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Original Paper

MiRNAs are Unlikely to be Involved in Retinoid Receptor Gene Regulation in Pancreatic Cancer Cells

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Key Words
Retinoid receptors • MiRNA • Pancreatic cancer

Abstract
Background/Aims: Retinoid receptors and retinoic acid were reported to be down-regulated in pancreatic duct adenocarcinoma (PDAC) compared to normal pancreas. Yet the mechanism of the down-regulation of retinoid receptors is not well defined. The aim of this study was to find out whether selected dysregulated miRNAs in PDAC are responsible for the decreased level of retinoid receptors. ***Methods:*** Bioinformatics, real-time PCR, western blot analysis as well as molecular manipulation with miRNA in cells of PDAC were carried out. ***Results:*** We first performed bioinformatics research to identify conserved target sequences for deregulated miRNAs within the 3'UTR region of retinoid receptor mRNA. This research revealed binding sites for miR-138, -27a, -27b, -206, -613, -9-5p, -27a/b-3p and -27a. Next, we investigated the expression of selected retinoid receptors and miRNAs in PDAC cell lines and in the Human Pancreatic Duct Epithelial (HPDE) cell line. Further, we investigated the effects of modifying expression levels of selected miRNAs using miRNA inhibitors or mimics. We demonstrated that none of these miRNAs can target the selected retinoid receptors *in vitro*. ***Conclusions:*** miR-27a, miR-27b, miR-9, miR10a and miR-10b were up-regulated in PDAC cells compared to HPDE cells. The up-regulation of these miRNAs was not responsible for the down-regulation of RARα, RARβ, RXRα and RXRβ in PDAC cells.

Introduction

Gene expression in benign and malignant cells can be regulated on various levels, such as epigenetic regulation (e.g. histone modifications, DNA methylation), transcriptional

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control (e.g. promoter regulation by transcription factors) or translational control by non-coding RNAs. miRNAs are small non-coding endogenous RNAs that regulate multiple biological processes by mechanisms that are still not fully understood [1]. These regulatory elements are first transcribed and then processed by Dicer and Drosha complexes into 21–23nt mature miRNAs [2]. The miRNA is incorporated into the RNA-induced silencing complex (RISC) containing Dicer and many other associated proteins including members of the Argonaute (Ago) protein family [3]. RISC regulates posttranscriptional mRNA expression (i.e. translation suppression) typically by binding to the 3' untranslated region (3'-UTR) of the complementary mRNA sequence, preventing the recognition of cap by eIF4E and subsequent binding of translational factors [4]. Therefore, there is some evidence proving that the translation activation by miRNAs is regulated through direct or indirect ways [5]. 3'UTR is not a unique target sequence for miRNAs. It has been shown that miRNAs also efficiently bind to the 5'UTR [6, 7] leading to activation [8-10] or repression [11, 12] of translation. However the precise mechanism by which miRNA binds to the 5'UTR is still to be deciphered.

Retinoic acid, the metabolite of vitamin A, is important for growth and differentiation of both healthy and cancerous cells. All-trans retinoic acid (ATRA), the major physiologically active form of vitamin A, regulates the expression of many genes [13]. Physiological effects of retinoid are mainly mediated by two families of nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). RAR and RXR are both ligand-dependent transcription factors, which belong to the nuclear hormone receptor superfamily. The RAR and RXR family comprise several isotypes: RARα, RARβ, RARγ and RXRα RXRβ, RXRγ [13].

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world. At present, only multimodal treatment including surgical resection can prolong survival of patients with this disease [14]. The reasons for this poor prognosis are early dissemination, lack of early specific symptoms and late diagnosis [15]. Additionally, PDAC is highly resistant to chemo- and targeted therapy, and withstands immunotherapy [16]. Therefore, new therapeutic approaches against PDAC are urgently needed. It has been shown that different miRNA promote growth and proliferation of PDAC cells [17-20] and predict poor clinical outcome of patients with PDAC [21].

Recently, ATRA and its derivatives have been reported to inhibit proliferation and migration of various cancer types including PDAC [22, 23]. Our previous studies showed that the concentration of ATRA and its derivatives as well as retinoid receptor expression are reduced in PDAC cancer tissues compared to normal pancreatic tissue [24, 25]. More importantly, the reduced expression of retinoid receptors correlates with the expression of markers of epithelial to mesenchymal transition and stemness of PDAC cells [24]. Besides, the expression of RARA and RXRB is found to be associated with a better overall survival in PDAC patients. Accordingly, the reduced level of retinoids and their receptors is an important feature of PDAC [24]. However, the mechanism of the down-regulation of retinoid receptors is not well defined.

The present study aimed to identify novel oncogenic-suppressive miRNAs as the regulators of retinoid receptors in PDAC.

Materials and Methods

Materials

Ampicillin, chloroform, isopropyl alcohol, ethanol, 0.4% trypan blue solution, EDTA, TEMED, ammonium persulfate and DMSO were purchased from Sigma (Darmstadt, Germany). Agarose was obtained from Gibco BRL (Idstein, Germany). Odyssey® Blocking Buffer was purchased from LI-COR (Lincoln, NE, USA). Kits: HiperFect transfection, RNeasy Mini, miScript II RT, miScript SYBR Green PCR, QuantiTect Reverse Transcription, QuantiTect SYBR Green PCR QuantiTect Primer Assays, miScript Primer Assays and QIAzol Lysis Reagent were from Qiagen (Hilden, Germany). Anti-RAR and anti-RXR antibodies were obtained from SantaCruz (Dallas, TX, USA). Anti-MET antibody was from Sigma (Darmstadt, Germany). IRDye® 680LT

Goat anti-Mouse, IRDye® 800CW Donkey anti-Rabbit, IRDye® 680CW Goat anti-Rabbit and IRDye® 800CW Donkey anti-Mouse antibodies were purchased from LI-COR (Lincoln, NE, USA). All miRNA products were purchased from Qiagen (Hilden, Germany). miRNA products and primers used are listed in the Table 1.

Table 1. miRNAs products and primers

miRNA mimic or inhibitors	*Sequence (5'-3')*
Syn-hsa-miR-9-5p miScript miRNA Mimic	5'UCUUUGGUUAUCUAGCUGUAUGA
Syn-hsa-miR-27a-3p miScript miRNA Mimic	5'UUCACAGUGGCUAAGUUCCGC
Syn-hsa-miR-27a-5p miScript miRNA Mimic	5'AGGGCUUAGCUGCUUGUGAGCA
Syn-hsa-miR-27b-3p miScript miRNA Mimic	5'UUCACAGUGGCUAAGUUCUGC
Syn-hsa-miR-27b-5p miScript miRNA Mimic	5'AGAGCUUAGCUGAUUGGUGAAC
Anti-hsa-miR-9-5p miScript miRNA Inhibitor	5'UCUUUGGUUAUCUAGCUGUAUGA
Anti-hsa-miR-27a-3p miScript miRNA Inhibitor	5'UUCACAGUGGCUAAGUUCCGC
Anti-hsa-miR-27a-5p miScript miRNA Inhibitor	5'AGGGCUUAGCUGCUUGUGAGCA
Anti-hsa-miR-27b-3p miScript miRNA Inhibitor	5'UUCACAGUGGCUAAGUUCUGC
Anti-hsa-miR-27b-5p miScript miRNA Inhibitor	5'AGAGCUUAGCUGAUUGGUGAAC
miRNA negative control Oligos	
Hs_miR-9_1 miScript Primer Assay	
Hs_miR-27a_1 miScript Primer Assay	
Hs_miR-27b_2 miScript Primer Assay	
Hs_miR-138_1 miScript Primer Assay	
Hs_miR-206_1 miScript Primer Assay	
Hs_miR-613_1 miScript Primer Assay	
Hs_RARA_1_SG QuantiTect Primer Assay (QT00095865)	
Hs_RARB_1_SG QuantiTect Primer Assay (QT00062741)	
Hs_RARG_1_SG QuantiTect Primer Assay (QT00000987)	
Hs_RXRA_1_SG QuantiTect Primer Assay (QT00005726)	
Hs_RXRB_1_SG QuantiTect Primer Assay (QT00061117)	
Hs_RXRG_1_SG QuantiTect Primer Assay (QT00007238)	

Cell lines

Human PDAC cell lines: Mia-PaCa-2 and Panc-1 derived from primary mesenchymal tumors and BxPc-3 and DanG derived from primary epithelial tumors were chosen for experiments. All cell lines were obtained from ATCC (Manassas, VA, USA). PDAC cells were maintained as a monolayer. Prior to passaging, trypsin and media were warmed up in a 37°C water bath. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged once or twice a week when a confluence of 80% was reached. Normal HPDEcells (kindly provided by Dr. Kong Bo, Technical University Munich, Germany) were maintained in Keratinocyte Basal Medium with supplements (Lonza, Clonetics KBM, Basel, Switzerland). The flask was incubated at 37° C in a humidified atmosphere of 5% CO2/95% air. Culture medium was replaced every two to three days. Cells were passaged when a confluence of 80% is reached. All cells were routinely checked for mycoplasma in conditioned cell medium using PCR according to internal SOPs and authenticated commercially by IDEX BioResearch (Ludwigsburg, Germany).

Total RNA and miRNA isolation from cultivated cells

Total RNA from cells was isolated using RNeasy Mini Kit following the manufacturer's instruction and described elsewhere [26]. RNA was quantified using a GeneQuant *pro* spectrophotometer (Eppendorf, Hamburg, Germany). The quality of isolated mRNA was evaluated by the A260/A280 value. To assess of RNA quality, the samples of total RNA were diluted 1:50, applied on the RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA) and analyzed with the Agilent Bioanalyzer 2100 (Aglient Technologies, Palo Alto, CA). All samples had RNA Integrity Numbers between 9.5 and 10.

Total miRNA from cells was isolated using QIAzol (Qiagen, Hilden, Germany) following the manufacturer's instruction (miRNeasy Mini kit, Qiagen, Hilden, Germany). In brief, cells were trypsinized at 37°C, collected and centrifuged at 300 × g for 5 minutes. The cell pellet was then washed with cold 1× PBS 3 times. Cells were then pelleted in a 1.5ml Eppendorf tube. By adding 700 µl QIAzol lysis reagent, cells were lysed after incubation at room temperature for 5 minutes. Then 140 µl chloroform was added and the tube was shaken vigorously for 15 seconds. After incubation at room temperature for 3 minutes, the tube was centrifuged at 8000 × g at 4°C for 15 min. The upper aqueous phase which contains miRNA was transferred into a new collection tube. Then 1.5 volumes of 100% ethanol was added and well mixed, before loading onto a RNeasy Mini spin column in a 2 ml collection tube for centrifugation at 8000 × g (RT, 1 min). The RNeasy Mini spin column which contains miRNAs was then washed with 700 µl RWT Buffer (included in the Qiagen kit) once and 500 µl RPE Buffer (included in the Qiagen kit) twice. 30-50µl RNase-free water was then added directly onto the RNeasy Mini spin column membrane and miRNAs were obtained by centrifugation at 8000 × g for 2min. RNA was quantified using a spectrophotometer. The quality of isolated miRNA was evaluated by the A260/A280 value. To determine the content of miRNA, each sample was diluted 1:50, applied on the Small RNA Nano LabChip (Agilent Technologies, Palo Alto, CA) and analyzed with the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The ratio miRNA to small RNA was in the range 1-7%.

Real time PCR analysis of mature miRNAs

Generation of the first strand cDNA from miRNA was done using miScript II RT Kit (Qiagen, Hilden, Germany). For this purpose, an equal amount of miRNA (500 ng) from each miRNA sample (in a final volume of 20 µl containing universal primer) was added into each reaction at the last step. The miRNA was placed on ice and the reverse transcription reaction was set up by mixing the following components from the kit: 4 µL of 5x miScript HiSpec Buffer, 2 µL of 10x Nucleics Mix, 2 µL of miScript Reverse Transcriptase Mix, variable volume of RNA template as well as RNase-free water. The end volume of each sample was 20 µL. The reagents were gently mixed and briefly centrifuged. Reverse transcription was carried out by incubation at 37°C for 60 minutes with the reaction terminated by incubation at 95°C for 5 minutes. After the reaction, each tube was diluted with 200 µL RNase-free water for later use in RT-PCR.

Expression of mature miRNAs was determined by miScript miRNA PCR Array (Qiagen, Hilden, Germany). For this purpose, 1µl cDNA was used per 25 µl reaction mixture containing 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10x miScript Universal Primer, 2.5 µl of 10x miScript Primer Assay and variable volume of RNase-free Water in a 96-well plate. The 96-well plate was briefly centrifuged for 1 minute to remove any bubbles. The reaction was then carried out for 40 cycles in StepOne™ Real-Time PCR System (Thermo Fisher, Darmstadt, Germany). The program included the following steps: 15 min initial activation at 95°C, 15 s denaturation at 94°C, 30 s annealing at 55°C and finally 30 s extension at 70°C. U6-snRNA was used as a house-keeping control for normalization. $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change of miRNA expression using the equation $2^{-\Delta\Delta Ct}=2^{-[(Ct\ gene\ of\ interest - Ct\ internal\ control)sample\ A - (Ct\ gene\ of\ interest - Ct\ internal\ control)sample\ B]}$ as described elsewhere [27], where internal control refers to the house-keeping control, sample A was a PDAC cell line and sample B was the HPDE cell line.

SYBR Green quantitative PCR for retinoid receptor

Genomic DNA was eliminated from the RNA samples before initiating reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with 500 ng of RNA from each sample. The reaction mixes for genomic DNA elimination (total volume of 14 µl) included 2 µl of 7x gDNA Wipeout Buffer and variable volume of Template RNA and RNase-free water. The reaction tubes were put directly on ice after incubation for 2 min at 42°C. After that, 6 µl of reverse-transcription master mix (1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5x Quantiscript RT Buffer and 1 µl of RT primer mix) was prepared per sample on ice. To each reverse transcription master mix tube, 14 µl of Template RNA after genomic DNA elimination reaction was added. In a PCR machine, reverse transcription was carried out for 15 min at 42°C and then Quantiscript Reverse Transcriptase was inactivated after 3 min at 95°C.

Expression of the retinoid receptor was determined by QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and described elsewhere [24]. Briefly, 1 µl of cDNA synthesized before was added to reaction mix in 96-well plate. The reaction mixture consisted of the following components: 12.5 µl 2x QuantiFast SYBR Green PCR Master Mix, 2.5 µl 10 x QuantiTect Primers and 9 µl RNAse-free water. The 96-well plate was briefly centrifuged for 1 minute to remove any bubbles. The cycling program was carried out in a StepOne™ Real-Time PCR System (Thermo Fisher, Darmstadt, Germany) with the following parameters: 5 min PCR initial activation step at 95°C followed by 40 two-step cycles of 10 s denaturation at 95ºC and 30 s combined annealing/ extension at 60ºC. GAPDH mRNA was used as a house-keeping gene for normalization. $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change of retinoid gene expression as described above.

Transfection of miRNAs

We used HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) to transfect miRNAs into pancreatic cancer cells. Transfection was done following manufacturer's manual. The initial step was to determine optimal miRNA transfection conditions for different cells. Briefly, 1×10^5 cells were suspended in 0.5 ml of growth medium and seeded into one well of a 24-well plate. Then appropriate amounts of miRNA and HiperFect Transfection Reagent were added into 100 µl culture medium without serum. The mixture was vortexed and incubated for 5 to10 min at room temperature to allow the formation of transfection complexes. Transfection was achieved by adding the mixture dropwise onto the cells in a well. The plate was gently swirled and incubated under normal growth condition (5% CO_2, 37°C, 90% humidity) for 48 to 72 h. After the incubation for 48 to 72 hours, cells were collected and miRNAs were isolated with the protocol as described before. The level of target miRNA in transfected cells was determined by RT-PCR and

compared with non-transfected cells. The same protocol was used for transfection of miRNA mimics and miRNA inhibitors.

Western blot analysis

Western blotting was performed as described elsewhere [26]. Briefly, cells grown in a 24 well-plate were washed with cold phosphate-buffered saline (PBS buffer) three times and lysed in RIPA extraction buffer (150 mM sodium chloride, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate) and 50mM Tris at pH 8.0) on ice for 15 min. The lysates were centrifuged at 14, 000 rpm for 20 min and the supernatant containing the total cell extract was stored at -80°C until used. The extracted protein was quantified using the BCA Protein assay kit (Thermo Fisher, Darmstadt, Germany). The proteins resolved by SDS-PAGE were transferred onto a PVDF membrane by semi-dry electrophoretic transfer. The membrane was first wetted in 100% methanol for 5 seconds and then equilibrated in transfer buffer. The blot was assembled on a semi-dry blotting apparatus from anode to cathode as follow: 2 Whatman papers soaked in transfer buffer, PVDF membrane, gel and finally 2 Whatman papers. Transfer of proteins was done for 1 hour by applying 1.8V per cm square of the blot. The membrane was then blocked for 1 hour at room temperature in TBS containing 0.1% Tween 20 and 5% BSA. The membrane was then incubated overnight with the primary antibody in blocking solution. Following three 10-min washes with 0.1% Tween 20 in TBS (TBST) the blot was incubated for 1 hour at room temperature with the fluorescence-conjugated secondary antibody diluted in Odyssey® Blocking Buffer (LI-COR, Lincoln, NE, USA) for 1 h at room temperature in the dark. The membrane was then washed 3 times at 10 min intervals with TBST buffer. Finally, the protein band was visualized in the Odyssey Reader machine (LI-COR, Lincoln, NE, USA). Densitometric values of the bands were calculated and normalized with respect to background by using GelAnalyzer 2010 software.

Bioinformatics analysis of the miRNA target

To identify potential target sequences in the 3'UTR of retinoid receptor mRNA, various public database websites were utilized: miRNA.org (http://www.microrna.org/microrna/getGeneForm.do), miRDB (http://www.mirdb.org/miRDB/) and TargetScanHuman (http://www.targetscan.org/vert_71/). For miRDB, miRNA target prediction program is based on support vector machines (SVMs), CLIP-ligation studies, a large microarray training dataset and a set of functional miRNAs annotated by integrating computational analyses with literature mining [28-30]. For Targetscan, 14 features (e.g., site type, 3′-supplementary pairing, local AU content, and distance from the closest 3′-UTR end), two miRNA features (TA and SPS) were selected and combined to develop the context++ model of miRNA targeting efficacy [31]. PicTar is also a computational method base on nuclMap, optimal free energy, alignments of highly probable nuclei and established score system [32]. Specific complementary target sequences were recorded for all predicted targets. In addition, another public database (miRTarBase, http://mirtarbase.mbc.nctu.edu.tw/) of experimentally validated miRNA targets was utilized to combine with the information of already published data.

Statistical analysis

Statistical analyses were performed using either Statistical Package for the Social Sciences (IBM SPSS Statistic 24) software or GraphPad Prism 7 (GraphPad Software, Inc.) software. Values were presented as means±SEM. Different statistical tests, such as one-way ANOVA and Pearson's correlation were utilized depending on specific data types, as indicated in the figures. Values were considered significantly different when p-values were less than 0.05.

Results

Prediction of retinoid receptor sequences targeted by miRNAs

In our previous study, we showed that the down-regulation of retinoid receptors in PDAC is negatively correlated with patient survival, which indicates that retinoid receptors could function as tumor suppressors [24]. To investigate whether retinoid receptor mRNAs are targeted by specific miRNAs, the first step is to predict the specific binding sites in the 3'UTRs. For this purpose, we integrated the predictions from different databases (Targetscan, mirbase, Pictar and miRDB) to increase the probability of finding all potential miRNA targets.

Table 2. Prediction of miRNA targets. [a]Predicted by miRNA.org and Targetscan, [b]Predicted by miRNA.org, Targetscan and miRDB, [c]Predicted by miRNA.org and miRDB, [d]Predicted by miRNA.org and miRDB, [e]Predicted by Targetscan, [f]Predicted by Targetscan, [g]Predicted by miRNA.org

	miR-9a	miR-10	miR-27a/b	miR-138	miR-206	miR-613
RARα	No	No	Yes[a]	Yes[b]	No	No
RARβ	No	No	No	No	Yes[c]	Yes[d]
RXRα	Yes[e]	No	Yes[f]	No	No	No
RXRβ	No	No	Yes[g]	No	No	No

As these predictive databases are based on different algorithms, the prediction of miRNA target sequences from a single database could result in high numbers of false positives. We used both the intersection or the union of miRNA targets predicted by the different algorithms. As the purpose of this research was to find the dysregulated miRNAs that can target retinoid receptors in PDAC, we first recruited dysregulated miRNAs and then used different algorithms to predict potential candidate miRNAs that target retinoid receptors. If the selected miRNA was predicted to target any of these receptors by any of these algorithms, it was recruited for validation in the next step. To avoid missing any possible miRNA targets, we integrated the predictions from the different algorithms. Some of the miRNAs, like miR-27a and miR-27b, were predicted to target more than one retinoid receptor by more than one algorithm (Table 2). In the aggregate, miR-138, miR-206, miR-613, miR-9, miR-27a/b and miR-613 can potentially target retinoid receptors.

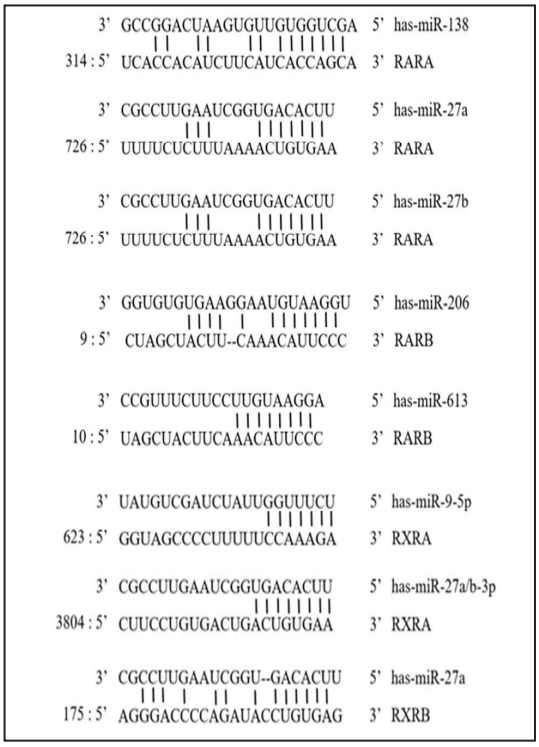

Fig. 1. Target sequence of selected miRNAs within 3' UTR of retinoid receptors predicted by integrated database.

To identify the specific targeted sites, each mRNA of the retinoid receptor was screened for complementarity to seed sequences of selected miRNAs. As a result, several 100% matches to target sequences were found: (1) miR-138 targets nt330-337 of the RARA-3'UTR; (2) miR-27a and miR-27b target nt740-747 of the RARA-3'UTR; (3) miR-206 targets nt22-30 of the RARB-3'UTR; (4) miR-613 targets nt21-29 of the RARB-3'UTR; (5) miR-9-5p targets nt637-644 of the RXRA-3'UTR; (6) miR-27a/b-3p targets nt2818-2826 of the RXRA-3'UTR (Fig. 1). In addition, a partial match to a target sequence was also identified: miR-27a targets nt177-196 (data not shown).

Basal expression of selected miRNAs and retinoid receptors in PDAC cell lines compared to HPDE cells

To find out whether there are correlations between the expression of selected miRNAs and these retinoid receptors, we investigated the basal expression levels of miR-27a and b, -206, -613, -138, -9, -10a and b in a HPDE cell line and 4 PDAC cell lines: BxPC-3, Panc-1, DanG, MiaPaCa-2. As one can see in Fig. 1, expression levels of these selected miRNAs varied considerably. Specifically, miR-27a increased by nearly 3 and 2 fold in BxPC-3 and MiaPaCa-2 cells respectively, compared to the expression level in HPDE cells. In contrast, the expression of miR-27a slightly decreases in PanC-1 and DanG cells (Fig. 2). Interestingly, the

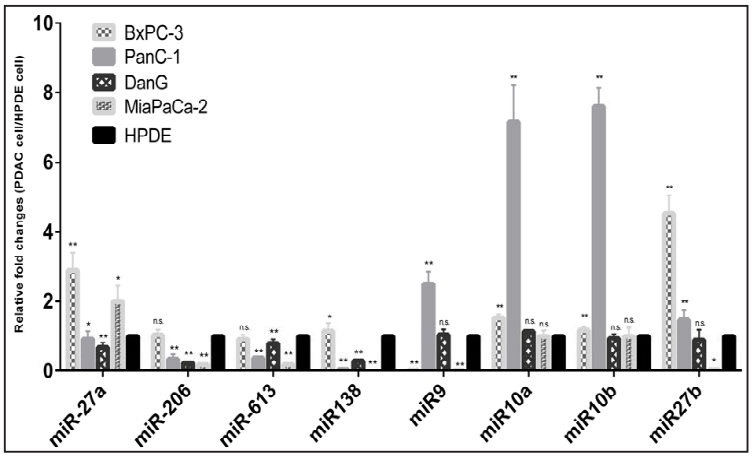

Fig. 2. miRNA expression in PDAC and HPDE cell lines determined by RT-qPCR. 500 ng of total RNA extracted from each cell line was used to determine miRNA expression levels by RT-qPCR. Relative expression levels were calculated by normalisation to the U6 expression level. An adjustment factor was applied to all miRNA relative expression levels so that the average value in HPDE cells was arbitrarily set to 1.0. The significant fold changes of miRNA in PDAC cell lines compared with HPDE cells are indicated with an asterisk (*) or double asterisks (**) when p values are <0.05 or <0.01 respectively after performing one-way ANOVA. n.s. indicates that there is no significant difference between the indicated PDAC group and the HPDE group.

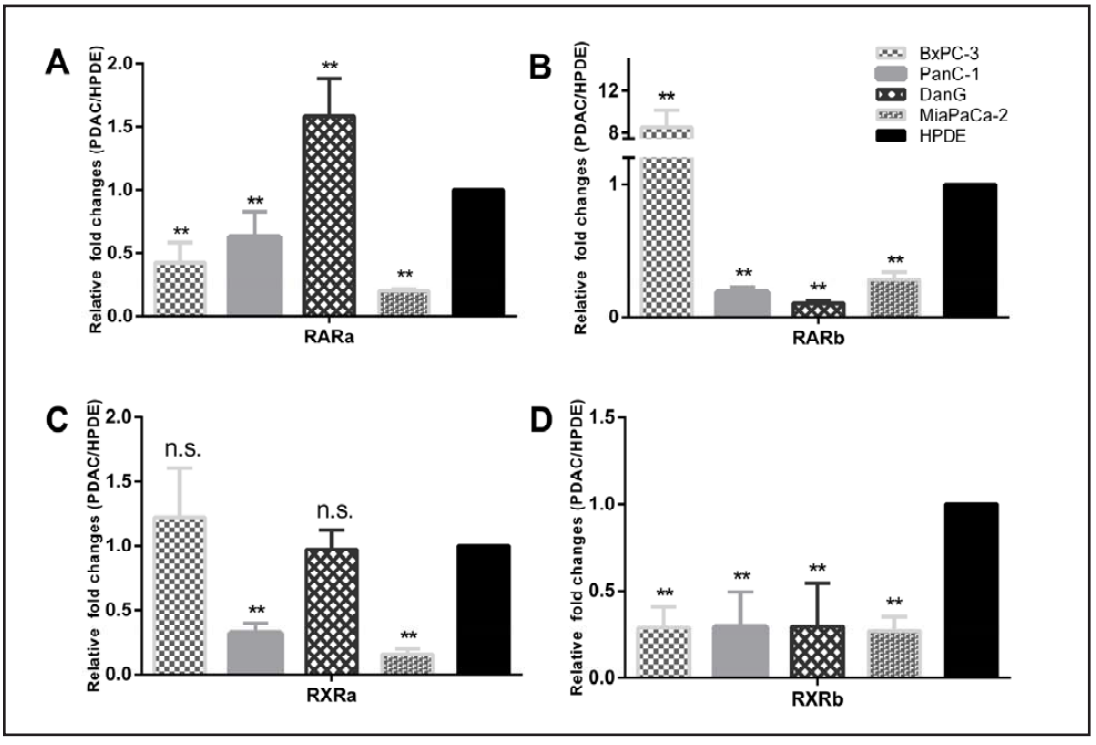

Fig. 3. Messenger RNA expression of retinoid receptors in PDAC cell lines and HPDE cell line determined by RT-qPCR. 500 ng of total RNA extracted from each cell line was used to determine mRNA expression levels by RT-qPCR. Relative expression levels were calculated by normalization to the GAPDH expression level. An adjustment factor was applied to all miRNA relative expression levels so that the average value in HPDE was arbitrarily set to 1.0. The significant fold changes of mRNAs in PDAC cell lines compared with HPDE cells are indicated with double asterisks (**) when p values are <0.01 after performing one-way ANOVA. n.s. indicates that there is no significant difference between the indicated PDAC group and the HPDE group.

basal expression of miR-206, miR-613 and miR-138 are significantly decreased in PanC-1, DanG and MiaPaCa-2 cells, but not significantly changed in BxPC-3 cells compared to the level in HPDE cells. In addition, miR-9, miR-10a and miR-10b are all high expressed in PanC-

Table 3. Correlations between expression levels of selected miRNAs and retinoid receptors in HPDE and PDAC cell lines. [a] Spearman's correlation coefficient, [b] Spearman's rank correlation test, *Significantly correlated with Spearman's correlation coefficient > +0.50 or < −0.50 and $P < 0.05$

	RARα	RARβ	RXRα	RXRβ
miRNA27a	-0.8[a]	+0.900*	+0.300	-0.600
	0.104[b]	0.037	0.624	0.285
miRNA206	+0.100	+0.700	+0.900*	+0.300
	0.873	0.188	0.037	0.624
miRNA613	+0.500	+0.500	+0.900*	+0.700
	0.391	0.391	0.037	0.188
miRNA138	+0.300	+0.600	+1.000*	+0.400
	0.624	0.285	0.000	0.505
miRNA9	+0.700	-0.600	0.000	+0.600
	0.188	0.285	1.000	0.285
miRNA10a	+0.051	-0.154	+0.154	-0.103
	0.935	0.805	0.805	0.870
miRNA10b	-0.462	+0.359	+0.051	-0.308
	0.434	0.553	0.935	0.614
miRNA27b	+0.000	+0.500	+0.700	+0.100
	1.000	0.391	0.188	0.873

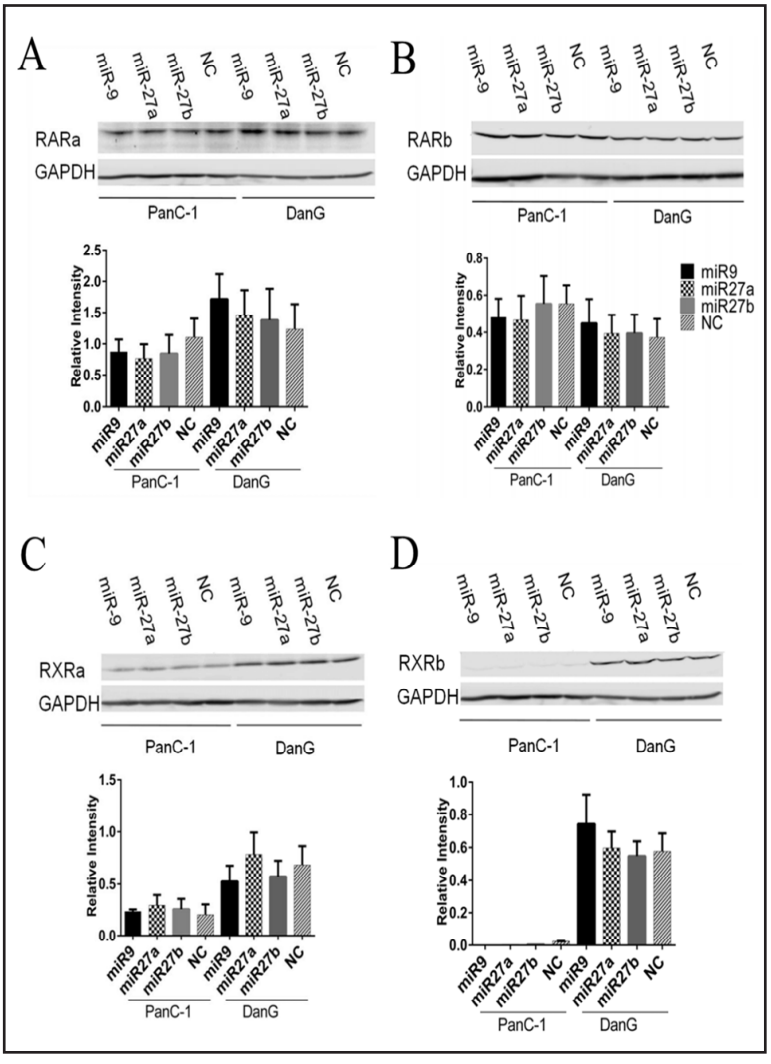

Fig. 4. Expression of retinoid receptors in Panc-1 and DanG cells transfected with miR27a, miR27b or miR9. (A) RARα, (B) RARβ, (C) RXRα and (D) RXRβ. 25 μg of total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by Western blotting. The membrane was incubated with rabbit polyclonal anti-RARa antibody as the primary antibody and IRDye® 680CW Goat anti-Rabbit as the secondary antibody. GAPDH quantity was used as the loading control. Densitometric values of the bands were calculated and normalized against background using GelAnalyzer 2010 software. Relative intensities (i.e. density ratio of target protein/GAPDH) are represented in bar diagrams, with each bar depicting the mean ± S.D. from 2 different analyses.

1 cells. For miR-27b, the expression levels increase significantly in BxPC-3 and PanC-1 cells with a fold change of 5 and 1.8 respectively, but decrease by 96% percent in MiaPaCa-2 cells (Fig. 2).

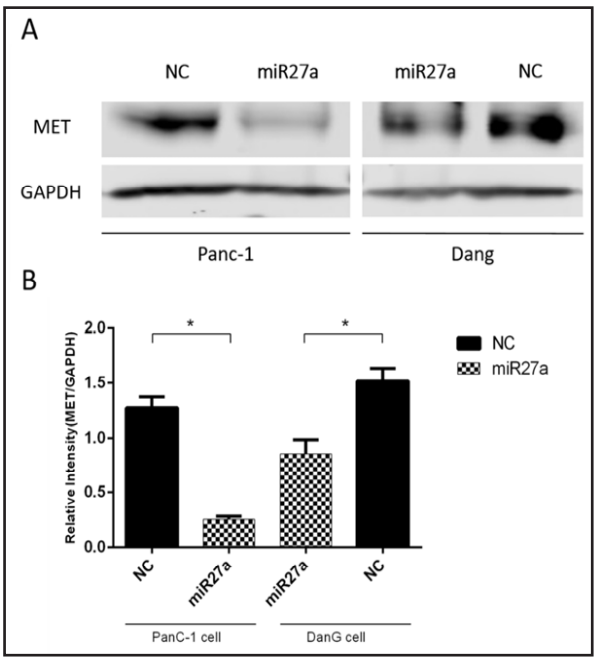

Fig. 5. Expression of MET in Panc-1 and DanG cells transfected with miR27a. (A) 25 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by Western blotting. The membrane was incubated with rabbit polyclonal anti-MET antibody and mouse polyclonal anti-GAPDH as primary antibodies and IRDye® 680CW Goat anti-Rabbit and IRDye® 680CW Goat anti-Mouse as secondary antibodies. GAPDH quantity was used as the loading control; (B) Densitometric values of the bands were calculated and normalized against background using GelAnalyzer 2010 software. Relative intensities (i.e. density ratio of target protein/GAPDH) are represented in a bar diagram, with each bar depicting the mean ± S.D. from 2 different analyses.

Next, we investigated the basal expression of RARα, RARβ, RXRα and RXRβ mRNAs in HPDE cells and PDAC cell lines. The results indicate that the expression of retinoid receptors varied in different cell lines: expression of RARα was significantly decreased in BxPC-3, PanC-1 and MiaPaCa-2 cells, but increased in DanG cells (Fig. 3). Similarly, RARβ was significantly decreased in PanC-1, DanG and MiaPaCa-2 cells when compared to the level in the HPDE cells (Fig. 3). However, the expression of RARβ was more than 8 times higher in BxPC-3 cells compared to HPDE cells (Fig. 3). In PanC-1 and MiaPaCa-2 cells, RXRα expressions were significantly down-regulated compared to HPDE cells. RXRβ expressions are decreased in all four PDAC cell lines compared to the HPDE cell line (Fig. 3).

No obvious correlation between selected miRNAs and retinoid receptor expression

Based on the basal expression of selected miRNAs and retinoid receptors in PDAC cell lines and HPDE cells, we next wanted to find out whether the expression of selected miRNAs correlated with the gene expression level of retinoid receptors. For this purpose, Spearman's rank correlation test was performed (Table 3). In HPDE cells and PDAC cell lines, expression levels of RARβ were positively correlated with the level of miR-27a. Furthermore, expression levels of RXRA were also shown to be positively correlated with miR-206, miR-613 and miR-138. However, no other significant correlations between the selected miRNAs and retinoid receptors were observed.

Despite the positive or absent correlations between retinoid receptors and miRNA expressions, we next wanted to investigate the effect of miR-27a, miR27b and miR9 levels on the gene expression of retinoid receptors due to the following facts: (1) the expression levels of miR-27a, miR27b, miR9, miR10a and miR10b in PDAC cell lines were significantly higher than that in HPDE cells; (2) The absolute expression levels of miR-27a, miR27b, miR9, miR10a and miR10b were not very low or even higher compared to U6 expression in PDAC cell lines and HPDE cells; (3) A real interaction between miR-27a and 3'UTP of RARA and RXRA has been demonstrated in rhabdomyosarcoma cells [33].

Transfection of PDAC cell lines with miR-27a, miR-27b and miR-9 does not influence retinoid receptor expression

To investigate the influence of miRNA on protein expression of the retinoid receptors, PDAC cells were transfected with miRNA mimics and incubated for 48 to 72 h under normal

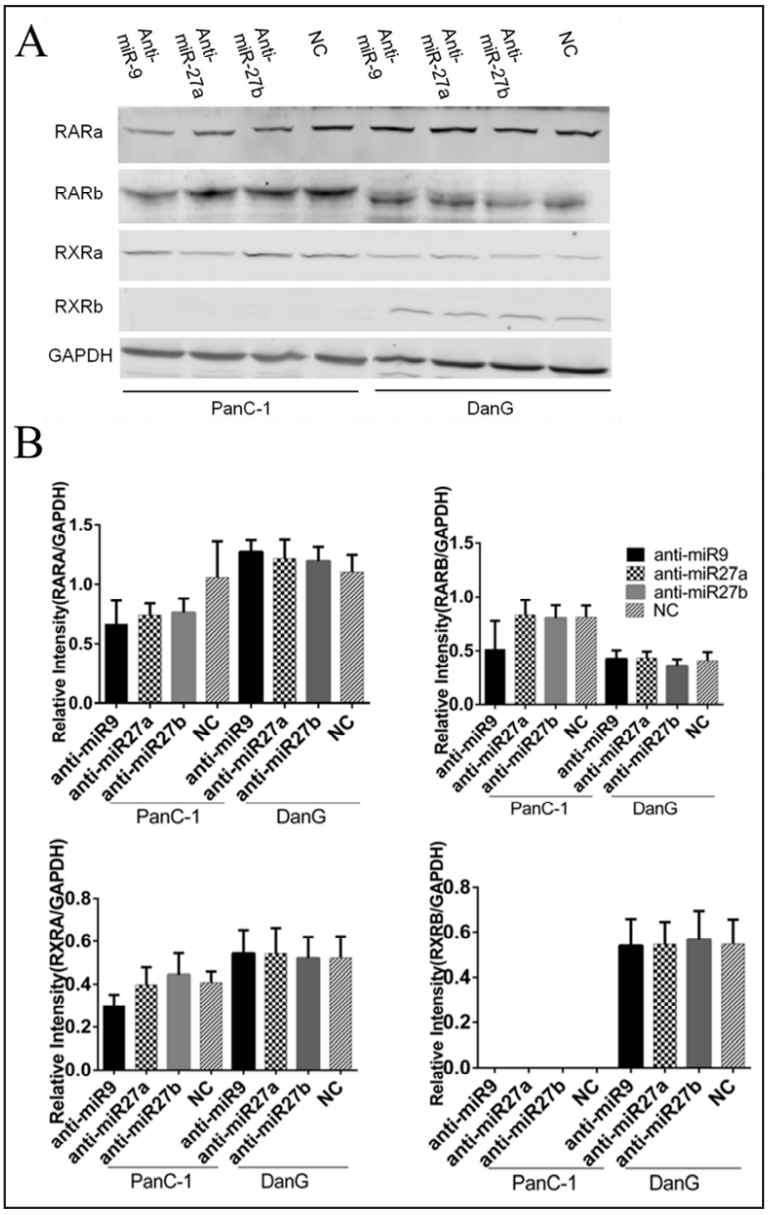

Fig. 6. Expression of retinoid receptors in Panc-1 and DanG cells transfected with anti-miR27a, anti-miR27b or anti-miR9. (A) 20 μg total protein extracted from Panc-1 and DanG cell lines were electrophoresed in 10% SDS-PAGE and followed by Western blotting. The membrane was incubated with rabbit polyclonal antibody (anti-RARa, anti-RARb, anti-RXRa or anti-RXRb) as the primary antibody and IRDye® 680CW Goat anti-Rabbit as the secondary antibody. GAPDH quantity was used as the loading control. (B) Expression of of various retinoid receptors depicted as relative intensities after the transfection of miRNA inhibitors. Densitometric values of the bands were calculated and normalized against background using GelAnalyzer 2010 software. Relative intensities (i.e. density ratio of target protein/GAPDH) are represented as bar diagrams, with each bar depicting the mean ± S.D. from 2 different analyses.

conditions for cell culture. Whole protein contents were isolated and Western blot analyses were carried out as described in the Material and Methods. Western blot analysis revealed that after transfection of miR-9, miR-27a or miR-27b, there were no significant inhibitions of retinoid receptor expressions in both PanC-1 and DanG cells compared with a negative control (Fig. 4).

To be sure that no technical problems have occurred, MET - an experimentally validated target of miR27a, was selected as a positive control [34]. The same amount of miR27a was transfected into PanC-1 and DanG cells with the same procedure, as mentioned above. After incubation for 48 to 72 h under normal conditions, whole protein contents were isolated and Western blot analyses were carried out as described in the Material and Methods. The analysis showed that transfection of miR27a mimics into PanC-1 and DanG cell lines led to a significant decrease in MET protein level (Fig. 5)

Transfection of miRNA inhibitors has no impact on retinoid receptor expressions

Finally, we investigated protein expression of retinoid receptors in PDAC cell lines after the transfection of miRNAs inhibitors. For this purpose, PDAC cells were transfected with

miRNA inhibitor and incubated for 48 to 72 h under normal conditions for cell culture. Whole protein contents were isolated and Western blot analyses were carried out, as described in the Material and Methods. The analyses revealed no significant changes in the expression of retinoid receptors in both PanC-1 cells and DanG cells compared with a negative control (Fig. 6).

Discussion

Consistent with our previous study, we showed in this report that retinoid receptors in PDAC cells are markedly down-regulated compared to normal pancreatic cells. The mechanisms by which retinoid receptors in cancer could be down-regulated include the following possibilities: (1) mutation or deletion of retinoid receptor genes; (2) transcriptional repression; (3) post-transcriptional repression. The fact that the retinoid receptor genes are not mutated in the majority of human cancer specimens indicates that epigenetic events could be involved. Studies have reported that RARβ can be repressed by hypermethylation and histone deacetylation in some types of cancer, resulting in an associated resistance to the growth inhibitory effects of retinoic acid in some cancer types [35-37].

Regulation of retinoid receptor genes on post-transcriptional level can also contribute to the dysregulated retinoid receptor expression in PDAC. Among these mechanisms, miRNA serves as a major pattern regulator of up to 30% of the protein-coding genes in mammals. Regulation by miRNAs result in the destabilization of target mRNA, translational repression and even activation [38].

In this study, we aimed to identify up-regulated miRNAs, which could target retinoid receptors in PDAC cell lines. We found that the selected up-regulated miRNAs (miR27a, miR27b and miR9) were not associated with the down-regulation of retinoid receptors. There were significant correlations between specific miRNA and retinoid receptors, such as the correlation between miR-613 and RXRA, but the correlation coefficient reveals that they are positively correlated rather than negatively correlated. As such, we cannot draw the conclusion that there are significant correlations that are meaningful for our experiments. Since a single miRNA has a lot of putative targets in a particular cell, we can assume that this pleotropic effect of miRNAs manifested in the modulation of expression of some proteins involved in the regulation of retinoid receptor expression.

However, the high expression of miR27, miR-21and miR-23a in PDAC patients was associated with aggressive tumor behavior and poor survival after PDAC resection. Specifically, miR-27a was found to target BTG2, ZBTB10 and Spry2, which are associated with cancer survival, growth and angiogenic responses in PDAC [39]. It was concluded that the triple combination of miR-21/23a/27a could be a prognosis factor of PDAC. These results showing that miR-27a is significantly up-regulated in PDAC cell lines are inconsistent with our results. Interestingly, Tombolan and his colleagues demonstrated that miR-27a is implicated in tumorigenesis as it targets RARα and RXRα expression in the 293T cell line [33]. A reason for this finding could be that specific miRNAs can have different effects in particular cell types and tumor entities.

Based on these findings, we conclude that miR-27a, miR-27b, miR-9, miR10a and miR-10b are up-regulated in PDAC cells compared to HPDE cells. The up-regulation of these miRNAs is not responsible for the down-regulation of RARα, RARβ, RXRα and RXRβ in PDAC cells.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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