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Novel miR-5582-5p functions as a tumor suppressor by inducing apoptosis and cell cycle arrest in cancer cells through direct targeting of GAB1, SHC1, and CDK2

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ABSTRACT

MicroRNAs (miRNAs) play pivotal roles in tumorigenesis as either tumor suppressors or oncogenes. In the present study, we discovered and demonstrated the tumor suppressive function of a novel miRNA miR-5582-5p. miR-5582-5p induced apoptosis and cell cycle arrest in cancer cells, but not in normal cells. GAB1, SHC1, and CDK2 were identified as direct targets of miR-5582-5p. Knockdown of GAB1/SHC1 or CDK2 phenocopied the apoptotic or cell cycle arrest-inducing function of miR-5582-5p, respectively. The expression of miR-5582-5p was lower in tumor tissues than in adjacent normal tissues of colorectal cancer patients, while the expression of the target proteins exhibited patterns opposite to that of miR-5582-5p. Intratumoral injection of a miR-5582-5p mimic or induced expression of miR-5582-5p in tumor cells suppressed tumor growth in HCT116 xenografts. Collectively, our results suggest a novel tumor suppressive function for miR-5582-5p and its potential applicability for tumor control.

1. Introduction

MicroRNAs (miRNAs), a class of endogenous small non-coding RNAs ~22 nucleotides in length, have received wide attention as important regulators of gene expression. They typically suppress gene expression at the post-transcriptional level by base-pairing with the sequences in the 3′-untranslated region (3′UTR) of target mRNAs, which results in mRNA degradation or translational repression [1,2]. In addition, some studies have shown that gene regulation by miRNA targeting can occur through miRNA binding to the 5′UTRs and the coding sequence (CDS) of target mRNAs as well [3,4]. A single miRNA can have a global impact on gene expression by simultaneously regulating multiple target genes. Growing evidence shows that miRNAs play an important role in a wide range of biological processes such as proliferation, development, differentiation, and apoptosis, and are thereby closely associated with various types of human diseases, especially cancer [5,6]. Aberrant expression of miRNAs is a characteristic feature of cancers and various miRNAs can function as oncogenes or tumor suppressors depending on the type of cancer by regulating the critical genes or signaling pathways involved in the tumorigenesis of the specific cancer type.

The most well-defined tumor suppressor miRNA is the miR-34 family, which is a direct target of the tumor suppressor TP53 [7]. miR-34 is called a master regulator of tumor suppression since it targets a broad range of proto-oncoproteins such as MYC, BCL2, CDK4, and MET, thereby exhibiting broad anti-oncogenic activity in various cancer types [8–11]. Additionally, several miRNAs have been demonstrated to function as a tumor suppressor, such as miR-29 and miR-193a-3p, which induce apoptosis by targeting p85α and MCL1, respectively [12,13]. In contrast, some miRNAs have been found to be expressed at high levels in various cancers and exert their oncogenic effects by targeting pro-apoptotic tumor suppressors. For example, the typical oncogenic miRNA miR-21 targets phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) [14,15], while miR-17-92 cluster targets BIM and PTEN [16]. These features of miRNAs acting as tumor suppressors or oncogenes make them attractive therapeutic tools or targets for cancer treatment. Accordingly, efforts are currently underway to

Abbreviations: CRC, colorectal cancer; miRNA, microRNA; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); GAB1, GRB2-associated binding protein 1; SHC1, SRC homology 2 domain containing 1; GRB2, growth factor receptor-bound protein 2; CDK2, cyclin-dependent kinase 2; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; qRT-PCR, quantitative real-time polymerase chain reaction.

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develop miRNA mimics and inhibitors as miRNA-based therapeutics for clinical use in cancer therapy [8,17].

In this study, we aimed to discover new miRNAs that can regulate the growth of cancer cells for future application to develop miRNA therapeutics, and identified miR-5582-5p as a novel miRNA inducing apoptosis and cell cycle arrest in cancer cells. This is, to our knowledge, the first report about the function of miR-5582-5p. Our results suggest that miR-5582-5p functions as a strong tumor suppressor and has a high potential for application to develop novel anti-cancer miRNA therapeutics.

2. Materials and methods

2.1. Cell culture and reagents

The human colorectal cancer (CRC) cell lines HCT116, SW480, DLD-1, HCT-15, the human non-small cell lung cancer cell lines A549 and H460, human colon fibroblast CCD-18co, and human pulmonary fibroblast (HPF) were obtained from American Type Culture Collection (ATCC, VA) and grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (10 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2. HCT116 (*TP*53$^{+/+}$) and HCT116 (*TP*53$^{-/-}$) cell lines were provided by Dr. B Vogelstein (Johns Hopkins University, MD). Z-VAD-FMK was purchased from Sigma-Aldrich (MO). Antibodies against GAB1, SHC1, CDK2, SOS1, BAX, BAK1, BCL2, E2F1, cyclin E, PCNA, and β-actin were purchased from Santa Cruz Biotechnology (CA). Antibodies against PARP, cleaved caspase-3, RB1, phospho-RB1, AKT, phospho-AKT, ERK, phospho-ERK, XIAP, BCL2L1, and GRB2 were purchased from Cell Signaling Technology (MA).

2.2. RNA oligonucleotides and transfection

A pool of synthetic miRNA mimics was synthesized by Genolution Pharmaceuticals (Seoul, Korea) as RNA duplexes designed from the sequences registered in miRBase database. The IDs of synthesized 267 miRNAs consisting of all the registered members of the release version 17 and part of the version 16 of the miRBase database are listed in Supplementary Table 1. An inhibitor of miR-5582-5p was also synthesized by Genolution Pharmaceuticals as a 2′-*O*-methyl-modified oligoribonucleotide single strand with a sequence of 5′-GCTATAACTTTAAGTGTGCCTA-3′. siRNA duplex oligonucleotides targeting GAB1, SHC1, and CDK2, and scrambled non-targeting negative control siRNA were purchased from Santa Cruz Biotechnology. For functional analyses, cells were transfected with the oligoribonucleotides of all the miRNA mimics, miR-5582-5p inhibitor, siRNA at a final concentration of 10 nM using G-fectin (Genolution Pharmaceuticals) according to the manufacturer's recommendation.

2.3. Expression vectors for GAB1, SHC1, and CDK2

The expression vectors for GAB1, SHC1, and CDK2 in Flag-tagged form were constructed using pCMV-Tag2B plasmid (Agilent Technologies, CA). The open reading frames (ORFs) of the proteins were amplified by PCR using the corresponding primer pairs (Supplementary Table 2) and subcloned in frame into the pCMV-Tag2B plasmid.

2.4. Determination of cell growth

Cells were seeded in 96-well plates (2.5×10^3 cells/well) and transfected with miRNAs. At 96 h after transfection, viable cells were quantified using an MTS method according to manufacturer's instruction (Promega, WI). The absorbance was read at 490 nm and data were obtained from 3 independent experiments. Cell growth was also determined by counting the number of cells excluding trypan blue.

2.5. Soft agar colony formation assay

The cells transfected with control or miR-5582-5p were suspended in 0.35% low-melting agarose/growth media and seeded on the top of solidified 0.5% low-melting agarose/growth media in 60 mm dishes (1000 cells/dish). The dishes were maintained in incubator at 37 °C for 2 weeks with addition of fresh growth media every week. Cells were fixed with 4% formaldehyde for 10 min and stained with 0.005% crystal violet, and colonies were counted.

2.6. Apoptosis analysis

Apoptosis in cultured cells was determined by flow cytometric method after Annexin V-FITC/ propidium iodide (PI) double staining using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instruction. The cells were then analyzed using a FACSCalibur apparatus. Analysis of apoptotic cells in xenograft tumor tissues was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay using DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer's instruction.

2.7. Cell cycle analysis

Cells were fixed in ice-cold 70% ethanol for 1 h and treated with RNase A (10 μg/mL) at 37 °C for 30 min after washing with PBS. The cells were then stained with PI (50 μg/mL) at room temperature for 1 h and cell cycle distribution was analyzed using a FACSCalibur apparatus.

2.8. Determination of intracellular ROS level

Intracellular ROS level was measured by staining cells with 2′-7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes). Cells were treated with DCF-DA at 5 μM in growth medium 48 h after transfection with control or miR-5582-5p and incubated at 37 °C for 1 h. The fluorescence intensity of cells was monitored by flow cytometry using a FACSCalibur apparatus (BD Biosciences, CA).

2.9. Reporter assay

To prepare the reporter constructs, DNA fragments of 3′UTR of target genes containing the putative miR-5582-5p binding sites were cloned in pGL3UC vector [12] as schematically depicted in Fig. 3C. The nucleotide sequences of primers for the amplification of the 3′UTR of target genes are listed in Supplementary Table 2. For generation of the mutant reporters, three nucleotide mutations were introduced into the putative miR-5582-5p binding sites using a QuikChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's recommendation. HCT116 cells were cotransfected with reporter plasmid (100 ng), pRL-CMV-Renilla plasmid (2 ng), and miRNA in 24-well plates using Lipofectamine 2000 (Invitrogen, CA). After 48 h of transfection, luciferase activity was measured using a Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. Firefly luciferase activity was normalized to Renilla luciferase activity. Experiments were carried out in triplicates.

2.10. RNA isolation and qRT-PCR analysis

Total RNA was extracted from cultured cells or frozen tumor tissues using Trizol reagent (Invitrogen) or RNeasy Mini kit (Qiagen, Germany), respectively, according to the instructions provided by manufacturers. Two micrograms of total RNA were used to determine the expression of mRNAs or primary miRNA transcript (pri-miRNA) using KAPA SYBR FAST one-Step qRT-PCR kit (KAPA Biosystems, MA) and iCycler real-time PCR detection system (Bio-Rad, CA) according to the