Biochemical and Biophysical Research Communications 406 (2011) 518–523

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-132 and miR-212 are increased in pancreatic cancer and target the retinoblastoma tumor suppressor

Jong-Kook Park [a], Jon C. Henry [b], Jinmai Jiang [a], Christine Esau [c], Yuriy Gusev [d], Megan R. Lerner [e], Russell G. Postier [f], Daniel J. Brackett [e], Thomas D. Schmittgen [a,*]

[a] College of Pharmacy, Ohio State University, Columbus, OH 43210, United States
[b] Department of Surgery, Ohio State University, Columbus, OH 43210, United States
[c] Regulus Therapeutics, Carlsbad, CA, United States
[d] Lombardi Cancer Center, Georgetown University, Washington, DC, United States
[e] Veterans Affairs Medical Center, Oklahoma City, OK, United States
[f] Department of Surgery, University of Oklahoma Health Sciences Center, Oklahoma City, OK, United States

ARTICLE INFO

Article history:
Received 9 February 2011
Available online 15 February 2011

Keywords:
microRNA
Retinoblastoma gene
β2 adrenergic receptor
Cancer

ABSTRACT

Numerous microRNAs (miRNAs) are reported as differentially expressed in cancer, however the consequence of miRNA deregulation in cancer is unknown for many miRNAs. We report that two miRNAs located on chromosome 17p13, miR-132 and miR-212, are over-expressed in pancreatic adenocarcinoma (PDAC) tissues. Both miRNAs are predicted to target the retinoblastoma tumor suppressor, Rb1. Validation of this interaction was confirmed by luciferase reporter assay and western blot in a pancreatic cancer cell line transfected with pre-miR-212 and pre-miR-132 oligos. Cell proliferation was enhanced in Panc-1 cells transfected with pre-miR-132/-212 oligos. Conversely, antisense oligos to miR-132/-212 reduced cell proliferation and caused a G_2/M cell cycle arrest. The mRNA of a number of E2F transcriptional targets were increased in cells over expressing miR-132/-212. Exposing Panc-1 cells to the β2 adrenergic receptor agonist, terbutaline, increased the miR-132 and miR-212 expression by 2- to 4-fold. We report that over-expression of miR-132 and miR-212 result in reduced pRb protein in pancreatic cancer cells and that the increase in cell proliferation from over-expression of these miRNAs is likely due to increased expression of several E2F target genes. The β2 adrenergic pathway may play an important role in this novel mechanism.

1. Introduction

Pancreatic adenocarcinoma (PDAC) cancer continues to be a lethal diagnosis for most patients with 43,140 new diagnoses and 36,800 deaths predicted for 2010 in the United States alone [1]. Despite much research on this disease over the past several decades, new and effective treatment regimens are lacking. Chemotherapy and radiation therapy remain mostly ineffective [2]. Therefore surgery remains the only attempt at a curative resection, but only 15–20% pancreatic cancer patients are eligible for surgery at the time of presentation and of those who undergo successful surgical resection the 5-year survival rate is a dismal 15–23% [3]. Novel treatment strategies are needed to combat this disease, however a better understanding of the molecular pathogenesis of PDAC must be achieved before new therapies may be developed.

microRNAs (miRNAs) are small, non-coding RNAs that post transcriptionally regulate protein levels by binding to the 3′ UTR of the mRNA [4]. miRNAs are differentially expressed in many solid tumors and often create a unique signature for each tumor type [5]. Our lab and others have demonstrated that miRNAs are linked to PDAC [6–9]. miR-212 was among the top differentially expressed miRNA precursors in PDAC with a 22-fold increased expression in the tumors [6]. miR-212 is located on chromosome 17p13.3, approximately 260 bp from a closely related miRNA, miR-132. Both miR-132 and miR-212 share the identical 5′ seed sequence, thus, would be expected to regulate the identical target genes.

miR-132 expression is increased in lung cancer [5,10], endocrine pancreatic tumors [5], squamous cell carcinoma of the tongue [11], breast cancer [12], and colorectal carcinoma [13,14]. Increased miR-212 expression was reported in colorectal carcinoma [13]. Decreased miR-132 expression is seen in osteosarcoma [15] while miR-212 has been shown to be down regulated in gastric cancers [16,17], non-small cell lung cancers [18], and head and neck squamous cell carcinoma [19]. The different levels of expression of these miRNAs in various cancers, highlight their diverse function in cells and the difficult task of determining their regulation and potential targets. To our knowledge, no one has taken an in depth look into the role of miR-132/-212 in PDAC.

* Corresponding author. Fax: +1 614 292 7766.
E-mail address: Schmittgen.2@osu.edu (T.D. Schmittgen).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.02.065

Several cancer-related targets of miR-132/-212 have been validated including c-Myc [16] and MECP2 [17] in gastric cancer, heparin binding EGF in head and neck carcinoma [19] and the antiapoptotic protein PED/PEA-15 in small cell lung cancer [18]. The only tumor suppressive target of miR-132/-212 to our knowledge is the pro angiogenic p120RasGAP that is decreased in endothelial cells of breast cancer due to upregulated miR-132 [12]. We report here that an important tumor suppressor, Rb1, is a target of miR-132/-212 in PDAC. Down regulation of pRb by miR-132/-212 leads to increased cell proliferation and cell cycle progression in Panc-1 cells. miR-132/-212 expression is increased by a β2 adrenergic receptor agonist, suggesting a novel mechanism for pancreatic cancer progression.

2. Materials and methods

2.1. Tissue procurement and isolation of protein and RNA

The tissue samples analyzed in this study were derived from patients undergoing a surgical procedure to remove a portion of the pancreas at the University of Oklahoma Health Sciences Center. The collection of samples conformed to the policies and practices of the facility's Institutional Review Board. Sections from each specimen were examined by a pathologist and graded histologically. RNA or protein was extracted from the tissues following pulverization in a cold mortar and pestle. Total RNA was isolated from the tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer. An RNA integrity number (RIN) of 6 or higher was used as the cutoff. For the protein samples, pulverized tissues were digested using Celytic™ M (Sigma–Aldrich, St. Louis, MO) with protease and phosphatase Inhibitor (Pierce, Rockford, IL) according to the manufacturer's guidelines.

2.2. Cell line

The human pancreatic cancer cell line Panc-1 was purchased from American Type Tissue Collection (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle Media (Invitrogen, Carlsbad, CA) with L-glutamine (Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS, HyClone). The cells were incubated at 37 °C under a humidified atmosphere with 5% carbon dioxide.

2.3. Cell proliferation assay

pre-miR-132 and -212 mimics and control oligonucleotide were purchased from Ambion (Austin, TX). Cells (1500/well) were plated 24 h prior to transfection with Lipofectamine 2000 (Invitrogen). Transfection with antisense oligonucleotides to miR-132 or miR-212 was conducted in the same manner. The cell proliferation assay was performed using the reagent WST-1 (Roche, Indianapolis, IN). All experiments were performed at least in triplicate. ASOs were chemically modified (100%) with a 2′-Omethoxyethyl and phosphorothioate backbone (2′-O-MOE-PS) and were provided by Regulus Therapeutics (Carlsbad, CA).

2.4. qPCR for miRNA and mRNA expression

One hundred nanogram of total RNA was primed using gene specific looped primers to miR-132 and miR-212. cDNA was then quantified with TaqMan miRNA Assays (Applied Biosystems, Foster City, CA). For mRNA expression analysis, cDNA was synthesized from 1 μg of total RNA using random primers. Gene expression analysis was performed by qPCR using the SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. For qPCR of both miRNA and mRNA, 18S rRNA was used as the reference gene and data were analyzed using the comparative C_T method. Primers used in this study are summarized in the Supplementary Information.

2.5. Luciferase reporter analysis

The full length Rb1 3′ UTR was cloned into the psiCHECK-2 Vector (Promega, Madison, WI) using standard techniques. The resulting recombinant plasmid of Rb1 was designated psiC2-Rb1. The mutant reporter construct Rb1-MUT was generated using QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Reporter vectors were assayed for luciferase expression using the Dual Luciferase Report Assay System (Promega) following the manufacturer's instructions. Twenty-four hour after transfection, relative luciferase activity was obtained by normalizing the renilla luciferase activity to the firefly luciferase activity. Primers used in this study were summarized in the Supplementary Information.

2.6. Protein extraction and immunoblotting

Protein was harvested using CelLytic™ M (Sigma–Aldrich) and 1× protease and phosphatase inhibitor (Pierce). Protein concentration was measured using the BCA Protein Assay Kit (Pierce). Thirty micrograms of total protein extract was separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blotting was performed for Rb1 (BD Pharmingen, San Diego, CA), cyclin A2, and cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin (Abcam, Cambridge, MA) was used as a loading control. Secondary horseradish peroxidase antibody was detected using ECL Western Blotting Analysis System (Amersham Biosciences, Piscataway, NJ).

2.7. BrdU cell proliferation assay

Non-isotopic bromodeoxyuridine (BrdU) incorporation assay (EMD chemicals, Gibbstown, NJ) was performed according to the manufacturer's instructions.

2.8. Cell cycle analysis

Antisense oligonucleotide effects on cell cycle were performed as previously described [20]. Data were analyzed using flow cytometry (FACS Calibur; Becton Dickinson), and cell cycle analysis software (Modfit; Verity, Topsham, ME). For each sample, 20,000 events were acquired.

3. Results

3.1. miR-132/-212 are increased in pancreatic cancer and target Rb1

We previously reported that 100 miRNA precursors were aberrantly expressed in pancreatic cancer [6]. As miR-212 was one of the most highly differentially expressed miRNAs in our prior study, we wished to further investigate its role in PDAC. qPCR was used to quantitate the expression of mature miR-132/-212 in 21 pancreas specimens (4 normal and 6 adjacent benign pancreas and 11 pancreatic adenocarcinomas). Both miR-132 and miR-212 were significantly up-regulated in each of the 11 pancreatic adenocarcinomas compared to the normal and benign tissues (Fig. 1A).

The TargetScan algorithm was used to look for potential targets of miR-132/-212. The human Rb1 3′ UTR has a miRNA binding sequence to both miR-132 and miR-212 (Supplementary Fig. 1A). To validate the interaction between the Rb1 3′ UTR and miR-132/-212, Panc-1 cells were co-transfected with psiC2-Rb1 vector and pre-miR-132, pre-miR-212 or control oligo. Luciferase expression