The deoxycholic acid targets miRNA-dependent CAC1 gene expression in multidrug resistance of human colorectal cancer

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ABSTRACT

There is evidence indicating that bile acid is a promoter of colorectal cancer. Deoxycholic acid modifies apoptosis and proliferation by affecting intracellular signaling and gene expression. We are interested in revealing the relationship between deregulated miRNAs and deoxycholic acid in colorectal cancer development. We found that miR-199a-5p was expressed at a low level in human primary colonic epithelial cells treated with deoxycholic acid compared with control, and miR-199a-5p was significantly down-regulated in colorectal cancer tissues. The miR-199a-5p expression in colorectal cancer cells led to the suppression of tumor cell growth, migration and invasion. We further identified CAC1, a cell cycle-related protein expressed in colorectal cancer, as a miR-199a-5p target. We demonstrated that CAC1 is over-expressed in malignant tumors, and cellular CAC1 depletion resulted in cancer growth suppression. HCT-8 cells transfected with a miR-199a-5p mimic or inhibitor had a decrease or increase in CAC1 protein levels, respectively. The results of the luciferase reporter gene analysis demonstrated that CAC1 was a direct miR-199a-5p target. The high miR-199a-5p expression and low CAC1 protein expression reverse the tumor cell drug resistance. We conclude that miR-199a-5p can regulate CAC1 and function as a tumor suppressor in colorectal cancer. Therefore, the potential roles of deoxycholic acid in carcinogenesis are to decrease miR-199a-5p expression and/or increase the expression of CAC1, which contributes to tumorigenesis in patients with CRC. These findings suggest that miR-199a-5p is a useful therapeutic target for colorectal cancer.

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1. Introduction

Colorectal cancer (CRC) is a malignant tumor of the digestive system that seriously threatens human health. Most colorectal cancers gradually develop as accumulating alterations in gene expression transform normal colonic epithelium cells to tumor cells. This transformation involves a multistep process, including genetic and epigenetic changes, which leads to the activation of oncogenes and inactivation of tumor suppressor genes in cancer cells (Barrett, 1993). Though we have already made progress in the diagnosis and therapy of CRC in the recent years, patient prognosis is still poor. Meanwhile, understanding the molecular mechanisms that maintain the malignant colorectal cancer cell growth remains incomplete. Colorectal cancer incidence is closely related to genetics; however, a variety of environmental, life-style and dietary factors have played an important role in the pathogenesis of colorectal cancer.

Among the most important risk factors is the consumption of a high-fat diet. High-fat diets lead to a change in the pattern of hepatic bile acid secretion, resulting in higher relative concentrations of potentially carcinogenic bile acids such as cholic, deoxycholic and lithocholic acids (Bernstein et al., 2011; Han et al., 2009).

Cholic, deoxycholic and lithocholic acids have been implicated in tumorigenesis. These acids can lead to activation of oncogenes and inactivation of tumor suppressor genes in colonic epithelial cells (Nagengast et al., 1995). Evidence collected to date indicates the involvement of microRNAs (miRNAs) in cancer initiation, development, and progression, and miRNAs are potentially useful as biomarkers in cancer diagnosis, prognosis and as additional therapeutic tools (Altomare et al., 2012; Liu and Tang, 2011). In fact, an increasing amount of evidence links miRNA deregulation to carcinogenesis in several human tumors including CRC (Zarate et al., 2012). Some studies have found that the deregulation of miRNAs is implicated in CRC pathogenesis, particularly by regulating the expression of oncogenes and tumor suppressor genes (Calin and Croce, 2006; Wu et al., 2011). Accumulating data suggest that some miRNAs may function as oncogenes or tumor suppressors (Chen, 2005). miRNAs comprise a class of small non-coding RNAs that, in general, negatively modulate the expression of complementary genes by translation inhibition or mRNA
degradation, thus playing an important role in regulating cell proliferation, apoptosis, and differentiation (Gregory and Shiekhattar, 2005). The dysregulation of miRNA expression may contribute to carcinogenesis by increasing the expression of proto-oncogenes or down-regulating tumor suppressors in CRC. However, because only a few miRNAs were reported to be involved in CRC (Scheperle et al., 2008), we are interested in revealing the relationship between deregulated miRNAs and deoxycholic acid (DCA) in CRC development. The influence of DCA on miRNA expression in CRC tissues has not been investigated.

In this study, we hypothesized that the cancer effects of DCA may be mediated in part via changes in miRNA expression. We performed miRNA microarray studies on human primary colonic epithelial cells (PCECs) treated with DCA and found significant alterations in the miRNA profile. These findings have uncovered a mechanism of DCA interaction with host gene expression that involves alterations in the miRNA profile, which alters cell proliferation.

We found that miR-199a-5p was expressed at a low level in PCECs treated with DCA compared with controls. Although decreased miR-199a-5p expression has been frequently demonstrated in other tumors (Hummel et al., 2011; Sakurai et al., 2011), a functional analysis and the translational relevance of miR-199a-5p has not been defined in CRC. We examined miR-199a-5p expression in CRC and found that miR-199a-5p was significantly down-regulated in CRC tissues when compared with adjacent non-tumor tissues.

These studies were undertaken to define the role of miR-199a-5p in CRC through the identification of new target genes and to analyze its biological function in CRC. We identified CAC1 as a miR-199a-5p target. CAC1 has been demonstrated to increase in colorectal cancer tissues, implicating a causal role of CAC1 in promoting tumor progression. CAC1 is activated by multiple extracellular signals, including growth factors, H2O2, and mitogens, leading to the activation of cyclin-dependent kinase 2 (CDK2), which, in turn, regulates cell proliferation and allows progression from the G1 to S phase of the cell cycle (Kong et al., 2009). We found that miR-199a-5p can inhibit CDK2 activity by down-regulating CAC1 expression. The up-regulation of miR-199-5p or down-regulation of CAC1 expression could suppress in vivo tumor growth. Thus, we postulated that abnormally expressed miR-199a-5p may partially contribute to tumorigenesis by the modulation of CAC1 expression in patients with CRC. In addition, we found that miR-199a-5p expression was significantly lower in multidrug-resistant tumor cells than in control cells, and the expression of CAC1 was significantly higher in multidrug-resistant tumor cells than in control cells. The high expression of miR-199a-5p and the low expression of CAC1 could reverse the tumor cell drug resistance.

The study provides experimental evidence that miR-199a-5p over-expression is able to inhibit CRC cell proliferation and reverse tumor cell drug resistance in vitro and in vivo, partly through suppressing the expression of CAC1 protein at the post-transcriptional level in CRC. Considering that miR-199a-5p is down-regulated in the majority of CRC, our results suggest that miR-199a-5p is a possible therapeutic approach for CRC.

2. Materials and methods

2.1. Colorectal cancer specimens

A total of 40 colorectal cancer tissues (diagnosis confirmed by pathology) and 40 adjacent normal tissues were obtained from The First Affiliated Hospital of Xi’an Jiaotong University between January 2007 and October 2010. Informed consent was obtained from each patient. The samples were obtained from 23 males and 17 females between the ages of 32–80, with an average age of 52.6. The samples consisted of 15 colon carcinomas and 25 rectum carcinomas. According to TNM standard, I:5, II:8; III:15, IV:12; The samples were snap-frozen in liquid nitrogen for 24 h and then stored at −80 °C.

2.2. Cell culture

The HCT-8 cell line was obtained from ATCC (Manassas, VA, USA). Cells were cultured in MEM media containing 10% fetal bovine serum (GIBCO) at 37 °C in a humidified atmosphere containing 5% CO2. The cells used in all experiments were in the logarithmic growth phase. The HCT-8/VCR cell line was obtained from KeyGEN Biotech (Nanjing, China). HCT-8/VCR cells were cultured in MEM media containing 2000 ng/ml vincristine (VCR) (Sigma–Aldrich) and 10% fetal bovine serum (GIBCO) at 37 °C in a humidified atmosphere containing 5% CO2.

Human primary colonic epithelial cells (PCECs) that were derived from freshly resected colonic surgical specimens were cultivated in DMEM supplemented with penicillin (100U/ml), streptomycin (10 mg/ml; both Sigma–Aldrich), and 10% fetal bovine serum (GIBCO) under a humidified atmosphere of 5% CO2 at 37 °C.

2.3. Deoxycholic acid treatment

The DCA concentration (Sigma–Aldrich) in experiments was determined by the MTT method, and the survival rates of the PCECs were measured when different concentrations of DCA (25, 50 and 100 μM) were added and cultured for 24 and 48 h. For the control group, the samples were cultured for 24 and 48 h without DCA addition. The low DCA dose induces cellular proliferation, and DCA concentrations greater than 50 μM cause cell death. Thus, we used the low DCA concentration, 25 μM, to treat the cells.

2.4. miRNA microarray

Small RNAs were isolated from untreated PCECs (control) and those treated with 25 μM Deoxycholic Acid for 48 h using the PureLink™ miRNA Isolation Kit (Invitrogen). Microarrays were produced using an LNA-based oligonucleotide probe library (miRCURY LNA array ready to spot v.7.1; Exiqon A/S). Two micrograms of sample RNA was directly labeled with Hy3 using the miRCURY LNA array labeling kit (Exiqon). We labeled 2 μg of the reference RNA with Hy5 using the LNA array labeling kit (Exiqon). The hybridization and washing of the microarray slides were performed as recommended by Exiqon. The microarray slides were scanned using the Agilent G2505B Microarray Scanner System (Agilent Technologies, Inc., USA), and the image analysis was performed using the ImaGene 8.0 software (BioDiscovery, Inc., USA). Each miRNA signal was converted to logarithm base 2, and a two-sample t test was conducted. Statistical analysis was conducted using MATLAB 7.0 (The MathWorks, Inc., USA).

2.5. Real-time PCR for miRNAs

Total RNA, including miRNAs, was isolated from pelleted PCECs, colorectal cancer tissues and adjacent normal tissues using TRizol reagent (Invitrogen) according to the manufacturer’s instructions, and it was enriched using an miRNAeasy mini-column (miRNAeasy Mini Kit, Qiagen, Hilden, Germany). Briefly, the samples were treated with Trizol and then chloroform. The mixture was centrifuged at 12,000 × g for 15 min at 4 °C. A 1.5-fold volume of 100% ethanol was then added to the aqueous layer. The mixture was
applied to a miRNeasy mini column (Qiagen) and processed according to the manufacturer’s recommendations. Real-time PCR was performed with an iCycler (Bio-Rad) using the miVana™ qRT-PCR miRNA detection kit (Ambion) with miR-199a-5p primers and U6 snRNA primers (Ambion) according to the manufacturer’s protocol. The level of each miRNA expression was measured using the 2−ΔΔCt method (Gramantieri et al., 2007).

2.6. Northern blot

Total RNA, including miRNAs, was isolated from pelleted PCECs, colorectal cancer tissues and adjacent normal tissues as mentioned above. One microgram of RNA was resolved on a 15% acrylamide 8 M urea gel, transferred onto nylon membranes, and UV cross-linked. A digoxigenin-labeled LNA probe for miRNA was prepared using a DIG-3′-end labeling kit (Roche). Hybridization was performed in hybridization solution (Roche) at 42 °C for 16 h. The membranes were washed in 2× SSC with 0.1% SDS at 42 °C twice for 5 min each and then in washing buffer [0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20] at room temperature for 5 min. After blocking in 1% Blocking reagent (Roche) for 30 min, the membranes were incubated with an antidigoxigenin antibody that was conjugated with alkaline phosphatase for 30 min. Following washing in the washing and detection buffers (0.1 M Tris, 0.1 M NaCl, pH 9.5), the membranes were incubated in CDP-Star (Roche), exposed and developed. U6 RNA was used as a loading control for normalization.

2.7. In situ hybridization

A miRNA locked nucleic acid (LNA) probe was prepared using the DIG-3′-end labeling kit (Roche) for the U6 positive control, miR-199a-5p, and a scrambled negative control. The tissue section was deparaffinized and hydrated, followed by treatment with proteinase K and refixation in 4% parafomaldehyde. These slides were prehybridized for 1 h and then hybridized with 200 nM miRNA LNA probe in a hybridization buffer (Roche) for 12 h. After three consecutive washes in 4× SSC/50% formamide, 2× SSC, and 0.1× SSC, the sections were treated with blocking buffer (Roche) for 1 h and incubated with the anti-DIG-AP Fab fragments (Roche) for 12 h. Following three washes in 1× maleic acid and 0.3% Tween 20 buffer followed by staining with NBT and BCIP (Promega, CA, USA), the sections were visualized under a microscope.

2.8. miRNA target prediction

Using four computer algorithms, including TargetScan, miRanda, PicTar and miRGen, we have identified CAC1 as a possible miR-199a-5p target. The sites were predicted based on the base-pairing of seed sequence matches.

2.9. Transfection

Precursor miRNA clones for miR-199a-5p (pEZX-MRD0-miR-199a-5p), the nontargeting control miRNA clone (pEZX-MR04-Control), the miRNA inhibitor (pEZX-AM04-anti-miR-199a-5p) and the miRNA inhibitor control clone (pEZX-AM04) were synthesized by GeneCopoeia Co., Ltd. (Rockville, MD USA). HCT-8 cells were transfected with these clones using HiPerFect transfection reagent (Qiagen) 24 h after plating, according to the manufacturer’s instructions. At 24 h post-transfection, the transfection medium was replaced. Their sequences were as follows: miR-199a-5p mature sequence: 5′–CCAGUGGUGCAGCUACCGUUCG-3′ and negative control oligonucleotide (control NC): 5′–CAGUACUUGUGUGUAGUACAA-3′.

2.10. Western blot

Total protein from colorectal cancer tissues, adjacent normal tissues, and HCT-8 cells transfected with these miRNA clones were extracted using RIPA buffer (1 mM MgCl2, 10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% NP-40). Protein lysates were collected after incubation on ice and brief centrifugation (13,000 × g), and protein expression was analyzed by Western blot. β-Actin served as a loading control. Equal amounts of protein lysates were run on a 10% SDS-PAGE gel. The protein was electro-transferred to a polyvinylidene fluoride (PVDF) membrane using a wet transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were pre-blocked with a milk blocking buffer (5% milk in Tris-buffered saline containing 0.01% Tween 20; TBS-T) and probed overnight. The level of protein expression was evaluated using primary antibodies against CAC1 (1:1500, GeneTex, GTX118514), CDK2 (H-298) (1:2000, Santa Cruz, SC-748), and MDR1 (D-11) (1:2000, Santa Cruz, SC-55510). Actin (1:19) (1:1000, Santa Cruz, SC-1612) was used as a loading control. Antibody binding was detected using the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ) according to the manufacturer’s protocol.

Membranes were stripped in between blots (no more than twice) by incubating them in an SDS stripping buffer containing β-mercaptoethanol (80 μl in 10 ml of buffer) for up to 15 min at 50 °C. This process was followed by a 2-h rinse in TBS-T.

2.11. Cloning of the 3′-UTR and luciferase reporter assay

The portion of the 3′-UTR of the human CAC1 gene containing the miR-199a-5p binding site was PCR amplified. The pMIR-CAC1-3′-untranslated region (UTR) luciferase vector containing the miR-199a-5p putative binding site in the multiple cloning site within the 3′-UTR of the lucerase gene in the pMIR-REPORT™ miRNA Expression Reporter Vector (Ambion, NY, USA) was constructed according to the manufacturer’s instructions. Mutagenesis of the miR-199a-5p seed sequence in the CAC1 3′-UTR region was synthesized using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The pMIR-CAC1-3′-UTR construct (200 ng) together with the β-gal expression vector pMIR-REPORT-β-gal (200 ng) (Ambion) was cotransfected with Pre-miR™ miRNA Precursor Molecules or negative control miRNA precursors (Ambion, NY, USA). Luciferase and β-gal enzyme assays were performed 48 h after transfection according to the manufacturer’s protocol. The firefly luciferase activity was normalized to β-gal expression for each sample.

2.12. Analysis of cell viability

HCT-8 cells were plated in 96-well plates at 4000 cells per well. After 24 h, the cells were transfected with miR-199a-5p, anti-miR-199a-5p, and negative controls. The MTT assay was used to determine the relative cell viability at 24, 48, and 72 h, and each experiment was performed in triplicate. In brief, at the indicated time points, the cell culture medium was replaced with 0.1 ml fresh medium containing 0.5 mg/ml MTT. HCT-8 cells were incubated at 37 °C for 1 h and resolved by 0.1 ml DMSO (Sigma–Aldrich). The absorbance was measured at 570 nm.

2.13. Apoptosis detection

Apoptosis analyses were performed as previously described (Gramantieri et al., 2007). The Annexin V FITC apoptosis detection kit (BD Pharmingen, San Jose, CA, USA) was used to detect apoptosis. A total of 1 × 105 cells were seeded in a 6-well plate and then transfected with miR-199a-5p, anti-miR-199a-5p, or negative controls and incubated for 48 h. After 48 h, the transfected
HCT-8 cells were collected and fixed in 70% ethanol at −20 °C for 16 h. Flow cytometry (FC) analysis of the cell cycle was performed as previously described using the FACSCalibur™ flow cytometer (BD Biosciences). The detection of apoptotic cells after doxorubicin treatment was performed according to the manufacturer’s instructions, and the cells were analyzed by FC. Each experiment was performed in triplicate.

2.14. Soft agar assay

A total of 2 × 10³ cells were seeded on 6-well plates in 2 ml of 0.3% agar layered onto 0.6% agar (Sigma–Aldrich). They were then transfected with or without the miRNA precursors or inhibitors and incubated. The cultures were grown at 37 °C in a 5% CO₂ atmosphere for 14 days. Cell colonies were counted and scored in a blinded fashion by two independent observers.

2.15. Matrigel matrix invasion assay

In vitro invasion assays were performed in BD BioCoat Matrigel chambers (Transwell, BD Biosciences, Heidelberg, Germany) as previously described (Shen et al., 2010). Briefly, 24 h after transfection, 4 × 10⁴ HCT-8 cells were seeded in the top chamber with a Matrigel coated filter, and 750 µl Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum was used as a chemoattractant. Inserts were incubated at 37 °C with 5% CO₂ for 24 and 48 h. After incubation, cells that remained on the upper side of the filters were mechanically removed. Cells that migrated to the lower side were fixed and stained with Giemsa (Sigma–Aldrich). The cells were counted in five fields for triplicate membranes at 10× magnification using an inverted optical microscope (Nikon ECLIPSE TS100, Nikon, Japan).

2.16. In vitro kinase assays

CDK2 was immunoprecipitated from equal amounts of protein (~500 µg) in whole cell lysates. Immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 mM HEPES pH 7.4, 15 mM MgCl₂, 20 mM EGTA, 50 mM NaF, 80 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 mM DTT, 1 mM pefabloc, 20 µg/ml leupeptin, and 1 mM cystine). In vitro kinase assay reactions were performed in 50 µl aliquots at 37 °C for 30 min in kinase buffer with 20 µM ATP, 5 µc of [γ-32P] ATP (6000 Ci mmol) and 0.2–1 µg of CDK substrate histone H1 (Roche). Reactions were terminated by the addition of 20 µl of SDS sample buffer, and the samples were subsequently subjected to 10% SDS-PAGE followed by autoradiography.

2.17. Tumorigenicity assays in nude mice

Healthy male Balb/c Nude mice (3 weeks old) were obtained from the Animal Centre of the Academia Sinica in Shanghai, China. Animals were maintained for 7 days in a conventional animal care unit before the start of the study. Animal experiments were performed according to ethical guidelines of animal experimentation. Twenty-four male Balb/c Nude mice were randomly allocated into four groups (n=6 mice/group) for treatment with different cells: miR-199-5p-, scramble- and NC-transfected HCT-8 cells. The cells (3 × 10⁶) were trypsinized, suspended in 200 µl physiological saline and inoculated by subcutaneous injection into the right flank of each mouse. After inoculation, the mice were observed for 5 h post-injection to ascertain that no health conditions occurred. Tumor growth was monitored by measuring the length (L) and width (W) of the tumor with calipers every 7 days, and the tumor volume was calculated using the following formula: Tumor volume = 1/2 (length × width²).

2.18. Immunohistochemistry examination

Subcutaneous tumors derived from the cells indicated in the previous section were excised from nude mice under ether anesthesia. The tumors were rinsed twice in physiological saline, fixed in 4% neutral-buffered formalin and embedded in paraffin. The sections were mounted on slides, deparaffinized with xylene, and rehydrated by graded alcohols. Immunohistochemical studies were performed on these samples. All staining steps were performed at room temperature, and samples were washed with PBS in between steps. Sections were then incubated with primary antibodies for 1 h. The primary antibodies used were CAC1 (1:400, GeneTex, GTX118514) and MDRI (D-11) (1:500, Santa Cruz, SC-55510). Biotinylated secondary antibodies (Santa Cruz) were applied for an additional hour following the removal of the primary antibodies. Staining was developed using the avidin-biotinylated horseradish peroxidase complex (Santa Cruz) and diaminobenzidine (Santa Cruz) following the manufacturer’s instructions. Pre-immune serum was used as a negative control.

2.19. Statistical analysis

All values are presented as the mean ± standard deviation (SD). Statistical significance was evaluated using ANOVA for multiple treatment groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Microarray analysis of miRNA expression at PCECs (control) and PCECs (treated with 25 µM deoxycholic acid) at 48 h

Clustering analysis demonstrated a significant difference between the control and DCA. In fact, the modulation of the miRNA expression profiles with DCA was striking. A large subset of the miRNAs was affected by DCA.

To examine the differential expression between the miRNAs in PCECs treated with DCA and the control, miRNA microarray analysis was performed using the miCURY LNA microarray from Exiqon. The scatter plot analysis is shown in Fig. 1A. Compared with the control, 13 miRNAs were up-regulated and 9 miRNAs were down-regulated at least 1.3-fold in PCECs treated with deoxycholic acid. We chose a 1.3-fold cutoff (P < 0.05) because a fold-change cutoff of 1.3 was considered a reasonable magnitude and the statistical P value is more important than the intensity for miRNA expression change analyses. In addition, false positive miRNAs were filtered out in this study using multiple test corrections as described in the methods section. The up-regulated miRNAs were hsa-miR-939, hsa-miR-22, hsa-miR-24, hsa-let-7d, hsa-let-7g, hsa-miR-30b, hsa-miR-30d, hsa-miR-106b, hsa-miR-29b, hsa-miR-629, mmu-miR-23b, hsa-miR-223 and hsa-miR-194. The nine down-regulated miRNAs were hsa-miR-1246, hsa-miR-519d, hsa-miR-451, mmu-miR-669f, mmu-miR-467e*, hsa-miR-143, hsa-miR-378*, hsa-miR-1827 and hsa-miR-199a-5p (Table 1).

To confirm the miRNA microarray results, 3 up-regulated miRNAs and 3 down-regulated miRNAs were randomly chosen for Northern blot analysis. Compared with the control, 3 miRNAs were up-regulated in PCECs treated with DCA, including hsa-miR-194, hsa-miR-629, and hsa-miR-30b; 3 miRNAs were down-regulated in PCECs treated with DCA, including hsa-miR-199a-5p, hsa-miR-1246, and hsa-miR-451 (Fig. 1B). We confirmed that these miRNAs were up-regulated or down-regulated in PCECs treated with DCA,
Fig. 1. Analysis of the miRNA microarray data and miRNA expression as verified by Northern blot. (A) Scatter plot representing miRNAs significantly altered in PCECs (control) and those treated with 25 μM deoxycholic acid. Red indicates the expression level of the up-regulated miRNAs, and green represents the expression level of the down-regulated miRNAs. (B) Northern blot analysis of the 6 differentially expressed miRNAs from the microarray data. After the density of each band was normalized to its corresponding U6 density, the ratio between control and DCA treatment is shown on the right. These results are in accord with the miRNA microarray data. (C) miR-199a-5p was down-regulated in human CRC tissues. miR-199a-5p expression was detected in 40 paired human CRC and non-neoplastic mucosa tissues by qRT-PCR. miR-199a-5p expression was normalized to that of U6 in each sample. (D) A Kaplan–Meier survival curve and log-rank test for patients with CRC classified as having either high or low miR-199a-5p expression. High miR-199a-5p expression (n = 11), group with the expression ratio ≥ the mean ratio. miR-199a-5p low expression (n = 29), group with the expression ratio < the mean ratio. miR-199a-5p expression had a significant (log-rank test, P < 0.05) relationship with patient survival. The results revealed that the patient survival rate of those with high miR-199a-5p expression was significantly higher than those with low miR-199a-5p expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
confirming the miRNA microarray data and suggesting that the carcinogenic effects of DCA are mediated by suppressing or inducing the expression of miRNAs that are up-regulated or down-regulated in colorectal cancer.

3.2. miR-199a-5p is down-regulated in CRC tissues

miR-199a-5p has been reported to be downregulated in some cancers including hepatocellular carcinoma, breast cancer, testicular cancer, and esophageal cancer. The role of miR-199a-5p in cancer development may be that of a tumor suppressor (Duan et al., 2012; Hummel et al., 2011; Cheung et al., 2011). However, whether miR-199a-5p is down-regulated in CRC and its role in CRC carcinogenesis are still elusive. To determine whether miR-199a-5p is involved in the regulation of CRC tumorigenesis, real-time PCR was used to assess miR-199a-5p expression in 40 human CRC and non-neoplastic mucosal tissues. As demonstrated in Fig. 1C, miR-199a-5p expression is significantly decreased in 29 of 40 (73%) tumor tissues compared with that in their matched non-neoplastic mucosal tissues. These results suggest that miR-199a-5p is down-regulated in CRC, and its down-regulation may participate in human CRC development.

The patients with CRC were divided into two groups according to the mean level of miR-199a-5p expression: miR-199a-5p low expressers (n = 29) and miR-199a-5p high expressers (n = 11). A statistically significant association between the miR-199a-5p expression level and clinicopathologic CRC characteristics is summarized in Table 2. miR-199a-5p expression was correlated with CRC TNM staging and differentiation. In 27 cases presenting with advanced stages III and IV, 4 (14.81%) of the cases had high levels of miR-199a-5p expression in CRC tissue, whereas in 13 early stages cases (stages I and II), 7 (53.84%) had high levels of miR-199a-5p expression. The miR-199a-5p expression in TNM stages I and II was higher than that in stages III and IV (P<0.05). In the 22 cases in the poorly differentiated group, 2 (9.09%) had high miR-199a-5p expression, while 9 (50.00%) of 18 cases of the highly and moderately differentiated group had high miR-199a-5p expression. miR-199a-5p expression in the highly and moderately differentiated group was higher than that in the poorly differentiated group (P<0.05). No correlation was observed between miR-199a-5p levels and age, gender, location, or pathologic type of CRC (P>0.05).

Kaplan–Meier analyses and log–rank tests were employed to calculate the effect of miR-199a-5p expression on overall patient survival. The results revealed that the patients with CRC with low miR-199a-5p expression had a significantly poorer prognosis compared with those with high miR-199a-5p expression. The 2-year survival rate in patients with high miR-199a-5p expression was 73%, which was significantly higher than those with low miR-199a-5p expression (55%) (Table 3). Statistical analysis revealed that the survival time between the high- and low-expressing miR-199a-5p groups was significantly different (P<0.05) (Fig. 1D). The results revealed that miR-199a-5p expression was correlated with the survival time for patients with CRC.

3.3. Detection of miR-199a-5p in normal and cancerous colorectal tissues by in situ hybridization (ISH)

Microarray and real-time PCR analyses demonstrated that miR-199a-5p was differentially expressed between control PCECs and those treated with deoxycholic acid. To examine the tissue distribution of miR-199a-5p expression, we investigated which of the specific cell types expressed miR-199a-5p by ISH. The specificity of the ISH procedure was verified using a scrambled control probe (Fig. 2A). The results demonstrated miR-199a-5p expression in the cytoplasm of mucous epithelial cells in normal colorectal tissues (Fig. 2B and E). Colorectal cancer tissue analysis also revealed miR-199a-5p expression in the cytoplasm of tumor cells (Fig. 2C, D, and F). In addition, ISH demonstrated miR-199a-5p expression in inflammatory cells including the plasma cells and lymphocytes in the lamina propria of colorectal cancer tissues. The labeling intensity among the different colorectal cancer tissues was variable. Consistent with our microarray observations, miR-199a-5p

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**Table 1**

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<td>1.5311809</td>
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<tr>
<td>mmu-miR-23b/mro-miR-23b</td>
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<td>1.6294935</td>
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<td>1.6372341</td>
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<tr>
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<td>1.9076207</td>
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<td>hsa-miR-106b/mmu-miR-106b/rno-miR-106b</td>
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<td>2.5477223</td>
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<tr>
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**Table 2**

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<th>N</th>
<th>miR-199a-5p</th>
<th>χ²</th>
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<td>Low</td>
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<td>11</td>
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<td>8</td>
<td>4</td>
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<tr>
<td>≥60</td>
<td>28</td>
<td>21</td>
<td>7</td>
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</tr>
<tr>
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<td>12</td>
<td>5</td>
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<tr>
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<tr>
<td>Rectal Cancer</td>
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<td>17</td>
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<tr>
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<td>14</td>
<td>5</td>
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<tr>
<td>Poorly differentiated cases</td>
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</tr>
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<td>7</td>
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<tr>
<td>III + IV</td>
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<td>23</td>
<td>4</td>
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**Table 3**

<table>
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<th>Pathological factors</th>
<th>Overall survival rate</th>
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<tr>
<td></td>
<td>6 months</td>
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<tr>
<td>Low expression</td>
<td>29/29</td>
</tr>
<tr>
<td>High expression</td>
<td>11/11</td>
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</table>
expression appeared to be reduced in the majority of tumors investigated relative to the normal mucosa.

3.4. CAC1 is directly targeted by miR-199a-5p

miR-199a-5p was significantly down-regulated after exposure to DCA for 48 h. The change in the expression of miR-199a-5p was the highest of all of the miRNAs assayed on the microarray. Thus, we suggest that miR-199a-5p may play an important role following DCA exposure. By categorizing the miR-199a-5p target genes, we determined that its potential cellular and molecular roles are involved in the cell cycle, cell death, cell-to-cell signaling and interaction, and cellular assembly and organization.

To further investigate the mechanisms responsible for the cell cycle arresting effect of miR-199a-5p in CRC, we identified the molecular targets of miR-199a-5p as miRNAs that...
Northern post-transcriptional vectors CAC1 (pMIR-REPORT-CAC1 sequences, function 2328 Y. miR-199a-5p 199a-5p, formed regulation HCT-8 To 3. Western blot was detected anti-miR-199a-5p, mainly putative and cotransfected that predicted functions CAC1, reduced. We cotransfected HCT-8 cells were transiently transfected with luciferase reporter vectors containing CAC1 3'-UTR sequences with mutated miR-199a-5p binding sites. We found that the luciferase activity of the MUT CAC1 3'-UTR reporter was not altered by miR-199a-5p co-transfection (Fig. 3A). This result confirms that the human CAC1 3'-UTR is directly targeted by miR-199a-5p expression.

To determine if miR-199a-5p regulates CAC1 expression in HCT-8 cell lines, the CAC1 protein levels were analyzed by immunoblotting in miR-199a-5p-HCT-8 and control cells. As shown in Fig. 3B, miR-199a-5p represses the expression of the CAC1 protein. We further compared CAC1 expression in protein samples from paired human CRC tissues and found that the CAC1 protein level was also up-regulated in CRC tissues with down-regulated miR-199a-5p (Fig. 3C), confirming that endogenously expressed CAC1 is regulated by miR-199a-5p expression.

Taken together, these data demonstrate that CAC1 is a direct target of miR-199a-5p and further suggest that miR-199a-5p may exert its cell cycle restraining effect by inhibiting CAC1 expression.

3.5. miR-199a-5p affects the HCT-8 cell invasion capability and the formation of cell colonies

The role of miR-199a-5p and its target genes in the modulation of the invasion capability and formation of cell colonies was investigated by matrigel invasion and soft-agar colony formation assays. The restoration of miR-199a-5p in HCT-8 cells caused a 40% decrease in the invasive potential. Accordingly, a 2.2-fold increase in the invading cells was observed following anti-miR-199a-5p in HCT-8 cells. These findings demonstrated that miR-199a-5p counteracts the invasive potential of CRC cells, and this event is, in part, likely related to CAC1 inhibition (Fig. 4A). Similarly, we also found that miR-199a-5p can inhibit the colony formation ability of HCT-8 cells compared with the controls. The restoration of miR-199a-5p in HCT-8 cells caused a 30% decrease in the formation of cell colonies (Fig. 4B).

3.6. miR-199a-5p inhibits DCA-induced CAC1 protein expression and cell proliferation

To investigate the effect of miR-199a-5p on DCA-induced CAC1 expression, HCT-8 cells were treated with 25 μM DCA and then transfected with miR-199a-5p and a control miRNA. We detected the CAC1 expression and found that the CAC1 protein level was up-regulated in DCA-induced HCT-8 cells and was down-regulated in HCT-8 cells treated with miR-199a-5p. The increase in CAC1 expression induced by DCA was partially reversed by the addition of miR-199a-5p (Fig. 5A).

We examined the role of miR-199a-5p on DCA-induced cell proliferation. HCT-8 cells were treated with multiple dilutions of DCA for 48 h, and a proliferation assay was performed. As shown in Fig. 5B, DCA dose-dependently induced cell proliferation when its concentration was less than 50 μM. Cells transfected with exogenous miR-199a-5p or the control for 24 h prior to 25 μM DCA exposure were also analyzed. The DCA-induced cell proliferation was reversed by miR-199a-5p addition (Fig. 5B). In contrast, control miRNA molecules (NC) demonstrated no effect on DCA-induced cell proliferation.
3.7. **miR-199a-5p affects the CDK2 kinase activity**

To determine whether CDK2 enzymatic activity may be affected by miR-199a-5p, we examined the CDK2 kinase activity. Lysates from miR-199a-5p-, anti-miR-199a-5p-, and siCAC1-transfected HCT-8 cells were subjected to immunoprecipitation with a CDK2 antibody and subjected to in vitro histone H1 phosphorylation assay. HCT-8 cells transfected with miR-199a-5p demonstrated a significant decrease in CDK2 kinase activity (Fig. 6A). The CAC1 protein levels remained decreased in miR-199a-5p-transfected HCT-8 cells. The CDK2 protein levels remained unchanged as shown in Fig. 6B. These results demonstrate that miR-199a-5p inhibited CAC1 expression and decreased CDK2 kinase activity (Fig. 6). However, we found that the reduced degree of CDK2 enzymatic activity was different between miR-199a-5p and siCAC1-treated cells, suggesting that the miR-199a-5p effect on CDK2 enzyme activity is not simply to inhibit CAC1 expression. It is possible that miR-199a-5p can affect the expression genes from other pathways that modulate CDK2 activity. Thus, further experiments are needed to verify this result.

3.8. Restoration of miR-199a-5p and/or CAC1 silencing in CRC cell lines reduces cell viability, promotes apoptosis, arrests the cell cycle and increases doxorubicin sensitivity in HCT-8/VCR cells

We investigated whether miR-199a-5p functions as a tumor suppressor in CRC and whether miR-199a-5p restoration and/or CAC1 silencing may modulate doxorubicin-induced apoptosis. miR-199a-5p was combined with doxorubicin treatment to determine if the miRNA mimic could promote apoptosis. G1/S cell cycle arrest and sensitized the effects of the traditional anti-cancer agent, miR-199a-5p and a control were transfected into HCT-8/VCR cells. Following doxorubicin treatment, flow cytometric analysis for Annexin V-positive cells revealed an increase in apoptosis upon miR-199a-5p restoration. Similarly, an increase of apoptotic cells was observed in CAC1 silenced cells (Fig. 7A and B). HCT-8/VCR cells transfected with miR-199a-5p demonstrated an increase in G1 phase (Fig. 7C). Additionally, a decrease in MDR1 was observed in miR-199a-5p-transfected cells (Fig. 7D). These data demonstrated...
that miR-199a-5p increases the doxorubicin sensitivity of CRC cells and is mediated, at least in part, by a decrease of CAC1.

3.9. miR-199-5p over-expression suppressed the progression of CRC in vivo

It has been reported that the miR-199-5p expression level is down-regulated in different tumor samples and has a prognostic significance for the overall survival of patients with CRC. In further investigations, the expression of miR-199-5p was up-regulated in HCT-8 cells with precursor miRNA clones (pEZX-MR04-miR-199a-5p), and the cells were subcutaneously injected into the posterior flank of male BALB/c Nude mice. Compared with the Scrambled- and NC-transfected groups, cells transfected with miR-199-5p or siCAC1 revealed a delayed tumor formation time and a significant reduction in the tumor size, suggesting a potential tumor suppressive effect of miR-199-5p in vivo (Fig. 8A). In addition, immunohistochemical analysis demonstrated that the levels of CAC1 and MDR1 in tumors from the miR-199-5p and siCAC1 group were lower than that from the NC groups (Fig. 8B). These results suggested that the up-regulation of miR-199-5p or down-regulation of CAC1 expression could suppress in vivo tumor growth.

4. Discussion

In this study, we hypothesized that the DCA carcinogenic effects may be mediated in part via changes in miRNA expression. We performed miRNA microarray studies on PCECs cells treated with DCA and found significant alterations in the miRNA profiles. miRNAs with more than a 1.3-fold differential expression were considered to be most likely important in CRC pathogenesis in this study. Among the deregulated miRNAs, miR-199a-5p is of particular interest because it is down-regulated in the majority of human malignancies. miR-199a-5p was linked to the modulation of different target genes, including c-Met, Smad1, and CD44, and its exogenous expression was reported to reduce the motility and...
viability of hepatocellular carcinoma, breast cancer, testicular cancer, and esophageal cancer (Duan et al., 2012; Hummel et al., 2011; Cheung et al., 2011).

Notably, with the help of a bioinformatic analysis, we found that the novel gene CAC1 is a hypothetical target of miR-199a-5p. miR-199a-5p targeted CAC1 through its specific miRNA response element within the CAC1 3′-untranslated region. CAC1 regulates cell proliferation and allows the progression of the cell cycle from G1 to the S phase (Kong et al., 2009, 2012). We found that the down-regulation of miR-199a-5p in the majority of CRC samples, together with the up-regulation of CAC1, supports this hypothesis because there is a causal link between these two molecules.

CAC1 has been mapped to human chromosome 10q26.1, a region frequently altered in human malignancies. CAC1 is highly conserved in mammals (NCBI database) and in other species, suggesting that this gene may play an important role in cell biology. Our previous study found that CAC1 is highly expressed in colorectal cancer tissues and cancer cell lines. CAC1 is expressed in a cell-cycle-dependent manner, and its expression is high in late G1 to S phase. CAC1 knockdown by RNAi inhibits cell proliferation and induces G1/S arrest. CAC1 interacts with CDK2 and promotes the kinase activity of the CDK2 protein; CAC1 is a novel cell cycle-associated protein capable of promoting cell proliferation (Kong et al., 2009, 2012).

We found that miR-199a-5p could control the cancerous properties of colorectal cancer cells through CAC1. Using HCT-8 colorectal cancer cells, we found that increasing miR-199a-5p expression inhibited the in vitro proliferative, migratory and invasive characteristics of colorectal cancer cells. It was also found that miR-199-5p could suppress the CRC progression in vivo, which was consistent with the results of the siCAC1 group, suggesting a potential tumor suppressive effect of miR-199-5p. Our results demonstrated that miR-199a-5p could bind to the 3′-UTR of CAC1 to inhibit CAC1 translation. When we down-regulated miR-199a-5p expression with anti-miR-199a-5p, the inhibitory effect of miR-199a-5p on CAC1 was reversed. This demonstrated that miR-199a-5p may function by inhibiting proliferation and promoting the cell cycle arrest of colorectal cancer cells, at least in part, via CAC1. Our results revealed that CAC1 appears to be a post-transcriptional target of miR-199a-5p.

**Fig. 8.** miR-199-5p negatively regulated the CRC progress in vivo. (A) Effect of siCAC1 and miR-199-5p on tumor growth in a nude mouse model. siCAC1-, miR-199-5p-, NC- and Scramble-transfected HCT-8 cells were subcutaneously injected into male BALB/c nude mice, and tumor growth was examined every 7 days. Photographs of dissected tumors from nude mice and a curve of the tumor growth rate are shown. These results are representative of four animals for each time point per group. These results demonstrated that miR-199-5p could also suppress the progression of CRC in vivo. (B) The levels of CAC1 and MDR1 in the transplanted tumors were detected by IHC. The levels of CAC1 and MDR1 in tumors from the miR-199-5p and siCAC1 groups were lower than those from the NC groups. Scale bar: 5 μm.
Thus, the identification of CAC1 as a miR-199a-5p target gene provides a possible explanation for why the suppression of miR-199a-5p can induce the growth of tumor cells by influencing their cell cycle progression. Thus, cell cycle deregulation may be a potential mechanism for miR-199a-5p-mediated tumorigenesis in CRC. Cell cycle modulation by miR-199a-5p resulted in increased G1 and decreased S phases. miR-199a-5p acts as an anti-oncogenic miRNA to inhibit the proliferation and metastasis of colorectal cancer cells in vitro and in vivo. However, further study is necessary to gain a full understanding of the underlying molecular mechanism.

Previous research found that CAC1 inhibition by RNAi increases sensitivity to chemotherapeutic agents by inhibiting the cell cycle. Accordingly, we investigated whether miR-199a-5p restoration and/or CAC1 silencing may modulate an increased sensitivity to doxorubicin-induced apoptosis. Following doxorubicin treatment, flow cytometric analysis for Annexin V-positive cells revealed an increase in apoptosis upon miR-199a-5p restoration. Similarly, an increase in apoptotic cells was observed in CAC1-silenced cells. As further evidence, a decrease in MDR1 expression was also observed in miR-199a-5p–transfected cells. This increased sensitivity to doxorubicin is, at least in part, mediated by CAC1-expression.

Our findings emphasize the role of miR-199a-5p as a modulator of the cell cycle; the proliferative, migratory and invasive capability; and the response to doxorubicin treatment. The identification of CAC1 as a miR-199a-5p target further emphasizes the role of this miRNA in CRC.

As described herein, hydrophobic bile acids can promote the tumorigenesis of colorectal tissues. We identified an important growth regulatory role for miRNAs in mediating the effects of DCA on host gene expression. These data suggest that miRNAs play an important role in hydrophobic bile acid carcinogenesis and that the abnormal expression mediated by DCA is a mechanism of its carcinogenic effects. The dysregulation of miRNA expression can influence carcinogenesis when the miRNA targets are tumor suppressors and oncogenes. Our findings support the idea that hydrophobic bile acid induction during colon cancer cell proliferation is dependent and mediated by deregulated miRNAs. These results have important implications for deregulated miRNAs and hydrophobic bile acid carcinogenesis.

Acknowledgments

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References


