Cancer Letters 319 (2012) 173–181

Contents lists available at SciVerse ScienceDirect

Cancer Letters

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

Increased Ras GTPase activity is regulated by miRNAs that can be attenuated by CDF treatment in pancreatic cancer cells

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ARTICLE INFO

Article history:
Received 13 December 2011
Received in revised form 9 January 2012
Accepted 10 January 2012

Keywords:
Ras GTPase activity
K-Ras
miRNAs
CDF
Xenograft mouse model

ABSTRACT

Ras gene is frequently mutated, and also associated with increased Ras expression and its GTPase activity (activity) in pancreatic cancer (PC), which could in part be due to deregulated expression of microRNAs (miRNAs) contributing to tumor aggressiveness. Here we report, for the first time, that Ras expression and its activity were significantly higher in MIAPaCa-2 cells compared to COLO-357 and BxPC-3 cell lines, which was correlated with loss of *let-7* family and *miR-143* expression in MIAPaCa-2 cells compared to COLO-357 and BxPC-3 cells. Whereas the expression of *miR-21*, a frequently up-regulated miRNA in solid tumors was up-regulated in MIAPaCa-2 cells and it was correlated with increased Ras expression and its activity. The miRNAs, *let-7i* and *miR-143* was found to target Ras, and forced re-expression of *let-7i* and *miR-143* inhibited Ras activity, cell proliferation and colony formation *in vitro*. We also found that the treatment of cells *in vitro* or treatment of MIAPaCa-2 induced tumors *in vivo* with CDF, a novel synthetic analog of curcumin, led to the re-expression of *let-7* and *miR-143*, and down-regulated *miR-21* expression, which was consistent with attenuation of Ras expression and its activity. Moreover, re-expression of *let-7i in vivo* resulted in decreased tumor growth and Ras activity. These results suggest that the loss of expression of *let-7* and *miR-143*, and increased expression of *miR-21* leads to increased expression of Ras and its GTPase activity, which could be attenuated by CDF treatment and, thus CDF could become a novel therapeutic agent for the treatment of PC.

1. Introduction

K-Ras is one of the most frequently mutated isoform of the *Ras* oncogene families that includes *H-Ras* and *N-Ras* that are involved in cellular signal transduction, and thus their activation is associated with increased cell growth and differentiation in many cancers including pancreatic cancer (PC) [1]. *K-Ras* mutation occurs often in colorectal, lung, multiple myeloma, and chronic pancreatitis patients, which subsequently leads to the development of PC [1,2]. Thus, *K-Ras* plays a key role in the development and maintenance of the malignant phenotype, and the most common mutation was found to be on codon 12 ($K\text{-}Ras^{G12D}$), which is related to the activation of Ras GTPase activity [3]. In addition to the activation of *Ras* due to mutation, the loss or the gain of expression of microRNAs (miRNAs) could also mediate deregulation in the expression of target genes that are important in human cancer [4,5]. Emerging evidence suggest that a number of miRNAs could function as either oncogenes or tumor suppressor [6,7], and the loss of one such miRNA is *miR-143* which function as a tumor suppressor in gastric cancer, and further showing that the re-expression of *miR-143* leads to the inhibition of cell growth [8]. The loss of *miR-143* expression has been reported in many other cancer types and that restorations of its expression has been shown to abrogate tumorigenesis [9–11]. Several groups, including ours have shown a significant down-regulation of *let-7* expression in PC as compared to normal or even histologically normal pancreas tissue adjacent to the tumor, and that the loss of expression was negatively correlated with differentiation, stage of the disease, and patients' survival [12–16].

The *let-7* family is known to negatively regulate the expression of *Ras*, and that the loss of expression of *let-7* has been shown to be associated with many cancers including PC [17,18]. Another report suggested that increased expression of Lin-28B which plays an important role in response to radiation in lung cancer cells was indeed mediated through the loss of expression of *let-7g* and consistent with up-regulation of K-Ras [19]. It has been reported

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doi:10.1016/j.canlet.2012.01.013

that the high mobility group A protein 2 (HMGA2) whose increased expression is responsible for increased cell growth and contributes to the acquisition of epithelial-to-mesenchymal transition (EMT) is in part co-operatively regulated through the activation of Ras signaling pathway in PC [20], and that the ectopic expression of *let-7* reduced HMGA2 expression leading to the inhibition of cell proliferation in lung cancer [21]. Other investigators have reported that *let-7* could regulate cell cycle, angiogenesis, migration, and all of which could indeed be due to inhibition of K-Ras protein expression [22,23]. To the best of our knowledge, there are no reports showing that a specific *let-7* family miRNAs especially *let-7i* could negatively regulate Ras expression and its GTPase activity that could play important roles in the regulation of tumor aggressiveness in PC.

Emerging evidence suggests that *miR-21* is the most frequently up-regulated miRNA in many solid tumors [12,24–27], and there are several *miR-21* targets such as PTEN, and PDCD4, which are also down-regulated by Ras in an AP-1 dependent manner in thyroid cell system [26]. In breast cancer, inhibition of *miR-21* led to decreased cell proliferation and migration *in vitro* and tumor growth *in vivo* [28]. We have also reported earlier showing over-expression of *miR-21* in both cell lines and in the plasma of patients diagnosed with PC, which was also linked with the loss of PTEN, Maspin, and TPM1 [12]. These and other evidence led to the recognition that targeted inactivation of Ras signaling is a suitable approach of PC therapy; however, Ras-targeting drug development has been disappointing [1], which further suggest that newer approaches must be developed for targeted inactivation of Ras expression and its GTPase activity with minimal toxicity. To that end, we have synthesized a novel analog of curcumin named CDF which has been shown to have potent anti-tumor activity mediated by multiple mechanisms including deregulation of miRNAs.

In the present study, we investigated the mechanistic role of several miRNAs especially *let-7* family, *miR-143* and *miR-21* in the aggressiveness of PC, and further tested whether these miRNAs mediate their biological activity through the regulation of Ras expression and its activity. During the search for putative miRNA as inhibitors of K-Ras, miRNA target prediction algorithms was utilized, and we found that *miR-143* possess a match for base pairing with the 3′-UTR of K-Ras with the context score percentile of 93 (TargetScanHuman 5.2). Although, miRNA target prediction for *let-7*i was not available by TargetScanHuman, numerous articles have reported *let-7* miRNA binding to 3′-UTR of K-Ras [29–33]. Thus, we investigated the putative role of *miR-143* and *let-7*i and their effects on the expression of Ras and its activity by transfecting *pre-miR-143* or *pre-miR-let-7i* in human PC cell lines. We found that re-expression of miRNAs by transfection of cells with *pre-miR-143* and *let-7*i led to decreased cell proliferation and clonogenicity, which was correlated with inhibition of Ras expression and its GTPase activity both *in vitro* as well as in a xenograft mouse model *in vivo*. Moreover, instead of transfection, our novel agent, CDF was able to re-express *let-7* and *miR-143*, and down-regulated *miR-21* leading to inhibition of cell growth *in vitro* and tumor growth *in vivo*, which was consistent with attenuation of Ras expression and its activity. Based on these results, we conclude the importance of specific miRNAs that could serve as key regulators of Ras activity, which could be attenuated by CDF, and thus CDF could be a novel agent for the treatment of human PC.

2. Materials and methods

2.1. Cells culture, drugs and reagents

Human pancreatic cancer cell lines COLO-357, MIAPaCa-2, and BxPC-3 were chosen for this study based on their expression level of Ras and its GTPase activity. The cell lines have been tested and authenticated using the core facility-Applied Genomics Technology Center at Wayne State University on March 13, 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). These cells were stored in multiple vials in liquid nitrogen for our use. CDF was synthesized as described in our earlier publications [34,35].

2.2. Ras GTPase activity

Total Ras GTPase activity was measured in both cell lines and in tumor remnants, lysed with beads coated with Raf1-RBD (Millipore, Billerica, Massachusetts). Briefly cells/tumor remnants were lysed in lysis buffer provided by the manufacturer and the total protein was quantitated, 200 μg of protein was used for the assay following the manufacture's protocol. Both positive and negative controls provided in the kit were used in the assay. Western blot was performed and the proteins were transferred to nitrocellulose membrane, and were incubated with anti-Ras provided with the kit for assessing the level of Ras expression.

2.3. Protein extraction and Western blot analysis

Total protein was extracted from all three PC cell lines and MIAPaCa-2 tumor remnants from xenograft model untreated and treated with CDF 1 μM or CDF 5 mg per mice and subjected to western blot analysis as described previously [36] to evaluate the expression of K-Ras and the data was adjusted against loading control using β-actin expression.

2.4. Anti-sense miR-21 oligonucleotide transfection

COLO-357, BxPC-3 and MIAPaCa-2 cells were plated in 6 well plates and incubated overnight. Cells were transfected with anti-sense *miR-21* oligonucleotide (ASO) or control miRNA using ExGen 500 (Fermentas, Hanover, MD) following the manufacture's protocol. Cells were treated with 1 μM of CDF for 48 h and tested the transfection effects of Ras expression by western blot and assayed for Ras GTPase activity.

2.5. TaqMan miRNAreal-time reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine the basal level of miRNAs (*let-7*a, *let-7*b, *let-7*c, *let-7*d, *let-7*e, *let-7*f, *let-7*i, *miR-21,* and *miR-143*) in all three PC cell lines, and in tumor samples from xenograft mouse model, we used TaqMan MicroRNA Assay kit (Applied Biosystems) following manufacturer's protocol. Cells were treated with CDF (0.5–1 μM) for 48 h and used for the study. Total RNA was extracted and 10 ng from each sample were reverse transcribed as described earlier [12]. Real-time PCR reactions were then carried out in a total volume of 10 μl reaction mixture as described earlier [12]. All reactions, including controls were performed in triplicate using StepOnePlus Real-Time PCR (Applied Biosystems). Relative expression of miRNAs was analyzed using C_t method and was normalized by *RNU48* expression.

2.6. Animal experiments

Female CB17 SCID mice (about 4 wks old) were purchased from Taconic Farms (Germantown, NY) and fed Lab Diet 5021 (Purina Mills, Inc., Richmond, IN). MIAPaCa-2 cells-derived pancreatospheres (5000 spheres) were injected subcutaneously in SCID mice bilaterally. Mice were randomized into two treatment groups ($n = 7$ per group): (1) untreated control; (2) CDF (5 mg/mice/day), intragastric once daily for 12 days. Tumor measurements were performed at multiple time points during the course of treatment. Tumor weights were calculated as described earlier [37]. All the animals were euthanized when the tumor weight in the control group reached approximately 2000 mg, and the tumor remnants were used for both western blot analysis and qRT-PCR.

2.7. Transfection of miRNA precursor miR-143 and let-7i

About 200,000 cells/well of COLO-357 or MIAPaCa-2 were seeded in six-well plates and transfected with *pre-miR-143*, or *pre-let-7i* and control miRNA (Ambion, Austin, TX) at a final concentration of 20 nM using DharmaFECT transfection reagent (Dharmacon), following the manufacturer's protocol, and as described previously [38]. After 48–72 h of transfection, the transfected cells were measured for cell growth by 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and clonogenic assay was done. The cells were also harvested separately for total RNA and protein extraction and the relative levels of miRNAs, and proteins were measured as described above.

2.8. Cell viability assay of transfected cells

Standard MTT assay was performed after transfection of cells for 72 h, and experiments were repeated three times. The color intensity was measured by TECAN's microplate fluorometer (TECAN, Research Triangle Park, NC) at 595 nm.

2.9. Clonogenic assay of transfected cells

Transfected cells were trypsinized and 1000 viable cells were plated in 100 mm petri dishes. The cells were then incubated for about 10–12 days at 37 °C in a 5% CO_2/5% O_2 /90% N_2 incubator. Colonies were stained with 2% crystal violet and quantitated.