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Up-regulation of miR-146a contributes to the inhibition of invasion of pancreatic cancer cells

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Abstract

Pancreatic cancer (PC) is an aggressive malignancy with high mortality and is believed to be in part due to its highly invasive and metastatic behavior, which is associated with over-expression of EGFR and activation of NF- κ B. Emerging evidence also suggest critical roles of microRNAs (miRNAs) in the regulation of various pathobiological processes including metastasis in PC and in other human malignancies. In the present study, we found lower expression of miR-146a in PC cells compared to normal human pancreatic duct epithelial (HPDE) cells. Interestingly, re-expression of miR-146a inhibited the invasive capacity of Colo357 and Panc-1 PC cells with concomitant down-regulation of EGFR and IRAK-1. Mechanistic studies including miR-146a re-expression, anti-miR-146 transfection, and EGFR knock-down experiment showed that there was a crosstalk between EGFR, MTA-2, IRAK-1, I κ B α and NF- κ B. Most importantly, we found that the treatment of PC cells with “natural agents” [3,3'-diinodolylmethane (DIM) or isoflavone] led to an increase in the expression of miR-146a and consequently down-regulated the expression of EGFR, MTA-2, IRAK-1 and NF- κ B, resulting in the inhibition of invasion of Colo357 and Panc-1 cells. These results provide experimental evidence in support of the role of DIM and isoflavone as potential non-toxic agents as regulators of miRNA, which could be useful for the inhibition of cancer cell invasion and metastasis, and further suggesting that these agents could be important for designing novel targeted strategy for the treatment of PC.

Keywords

microRNA; invasion; pancreatic cancer

Introduction

Pancreatic cancer (PC) is an aggressive malignancy with one of the worst outcomes among all cancers. For all stages combined, the 5-year relative survival rate is only 5% (1). The high mortality of PC could be partly due to the ability of PC cells to acquire invasive characteristics during early stages of carcinogenesis. Thus, most patients diagnosed with PC

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are un-resectable and usually die from metastatic disease even after treatment with existing therapies. Therefore, there is a dire need for understanding the molecular mechanism(s) involved in the aggressiveness of PC and its high propensity for metastasis, and further novel approaches are needed for developing novel therapeutic strategies for the inhibition of tumor aggressiveness and metastasis so that the patients diagnosed with PC could be treated with better outcome.

It has been reported that over-expression of EGFR and activation of NF- κ B are common in PC (2, 3). Elevated expression of EGFR and its ligand correlates with large tumor size, advanced clinical staging, and decreased survival period in PC (4). The constitutive activation of NF- κ B, which regulates important genes and thereby affects many cellular processes, are also known to contribute to the aggressive behavior of PC (3, 5). However, the molecular mechanism(s) involved in the over-expression of EGFR and activation of NF- κ B in PC have not been fully elucidated.

Recently, emerging evidence indicates the critical role of microRNAs (miRNAs) in the regulation of various biological and pathological processes including metastasis (6). These small, non-coding molecules elicit their regulatory effects by imperfectly binding to the 3' untranslated region (3'UTR) of target mRNA, causing either degradation of mRNA or inhibition of their translation to functional proteins (7). The expression of miRNAs has been recognized as integral components of many normal biological processes involving cell proliferation, differentiation, apoptosis, and stress resistance (8). More importantly, it has been recently suggested that aberrant up-regulation or down-regulation of specific miRNAs and their targets in various types of cancer is associated with the development and progression of cancer (9-11). Moreover, miR-146a has been found to be lost in metastatic prostate cancer (12) and its expression reduces the metastatic potential of breast cancer cells through repression of IRAK1 (interleukin 1 receptor-associated kinase 1) and subsequent inhibition of NF- κ B activity (13, 14). In addition, miR-146a could target EGFR based on predicted base pairing by using miRBase analysis (15). Therefore, it is important to unravel the relationship between miR-146a and the signaling of EGFR and NF- κ B, and find novel agents that could regulate miR-146, EGFR and NF- κ B, and which could be useful for the inhibition of PC progression and metastasis.

We have previously found that the dietary compounds including isoflavone genistein and 3,3'-diindolylmethane (DIM) could enhance the anti-tumor activity of chemotherapeutic agents through the inhibition of NF- κ B (16, 17). However, the precise molecular mechanisms by which these dietary compounds regulate NF- κ B have not been fully elucidated. In this study, we assessed the expression pattern of miRNAs in the normal pancreatic duct epithelial cells, and pancreatic cancer (PC) cells. We also investigated whether the treatment of PC cells with either B-DIM (BioResponse formulated DIM with greater bioavailability) or G2535 (a mixture of genistein and other isoflavones) could alter the expression of miRNAs and their targets that are involved in the processes of invasion and metastasis. We found that B-DIM and G2535 could inhibit invasion of PC cells through the induction of miR-146a and with corresponding down-regulation of its targets including IRAK-1/NF- κ B, EGFR, and MTA-2 (metastasis-associated protein 2), suggesting that re-

expression of specific miRNA by non-toxic “natural agents” could be useful strategy for the treatment of PC in the future.

Materials and Methods

Cell lines, reagents, and antibodies

Colo357 and Panc-1 pancreatic cancer cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a 5% CO₂ atmosphere at 37°C. Human pancreatic duct epithelial (HPDE) cells were obtained from MD Anderson Cancer Center (a generous gift of Dr. Paul J. Chiao) and cultured in keratinocyte serum-free medium supplied with 5 ng/mL of epidermal growth factor and 50 µg/mL of bovine pituitary extract (Invitrogen) (18). The cell lines have been tested and authenticated in core facility Applied Genomics Technology Center at Wayne State University. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). B-DIM [BioResponse, Boulder, CO; known as BR-DIM and referred as B-DIM with higher bioavailability *in vivo* (19)] was generously provided by Dr. Michael Zeligs and was dissolved in DMSO to make a 50 mM stock solution. Isoflavone mixture G2535 (70.54% genistein, 26.34% diadzin and 0.31% glycitein manufactured by Organic Technologies and obtained from NIH) was dissolved in DMSO to make a stock solution containing 50 mM equivalent to genistein. The concentration of isoflavone we described in this article all refer to the concentration of genistein in the isoflavone mixture. The EGFR tyrosine kinase inhibitor, erlotinib, was a generous gift from OSI Pharmaceuticals (Melville, NY). Anti-EGFR (Cell Signaling, Danvers, MA), anti-phospho-EGFR Tyr992 (Cell Signaling), anti-IRAK-1 (Santa Cruz, Santa Cruz, CA), anti-MTA-2 (Santa Cruz), anti-NF-κB p65 (Millipore, Billerica, MA), anti-IκBα (Cell Signaling), anti-phospho-IκBα (Cell Signaling), and anti-β-actin (Sigma, St. Louis, MO) primary antibodies were used for Western Blot analysis.

RNA isolation

Total RNA was extracted by using the *mirVana*TM miRNA Isolation Kit (Ambion Inc, Austin, TX) following the protocol provided by the manufacturer. Briefly, Colo357 and Panc-1 cells were treated with 25 µM B-DIM or 25 µM G2535 for 48 hours. Control cells received 0.05% DMSO. Then, the PC cells from each condition, and the HPDE cells were subject to RNA extraction following the procedure we reported before (20).

miRNA array and data analysis

Five micrograms of each total RNA sample from HPDE, Colo357, and Panc-1 cells were sent to a service provider for completion of the miRNA arrays (LCSciences, Houston, TX). In LCSciences, the total RNA samples were enriched for microRNAs and the miRNA arrays were performed on µParaFloTM microfluidic chips (version 10.0), each of which has a miRNA probe region with multiple repeat regions that detect 711 miRNAs. Multiple control probes are also included on the arrays for assessing various chip and assay qualities such as uniformity and specificity. Chips were scanned and the signal intensity data was obtained. Then, the data was analyzed by subtracting the background and normalizing the signals to

balance the intensities of transcripts in normal, cancer, control, and treatment samples so that differential expression ratios can be calculated. The ratio of the two sets of detected signals such as normal and cancer, control and treatment was calculated. The *t* test was also performed to calculate *p* value. The predicting target genes for various miRNAs were also analyzed using computerized analysis (microrna.org; mirbase.org).

miRNA Real-time RT-PCR

To verify the alteration in the expression of miR-146a that was found in miRNA array analysis, we conducted real-time miRNA RT-PCR analysis using miR-146a TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA) following the procedure we reported before (20).

Real-time RT-PCR using mRNA

Real-time RT-PCR analysis was also conducted to measure the expression of EGFR in PC cells with and without 25 μ M B-DIM or 25 μ M G2535 treatment following the procedure we reported before (20). The *t* test was also performed to calculate *p* value for EGFR mRNA and miR-146a data.

Western Blot analysis

We also conducted Western Blot analysis to verify the alterations in the protein expression of genes, which are targets of miR-146a. Colo357 and Panc-1 PC cells were treated with or without 25 μ M B-DIM or 25 μ M G2535 for 48 hours. After treatment, the cells were lysed in 62.5 mM Tris-HCl and 2% SDS, and protein concentration was measured using BCA protein assay (PIERCE, Rockford, IL). The proteins were subjected to 10% or 14% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with specific primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase (Bio-rad, Hercules, CA). The signal was detected using the chemiluminescent detection system (PIERCE). The signal of each target protein expression was scan and quantified by using AlphaEaseFC (Alpha Innotech, San Leandro, CA). The Ratios of detected target proteins against β -actin were calculated with standardizing the ratio of each control to the unit value.

Re-expression and inhibition of miR-146a in PC cells

Colo357 and Panc-1 cells were seeded in 6 well plates and transfected with miR-146a, miRNA negative control, anti-miR-146, or anti-miR negative control (Ambion) at a final concentration of 20 nM using DharmaFact Transfection Reagent (Dharmacon, Lafayette, CO). After 3 days of transfection, the cells were split and transfected repeatedly with the miRNA or anti-miRNA every 3-4 days for indicated period of times. Total RNA from each samples were then extracted using the Trizol reagent (Invitrogen). One microgram of RNA was subject to RT-PCR using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) as described earlier. Total proteins from each sample were also extracted and subject to Western Blot analysis as described earlier. Cytoplasmic and nuclear proteins were also extracted and subjected to Western Blot analysis and electrophoretic mobility shift assay.

Inhibition of EGFR expression by siRNA in PC cells

Colo357 and Panc-1 cells were seeded in a 6 well plate (1.2×10^5 cells per well) and incubated at 37°C for 24 hours. The cells were then transfected with EGFR siRNA (Santa Cruz) or control RNA duplex by DharmaFact Transfection Reagent (Dharmacon) for 72 hours. Then, the total, cytoplasmic, and nuclear proteins were extracted. The proteins were subjected to Western Blot analysis.

Electrophoretic mobility shift assay (EMSA)

EMSA was conducted to measure the DNA binding activity of NF- κ B in miR-146a transfected or B-DIM and G2535 treated Colo357 and Panc-1 cells. Briefly, after transfection or treatment, nuclear proteins from each sample were extracted and subject to EMSA following the standard procedure we reported before (17).

Invasion assay

The invasive capacity of Colo357 and Panc-1 cells with miR-146a or anti-miR-146 transfection or after different treatments were tested by using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to the manufacturer's protocol with minor modification. Briefly, Colo357 and Panc-1 cells were transfected with miR-146a or anti-miR-146 for 5 days as described above and/or treated with 25 μ M B-DIM or 25 μ M G2535 for 2 days. Then, the transfected or treated Colo357 and Panc-1 cells (5×10^4) with serum free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 24 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with 4 μ g/ml Calcein AM in Hanks buffered saline at 37°C for one hour. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

Growth inhibition assay

Panc-1 and Colo357 cells were transfected with miR-146a, anti-miR-146, or miRNA negative control for 5 days as described. Then, the transfected cells were seeded in 96 well plates and treated with 1 μ M erlotinib, 12.5 μ M B-DIM or 12.5 μ M G2535 for 2 days. After treatment, the cells were subjected to cell proliferation assay using Cell Proliferation Reagent WST-1 assay (Roche, Indianapolis, IN) following the manufacturer's protocol. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 450 nm.

Results

PC cells showed down-regulation of miR-146a, and the treatment of PC cells with B-DIM and G2535 showed increased expression of miR-146a

To investigate the difference in miRNA expression between normal human pancreatic duct epithelial cells (HPDE cells) and PC cells, we conducted miRNA array. The results from

miRNA array were validated by miRNA RT-PCR analysis. By miRNA array and RT-PCR, we found that the expression level of miR-146a in HPDE cells was higher than that in Colo357 and Panc-1 PC cells (Table 1, Fig. 1A), suggesting that PC cells had lower expression of miR-146a compared to normal cells. Importantly, we also found that the miR-146a expression was up-regulated upon 25 μ M B-DIM or 25 μ M G2535 treatments of Colo357 and Panc-1 cells (Table 1, Fig. 1B and 1C). These results suggest that miR-146a could be an inhibitory molecule for cancer development and progression and that B-DIM and G2535 could inhibit the progression of PC through induction of miR-146a expression. Because miRNAs regulate expression of target genes, we further tested the expression of miR-146a target genes after re-expression or inhibition of miR-146a by transfection studies in PC cells.

Re-expression of miR-146a led to the inhibition of EGFR signaling in PC cells

In order to investigate the role of miR-146a in the regulation of cellular signaling, we transfected Colo357 and Panc-1 cells with miR-146a pre-microRNAs and cultured for a period of up to 9 days. We found that the re-expression of miR-146a in the Colo357 and Panc-1 cells resulted in the down-regulation of EGFR both at the mRNA and protein levels (Fig. 2A and 2B). Importantly, we found that phosphorylated EGFR at Tyr992, which is responsible for the binding of the Src homology 2-containing phosphotyrosine phosphatase (SHP2) and positively affects receptor tyrosine kinase signaling (21), was also down-regulated after miR-146a transfection (Fig. 2B). To confirm the effects of miR-146a on EGFR, we transferred anti-miR-146 into PC cells and found that inhibition of miR-146 increased the levels of EGFR and p-EGFR (Fig. 2B). These results suggest the inhibitory effects of miR-146a on EGFR signaling. Because miR-146a could also regulate the expression of IRAK-1 which could activate NF- κ B signaling, we investigated the effects of miR-146a on IRAK-1/NF- κ B signaling pathways.

Re-expression of miR-146a led to the inhibition of IRAK-1/NF- κ B signaling and MTA-2 expression in PC cells

After we transfected miR-146a into Colo357 and Panc-1 cells, we found that the re-expression of miR-146a also caused down-regulation of IRAK-1 expression in the PC cells (Fig. 2C). Moreover, the level of NF- κ B was also decreased after miR-146a transfection (Fig. 2C), suggesting that miR-146a could regulate IRAK-1/NF- κ B signaling. Interestingly, we found that miR-146a inhibited the expression of MTA-2 (Fig. 2D), which is one of the computerized predicted miR-146a target genes and associated with cancer cell invasion and metastasis, suggesting that our findings are highly novel. These results were also confirmed by the observations that the expression of IRAK-1, NF- κ B, and MTA-2 was up-regulated by anti-miR-146 (Fig. 2C and 2D). Because we observed the up-regulation of miR-146a after B-DIM and G2535 treatment, we further investigated whether the treatment of PC cells with B-DIM and G2535 could achieve similar effects as seen with re-expression of miR-146a by transfection experiments.

B-DIM and G2535 treatment suppressed EGFR, IRAK-1/NF- κ B, and MTA-2 in PC cells

By RT-PCR and Western Blot analysis, we found that 25 μ M B-DIM or 25 μ M G2535 did inhibit the expression of EGFR at mRNA and protein levels in Colo357 and Panc-1 cells

(Fig. 3A and 3B). Moreover, B-DIM and G2535 decreased the level of phosphorylated EGFR at Tyr992 site in Colo357 and Panc-1 cells (Fig. 3B). Similar to the effects of miR-146a re-expression, B-DIM and G2535 also decreased the expression of IRAK-1, NF- κ B, and MTA-2 in Colo357 and Panc-1 cells (Fig. 3C and 3D). These results suggest that the down-regulation of EGFR, IRAK-1, NF- κ B, and MTA-2 by B-DIM and G2535 could be mediated via the up-regulation of miR-146a as seen earlier (Fig. 1B and 1C). Because EGFR and IRAK-1 could alter the activity of NF- κ B, we further investigated the relationship between EGFR, IRAK-1, NF- κ B, and MTA-2 as presented below.

Cross-regulation between miR-146a, EGFR, IRAK-1, I κ B α , NF- κ B, and MTA-2

As shown in Fig. 4A, the re-expression of miR-146a inhibited the expression of cytoplasmic EGFR and decreased the level of cytoplasmic phosphorylated I κ B α and nuclear NF- κ B whereas the expression of cytoplasmic I κ B α was increased after miR-146a transfection. In order to confirm whether these effects of miR-146a were mediated through the down-regulation of EGFR, we conducted EGFR siRNA silencing experiments. We found that down-regulation of EGFR by EGFR siRNA also caused increased levels of cytoplasmic I κ B α and decreased the levels of cytoplasmic phosphorylated I κ B α and nuclear NF- κ B (Fig. 4A). Interestingly, we also found inhibition of MTA-2 expression by EGFR siRNA (Fig. 4B). These results suggest that there are cross-regulations between EGFR, I κ B α , NF- κ B, and MTA-2 signaling. More importantly, we found that B-DIM and G2535 also increased the level of cytoplasmic I κ B α and decreased the level of cytoplasmic EGFR, phosphorylated I κ B α and nuclear NF- κ B (Fig. 4C). By EMSA, we observed that the NF- κ B DNA binding activity was inhibited by the re-expression of miR-146a and similar findings were also obtained by the treatment of PC cells with B-DIM or G2535 (Fig. 4D), which is consistent with our data obtained from Western Blot analysis. These results suggest that the inhibition of NF- κ B by B-DIM and G2535 could be partly mediated via the up-regulation of miR-146a and subsequent down-regulation of EGFR. Because EGFR, NF- κ B, and MTA-2 all are associated with invasion and metastasis in cancer cells, we tested the invasive capacity of Colo357 and Panc-1 cells after re-expression or inhibition of miR-146a by transfection studies as well as after treatment of PC cells with B-DIM and G2535.

miR-146a transfection or B-DIM and G2535 treatment inhibited the ability of PC cells invasion

By invasion assay, we found that the invasive capacity of Colo357 and Panc-1 cells was significantly inhibited by the re-expression of miR-146a either by miR-146a transfection or by the treatment of PC cells with B-DIM or G2535 (Fig. 5A and 5B). However, anti-miR-146 increased the invasion of PC cells and restored invasive capacity of PC cells treated with B-DIM and G2535 (Fig. 5A and 5B). We further treated PC cells with erlotinib, one of the EGFR inhibitors, after miR-146a or anti-miR-146 transfection and found that the level of miR-146 influenced the inhibitory effect of erlotinib, B-DIM, and G2535 on cell growth (Fig. 5C), suggesting the relationship between miR-146a and EGFR. These results clearly suggest that B-DIM and G2535 could inhibit invasion and metastasis of PC cells, which is in part due to the up-regulation of miR-146a, and subsequent down-regulation of EGFR, NF- κ B, and MTA-2 signaling.

Discussion

Although recent years have witnessed the deregulated expression of miR-146, which has been implicated both in the development of various cancers and in the negative regulation of inflammation induced via the innate immune response (22, 23), controversies exist regarding the effects of miR-146a on cancer development and progression. The elevated miR-146a levels has been found in papillary thyroid carcinoma (23) and cervical cancer (24) whereas reduced miR-146a expression was associated with hormone-refractory prostate cancer (12). In our study, we found lower levels of miR-146a in pancreatic cancer (PC) cells compared to normal human pancreatic duct epithelial cells (HPDE cells) although we recognize that HPDE cell may not be a perfect control for comparing miRNA expression with pancreatic glandular cancer cells. Most importantly, we found that the re-expression of miR-146a could reduce the invasive capacity of both Colo357 and Panc-1 PC cells, suggesting that the regulated expression of miR-146a and its targeted genes could be exploited for designing novel strategies for the treatment of PC in the future. Our finding is consistent with recent reports showing that up-regulation of miR-146a inhibited cancer cell metastasis in breast (13, 14, 25) and prostate (12) cancers. These results collectively suggest that miR-146a could exert its critical effects in the later stages of cancer rather than the initial stage, and thus it is tempting to speculate that novel strategies by which one could induce higher levels of miR-146a in cancer cells could be useful for suppressing tumor progression by inhibiting invasion, and metastasis.

The molecular mechanism(s) involved in the miR-146a mediated inhibition of metastasis are still unclear. From our results, we believe that miR-146a inhibits invasion and metastasis partly through the regulation of EGFR and NF- κ B signaling (Fig. 5D). From Sanger database, it has been found that EGFR and MTA-2 are predicted targets of miR-146a. Hurst et al. recently reported that miR-146a and miR-146b down-regulated the expression of EGFR, and inhibited breast cancer metastasis (14); however, how EGFR regulates cancer metastasis is unknown. In our study, we found that the re-expression of miR-146a by transfection suppressed the expression of EGFR and MTA-2 in PC cells. Furthermore, down-regulation of EGFR by siRNA could also decrease MTA-2 expression, suggesting their cross-talks. MTA (metastasis-associated gene or protein) is a newly discovered family of cancer gene that is associated with tumor progression and metastasis (26). MTA-2 could deacetylate and inactivate the tumor suppressor p53 protein (27). High expression of MTA-2 was associated with more aggressive behaviors of ovarian epithelial cancer (28). Therefore, miR-146a could directly inhibit the expression of MTA-2 or indirectly down-regulate MTA-2 through the inhibition of EGFR, leading to the suppression of cancer cell invasion and metastasis as partly supported by our results (Fig. 5D). Moreover, it has been reported that EGFR could regulate NF- κ B signaling (29), thus, miR-146a could indirectly down-regulate NF- κ B signaling by suppressing EGFR, resulting in the inhibition of cancer cell invasion and metastasis. However, further in-depth mechanistic studies are warranted based on our exciting results.

NF- κ B is activated in a wide-array of human cancers, and it is thought to be involved in promoting invasion, angiogenesis and metastasis in various cancers (30, 31). It has been reported that NF- κ B could be activated by IRAK-1 (32, 33) which is a target of miR-146a

(13). Therefore, miR-146a mediated inhibition of invasion could be via the down-regulation of IRAK-1 and subsequent inactivation of NF- κ B. Indeed, we found that the expression of IRAK-1 was significantly inhibited by miR-146a transfection. We also observed increased levels of cytoplasmic I κ B α and decreased levels of cytoplasmic phosphorylated I κ B α and nuclear NF- κ B after re-expression of miR-146a or EGFR siRNA transfection, suggesting that miR-146a could regulate I κ B/NF- κ B signaling through the inhibition of both IRAK-1 and EGFR (Fig. 5D). Our results are also consistent with the findings reported by other investigators showing that IRAK-1 could inhibit I κ B α leading to the activation of NF- κ B (32, 33), and that EGFR or Her-2/neu are known activators of NF- κ B through the phosphorylation of I κ B α mediated via by Akt (34) and the degradation of I κ B α by calpain (35). Therefore, the inactivation of NF- κ B could be another molecular mechanism by which miR-146a inhibits cancer cell invasion and metastasis.

The inhibition of cancer cell invasion and metastasis should be an important strategy for the successful treatment of PC, and thus any novel strategies by which the invasive and metastatic ability of PC cells could be inhibited should be useful for improving the devastating outcome of patients diagnosed with PC. We and others previously reported that isoflavone and DIM (non-toxic “natural agents”) could inhibit invasion and metastasis of prostate, breast, ovarian, and bladder cancer cells (36-41); however, the molecular mechanism(s) involved has not been fully elucidated. The data presented in our current manuscript showed, for the first time, that the non-toxic “natural agents” such as B-DIM and isoflavone could up-regulate the expression of miR-146a, which in turn down-regulated the expression of EGFR, MTA-2, IRAK-1, and NF- κ B, resulting in the inhibition of invasion of PC cells (Fig. 5D). These results clearly suggest that instead of re-expression of miR-146a by transfection studies, one could simply treat PC cells with non-toxic “natural agents” that will result in the induction of miR-146a expression, and thus we believe that such a strategy could be useful for the inactivation of multiple genes downstream of miR-146a toward PC therapy, especially because EGFR targeted therapy (gefitinib, erlotinib or antibodies to EGFR) has essentially been failed in clinical trials.

In conclusion, our results clearly showed that B-DIM and isoflavone could up-regulate miR-146a and inhibit cancer cell invasion, which was in part due to down-regulation of EGFR and NF- κ B, the molecular targets that are commonly activated in PC. These results suggest that further *in vivo* studies and clinical trials are needed to establish whether these agents could also be useful in combination with conventional chemotherapeutics or conventional targeted agents for the treatment of PC for which there is no effective and curative therapy.

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Figure 1.

Real-time RT-PCR showed that the expression of miR-146a was higher in HPDE cells compared to PC cells (A) and that treatment of Colo357 (B) and Panc-1 (C) cells with 25 μ M B-DIM or 25 μ M isoflavone induced the expression of miR-146a compared to vehicle control. (Columns, mean from three independent experiments; bars, SEM; *, $P < 0.05$.)

Figure 2.

Re-expression of miR-146a by transfection resulted in the down-regulation of EGFR expression and phosphorylation in Colo357 and Panc-1 cells as assessed by real-time RT-PCR (A. Columns, mean from three independent experiments; bars, SEM; *, $P < 0.05$.) and Western Blot analysis (B). The expression of IRAK-1, NF-κB (C), and MTA-2 (D) was also down-regulated in PC cells after miR-146a transfection. However, the expressions of EGFR, p-EGFR (B), IRAK-1, NF-κB (C), and MTA-2 (D) were up-regulated by anti-miR-146 transfection. (c: control miRNA transfection; 146: miR-146a transfection; Anti: Anti-miR-146 transfection; 3D, 5D, 6D, 9D: transfection for 3, 5, 6, or 9 days; The Ratios of detected target proteins against β-actin were shown under each Western Blot image).

Figure 3.

25 μ M B-DIM or 25 μ M isoflavone (G2535) treatment for 2 days decreased EGFR expression and phosphorylation in PC cells as assessed by real-time RT-PCR (**A**. Columns, mean from three independent experiments; bars, SEM; *, $P < 0.05$.) and Western Blot analysis (**B**). The expression of IRAK-1, NF- κ B (**C**), and MTA-2 (**D**) was also down-regulated in PC cells after B-DIM or isoflavone treatment. (c: control miRNA transfection; BD: B-DIM treatment; Iso: isoflavone treatment).

Figure 4.

miR-146a or EGFR siRNA transfection increased the level of I κ B α (A) and decreased the levels of EGFR, p-I κ B α , NF- κ B, and MTA-2 (B) in PC cells. 25 μ M B-DIM or 25 μ M isoflavone treatment increased the level of I κ B α and decreased the levels of EGFR, p-I κ B α , and NF- κ B in PC cells. EMSA (D) showed that NF- κ B DNA binding activity in PC cells was inhibited by the re-expression of miR-146a or the treatment of PC cells with 25 μ M B-DIM or 25 μ M isoflavone. (c: control; 146: miR-146a transfection; siE: EGFR siRNA transfection; BD: B-DIM treatment; Iso: isoflavone treatment; 2D: treatment for 2 days; 5D, 9D: transfection for 5 or 9 days; The Ratios of detected target proteins against β -actin were shown under each Western Blot image).

Figure 5.

Invasion assay showed that the re-expression of miR-146a by transfection studies or by the treatment of PC cells with 25 μM B-DIM or 25 μM isoflavone significantly inhibited the invasion of PC cells through matrigel matrix membrane. However, inhibition of miR-146 increased the invasion of PC cells and restored invasive capacity of PC cells treated with B-DIM and G2535. (A. Panc-1 cells; B. Colo357 cells; a. negative control miRNA transfection; b. transfected with anti-miR-146; c. transfected with miR-146a; d. treated with 25 μM B-DIM; e. transfected with anti-miR-146 followed by B-DIM treatment; f. treated with 25 μM isoflavone; g. transfected with anti-miR-146 followed by G2535 treatment; x200). The graphs showed the value of fluorescence from the invaded PC cells. The values indicated the comparative number of invaded PC cells. (Columns, mean from two independent experiments; bars, SEM; $P < 0.05$ each group compared to control.) C: The level of miR-146 influenced the inhibitory effect of erlotinib, B-DIM, and G2535 on cell proliferation. (Columns, mean from three independent experiments; bars, SEM) D: Diagram showing the hypothetical cross-regulation between miR-146a, EGFR, MTA-2, IRAK-1, IκB, and NF-κB in the inhibition of cancer cell invasion and metastasis by B-DIM and isoflavone.

Table 1
Differential expression level (average signal) of miR-146a in pancreatic duct epithelial cells and pancreatic cancer cells treated with B-DIM or G2535 tested by miRNA array

Cell line	Control	B-DIM treatment	G2535 treatment
Panc-1	1829.54±102.80*	2408.24±97.82	2732.86±105.91
Colo357	2564.36±133.84	4541.72±239.33	8758.75±429.05
HPDE	3066.60±145.00		

* means±SD