression of UHRF1 in CRC tissues. Then a miRNA was predicted and confirmed to modulate the expression of UHRF1. Our results revealed that UHRF1 is a target of miR-9, which has a vital role in regulating CRC cell proliferation and apoptosis.

Materials and Methods

Cells. HCT116 and HT29 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in McCoy’s 5A (modified) medium supplemented with 10% FBS and 50 U/mL penicillin and streptomycin sulfate.

Tissue specimens. A total of 38 patients with CRC who underwent surgical resection between 2008 and 2009 at Nanjing First Hospital (Nanjing, China) were recruited to the study. Patients who received preoperative chemotherapy were excluded. Specimens of cancer tissues and adjacent normal tissues were collected from patients with informed consent. All patients had a pathological diagnosis of CRC, and each patient experiment were: 5'-CCACATCGTCTACACAGC-3' (forward), and 5'-GGTCCACATCATCCTCACAGC-3' (reverse) for UHRF1; and 5'-GCACCGTCAGGGCTGAAC-3' (forward) and 5'-TGTTGAGACGCAGCCAGTGA-3' (reverse) for GAPDH. The mature miR-9 expression was determined using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA).

RNA extraction and quantitative real-time RT-PCR. Total RNA containing small RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s recommendations. cDNA was synthesized with the Reverse Transcription System (Promega, Madison, USA). Quantitative RT-PCR analyses were carried out to detect mRNA expression using SYBR Premix Ex Taq II (TaKaRa, Dalian, China). GAPDH was used as a reference gene to normalize the results. Primers used in this experiment were: 5'-CCACATCGTCTACACAGC-3' (forward), and 5'-GGTCCACATCATCCTCACAGC-3' (reverse) for UHRF1; and 5'-GCACCGTCAGGGCTGAAC-3' (forward) and 5'-TGTTGAGACGCAGCCAGTGA-3' (reverse) for GAPDH. The mature miR-9 expression was determined using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry. Sections were incubated at 37°C with the primary antibodies anti-UHRF1 (1:50, Abcam, Cambridge, UK) for 2 h. After rinsing with PBS, slides were incubated with secondary antibody for 30 min and washed again with PBS for 10 min. The protein was visualized by EnVision Detection Systems (Dako, Glostrup, Denmark). Semi-quantitative analysis of staining results of individual tissue array cores was carried out by two independent observers without prior information of the clinicopathological features. The frequency of UHRF1-positive staining cells was scored on the basis of the percentage of positive cells as: negative, 0%; 1, 1–25%; 2, 26–50%; and 3, >51%. The intensity of UHRF1 expression was scored as: 1, weak; 2, moderate; and 3, strong. The scores were calculated to evaluate the UHRF1 expression levels and the levels were classified as low (<2) or high (>2).

Vector constructs and transfection. The miRNA expression vectors for pre-miR-9 were obtained from GeneChem (Shanghai, China). The human pre-miR-9 sequence was amplified from human genomic DNA using specific primers (forward, GGGCCCGCTCTAGACTCGAGAATTTGCATGTGCATATG TG; reverse, CGGGCCGCTCTAGACTCGAGATCAAAAAAGGC ACATCGGAGGCTGATC). The amplicon was cloned into the GV259 (GeneChem) at the XhoI and BamHI sites. All cloned constructs were fully sequenced. HCT116 and HT29 cells were infected with the pre-miR-9 lentivirus or control viruses lacking the miR-9 sequence (MOI = 50). After being cultured for 72 h, cells were detected by fluorescence microscopy. A stable infection efficiency of approximately 100% was attained. Expression of the mature miR-9 was verified by TaqMan real-time PCR. Transient transfection of miR-9 inhibitor (Ambion, Austin, TX, USA) was performed using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen).

In situ hybridization. Sections of paraffin and formaldehyde-fixed tissue were cut from CRC patients. In situ hybridization was carried out using the microRNA Hybridization Kit (Foco, Guangzhou, China) according to the manufacturer’s instructions. Briefly, the sections were incubated with 4% (v/v) para-formaldehyde for 15 min at room temperature. Afterwards, the slides were washed and incubated with prehybridization solution for 2 h at 55°C. Digoxin-labeled miR-9 probe and U6 snRNA probe were added to each tissue section and hybridized for 24 h at 37°C. After washing with PBS, blocking was carried out for 1 h at 37°C, and then the anti-digoxigenin Rhodamine conjugate was added to the sections. To stain the cell nucleus, the sections were counterstained with 20 μL DAPI.

Fig. 1. Expression of UHRF1 was investigated in patients with colorectal cancer (CRC). (a) Immunohistochemical analysis of UHRF1 in cancer tissues and adjacent normal tissues. (b) Kaplan–Meier overall survival analysis for CRC patients.
Fig. 2. MicroRNA-9 (miR-9) expression levels are downregulated in colorectal cancer (CRC) tissues. (a) Representative in situ hybridization (ISH) images showing miR-9 (red) and control U6 (green) in CRC tissues and normal tissues, respectively. Scale bar = 100 μm. (b) Quantitation of miR-9 and control U6 expression in CRC tissues and matched normal tissues. (c) Kaplan–Meier analysis of the correlation between miR-9 expression and the overall survival of patients with CRC. H, high; L, low.

Fig. 3. MicroRNA-9 (miR-9) inhibits human colorectal cancer cell proliferation and growth. (a, b) After transfection with pre-miR-9 and pre-miR-control (pre-miR-cont) for 72 h in HCT116 and HT29 cells, miR-9 expression was detected by RT-PCR. (c, d) Upregulation of miR-9 repressed cell proliferation as determined by CCK-8 assays. (e, f) Colony formation was carried out to analyze cell proliferation. *P < 0.05. OD, optical density.
considered significant at expression levels of miR-9 and UHRF1. A difference was carried out to determine the correlation between the log

cells in 24-well plates, together with 100 nM miR-9 or miR-

The number of colonies in each well was counted. Colonies were fixed with 4% paraformaldehyde for 5 min, and then stained with Giemsa solution (KeyGEN BioTech, Nanjing, China) for 15 min after being washed twice with PBS. Colony formation assay. Cells (2000 per well) were placed in 6-cm culture dishes in triplicate and cultured for 2 weeks. The colonies were fixed with 4% paraformaldehyde for 5 min, and then stained with Giemsa solution (KeyGEN BioTech, Nanjing, China) for 15 min after being washed twice with PBS. The number of colonies in each well was counted.

Flow cytometry analysis. Using the BD FACSCalibur (BD Biosciences, San Jose, CA, USA), transfected cells were incubated with an annexin V/7-AAD kit (BioLegend, San Diego, CA, USA) for 15 min at room temperature to detect the apoptosis.

Luciferase reporter assay. The wild-type or mutant 3′-UTR of UHRF1 containing the putative miR-9 binding site were cloned into pGL3 promoter (GeneChem), individually. One microgram vectors were cotransfected into HCT116 and HT29 cells in 24-well plates, together with 100 nM miR-9 or miR-control using Lipofectamine 2000. After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Normalized luciferase activity was reported as firefly/Renilla luciferase activity. Each transfectant was assayed in triplicate.

Statistical analysis. Statistical analyses were carried out using spss 16.0 for Windows (SPSS, Chicago, IL, USA). Student’s t-test was used for comparison between the two groups. Survival curves were plotted by the Kaplan–Meier method and compared with the log–rank test. Spearman’s correlation analysis was carried out to determine the correlation between the expression levels of miR-9 and UHRF1. A difference was considered significant at \( P < 0.05 \).

Results

UHRF1 significantly overexpressed in human CRC tissues. The localization and expression of UHRF1 was determined by immunohistochemistry in 38 CRC samples. Strong staining was found mainly in nucleus. Immunohistochemistry showed that UHRF1 expression was more frequently present in CRC tissues than that in the adjacent normal tissues. Fourteen of 38 cancer lesions (36.8%) were stained positive and four adjacent normal tissues were positive (10.5%). Nine cases (23.7%) were scored as strong expression and five cases (13.2%) as moderate expression. Representative result of UHRF1 staining is shown in Figure 1(a). Kaplan–Meier survival analysis revealed that overall 5-year survival was significantly lower in patients with high UHRF1 expression as compared to patients with low UHRF1 expression (Fig. 1b).

MicroRNA-9 expression is downregulated in CRC tissues and associated with prognosis CRC. The expression of miR-9 was analyzed in CRC tissues \((n = 38)\) and corresponding normal tissue samples by in situ hybridization and RT-PCR. Strikingly, in situ hybridization results showed that miR-9 expression level was remarkably reduced in CRC tissues compared with matched normal tissues, whereas U6 snRNA expression levels remained unchanged (Fig. 2a). In agreement with the results of the in situ hybridization, the RT-PCR results showed that the expression levels of miR-9 were significantly lower in the CRC specimens than those in the adjacent normal speci-

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mens \((P < 0.0001)\) (Fig. 2b). Kaplan–Meier survival analysis suggested that patients with low miR-9 expression were significantly associated with a poor clinical outcome (Fig. 2c).

Overexpression of miR-9 inhibits proliferation of CRC cells in vitro. To determine whether miR-9 was involved in CRC tumorigenesis, stable HCT-116 and HT29 cell lines expressing pre-miR-9 were established. After pre-miR-9 transfection for 72 h in HCT-116 and HT29 cells, the expression levels of miR-9 were substantially increased, approximately 57-fold and 45-fold, respectively, compared to pre-miR control (Fig. 3a,b). The cell proliferation assays suggested that upregulation of miR-9 can significantly inhibit CRC cell proliferation \((P < 0.05);\) Fig. 3c,d). Additionally, the plate colony formation assay was used to assess the effect of miR-9 expression on proliferation of CRC cell lines in vitro. Similarly, colony formation was significantly reduced in the miR-9 upregulated cells \((P < 0.01);\) Fig. 3e,f). Together, the results suggested that ectopic expression of miR-9 can significantly inhibit CRC cell proliferation.

MicroRNA-9 promotes CRC cells apoptosis in vitro. To detect the effects of miR-9 expression on HCT-116 and HT29 cell apoptosis, we used an annexin V/7-AAD kit to examine the apoptosis of these cells. After transfection with miR-9 for 72 h, the effect of miR-9 on apoptosis of HCT-116 and HT29 cells was evaluated by flow cytometry. It was found that the proportion of apoptotic cells was significantly increased in pre-miR-9-transfected CRC cells compared with control cells (Fig. 4).

UHRF1 is a direct target of miR-9. Next, the UHRF1 expression in pre-miR-9-infected CRC cells was determined. The results showed the expression of UHRF1 significantly decreased in stable pre-miR-9-transfected cells, whereas UHRF1 was upregulated after blocking expression of miR-9 with antagonistic oligonucleotide in both cell lines (Fig. 5a,b). To further analyze the relationship between miR-9 and UHRF1, we used two computational algorithms (miRanDa and TargetScan) to predict the targets gene of miR-9. The bioinformatic methods suggested that miR-9 could target the 3′-UTR region of UHRF1. To validate the prediction, the wild-type or mutated 3′-UTRs of UHRF1 were cloned into luciferase reporter vector (Fig. 5c). Dual luciferase reporter assay showed that, compared to miRNA control, miR-9 led to a significant relative luciferase activity reduction in the wild-type UHRF1 3′-UTR plasmid. However, luciferase activity was not reduced in the 3′-UTR with mutant binding sites (Fig. 5d). These results showed that miR-9 may restrain UHRF1 expression by targeting its 3′-UTR.

Downregulation of UHRF1 inversely correlates with miR-9 expression in CRC. To further explore the relationship between miR-9 and UHRF1, we also used quantitative RT-PCR to analyze the expression of UHRF1 in 15 patients with CRC. These results showed that UHRF1 mRNA expression was higher in CRC tissues than that in corresponding normal tissues (Fig. 6a). Spearman’s correlation analysis showed that the UHRF1 expression was inversely correlated with miR-9 expression in CRC \((r = -0.606, P = 0.0004);\) Fig. 6b).

Discussion

Epigenetic alteration, such as DNA methylation and histone modification, inactivates various tumor suppressor genes.\(^{(30)}\) It...
is generally agreed that UHRF1 is involved in gene epigenetic abnormalities and exerts its role as an oncogene in cancer development. Overexpressed UHRF1 could bind to methylated promoter regions of various tumor suppressor genes and suppress the expression of these genes including p16INK4A, p14ARF, RARβ, FHIT, APC, DAPK, and p21WAF1. In the UHRF1 regulation axis, tumor suppressor p53 could downregulate UHRF1 through upregulation of p21WAF1 and subsequent deactivation of E2F1. Thus, due to the absence of intact p53 in most cancer cells, dysregulation of UHRF1 expression has been observed in several cancers. In addition, UHRF1 was reported to be associated with tumor stage and to predict poor prognosis. Consistent with previous studies, we also found that UHRF1 was upregulated in CRC. Patients with a high level of UHRF1 expression tended to have lower overall survival rates compared with those with low expression. The above results were obtained from only 38 CRC patients. To validate these results, more research on a larger population is warranted.

Increasing evidence has shown that miR-9 is confirmed to be downregulated in many types of cancers and considered to be an important mediator in cancer development through its ability to regulate different target genes. In oral squamous cell carcinoma, miR-9 could inhibit the proliferation of cancer cells by suppressing expression of CXC chemokine receptor 4. In acute myeloid leukemia, miR-9 exerts its effects through the cooperation with let-7 to restrain the oncogenic LIN28B/HMGAl axis. In the present study, we showed that miR-9 was underexpressed in CRC tissues. Lentivirus-mediated overexpression of miR-9 could suppress the expression of UHRF1. Cell proliferation was also suppressed in pre-miR-9 transfected cells. Furthermore, restoration of miR-9 expression significantly induced cell apoptosis. Interestingly, the expression level of miR-9 was inversely correlated with UHRF1 expression in CRC tissues. Finally, we identified that UHRF1 is regulated by miR-9 in CRC by dual luciferase reporter assay.

In conclusion, our studies provide insight into the mechanism that miR-9 could function as a tumor-suppressive miRNA by repressing UHRF1 expression. Understanding the relationship between UHRF1 and miR-9 will not only increase our understanding of CRC tumorigenesis, but may also allow the development of a novel therapeutic strategy based on upregulation of miR-9.

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Disclosure Statement

The authors have no conflict of interest.

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