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Original Articles

Epigenetic silencing of miR-490-3p promotes development of an aggressive colorectal cancer phenotype through activation of the Wnt/β-catenin signaling pathway

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ABSTRACT

The Wnt/β-catenin pathway is known to contribute to colorectal cancer (CRC) progression, although little is known about the contribution of β-catenin on this process. We investigated the role of miR-490-3p, which was recently reported to suppress tumorigenesis through its effect on Wnt/β-catenin signaling. We found that hypermethylation of the miR-490-3p promoter down-regulates miR-490-3p expression in CRC tissue. Gain- and loss-of-function assays in vitro and in vivo reveal that miR-490-3p suppresses cancer cell proliferation by inducing apoptosis and inhibits cell invasiveness by repressing the initiation of epithelial-to-mesenchymal transition (EMT), a key mechanism in cancer cell invasiveness and metastasis. The frequently rearranged in advanced T-cell lymphomas (FRAT1) protein was identified as a direct target of miR-490-3p and contributes to its tumor-suppressing effects. miR-490-3p appears to have an inhibitory effect on β-catenin expression in nuclear fractions of CRC cells, whereas FRAT1 expression is associated with the accumulation of β-catenin in the nucleus of cells, which could be weakened by transfection with miR-490-3p. Our findings suggest that the miR-490-3p/FRAT1/β-catenin axis is important in CRC progression and provides new insight into the molecular mechanisms underlying CRC. They may help to confirm the pathway driving CRC aggressiveness and serve for the development of a novel miRNA-targeting anticancer therapy.

Introduction

Colorectal cancer (CRC) is one of the most common human malignant tumors worldwide. Arising from the transformation of epithelial cells in the colon or rectum into malignant cells [1–3], the foundation of CRC pathogenesis is in the progressive accumulation of mutations in oncogenes and tumor-suppressor genes, such as *APC* and *KRAS* [4]. Approximately 50% of CRC cases involve recurrence and metastasis following radical surgery [5]. A process known as epithelial to mesenchymal transition (EMT) is a key mechanism in the invasiveness and metastatic drive in most cases of carcinoma. During EMT, the cells develop increased mobility and initiate migratory characteristics, which contribute to the concomitant loss of adhesion and increase in mesenchymal components [6]. Targeting driver pathways may prove to be the best option for tailoring therapy for patients with metastatic cancer and improving their rate of survival [7]. By improving our understanding of the molecular mechanisms underlying CRC progression, we may be able to develop effective therapeutic interventions and methods of managing this disease.

A family of small non-coding RNAs known as microRNAs (miRNAs) participates in the regulation of gene expression by binding with complementary sites within the 3′-untranslated region (3′UTR) of targeted genes [8–11]. A growing body of evidence suggests that miRNAs regulate approximately 60% of the protein-coding genes [12]

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that participate in several bioprocesses, including developmental timing, cell death, cell proliferation, apoptosis [11,13], and EMT [14,15]. Recently, several miRNAs were found to be involved in CRC metastasis: both miR-200 and miR-29c [16], for example, suppress CRC metastasis, whereas miR-106b promotes it [17]; additionally, miR-99b-5p may suppress liver metastasis in CRC [18]. The pathway(s) driving miRNAs to regulate CRC metastasis, however, are still not clear. In addition, the mechanism for miRNAs silencing in cancer also needs to be explored, among which epigenetic regulation is an important cause. Epigenetic silencing is involved in several aberrant expression of miRNAs through methylation of their promoter region, including miR-210 [19] and miR-622 [20]. The newly discovered miRNA miR-490-3p plays an important role in the carcinogenesis of several types of tumors, such as gastric [21], hepatocellular [6], and ovarian carcinoma [18]. Its mechanism underlying the development and progression of CRC remains unknown. We investigated the role of miR-490-3p in the genesis and metastasis of CRC in both in vitro and in vivo studies. We also sought to analyze the signaling pathways regulated by potential targets of miR-490-3p and clarify the functional significance of its downstream targets.

Materials and methods

Cell lines and tumor tissue samples

The CRC cell lines NCM460, HT29, HCT116, LS174T, RKO, SW480, SW620, and LOVO were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained as previously described. All cells were authenticated by short tandem repeat (STR) profiling before receipt and were propagated for less than 6 months after resuscitation. A human CRC cell subline with unique liver metastatic potential, designated SW480/M5, was established in our laboratory [22] and used in the analysis. These cells were grown in RPMI Medium 1640 (Thermo Fisher Life Technologies Corporation; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Fresh primary CRC specimens and paired noncancerous colorectal tissue specimens were provided by the Department of General Surgery in Zhujiang Hospital of Southern Medical University in Guangdong, China. Each patient had a diagnosis of primary CRC and had undergone elective surgery for CRC in Zhujiang Hospital between 2010 and 2013. The pathological diagnosis was made in the Department of Pathology in Zhujiang Hospital. The study was approved by the Ethics Committee of Southern Medical University, and all aspects of the study comply with the criteria of the Declaration of Helsinki. The Committee approved the collection of tissue without requiring informed consent, given that the data would be analyzed anonymously.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using Trizol (Invitrogen; Carlsbad, California). To quantify the expression of miR-490-3p, we subjected the total RNA to polyadenylation and reverse transcription (RT) using an NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen). Real-time polymerase chain reaction (PCR) analysis was carried out using an SYBR Green PCR master mix (Applied Biosystems; Foster City, California) on an ABI 7500HT system. *GAPDH* or *U6* snRNA was used as an endogenous control. All samples were normalized to internal controls, and fold changes were calculated through relative quantification ($2^{-\Delta\Delta CT}$). Real-time PCR for target genes was performed as previously described [2]. The primers used are shown in Supplementary Table S1.

Methylation-specific PCR and DNA demethylation treatment

Genomic DNA was exposed to bisulfite using an EZ DNA Methylation Kit (Zymo Research; Irvine, California) then subjected to methylation-specific PCR (MSP) using primers specific to the methylated (Forward: TAGATTAGTTAAGTGGTTGA-GACGA; Reverse: ATAAAAATAAAACGAAAAAAACGAA) or unmethylated (Forward Primer: TAG-ATTAGTTAAGTGGTTGAGATGA; Reverse: TAAAAATAAAACAAAAAAA-ACAAA) CHRM2/miR-490-3p promoter, as previously described [1]. Cells were incubated with 5-AdC (2 μM) and allowed to grow to approximately 40% confluency over 72 h at 37 °C.

Western blot analysis

Protein expression was assessed by immunoblot analysis of cell lysates (20–60 μg) in RIPA buffer in the presence of rabbit antibodies to E-cadherin; mouse antibodies to β-catenin, fibronectin, vimentin, and β-actin (1:500) (Santa Cruz Biotech; Santa Cruz, California); rabbit antibodies to N-cadherin (1:1000) (Cell Signaling Technology; Danvers, Massachusetts); the frequently rearranged in advanced T-cell lymphomas (FRAT1) protein (1:1000) (Abcam; Cambridge, UK), and histone h3(k4) (1:500) (Bioworld; Minneapolis, Minnesota).

miRNA target validation

The 2651-bp full-length FRAT1 3′ untranslated region (3′UTR) was amplified by PCR and cloned downstream of the firefly luciferase gene in the psiCHECK-2 vector (Promega; Madison, Wisconsin). This vector was named wild-type (wt) 3′UTR. Site-directed mutagenesis of the miR-490-3p binding site in the FRAT1 3′UTR was carried out using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and named mutant (mt) 3′UTR. For reporter assays, the wt or mt 3′UTR vector and miR-490-3p mimic or inhibitor were cotransfected. Luciferase activity was measured at 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). The primers used to amplify 3′UTR of FRAT1 were as shown in Supplementary Table S2.

Preparation of lentiviral vectors

A DNA fragment corresponding to pre-miR-490-3p and the flanking sequence was amplified from human genomic DNA and then cloned into a pGLV3/H1/GFP + puro lentiviral vector (http://www.genepharma.com). The production, purification, and titration of lentivirus were performed as previously described [3]. The packaged lentiviruses were named LV-miR-490-3p. The empty lentiviral vector LV-con was used as a control.

miRNA target prediction and signaling pathway analysis

Potential target genes of the down-regulated miRNAs were predicted using the miRWalk database, which includes three prediction programs (miRanda, miRWalk, and TargetScan). A gene was considered a putative target if its fold change was >2 in the chip microarray and it was predicted by all three programs. A list of target genes was analyzed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov), and the signaling pathways in which the target genes may be involved in were identified using the KEGG pathways program (http://www.genome.ad.jp/kegg). The primers used are shown in Supplementary Table S3.

Statistical analysis

Data were analyzed using SPSS version 19.0 software (SPSS; Chicago, Illinois). The Student *t*-test and the one-way ANOVA test were carried out for quantitative RT (qRT)-PCR. Analysis using CCK-8 (Dojindo) was applied to calculate the tumor growth curve. The significance of the correlation of the expression of miR-490-3p and histopathological factors was determined using Pearson's chi-squared (χ^2) test. Kaplan–Meier plots were used to estimate the prognostic relevance of miR-490-3p in univariate analysis. Multivariate analysis was performed by applying Cox proportional hazards test. Statistical significance was established at $P < .05$.

Results

miR-490-3p is down-regulated in human CRC cancer

Real-time PCR studies of miR-490-3p transcripts in CRC tissues and cell lines revealed that miR-490-3p expression decreased as much as 36-fold in 29 of all 41 CRC samples compared with controls (Supplementary Fig. S1). miR-490-3p expression in CRC tissue was significantly lower than in normal adjacent tissue ($P = .012$, Fig. 1A), as it was in metastatic CRC (mCRC) tissue compared with nonmetastatic CRC (nmCRC) tissue ($P = .0303$, Fig. 1A). miR-490-3p expression was also lower in all eight CRC cell lines compared with the normal human colon epithelial cell line NCM460 (Fig. 1B).

miR-490-3p gene promoter is hypermethylated in CRC cancer

In our investigation of the upstream mechanism involved in miR-490-3p down-regulation in CRC cancer, we found that exposure to the DNA demethylating agent 5-aza-2′-deoxycytidine restored miR-490-3p expression in HCT116, SW480, and HT29 cells (Fig. 1C). This suggests the involvement of promoter hypermethylation in miR-490-3p activity. Using MSP, we frequently observed hypermethylation of the miR-490-3p promoter in human CRC cancer cell lines (Fig. 1D). The rate of DNA methylation in the promoter region of miR-490-3p was also substantially higher in human CRC cancer tissue compared with noncancerous adjacent tissue ($P = .047$, Fig. 1E).

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