Personalized RNA Medicine for Pancreatic Cancer

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Abstract

Purpose: Since drug responses vary between patients, it is crucial to develop pre-clinical or co-clinical strategies that forecast patient response. In this study, we tested whether RNA-based therapeutics were suitable for personalized medicine by using patient-derived-organoid (PDO) and patient-derived-xenograft (PDX) models.

Experimental Design: We performed microRNA (miRNA) profiling of PDX samples to determine the status of miRNA deregulation in individual pancreatic ductal adenocarcinoma (PDAC) patients. To deliver personalized RNA-based-therapy targeting oncogenic miRNAs that form part of this common PDAC miRNA over-expression signature, we packaged antimiR oligonucleotides against one of these miRNAs in tumor-penetrating nanocomplexes (TPN) targeting cell surface proteins on PDAC tumors.

Results: As a validation for our pre-clinical strategy, the therapeutic potential of one of our nano-drugs, TPN-21, was first shown to decrease tumor cell growth and survival in PDO avatars for individual patients, then in their PDX avatars.

Conclusions: This general approach appears suitable for co-clinical validation of personalized RNA medicine and paves the way to prospectively identify patients with eligible miRNA profiles for personalized RNA-based therapy.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer with a very poor prognosis that progresses rapidly. Multiple therapeutic trials developed to date, such as with chemotherapy, radiation, and antistromal therapies, have only resulted in small advances, and survival after diagnosis is generally less than a year (1). Clearly, novel therapeutics based on a better understanding of this disease combined with new drug delivery systems are desperately needed. Here, we tested a preclinical strategy with a novel RNA-based therapeutic tool for personalized PDAC treatment.

miRNAs are small (~18–25 nucleotides) RNAs that can bind target miRNAs in a sequence-specific fashion to induce their posttranscriptional downregulation. Several studies have already identified miRNAs with significantly altered expression between normal pancreas and PDAC tissues; among them, one of the more relevant is miR-21 (2–5). Our laboratory previously generated a mouse model of miR-21 overexpression and revealed that the mice develop tumors in the tissue where miR-21 is overexpressed and that the tumors depend on the continued expression of miR-21 for their survival (6). In addition, our group has already proposed a novel antimiR delivery platform that effectively inhibits oncomiR-155 in another mouse model introducing a new paradigm in the use of anti-miRs as anticancer drugs (7). These lines of evidence highlight the role of “oncomiR addiction” in maintenance of the tumor phenotype in cancer and open avenues for new therapeutic options (8).

Pancreatic cancer is hypovascularized and largely not penetrable, making therapeutic drug delivery a current challenge (9, 10). RNAs are often large and charged, preventing them from easy internalization in the cell. In addition, instability in circulation and rapid renal clearance make the delivery of small RNAs to the tumor site particularly difficult (11). Previous studies testing direct miRNA-based therapy for pancreatic cancer in mouse models have used viral-based vectors, prior antimiR transfection of tumor cells before inoculation to mouse models, or delivery through intratumoral injection (12–16). To date, direct use of antimiRs has failed to target miRNAs in vivo in PDAC (12). We hypothesized that targeted nanoparticles encapsulating oligonucleotide analogues will reduce the rapid clearance of antimiRs and...
increase their delivery and internalization into the PDAC tumor site to prevent the growth of PDAC tumors. In this new strategy, we used nanoparticles containing ‘tandem’ peptides combining a cell-penetrating peptide transportan and tumor-penetrating peptide iRGD (17–19) that have been previously shown to deliver siRNAs to the tumor site in a model of ovarian tumor (20). This particle complexes with RNA through charge and hydrophobic interactions to form tumor-penetrating nanocomplexes (TPN; ref. 21). Because iRGD has been recently demonstrated to enhance delivery and biodistribution of chemotherapy in PDAC (22), we tested the efficacy of TPNs in the delivery of antimiRs to PDAC.

It is accepted that there is a strong disconnect between preclinical work performed in cell lines and xenografts and success of clinical trials (23). For this reason, we tested our miRNA-based therapeutic on patient-derived-xenograft (PDX) samples and patient-derived-organoids (PDO) to better capture human tumor heterogeneity and potentially predict patient response. In our study, through encapsulation in TPN, we successfully delivered antimiR therapeutics to the tumor site for PDAC. We show, for the first time, the potential for a new therapeutic precision medicine strategy that monitors levels of oncomiRs in individual patient tumor samples and that tests RNA-based therapeutic nano-drugs in patient avatars to predict future clinical response in pancreatic cancer.

Results
Clinically relevant oncomiRs form part of a tumor signature in human PDAC patient samples
To identify a PDAC miRNA signature, we worked with an initial set of 27 PDAC snap-frozen tumor samples from PDXs that maintain tumor architecture and clonal diversity (24, 25). This set has known gemcitabine resistance status (24) and KRAS mutation status (Supplementary Table S1A). To determine miRNA expression patterns in PDAC tumors, we conducted a Firefly microRNA assay of our PDAC set compared with 3 normal pancreatic samples. For these assays, 46 miRNAs already known to be associated with KRAS, with pancreatic cancer, or similar diseases were preselected and profiled from our PDAC samples (Supplementary Table S1B). Associated bioinformatics analysis of our profiling revealed the deregulated miRNA expression profile of PDX samples compared with normal samples presented as a heat-map of fold change (Fig. 1A; Supplementary Fig. S1A). An ANOVA test comparing control samples with PDX tumor samples revealed 9 miRNAs significantly deregulated with an adjusted P ≤ 0.01 (dark dot; miR-217 is out of volcano plot) and additional 4 miRNAs significantly deregulated with raw P ≤ 0.01 (blue dot; Fig. 1B).

To functionally validate the oncomiR signature revealed by the PDAC tumor sample analysis, we developed PDAC 3D models, which formed compact and round spheroids after 7 to 8 days in culture (Fig. 1C). We also generated human tumor organoids from 5 independent PDX tumors (Panc 286, Panc 281, Panc 219, PDO 030, and PDO 017) that we characterized and used later as an additional PDAC model for functional tests (Figs. 5 and 6). Firefly microRNA assay was performed on PDAC cell lines, 3D cultures models, and PDO to analyze their miRNA signature (Supplementary Fig. S1B). Interestingly, an ANOVA test comparing all tumor samples (cell lines, 3D models, and PDO) with control samples (including normal pancreatic cell lines and pancreatic organoid from normal early progenitor) revealed that 6 miRNAs (miR-21-5p, miR-146a-5p, miR-196a-5p, miR-196b-5p, let-7i-5p, and miR-29b-5p) from the 13 significantly deregulated miRNAs from PDX profiling are consistently upregulated between PDX and cell line/organoid profiles (Fig. 1D). This group of 6 miRNAs forms part of a PDAC signature and reveals a list of potent PDAC oncomiRs that are commonly upregulated in both PDX cell lines and PDO models. Additional data from a set of 191 patients (prognostic miRNA database; ref. 26) showed that of these 6 oncomiRs, high miR-21, 196a, 196b, and let-7i expression levels in patients are strongly associated with low survival in pancreatic cancer (Fig. 1E) and reveal miR-21-5p, miR-196a, and miR-196b as a significant potential clinically relevant regulators of PDAC initiation and progression and key miRNAs to detect and target in PDAC using an RNA-based medicine approach.

OncomiR inhibition limits PDAC 3D model growth
To test the therapeutic potential of oncomiR inhibition for personalized PDAC therapy for the remainder of this study, we selected one of the 4 oncomiRs that emerged from our analysis above. We chose to first focus on miR-21-5p for our “proof-of-concept” study as it has previously been reported to be prognostic in PDAC (27) and because from this set of patient tumor samples, PDAC cell lines, and organoids, it is part of 8 miRNAs (miR-21-5p, let-7b-5p, let-7a-5p, let-7c-5p miR-22-3p, miR-30b-5p, miR-103-3p, and miR-92-3p) found in relative high abundance. First of all, overexpression of miR-21-5p via the firefly profiling was confirmed in all of the PDAC models by qRT-PCR (Supplementary Fig. S2A).

Analysis of each tumor sample’s miR-21-5p expression showed that all the 27 tumor samples upregulated miR-21-5p (Fig. 2A), which we confirmed by qRT-PCR (Fig. 2B). To support the relevance of this miR-21-5p profile, we tested mRNA expression for two PDAC miR-21-5p direct targets, the tumor suppressors programmed cell death 4 (PDCD4) and phosphatase and tensin homolog (PTEN) in these PDX samples (28). PDCD4 and PTEN mRNA expression in tumor samples revealed a significant inverse correlation with miR-21-5p expression for PDCD4 as well as a strong tendency toward downregulation for PTEN (Fig. 2C and D), supporting a key role played by the oncomiR miR-21-5p in PDAC. To investigate the role of miR-21 in PDAC growth, we functionally inhibited miR-21-5p by generating a GFP-stable cell line where miR-21 activity was inactivated via a LentimiriRa-Off-hsa-miR-21-5p (Lenti-21). Loss of activity of miR-21 was validated by measuring the rescue of miRNA expression of miR-21 targets, including PDCD4 and PTEN (Supplementary Fig. S2B), compared with a Lenti-empty vector control.

Lenti-transduced cells were plated to promote spheroid formation as for Fig. 1C and their relative growth rate studied. Interestingly, whereas cells transduced by the lentivirus control (Lenti-control) grew with the same kinetics as untransfected cells, Lenti-21 spheroids showed a decrease in spheroid number and in size (Fig. 2E). Metabolic activity measurements of these 3D cultures showed that miR-21 inhibition decreased 3D viability by 50%, suggesting that miR-21 inhibition may promote PDAC cell death (Fig. 2F). To validate this idea, we stained for apoptosis by using a specific dye that is cleaved in the nucleus under high levels of activated caspase-3. Analysis of the staining showed a...
Figure 1.

A, A heatmap of miRNA expression in PDAC PDX tumor samples ($n = 27$ cases) compared with normal pancreas samples ($n = 3$ control). Normalized miRNA signal intensities are presented as fold change (log$_{10}$ of the ratio between a probe value to the average of all the other samples for that probe). Green, highly expressed miRNA; red, lowly expressed miRNA. miRNA profiling was done through the firefly circulating miRNA assay and normalized using two miRNAs that are not significantly deregulated between all samples, miR-22-3p and miR-30b-5p defined by the geNorm-like algorithm. B, Volcano plot of statistical significance against fold change between tumor samples group and normal samples group, demonstrating the most significantly deregulated miRNAs. Significantly deregulated miRNAs are classified by adjusted $P$ value (black dot) and raw $P$ value (blue dot; $<0.01$). C, Representative phase-contrast pictures of PDAC 3D sphere models from PANC1 and BxPC3 cell lines and PDO Panc 286 and Panc 281. Scale bar, 1,000 μm. D, Overview of the top commonly deregulated miRNAs from PDX and cell line/organoid profiling. The list of miRNAs was generated by matching the 14 miRNAs significantly deregulated ($<0.01$) from PDX profiling with the 14 miRNAs most deregulated (ranked by $P$ value) from cell line-organoid profiling. miR-21-5p is the only miRNA significantly upregulated in both profiling experiments: (+) is used for upregulated miRNAs and (−) for downregulated miRNAs. E, PROGmiR representation of miR-21-5p, miR-146a-5p, miR-196b-5p, let-7i-5p, miR-196a-5p, and miR-29b-5p expression and overall survival in pancreatic adenocarcinoma. High miR-21-5p, miR-196b-5p, let-7i-5p, and miR-196a-5p expression are clinically correlated with decreased overall survival in pancreatic cancer. *, $P = 0.01$–0.05; **, $P = 0.001$–0.01; ***, $P < 0.001$; ****, $P < 0.0001$; N.S., not significant, two-tailed t test.
significant increase of the caspase-3 activity in the Lenti-21 spheroids at the end of the 9-day assay (Fig. 2G).

miR-21-5p has been previously described to promote pancreatic cancer cell invasion by indirectly increasing the mRNA expression of metalloproteinase-2 and 9 (29). We observed that PANC-1 transduced by Lenti-21 expressed a lower level of these two MMPs, supporting the PDAC growth delay phenotype in 3D culture (Fig. 2H; ref. 29).

Because it is currently not possible to inhibit miR-21 by transduction of a stable lentiviral construct for human therapy, we tested whether miR-21 inhibition by transfection of a mirVana miRNA inhibitor would show similar effects in PDAC cells. In PANC-1, BxPC3, and PL-45, we obtained, respectively, 95.2%, 87.3%, and 64.6% decrease in miR-21 expression at the dose of 50 nmol/L (Supplementary Fig. S2C). The mirVana miRNA inhibitor was used to inhibit miR-21 in the remaining
parts of our study. Through utilization of this anti–miR-21 strategy, we demonstrated that the targeting of relevant oncomiRs counters PDAC tumor progression and reveals the potent role of oncomiR inhibition.

TPNs promote potent delivery of RNA-based therapeutics to PDAC

To optimize delivery and targeting of RNA medicine therapeutic compounds to the tumor site, we encapsulated antimiRs in TPN that had been described to increase tumor targeting through sequential binding to integrins and NRP1 by tumor-penetrating peptide iRGD (20, 22). iRGD specifically binds to αvβ3 integrins, and following proteolytic cleavage, initiates transcytosis and internalization through a semaphorin receptor NRP1 present on tumor cells (17, 30). TPN consists of a C-terminal cell-penetrating peptide iRGD and a N-terminal fatty acid group used to facilitate hydrophobic interactions for self-assembly of nanoparticles. Here, consistent with our prior approach, we selected anti–miR-21 RNA that self-associated with and condensed into nanoparticles, called TPN-21 (Fig. 3A). As it has already been described that cell-penetrating peptide transportan and tumor-penetrating peptide iRGD do not bind and penetrate in low integrins-NRP1 pancreatic cells in vitro and in vivo (18, 19, 21), we first validated the expression of the two binding proteins targeted by TPN-21 in PDAC cells by performing Western blots. Semiquantitative analysis of their expression showed that all the tumor cell lines expressed elevated levels of both receptors (integrins and NRP1), compared with normal pancreatic duct epithelial cell H6c7 (normalized to 1; Fig. 3B). This result indicated that iRGD-containing TPNs should specifically bind integrins/NRP1 positive PDAC tumor models as confirmed in Supplementary Fig. S3A. Next, we demonstrated the capacity of TPN-21 (100 nmol/L) administration without a transfaction agent to inhibit miR-21 expression in PDAC cells at a comparable level to conventional anti–miR-21 transfection (50 nmol/L; Supplementary Fig. S3B).

To test TPN in a 3D PDAC model, we first validated the specific binding of the tumor-penetrating peptide (iRGD) in this context. PDAC spheres were incubated with iRGD-TAMRA for 2 hours and observed under a fluorescence microscope. We observed a strong fluorescent signal on the sphere surface corresponding to iRGD-TAMRA binding after 2- to 4-hour incubation (Fig. 3C). In addition to surface binding, it was important to assess whether the TPNs could be internalized into the sphere. For this purpose, TAMRA-TPN delivery was monitored by confocal microscopy. Z-stack confocal images (Fig. 3D) revealed binding and lumen internalization of TPN-21 (TAMRA-TPN-21) into spheres generated with two different PDAC samples.

As previously observed after targeting the stable cell lines to inhibit miR-21 (Fig. 2E), repeated administration of TPN-21 (100 nmol/L) following an every-other-day treatment plan (Supplementary Fig. S3C) reduced the number and size of 3D models of PDAC (Fig. 3E). Analysis of miR-21 expression by qRT-PCR on treated spheres revealed a 70% knockdown of miR-21, indicating successful delivery and functional activity of the anti–miR-21 compound in the 3D model (Fig. 3F). It has already been described that use of tumor-penetrating peptide iRGD accumulates in a tumor-specific manner in PDAC (18). Here, to validate the in vivo homing behavior of iRGD-TPN-21, we generated a PDAC orthotopic model in mice and performed a single intravenous injection of TAMRA-TPN-21 allowing us to assay for TPN-21 fluorescence in mouse organs. Analysis of TPN-21 biodistribution 6 hours postinjection was performed by analyzing tissue fluorescence. As commonly found for nucleic acid delivery, after 6 hours, the highest fluorescent accumulation remains in the liver (31), but relatively high levels of fluorescence were also detected in the tumor site, compared with other organs from the reticuloendothelial system (spleen and lung; Fig. 3G). Altogether, these data suggest that iRGD-TPN-21 will promote pancreatic tumor–selective delivery of anti–miR-21 therapy to the PDAC tumor site.

RNA-based therapy reduces RC model tumor growth and restores PTEN and PDCD4 expression

To explore the clinical potential of the results observed in 3D models, we used a mouse model to test TPN-21 therapy efficiency in vivo. We first focused on the D8-175 mouse cell line (mPDAC) derived from Pdcl-Cre; KrasG12D;Tp53R172H (RCP) mice (32) that bear the most common mutations found in PDAC patients (KRAS and TP53). We demonstrated that mPDAC cells highly expressed the two TPN-21 receptors of TPN (integrins and NRP1; Fig. 3B). We observed an 80-fold upregulation of miR-21-5p by qRT-PCR in mPDAC compared with normal mouse pancreas (Fig. 4A). We also validated that use of anti–miR-21 promotes apoptosis on mPDAC cells by analyzing the costaining of active caspase-3 (apoptotic cells) and DAPI (total cells) and observed a 2-fold change in apoptotic rate after anti–miR-21 treatment (Fig. 4B).

To test whether use of RNA-based therapy can disrupt tumor maintenance and be a successful approach for PDAC patients, we first tested antimiR treatment through TPN-mediated delivery in vivo. We generated a PDAC mouse model by injection of 5 × 10^6 mPDAC cells/flank in NOD/SCID mice (Fig. 4C). Animals bearing mPDAC allograft were intravenously injected with TPN-control or TPN-21 (5 mg/kg of antimiR) 2 times per week over 36 days, starting when the tumor volume reached approximately 150 mm in diameter. Tumor size measurements revealed that delivery of anti–miR-21 significantly reduced mPDAC tumor growth after only 1 injection (Fig. 4D). As expected from our 3D analysis (Fig. 3E), repeated, systemic administration of the TPN-21 strongly slowed tumor growth along the entire treatment course from day 1 to day 36 and resulted in a 51.1% suppression of tumor growth (15.07 vs. 30.83 relative tumor burden size vs. baseline) at the end of the treatment (Fig. 4E). Interestingly, tumor growth is most affected in the first 24 days of treatment with TPN-21 compared with TPN-control (4.25 vs. 14.37 relative tumor burden vs. baseline), suggesting a more potent effect of anti–miR-21 in the early stages of tumor growth.

To validate miR-21 targeting to the tumor site following TPN-21 therapy, mPDAC tumors were collected at the end of the treatment, and miR-21 expression was determined by qPCR. We found that miR-21 expression was significantly decreased in the mPDAC tumors group treated with TPN-21 compared with the mPDAC tumors group treated with TPN-control (Fig. 4F). As a consequence of miR-21 inhibition, we observed a significant restoration of miR-21 targets, PTEN and PDCD4 (Fig. 4G). Altogether, these data showed that use of TPN-21 allowed delivery of anti–miR-21 into the tumor site promoting inhibition of miR-21 expression and upregulation of tumor suppressor PTEN and PDCD4, which correlated with reduced tumor growth.

On the basis of our result, we demonstrate that use of TPN approach is suitable for safe and potent delivery of RNA-based therapy (antimiR oligonucleotides) compound to the tumor site.
Figure 3.

A, Representation of TPN loaded with anti-miR-21 (TPN-21). B, Western blot analysis of two receptors (NRPI and integrins) bound by the TPN. Column 1 (H6c7 normal pancreatic cell lines), columns 2–7 (PDAC human and mouse cell lines). Western blot band corresponding to NRP1 and integrins have been quantified against tubulin levels and normalized against normal cell lines (1). C, TAMRA-TPN-21 (red) binding in PDAC 3D models after 1 hour of treatment. Nuclei were stained with DAPI. Scale bars, 100 μm (PANC-1) and 50 μm (BxPC3). D, Z-stack confocal images show binding of TAMRA-TPN-21 to the PANC1 and BxPC3 spheroid and internalization into the lumen after 2-hour treatment. E, Quantification of number and size of 3D sphere after TPN-21 treatment and representative picture of Bxpc3 and PANC1 3D models after repeated TPN-21 treatments. Sphere size was measured through imageJ software on a total of 35 spheroids and is presented in arbitrary units (UA). F, qPCR analysis of miR-21-5p expression level after TPN-21 treatment. G, Tumor biodistribution of fluorescent-TPN-21 in organs (1, liver; 2, spleen; 3, lung; 4, kidney; 5, heart) and tumor site (6, pancreas) after 48-hour injection in an orthotopic KC model of PDAC (n = 2). For qPCR, each miRNA sample was normalized on the basis of its 18s content and on the basis of GAPDH for mRNAs. Error bars, mean ± SD. *, P = 0.01–0.05; **, P = 0.001–0.01; ***, P < 0.001; ****, P < 0.0001; N.S., not significant, two-tailed t test; n = 3 biological replicates and n = 2 biological replicates for Western blot analysis.
TPN-21 impacts human PDO growth and predicts PDX response

To study patient responses to the TPN-21 strategy, we generated human tumor organoids from PDX tumors. We selected one patient sample that has been studied extensively and shows a high degree of concordance between the human sample biopsy and the biopsy growing in mice (24). In addition, this PDX has been characterized as gemcitabine resistant (Supplementary Table S1B) with a poor therapeutic outcome. To generated PDO 286, fresh PDX tumor fragments were minced and processed accordingly to protocol (33) and grown in Matrigel (Fig. 5A). These organoids formed as multilayers of cells with diameters between 50 and 150 μm. They were largely filled with tumor cells, but also exhibited the presence of microlumens. The general morphology of organoids shown by H&E resembled the tumor tissues in vivo (Fig. 5B). In addition, we showed by IHC that PDO 286 expressed both TPN receptors (Fig. 5B). Before testing our RNA-based strategy

Figure 4.

A, qRT-PCR of miR-21-5p expression level in the PDAC (mPDAC) cell line compared with normal pancreas. B, Nucview staining (red) normalized to total cells stained with DAPI (blue) reports significant increase of the caspase-3 activity after TPN-21 treatment in mPDAC cells. C, mPDAC flank tumor model. D, Relative tumor burden after (1, 3, 5, and 10 intravenous injection of TPN-control n = 7 or TPN-21 n = 10 (5 mg/kg)). E, Relative tumor burden after intravenous injection of TPN-control or TPN-21 (5 mg/kg) over the 36 days. F, qPCR analysis of miR-21-5p expression level in the tumor site at the end of treatment (TPN-control n = 5; TPN-21 n = 5). G, qPCR analysis of PTEN and PDCD4 expression level at the end of treatment (TPN-control n = 5; TPN-21 n = 5). For qPCR, each mRNA sample was normalized on the basis of its 18s content and on the basis of b-actin for mRNAs. Error bars, mean ± SD. *, P = 0.01–0.05; **, P = 0.001–0.01; ***, P < 0.001; ****, P < 0.0001; N.S., not significant, two-tailed t test; n = 3 biological replicates.
on PDO, we first validated that the general miRNA profile was maintained between this PDX and this PDO. By statistically analyzing the miRNA profile of PDX 286 and PDO 286, we observed a general concordance of miRNA expression levels, with strong correlations (Spearman $\rho = 0.75$) and high statistical significance ($P = 2.7\times10^{-8}$) between the two samples (Fig. 5C). We also validated that the most deregulated miRNAs from both profiles (miR-21-5p, miR-146a-5p, miR-196b-5p, let-7i-5p, ...
miR-196a-5p, miR-29b-5p; Fig. 1D) are also commonly up-regulated in PDO 286 relative to their own control (Fig. 5C).

To investigate the binding and internalization of TAMRA-TPN into the PDO, PDO 286 was incubated with TAMRA-TPN for 2 hours and observed under a fluorescence microscope. We validated that the fluorescent peptide bound 90% of the organoids after 2 hours (Fig. 5D). To test TPN-21 on this patient avatar, we performed repeated administration of TPN-21 (100 nmol/L) following an every-other-day treatment plan (Supplementary Fig. S3C). We counted the number of organoids growing per well at days 3, 5, 7, and 9 of treatment. A delay in organoid growth was apparent after only 1 treatment, reaching a maximum effect at 9 days (Fig. 5E). This observation showed that TPN-21 induced a strong inhibitory effect on tumor growth starting from early times (Fig. 5E). To test this effect, we used a dose-response study by treating PDO with TPN-21 or TPN-control for 9 days at different concentrations (12.5, 25, 50, and 100 nmol/L) following the same every-other-day treatment plan (Supplementary Fig. S3C). We observed that repeated administration of TPN-21 significantly decreased the number of organoids per well from the dose of 25 nmol/L compared with TPN-control, whereas at the 100 nmol/L dose, we observed a decrease of 50% of the number of organoids (Fig. 5F). In parallel, we performed a metabolic activity measure by using MTT on the organoids, which confirmed significant inhibition of their viability (42%) after 4 repeated administrations of 100 nmol/L TPN-21 (Fig. 5F).

For the PDO screen for personalized RNA-based therapy (PDAC) or tumor microenvironment where drug delivery is constrained by tumor type has limited their therapeutic use to liver and kidney disease (35–37). The work described here demonstrates a targeted approach to deliver RNA-based therapy including anti-miR-21, and gemcitabine in PDAC, use of TPN-21 therapy sensitized resistant PDOs to gemcitabine treatment by reducing organoid size and viability (Supplementary Fig. S5A and S5B; ref. 34).

More interestingly, the 2 “low-miR-21” PDOs (PDO 030 and PDO 017) did not show sensitivity to TPN-21 therapy under these conditions (Fig. 6F–I). These results suggest that the patients (PDO/PDX) that do not overexpress miR-21 will potentially not respond to TPN-21 therapy. Altogether, these results demonstrate that a rapid screen of PDO is capable of evaluating and predicting patient response to TPN-21 therapy and suggest that miR-21 overexpression in patient samples could be used as a biomarker for predicting favorable outcome to TPN-21 therapy. In addition, our study conducted on PDO 286 and PDO 219 strongly suggests the favorable therapeutic potential of TPN-21 use for gemcitabine-resistant patients.

Discussion

RNA therapeutics for cancer, including anti–miR-21, have been discussed for the past few years, but their reported lack of delivery to most disease tissues has limited their therapeutic use to liver and kidney disease (35–37). The work described here demonstrates a targeted approach to deliver RNA-based therapy including antimiRs and potentially mimics, to a specific tumor type (PDAC) or tumor microenvironment where drug delivery is routinely poor. By packaging RNA-based oligonucleotides in TPNs targeting specific PDAC tumor receptors, we propose safe and efficient delivery of RNA therapy, paving the way for the use of TPN-21 as a therapeutic anticancer agent for PDAC. We demonstrated that TPN-21 strongly restrains PDO growth, even where gemcitabine failed, and limits PDAC growth in vivo (Figs. 5E–G and 6B). Currently, numerous personalized models that help
Figure 6.
A, Representative pictures and qPCR analysis of miR-21-5p expression levels in 4 PDO (Panc 286, Panc 219, PDO 030, and PDO 017). Scale bar, 1,000 μm. B, D, F, and H, PDO metabolic activity measured from MTT assay after repeated TPN-21 treatments and representative pictures of PDO 286, PDO 219, PDO 030, and PDO 017. Scale bar, 1,000 μm. C, E, G, and I, Quantification of organoid size after TPN-21 treatment. Organoids size was measured through ImageJ software on a total of 35 spheroids and is presented in arbitrary units (UA). For qPCR, each miRNA sample was normalized on the basis of its 18s content.

* P = 0.01–0.05; ** P = 0.001–0.01; *** P < 0.001; **** P < 0.0001; N.S., not significant, two-tailed t test.
guide precision medicine are emerging (33, 38–40). In this study, through the utilization of PDO as a rapid screen for TPN-21, we show for the first time the potential of personalized models for RNA-based precision medicine therapy in PDAC. By working with patient avatars, we have shown the potential to harness the ability of PDO and PDX models to predict clinical outcomes of patients and thus align preclinical to patient response to TPN-21 therapy, a key goal in personalized medicine (41).

Our work reveals that for PDAC, miR-21 is both a companion diagnostic and the therapeutic target, a therapeutic. Upregulation of miR-21 in tumor and stromal tissues is currently clearly associated with several gastrointestinal tumor types (pancreatic cancer, esophageal, gastric cancer, liver cancer; refs. 42, 43) and resistance to multiple anticancer agents (44) and is a promising noninvasive biomarker for pancreatic cancer risk and detection (24, 45, 46). As miR-21 is already a detectable biomarker for PDAC (27, 47, 48), in a future phase I clinical trial, we propose to prospectively identify patients presenting with a similar miRNA signature that could be eligible for this biomarker-based RNA therapy.

In the near future, additional antimiRs (and miRNA mimics) could be packaged into the TPN system and tested on PDO, including the 3 key miRNAs deregulated from our PDAC profiling (TPN-7i, TPN-196a, TPN-196b for anti-let-7i, anti-miR-196a, and anti-miR-196b). Use of miRNA-based therapy for PDAC treatment is very novel and is positioned at the forefront of current PDAC and RNA medicine therapeutic challenges. This general approach, described here through this successful proof of principle using anti-miR-21 theranostics, shows very high potential not only for clinical significance but in the establishment of integrative screening methodologies with broad applicability to other cancers and lethal diseases with notoriously dismal clinical outcomes.

Materials and Methods

miRNA profiling

For miRNA analysis, total RNA was isolated from frozen PDO tissue samples using the miVana miRNA Isolation Kit following standard protocol (Invitrogen). Total RNA samples were sent to Abcam Firefly group for miRNAs FireFly analysis. Data analysis and plotting heatmap and volcano plots were done through FireFly Analysis Workbench software for multiplex miRNA assays. For PDO tissue sample data analysis, miRNAs were normalized using miR-22-3p and miR-30b-5p and were defined by the geNorm-like algorithm for cell lines, 3D model, and organoids, miRNAs were normalized using miR-181b-5p, miR-103-3p, and miR-30b-5p, which were defined by the geNorm-like algorithm.

Tumor mouse models

For generation of PDO mouse models, tumor organoids were grown in 3D tissue culture for 16 days and then dissociated with collagenase/dispase, Accutase. Disassociated cells were resuspended in organoid growth media supplemented with 50% Matrigel and 10 μmol/L Y27632 at final concentration 2.5 × 108 cells/mL, and the suspension was kept on ice all the time. Six-week-old nude mice were injected with 100 μL cell suspension on the right flank of each mouse. Tumor growth was monitored twice a week by observation and palpation. We defined approximately 100 mm3 tumors as the starting point to perform a treatment trial. Mice were treated 2 times a week for a total of 4 intravenous injections with either TPN-21 or TPN-control (5 mg/kg oligonucleotides) or saline solution as an additional control. At the end of the treatment, mice were sacrificed, tumors were collected, and samples were processed appropriately. All procedures were conducted following an institutionally approved animal IACUC protocol. We conducted therapeutic trials in a subcutaneous model of pancreatic cancer driven by D8-175 cell line. Bilateral flank allografts were implanted on 6-week-old NCR/nude mice each seeded with 5 × 105 cells. Mice were divided into groups of 5, with nearly equal average and standard deviation of tumor burden at the start of treatment (~150 mm3 total tumor burden per mouse). TPN-21 or TPN-control (5 mg/kg oligonucleotides) or saline solution was administered intravenously twice every week until the tumor burden reached or exceeded 1 cm according to the approved animal operation protocol. For biodistribution study, D8-175 cells carrying stably expressed luciferase were suspended in PBS and directly injected into the parenchyma of pancreatic tails of nude mice (5–6 weeks) at 5 × 105 cells in 100 μL per mouse. The rate of tumor formation was tracked using IVIS system every week after surgical operation. To investigate biodistribution of TPNs in this orthotopic PDAC model, 100 μL of fluorescence labeled nanoparticles (TAMRA-TPN-21) carrying 7.5 μmol/L of miR-21 antagonists were injected through intravenous administration into tumor-bearing mice. Four hours after TAMRA-TPN-21 injection, mice were sacrificed; major organs (liver, spleen, lung, kidney, hear, tumor) were extracted and scanned using a LI-COR Odyssey Near-Infrared Imaging System. Fluorescence intensity of each organ was measured and quantified using ImageJ.

qPCR

3D models and organoids were separated from Matrigel by washing with PBS. Cell lines, 3D models, and organoids were lysed using TRIzol, and RNA extraction was done using the miVana miRNA Isolation Kit. cDNA synthesis was performed using miScript II RT Kit (Qiagen) with 250 ng RNA as input. qRT-PCR was performed using the Roche480 Light Cycler miRNA system as per the manufacturer’s instructions, and gene expression was normalized to U6 and/or 18s. For miRNA analysis, cDNA synthesis was performed using Verso cDNA Synthesis Kit (Life Technologies) using 500 to 1,000 ng RNA as input. cDNA was used for SYBR Green–based real-time PCR. Gene expression was normalized to GAPDH and or β-actin. Hsa-miR-21-5p and U6 miScript Primer and 18s, MMP-2, MMP-9, GAPDH, β-actin, PTEN, and PDCD4 QuantiTect Primer are from Qiagen.

Cell lines and antimiR transfection

PANC-1, PL-45, BxPc3, and Capan-2 were obtained from the ATCC and cultured at 37°C with 5% CO2 in DMEM, RPMI1640, and McCoy’s 5a Medium Modified supplemented with 10% FBS and 1% penicillin/streptomycin. H6c7 (human pancreatic duct epithelial cell line) and ECA001 were obtained from Kerafast and maintained in Keratinocyte Basal Medium + supplied supplements (Lonza, Clonetics KBM, cat#CC-3111). D8-175 is a cell line derived from Pdx1-Cre; KrasG12D; Trp53−/− (KPC) mice and was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cell lines were tested for mycoplasma using the Lonza MycoAlert Detection Kit. For RNA-based functional experiments, miVana miRNA inhibitor for hsa-miR-21-5p and miVana miRNA Inhibitor, negative control #1 (Life Technologies) was used at 50 nmol/L and transfected into cells using RNAiMAX (invitrogen), as per the manufacturer’s protocol.
3D models and organoids
Snap-frozen PDAC tumor samples used in this work were obtained from the M. Hidalgo’s PDX collection. The pancreatic cancer PDXs were generated as described in refs. 10 and 25. The KRAS mutational status and sensitivity to gemcitabine were routinely analyzed for most of the PDX models within the collection. To generated PDAC 3D cell culture models, 2,000 PANC-1 or BxPC3 cells were mixed with Geltrex Matrix (Thermo Fisher Scientific) and DMEM or RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin as 1:1 ratio and plated on nontissue culture treated 96-well plates (BD Falcon). After the matrix solidified, 100 µL medium was added on the well, and spheroids were grown under standard culture conditions (5% CO₂, 37°C). Normal human pancreatic progenitors were derived from human pluripotent stem cells through a two-stage induction protocol, in DMEM based media including B27, insulin, hydrocortisone, and other factors, as described previously (33). Tumor organoids and PDX mouse models were generated as described by Huang and colleagues (2015). Fresh PDX tumor fragments were minced with a no. 22 blade into 1 to 2 mm small pieces then digested with 1 mg/mL collagenase/dispase for 30 minutes, follow by Accutase digestion for 40 minutes. The slurry was resuspended in DMEM and then filtered through a tissue strainer and centrifuged at 1,500 rpm for 5 minutes. After aspirating the supernatant, we resuspended the cell pellet with organoid growth medium supplemented with 5% Matrigel and 10 µmol/L Y27632. Organoids were grown under standard culture conditions (5% CO₂, 37°C).

To perform a dose–response study, organoids were treated with TPN-21, TPN-control, or gemcitabine (HCl, Calbiochem; 12.5, 25, 50, and 100 nmol/L) following an every-other-day treatment plan. Organoids were tested with gemcitabine at 100 nmol/L alone or combined with TPN-21 following an every-other-day treatment plan.

Viability and apoptotic assay
Viability assay on 3D models was performed by using MTT reagents from Sigma (M2128) according to the manufacturer's recommendations. The apoptosis assay was performed by using Nuview 530 Caspase-3 Substrate (Biotium). Briefly 3D models were incubated with Nuview reagent (1/500) and Hoechst (1/1,000) for 30 to 60 minutes, and the red signal corresponding to caspase–3–positive structures was counted and normalized to the total number of structures. Viability and apoptosis of 3D models and organoids after treatment was conducted on 3 independent wells well per condition in each experiment for 3 independent experiments.

Western blotting
The H6c7 normal pancreatic cell line and PDAC human and mouse cell lines were cultured and lysed in 1× RIPA buffer with protease and phosphatase inhibitor (Thermo Fisher Scientific) for 30 minutes on ice. The whole-cell lysates were then clarified by centrifugation for 25 minutes at 15,000 rpm at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Equal amounts (10 µg) of protein samples were fractionated by a Novex 4% to 20% Tris-Glycine gel (Thermo Fisher Scientific), transferred to nitrocellulose membranes (Thermo Fisher Scientific), and blocked in Odyssey blocking buffer (LI-COR Biosciences). Integrin was probed by primary rabbit antibody (1:1,000; Abcam, ab52971), NRP-1 was stained using primary rabbit antibody (1:500) (Abcam, ab81321), and tubulin was measured using primary rabbit antibody (Cell Signaling Technology, #2144). The desired bands were detected with labeling with anti-rabbit (1:10,000) IgG–IRDye 680 secondary antibodies and visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

TPN and binding assay
All peptides in this study are produced by solid-phase peptide synthesis CPC Scientific. Tandem peptide (pTP–iRGD) has the sequence CH3(CH)15-GWTLNSAGYLLGKINLKALAALAKKIL–C14). The desired concentration was obtained by CPC Scientific. mirVana miRNA inhibitor against hsa-miR-21-5p and non-targeting controls were obtained from Thermo Fisher Scientific. PEGylation method of the tandem peptide was described previously (49). To form the TPNs in solution, miRNA inhibitor, PEGylated pTP–iRGD, and pTP–iRGD were resuspended in nuclease-free water and mixed with the miRNA inhibitor, PEG-containing component, and peptide in 1:2.5:15 molar ratios, first by thoroughly mixing the miRNA with the PEGylated pTP–iRGD and by subsequently mixing in the peptide to create a concentrated solution of TPNs that were adjusted to the desired dilution and buffer composition with appropriate diluent. Binding of iRGD-TAMRA on cells was done at 4°C on ice. The H6c7 normal pancreatic cell line and PDAC human and mouse cell lines (PANC1 and D8-175) were placed for 30 minutes on ice before addition of iRGD-TAMRA (10 nmol/L). After 15-minute treatment with iRGD-TAMRA, cells were washed 3 times with PBS and fixed with 4% PFA and stained with DAPI.
Statistical Analysis
Statistical analysis was achieved by using a two-tailed unpaired t test using GraphPad Prism software. Two-way ANOVA has been done for miRNA profiling assays. All graphs show means ± SD (\( P = 0.01–0.05 \); **\( P = 0.001–0.01 \); ***\( P < 0.001 \); ****\( P < 0.0001 \); N.S., not significant). For qRT-PCR data, means and SD were calculated at the ddCt level before being converted to fold changes as presented in the graphs (50).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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