Re-expression of miR-200 by novel approaches regulates the expression of PTEN and MT1-MMP in pancreatic cancer

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Abbreviations: EMT, epithelial to mesenchymal transition; miRNAs, micro-RNAs; MT1-MMP, membrane type-1 matrix metalloproteinase; PC, pancreatic cancer; PTEN, phosphatase and tensin homolog.

Introduction

It has been estimated that 43,920 people will be newly diagnosed with pancreatic cancer (PC) in 2012, and it is the fourth leading cause of cancer-related deaths in the USA (1). Although research effort has advanced toward targeted therapies, late diagnosis and/or diagnosis with metastatic therapy-resistant disease has made PC the leading cause of high mortality. Hence, better understanding of the underlying mechanisms involved in therapeutic resistance and findings ways to overcome drug resistance is critical for improving the dismal survival statistics and therapeutic efficacies in PC patients.

Amongst the multiple matrix metalloproteinases activated and expressed in a wide range of tumors, matrix metalloproteinase 14 (MMP-14), also known as membrane type-1 matrix metalloproteinase (MT1-MMP), is believed to play a crucial role in facilitating the tumor environment, inhibiting the processes of EMT and metastasis (20). In lung cancer cell lines derived from mice, IGF-1 receptor controls tumor cell invasion through MT1-MMP activation mediated by antagonism of PI3K signaling (15). Moreover, in H-59 cells of lung carcinoma, IGF-1 receptor controls tumor cell invasion through MT1-MMP activation mediated by activation of PI3K/Akt/mTOR signaling (16). Thus, the loss of PTEN expression and increased MT1-MMP expression could have a significant impact on the regulation of cell growth, invasion, migration and aggressiveness of PC cells. Therefore, it is vital to find novel agents that could mechanistically regulate MT1-MMP and PTEN expression in PC, which would likely advance our knowledge in designing novel and improved therapies for the treatment of PC.

Emerging evidence suggest that micro-RNAs (miRNAs), highly conserved and small non-coding regulatory RNAs, play a major role in the regulation of gene expression through post-transcriptional repression, and appear to be important in PC. The expression of miR-200 family has been established by us and others both in vitro and in vivo as one of the most intensively studied epithelial to mesenchymal transition (EMT)-related miRNAs that target multiple genes (12,17–20). Recent evidence has also shown down-regulation of miR-200 family by ZEB1 due to suppression of stemness-inhibiting miRNAs in the 38 different carcinoma cell lines of the NCI-60 cell line panel (20). In lung cancer cell lines derived from mice, miR-200 altered the tumor environment, inhibiting the processes of EMT and metastasis (21). These findings suggest that the expression of miR-200 in PC is closely correlated with stemness, metastasis and EMT, which is due to targeting multiple genes. Hence, re-expression of miR-200 family either by transfection with its precursors or treatment by novel agents (natural agents) could have the potential for the inhibition of EMT and stemness markers, suggesting that such a strategy could become a novel therapeutic approach for the treatment of PC.

Although the loss of PTEN has been shown to regulate miR-21 expression, the extent to which it is affected through modulation of miR-200 and its role in the deregulation of MT1-MMP and PTEN has not been previously examined. Therefore, the aim of the current study was to investigate the interplay between the expression of MT1-MMP and PTEN deregulation mediated through the expression of miR-200 in PC cell lines. We further mechanistically investigated the putative role of miR-200c and its effects on the expression of MT1-MMP and PTEN by transfecting pre-miR-200c (precursor) or ASO-miR-200c (inhibitor) in human PC MiaPaCa-2 cell line. We found that the re-expression of pre-miR-200c led to decreased cell migration and clonogenicity, which was associated for angiogenesis and metastasis (4–6). According to another report, binding of endothelial cells to extracellular matrix shows the existence of two phases of MMP regulation, one through rapid inhibition of pro-MMP-2 activation, through inhibition of MT1-MMP, and the other by slower response of cell spreading and changes in the cytoskeleton to suppress the levels of MT1-MMP mRNA and protein (7). The over-expression of MT1-MMP has also been associated with metastatic behavior of virtually all types of cancers (8,9), including PC in K-Ras transgenic mouse model (10) and in biopsies from triple-negative breast cancer (11). However, little is known about the expression of MT1-MMP and the underlying mechanisms involved in human PC, suggesting that understanding the regulation of MT1-MMP and finding ways to inhibit its expression would be important for designing novel therapies for PC.

Besides the deregulation of MT1-MMP, the loss of expression of phosphatase and tensin homolog (PTEN), which is a ubiquitous tumor suppressor gene, has been shown to correlate with tumor aggressiveness and was also associated with up-regulation of miRNA expression such as miR-21 (12,13). Additionally, over-expression of MT1-MMP was found to be associated with loss of PTEN expression in prostate cancer cells derived from mice through the activation of the PI3K/Akt pathway (14); whereas in renal cell carcinoma, loss of PTEN induces HIF-2α transcriptional activity through antagonism of PI3K signaling (15). Moreover, in H-59 cells of lung carcinoma, IGF-1 receptor controls tumor cell invasion through MT1-MMP activation mediated by antagonism of PI3K/Akt/mTOR signaling (16). Thus, the loss of PTEN expression and increased MT1-MMP expression could have a significant impact on the regulation of cell growth, invasion, migration and aggressiveness of PC cells. Therefore, it is vital to find novel agents that could mechanistically regulate MT1-MMP and PTEN expression in PC, which would likely advance our knowledge in designing novel and improved therapies for the treatment of PC.

In conclusion, we investigated the putative role of miR-200 family in the PC cell lines. It was found that miR-200 exerted regulatory effect on the expression of MT1-MMP and PTEN. Moreover, ASO-miR-200c led to decreased cell migration and clonogenicity, which was associated with miR-200 family. Thus, the present study possibly provides a new therapeutic approach for the treatment of PC.
with down-regulation of MT1-MMP and re-expression of PTEN. Moreover, instead of transfection, our novel agents, BR-DIM and CDF, were able to cause re-expression of miR-200c and down-regulated the expression of MT1-MMP, which was consistent with the up-regulation of PTEN expression.

Materials and methods

Cells, culture, drugs and reagents

Human PC cell lines AsPC-1, BxPC-3, COLO-357, MIAPaCa-2, MIAPaCa-GR (gemcitabine-resistant) and PANC-1 were chosen for this study. The cell lines have been tested and authenticated using the core facility—Applied Genomics Technology center at Wayne State University, on 13 March 2009. The method used for testing was short tandem repeat profiling using the PowerPlex® 16 System from Promega (Madison, WI, USA). These cells were stored in multiple vials in liquid nitrogen for our use. CDF was synthesized as described in our earlier publications (22,23), and BR-DIM, a formulated DIM with higher bioavailability (24), was obtained from Dr. Michael Zeligs (BioResponse, LLC, Boulder, CO, USA). Both of these novel agents have been extensively used in our laboratory for the treatment of most cancer cells in vitro and in vivo including PC, as shown by many of our published papers (12,24–31).

Protein extraction and western blot analysis

We initially tested a range of concentrations for BR-DIM (10–50 µM) and CDF (0.5–2 µM), and found that 25 µM of BR-DIM and 0.5–1 µM of CDF was optimal for further studies. Based on these initial results, MIAPaCa-2, MIAPaCa-2-GR and BxPC-3 cells were treated with BR-DIM (25 µM) or CDF (0.5–1 µM) for all subsequent assays for 24, 48 and 72h. Light microscopic pictures were taken at every time point with both BR-DIM and CDF treatment. Total protein was extracted from untreated and both BR-DIM- and CDF-treated cells at 24, 48 and 72h and were loaded with 50 µg of protein and subjected to western blot analysis as described previously (32) to evaluate the expression of PTEN and MT1-MMP. The data were adjusted against loading control using β-actin expression.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine the basal level of miR-200 family (miR-200a, miR-200b, miR-200c) in all six PC cell lines, and also cells treated with BR-DIM (25 µM) or CDF (0.5–1 µM) for 24h, we used TaqMan MicroRNA (miRNA) Assay kit (Applied Biosystems) and the available primer and probe for miR-200 family from Applied Biosystems, following manufacturer’s protocol. Total RNA was extracted and 10 ng from each sample were reverse transcribed as described earlier (33). The expression of miR-200a, 200b and 200c of untreated and treated with BR-DIM and CDF were then carried out in a total volume of 10 µl reaction mixture by qRT-PCR as described earlier (33). All reactions, including controls and the experiments, were performed in triplicate, using StepOnePlus Real-Time PCR (Applied Biosystems). Relative expression of miRNAs was analyzed using C method and was normalized by RNU48 expression.

Pre-miR-200c and antisense miR-200c oligonucleotide transfection

MIAPaCa-2 cells were plated in 6-well plates and incubated overnight. Cells were transfected with either control miRNA or pre-miR-200c or ASO-miR-200c (Ambion, Austin, TX, USA) at a final concentration of 20 nM, using DharmaFECT transfection reagent (Dharmacon), followed by BR-DIM or CDF treatment for 48–72h. After 24h of transfection, the medium was changed to avoid cell death during transfection. Transfected cells were then tested for wound healing and clonogenic assay, and also harvested for the extraction of total RNA and protein using standard methods.

Wound healing assay of transfected cells

Wound healing assay was performed to examine the capacity of cell migration and invasion, as described previously (13). Briefly, after the cells grew in about 80–90% confluence in 6-well plates, the wound was generated by scratching the surface of the plates with a 200 µL pipette tip. The cells were then transfected with either control miRNA, pre-miR-200c or ASO-miR-200c, followed by either BR-DIM or CDF treatment for 18h. Photographic images were taken at 0h and 18h using a microscope (Nikon ECLIPSE TS100).

Clonogenic assay of transfected cells

Transfected cells as described above 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 scanned for images.

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.
Results

MT1-MMP and PTEN expression in PC cells was associated with deregulated expression of miRNAs

MT1-MMP is often expressed in tumor cells with significant invasive properties, and is associated with poor prognosis of patients. In contrast, PTEN, a well-known tumor suppressor gene, has been reported to be lost in tumors (12). We examined the basal level of MT1-MMP and PTEN expression in six human PC cell lines. MT1-MMP expression was highly elevated in AsPC-1, PANC-1 and MIAPaCa-2-GR cells, and moderately elevated in MIAPaCa-2 cells compared with BxPC-3 and COLO-357 cells (Figure 1A). In contrast, the expression of PTEN was significantly lower in PANC-1 and MIAPaCa-2-GR cells. Similarly, miRNA-200 family were differentially expressed in all six cell lines of which MIAPaCa-2, MIAPaCa-2-GR and PANC-1 cells showed significantly lower expression of miR-200a, miR-200b and miR-200c, compared with AsPC-1, BxPC-3 and COLO-357 cells (Figure 1B). The loss of expression of miR-200 family was correlated with the level of PTEN expression. For our subsequent studies, three cell lines (BxPC-3, MIAPaCa-2 and MIAPaCa-2-GR) were chosen as documented below.

Light micrographic pictures

Figure 2A and 2B demonstrate the morphological differences in MIAPaCa-2 and MIAPaCa-2-GR cells. The MIAPaCa-2 cells were exposed to gemcitabine every other week for a period of 6 months which led to this mesenchymal phenotype as reported earlier (12,34). MIAPaCa-2 and MIAPaCa2-GR cells treated with either BR-DIM or CDF for 24, 48 and 72 h (Figure 2A and 2B) were photographed and were subsequently used for all our experiments. BxPC-3 cells were also treated similarly (images not shown) and were used for all our subsequent experiments as shown below.

Re-expression of miR-200 family was achieved by BR-DIM treatment

We determined the expression levels of miR-200 family (miR-200a, miR-200b and miR-200c) after treatment with 25 µM of BR-DIM for 24 h in MIAPaCa-2, MIAPaCa-2-GR and BxPC-3 cells. The expression level was determined by real-time RT-PCR. We found a significant up-regulation in the expression of miR-200a, miR-200b and miR-200c in all three cell lines treated with BR-DIM (Figure 3A).

Re-expression of miR-200 family was achieved by CDF treatment

We also determined the expression levels of miR-200a, miR-200b and miR-200c after treatment of PC cells with 0.5–1 µM of CDF for 24 h and assessed by real-time RT-PCR. We found a significant up-regulation in the expression of miR-200a, miR-200b and miR-200c in all three cell lines treated with CDF (Figure 3B). To further validate whether the protein expression of MT1-MMP and PTEN could be altered by either BR-DIM or CDF treatment, we investigated the effect of treatment in all three cell lines by western blot analysis as presented below.

Modulation of MT1-MMP and PTEN expression by BR-DIM and CDF

BxPC-3, MiaPaCa-2 and MiaPaCa-2-GR cells were used to evaluate the effects of BR-DIM and CDF treatment on the expression of MT1-MMP

![Fig. 2. The time-dependent effect of treatment with both BR-DIM (A) and CDF (B). Light photomicrographs of MIAPaCa-2 and MIAPaCa-2-GR cell lines untreated and treated with BR-DIM (25 µM) for 24, 48 and 72 h and untreated and treated with CDF 1 µM for 24, 48 and 72 h. MIAPaCa-2 cells were exposed to gemcitabine and the paired cell line was called MIAPaCa-2 and MIAPaCa-2-GR based on their changes in morphology from epithelial-like to mesenchymal-like phenotype. Each experiment was repeated, at least, three times independently.](image-url)
and PTEN. Cells were treated with 25 µM BR-DIM or 0.5–1 µM CDF in a time-dependent manner for 24, 48 and 72h. Expression of MT1-MMP proteins was significantly reduced in all three cell lines treated with either BR-DIM or CDF when compared with untreated control (Figure 4). In contrast, the expression of PTEN, a tumor suppressor gene, was found to be decreased in MIAPaCa-2-GR cells compared with BxPC-3 or MIAPaCa-2 cells and was significantly enhanced with both BR-DIM and CDF treatment in all three cell lines. These results suggest that BR-DIM and CDF could be effective for re-expression of PTEN. To further validate whether miR-200 indeed could target the MT1-MMP, or PTEN expression, we chose to investigate the effect of transfection of miR-200c with both precursor and antisense oligonucleotide in MIAPaCa-2 cells, and also treated the cells with BR-DIM and CDF as presented below.

**MT1-MMP and PTEN expression are regulated by miR-200c** and affecting wound-healing capacity and colony formation of MIAPaCa-2 cells

In order to test whether miR-200c expression could regulate MT1-MMP and PTEN expression, we over-expressed miR-200c with pre-miR-200c in MIAPaCa-2 cells, which express relatively low basal levels of miR-200c compared with BxPC-3 cells. MIAPaCa-2 cells were seeded in
down-regulation of MT1-MMP and up-regulation of PTEN in all for 72 h (MIAPaCa-2 and MIAPaCa-2-GR cells with 25 µM of BR-DIM and PTEN as determined by western blot analysis of BxPC-3, expression (Figure 5D), and led to a significant decrease in colony formation (Figure 5C). Interestingly, the re-expression of compared with control (Figure 5A), increased expression of miR-200c with simultaneous increase in PTEN expression by transfection as shown in Figure 6A, which was correlated with wound-healing capacity and clonogenic growth as presented below.

Transfection of antisense miR-200c in MIAPaCa-2 cells increased cell migration and colony formation, and caused altered protein expression of MT1-MMP and PTEN

We investigated the consequence of inactivation of miR-200c expression by transfecting the cells with ASO-miR-200c transfection and assessed the expression of MT1-MMP and PTEN and correlated our findings with wound-healing capacity of the cell changed to mesenchymal morphology of cells with ASO-miR-200c transfection and assessed the expression of miR-200b and miR-200c for further mechanistic studies in MIAPaCa-2 cells. We found that the loss of PTEN was directly correlated with low expression of miR-200c, and that the forced over-expression of miR-200c with precursor or treatment of cells with our novel agents especially CDF resulted in increased PTEN expression, suggesting that miR-200c could regulate the expression of PTEN by translational regulation, and thus we believe that deregulation of miRNAs could become a new strategy for the treatment of PC.

Previous studies have shown that PC cell lines exhibit significantly lower levels of expression of miR-200 family, which was associated with increased EMT, suggesting that miR-200 play an important role in several key aspects of tumor initiation and progression (12,41). Others have reported down-regulation of miR-200 family expression through Smad signaling-dependent manner during the progression of renal fibrosis (42). Moreover, the loss of p53 was correlated with decrease in miR-200c expression and an increase in EMT and sterness markers in a cohort of breast tumors (43). These evidences clearly suggest the role of miR-200 expression in tumor aggressiveness. In our current study, we found that miR-200 expression was drastically down-regulated in aggressive PC cell lines, and that the re-expression of miR-200c using pre-miR-200c transfection of MIAPaCa2-2 cells led to decreased expression of MT1-MMP with concomitantly increased expression of PTEN. Interestingly, these effects were further pronounced by treatment of cells with both BR-DIM and/or CDF treatment, suggesting that these agents especially CDF could be useful for deregulation of important molecular events that are associated with tumor aggressiveness. In contrast, following further knockdown of miR-200c by ASO-miR-200c, we found a marked increase in MT1-MMP expression, which resulted in the down-regulation of the role of miRNAs involved in tumor growth and metastasis, and their deregulation either by over-expression or knockdown by precursors and inhibitors is an emerging area of research. Computational algorithms have been the major methods in predicting miRNA targets based on the base pairing of miRNA and target gene 3’-UTR (40). Based on our experimental evidence and TargetScanHuman 5.2, we found miR-200b and miR-200c to possess a match for base pairing with the 3’-UTR of PTEN. Based on our initial finding, we chose miR-200c for further mechanistic studies in MIAPaCa-2 cells. We found that the loss of PTEN was directly correlated with low expression of miR-200, and that the forced over-expression of miR-200c with precursor or treatment of cells with our novel agents especially CDF resulted in increased PTEN expression, suggesting that miR-200c could regulate the expression of PTEN by translational regulation, and thus we believe that deregulation of miRNAs could become a new strategy for the treatment of PC.
of tumor suppressor gene PTEN in PC cell lines. Moreover, the inactivation in the expression of miR-200c resulted in the acquisition of EMT phenotype and tumor cell aggressiveness, which is consistent with our previous findings (12). Based on our observations, we conclude the importance of the miR-200c miRNA because it may serve as the key regulators of MT1-MMP and PTEN expression, and that the restoration of PTEN expression and down-regulation of MT1-MMP could be easily achieved with both CDF and BR-DIM treatment, which was mediated through re-expression of miR-200c although CDF was found to be superior than BR-DIM.

We have previously demonstrated an increase in MT1-MMP expression in K-ras Cre-mediated activation of a mutant K-ras allele (Kras\textsuperscript{G12D}) and deletion of a conditional Ink4a/Arf tumor suppressor allele in transgenic mouse model (10), which led to tumor initiation and progression. Another recent study reported that dimerization of MT1-MMP is required to promote cell

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**Fig. 5.** Re-expression of miR-200c by pre-miR-200c precursor transfection in MIAPaCa-2 cells led to change in morphology of cells (A). Transfection of miR-200c or treatment of MIAPaCa-2 cells by BR-DIM or CDF increased expression of miR-200c as assessed by qRT-PCR (B), decreased in cell migration (C), decreased levels of MT1-MMP, and up-regulation of PTEN by western blot analysis (D) decreased clonogenicity as determined by colony formation assay (E) as compared with control cells. Each experiment was repeated, at least, three times independently.
invasion in a collagen-enriched environment (44). RNA silencing of endogenous MT1-MMP expression in fibrosarcoma and gastric carcinoma cell lines down-regulated only MT1-MMP expression, but not other MMPs which caused significant inhibition in the migration and invasion of tumor cells (45). Our observation in this study showed that over-expression of MT1-MMP increased cell migration in aggressive PC cell lines, which was significantly reduced by the treatment of cells with our novel agents BR-DIM.

Fig. 6. Inhibition of miR-200c by ASO-miR-200c transfection led to change in morphology of cells (A), decreased expression of miR-200c as assessed by qRT-PCR (B), increased in cell migration (C), increased levels of MT1-MMP, and inhibition of PTEN by western blot analysis (D) increased clonogenicity as determined by colony formation assay (E) as compared with control cells. All the above changes were rescued by BR-DIM or CDF treatment. Each experiment was repeated, at least, three times independently.
and CDF. In conclusion, our current findings clearly suggest that increased expression of MT1-MMP and decreased expression of PTEN is in part due to loss of expression of miR-200c in PC. Moreover, we have provided experimental evidence, supporting that targeted re-expression of miR-200c by BR-DIM and CDF led to decreased expression of MT1-MMP and causing re-expression of PTEN, resulting in reducing tumor cell aggressiveness. Hence these agents, especially CDF, could serve as a novel approach for the treatment of PC.

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