miRNAs As Diagnostic and Prognostic Biomarkers in Pancreatic Ductal Adenocarcinoma and Its Precursor Lesions: A Review

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ABSTRACT: Pancreatic ductal adenocarcinoma (PDAC), a rare but lethal tumor, is difficult to diagnose without performing an invasive procedure. miRNAs are known to be deregulated in PDAC patients, and recent studies have shown that they can be used as diagnostic and prognostic of the disease. The detection of miRNAs in samples acquired through minimally or noninvasive procedures, such as serum, plasma, and saliva, can have a positive impact on the clinical management of these patients. This article is a comprehensive review of the major studies that have evaluated the expression of miRNAs as biomarkers in pancreatic cancer and its premalignant lesions.

KEYWORDS: pancreatic cancer, miRNAs, biomarkers, circulating biomarkers

Introduction

Among all cancer types, pancreatic ductal adenocarcinoma (PDAC), although rare, is considered one of the most lethal tumors, being responsible for 3% of all new cancer cases and 7% of all cancer-related deaths. PDAC is also the fourth highest cause of cancer-related death in the United States; moreover, the National Cancer Institute estimates that in 2015 ~48,960 people will be diagnosed with pancreatic cancer and that 40,560 of them will die because of the disease.[1] Several of PDAC's associated features make it a devastating and lethal disease, for example, the early dissemination of tumor-derived cells in the blood stream,[2] substantial morbidity associated with disease progression (which often renders the patient unsuitable for surgery or even nonsurgical disease-specific treatments),[3] and widespread tumor resistance to most forms of current treatment.[4] The only curative approach for PDAC patients is surgical resection, and only patients with localized (early stage) tumors are eligible for this therapy.[5]

At present, there is no detection method for the diagnosis of early stage PDAC; indeed, pancreatic cancer is usually a silent disease that only becomes apparent after tumor invasion of the surrounding tissues or metastatic seeding of distant organs. Therefore, the discovery of new diagnostic and prognostic biomarkers in pancreatic cancer is particularly important for patient survival. The only tumor markers for pancreatic cancer currently being used in a clinical setting are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), both of which present limitations because of low sensitivity and specificity. Besides their inability to discriminate malign and benign disease, these biomarkers are not specific tumor markers; in fact, the serum levels of these markers show variation in many diseases.

miRNAs have recently emerged as promising biomarkers in PDAC because these molecules show tissue-specific expression patterns[6] and are stable in circulating samples that can be easily obtained; hence, they enable disease screening in high-risk patients (as diagnostic biomarkers) and evaluation of several disease parameters (as prognostic biomarkers).[7]

In 1993, using *Caenorhabditis elegans* as a model, Lee et al.[8] identified a gene that codes for small noncoding RNAs, and further studies have demonstrated that these small RNAs can regulate protein translation by binding to the 3′-UTR and consequently inhibit mRNAs.[9] In the following years, these small noncoding RNA genes were described in different species, including humans, and they were named microRNAs (miRNAs or miRs).[10,11] Nowadays, miRNAs are well-known cancer biomarkers; they can fulfill this role because of their deregulation in virtually all tumors and presentation of tumor-specific profiles.

The aim of the present article is to review the major studies that have evaluated the expression of miRNAs as biomarkers in pancreatic cancer and its premalignant lesions. The article is structured into five main sections. The first section, miRNAs and cancer, introduces the early studies in this field; the next three sections explore the differential expression of miRNAs in premalignant lesions and PDAC. The final section highlights the recent findings related to miRNAs as useful circulating biomarkers. The discussion of the biogenesis, mechanism of action, and editing of miRNAs is beyond the scope of this article, but excellent reviews on these topics can be found elsewhere.[12–16]

miRNAs and Cancer

An association between miRNAs and cancer was proposed for the first time in 2002, when Calin et al.[17] demonstrated that a region commonly deleted in B-cell chronic lymphocytic leukemia (B-CLL) corresponded to miR-15 and miR-16 genes. The loss of this region (13q14) is the most frequent chromosomal abnormality in CLL, and this loss also occurs in other tumors, which indicates that tumor-suppressor genes could be located in this region. Calin et al also showed that both miRNA genes are deleted or downregulated in 68% of CLL cases. In 2005, Cimmino et al.[18] demonstrated that miR-15 and miR-16 negatively regulate *BCL2*, which encodes the antiapoptotic protein Bcl2. The absence of miR-15 and miR-16 in CLL promotes Bcl2 superexpression, which inhibits apoptosis and contributes to the establishment and progression of the malignant phenotype. Two years after their study describing the relationship between miRNAs and cancer, Calin et al.[19] published a paper in which genome-wide miRNA microarray profiling was used to show, for the first time, the potential importance of miRNAs in the diagnosis and prognosis of human malignancies.

In a comprehensive study, in which bead-based flow-cytometric profiling technology was applied, the authors demonstrated that a relatively small number of miRNAs could provide a large amount of diagnostic information because the expression patterns indicated not only different human cancers types but also differentiation states. The authors also observed patterns of gene expression for each type of tumor that reflected distinct mechanisms of transformation.[6] The feasibility and utility of monitoring the expression of miRNAs in human cancer was confirmed in this study and reinforced by others, because unlike mRNAs, miRNAs remained largely intact in routinely obtained, formalin-fixed, paraffin-embedded (FFPE) tissues.[20] Following the publication of this paper, several studies have shown that miRNA expression patterns are associated with different steps of carcinogenesis, and it is now well known that miRNAs can act as onco-miRNAs or tumor-suppressor miRNAs. In the first scenario, the overexpression of an miRNA that acts as an onco-miRNA would contribute to tumor formation through the negative regulation of a tumor-suppressor mRNA. Increased levels of these mature miRNAs could occur because of miRNA gene amplifications,

constitutive promoter activation, increased efficiency in miRNA processing, or increased stability of the miRNA. In the second scenario, the reduction or absence of an miRNA that acts as a tumor suppressor can lead to tumor formation because low expression levels of the miRNA promotes the overexpression of mRNAs that encode oncoproteins, and this favors the development of other features necessary for tumor progression, such as increased proliferation, invasiveness, and angiogenesis. The reduction or elimination of an miRNA can be caused by defects at any stage of miRNA biogenesis.[12] Although the correlation between miRNA and cancer was clear in 2005, the first study to show that the deregulation of a single-miRNA gene could lead to cancer was published in 2006; specifically, this study investigated a lymphoblastic leukemia/high-grade lymphoma in mice.[21]

In light of the central role that miRNAs play in carcinogenesis, they can be considered new regulators of cancer hallmarks.[22] Several examples can be used to illustrate the importance of miRNA regulation in all tumorigenic steps, such as cell proliferation, apoptosis, replicative potential, angiogenesis, immune response, tumor invasion, metastatic potential, and genomic instability. Indeed, in one elegant study, high-throughput miRNA profiling was applied to compare miRNA expression levels in premalignant stages as well as tumors and metastasis, and each stage was found to correlate with a distinct miRNA expression signature. Moreover, the wide variety of altered miRNAs was shown to reflect distinct functional roles in the acquired capabilities necessary for tumor formation and progression, the hallmarks of cancer, in a mouse model of pancreatic neuroendocrine tumors.[23,24]

According to Volinia et al.[25], despite the evident molecular differences between tumor types, some tumors present similar miRNA expression patterns, including prostate, colon, gastric, and pancreatic cancers, whereas others, such as lung and breast cancers, presented different signatures. Although some miRNAs are typical in one tumor type or tissue, other miRNAs, such as miR-21, miR-191, and miR-17-5p, are significantly overexpressed in several different tumor types. The study of Volinia et al showed that the most common miRNA alteration in solid tumors involves an increase in the expression levels, whereas loss of expression is less common and usually tissue specific. Finally, the authors' data provided further evidence to suggest that miRNAs function either in a dominant or recessive fashion by controlling the expression of protein-coding tumor suppressors and oncogenes.

Differential Expression of miRNAs in Premalignant Pancreatic Lesions: Pancreatic Intraepithelial Neoplasia

Most authors in the field propose that there are three possible precursor lesions to PDAC: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN), and a less prevalent precursor lesion known as mucinous cystic neoplasm (MCN). All of these alterations harbor

different types of dysplasia and involve a stepwise accumulation of genetic alterations that leads to the progression of the lesions from benign to malignant neoplasms.[26]

PanINs are found in the smaller caliber pancreatic ducts and are the most common and extensively studied of the premalignant lesions. The classic PDAC progression model is based on a sequence of molecular and histological alterations, which involves the transformation of the normal pancreatic duct in low-grade dysplasias (PanIN-IA and -IB) to high-grade dysplasias (PanIN-II and -III).[27] PanIN-IA and -IB are characterized by an increase in the ductal cells with abundant mucin production; however, in PanIN-IB, the papillary architecture replaces the flat architecture. While the lesions progress, the cells acquire moderated nuclear alterations (PanIN-II) with abnormal mitosis and lumen invasion, similar to those from in situ carcinoma (PanIN-III, when cells also present a severe nuclear alteration).[28]

The progression of premalignant lesions to invasive neoplasia is the result of successive genetic changes in a relatively predictable order in tumor-suppressor genes, oncogenes, and DNA repair genes.[27] The evaluation of miRNA expression in low- and high-grade PanINs is aimed at identifying biomarkers in the early stages of pancreatic carcinogenesis. Indeed, a PanIN progression model based on miRNA differential expression was described, showing that different miRNA expression profiles correlate with each stage of premalignant lesions.[29] In particular, the study investigated the expression profile of 735 human miRNAs in PanINs, PDAC, and normal tissues, and it included a principal component analysis demonstrating that miRNA expression profiles could be used to not only classify different PanIN stages but also correctly discriminate between PanIN and normal pancreatic (ductal) tissue. Sixty-five miRNAs were differentially expressed in precursor lesions relative to normal cells, and among these, 13 overexpressed miRNAs (miR-146a, -182, -193a-3p, -193b, -200a, -200b, -200c, -21, -29b, -425, -486-3p, -708, and -874) and one underexpressed miRNA (miR-296-5p) were selected for validation by quantitative polymerase chain reaction (qPCR) in an independent cohort (all overexpressed levels were confirmed). An interesting finding of this study was the identification of miR-196b as the most highly expressed miRNA in PanIN (compared with expression in normal cells); it was also highly expressed in PanIN-III (compared with expression in PanIN-I and -II) and expressed at even greater levels in PDAC samples. These findings led to the conclusion that miRNA expression levels can be used to discriminate patients with low-grade premalignant lesions from patients with high-grade lesions and PDAC.[29]

The same miRNA was also overexpressed in serum samples from humans and transgenic KPC mice (a mouse model of PDAC that expresses both oncogenic KRAS and mutant p53 in pancreatic cells). By analyzing the expression profiles of five miRNAs that were differentially expressed in PDAC (miR-21, -155, -196a, -196b, and -210) in KPC mice, Slater et al.[30]

found that only miR-196a and -196b were significantly overexpressed. They confirmed this finding in human serum samples and found that the expression levels of miR-196a and -196b, when measured together, discriminated PanIN-II, -III, and PDAC patients from healthy controls. This panel of miRNAs reached a perfect sensitivity and specificity (100%) when used to discriminate healthy controls from high-grade PanIN patients; thus, these miRNAs are promising biomarkers for early diagnosis. In human microdissected PanIN samples, miR-155 and miR-21 were evaluated by quantitative real-time PCR (qRT-PCR): only miR-155 expression was significantly different in high-grade PanINs versus nontumoral tissues; however, no difference in miR-155 expression was observed when PanIN-I lesions were compared with nontumoral tissue.[31]

The expression levels of a panel of five miRNAs (miR-10b, -21, -148a, -196a, and -217) were analyzed in PanINs and PDAC by Xue et al.[32] The authors corroborated the results of previous studies; they found that miR-196a was overexpressed in PanIN and PDAC compared with its expression in benign pancreatic tissue. Furthermore, miR-10b was upregulated in PanIN, miR-21 was upregulated in PDAC, and miR-217 was underexpressed in PDAC samples (compared with their expression in normal tissue). miR-148a expression was inversely correlated with PDAC progression because its levels were found to be high in normal pancreatic tissue samples and low in PDAC samples [with intermediate levels observed in chronic pancreatitis (CP) and PanINs]. These findings suggested that miR-148a could be a good marker of disease progression.

Because miR-21 is a well-known oncogenic miRNA in many cancers,[33] including PDAC, its presence is common in various miRNA panels. duRieu and cols found that together with other miRNAs (eg, miR-221, -222, -200, and -205), miR-21 was found to be overexpressed in PanIN, with levels increasing as the lesion progresses and reaching its highest levels in PanIN-II and -II. In this study, the authors also identified the overexpression of let-7 (a well-characterized miRNA that inhibits cell proliferation) in PanIN-II and -III as well as its underexpression in PDAC (as expected, according to Torrisani et al.[34] and Ali et al.[35,36]), thereby characterizing this miRNA as a potential biomarker for differential diagnosis in tissue samples.[37]

Finally, in another study, the expression levels of miR-145 were also correlated with PanIN progression. PanIN-I showed high levels of miR-145, whereas PanIN-II and -III as well as PDAC tissues presented with low or absent miR-145 expression.[38]

Differential Expression of miRNA in Premalignant Pancreatic Lesions: Mucinous Lesions

In addition to PanINs, two other premalignant lesions, IPMNs and MCNs (both containing a mucinous component), can play an important role in pancreatic carcinogenesis.

IPMNs are the most common cystic precursor lesions of PDAC. These lesions are mucin-producing epithelial

neoplasms that arise within the main pancreatic duct or one of its branches. They are considered potentially malignant and, being composed of columnar cells, are macroscopically visible via imaging technology.[39] IPMNs are organized into three categories depending on the degree of cytoarchitectural atypia. Lesions with low-grade dysplasia are characterized by a single layer of well-polarized cells, small pleomorphism, and rare mitoses. Intermediate-grade dysplasia shows nuclear stratification, loss of polarity, and crowding. Finally, IPMN lesions with high-grade dysplasia show severe dysplastic epithelial changes, marked loss of polarity, and loss of differentiated cytoplasmic features.[40]

MCNs of the pancreas are single spherical lesions macroscopically visible by imaging (mean diameter: 6–10 cm); they are most often found in the pancreatic body and tail, and they do not communicate with the pancreatic ductal system.[41,42] According to the grade of intraepithelial dysplasia, the World Health Organization has classified MCNs into three categories: MCNs with low, intermediate, and high-grade dysplasia.[42]

The potential of miRNAs as biomarkers in mucinous lesions has been evaluated in several studies, given that once these lesions are diagnosed by imaging there is neither a way to evaluate the level of epithelial dysplasia nor a method to predict whether the lesion will progress to adenocarcinoma and how long this might take. The potential of miRNAs as biomarkers in mucinous lesions has been evaluated in several studies.

The first study to evaluate miRNA expression in IPMNs was published in 2009 by Habbe et al, and it included a sample set containing 13 IPMN adenomas, 31 borderline IPMNs, and 20 IPMNs with carcinoma in situ, plus 54 matched non-neoplasic pancreatic tissue samples. First, the authors conducted a pilot study in which the expression of 12 miRNAs (described in the literature as deregulated in pancreatic cancer) was compared in 15 IPMN samples and matched normal pancreatic tissues. Expressions levels for 10 of these miRNAs differed significantly between cases and controls. miR-21 and miR-155 had the highest fold-changes, 12.1 and 11.6, respectively. The expression levels of these two miRNAs were subsequently evaluated by using locked nucleic acid in situ hybridization in 64 IPMN lesions and 54 controls, which confirmed their overexpression in the IPMN samples. Expression levels of these two miRNAs were also assessed in pancreatic juice; miR-155 was overexpressed in 60% of all cases, with 20-fold or greater relative-fold expression, whereas miR-21 was also highly expressed in the cases relative to the controls, but not to the extent of miR–155. Although these were interesting results, none reached statistical significance.[43] miR-21 and miR-155, together with miR-101, were also analyzed in a multicenter study of 65 invasive IPMNs, 16 noninvasive IPMNs, and 5 normal pancreatic tissues. The expression levels of the first two miRNAs correlated with an increase in disease severity; specifically, they were overexpressed in

invasive IPMN samples, expressed at lower levels in benign tissue, and almost absent in normal pancreatic cells. Similar to findings in PDAC studies, IPMN patients with high miR-21 levels had a significantly shorter overall survival (17.2 months) compared with patients who had low miR-21 levels (53.3 months). miR-101 showed an opposite expression pattern: it was equally expressed in normal pancreas tissues and benign IPMNs, but it was underexpressed in invasive IPMN samples, which suggests that miR-101 acts as a tumor suppressor.[44] Nakahara et al further investigated the role of miR-101 in pancreatic carcinogenesis.[45] With the aim of understanding whether this miRNA regulates the expression of the *EZH2* gene, which is suspected to act as an oncogene by downregulating tumor-suppressor genes,[46] the authors assessed expression of miR-101 using qPCR in five malignant tumors and five benign IPMN samples. Its expression was significantly lower in malignant IPMN than in benign samples. Thus, together with other findings, this study suggested that *EZH2* blockage by miR-101 is an important step in pancreatic carcinogenesis, and this miRNA could therefore represent a potential therapeutic target.

In a study that included several pancreatic lesions, the expression of 866 mature miRNAs was assayed in an attempt to distinguish between pancreatic tumors and lesions with low and high malignant potential. To confirm the microarray findings in this study, levels of miR-21, -126 and -16 (ie, miRNAs with known relevance to pancreatic carcinogenesis) were also measured by qRT-PCR. Expression levels of miR-21 did not differ between benign cystic tumors (comprising IPMN, MCN, and serous microcystic adenoma), PDAC, and carcinoma ex-IPMN (which is an invasive malignancy that arises from IPMN). The expression of miR-126 did not differ between IPMN, MCN, and the other groups, whereas miR-16 was overexpressed in IPMN samples relative to PDAC samples.[47] Ryu et al used a categorization strategy in their study, which involved investigating a panel of miRNAs, including two recurrently analyzed miRNAs (miR-21 and miR-155) plus miR-221, miR-17-3p, and miR-191 to discriminate mucinous (IPMN and MCN) from nonmucinous lesions [serous cystadenomas (SCAs) and other benign cysts]. Although the first four miRNAs in the aforementioned list could be used to discriminate between the groups, none of the miRNAs allowed differentiation between IPMN and MCN.[48] Using a slightly different approach in which cystic lesions were categorized into three risk categories (benign, corresponding to SCAs; premalignant, corresponding to IPMN and MCN; and malignant, corresponding to adenocarcinoma), Henry et al.[49] showed that a panel of nine miRNAs could be used to correctly classify these groups. In another study, Lee et al identified a MCN classifier, which included a panel of four miRNAs (miR-10b-5p, miR-202-3p, miR-210, and miR-375) whose expression levels could be employed to discriminate MCN from other pancreatic cystic neoplasms and PDAC with a sensitivity and specificity of 100%.[50]

Matthaei et al.[51] used a DiffPairs approach to identify novel biomarkers and analyze the expression profiles of 750 miRNAs in IPMN and aspirated cystic fluid samples, thereby identifying 26 and 37 candidate miRNAs for these respective samples. By using these candidate miRNAs, the authors were able to distinguish between high-risk lesions (high-grade IPMNs) and low-risk lesions (low-grade IPMNs and SCA). By narrowing their analysis down to a panel of nine miRNAs, they were able to discriminate both groups with a sensitivity of 89% and a specificity of 100%; furthermore, they could distinguish low-grade IPMNs/SCA from unusual cysts as cystic pancreatic neuroendocrine tumors and solid pseudopapillary tumors. Using a similar approach, Lubezky et al.[52] analyzed 846 human miRNAs in 55 specimens, including low-, moderate-, and high-grade IPMNs, PDAC, and normal pancreatic tissues. Fifteen miRNAs were differentially expressed in benign lesions (low- and moderate-grade IPMNs) compared with malignant lesions (high-grade IPMN and invasive carcinoma arising in IPMN), including overexpression of miR-155, miR-708, miR-424*, miR-21, miR-503, miR-214*, miR-150, miR-146a, and miR-21* and underexpression of miR-130b, miR-375, miR-148a, miR-216b, miR-216a, and miR-217.

More recently, studies used high-throughput methods to reveal new deregulated miRNAs in premalignant lesions, including miR-100, miR-99a, miR-99b, miR-342-3p, and miR-130a as well as miR-126. In microarray analysis, all of these miRNAs were underexpressed in high-risk IPMNs, relative to low-risk IPMNs, and this trend was confirmed by qRT-PCR in a different cohort. Moreover, an miRNA profile composed of miR-99b, miR-130a, and miR-342-3p reached an area under curve (AUC) value of 0.74 in the validation phase.[53] Wang et al.[54] employed next-generation sequencing (SOLiD platform) to identify patients with low-grade and high-grade dysplasia, and they found 13 overexpressed and 2 underexpressed miRNAs in cystic fluid samples from patients with IPMN and MCN (compared with expression in pancreatic tumor samples). However, these results were not statistically significant, perhaps because of small sample sizes. Only miR-216 was significantly overexpressed in high-risk patients; therefore, this single miRNA discriminated low-risk patients from high-risk patients.

Unfortunately, authors have used divergent nomenclature to categorize IPMN lesions in various stages, which hampers comparisons between different studies. A schematic progression model of precursor lesions (PanINs and IPMNs) and miRNAs deregulated in each stage can be seen in Figure 1.

Differential Expression of miRNAs in PDAC

More than 90% of patients diagnosed with pancreatic cancer die from the disease,[55] and accurate diagnostic and prognostic biomarkers are absent. Therefore, identification of new biomarkers that provide informative data regarding diagnosis and prognosis, as well as elucidate important aspects of tumor biology, is valuable for providing appropriate patient management.

In 2006, Lee et al.[56] used qRT-PCR in an effort to provide insights into deregulation of miRNAs in pancreatic cancer. Consequently, they published the first study comparing expression patterns of miRNA precursors in PDAC tumors, adjacent benign tissues, CP specimens, normal pancreatic tissues, and pancreatic cancer cell lines. The expression profile of 201 miRNAs showed a specific pattern for each sample type, with tumors, normal tissues (obtained from normal pancreas),

| References	Underexpressed	Overexpressed	Normal pancreas
Yu et al., 2012; Khan et al., 2014		miR-21, miR-155, miR-145, miR-182, miR-200a, miR-200b, miR-200c,	**PanIN-I**
Yu et al., 2012; Ryu et al., 2010; Rieu et al., 2010; Khan et al., 2014	miR-296-5p	let-7, miR-21 miR-155, miR-145, miR-182, miR-200, miR-200a, miR-200b, miR-200c, miR-205, miR-221, miR-222	**PanIN-II**
Yu et al., 2012; Ryu et al., 2010; Rieu et al., 2010; Khan et al., 2014	miR-125b miR-126, miR-218, miR-296-5p, miR-452	let-7, miR-18a miR-21, miR-155, miR-145, miR-182, miR-183*, miR-196b, miR-200, miR-205, miR-221, miR-222, miR-338-3p, miR-486-3p	**PanIN-III**

Figure 1. (*Continued*)
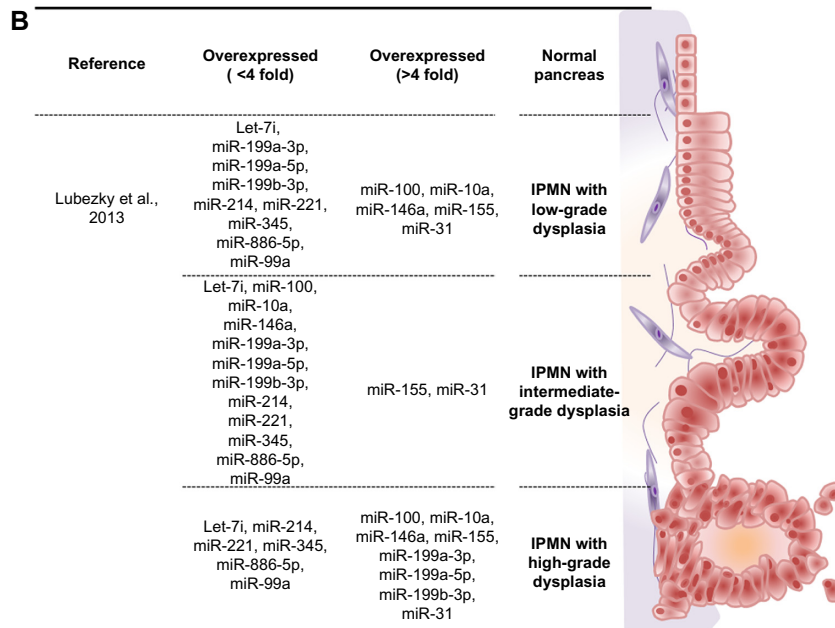
Figure 1. PanIN (**A**) and IPMN (**B**) progression models and corresponding miRNA differential expression. Normal duct cells (up) accumulate several histological and molecular abnormalities, which leads to an invasive PDAC (down).

and benign tissues (adjacent non-tumoral tissues, classified as normal tissues) profiled correctly (and differentially); a finding that reinforced the hypothesis that miRNA expression profiles may generate a unique molecular signature for each tissue. Although some miRNAs were exclusively expressed in pancreatic tumors cells (eg, miR-221, -376a, and -301), some of the most aberrantly expressed miRNAs (eg, miR-155, -21, -221) also showed abnormal expression in other cancer types. The correct categorization of different tissue types was also possible using a panel of 25 differentially expressed miRNAs (21 overexpressed and 4 underexpressed), as demonstrated by Bloomston et al.[57] These authors also showed that miRNA expression patterns could differentiate PDAC from normal pancreatic tissue and CP in 90% of all tested cases. A second panel containing 15 overexpressed and 8 underexpressed miRNAs differentiated PDAC tumors from CP with an accuracy of 93%. The authors also investigated whether absolute levels of miRNA expression could be applied to discriminate between short-term and long-term survivors with node-positive diseases, and statistical analyses revealed a subgroup of six overexpressed miRNAs in patients with long-term survival. Specifically, two miRNAs could be used to reliably predict survival: patients with increased miR196a-2 and miR-219 expression showed short-term survival (median survival of 14.3 and 13.6 months, respectively), whereas patients with low expression of these miRNAs showed long-term survival (26.5 and 23.8 months, respectively). It is interesting to note that nodal status, T stage, and histologic grade were not predictive of survival, which reinforces the specific usefulness of miRNA profiles in diagnosis and prognostic analyses. By using a set of 94 miRNAs, Szafranska et al.[58] found that the global miRNA

expression in CP tissues is intermediate between normal and PDAC tissues, and they showed that expression of this panel clearly distinguished PDAC from normal and CP tissues. This study aimed to establish a pancreatic miRNome, ie, the first comprehensive miRNA expression profile in normal pancreatic tissue, CP, and PDAC. The authors found that 84 miRNAs were differentially expressed between normal pancreatic tissues and PDAC samples. Among these miRNAs, 41 were downregulated and 32 were upregulated (at least two-fold), while 11 other miRNAs were strongly enriched in PDAC.

Zhang et al.[59] analyzed expression levels of 95 miRNAs (chosen based on their involvement in cancer biology, cell development, and apoptosis) not only in PDAC and normal pancreatic tissues but also in pancreatic cell lines. They demonstrated that expression patterns in pancreatic cancer tissues and pancreatic cancer cell lines were significantly different from those observed in a normal human pancreatic ductal epithelial cell line and normal pancreatic tissues obtained from patients. They also showed that each pancreatic cancer tissue and cell type presented a unique profiling pattern, which indicated that significant intertumoral heterogeneity exists. This study identified several novel miRNAs associated with pancreatic cancer and revealed that eight miRNAs (miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95) were significantly upregulated in most PDAC tumors from patient samples and cell lines.

A meta-analysis conducted by Ding et al.[60] corroborated the findings of most published articles when it revealed that multiple-miRNA profiling assays are more accurate than single-miRNA profiling assays for diagnosing PDAC (AUC 0.92% and 0.82%, respectively).

Several recent studies have demonstrated the deregulation of miRNA expression in pancreatic cancer, which highlights their utility as diagnostic and prognostic markers. Different methodologies have been used to assess miRNA expression, including northern blotting,[61] qRT-PCR assays developed for the amplification of precursor molecules,[62] miRNA array,[63] modified Invader assays,[64] qRT-PCR developed to assay mature miRNA[65] (either by using TaqMan or SYBR Green strategies), and analysis of RNA-seq data.[66]

Regardless of the methodology used, certain miRNAs appear to have a more central role in pancreatic carcinogenesis. For example, miR-21 is consistently reported as being overexpressed in PDAC,[67,68] and studies suggest that this miRNA contributes to cell proliferation, invasion, and chemoresistance in pancreatic cancer.[69] Indeed, PDAC patients whose tumors present low miR-21 expression have a better response to adjuvant treatment,[70] and lymph node-negative patients with high (tumoral) miR-21 expression levels have shorter survival times than similar patients with low miR-21 expression levels.[67] Similar results have been reported when progression-free survival was analyzed.[68]

miR-34a is an important component of the p53 pathway and acts as a tumor suppressor; it is frequently underexpressed in PDAC cell lines,[71–73] and its expression is known to decrease according to several mechanisms.[71,74,75] In a p53-mutant pancreatic cancer cell line, restoration of miR-34a expression restored the tumor-suppressing function of p53 and sensitized the tumor cells to chemotherapy and radiation.[76] Recent studies in PDAC patients showed that patients with high miR-34a expression levels had a better survival following resection with curative intent.[77]

Overexpression of miR-155 is common in several different tumor types,[25,78,79] and this miRNA was found to be upregulated by approximately three-fold in 11 of 21 PDAC cell lines.[80] One of its targets, the p53-regulated stress-induced gene *TP53INP1*, is downregulated in PDAC and correlates with tumor progression.[81] High miR-155 expression levels are observed in the tumors of patients with worse survival curves.[82]

Several other miRNAs have been associated with pancreatic carcinogenesis and are briefly discussed here. miR-196a has been shown to be virtually absent in normal pancreatic tissue, but it increased by 14-fold in PDAC tissues and cell lines. In addition, miR-196a expression levels correlated well with survival and disease progression, with levels already altered in PanINs and in all other stages up to PDAC.[57,83] miR-200b is overexpressed in 70% of all PDAC cases; levels increased up to 43-fold in PDAC tissues relative to non-tumoral pancreatic tissues.[59] The miR-200 family of miRNAs mainly targets two transcription factors, ZEB1 and ZEB2, which negatively regulate E-cadherin; this ultimately promotes progression of the epithelial-to-mesenchymal transition (EMT), which ensures mobility to tumor cells and favors metastasis.[84,85] Khan et al.[38] showed (through an invasion assay) that miR-145

reduces the amount of invading cells and is also able to enhance the effects of gemcitabine in PDAC cell lines. miR-145, which negatively regulates the MUC13 protein (which increases tumorigenic cell signaling pathways in PDAC[86]), is virtually absent in pancreatic cancer tissues, but it is highly expressed in normal pancreatic cells. miR-125b is overexpressed in a gemcitabine-resistant pancreatic cancer cell line (BxPC3-GZR) and advanced PDAC tumor tissues, and its suppression also causes a partial reversal of the mesenchymal phenotype and increases the response to gemcitabine treatment in BxPC3-GZR and PANC-1 cell lines.[66] Finally, deregulation of another miRNA, miR-376a, in PDAC was first described in 2006 when its expression was shown to be increased by seven-fold in PDAC tissues. The exclusive expression of this miRNA in pancreatic tumor cells (it was not found in benign pancreatic acini or stromal cells) suggested that it could be a good PDAC biomarker.[56] Years later, overexpression of miR-376a was also observed in rats in a study that showed that serum levels of this miRNA reflected tumoral status.[87]

Circulating miRNAs as Biomarkers in PDAC

It is often difficult to diagnose pancreatic cancer because of limited access and the need for invasive diagnostic procedures; thus, substantial research effort has been applied to the identification of diagnostic and prognostic biomarkers in circulating samples. In 2008, two different studies showed the presence of miRNAs in serum and plasma samples, thereby highlighting the potential of miRNAs as minimally invasive biomarkers for diagnostic as well as prognostic purposes.[88,89] In addition, Mitchell et al.[7] demonstrated that miRNAs are stable in plasma because they are protected against endogenous RNase activity. In an experiment with prostate cancer xenografts, miRNAs were shown to originate from tumors and enter circulation, where their expression levels could be used to robustly distinguish xenografted mice from controls. In humans, serum levels of miR-141 distinguished patients with prostate cancer from healthy controls, and this finding led the authors to propose that measurement of tumor-derived miRNAs in serum or plasma would be a useful method for blood-based detection of human cancer. Several more recent studies have demonstrated that circulating miRNAs are included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles (up to 1 μm), and exosomes (small membrane vesicles of endocytic origin, 50–100 nm in size), or protected by Argonaute protein 2.[90–92] Therefore, they are highly stable and could be used as biomarkers for different tumor types,[93] and different sampling strategies could be employed, including minimally invasive procedures to obtain serum and plasma and noninvasive procedures to obtain samples such as saliva.[94]

In PDAC, miRNAs were reported as useful circulating biomarkers for the first time in 2009 when Wang et al demonstrated that a panel of four miRNAs (miR-21, -210, -155, and -196a) overexpressed in PDAC tissues could also be detected in the plasma of PDAC patients and that these

miRNAs clearly distinguished patients with and without cancer (sensitivity of 64% and specificity of 89%).[95] Another study measured the plasmatic levels of miR-210, (which increase in hypoxia situations[96]) and found that expression of this miRNA significantly increased (four-fold) in PDAC patients relative to controls, confirming the potential of miRNAs as diagnostic biomarkers in PDAC.[97] miR-21, miR-155, and miR-196a were also evaluated by Kong et al.[98], who investigated a larger panel of miRNAs (miR-21, -155, -196a, -181a, -181b, -221, and -222). Using serum samples, they found that miR-21, -155, and -196a could be used to correctly discriminate PDAC patients from controls. Circulating levels of miR-196a were also shown to predict prognosis: patients with unresectable PDAC (stages III and IV) had significantly higher miR-196a levels, whereas patients with low miR-196a expression levels were in the early stages of the disease (stages I and II). Finally, serum miR-196a levels were found to be a good predictor of median survival time in PDAC patients; patients with high-level miR-196a expression had a median survival of 6.1 months, whereas patients with low miR-196a levels had a median survival of 12 months. Morimura et al.[99] evaluated miRNA expression levels in the plasma and tissue of the same patients. They analyzed the expression levels of the miR-17–92 cluster in the tissue and plasma of PDAC patients and healthy controls and found that, within the cluster and considering concordant expression levels in plasma and tissue, miR-18a expression distinguished patients with and without the disease.

In a large study performed in 2012, Liu et al.[100] identified 44 overexpressed miRNAs and 19 underexpressed miRNAs in the serum of PDAC patients (where expression was compared with age- and sex-matched cancer-free controls). Surprisingly, some of the miRNAs reported as deregulated in this study were different from those described in previous studies that analyzed circulating samples from PDAC patients. Among these miRNAs, only seven (miR-20a, -21, -24, -25, -99a, -185, and -191) were expressed at significantly different levels in the serum of PDAC patients versus controls, and these could be used to distinguish cancer-affected patients from CP patients. Patients who expressed high levels of miR-21 had a lower survival rate than those with low miR-21 expression levels. In addition, a prospective study used this seven-miRNA panel to screen and follow 55 clinically suspected cases of PDAC across several months. With the pathological diagnosis considered as a standard, the seven-miRNA panel could be employed to identify PDAC patients with an accuracy of 83.6%, which was significantly higher than the accuracy of CA19-9 (56.4%), the only currently available peripheral biomarker for PDAC, and CEA (36.4%) for the same sample set. In the same year, another group also described plasmatic levels of two miRNAs (miR-196a and miR-16) as early and independent biomarkers for PDAC, more effective than CA19-9 for early diagnosis. Furthermore, the combination of miRNAs with CA19-9 significantly improved the diagnostic accuracy of the miRNA panel or CA19-9 alone.[101]

In contrast to these previous studies, Cote et al.[102] reported that miR-21 expression levels in plasma were not significantly different between PDAC patients and healthy controls. Through measurement of 10 candidate miRNAs in plasma and bile aspirates of PDAC patients, CP patients, and healthy controls, the authors identified a panel of five miRNAs (miR-10b, miR-155, miR-160b, miR-30c, and miR-212) that could distinguish cases from controls with accuracy in both sample types. When considering only plasma samples, seven miRNAs (miR-10b, -30c, -106b, -155, -181b, -196a, and -212) showed significantly different expression across all three groups and between individuals with PDAC and CP.

Although the use of miRNA panels seems to be the most effective approach to diagnosis and prognosis, some individual miRNAs also show potential. For example, circulating levels of miR-182 were shown to be increased in PDAC patients compared to those in patients with CP and healthy controls, and this trend was significantly associated with advanced clinical stages and lymph node metastases. Moreover, this single miRNA was able to predict outcome after pancreatectomy; PDAC patients with high levels of miR-182 had shorter disease-free survival and overall survival compared with those of patients with low miR-182 levels.[103] A recent study from our group[104] also revealed that the expression of two miRNAs (miR-21 and miR-34a) in serum samples could individually discriminate PDAC and healthy controls. Indeed, when both variables were considered together, only a discrete improvement was observed.

In the last few years, a growing number of researchers have employed high-throughput methodologies to analyze hundreds of miRNAs and collect data that are considered more robust. One of the first studies to achieve this was a multicenter study in which 245 samples (129 tissue and 116 blood samples) from PDAC patients, CP patients, and healthy controls were assessed for the expression of 863 miRNAs using the Geniom Biochip miRNA *Homo sapiens*. In blood samples, 87 miRNAs differentiated PDAC patients from healthy controls, whereas 18 miRNAs distinguished CP patients from controls. Interestingly, the study did not identify a single circulating miRNA that could be used to distinguish between PDAC and CP patients.[105] In the second study with a case–control design, 754 miRNAs were analyzed in the whole blood of 409 PDAC patients, 25 CP patients, and 312 healthy controls (organized into discovery, training, and validation cohorts) with the aim of finding an miRNA panel suitable for diagnosis of PDAC. Using a TaqMan human microRNA assay to access miRNA expression, two diagnostic panels (indexes I and II) were created that comprised 4 (miR-145, miR-150, miR-223, and miR-636) and 10 (miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, and miR-885-5p) miRNAs, respectively. Both panels provided satisfactory results, and when they were combined with CA19-9, index I had an AUC of 0.94 (85% sensitivity and 98% specificity) and index II had an AUC of 0.93 (85% sensitivity and 90% specificity). Furthermore, the

AUC for the combination of CA19-9 and miRNA panels was significantly higher than that for CA19-9 used alone.[106]

Finally, Lin et al.[107] analyzed differential expression of 1,711 miRNAs in the serum of six PDAC patients and six controls, and they demonstrated that 22 miRNAs were upregulated, while 23 were downregulated. Among these miRNAs, they chose the eight most deregulated (upregulated: miR-1238, miR-296–3p, miR-4290, and miR-483-5p; downregulated: miR-1280, miR-492, miR-595, and miR-663a) for validation in a larger cohort of 49 PDAC and 27 control serum samples. Receiver operating characteristic curve analysis showed that miR-492 and miR-663a yielded the largest AUCs, 0.787 and 0.870, respectively. In an analysis that was even more comprehensive, Kojima et al.[108] performed a 3D-Gene assay, ie, a sensitive microarray developed to detect 2,555 human miRNAs registered in the miRBase (release 20; http://www.mirbase.org/), using 100 serum samples from PDAC patients and 150 from healthy controls, in addition to other digestive cancers samples. Notwithstanding the finding that miR-6836-3p showed high sensitivity and specificity when used alone (89.4% and 82.3%, respectively), the authors found that a combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) had an even higher sensitivity (80.3%) and specificity (97.6%). As expected, CA19-9 and CEA showed lower sensitivities (65.6% and 40%, respectively) and specificities (92.9% and 88.6%, respectively) in the same sample cohort. These results suggest that the assessment of miRNA expression levels, when miRNAs are used alone or organized into different panel types, is clinically valuable for identifying patients with pancreatic tumors who could benefit from surgical intervention, chemotherapy, or radiotherapy.

Besides plasma and serum biomarkers, some authors have focused on salivary biomarkers because saliva is composed of a complex combination of enzymes, hormones, antibodies, etc., which makes saliva samples as informative as blood in certain clinical situations.[109] Moreover, obtaining a saliva sample is noninvasive and involves low cost, and several studies have shown that salivary molecules can be useful as cancer biomarkers.[110–113] In 2010, Zhang et al.[114] demonstrated that a panel of four salivary mRNAs (KRAS, MBD3L2, ACRV1, and DPM1) distinguished PDAC patients from CP patients and healthy controls with 90% sensitivity and 95% specificity. More recent studies have shown that miRNAs can be detected in saliva samples and used as biomarkers.[94,115] Indeed, Xie et al.[116] performed a pilot study using the saliva samples of eight PDAC patients and eight healthy controls, which were analyzed with the human miRNA microarray (Agilent; capable of profiling the expression of 2,006 miRNAs). Considering certain criteria, the authors chose the 10 most deregulated miRNAs and tested them by using qPCR in the same cohort. Among the miRNAs, miR-3679-5p and miR-940 showed significant differential expression between the two groups, and when these were assayed in a larger cohort, miR-3679-5p

was found to be downregulated and miR-940 upregulated in PDAC patients compared with expression levels in healthy controls. Both miRNAs reached satisfactory sensitivities (82.5% and 90%, respectively) but not specificities (45% and 40%, respectively). In a recent study, miRNA expression was assayed in saliva samples and similar issues with specificity were not observed; specifically, miR-21, miR-23a, miR-23b, and miR-29c were significantly upregulated in PDAC patients compared with controls, and their specificity was 100%, while their sensitivities were variable (71.4%, 85.7%, 85,7%, and 57%, respectively).[117] Another pilot study using salivary samples did not obtain statistically significant results and found (using qRT-PCR) high Ct values, indicating an almost undetectable expression of the miRNAs assayed.[104]

Conclusions

Usually, early stage pancreatic cancer is a silent disease; it becomes apparent only after tumor invasion of surrounding tissues or metastatic seeding of distant organs. Suspected PDAC cases are primarily identified through imaging (eg, ultrasonography, computed tomography, and magnetic resonance), but this approach only identifies a pancreatic mass, which could be related to nonmalignant pancreatic disorders. Thus, imaging is usually insufficient for providing the final diagnosis of malignancy, CP, or other benign pancreatic lesions. Definitive diagnosis is usually obtained through invasive procedures, such as biopsies, which are usually accomplished by endoscopic ultrasonography, with diagnostic confirmation only possible in some cases after a laparotomy; thus, the definitive diagnosis of PDAC or pancreatic precursor lesions is almost impossible when using only noninvasive procedures.[118,119]

The biomarkers most commonly used in clinical settings, CEA and CA19-9, are not sufficiently accurate for the detection of pancreatic cancer, but they can be useful in follow-ups to assess disease progression in patients who have been already diagnosed. Therefore, the identification of highly sensitive and specific diagnostic and/or prognostic biomarkers is important for avoiding multiple surgical procedures and helping to distinguish, without invasive procedures, patients with and without cancer as well as patients with different premalignant lesions.

Several different approaches have revealed a number of miRNAs that could be used as biomarkers in PDAC, but it is important to also highlight some limitations. Only a few miRNAs have been appropriately validated in independent cohorts or investigated for their function in carcinogenesis. The wide variety of analytical methods employed in different studies is a barrier to comparing existing published results, a fact that reinforces the need to confirm the differential expression of miRNAs encountered in distinct populations. In some studies, sample size is also an issue, and the low prevalence of pancreatic cancer and its precursor lesions should stimulate efforts to conduct more collaborative projects. Furthermore, the use of circulating miRNAs as biomarkers is also limited by their heterogeneous origin. Blood and endothelial cells are

Table 1. Circulating miRNAs deregulated in PDAC patients.

ORIGIN	miRNA	REGULATION	REFERENCE
Serum	miR-125a-3p, miR-492, miR-595, miR-663a, miR-1280, miR-4294, miR-4476, miR-4530, miR-6880-5p	Down	Lin et al, 2014; Kojima et al, 2015
	miR-21, miR-155, miR-196a, miR-296-3p, miR-483-5p, miR-1238, miR-4290, miR-6075	Up	Kong et al, 2011; Lin et al, 2014; Kojima et al, 2015
Plasma	miR-10b, miR-16, miR-18a, miR-20a, miR-21, miR-24, miR-25, miR-30c, miR-99a, miR-106b, miR-155, miR-181b, miR-182, miR-185, miR-191, miR-196a, miR-210, miR-212	Up	Wang et al, 2007; Ho et al, 2010; Morimura et al, 2011; Liu R et al, 2012; Liu J et al, 2012; Cote et al, 2014; Chen et al, 2014
Whole Blood	miR-31, miR-31*, miR-93, miR-126*, miR-150, miR-663a, miR-935,	Down	Schultz et al, 2014
	miR-34a, miR-122, miR-145, miR-199b-5p, miR-582-3p, miR-769-5p, miR-885-5p	Up	Schultz et al, 2014
Saliva	miR3679-5p	Down	Xie et al, 2015
	miR-21, miR-23a, miR-23b, miR-29c, miR-940	Up	Xie et al, 2015; Humeau et al, 2015

the origin of most of the cell-free miRNAs in the blood, and this could be a confounding factor in the search for tumor-specific miRNAs. Indeed, circulating miRNAs are only stable (ie, protected against RNase activity) because of their association with other structures (eg, apoptotic bodies, exosomes, high-density lipoprotein vesicles, and mainly proteins from the Argonaute family[90–92,120]). This primordial interaction adds another bias if we consider that differences in miRNA expression levels could be because of differential degradation in different environments. The future perspectives in the miRNA field should focus on overcoming these limitations to find a robust panel of miRNAs for clinical use.

As shown in this review, miRNAs, considered either individually or in panels, are key players in a wide variety of applications. Although making robust comparisons is difficult because of the different analytical approaches used to date, some miRNAs have been consistently reported as biomarkers, either for diagnostic or prognostic purposes. For example, miR-21, miR-155, and miR-196 can be used to distinguish between normal, premalignant, and PDAC tissues in a tissue-approach, and they discriminated healthy controls from PDAC patients in serum and plasma samples in more than one independent study. Other deregulated miRNAs in different sample types are shown in Table 1. Despite the aforementioned limitations, substantial progress has been made toward the discovery of novel diagnostic and prognostic PDAC biomarkers, and miRNAs show particular promise in this field.

Author Contributions

Conceived and designed the experiments: BA. Analyzed the data: BA, CG. Wrote the first draft of the manuscript: BA, CG. Contributed to the writing of the manuscript: BA, CG, PAP. Agree with manuscript results and conclusions: BA, CG, PAP. Jointly developed the structure and arguments for the paper: BA, CG, PAP. Made critical revisions and

approved final version: BA, CG, PAP. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin*. 2015;65(1):5–29.
2. Rhim AD, Mirek ET, Aiello NM, et al. EMT and dissemination precede pancreatic tumor formation. *Cell*. 2012;148(1–2):349–61.
3. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet*. 2004;363(9414):1049–57.
4. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324(5933):1457–61.
5. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med*. 2014;371(22):2140–1.
6. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834–8.
7. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105(30):10513–8.
8. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 1993;75(5):843–54.
9. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*. 1993;75(5):855–62.
10. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science*. 2001;294(5543):853–8.
11. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*. 2001;294(5543):862–4.
12. Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6(4):259–69.
13. Nilsen TW. Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet*. 2007;23(5):243–9.
14. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281–97.
15. Warnefors M, Liechti A, Halbert J, Valloton D, Kaessmann H. Conserved microRNA editing in mammalian evolution, development and disease. *Genome Biol*. 2014;15(6):R83.
16. Gong J, Wu Y, Zhang X, et al. Comprehensive analysis of human small RNA sequencing data provides insights into expression profiles and miRNA editing. *RNA Biol*. 2014;11(11):1375–85.
17. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*. 2002;99(24):15524–9.
18. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*. 2005;102(39):13944–9.

19. Calin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci U S A*. 2004;101(32):11755–60.

20. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA. 2007;13(10):1668–74.

21. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci U S A*. 2006;103(18):7024–9.

22. Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. *Cancer Lett*. 2009;285(2):116–26.

23. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57–70.

24. Olson P, Lu J, Zhang H, et al. MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer. *Genes Dev*. 2009;23(18): 2152–65.

25. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103(7):2257–61.

26. Maitra A, Hruban RH. Pancreatic cancer. *Annu Rev Pathol*. 2008;3:157–88.

27. Hruban RH, Wilentz RE, Kern SE. Genetic progression in the pancreatic ducts. *Am J Pathol*. 2000;156(6):1821–5.

28. Schneider G, Siveke JT, Eckel F, Schmid RM. Pancreatic cancer: basic and clinical aspects. *Gastroenterology*. 2005;128(6):1606–25.

29. Yu J, Li A, Hong SM, Hruban RH, Goggins M. MicroRNA alterations of pancreatic intraepithelial neoplasias. *Clin Cancer Res*. 2012;18(4):981–92.

30. Slater EP, Strauch K, Rospleszcz S, et al. MicroRNA-196a and -196b as potential biomarkers for the early detection of familial pancreatic cancer. *Transl Oncol*. 2014;7(4):464–71.

31. Ryu JK, Hong SM, Karikari CA, Hruban RH, Goggins MG, Maitra A. Aberrant microRNA-155 expression is an early event in the multistep progression of pancreatic adenocarcinoma. *Pancreatology*. 2010;10(1):66–73.

32. Xue Y, Abou Tayoun AN, Abo KM, et al. MicroRNAs as diagnostic markers for pancreatic ductal adenocarcinoma and its precursor, pancreatic intraepithelial neoplasm. *Cancer Genet*. 2013;206(6):217–21.

33. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. *J Cell Mol Med*. 2009;13(1):39–53.

34. Torrisani J, Bournet B, du Rieu MC, et al. Let-7 microRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression. *Hum Gene Ther*. 2009;20(8):831–44.

35. Ali S, Almhanna K, Chen W, Philip PA, Sarkar FH. Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. *Am J Transl Res*. 2010;3(1):28–47.

36. Ali S, Saleh H, Sethi S, Sarkar FH, Philip PA. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. *Br J Cancer*. 2012;107(8):1354–60.

37. du Rieu MC, Torrisani J, Selves J, et al. MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. *Clin Chem*. 2010;56(4):603–12.

38. Khan S, Ebeling MC, Zaman MS, et al. MicroRNA-145 targets MUC13 and suppresses growth and invasion of pancreatic cancer. *Oncotarget*. 2014;5(17):7599–609.

39. Hruban RH, Takaori K, Klimstra DS, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am J Surg Pathol*. 2004;28(8):977–87.

40. Adsay NV, Fukushima N, Furukawa T, et al. Intraductal neoplasm of the pancreas. Bosman FT, Carneiro F, Hruban RH, Theise ND, Editors. *WHO Classification of Tumors of the Digestive System*. 4th ed. Lyon: WHO Press; 2010:304–13.

41. Fukushima N, Fukayama M. Mucinous cystic neoplasms of the pancreas: pathology and molecular genetics. *J Hepatobiliary Pancreat Surg*. 2007;14(3):238–42.

42. Zamboni G, Fukushima N, Hurban RH, Kloppel G. Mucinous cystic neoplasms. Bosman FT, Carneiro F, Hruban RH, Theise ND, Editors. *WHO Classification of Tumors of the Digestive System*. 4th ed. Lyon: WHO Press; 2010:300–3.

43. Habbe N, Koorstra JB, Mendell JT, et al. MicroRNA miR-155 is a biomarker of early pancreatic neoplasia. *Cancer Biol Ther*. 2009;8(4):340–6.

44. Caponi S, Funel N, Frampton AE, et al. The good, the bad and the ugly: a tale of miR-101, miR-21 and miR-155 in pancreatic intraductal papillary mucinous neoplasms. *Ann Oncol*. 2013;24(3):734–41.

45. Nakahara O, Takamori H, Iwatsuki M, et al. Carcinogenesis of intraductal papillary mucinous neoplasm of the pancreas: loss of microRNA-101 promotes overexpression of histone methyltransferase EZH2. *Ann Surg Oncol*. 2012; 19(suppl 3):S565–71.

46. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J*. 2003;22(20):5323–35.

47. Jiao LR, Frampton AE, Jacob J, et al. MicroRNAs targeting oncogenes are down-regulated in pancreatic malignant transformation from benign tumors. *PLoS One*. 2012;7(2):e32068.

48. Ryu JK, Matthaei H, Dal Molin M, et al. Elevated microRNA miR-21 levels in pancreatic cyst fluid are predictive of mucinous precursor lesions of ductal adenocarcinoma. *Pancreatology*. 2011;11(3):343–50.

49. Henry JC, Bassi C, Giovinazzo F, Bloomston M. MicroRNA from pancreatic duct aspirate differentiates cystic lesions of the pancreas. *Ann Surg Oncol*. 2013;20(suppl 3):S661–6.

50. Lee LS, Szafranska-Schwarzbach AE, Wylie D, et al. Investigating microRNA expression profiles in pancreatic cystic neoplasms. *Clin Transl Gastroenterol*. 2014;5:e47.

51. Matthaei H, Wylie D, Lloyd MB, et al. miRNA biomarkers in cyst fluid augment the diagnosis and management of pancreatic cysts. *Clin Cancer Res*. 2012;18(17):4713–24.

52. Lubezky N, Loewenstein S, Ben-Haim M, et al. MicroRNA expression signatures in intraductal papillary mucinous neoplasm of the pancreas. *Surgery*. 2013;153(5):663–72.

53. Permuth-Wey J, Chen YA, Fisher K, et al. A genome-wide investigation of microRNA expression identifies biologically-meaningful microRNAs that distinguish between high-risk and low-risk intraductal papillary mucinous neoplasms of the pancreas. *PLoS One*. 2015;10(1):e0116869.

54. Wang J, Paris PL, Chen J, et al. Next generation sequencing of pancreatic cyst fluid microRNAs from low grade-benign and high grade-invasive lesions. *Cancer Lett*. 2015;356(2 pt B):404–9.

55. Neuzillet C, Sauvanet A, Hammel P. Prognostic factors for resectable pancreatic adenocarcinoma. *J Visc Surg*. 2011;148(4):e232–43.

56. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer*. 2007;120(5):1046–54.

57. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA*. 2007;297(17):1901–8.

58. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*. 2007;26(30):4442–52.

59. Zhang Y, Li M, Wang H, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*. 2009;33(4):698–709.

60. Ding Z, Wu H, Zhang J, Huang G, Ji D. MicroRNAs as novel biomarkers for pancreatic cancer diagnosis: a meta-analysis based on 18 articles. *Tumour Biol*. 2014;35(9):8837–48.

61. Wang X, Zhao J, Huang J, Tang H, Yu S, Chen Y. The regulatory roles of miRNA and methylation on oncogene and tumor suppressor gene expression in pancreatic cancer cells. *Biochem Biophys Res Commun*. 2012;425(1):51–7.

62. Schmittgen TD, Jiang J, Liu Q, Yang L. A high-throughput method to monitor the expression of microRNA precursors. *Nucleic Acids Res*. 2004;32(4):e43.

63. Babak T, Zhang W, Morris Q, Blencowe BJ, Hughes TR. Probing microRNAs with microarrays: tissue specificity and functional inference. *RNA*. 2004;10(11):1813–9.

64. Allawi HT, Dahlberg JE, Olson S, et al. Quantitation of microRNAs using a modified invader assay. *RNA*. 2004;10(7):1153–61.

65. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res*. 2005;33(17):5394–403.

66. Bera A, VenkataSubbaRao K, Manoharan MS, Hill P, Freeman JW. A miRNA signature of chemoresistant mesenchymal phenotype identifies novel molecular targets associated with advanced pancreatic cancer. *PLoS One*. 2014;9(9):e106343.

67. Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg*. 2008;12(12):2171–6.

68. Giovannetti E, Funel N, Peters GJ, et al. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res*. 2010;70(11): 4528–38.

69. Moriyama T, Ohuchida K, Mizumoto K, et al. MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. *Mol Cancer Ther*. 2009;8(5):1067–74.

70. Hwang JH, Voortman J, Giovannetti E, et al. Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS One*. 2010;5(5):e10630.

71. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell*. 2007;26(5):745–52.

72. Tarasov V, Jung P, Verdoodt B, et al. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle*. 2007;6(13):1586–93.

73. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ*. 2010;17(2):193–9.

74. Calhoun ES, Hucl T, Gallmeier E, et al. Identifying allelic loss and homozygous deletions in pancreatic cancer without matched normals using high-density single-nucleotide polymorphism arrays. *Cancer Res*. 2006;66(16):7920–8.

75. Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle*. 2008;7(16):2591–600.

76. Ji Q, Hao X, Zhang M, et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One*. 2009;4(8):e6816.

77. Jamieson NB, Morran DC, Morton JP, et al. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. *Clin Cancer Res*. 2012;18(2):534–45.

78. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102(52):19075–80.

79. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A*. 2005;102(10):3627–32.

80. Kent OA, Mullendore M, Wentzel EA, et al. A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells. *Cancer Biol Ther*. 2009;8(21):2013–24.

81. Gironella M, Seux M, Xie MJ, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A*. 2007;104(41):16170–5.

82. Greither T, Grochola LF, Udelnow A, Lautenschläger C, Würl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. *Int J Cancer*. 2010;126(1):73–80.

83. Szafranska AE, Doleshal M, Edmunds HS, et al. Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. *Clin Chem*. 2008;54(10):1716–24.

84. Li Y, VandenBoom TG 2nd, Kong D, et al. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res*. 2009;69(16):6704–12.

85. Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem*. 2008; 283(22):14910–4.

86. Chauhan SC, Ebeling MC, Maher DM, et al. MUC13 mucin augments pancreatic tumorigenesis. *Mol Cancer Ther*. 2012;11(1):24–33.

87. Yabushita S, Fukamachi K, Tanaka H, et al. Circulating microRNAs in serum of human K-ras oncogene transgenic rats with pancreatic ductal adenocarcinomas. *Pancreas*. 2012;41(7):1013–8.

88. Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008;141(5):672–5.

89. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18(10):997–1006.

90. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One*. 2012;7(3):e30679.

91. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*. 2010;101(10):2087–92.

92. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011;108(12):5003–8.

93. Albulescu R, Neagu M, Albulescu L, Tanase C. Tissular and soluble miRNAs for diagnostic and therapy improvement in digestive tract cancers. *Expert Rev Mol Diagn*. 2011;11(1):101–20.

94. Patel RS, Jakymiw A, Yao B, et al. High resolution of microRNA signatures in human whole saliva. *Arch Oral Biol*. 2011;56(12):1506–13.

95. Wang J, Chen J, Chang P, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)*. 2009;2(9):807–13.

96. Kulshreshtha R, Ferracin M, Wojcik SE, et al. A microRNA signature of hypoxia. *Mol Cell Biol*. 2007;27(5):1859–67.

97. Ho AS, Huang X, Cao H, et al. Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. *Transl Oncol*. 2010;3(2):109–13.

98. Kong X, Du Y, Wang G, et al. Detection of differentially expressed microRNAs in serum of pancreatic ductal adenocarcinoma patients: miR-196a could be a potential marker for poor prognosis. *Dig Dis Sci*. 2011;56(2):602–9.

99. Morimura R, Komatsu S, Ichikawa D, et al. Novel diagnostic value of circulating miR-18a in plasma of patients with pancreatic cancer. *Br J Cancer*. 2011;105(11):1733–40.

100. Liu R, Chen X, Du Y, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem*. 2012;58(3):610–8.

101. Liu J, Gao J, Du Y, et al. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer*. 2012;131(3):683–91.

102. Cote GA, Gore AJ, McElyea SD, et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. *Am J Gastroenterol*. 2014;109(12):1942–52.

103. Chen Q, Yang L, Xiao Y, Zhu J, Li Z. Circulating microRNA-182 in plasma and its potential diagnostic and prognostic value for pancreatic cancer. *Med Oncol*. 2014;31(11):225.

104. Alemar B, Izetti P, Gregório C, et al. miRNA-21 and miRNA-34a are potential minimally invasive biomarkers for the diagnosis of pancreatic ductal adenocarcinoma. *Pancreas*. 2015.

105. Bauer AS, Keller A, Costello E, et al. Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One*. 2012;7(4):e34151.

106. Schultz NA, Dehlendorff C, Jensen BV, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA*. 2014;311(4):392–404.

107. Lin MS, Chen WC, Huang JX, Gao HJ, Sheng HH. Aberrant expression of microRNAs in serum may identify individuals with pancreatic cancer. *Int J Clin Exp Med*. 2014;7(12):5226–34.

108. Kojima M, Sudo H, Kawauchi J, et al. MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. *PLoS One*. 2015;10(2):e0118220.

109. Lee YH, Wong DT. Saliva: an emerging biofluid for early detection of diseases. *Am J Dent*. 2009;22(4):241–8.

110. Franzmann EJ, Reategui EP, Carraway KL, Hamilton KL, Weed DT, Goodwin WJ. Salivary soluble CD44: a potential molecular marker for head and neck cancer. *Cancer Epidemiol Biomarkers Prev*. 2005;14(3):735–9.

111. Li Y, St John MA, Zhou X, et al. Salivary transcriptome diagnostics for oral cancer detection. *Clin Cancer Res*. 2004;10(24):8442–50.

112. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT. The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin Cancer Res*. 2000;6(6):2363–70.

113. Brooks MN, Wang J, Li Y, Zhang R, Elashoff D, Wong DT. Salivary protein factors are elevated in breast cancer patients. *Mol Med Rep*. 2008;1(3):375–8.

114. Zhang L, Farrell JJ, Zhou H, et al. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology*. 2010;138(3):e941–7.

115. Park NJ, Zhou H, Elashoff D, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin Cancer Res*. 2009;15(17):5473–7.

116. Xie Z, Yin X, Gong B, et al. Salivary microRNAs show potential as a noninvasive biomarker for detecting resectable pancreatic cancer. *Cancer Prev Res (Phila)*. 2015;8(2):165–73.

117. Humeau M, Vignolle-Vidoni A, Sicard F, et al. Salivary microRNA in pancreatic cancer patients. *PLoS One*. 2015;10(6):e0130996.

118. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362(17):1605–17.

119. Mayo SC, Austin DF, Sheppard BC, Mori M, Shipley DK, Billingsley KG. Evolving preoperative evaluation of patients with pancreatic cancer: does laparoscopy have a role in the current era? *J Am Coll Surg*. 2009;208(1):87–95.

120. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13(4):423–33.