Original Articles

IFN-γ-mediated IRF1/miR-29b feedback loop suppresses colorectal cancer cell growth and metastasis by repressing IGF1

Li Yuan [a,1], Chang Zhou [b,1], Yanxia Lu [a], Min Hong [a], Zuoyang Zhang [a], Zheying Zhang [a], Yaya Chang [a], Chao Zhang [a], Xuenong Li [a,*]

[a] Department of Pathology, School of Basic Medical Sciences, Southern Medical University, NO.1838 Guangzhou Road, Guangzhou 510515, China
[b] Department of Anatomy and Histology, Guangdong Pharmaceutical University, No. 280 Outer Ring East Road, Guangzhou Higher Education Mega Center, Guangzhou 510006, China

ABSTRACT

To investigate the clinicopathological significance and underlying mechanism of microRNA-29b (miR-29b) in colorectal cancer (CRC), the role of miR-29b was investigated using in vivo and in vitro assays. Luciferase reporter assays were conducted to determine the association between miR-29b and the insulin-like growth factor 1 (IGF1) 3′ untranslated region (3′UTR). Chromatin immunoprecipitation (ChIP) assays were employed to assess the direct binding of interferon regulatory factor 1 (IRF1) to miR-29b. We found that interferon (IFN)-γ could induce miR-29b by recruiting IRF1 to binding sites in the miR-29b promoter. A low level of miR-29b was significantly associated with an aggressive phenotype. MiR-29b inhibited CRC cell growth and invasion. IGF1, an activator of PI3K/Akt signaling, was confirmed as a novel target of miR-29b. Moreover, miR-29b increased IRF1 expression, and the inhibition of miR-29b suppressed IFN-γ-induced apoptosis. We elucidated the potential signaling pathway, IFN-γ/IRF1/miR-29b/IGF1, and its implication for CRC tumorigenesis. A positive feedback loop between IRF1 and miR-29b may contribute to the sensitivity of CRC cells to IFN-γ. Targeting miR-29b may provide a strategy for blocking CRC growth and metastasis.

© 2015 Elsevier Ireland Ltd. All rights reserved.

Introduction

Interferons (IFNs) are antiviral cytokines that have significant effects on anti-tumorigenesis and inflammatory reactions. After binding to the cognate cell surface receptor, IFN-γ induces gene expression through activation by tyrosine and serine phosphorylation of the JAK/STAT pathway [1]. IFN-γ constitutes a major part of the innate immune response, but it is also recognized for its anti-tumor effects on cancer cells, including colon cancer [2–4]. Recently, IFNs have been found to regulate the expression of microRNAs (miRNAs) through signal transducers, which may influence IFN-induced anti-tumor activity [5]. The signaling cascades involving the IFN-α/β/signal transducer and activator of transcription 3 (STAT3)/miR-21 and others have been confirmed to mediate growth inhibition of cancer cells in several types of cancer [6,7]. In miRNA microarrays, miR-29b has been shown to be one of the top-10 dynamically up-regulated miRNAs after IFN-γ treatment [8].

It is now well accepted that miRNAs are a class of small non-coding RNAs that have recently emerged as key players in regulating cancer progression, metastasis and immunity [9,10]. Among these, miR-200c inhibited growth and metastasis in colon cancer through the down-regulation of Sox2 [11]. MiR-216a has emerged as a multifunctional miRNA involved in the regulation of growth and metastasis in lung cancer [12]. Tumor-suppressing and immune-modulating properties have been attributed to MiRNA-29, a microRNA family including three mature members miR-29a, miR-29b and miR-29c [13]. miR-29s are down-regulated in many types of cancer and have consequently acted as tumor suppressors, although in a few cases, ontogenetic roles have also been reported [14,15]. Moreover, miRNAs could be of help in the selection of chemotherapy strategies for patients with cancer. MiR-31-3p has recently been identified as a new biomarker whose expression level allows for the identification of patients with wild-type KRAS metastatic CRC who are more likely to respond to anti-EGFR therapy [16]. However, no relevant data have been found regarding the functions of miR-29b in the control of CRC tumorigenesis and therapy.

Members of the miR-29 family are involved in cancer suppression [17]. IFN/STAT1/miR-29 has been confirmed to exhibit anti-proliferative activities in melanoma [18]. In addition to inducing STAT3 expression, IFNs have been found to activate the RANTES promoter through nuclear factor kappaB and IRF1 [19]. Furthermore, another report has suggested that an IFN-γ-type signature enhanced the expression of IRF1 and predicted better responsiveness

to anti-cancer therapy [20]. IRF1 is a nuclear transcription factor that mediates interferon and other cytokine effects and appears to have antitumor activity in vitro and in vivo in cancer cells [21]. Because IRF1 has also been implicated in IFN-γ action and binds to specific DNA sequences in several promoters to initiate the transcription of specific genes [22], we examined the promoters of miR-29b and identified a potential IRF1 binding site in the miR-29b promoter.

In this work, we found that IRF1 regulated IFN-induced miR-29b transcription in CRC cells, and in a positive feedback loop, miR-29b upregulated IRF1 expression. Moreover, we provided evidence that the tumor-suppressing properties of miR-29b are achieved through the negative regulation of IGF1, which is a locally acting growth factor that can activate the PI3K/Akt or MAPK pathway and plays a role in colonic carcinogenesis [23]. We therefore attempted to focus on the IFN-γ/IRF1/miR-29b/IGF1/PI3K/Akt-related signaling pathways to explain the mechanism of miR-29b-modulated proliferation and metastasis in CRC and to open up new connections among miRNAs, interferon signaling and CRC, which may lead to novel concepts for potential treatment options in the future.

Materials and methods

Clinical specimens

Colorectal cancer tissues and matched adjacent normal mucosa of 41 patients were collected from fresh surgical specimens, frozen in liquid nitrogen, and stored at –80 °C until further analysis. All tissues had been histologically confirmed to be an adenocarcinoma of the colon. All tissues and classifications were performed based on the system of the International Union Against Cancer. The research protocol was approved by the Ethics Committee at Nanfang Hospital.

Cell culture and animals

Colorectal cancer cell lines HCT116, HT29, LS174T, SW480, SW620 and HEK293 were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and authenticated according to the ATCC recommendations. The CRC cell lines were cultured in RPMI 1640, and the HEK293A and HEK293FT cell lines were cultured in DMEM (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) at 37 °C in a humidified incubator of 5% CO_2. IFN-γ was purchased from PeproTech. Female 4–6 week-old athymic BALB/c nu/nu mice were purchased from and maintained at the Central Laboratory of Animal Science of Southern Medical University. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at our university.

Immunofluorescence staining

Cells were incubated with the primary antibody anti-IRF1 rabbit polyclonal antibody (Proteintech Group, Chicago, MA, USA) overnight at 4 °C. Then, the cells and sections were stained with DyLight-conjugated goat anti-rabbit IgG (ZSGB-bio, Beijing, China). Nuclear staining for total cell counting was performed by a 5 min addition of 1 mg/ml of DAPI, and the fluorescence signals were analyzed by recording and merging single-stained images using a confocal Olympus microscope.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's instructions. The expression of miR-29b was quantified by quantitative RT-PCR using an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD) according to the manufacturer's instructions. U6 was used as an endogenous control. The primer sequences used for qRT-PCR or PCR are listed in Supplementary Table S1.

Construction of plasmids and transfection

shIRF1 and shCON were constructed using the GV118 vector purchased from Genechem (Nanjing, China). The sequences used for transfection are listed in Supplementary Table S2. The miR-29b precursor was amplified by PCR and ligated to the PLVTHM lentivector (Addgene) as PLV-miR29b. The recombinant PLV-miR29b plasmid and packing plasmid psPAX2 and pMD2.G were cotransfected into HEK293FT cells. The controls were packed with PLVTHM empty vectors. After 48 hours of transfection, the supernatant containing the lentivirus was collected. The miR-29b-down-lentivirus (miR-29bsh) with a GFP gene was purchased from Genechem. After 7 days of lentivirus infection, the miR-29b-overexpressed or suppressed cells were sorted by flow cytometry for GFP and confirmed by qRT-PCR. The IGF1 3′UTR covering the 2

binding sites of miR-29b and IGF1-CDS was generated by PCR and subcloned into psiCHECK2 and pcDNA3.0 (Promega, Madison, W, USA), respectively. Mutant constructs in the 2 miR-29b binding sites of IGF1 3′UTR were generated using a KOD-Plus-mutagenesis kit (Toyobo, Japan). For the binding of IRF1 to the miR-29b promoter, the coding region of IRF1 and the promoter of miR-29b were amplified by PCR and then inserted into the pcDNA3.0 and PGL3 luciferase reporter vectors (Promega), respectively. All the primers used to generate these constructs are listed in Supplementary Table S3.

Luciferase activity assay

Cells were seeds in triplicate in 24-well plates (1×10^5/well) and cultured for 24 hours. Co-transfections of IGF1-3′UTR or mut IGF1-3′UTR plasmids with miR-29b mimics (Genepharma, Shanghai, China) into cells were accomplished using Lipofectamine2000 (Invitrogen, Foster, CA, USA). For the binding of IRF1 to the miR-29b promoter, PGL3-Luc, pcDNA3.0-IRF1 or the control-luciferase plasmid was cotransfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000. Luciferase activity was measured 48 hours after transfection by the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP) assay

According to the ChIP assay kit (Millipore, Temecula, CA, USA) protocol, SW480 and SW620 cells were lysed using sodium dodecyl sulfate lysis buffer, and DNA was sheared by sonication to lengths between 200 and 1000 bp. Protein–DNA complexes were precipitated by anti-IRF1 antibody (Abcam, Boston, USA) and control IgG, respectively, followed by the elution of the complex from the antibody. PCR was performed with primers specific for miR-29b.

Wound healing assay

To assess the migration ability of CRC cells upon miR-29b transfection, we performed a wound-healing assay. Cells were grown to 80% confluence, seeded into 6-well plates and transfected as described above. Cells were allowed to grow to confluence, and the monolayer was scratched with a 100 µl pipette tip. The remigration of cells to close the wound was assessed by live cell imaging. The percentage of open area was measured with the software at defined time points (1, 24, and 48 h). All values were normalized to the percentage of open area at 1 h.

Flow cytometry

A flow cytometer was used to assess cell apoptosis with an AnnexinV-FITC or AnnexinV-APC Apoptosis Detection kit (Keygene, Nanjing, China). At 24 h post-transfection, the cells were harvested and washed twice with cold PBS prior to 10^6 cells being resuspended in 200 µl binding buffer supplemented with 10 µl Annexin-V-FITC (or Annexin-V-APC) and 5 µl PI (or 7-AAD). The cells were then incubated in the dark for 10 min. Subsequently, 300 µl binding buffer was added, and a flow cytometric analysis was performed.

Soft-agar colony formation assay

For this assay, 1×10^4 cells were suspended in a 2 ml complete medium containing 0.33% agar (Sigma, MO, USA).Then, the agar-cell mixture was plated on top of a bottom layer with a 1% complete medium mixture, and 14 days later, the colonies were measured with an ocular micrometer. Colonies that were larger than 0.1 mm in diameter were counted.

Western blot analysis

Proteins were extracted and separated in SDS-PAGE gels, transferred onto PVDF membranes, and blotted according to standard methods using anti-IGF1, anti-caspase3, anti-caspase9, anti-tubulin (Proteintech Group), anti-p-Akt, anti-Akt, anti-p-PI3K, anti-PI3K (Cell Signaling Technology, MA, USA), anti-IRF1 (Abcam and Proteintech Group), and anti-GAPDH (ZSGB-bio), followed by their respective horseradish–peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence reaction performed according to the manufacturer's instructions (Alpha Innotech, San Leandro, CA, USA).

Animal model

SW480-PLVTHM, SW480-PLV-miR29b, HCT116-NC, and HCT116-miR-29bsh (1×10^7 cells) were injected subcutaneously into the left or right flank of nude mice (n = 5 per group). All mice were housed and maintained under specific pathogen-free conditions and scarified after 27 days of injection. Tumor size was measured, and tumor volume was determined by the formula (length × width^2)/2. Then, the fluorescence emitted by cells was imaged through a whole-body GFP imaging system (Lighttools, Encinitas, CA, USA). The subcutaneous tumors were cut into small pieces