MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells

Sanjeev K.Srivastava1, Arun Bhardwaj1, Seema Singh1, Sumit Arora1, Bin Wang2, William E.Grizzle3 and Ajay P.Singh1,4.  

1Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, 1660 Springhill Avenue, Mobile, AL 36604-1405, USA; 2Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-1150, USA; 3Department of Mathematics and Statistics, University of South Alabama, Mobile, AL 36688-0002, USA; 4Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL 36688-0002, USA.  

*To whom correspondence should be addressed. Tel: +1 251 445 9843; Fax: +1 251 460 6994; Email: asingh@usouthal.edu

Pancreatic cancer (PC) has the worst prognosis among all cancers due to its late diagnosis and lack of effective therapies. Therefore, identification of novel gene targets, which are differentially expressed in PC and functionally involved in malignant phenotypes, is critical to achieve early diagnosis and development of effective therapeutic strategies. We have shown previously that MUC4, an aberrantly overexpressed transmembrane mucin, promotes growth, invasion and metastasis of PC cells, thus underscoring its potential as a clinical target. Here, we report a novel microRNA (miRNA)-mediated mechanism underlying aberrant expression of MUC4 in PC. We demonstrate that the 3’ untranslated region of MUC4 contains a highly conserved miRNA-150 (miR-150) binding motif and its direct interaction with miR-150 downregulates endogenous MUC4 protein levels. We also show that miR-150-mediated MUC4 downregulation is associated with a concomitant decrease in human epidermal growth factor receptor 2 and its phosphorylated form, leading to reduced activation of downstream signaling. Furthermore, our findings demonstrate that miR-150 overexpression inhibits growth, clonogenicity, migration and invasion and enhances intercellular adhesion in PC cells. Finally, our data reveal a downregulated expression of miR-150 in malignant pancreatic tissues, which is inversely associated with MUC4 protein levels. Altogether, these findings establish miR-150 as a novel regulator of MUC4 and a tumor suppressor miRNA in PC.

Introduction

Pancreatic cancer (PC) is a highly lethal malignancy and has the worst prognosis among all cancers. Currently, it is the fourth leading cause of cancer-related deaths in the USA (1). The collective median survival for all patients with PC is 2–8 months, and only 1–4% of all patients survive 5 years after diagnosis (2). Such a grim prognosis of PC is explained by the fact that at the time of diagnosis, majority of patients have already developed an aggressive form of the disease thus limiting the potential for therapeutic intervention (3). Even small adenocarcinoma of pancreas at diagnosis is genetically advanced and carries numerous genetic and epigenetic aberrations that cooperatively act to confer aggressive malignant phenotypes (4,5). Recent years have witnessed important advances in our understanding of the molecular progression of PC, and several important targets have been identified and experimentally tested for their functional participation in the disease processes (4,6,7).

MUC4 is a high-molecular weight glycoprotein that belongs to the family of membrane-bound mucins (8). It is overexpressed in pancreatic adenocarcinomas and tumor cell lines while remains undetectable in the normal pancreas (9). Expression analysis of MUC4 in increasing grade pancreatic intraepithelial neoplasias and malignant lesions demonstrated a positive correlation of MUC4 with disease progression (10). Importantly, in our earlier studies, we have shown a pathogenic role of MUC4 in promoting pancreatic tumor growth and metastasis (11,12). Furthermore, aberrant MUC4 expression is also reported in other malignancies indicating its clinical relevance as a target for therapeutic intervention (8,13). However, there is still little known about the molecular mechanisms that regulate MUC4 expression and whose perturbation ultimately leads to its aberrant expression during cancer initiation and progression (8,14).

Recently, a novel class of endogenous small non-coding gene regulatory RNAs, termed as microRNAs (miRNAs or miRs), has gained significant attention (15). These small molecules exert their regulatory effects by base pairing with partially complementary messenger RNAs (miRNAs) and function by two mechanisms: degrading target mRNA or inhibiting their translation (16,17). It is now well established that miRNAs play critical roles in the development of cancer by altering the expression of oncogenes and tumor suppressor genes (15,16). In the present study, we have investigated the role of microRNA-150 (miR-150) in the regulation of MUC4 expression in PC cells. Our findings demonstrate that 3’ untranslated region (UTR) of MUC4 contains putative binding site for mirR-150, which is highly conserved across several mammalian species. Furthermore, we experimentally show that miR-150 directly targets the 3’ UTR of MUC4 to suppress its expression. Downregulation of MUC4 by miR-150 also leads to a concomitant decrease in human epidermal growth factor receptor 2 (HER2), an interacting partner of MUC4 (18), and its phosphorylated form leading to reduced activation of downstream signaling molecules. Our findings also demonstrate that miR-150 overexpression leads to reduced growth, clonogenicity, migration and invasion in PC cells. Finally, our data reveal a discordant expression of MUC4 at the transcript and protein levels, which is inversely associated with miR-150 expression in malignant clinical specimens. Altogether, our study characterizes a novel miRNA-mediated mechanism of MUC4 regulation and suggests tumor suppressive actions of miR-150 in PC cells.

Materials and methods

Cell lines and pancreatic tissue specimens

HPAF, Panc10.05 and Colo357 PC cell lines were maintained as monolayer cultures in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 μM each of penicillin and streptomycin (Invitrogen) in a humidified atmosphere of 5% CO2 at 37°C. All the cells were tested and determined to be free from mycoplasma contamination every alternate month. Frozen pancreatic tissue samples (normal and malignant) were obtained through cooperative human tissue network (CHTN) at the University of Alabama at Birmingham (UAB) under an Institutional Review Board (IRB)-approved protocol.

Transfection

HPAF, Panc10.05 and Colo357 cells were seeded at 3 x 105 cells per well in six-well plates. After the cells reached 60–70% confluence, they were transfected with miR-150 mimics (Catalog # AM17100) or non-targeting control (miR-NC) mimics (Catalog # AM17111) (Ambion, Austin, TX) at the concentrations ranging from 50 to 150 nM using Lipofectamine 2000 (Invitrogen) as transfection reagent. As per the supplier, these miRNA mimics are small interfering RNA (siRNA)-like structures. The mature miRNA is paired with its complement to form a 21-mer antiparallel duplex with 3’ overhangs similar to

Abbreviations: ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HER2, human epidermal growth factor receptor 2; miRNA, microRNA; mir-150, miRNA-150; mRNA, messenger RNA; PC, pancreatic cancer; PCR, polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region.

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miR-150 targets MUC4 expression

Fig. 1. miR-150 negatively regulates the expression of MUC4. (A) Identification of a putative miR-150-binding site in the MUC4 3′ UTR at position. Eight bases (71 through 78) of the MUC4 3′ UTR are perfect matches (seed sequence) for the miR-150. (B) Comparison of the MUC4-binding element among mammals demonstrates a high degree of conservation. (C and D) Posttranscriptional regulation of MUC4 by miR-150. HPAF, Panc10.05 and Colo357 PC cells treated with different concentration of miR-150 or non-targeting control (miR-NC) mimic for 48 h. Mock-transfected cells represent cells treated with Lipofectamine 2000 alone. Expression of MUC4 was examined at mRNA (C) and protein (D) levels by quantitative reverse transcription–PCR and western blot analyses, respectively. GAPDH (for RNA) and β-actin (for protein) were used as internal controls. Amplified products from one of replicate wells of MUC4 and GAPDH quantitative PCR were also run on 1% agarose gel (C). Intensities of the immunoreactive bands in western blots were quantified by densitometry (D). Bars represent relative MUC4 expression after normalization with the relative internal control ± SD, *P < 0.05.

RNA isolation and reverse transcription–quantitative real-time polymerase chain reaction assay

Total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using 1 µg of total RNA and the High Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a 10 µl reaction volume following manufacturer’s instruction. Quantitative real-time polymerase chain reaction (PCR) was performed using 5 µl (for MUC4) and 2.5 µl (for GAPDH) of 1:10 dilution first-strand complementary DNA in 96-well plates using SYBR Green Master Mix (Applied Biosystems) on an iCycler system (Bio-Rad Laboratories, Hercules, CA). To examine the expression level of mature miR-150, we followed the strategy developed by Chen et al. (19). In brief, we first designed stem–loop RT primers by incorporating a stem–loop extension (at the 3′ end) to the six nucleotides reverse complementary sequence of the 3′ end of miRNA. Later, 5′ sequence of this extension was used to design a universal reverse primer. Forward primer was designed by excluding the last six nucleotides at the 3′ end of miR-150. We added six nucleotides at the 5′ end of forward primer to increase the melting temperature. U6 small nuclear RNA was used as an internal control. Threshold cycle (Ct) values for MUC4 and miR-150 were normalized against Ct values for GAPDH and U6 small nuclear RNA, respectively, and a relative fold change in expression with respect to a reference sample was calculated by the 2−ΔΔCt method. Sequence detail for all the primers used is provided in supplementary Table 1, available at Carcinogenesis Online. The thermal conditions for the real-time PCR were as follows: cycle 1: 95°C for 10 min, cycle 2 (x40): 95°C for 10 s and 58°C for 45 s.

Western blot analysis

Protein extraction and western blotting were performed as described earlier (2,12). In brief, 15–80 µg of protein lysates were resolved by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels. For immunodetection of MUC4, we resolved protein lysates on 2% sodium dodecyl sulfate–agarose gel due to its large size (12). Resolved proteins were transferred onto polyvinylidene difluoride membranes and subjected to standard immunodetection procedure using specific antibodies against: MUC4 (8G7), pY1248-HER2 (mouse monoclonal), extracellular signal-regulated kinase (ERK) 1/2, focal adhesion kinase (FAK) and pFAK (rabbit monoclonal) (Epitomics, Burlingame, CA), and β-actin (mouse monoclonal) (Sigma–Aldrich, St Louis, MO). All secondary antibodies (Santa Cruz Biotechnology) were used at 1:2000 dilutions. Proteins were visualized with the SuperSignal West Femto Maximum sensitivity substrate kit (Thermo Scientific, Logan, UT) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).
Fig. 2. MUC4 is a direct target of miR-150. (A) Schematic representation of firefly luciferase reporter construct containing MUC4 3′ UTR with either wild-type (WT) or mutant (MUT) miR-150 target site. MUT-3′ UTR construct carries three nucleotides (74–76) variation in the seed matching region of the target site to disrupt binding of miR-150. (B) Luciferase reporter assay to examine the miR-150-mediated control of gene expression. HPAF, Panc10.05 and Colo357 cells (0.5 × 10^5 cells per well) were transiently cotransfected for 24 h with reporter plasmids (200 ng, WT or MUT) and 100 nM of miR-150 or miR-NC mimic. Subsequently, protein lysates were made and luciferase (Firefly; test and Renilla, transfection efficiency control) activity assessed using a dual-luciferase assay system. Data are presented as normalized fold change in luciferase activity (mean ± SD; n = 3, *P < 0.05).
miR-150 targets MUC4 expression

Fig. 3. miR-150 represses HER2 expression and its downstream signaling in PC cells. HPAF, Panc10.05 and Colo357 cells were treated with either miR-150 mimic or non-targeting control (miR-NC) mimic for 48 h. Immunoblotting was performed for MUC4, HER2, p-HER2, ERK1/2, pFAK, pFAK and β-actin (as loading control) proteins followed by densitometry of immunoreactive bands. Normalized densitometric values are indicated at the bottom of the bands. Data show a parallel decrease in HER2 and its phosphorylated form (pY1248-HER2) with MUC4 and consequently decreased phosphorylation of its downstream effector molecules.

(www.microrna.org). As shown in Figure 1A, in silico analysis revealed a putative 8-mer-binding site for miR-150 in the 3′ UTR of MUC4 transcript. We also observed that miR-150 complementary site in the MUC4 3′ UTR is highly conserved across several mammalian species (Figure 1B), which further suggested the MUC4 targeting ability of miR-150. Next, we experimentally tested the role of miR-150 in MUC4 regulation in three aggressive and metastatic pancreatic adenocarcinoma cell lines, i.e. HPAF, Panc10.05 and Colo357, which express high levels of MUC4. The cells were transiently transfected with miR-150 mimic and the expression of MUC4 was examined. Our data revealed no apparent change in MUC4 expression at the transcript level (Figure 1C), whereas it was significantly downregulated at the protein level (Figure 1D) in miR-150 mimic transfected cells as compared with non-targeting control (miR-NC) mimics transfected cells. Our data suggest that miR-150 downregulates MUC4 expression through a posttranscriptional mechanism.

miR-150 directly targets 3′ UTR of MUC4

We next determined whether MUC4 is a direct target of miR-150 and if the repression of MUC4 occurs due to the interaction of miR-150 with the predicted binding site in its 3′ UTR. For this, we cotransfected the PC cells with miR-150 or miR-NC (non-targeting control mimic) and a firefly luciferase reporter plasmid containing a region of MUC4 3′ UTR harboring miR-150 target site. As a control, we also generated MUC4 3′ UTR mutant in the miR-150 target region to disrupt its binding (Figure 2A) and used in cotransfection of PC cells with miR-150 or miR-NC. Luciferase activity was measured after 24 h of transfection (Figure 2B). Our data demonstrated that relative luciferase unit was decreased (>70%) in wild-type 3′ UTR transfected PC cells that were cotransfected with miR-150 mimic as compared with that cotransfected with miR-NC. Furthermore, cells transfected with MUT 3′ UTR were resistant to the suppressor activity of miR-150 (Figure 2B). Thus, our data strongly suggest that miR-150 negatively regulates the expression of MUC4 by directly targeting the 3′ UTR of MUC4 transcript.

miR-150 represses the expression of HER2, an interacting partner of MUC4, and its downstream signaling

Earlier, it has been shown that MUC4 interacts with HER2 and positively regulates its expression by increasing its stability (18). Thus, we investigated the effect of miR-150 restoration on the expression of HER2. Our immunoblot data demonstrate that the expression of HER2 and its phosphorylated form (pY1248-HER2) was decreased following restoration of miR-150 in all the three PC cell lines (Figure 3). We next examined the activation status of ERK or p42/44 MAPK and FAK, which are among the downstream mediators of HER2 signaling (18). Our data demonstrated a decreased phosphorylation of ERK and FAK in miR-150-transfected cells as compared with controls, whereas there was no change in the expression of total ERK and FAK. In parallel findings, we observed similar effects on the expression of HER2 and its downstream targets following MUC4 silencing (supplementary Figure 1 is available at Carcinogenesis Online). Altogether, our data suggest that miR-150 represses HER2 and its downstream signaling through MUC4 downregulation in PC cells.

miR-150 overexpression inhibits growth and clonogenicity of PC cells

MUC4 overexpression has been associated with enhanced tumorigenic potential of PC cells (11,12). Therefore, we first studied the effect of miR-150 on the growth of three PC cell lines. Our data demonstrated that relative cell growth was significantly inhibited in miR-150 mimic transfected HPAF (~51.2%), Panc10.05 (~58.9%) and Colo357 (~64.3%) PC cells on day 5 as compared with their respective control (miR-NC transfected) cells (Figure 4A). We next examined the effect of miR-150 restoration on the anchorage-dependent clonogenic ability of the PC cells. In our assay, we observed that the clonogenic ability was decreased by ~65.4, 73.8 and 78.0% in miR-150-transfected HPAF, Panc10.05 and Colo357 cells, respectively, as compared with their respective controls (Figure 4B). In parallel, we also investigated the effect of MUC4 silencing on growth and clonogenicity of PC cells to validate if the effect of miR-150 was indeed mediated through MUC4 downregulation. Although we observed a decrease in the growth and clonogenicity of PC cells following silencing of MUC4 (supplementary Figure 2A and B is available at Carcinogenesis Online), it was relatively less as compared with the effects caused by miR-150. Our findings, thus suggest that miR-150-mediated inhibition of growth and clonogenicity in PC cells may, in part, be due to downregulation of MUC4 expression.

Ectopic expression of miR-150 suppresses the malignant behavior of PC cells

Agressiveness of a cancer cell is determined by its capacity to invade through the basement membrane. As MUC4 was shown previously to potentiate migration and invasion (11,12), we investigated the role of miR-150 on these malignant behavioral properties in PC cells. Cell motility assay was performed by following the migration of tumor cells under chemotactic drive in a Boyden's chamber. We observed that the number of migrated cells was significantly decreased in the miR-150-transfected HPAF, Panc10.05 and Colo357 cells as compared with their respective control (miR-NC transfected) cells (Figure 5A). To determine the effect of miR-150 on invasive capacity of PC cells, an in vitro Matrigel invasion assay was performed. Our data demonstrated decreased invasiveness of the HPAF (2.2-fold), Panc10.05 (2.3-fold) and Colo357 (2.7-fold) cells as compared with their respective controls (Figure 5A). To determine the effect of miR-150 on invasive capacity of PC cells, an in vitro Matrigel invasion assay was performed. Our data demonstrated decreased invasiveness of the HPAF (2.2-fold), Panc10.05 (2.3-fold) and Colo357 (2.7-fold) PC cells transfected with miR-150 as compared with the cells transfected with miR-NC (Figure 5A).
miR-150 is downregulated and inversely correlated with MUC4 in PC

To obtain a clinical evidence of an inverse correlation between miR-150 and MUC4, we examined their expression in a set of 20 human PC tissue specimens along with seven NP tissues. We observed that majority of PCs exhibited downregulated expression of miR-150 as compared with NPs, except one (NP3) as examined in a reverse transcription–quantitative PCR assay (Figure 6A). A t-test analysis showed that the mean expression level of miR-150 in the NPs (69.93) was significantly higher than that of the PCs (21.68) with \( P < 0.00001 \). As shown in supplementary Figure 3A (available at 

Carcinogenesis Online). Our study confirms the clinical relevance of our experimental data on miR-150-mediated MUC4 regulation in PC cells.

Discussion

In the present study, we investigated the role of miR-150 as a novel regulator of MUC4 mucin and determined the functional consequences of its overexpression on the phenotype of PC cells. In silico analysis revealed that 3' UTR of human MUC4 gene contains an 8-mer target site of miR-150 and miR-150/MUC4 target relationship is conserved across several mammalian species. We also demonstrated that ectopic expression of miR-150 in MUC4-expressing HPAF, Panc10.05 and Colo357 PC cell lines resulted in successful downregulation and reduction of MUC4 expression in clinical specimens.

miRNAs are posttranscriptional regulators of gene expression exerting their action through partial complementary elements in the 3' UTR of their target mRNAs (15, 17). Most animal miRNAs are evolutionarily conserved and often exist in clusters (20). Numerous miRNAs have been reported to be differentially expressed in PC as compared with the normal pancreas (21, 22), suggesting their involvement in PC pathogenesis. In this study, we identified miR-150 as an evolutionarily conserved novel regulator of MUC4 mucin, which is downregulated in a significant proportion of pancreatic tumors. An
aberrant expression of miR-150 has also been reported in other malignancies. Although miR-150 is downregulated in lymphoma and leukemia (23,24), its expression is upregulated in gastric and colorectal malignancies (25,26). Furthermore, miR-150 is shown to promote gastric cell proliferation (26) while it acts as a tumor suppressor in malignant melanoma (24). Among the important targets of miR-150 that has been experimentally validated are c-Myb, P2X7 and EGR2, of which latter two mediate its tumor promoting functions (26–28). Therefore, these studies along with our findings indicate cell type-specific and/or context-dependent functions of miR-150.

MUC4 is frequently deregulated in a wide variety of cancers (29), and its overexpression has been associated with pancreatic tumor growth and metastasis (11,12). Furthermore, recent data also indicate its pathological involvement in other malignancies and inflammatory diseases (30–33). MUC4 and its rat homolog (SMC/Muc4) have been shown to regulate a variety of cell processes such as proliferation, apoptosis, migration, invasion and differentiation, sometimes in a context-dependent manner (8,11,13,14). In majoriy of studies, MUC4/Muc4 has been shown to modulate cell signaling by either acting as an intramembrane ligand for ErbB2 (rat Muc4) or by modulating HER2 expression through enhanced stability (18,29). However, an indirect role of MUC4 in cell phenotype has also been predicted through surface interference (steric hindrance) due to its large molecular size (13,34). MUC4 may thus alter intercellular interaction of surface adhesion proteins and impact cell signaling by indirect mechanism. In other instance, MUC4 may also facilitate tumor cell–endothelial cell interactions through presence of selectin ligands (Sialyl Lewis x/a glycotopes) on the core protein (13,34). Thus, identification of miR-150 as a novel regulator of MUC4 may have important therapeutic implications in pancreatic and other malignancies.

miR-150 targets MUC4 expression

In growing body of evidence now suggests that miRNAs act either as oncogenes or tumor suppressors in a variety of cancers (35). miRNAs have been implicated in a broad range of biological processes including cell proliferation, apoptosis, differentiation, metabolism and migration (27,36–39). Our investigation revealed that miR-150 overexpression decreases pancreatic tumor cell growth, clonogenicity and suppresses the malignant behavioral properties. These effects are consistent with previously reported role of MUC4 and thus downregulation of MUC4 may underlie such functional consequences of miR-150 overexpression. However, in other studies, several additional targets of miR-150 have also been established (26,28), and some may still be pending experimental validation, these targets along with MUC4 may also mediate miR-150 action in PC. In fact, this notion is further substantiated by the fact that we observed a more potent effect of miR-150 restoration as compared with that observed upon silencing of MUC4 alone (supplementary Figure 2A and B is available at Carcinogenesis Online). Therefore, it will be of interest to
identify novel targets of miR-150 and/or examine the functional role of already characterized targets in PC progression. Nonetheless, our study provides a clear evidence for a role of miR-150 as a tumor suppressor and establishes MUC4 downregulation as a plausible mechanism. In our earlier studies, we have shown that MUC4 expression is associated with the activation of FAK and ERK pathways through modulation of HER2 expression in pancreatic and ovarian cancer cells (18,32). Consistent with this, we observed inhibition of HER2 and its downstream signaling (FAK and ERK) in miR-150 overexpressing PC cells and this effect could partly mediate the tumor suppressive action of miR-150.

In summary, we have validated the role of miR-150 as a negative regulator of MUC4 in three PC cell lines. As a consequence, we also observe repression of HER2 and its downstream signaling. These molecular changes, at least in part, are responsible for the decreased growth, clonogenicity, migration and invasion and enhanced homotypic interactions of PC cells. Furthermore, we observed discordance in the expression of MUC4 at transcript and protein levels that correlated with dysregulated expression of miR-150.

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