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MicroRNA Regulation of K-Ras in Pancreatic Cancer and Opportunities for Therapeutic Intervention

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Abstract

The Ras family of GTPases is involved in cell proliferation, cell survival, and angiogenesis. It is upregulated in several cancers, including pancreatic cancer (PC) and leads to uncontrolled growth and aggressiveness. PC is well known to be a lethal disease with poor prognosis, plagued by limited therapeutic modalities. MicroRNAs (miRNAs), which are short non-coding RNA molecules, have recently emerged as regulators of signaling networks and have shown potential to target pathway components for therapeutic use in several malignancies. K-Ras mutations are widespread in PC cases (90%), with mutations detectable as early as pancreatic intraepithelial neoplasias and in later metastatic stages alike; therefore, these mutations in K-Ras are obvious drivers and potential targets for PC therapy. Several K-Ras targeting miRNAs have lately been discovered, and many of them have shown promise in combating pancreatic tumor growth *in vitro* and in mouse models. However, the field of miRNA therapy is still in its infancy, and miRNA mimics or anti-miRNA oligonucleotides that target Ras pathway have thus far not been evaluated in PC patients. In this review, we summarize the role of several miRNAs that regulate oncogenic K-Ras signaling in PC, with their prospective roles as therapeutic agents for targeting K-Ras pathway.

Introduction

Ras belongs to a family of small guanosine triphosphatases (GTPases) that acts as a molecular switch by transitioning between the active GTP-bound Ras protein and the inactive GDP-bound state, thus coupling cues from cell membrane-bound growth factor

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receptors to intracellular signaling pathways. Ras is one of the most frequently mutated genes in human tumors [1]. In fact, aberrant Ras signaling can cause malignant transformation, a property which initially led to identification of the *RAS* genes as retroviral oncogenes in the 1960s from the genome of Harvey and Kirsten rat sarcoma (Ras) viruses [2]. Ras proteins relay signals from diverse stimuli, including growth factors, cell stress, and interaction with extracellular components via receptor tyrosine kinases (RTKs), integrins, or ion channels; they function upstream of diverse signaling pathways associated with cancer including the Raf/Mek/Erk, PI3K/PTEN/Akt pathways [3] (Figure 1). Signaling pathways triggered in response to activated Ras regulate diverse physiological responses, including cell cycle progression, cell proliferation, migration, adhesion, cytoskeletal changes, apoptosis, and senescence [4].

In untransformed cells harboring wild-type Ras, the Ras pathway is tightly regulated at multiple levels. First, the ratio of GTP-bound Ras (active) to GDP-bound Ras (inactive) depends on levels and activity of GAPs (GTPase activating proteins) and GEFs (Guanine nucleotide exchange factors); GAPs promote GTP hydrolysis and conversion to the inactive GDP-bound state, whereas, GEFs promote recycling to the active, GTP-bound state by reducing the affinity of Ras for GDP [5]. Second, scaffolds and adaptors proteins act as regulators of the Ras/Mapk pathway by controlling proper localization of signaling molecules, such as SPRED1, critical for membrane localization of GAPs [5, 6]. Third, Ras signaling can also be modulated by the microRNAs (miRNAs) that constitute an additional layer of gene regulation via affecting translation of Ras pathway components, scaffolds, and/or regulators [6–8].

Activating point mutations in Ras proteins result in constitutive activation of Ras signaling in cancer, due to higher amounts of active GTP-bound Ras. Following the main biochemical differences between oncogenic Ras mutants and wild-type Ras render higher constitutive pathway activation by Ras mutants: 1) point mutations in Ras reduce the rate of intrinsic GTPase activity of Ras proteins by one order of magnitude; 2) activity of GAP to hydrolyze GTP is reduced or eliminated; 3) certain isoforms may have a higher affinity for downstream signaling proteins such as Raf [9].

Pancreatic cancer (PC) is the third-leading cause of cancer death in the United States. On average, of 50,000 people diagnosed, in the US alone, more than 45,000 patients will succumb to PC each year [10]. Despite available therapies, prognosis remains poor. Our inability to target K-Ras, the driver mutation for PC, is one handicap to successfully improving survival in this disease. MicroRNAs (miRNAs) have recently emerged as regulators of critical signaling networks including the Ras pathway, and have been targeted for therapy in several cancers [11–15]. As a result of extensive preclinical studies in PC conducted to elucidate the crucial role miRNAs play in the initiation, progression, and chemoresistance of the disease, it is clear that miRNAs can be exploited to develop novel therapeutic approaches against it [16].

Here, we discuss the microRNA-mediated regulation of Ras signaling in cancer. We briefly discuss Ras mutations in cancer generally, and the status of K-Ras mutations in pancreatic cancer. We describe the means by which miRNAs can regulate signaling, and present

specific examples of how K-Ras targets miRNAs in various cancers. The focus is on miRNAs targeting K-Ras in PC, their functions and regulation, and opportunities for therapeutically exploiting miRNAs in pancreatic cancer, along with modifications and delivery systems that have been used to make miRNAs more amenable for therapy.

K-Ras pathway in pancreatic cancer

Ras mutations in cancer

Somatic mutations in any one of the three canonical RAS family genes (*K-RAS, N-RAS and H-RAS*) are among the most common events in human tumorigenesis. Oncogenic Ras mutations in human *K-RAS* gene are found within two hotspots, located in codons 12 and 61 of their highly conserved coding sequences [1]. Mutations in these three *RAS* genes are observed in up to about 30% of all human tumors. Oncogenic mutations predominantly affect *K-RAS* locus, with 25–30% of human tumors screened harboring K-Ras mutations [1, 17], which appear early in the course of tumor progression. Whereas, only 8% and 3% of samples screened harbor oncogenic mutations in the N-Ras and H-Ras genes, respectively. Mutations in Ras family members across different cancers have been studied extensively over the past three decades, and there appears to be an association of specific Ras isoform mutations with particular types of tumors. For instance, the majority of pancreatic ductal adenocarcinomas and significantly high percentages of lung and colon cancers harbor mutations in *K-RAS* gene. However, K-Ras mutations in bladder cancers are rare, where H-Ras is the most frequently detected mutated isoform. By contrast, hematopoietic malignancies as well as malignant melanomas have a high incidence of N-Ras mutations, whereas the rate of K-Ras or H-Ras mutations in these cancers is marginal [1, 17].

K-Ras in pancreatic cancer

Pancreatic adenocarcinomas are among the worst prognostic tumors in people, and hold the highest reported incidence of K-Ras mutations, ranging in the literature from 69% to 95% [1]. Evidence suggests that K-Ras mutations are drivers for pancreatic adenocarcinomas; they represent an early event in tumor evolution, and even samples from chronic pancreatitis, considered a risk factor for pancreatic cancer, harbor K-Ras mutations [18]. The majority of mutations affect K-Ras codon 12 and result in a replacement of glycine with either aspartic acid, arginine, or valine.

It is clear that K-Ras plays a significant role in pancreatic tumorigenesis, and there appears to be an association between K-Ras mutations and poor prognosis of pancreatic cancer. Recent studies suggest that non-resectable pancreatic tumors harboring K-Ras mutations have a worse prognosis and that detection of K-Ras mutations in cancer adjacent normal tissue surrounding the surgical margins of resected pancreatic tumors is associated with shorter survival [19, 20]. Of note, the type of K-Ras mutation leading to a particular amino acid replacement has also been reported to influence the aggressiveness of the disease. For instance, K-Ras G12R and G12A mutations in pancreatic tumors were reported to have worse survival rates compared to tumors bearing G12V or G12S mutations [21, 22]. Since K-Ras mutations are highly prevalent across pancreatic tumors and likely contribute to cancer progression, they have been explored as a therapeutic target. Targeting K-Ras in cell

lines and mouse xenograft models have yielded promising results for combating PC [23–25]. However, clinical trials with farnesyltransferase inhibitors, aimed at blocking posttranslational modifications of K-Ras proteins, have not been successful [26–28]. Therefore, it is important to understand the nuances of Ras signaling in normal versus malignant conditions to be able to exploit these differences for treatment. Moreover, alternative strategies of targeting oncogenic K-Ras must be explored.

Interactions between miRNAs and K-Ras in PC

Regulation of signaling via miRNAs

miRNAs are small (19–25 nucleotide in length), non-coding RNAs predominantly transcribed by RNA polymerase II in humans [29]. Mature miRNAs participate in translation repression of mRNA transcripts by being incorporated into multiprotein complexes termed RNA-induced silencing complexes (RISCs) [30]. One of the stands of the mature miRNA duplex is retained in RISC based on relative thermodynamic stability. The RISC then scans across the mRNA targets, and selectively arises from sequence complementarity in the 3′ untranslated region of mRNAs. The miRNA seed sequence that consists of the miRNA sequence between bases 2–8 at the 5′end of the miRNA plays a dominant role in determining mRNA target specificity [29]. miRNAs can then cause translational repression based on sequence complementarity by: a) blocking translation initiation; b) degrading mRNA; and c) site-specific cleavage of the target mRNA [29, 31].

Multiple miRNAs can regulate translation of a single mRNA transcript and, conversely, a single miRNA may target many different protein-coding transcripts involved in various signaling pathways. Of note, the collaborative action of miRNAs can determine the expression of pathway components, thereby modulating activity of key signaling networks, including the Ras/Raf/Mapk pathway [29, 31]. Indeed, core pathway components such as H-Ras, K-Ras, and N-Ras are targeted by let-7 [32] (Figure 1). miRNAs can also affect critical pathway regulatory proteins, including GAP RAS p21 protein activator1 (RASA1), and other inhibitory proteins such as Sprouty-Related EVH1 Domain Containing 1 (SPRED1), the sprout homolog Sprouty RTK Signaling Antagonist 1 (SPRY1), and tumor suppressor Phosphatase and Tensin homolog (PTEN) that maintain proper spatio-temporal control of Ras/Erk signaling. miR-206 and/or miR-21 collaboratively target RASA1, SPRED1, and SPRY1, whereas miR-21 individually targets PTEN [6, 33, 34]. In addition, miRNAs can regulate expression of upstream drivers and downstream effectors and/or regulators. Examples include the β1 integrin gene (ITGB1) targeted by miR-9-3P [35] and the tumor-suppressor programmed cell death 4 (PDCD4) targeted by miR-206/21 [36, 37]. Further, various miRNAs exhibit differential expression during different stages of PC development [38]; thereby affecting regulation of signaling in PC.

miRNAs targeting Ras

Ras family proteins, including H-Ras, K-Ras and N-Ras, are targeted and regulated by multiple miRNAs. The miRNA regulation of Ras proteins was first identified in 2005 by Johnson et al., who identified the Ras genes as the targets of the let-7 family miRNAs in lung tumors [7]. Later, several studies identified other miRNAs that specifically target Ras

proteins in various cancers. As noted above, oncogenic *K-RAS* is the major driver and tumor maintenance gene in PC; hence, understanding the regulatory elements that control K-Ras is essential to uncover novel therapeutic targets. However, very few studies have documented miRNA regulation of the Ras family proteins in pancreatic cancer. Ten miRNAs that directly target K-Ras in PC include miR-96, miR-126, miR-143, miR-145, miR-193b, miR-206, miR-217, miR-3923, Let-7a, and Let-7b (Figure 2 and Table 1) [39–47].

Tumor suppressor function of K-Ras targeting miRNAs

Let7—The human Let-7 miRNA family consists of 13 miRNAs including Let-7a and Let-7b. Let-7 family miRNAs are involved in development as they regulate cell proliferation and differentiation by directly targeting various oncogenes such as MYC, RAS and HMGA2 [48]. The deregulation of Let-7 miRNAs is observed in several cancers [49]. Further, Let-7 expression levels are significantly down-regulated in K-Ras-mutated cancers such as lung, colorectal and pancreatic cancers compared to their normal counterparts. Of interest, the 3′-UTR of K-Ras mRNA has a binding site for Let-7, and a study from our lab showed that ectopic expression of Let-7b in PC cells leads to suppression of K-Ras expression, further providing evidence of Let-7b mediated regulation of K-Ras [44]. Torrisani et al. showed that expression of Let-7 was reduced in PC patients, and that restoration of Let-7 expression inhibited PC cell growth and down-regulated K-Ras expression. However, the group also reported that PC cells with Let-7 overexpression failed to inhibit cancer progression *in vivo,* suggesting that inefficient miRNA delivery limits the use of miRNAs as novel therapeutic targets *in vivo* [50].

The role of miRNAs in the regulation of K-Ras was also studied in radio-sensitization. One study demonstrated that upregulation of Let-7a decreases K-Ras expression, and that suppression of the Let-7a repressing protein, Lin28, leads to loss of K-Ras expression and radio-sensitizes PC cells [51].

miR-193b—Aberrant expression of miR-193b is commonly observed in several cancers. The tumor suppressor function of miR-193 was also frequently reported in various cancers, including PC. A study showed that expression of miR-193b is significantly reduced in PC tissues compared to healthy tissue. Transfection of PC cell lines with miR-193b resulted in inhibition of PC cell proliferation and invasive capacity, suggesting the tumor suppressive role of miR-193b. Further, miR-193b directly targets K-Ras by binding its 3′-UTR region, leading to inhibition of downstream Akt and ERK signaling, which results in inhibition of PC cell growth and proliferation [40]. Overexpression of miR-193b also abrogates PC aggressiveness by inducing apoptosis and targeting the cell cycle.

miR-206—An analysis of the miRNA expression using data set from the GEO (Gene Expression Omnibus) database showed that miR-206 is significantly down-regulated in PC tissue compared to healthy tissue,[41] and miR-206 was shown to inhibit NF-kB signaling to reduce tumor growth by directly targeting the *K-RAS* oncogene and thereby exerting a tumor suppressive function to inhibit PC cell growth, migration, and invasion [41]. The group also demonstrated that miR-206 inhibits pro-angiogenic and pro-inflammatory molecules by directly targeting the K-Ras-NF-kB axis.

miR-96—miR-96 is a member of the miR-183-96-182 cluster, and the miRNAs in this cluster are associated with tumorigenesis [52]. Deregulation of this cluster is frequently observed in several cancers, including PC. The miR-183-96-182 cluster miRNAs can act as either oncomirs or as tumor suppressors, depending on context. For instance, the expression of miR-96 is increased in bladder, lung, prostrate, hepatocellular, and colorectal cancers,[53] but its expression is significantly down-regulated in PC. miR-96 has been shown to suppress the expression of K-Ras by binding to its 3′UTR region, thereby act as a tumor suppressor in PC. Tanaka and colleagues showed that EVI1 oncoprotein is upregulated in pancreatic neoplasms, and potentially inhibits the expression of miR-96 to exert its oncogenic function [45, 54].

miR-143/145—The tumor suppressing role of miR-143/145 cluster was first identified in colorectal cancer. Chen and colleagues first reported the inhibitory effect of miR-143 on K-Ras translation in colorectal cancer [55]. Later, the tumor suppressive function of this cluster was reported in other cancers, including PC. Kent et al. have shown that K-Ras mutant PCs have significantly decreased expression levels of miR143/145 because activated K-Ras inhibits the miR143/145 promoter through Ras Responsive Element Binding Protein 1 (RREB1). The group also demonstrated that K-Ras and RREB1 are direct targets of miR143/145, suggesting a feed-forward mechanism for uninterrupted activation of K-Ras signaling [42]. The loss of miR-143 also led to increased Ras GTPase activity in PC cells, suggesting an association of Ras with miR-143/145.

miR-217/216—A comparative study showed that normal human pancreatic tissues express increased levels of miR-217 and miR-216, compared to PC tissues [56]. Expression of miR-217 and miR-216 cluster is also significantly downregulated in pancreas tissue from a K-Ras mutated mice model [57]. Zhao et al. reported that miR-217 levels are downregulated in pancreatic ductal adenocarcinoma (PC) and that ectopic overexpression of miR-217 resulted in inhibition of PC tumor growth by directly targeting K-Ras [46]. Overall, miR-217 and miR-216 can regulate K-Ras and act as tumor suppressors.

miR-126—The human miR-126 is located on chromosome 9 within the seventh intron region of the EGFL7 gene. Studies show that miR-126 regulates various functions, including angiogenesis, inflammation, tumor growth and tumor suppression [39]. In pancreatic cancer, the anti-oncomir, miR-126 has been shown to be downregulated, that it directly targets K-Ras, and that it acts as a tumor suppressor in normal pancreas [58].

miR-3923—It was recently shown that miR-3923 has a binding site on 3′-UTR of K-Ras mRNA, and that the resultant binding leads to suppression of K-Ras expression [43].

Upstream regulation of K-Ras targeting miRNAs

Finding upstream regulators or mechanisms involved in the activation of K-Ras targeting miRNA genes is crucial for the development of efficient miRNA therapeutic strategies. However, very little is known about the upstream signaling that mediates regulation of miRNAs by K-Ras in PC.

The presence of hundreds of miRNA genes has recently been reported in various studies [59]. Most of these miRNA genes are located in intergenic regions of chromosomes. As mentioned above, the RNA polymerase II predominantly transcribes miRNA genes [60]. However, knowledge about the mechanisms that activate transcriptional factors, which in turn regulate miRNA gene transcription, is limited.

It has been repeatedly documented that K-Ras signaling regulates miRNA gene transcription, suggesting that K-Ras is not only a target of miRNAs, but can also regulate miRNAs to exert a function related to either PC repression or promotion (Figure 3). Yuan and colleagues suggest that K-Ras, through the NF-kB pathway, activates transcription factor YY1, which in turn represses a tumor suppressor gene, miR-489 [61]. In another study, oncogenic K-Ras has been shown to activate miR-137, which targets KDM4A mRNA and leads to activation of p53. During senescence, p53 is activated due to KDM4A downregulation that occurs through activation of miR-137 by oncogenic K-Ras [62]. During the initiation of PC, activated oncogenic K-Ras can trigger cellular senescence, a tumor suppressor response. In another example of K-Ras-mediated PC promotion, oncogenic K-Ras activation leads to transactivation of miR-31 and results in enhanced migration and invasion of PC [63]. Likewise, mutant K-Ras activation induces miR-155 and contributes to the promotion of PC by increasing ROS levels [64]. Moreover, Kent et al. demonstrated that K-Ras inhibits the miR143/145 tumor suppressor genes in PC. They identified a feed-forward mechanism in which the same miRNAs target and inhibit K-Ras in normal pancreatic tissues [42].

miRNAs for pancreatic cancer therapy

miRNAs as Therapeutic Targets for Cancer

Ras proteins play a critical role in driving multiple signaling pathways in several cancers, and therefore is an obvious choice for targeting [65]. Increasing evidence shows that inhibiting Ras via modulation of Ras targeting miRNAs can impede tumor growth and aggressiveness [47]. For example, ectopic expression of miR-34 was shown to inhibit cell proliferation and migration *in vitro,* and in lung cancer significantly suppress tumor growth *in vivo* by targeting K-Ras [11]. Similarly, miR-31 was found to significantly decrease cell proliferation *in vitro* and tumor size *in vivo* upon downregulation, accomplished by directly targeting the RAS p21 GTPase activating protein 1 (RASA1), a regulator of the RAS-MAPK pathway in colorectal cancer [14]. Shin et al. found that miR-181a directly targets K-Ras and showed that ectopic overexpression of miR-181a significantly suppresses cell proliferation and colony formation in squamous cell carcinoma [13]. Supporting previous observations, another study demonstrated that overexpression of miR-451 *in vivo* significantly reduced tumor growth by directly targeting Ras-related protein 14 (RAB14) in human non-small cell lung cancer cells [15]. Numerous studies, including the few mentioned above, show that the Ras pathway is a highly viable target for development of potential therapeutic approaches towards cancer, and that miRNA can be an effective facilitator to do so. Considering the importance of the Ras pathway for PC, miRNA-based targeting of K-Ras represents a vast opportunity for therapy that to date has largely been left unharvested. Yet there are few reports on miRNA-based targeting of K-Ras in PC, as discussed below.

A recent study showed that miR-143, miR-145, or miR-34a, delivered via nanoparticles in mouse models of PC, significantly inhibited tumor growth [12]. Another study demonstrated that antisense oligonucleotides can be used to inhibit inherently over-expressed miR-21 and miR-221, and that it can enhance gemcitabine based therapy in PC [66]. A similar approach demonstrated that inhibition of miR-132 and miR-212 by antisense miRNA oligonucleotides resulted in inhibition of growth of pancreatic tumors [67] via their action on the retinoblastoma tumor suppressor (Rb1). Another example of miRNA inhibiting PC is miR-10a, which when repressed was able to inhibit tumor growth and metastasis [68]. Another mode for miRNA therapy is to use viral vector delivery, as suggested by delivery of miR-145 or miR-143, which effectively inhibited PC development [69]. Adenovirus-mediated delivery of miR-143 and miR-150 also was reported to provide a significant inhibition of PC cell growth and metastasis [50, 70, 71]. These studies show that multiple modalities can be applied to miRNAs for developing a Ras-targeted therapy for PC. However, so far very limited studies have successfully targeted the Ras pathway. Although a recent study showed that restoration of Let-7 miRNA, a known tumor suppressor, strongly reduced proliferation of pancreatic cancer cell lines by downregulating K-Ras and inhibiting mitogen-activated protein kinase activation [50].

Mutant K-Ras is found in over 90% of refractory PC cases and acts as a molecular switch to activates Rho GTPase signaling, which in turn launches an array of molecules and oncogenic microRNAs that promote tumor survival. A recent study evaluated the effect of inhibition of PAK4 (p21 activated kinase 4), a Rho GTPase effector protein, on p-Bad, a prosurvival protein and oncogenic miRNA. They reported that when NAMPT (Nicotinamide phosphoribosyltransferase) and PAK4 modulators (KPT-9274 and KPT-9307) were inhibited in PC cells, proliferation decreased, and that this decrease was due to inhibition of Bad phosphorylation and up-regulation of tumor suppressive miRNAs (miR-145, let-7c, let-7d, miR-34c, miR320, and miR-100). This report highlights how stimulating an increase in tumor suppressive miRNAs that target the Ras pathway could prove a promising strategy to target therapy resistant PC [72].

Yu et al. recently reported the impact of targeting K-Ras directly using miRNA in PC. They showed that miR-96 directly targets the *K-RAS* oncogene and thereby acts as a tumor-suppressing miRNA. The study used a synthetic miRNA precursor to allow ectopic expression of miR-96. This restoration of miR-96 led to an inhibition of K-Ras and a consequent decrease in Akt signaling, which, in turn, triggered apoptosis in cells, decreased cancer cell invasion and migration *in vitro* and inhibited tumor growth *in vivo*. Further, the study found an association of elevated K-Ras levels with decreased expression or deletion of miR-96 in human clinical specimens. The study highlights the role of miR-96 in the regulation of K-Ras, and provides a basis to devise novel therapeutic strategies for the treatment of PC and other K-Ras-driven cancers [73].

As mentioned above, a recent study by Zhao et al. found that overexpression of miR-217 in PC cells inhibits tumor cell growth and anchorage-independent colony formation *in vitro,* and that it decreased tumor growth in a nude mouse xenograft model *in vivo*. Their *in silico* predictions revealed that K-Ras was a potential direct target for miR-217; a dual-luciferase reporter gene assay proved that K-Ras was indeed a direct target of miR-217. The authors

reported that ectopic overexpression of miR-217 decreased K-Ras protein levels and reduced downstream activation of the AKT pathway. Furthermore, the study reported that miR-217 expression was negatively correlated with K-Ras expression in PC cell lines. The authors concluded that miR-217 acts as a tumor suppressor, and its downregulation can upregulate K-Ras in PC. This suggests that by targeting the oncogenic K-Ras pathway, miR-217 has potential as a therapeutic agent for miRNA PC therapy [74].

Strategies to target miRNAs

Thus far and despite multiple attempts, very little success has been achieved in targeting the Ras molecule or pathway in pancreatic cancer, but limited studies have been conducted using miRNA to do so. Currently, no clinical trials for miRNA targeting of K-Ras are underway, although it could be the case that since the field of miRNA investigation is blooming now, greater potential applications will likely be seen in the near future. The discovery of deregulated miRNAs as new molecular targets in PC certainly warrants the need for appropriate innovative tools to access these miRNAs (Figure 2).

One strategy utilize for targeting oncogenic miRNAs is the use of Anti-miRNA oligonucleotides (AMOs). These compounds are molecular tools that can tightly bind and inactivate the miRNA and induce miRNA silencing either *in vitro* or *in vivo* [75, 76]. For example, it has been shown that anti miRNA approaches have the capacity to specifically inhibit mutated K-Ras G12D, discriminating it from the wild type K-Ras to downregulate oncogenic signaling [77]. Another, similar approach to modulate intracellular miRNA levels is miRNA replacement therapy. Here, lower endogenous levels of tumor suppressor miRNAs are supplemented with oligonucleotide mimics [76]. These mimics, due to their unique characteristics (low size, low immunogenicity, and high target affinity) are promising tools to manipulate miRNA functions [76]. For instance, miR-489, tumor suppressor miRNA is downregulated by K-Ras signaling in PC, which consequently facilitates PC metastasis [61]. Therefore, miR-489 could prove to be a potential therapeutic target using miRNA mimetic strategies.

However, there are several critical hurdles to overcome with this approach. Limitations to therapy designs using miRNAs include lack of cell specificity, reduced *in vivo* stability, varied bio-distribution, disruption and saturation of endogenous RNA machinery, and potential side effects, all of which impede appropriate delivery of miRNA-targeting agents or miRNA mimics into target cells [78]. To overcome these hurdles and translate results to clinical applicability, appropriate methodologies, such as oligonucleotides modification and ingenuous delivery systems, have to be used and designed.

Chemical modifications of oligonucleotides are designed to confer resistance to nucleases and increase binding affinity to target molecules, resulting in improved performance [79]. One successful example of this approach is Locked Nucleic Acid (LNA™) nucleosides. These miRNA expression modulators are nucleic acid analogs containing one or more LNA nucleotide monomers, which are modified nucleotide RNA containing a bicyclic furanose unit locked in an RNA-mimicking sugar conformation. This restriction in conformation increases hybridization affinity towards complementary target, single-stranded RNA, and has been proven both *in vitro* and *in vivo* [78, 80, 81].

Delivery system modification for miRNA

To allow efficient delivery of AMOs or miRNA mimics to target cells that will result in successful translation of new therapeutic methodologies, novel delivery systems have to be employed. Considering this need, multiple new delivery platforms have been generated (Figure 2).

For instance, downregulated miR-29 was augmented using microRNA replacement therapy via a cationic liposome-based system, consisting of DOTAP, cholesterol, and D-α-Tocopherylpolyethyleneglycol 1000 succinate (vitamin E TPGS), to successfully deliver miR-29b *in vitro* and *in vivo* in lung cancer [82]. This approach led to a reduction of expression of key targets of miR-29b, along with inhibition of cell growth and clonogenic potential of non-small carcinoma cells *in vitro.* Such systemic delivery of lipoplexes containing miR-29b also led to an increase in available concentration of this miRNA in tumor. Consequent downregulation of the downstream target mRNA in the tumor then was seen to significantly inhibit tumor growth *in vivo* [82]. Similarly, co-delivery of Let-7b and Vismodegib (GDC-0449), a Hedgehog signaling antagonist using methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol-graft tetraethylpentamine) (mPEG-b-PCC-g-DC-g-TEPA) micelles (which can encapsulate GDC-0449 and bears pendant cationic chains to form complexes with Let-7b) resulted in synergistic decrease in tumor growth in subcutaneous athymic nude mice PC tumor model [83]. The entrapment of Let-7b into these micelles offers advantages of its improved *in vivo* stability, enhanced mean residence time, and ensures similar biodistribution of both GDC-0449 and Let-7b [83].

Another study reported that exosomes, small endosome-derived vesicles, could be used to deliver miRNAs to target cells. The authors demonstrated that Let-7a miRNA could be delivered to EGFR-expressing breast cancer cells *in vivo*. The study used exosomes from modified donor cells that expressed the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide to allow targeting EGFR-positive breast cancer cells [84]. Administration of exosomes loaded with let-7a miRNA, intravenously to EGFR-expressing breast cancer mouse model, led to a significant inhibition of tumor growth [84], since exosomes are natural carriers of miRNA *in vivo*.

Viral vectors are another mode of delivery systems known to be highly effective and often used in gene therapy against cancer. Oncolytic adenoviruses have frequently been used as vehicles for delivery of genes for therapeutic purposes against cancer because of their tumor-restricted replication [85].

Lou et al. demonstrated the utility of a combinatorial approach in a hepatocellular carcinoma xenograft mouse model by co-expressing miRNA-34a and IL-24 to achieve a synergistic antitumoral effect [85]. This study revealed that miRNA-34a and IL-24 could be co-expressed after transduction with oncolytic adenovirus (AdCN205-IL-24-miR-34a). Moreover, administration of this AdCN205-IL-24-miR-34a significantly inhibited growth of tumor cells *in vitro*, and resulted in absolute tumor regression without tumor recurrence *in vivo* [85].

Conclusions and perspectives

K-Ras mutation is a driving factor in PC. miRNAs have recently emerged as crucial regulators of oncogenic molecules, including K-Ras. A tight miRNA regulation of K-Ras is possible because K-Ras mRNA has binding sites for various miRNAs in its 3′-UTR region. Several miRNAs have been shown to directly regulate K-Ras (miR-96, miR-126, miR-143, miR-145, miR-193b, miR-206, miR-217, miR-3923, Let-7a, and Let-7b). Of interest, all these miRs function as tumor suppressors and their expression levels are significantly reduced in PC. In normal healthy tissues, expression of K-Ras and its targeting miRNAs is tightly regulated; whereas, under cancerous conditions, activated K-Ras transcriptionally downregulates the tumor suppressor miRNAs by a feed-forward mechanism, and promotes aggressiveness of the tumor.

Although there is enough evidence for miRNA-mediated regulation of K-Ras, further studies are needed to unveil other miRNAs that regulate K-Ras. At the same time, it is crucial to investigate mechanisms that regulate K-Ras targeting miRNA genes in PC that will help in designing miRNA-based K-Ras targeting therapeutic strategies. Based on evidence from previous studies, it is possible that K-Ras mediated signaling is the one that itself regulates K-Ras targeting miRNAs. In normal healthy pancreas tissues, K-Ras activity is tightly regulated and inhibited by miRNAs; it is extremely important to investigate whether mutant K-Ras inhibits its killer miRNA under cancerous conditions. Over-expression of K-Ras targeting miRNAs in PC may radio-sensitize resistant cells to radiation treatment. This was proved regarding Let7, and it is essential to investigate whether the upregulation of other K-Ras targeting miRNAs can sensitize PC cells for radiation treatment.

With an aim to develop new therapeutic strategies that provide higher efficacy and lower side effects, multiple methodologies have been explored for efficient and successful delivery of miRNA mimics or AMOs into target cells. Several clinical studies have highlighted the importance of exploring miRNA as a therapeutic tool against pancreatic cancer [86, 87]. Considering the oncogenic role of Ras mutations in PC and our present inability to target Ras mutations with standard approaches aimed at inhibiting post translational modifications of Ras for therapy [27, 88], it is logical to put tremendous effort towards using miRNAs to target Ras. However, optimisation and execution of miRNA therapeutic approaches lag behind other currently available therapies [12, 89]. Additional research is needed to integrate miRNA-based therapy into standard anticancer therapy. Currently available data is not enough, and intense research efforts are required to understand and predict the role of these therapies in improving patient outcome and overall survival [90].

It is now common knowledge that not all patients respond in same way to prescribed anticancer therapies, owing to the immense heterogeneity in PC among patients, and therefore the best treatment approach will be tailored to each patient. miRNAs provide a scope for developing personalized therapeutic approaches against PC, since specific miRNAs in individual patients can be identified easily and quickly in bodily fluids to determine the best therapeutic approach [78, 90]. Thus, rather than concentrating on chemotherapeutic agents, a more effective approach may be a combination of miRNA

therapy along with chemotherapy tailored to meet the specific disease presentation of each patient.

This review suggests a strong association between miRNAs and K-Ras while providing evidence for miRNA-mediated regulation of K-Ras in PC. It is hoped that this information will help provide a comprehensive understanding of miRNA regulation of K-Ras, and that it will pave the way for development of miRNA-based targeted therapies for PC.

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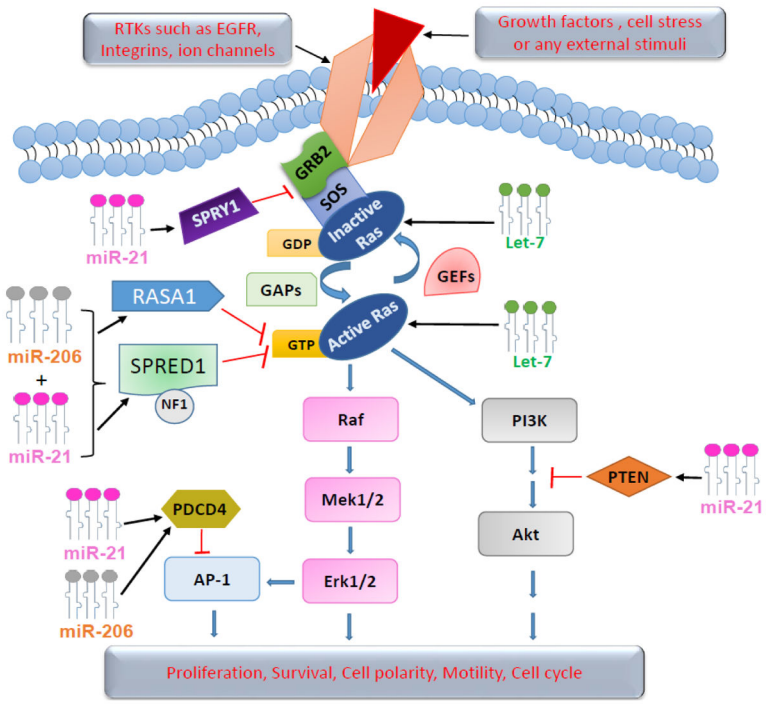

Figure 1. Oncogenic Ras signaling and miRNAs targeting Ras pathway components in cancer

Ras functions as a membrane bound molecular mediator between diverse external stimuli such as growth factors or cell stresses, which act via receptor tyrosine kinases, integrins or ion channels and intracellular signaling pathways including Ras-Erk and PI3K-Akt pathway. Ras GTPases behave as molecular switches by transitioning between inactive GDP-bound state and active GTP-bound state. In cancer, Ras signaling is constitutively activated owing to higher Ras-GTP levels leading to greater survival, proliferation, and motility of cancer cells. MicroRNAs (miRNAs) can target various Ras pathway components. The let-7 miRNA targets each of the Ras family GTPases including K-Ras, H-Ras, and N-Ras. Repressors of Ras-Erk signaling RASA1 (GAP RAS p21 protein activator1) and SPRED1 (Sprouty-Related EVH1 Domain Containing 1) are co-targeted by miR-206/21. The GAP protein NF1 is indicated as a likely catalytic partner of SPRED1. Another repressor of this signaling pathway, SPRY1 (Sprouty RTK Signaling Antagonist 1) and PI3K–Akt pathway repressor, Phosphatase and tensin homolog (PTEN) are targeted by miR-21 alone. Downstream pathway effector, tumor-suppressor programmed cell death 4 (PDCD4) is targeted by miR-21 and miR-206. Raf: Raf/mil family of serine/threonine protein kinases; Mek: Mitogen activated protein kinase; Erk: Extracellular signal-regulated kinases; PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase; Akt: v-akt murine thymoma viral oncogene homolog.

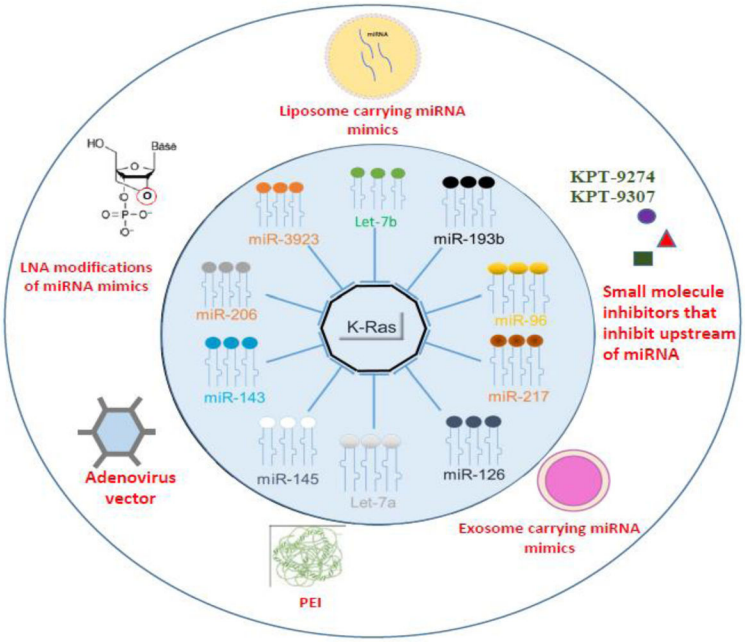

Figure 2. K-Ras targeting miRNAs in Pancreatic Cancer and therapeutic approaches

Various miRNAs, miR-96, miR-126, miR-143, miR-145, miR-193b, miR-206, miR-217, miR-3923, Let-7a and Let-7b directly target K-Ras in pancreatic cancer. The outer circle in the figure depicts the approaches that are being used to utilize miRNAs as therapeutic tools in pancreatic cancer. Liposomes, PEI (polyethylenimine) and Exosomes delivery system are being used to carry miRNA mimic of Let-7, Let-7a, miR-143, miR-145 which may or may not be LNA (locked nucleic acid) modified. Small molecule inhibitors KPT-9274 and KPT-9307 inhibit their targets and cause an eventual inhibition of Let-7 and miR-145. Adenovirus vectors carrying miR-143 and miR-145 can be used target PC.

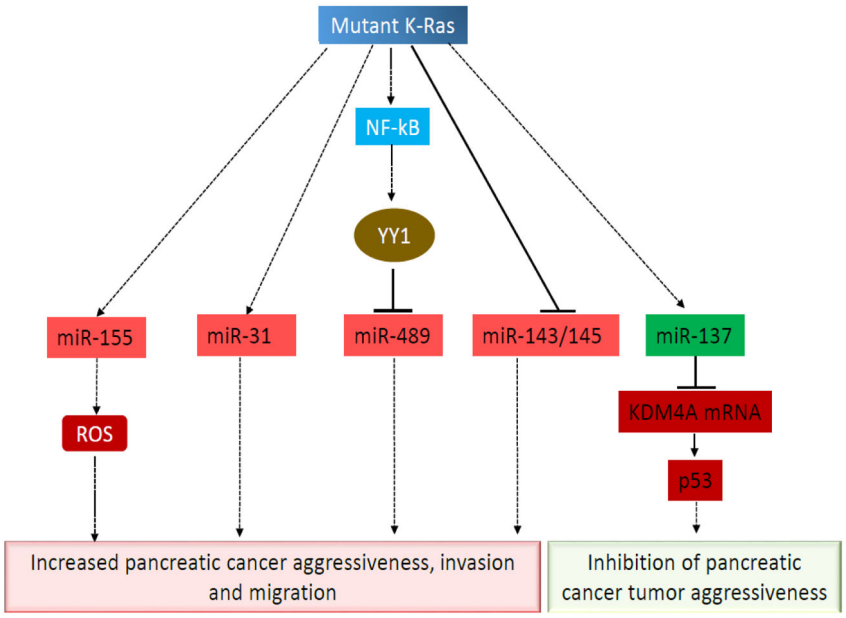

Figure 3. Regulation of miRNAs by K-Ras in Pancreatic Cancer

Mutant K-Ras induces ROS by the activation of miR-155 leading to the aggravation of Pancreatic Cancer. Other microRNAs, miR-31 and miR-143/145 are induced by K-Ras, resulting in pancreatic cancer invasion and migration. Further, K-Ras inhibits a tumor suppressor gene, miR-489 through NF-kB mediated activation of miR-489 inhibiting transcription factor, YY1. The mutant K-Ras also activates miR-137, which increases p53 expression by inhibiting KDM4A mRNA leading to the suppression of pancreatic cancer growth.

Table 1

K-Ras regulating miRNAs in pancreatic cancer

Name of miRNA	Common targets	Expression status in normal tissue	Expression status in PDAC	Function	Reference
Let7	K-Ras	Upregulated	Downregulated	Tumor suppressor, regulating glucose homeostasis and insulin sensitivity	Torrisani et al. 2009
Let-7a	K-Ras	Upregulated	Downregulated	Upregulation of Let7a targets K-Ras and radio-sensitizes PDAC cells	Oh et al. 2010
Let-7b	K-Ras	Upregulated	Downregulated	Overexpression leads to suppression of K- Ras expression	Rachagani et al. 2015
miR- 193b	K-Ras	Upregulated	Downregulated	Directly targets K-Ras and thus inhibits K-Ras mediated Akt and ERK signaling network	Jin et al. 2015
miR- 206	K-Ras	Upregulated	Downregulated	Inhibits NK-kB signaling by targeting K-Ras	Keklikoglou et al. 2015
miR-96	K-Ras	Upregulated	Downregulated	Targets K-Ras; and EVI1 oncoprotein inhibits miR-96 in PDAC.	Tanaka et al. 2014
miR- 143/145	K-Ras; c- Myc	Upregulated	Downregulated	Targets K-Ras and RREB1; Activated K- Ras through RREB1 inhibits miR-143/145; A feed forward loop	Kent et al. 2010
miR- 216/217	K-Ras; SIRT1	Upregulated	Downregulated	Tumor suppressors and targets K-Ras	Zhao et al. 2014
miR- 126	K-Ras; CRK; ADAM9	Upregulated	Downregulated	Targets K-Ras and act as anti-oncomir; Regulates EMT	Frampton et al. 2012; Shin et al. 2012
miR- 3923	K-Ras	Upregulated	Downregulated	Directly targets K-Ras	Li et al. 2016