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

Shadan Ali¹, Aamir Ahmad², Amro Aboukameel¹, Bin Bao², Subhash Padhye³, Philip A. Philip¹, and Fazlul H. Sarkar^{1,2}

¹Department of Oncology, Wayne State University School of medicine, Detroit, Michigan

²Pathology, Karmanos Cancer Institute, Wayne State University School of medicine, Detroit, Michigan

³Interdisciplinary Science & Technology Research Academy, Abeda Inamdar Senior College, Azam Campus, Pune 411 001, India

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.

Keywords

Ras GTPase activity; K-Ras; miRNAs; CDF; xenograft mouse model

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Address for Correspondence: Fazlul H. Sarkar Ph.D. Department of Pathology, Karmanos Cancer Institute Wayne State University School of Medicine 740 Hudson Webber Cancer Research Center, 4100 John R Street Detroit, MI 48201 Tel: 313-576-8327 Fax: 313-576-8389 fsarkar@med.wayne.edu.

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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-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 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-7i* 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-7i* 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-7i* 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 5mg 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 μ M 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 miRNA Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To determine the basal level of miRNAs (*let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7i*, *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 μ M) for 48h 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 μ l 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 2,000 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 48–72h of transfection, the transfected cells were measured for cell growth by 3-(4,5-dimethylthiazol-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% CO₂ /5% O₂ /90% N₂ incubator. Colonies were stained with 2% crystal violet and quantitated.

2.10. Transfection of miRNA precursor *let-7i* in vivo

Effect of *pre-let-7i* was studied by transfection of expression plasmids with *pre-let-7i* (Origene Technologies) in MIAPaCa-2 cells prior to injection in the mice. Empty plasmid was transfected as vector control in MIAPaCa-2 cells. Stably transfected cells were selected

by adding 800 µg/ml G418 (Sigma, St Louis, MO) in the normal media. About one million MIAPaCa-2 cells (vector control and *let-7i* –transfected) were injected subcutaneously bilaterally in female ICR-SCID mice. Tumor measurements were performed at multiple time points between parental and *pre-let-7i* transfected group for four weeks with 5 animals in each group. Tumor weights were calculated. Animals were euthanized at the end of 4 weeks and RNA and protein was extracted from the tumor tissue of both groups to measure the *let-7i* miRNA expression and Ras GTPase activity levels.

2.11. Statistical Methods

Comparisons of treatment outcome were tested for statistical difference by the paired t-test. Statistical significance was assumed at a p value of < 0.05.

3. Results

3.1. Ras expression and its GTPase activity in PC cells was associated with deregulated expression of miRNAs

We examined the basal level of Ras expression and its GTPase activity in three human PC cell lines. Ras expression and activity was highly elevated in MIAPaCa-2 cells (mutant KRas), and moderately elevated in COLO-357 cells (mutant K-Ras) compared to wild type K-Ras BxPC-3 cells (Figure 1A). Similarly, several miRNAs were differentially expressed in all three cell lines tested of which *let-7* family (*let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7i*), and *miR-143* was significantly lower in MIAPaCa-2 cells compared to COLO-357 and BxPC-3 cells (Figure 1B, 1C). The loss of expression of *let-7* family especially *let-7i* and *miR-143* was correlated with the level of Ras expression and its GTPase activity. In contrast, the expression of *miR-21* was significantly higher in MIAPaCa-2 cells compared to COLO-357 and BxPC-3 cells (Figure 1C).

3.2. Re-expression of *let-7* family, and *miR-143* was achieved by CDF treatment

We determined the expression levels of *let-7* family (*let-7a*, *let-7e*, *let-7f*, *let-7i*), and *miR-143* after treatment of PC cells with 0.5 µM-1 µM of CDF for 48h in all three cell lines (COLO-357, MIAPaCa-2, and BxPC-3 cells). The expression level was determined by real time RT-PCR. We found a significant up-regulation in the expression of *let-7i* in all three cell lines treated with CDF in a dose dependent manner (Figure 2). Since the basal level of *miR-143* was undetectable in MIAPaCa-2 cells, we chose MIAPaCa-2 pancreatospheres tumor derived cells and treated with CDF to see the effect on treatment. We observed a significant up-regulation of *miR-143* in pancreatospheres treated with CDF (Figure 2). To further validate whether *miR-21* indeed could target the Ras expression and its GTPase activity, we investigated the effect of transfection of *miR-21* anti-sense oligonucleotide in all three cell lines and further treated with CDF as presented below.

3.3. Transfection of anti-sense *miR-21* in PC cells reduced Ras expression and its GTPase activity

COLO-357, BxPC-3, and MIAPaCa-2 cells were used to evaluate the effect of *miR-21* anti-sense oligonucleotide transfection on the expression of Ras and its GTPase activity. The cells were transfected with anti-sense *miR-21* for 24h followed by CDF treatment for 48h. Both positive and negative controls were included in the assay. The expression of Ras and its GTPase activity was reduced in anti-*miR-21* transfection in all three cell lines; however, the effect was much more pronounced in MIAPaCa-2 cells (Figure 3C). These findings suggest that K-Ras oncogene is a target of *miR-21*, and our synthetic agent CDF was capable of further down-regulation in the expression of Ras and its GTPase activity. In order to

recapitulate many important aspects of the human pancreatic tumor, a xenograft animal model was chosen for *in vivo* studies.

3.4. CDF inhibited pancreatospheres-induced tumor growth and inhibited the expression of Ras and its GTPase activity resulting from the re-expression of miRNAs in tumor remnants *in vivo*

In our previous reports, we have documented the reduction of tumor weight by CDF in MIA PaCa-2 cells induced tumors in a mouse model [39]. In this study, we examined the effects of CDF on tumors derived from pancreatospheres of MIA PaCa-2 cells implanted in mice. CDF treatment group showed significant inhibition in tumor growth compared to untreated controls (Figure 4A). The mice did not show any weight loss during the treatment period, suggesting that this treatment had no major adverse effects on animals. We further tested the effects of treatment on Ras expression and its GTPase activity in tumor remnants. We found that the expression of Ras and its GTPase activity was highly elevated in pancreatospheres derived tumors (Figure. 4B) and as expected, treatment of these tumors in mice with CDF for 12 days showed decreased expression of Ras and its GTPase activity (Figure 4B).

Moreover, the expression level of *let-7a*, *let-7e*, *let-7f*, and *let-7i*, miRNAs were significantly up-regulated in CDF treated groups (Figure 4C). The pancreatospheres induced tumor and also tumor-derived cells from pancreatospheres showed significant up-regulation of *miR-143* when treated with CDF (Figure 4D). In order to investigate the functional roles of *miR-143* and *let-7i* in PC, we examined their effects on cell growth, colony formation, the levels of Ras expression and its GTPase activity, and the expression of miRNAs using COLO-357 and MIA PaCa-2 cells transfected with *miR-143*, and *let-7i* precursor as presented below.

3.5. Ras expression and its GTPase activity are regulated by *miR-143* and affecting cell proliferation, and colony formation of COLO-357 cells

Ras oncogene is known to regulate cell proliferation and transformation [40]. In order to test whether *miR-143* expression could regulate Ras expression and its activity, we over-expressed *miR-143* with *pre-miR-143* in COLO-357 cells which expresses relatively low basal levels of *miR-143* compared to BxPC-3 cells. COLO-357 cells were seeded in both 96 and 6 well plates and were transfected with *pre-miR-143* precursor for 48h followed by treatment with CDF. Ectopic expression of *miR-143* with *miR-143* precursor led to increased expression of *miR-143* compared to control as tested by qRT-PCR (Figure 5A), which was expected. Interestingly, the re-expression of *miR-143* led to decreased expression of Ras and its GTPase activity (Figure 5B).

Moreover, over-expression of *miR-143* significantly inhibited cell viability (Figure 5C), and clonogenicity (Figure 5D), and this was further inhibited after CDF treatment compared to control. Since a gene can be a target of more than one miRNAs, we investigated other *K-Ras* targeting miRNAs that can serve as a mechanism for regulating the expression of Ras and its GTPase activity for which we chose *let-7i* because its expression was completely lost in MIA PaCa-2 cells.

3.6. Ras expression and its GTPase activity are regulated by *let-7i* and affecting cell proliferation and colony formation

Since MIA PaCa-2 cells showed relatively lowest levels of *let-7i* expression compared to COLO-357 and BxPC-3 cells, we chose MIA PaCa-2 cells for assessing the effect of re-expression of *let-7i* by transfection experiments. Cells were seeded in both 96 and 6 well plates and were transfected with *let-7i* precursor for 48h followed by CDF treatment.

Transfection of cells with *let-7i* precursor showed increased expression of *let-7i* as determined by qRT-PCR compared to control (Figure 6A), and these results were correlated with decreased expression of Ras and its GTPase activity (Figure. 6B). Transfection of cells with *let-7i* precursor led to a significant inhibition of cell viability (Figure 6C), and colony formation (Figure 6D), and these effects were much more pronounced after CDF treatment.

3.7. Stably transfected cells with *pre-let-7i* showed decreased tumor growth rate, and it was correlated with increased expression of *let-7i* and decreased expression of Ras and its GTPase activity

In order to further confirm our *in vitro* results suggesting that the loss of *let-7i* is associated with increased levels of Ras expression and its GTPase activity, we chose MIAPaCa-2 cells for assessing tumor growth after re-expression of *let-7i*. We generated stable clones of MIAPaCa-2 that over-expressing *let-7i* which showed reduced levels of Ras and its GTPase activity for *in vivo* study. We carried out *in vivo* experiment by injecting stably transfected *pre-let-7i* cells and also vector-transfected control in MIAPaCa-2 cells implanted subcutaneously in SCID mice which led to a differential tumor growth rate (Figure 7). Interestingly, re-expression of *let-7i* in the tumor specimens showed increased expression of *let-7i* (Figure 7B), and the reduced tumor growth was consistent with significant reductions in the expression of Ras and its GTPase activity compared to parental MIAPaCa-2 used as control.

4. Discussion

Since *K-Ras* mutation is frequently observed in human pancreatic tumors and cell lines, it has been the focus for the development of Ras inhibitors for therapy. Although effective clinical responses regarding K-Ras targeted therapy have not been validated yet, the goal of our study was to examine the role of specific miRNAs that may regulate the expression of Ras and its activity and further determine whether CDF, a newly developed analog of curcumin, could be useful for targeted inactivation of Ras expression and its activity in PC cells *in vitro* and in mouse xenograft tumor model *in vivo*. In this study, initially we investigated the basal level of expression of Ras and its GTPase activity in a panel of three PC cell lines and also in xenograft mouse model. The results showed highly elevated expression of Ras and its GTPase activity in K-Ras mutant human PC cell lines and also in tumors in animal compared to low levels in the K-Ras wild type PC cells (BxPC-3 cells). Previous studies have shown that pancreatic and lung cancer cell lines exhibit significantly lower level of expression of *let-7* family, which was associated with increased expression of K-Ras protein compared to normal fibroblasts, suggesting that *let-7* could negatively regulate K-Ras expression and its activity [17;18]. In colorectal cancer cell lines, *let-7* was not only involved in cell growth, but also modulated tumor sensitivity to chemotherapeutic drugs [41]. Our findings clearly show, for the first time, that re-expression of *let-7* family especially *let-7i* could regulate the expression of Ras and its GTPase activity resulting in the inhibition of tumor growth.

During our investigation we found that multiple miRNAs could potentially target Ras activity in PC cells and we found loss of expression of miRNA *let-7* family (*let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, and *let-7i*), *miR-21*, and *miR-143* in MIAPaCa-2 cells showing highest levels of Ras expression and its GTPase activity. We also found that CDF could be useful for the re-expression of the lost miRNAs, which in turn reduced the levels of Ras and its GTPase activity both in PC cells *in vitro* and in tumor *in vivo*. Numerous reports have shown that Ras, a well known oncogene has been known to be directly targeted by *let-7*, *miR-143*, and *miR-21*. Its over-activity, either by mutation or upstream signaling, often plays important roles in transformation and progression of cancer stem like cells in various cancers, including pancreatic cancer. The deregulation of miRNAs could be associated

either with chemo-resistance or chemo-sensitivity. CDF has the capacity of sensitizing cancer cells because of better cellular uptake and retention by cancer cells. It has also been proven to markedly diminish the number of stem cells *in vitro* and *in vivo*. Although the exact mechanism by which CDF leads to alterations in the expression of *let-7i*, *miR-143* and *miR-21* is not yet known, our data show for the first time that CDF could re-express the lost miRNAs and down-regulate the miRNA that is over-expressed in pancreatic cancer. CDF-mediated alteration in the expression of miRNAs leads to the down-regulation in the expression and the GTPase activity of Ras, which is a novel finding. Unlike loss of expression, we found increased expression of *miR-21* in MIAPaCa-2 cells relative to other cell lines and that CDF was able to down-regulate *miR-21* expression which was correlated with decreased levels of Ras expression and its GTPase activity. These results are consistent with our previous findings showing that over-expression of *miR-21* is associated with tumor aggressiveness [12] and that CDF could function as a novel anti-tumor agent by targeting *miR-21* expression [39] and thereby reducing the expression of Ras and its activity.

Previous reports have used transgenic mouse models for targeting different organs for the development of tumors, and have shown that the activation of *K-Ras* could function as an oncogenic signaling for multiple tumor system including PC [3;42]. Expression of a mutant *K-Ras* in cancer cells indicates a crucial role in the development of malignant phenotype, and has a defined role in oncogenesis, in regulating cell growth, differentiation and early stages of tumorigenesis in mouse model [43]. We investigated the tumor inducing activity of K-Ras mutant MIAPaCa-2 cells derived pancreatospheres in mice, which show cancer stem-like cells (CSC) characteristics. Consistent with the above findings from previous investigators, our *in vivo* results also showed significant increase in tumor formation induced by pancreatospheres derived from MIAPaCa-2 cells in mice. The increase in tumor growth was reduced by CDF, which could indeed be due to attenuation of Ras expression and its activity mediated by re-expression of *let-7* family of miRNAs (*let-7a*, *let-7e*, *let-7f*, *let-7i* family), and *miR-143*. The inhibition of tumor growth by CDF was consistent with our previous findings [39].

Among the several *let-7* family miRNAs tested both *in vitro* and *in vivo*, *let-7i* showed significantly lower expression in both MIAPaCa-2 cells and pancreatospheres derived tumors. Hence, we tested whether the re-expression of *let-7i* using *pre-let-7i* transfection of MIAPaCa-2 cells alone or in combination with CDF treatment could optimally suppress cell growth, colony formation, and whether this effect could be associated with inactivation of Ras expression and its activity. Similarly, in light of the potential role of *miR-143* in the regulation of Ras expression and its activity, we also investigated the consequence on the re-expression of *miR-143* by transfecting *pre-miR-143* in COLO-357 cells, and further determined its direct impact on Ras expression and its activity. Interestingly, we found suppressed cell growth, colony formation, and Ras activity in both MIAPaCa-2 and COLO-357 cells transfected with either *let-7i* or *miR-143* precursor, and that this effect was much more pronounced after CDF treatment.

To further support our *in vitro* findings, an *in vivo* tumor model was used to assess the tumor growth capacity of stably transfected MIAPaCa-2 cells with *pre-let-7i* miRNA. Inhibition of tumor growth in the xenograft mouse model suggests that *let-7i* re-expression would be a novel strategy for the treatment of PC, which is mediated by inactivation of Ras expression and its activity. Our data provided mechanistic evidence for the first time showing that re-expression of *let-7i* will regulate the expression of Ras and its GTPase activity, which could be accomplished either by re-expression by transfection or treatment of cells with CDF. In summary, elevated levels of Ras expression and its GTPase activity plays a crucial role in the development and progression of PC, which is regulated by multiple miRNAs specifically *let-7i*, *miR-143*, and *miR-21* and these processes could be attenuated by CDF. Therefore, we

suggest that our novel agent, CDF, could serve as an innovative approach for the treatment of patients diagnosed with PC.

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Figure 1.

Ras GTPase activity and basal levels of K-Ras expression in human pancreatic cancer (PC) cell lines COLO-357, BxPC-3 and MIAPaCa-2 cells. Ras expression and its GTPase activity were significantly higher in MIAPaCa-2 cells followed by COLO-357 and BxPC-3 (A). Relative expressions of *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7i* (B), *miR-21* and *miR-143* (C) as assessed by qRT-PCR in COLO-357, BxPC-3 and MIAPaCa-2 cells. P values represent comparison between BxPC-3 and MIAPaCa-2 cells as calculated by the paired *t* test.

Figure 2.

Comparative level of expression of *let-7a*, *let-7e*, *let-7f*, *let-7i*, and *miR-143* in untreated and treated cells with CDF (0.5– 1 μ M) for 48h in COLO-357 (A), BxPC-3 (B) and MIAPaCa-2 cells (C). There was a significant up-regulation of *let-7i*, and *miR-143* expression with CDF treatment. P values represent comparison between untreated and CDF 1 μ M treated cells as calculated by the paired *t* test.

Figure 3.

Ras GTPase activity and the levels of K-Ras expression in human PC cell lines after *miR-21* anti-sense transfection; COLO-357 (A), BxPC-3 (B) and MIAPaCa-2 cells (C) by western blot analysis. A significant inhibition of Ras GTPase activity and its expression was observed in MIAPaCa-2 and COLO-357 cells transfected with anti-sense *miR-21*. No such effect was observed in control miRNA transfected cells.

Figure 4.

CDF exhibited anti-tumor activity in MIAPaCa-2 pancreatospheres induced tumors in a xenograft mouse model (A), which was consistent with inhibition of Ras expression and its GTPase activity (B), and re-expression of *let-7* family (C) and *miR-143* expression (D) in tumor remnants. The arrow indicates starting day of the treatment. P values were calculated by the paired *t* test.

Figure 5.

Re-expression of *miR-143* by *pre-miR-143* precursor or CDF treatment in COLO-357 cells showed increased expression of *miR-143* as assessed by qRT-PCR (A), and corresponding decrease expression of Ras and its GTPase activity as assessed by western blot (B) compared to control cells. The above treatment also led to reduced cell viability as assessed by MTT assay (C) and clonogenicity as assessed by colony formation assay (D).

Figure 6. Re-expression of *let-7i* by *pre-let-7i* precursor transfection or treatment of MIPaCa-2 cells by CDF led to increased expression of *let-7i* as assessed by qRT-PCR (A), and showed decreased levels of Ras expression and its GTPase activity (B) compared to control cells. The above treatment also led to reduced cell viability as assessed by MTT assay (C) and clonogenicity as determined by colony formation assay (D).

Figure 7. Over-expression of *let-7i* using stably transfected *pre-let-7i* in MIAPaCa-2 cells *in vivo* showed decreased tumor growth rate in a subcutaneous tumor xenograft model (A), consistent with increased levels of *let-7i* expression (B), which was associated with decreased levels of Ras expression and its activity (C) compared to control vector MIAPaCa-2 cells. n=5 mice for each group.