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REVIEW

Circulating RNAs as new biomarkers for detecting pancreatic cancer

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Abstract

Pancreatic cancer remains difficult to treat and has a high mortality rate. It is difficult to diagnose early, mainly due to the lack of screening imaging modalities and specific biomarkers. Consequently, it is important to develop biomarkers that enable the detection of early stage tumors. Emerging evidence is accumulating that tumor cells release substantial amounts of RNA into the bloodstream that strongly resist RNases in the blood and are present at sufficient levels for quantitative analyses. These circulating RNAs are upregulated in the serum and plasma of cancer patients, including those with pancreatic cancer, compared with healthy controls. The majority of RNA biomarker studies have assessed circulating microRNAs (miRs), which are often tissue-specific. There are few reports of the tumor-specific upregulation of other types of small non-coding RNAs (ncRNAs), such as small nucleolar RNAs and Piwi-interacting RNAs. Long ncRNAs (lncRNAs), such as HOTAIR and MALAT1, in the serum/plasma of pancreatic cancer patients have also been reported as diagnostic and prognostic markers. Among tissue-derived RNAs, some miRs show increased expression even in pre-cancerous tissues, and their expression profiles may allow for the discrimination between a chronic inflammatory state and carcinoma. Additionally, some miRs and lncRNAs have been reported with significant alterations in expression according to disease progression, and they may thus represent potential candidate diagnostic or prognostic biomarkers that may be used to evaluate patients once detection methods in peripheral blood are well established. Furthermore,

recent innovations in high-throughput sequencing techniques have enabled the discovery of unannotated tumor-associated ncRNAs and tumor-specific alternative splicing as novel and specific biomarkers of cancers. Although much work is required to clarify the release mechanism, origin of tumor-specific circulating RNAs, and selectivity of carrier complexes, and technical advances must also be achieved, such as creating a consensus normalization protocol for quantitative data analysis, circulating RNAs are largely unexplored and might represent novel clinical biomarkers.

Key words: MicroRNA; Non-coding RNA; Pancreatic cancer; Biomarkers; Early detection

Core tip: In this review, we summarize the latest findings on circulating RNAs in serum with a focus on their clinical use as novel diagnostic and prognostic biomarkers for pancreatic cancer. In addition, we summarize the current issues that need to be addressed to enable the clinical use of these circulating RNAs. This review will allow readers to concisely understand the current status and issues about the use of serum circulating RNAs as novel biomarkers for pancreatic cancer.

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INTRODUCTION

Pancreatic cancer remains an intractable disease and is the fourth leading cause of cancer death, with an increasing prevalence in the United States. The incidence and death rate of pancreatic cancer are high, and the 5-year survival rate remains only 6%[1]. More than half of pancreatic cancer patients are diagnosed at an advanced stage due to the lack of methods for its early detection. At present, we cannot expect a dramatic innovation in imaging modalities that will enable the detection of early stage cancer or pre-cancerous lesions using convenient, inexpensive methods[2], such as chest X-rays in lung cancer, mammography in breast cancer, or gastroendoscopy in gastric cancer.

Circulating biomarkers are useful screening tools because of the ease and relatively non-invasive nature of their collection. Among biochemical tests, serum CA19-9 is used widely as a circulating biomarker of pancreatic adenocarcinoma, but its utility is limited to monitoring the response to chemotherapy or surgical dissection because its sensitivity and specificity are insufficient for its use as a diagnostic biomarker of early stage pancreatic cancer[3]. Other serum protein markers have been suggested to enhance the predictability of pancreatic cancer, such as CEACAM1[4], MUC-1[5], REG4[6], TIMP-1[7], DJ-1[8], but none of them are in routine clinical use. Therefore, it might be useful to examine other molecules, such as nucleic acids, as novel biomarkers for the early detection of pancreatic cancer. Nucleic acids have several advantages as follows: their amplification is technically easy and they are less affected by degeneration or modification than protein- or carbohydrate-based tumor makers. Consequently, circulating nucleic acids have attracted increasing attention as novel tools in cancer diagnosis.

CIRCULATING NUCLEIC ACID AND CANCER

In the first study of circulating nucleic acids in cancer patients, Leon *et al*[9] detected free circulating DNAs in serum by radioimmunoassay. The polymerase chain reaction (PCR) technique has been used to isolate tumor-derived mutated sequences of the Kras and Nras genes from the serum and plasma of cancer patients[10,11]. Because Kras mutations are frequently observed in lung and gastrointestinal cancers in addition to nearly 100% of pancreatic cancers[12], many researchers are interested in the detection of mutated Kras in plasma[13]. More recently, genome-wide high-throughput sequencing of circulating DNA has been demonstrated as a potential detection tool, as well as a predictor of chemosensitivity[14,15].

CIRCULATING mRNAs

Since the late 1990s, quantitative reverse transcriptase (qRT)-PCR has been used to detect circulating RNAs as cancer diagnostic markers. Tumor-derived circulating RNAs have been detected in whole blood, plasma, or serum from patients with pancreatic cancer, gastric cancer[16], nasopharyngeal carcinoma[17], and melanoma[18]. Although RNA species in the bloodstream have been considered to be more fragile than DNA because of the high RNase level in blood[19], plasma RNAs have been found to be unexpectedly stable against RNase degradation[20,21].

Since the early 2000s, various types of messenger RNAs (mRNAs) that are upregulated in cancer tissues have been detected in the peripheral blood of patients with lung cancer[22], breast cancer[23,24], melanoma[21], hepatocellular carcinoma[25], colorectal cancer[26-28], prostate cancer[29], gastric cancer[30], glioblastoma[31], chronic myelogenous leukemia[32], and pancreatic cancer[33-35]. In particular, many studies have reported circulating hTERT mRNA in the serum or plasma as a marker of various cancers[36-41], because telomerase

activity, which maintains telomere length and prevents eukaryotic cells from senescence, is upregulated in a wide variety of cancers, whereas it is mostly suppressed in non-cancer tissues[42]. Some studies have achieved higher sensitivity and specificity by evaluating a combination of several tumor-specific mRNAs rather than circulating DNA or carbohydrate tumor markers[40,43,44].

In pancreatic cancer, Funaki *et al*[16] have detected carcinoembryonic antigen (CEA) mRNA by RT-PCR in the whole blood of pancreatic cancer patients. Clarke *et al*[33] have detected epidermal growth factor receptor (EGFR) mRNA in the serum and Ishizone *et al*[34] have detected alpha 1,4-N-Acetylglucosaminyltransferase (α4GnT) mRNA in the mononuclear cell fraction of peripheral blood from pancreatic cancer patients. Further, Kang *et al*[35] have recently demonstrated that serum type Ⅵ collagen (COL6A3) mRNA is a good marker of pancreatic cancer because it undergoes tumor-specific alternative splicing, which is expected to result in high specificity.

CIRCULATING mRNAs AND PANCREATIC CANCER

MicroRNAs (miRs) are small, single-stranded non-coding RNAs (ncRNA) consisting of 18-22 nucleotides that regulate the post-transcriptional expression of multiple genes[45]. Because miRs play important roles in controlling cell proliferation, differentiation, and apoptotic induction by targeting the mRNAs of various genes and a single miR is able to control the expression of hundreds of genes[46,47], miR dysregulation may affect cancer development, as well as the expression of oncogenes or onco-suppressor genes. There have been many reports on the aberrant over-expression or downregulation of miRs in various cancer tissues and subsequent alternations in the expression of their target genes, which are involved in proliferation and malignant transformation. In addition, miR expression is tissue specific[48], and alteration in specific miRs have been associated with cancer development[49,50]. Consequently, an altered miR expression profile could be a biomarker of malignant tumors, as well as an attractive therapeutic target in cancer[51,52].

In 2008, two studies demonstrated that miRs are released into the circulation in a remarkably stable form, even after freeze/thaw cycling or room temperature incubation, and suggested that circulating miRs carry disease-specific signatures that can be exploited as non-invasive biomarkers[53,54].

Regarding pancreatic cancer, several studies have described tumor-derived miRs in the circulation as diagnostic or prognostic biomarkers (Table 1). Wang *et al*[55] have analyzed the expression of miRs in the plasma of patients with pancreatic ductal adenocarcinoma and have identified miRs-21, miR-210, miR-155, and miR-196a, which have been reported to be upregulated in pancreatic cancer tissue and cell lines[56-67], as candidate biomarkers. Similarly, miR-200a/b, miR-18a, miR-221, and miR-196a/b have been found to be upregulated in the serum/plasma in parallel with cancer tissues[55,58-83]. Recently, comprehensive sequencing and microarray analyses have been performed to identify other circulating miRs, and combined analyses of the expression of several miRs have achieved high detectability with high sensitivity and specificity[73,75,77,78,81,82,84].

OTHER CIRCULATING ncRNAs AND CANCER

Small ncRNAs

NcRNAs are divided into two families according to their lengths, small ncRNAs (up to 200 bases) and large ncRNAs (over 200 bases). Almost all of the studies examining circulating RNAs have focused on miRs because of the existence of established methods for quantifying their expression, such as microarray kits and qRT-PCR. There are currently few reports of other small ncRNA family members (Table 2), such as small nucleolar RNA (snoRNA)[85-88], small nuclear RNA (snRNA), and Piwi-RNA (piRNA)[89,90], in cancer tissues. SnoRNAs, which function as guide RNAs for the post-transcriptional modification of ribosomal RNAs and some spliceosomal RNAs[91], are deregulated in various cancer tissues and induce a tumor-promoting phenotype[85-88,92]. Using comprehensive next-generation sequence analysis, Liao *et al*[93] have found that the plasma snoRNA SNORD33/66/76 might serve as a diagnostic biomarker for non-small-cell lung cancer.

PiRNAs interact with a subset of Argonaute proteins related to Piwi (Pelement induced wimpy testis in Drosophila) and maintain genomic integrity by epigenetically silencing transposons *via* DNA methylation, especially in germline stem cells. They have recently even been identified outside of the germline and in human cancer cells[89,90] and may be valuable biomarkers for detecting circulating gastric cancer cells[94].

SnRNAs are found within the splicing speckles of the cell nucleus and function as guides for pre-mRNA splicing in association with small nuclear ribonucleoproteins[95]. Several types of snRNA, including U2 snRNA and U6 snRNA, have been frequently used as housekeeping genes for normalization in qRT-PCR. However, it has also been reported that circulating U2 snRNA (RNU2-1) might be a useful diagnostic biomarker in pancreatic, colorectal, lung, and ovarian cancers[84,96,97].

Long ncRNAs

High-throughput RNA sequencing techniques have revealed that long non-coding RNAs (lncRNAs), which are not translated into proteins, are transcribed as

Table 1 Circulating RNA specifically deregulated in pancreatic cancer

Target candidate	Up/ down	Sample	Number of patients	Extraction method	Quantification method	Target selection	Normalization	Potential value	Ref.
mRNA									
CEA mRNA	Up	Whole blood	9 PK, 9 HC	AGPC	RT-PCR	Pre	ACTB	D	[16]
EGFR mRNA	Up	Serum	11 PK, 23 HC	N/A	RT-PCR	Pre	B2M	D	[33]
4GnT mRNA	Up	Whole blood	55 PK, 10 CP, 70 HC	RNeasy Mini kit	RT-PCR	Pre	GAPDH	D	[34]
COL6A3 mRNA	Up	Serum	44 PK, 46 BT, 30 HC	PureYield RNA Midiprep	qRT-PCR	Pre	GAPDH	D/P	[35]
miRNA									
mIR-155	Up	Plasma	49 PK, 36 HC	TRIzol LS	Taqman	Pre	miR-16	D	[55]
miR-196a	Up							D	
miR-21	Up							D	
miR-210	Up							D	
miR-155	Up	Pancreatic juice	16 PK, 5CP	mirVana PARIS kit	Taqman	Pre	miR-199 U6-snRNA	D	[68]
miR-21	Up							D	
miR-196a	Up	Serum	35 PK, 15 CP, 15 HC	TRI Reagent BD	Taqman	Pre	cel-miR-39	D/P	[69]
miR-200a	Up	Serum	45 PK, 11 CP, 32 HC	mirVana miR isolation kit	Taqman	Pre	miR-16	D	[70]
miR-200b	Up							D	
miR-210	Up	Plasma	11 PK, 14 HC	Boiling	Taqman	Pre	cel-miR-54	D	[71]
miR-18a	Up	Plasma	36 PK, 30 HC	mirVana PARIS kit	Taqman	Pre	synthetic reference panel	D/T	[72]
miR-16	Up	Plasma	140 PK, 111 CP, 68 HC	TRI Reagent BD	Taqman	Pre	cel-miR-39	D	[73]
miR-196a	Up							D	
miR-185	Up	Serum	80 PK, 129 HC	TRIzol LS	Taqman	Sequence	serum volume	D	[74]
miR-191	Up							D	
miR-20a	Up							D	
miR-21	Up							D/P	
miR-24	Up							D	
miR-25	Up							D	
miR-99a	Up							D	
miR-1290	Up	Serum	41 PK, 38 BT, 35 CP, 19 HC	mirVana PARIS kit	Taqman	Taqman mA	miR-16	D	[75]
miR-221	Up	Plasma	47 PK, 30 HC	mirVana PARIS kit	Taqman	Pre	synthetic reference panel	D/T/P	[76]
miR-375	Down							D/T/P	
miR-375	Up	Plasma	48 PK, 47 HC	Total RNA purification kit (Norgen)	Taqman	Affymetrix mA	cel-miR-39,cel-miR-54, cel-miR-238	D	[77]
miR-27a3p	Up	Whole blood	129 PK, 103 BT, 60HC	Trizol	Taqman	Sequence	U6 snRNA	D/S	[78]
miR-196a	Up	Serum	19 PK, 10 CP, 20 BT, 10 HC	miReasy RNA extraction kit	Taqman	Pre	miR-24	D	[79]
miR-196b	Up							D	
miR-205	Up	Pancreatic juice	50 PK, 19 CP, 19 HC	TRIzol LS	Taqman	Agilent mA	U6 snRNA	D/P	[80]
miR-210	Up							D/P	
miR-492	Up							D/P	
miR-1427	Up							D/P	
miR-22	Up	Plasma	11 PK, 11 HR, 11 HC	TRI Reagent BD	Taqman	custom mA	miR-3196	D	[81]
miR-642b	Up							D	
miR-885-5p	Up							D	
Multi gene index		Whole blood	409 PK, 25 CP, 312 HC	PAXgene blood RNA	Taqman	Taqman mA	ath-miR159a	D	[82]
miR-483-3p	Up	Plasma	32 PK, 12 BT, 30 HC	mirVana PARIS kit	Taqman	Pre	miR-16	D	[83]
miR-21	Up							D	
snRNA									
U2 snRNA	Up	Serum	80 PK, 129 HC	mirVana miR isolation kit	qRT-PCR	Agilent mA	cel-miR-54	D	[84]

Reference were searched in PubMed database using the following search terms: "pancreatic cancer", "non-coding RNA", and "circulating" or "serum" or "plasma" or "blood". PK: Pancreatic cancer; BT: Benign tumor; CH: Chronic pancreatitis; HR: High-risk patients; HC: Healthy control; AGPC: Acid-guanidinium-phenol-chroroform method; N/A: Not available; Taqman: Taqman qRT-PCR; Pre: Previously reported in pancreatic cancer tissues; mA: MiRNA microarray; D: Diagnostic marker; P: Prognostic marker; S: Staging marker; T: Treatment marker.

Table 2 Other circulating non-coding RNAs detected in cancer patients

Target candidate	Up/ down	Cancer type	Sample	Number of patients	Extraction method	Quantification method	Normalization	Target selection	Ref.
Small ncRNA									
SNORD33/66/76	Up	Lung	Plasma	37 Ca, 26 HR, 22 HC	mirVana miR isolation kit	SYBR qRT-PCR	U6 snRNA	mA	[93]
piR-651	Up	Gastric	Whole blood	93 Ca, 32 HC	TRizol	SYBR qRT-PCR	U6 snRNA	Pre	[94]
piR-823	Up								
U2snRNA	Up	Pancreatic	Serum	80 Ca, 129 HC	mirVana miR isolation kit	qRT-PCR	cel-miR-54	mA	[84]
U2snRNA	Up	Colorectal		132 Ca, 129 HC					
U2snRNA	Up	Lung	Serum	62 Ca, 51 BT, 45 HC	miRNeasy mini kit	LNA qRT-PCR	cel-miR-39	mA	[96]
U2snRNA	Up	Ovarian	Serum	119 Ca, 35 HC	mirVana PARIS kit	qRT-PCR	cel-miR-54	mA	[97]
Long ncRNA									
H19	Up	Gastric	Plasma	43 Ca, 34 HC	N/A	pre-amplification qRT-PCR	N/A	Pre	[107]
MALAT1	Up	Prostate	Plasma	87 Ca, 82 HR, 23HC	mirVana PARIS kit	Taqman	Input amount	Pre	[108]
HULC	Up	Hepato-cellular	Plasma	30 Ca, 20 HC	TRIzol	qRT-PCR	GAPDH	Pre	[110]
TUG1 lincRNA	Up	Multiple myeloma	Plasma	62 MM, 40 HC	TriPure	qRT-PCR	GAPDH	Pre	[111]
LincRNA-p21	Down	CLL		68 CLL, 40 HC					

Reference were searched in PubMed using the following search terms: "cancer", "non-coding RNA"or "snoRNA" or "snRNA" or "piRNA" or "lncRNA", and "circulating" or "serum" or "plasma" or "blood". lincRNA: Long intergenic noncoding RNA; CLL: Chronic lymphocytic leukemia; MM: Multiple myeloma; Ca: Cancer patients; BT: Benign tumor; HR: High-risk patients; HC: Healthy control; N/A: Not available; Taqman: Taqman qRT-PCR; Pre: Previously reported in cancer tissue; mA: MiRNA microarray.

Table 3 Non-coding RNAs specifically upregulated in pancreatic cancer tissue

Target candidate	Up/ down	Sample	Number of patients	Extraction method	Quantification method	Normalization	Target selection	Potential value	Ref.
HOTAIR	Up	Tissue	36 PK, 36 HC	mirVana RNA isolation kit	qRT-PCR	GAPDH	Pre	P	[112]
MALAT-1	Up	Tissue	126 PK, 15 HC	TRIzol	qRT-PCR	N/A	Pre	D/P/S	[113]
MEG3	Up	Tissue	31 PNET, 7 HC	RNeasy	qRT-PCR	GAPDH	Pre	D	[114]
Gas5	Up	Tissue	23 PK, 23 AN	TRIzol	qRT-PCR	ACTB	Pre	D	[115]
HULC	Up		304 PK, 304 AN	TRIzol	qRT-PCR	GAPDH	Pre	D/P	[116]
LOC285194	Down	Tissue	85 PK, 85 AN	TRIzol	qRT-PCR	GAPDH	Pre	D/P	[117]
PPP3CB intronic lncRNA	Up	Tissue	11 PK, 7 metastasis	TRIzol	qRT-PCR	HMBS	mA	D/S	[118]
MAP3K1	Up								
DAPK1	Up								
BC008363	Up	Tissue	30PK, 30 AN				mA	D/P	[119]
ENST00000480739	Down		35 PK, 35 AN	TRIzol	qRT-PCR	ACTB	mA	D/P	[120]
HSATII	Up	Tissue	11 PK, 2 HC	TRIzol	Sequence	Input amount	Sequence	D/P	[121]

Reference were searched in PubMed using the following search terms: "pancreatic cancer", "long non-coding RNA" or "lncRNA" or "ncRNA". PK: Pancreatic cancer; AN: Adjacent normal tissue; HC: Healthy control; mA: cRNA microarray; N/A: Not available; Pre: Previously reported in cancer tissue or cell line; D: Diagnostic marker; P: Prognostic marker; S: Staging marker.

frequently as protein-coding mRNAs. Most lncRNAs are thought to have biological functions, and increasing numbers of cancer-associated lncRNAs, such as HOTAIR[98], MALAT-1[99], ANRIL[100], H19[101], PTCSC3[102], and PCA3[103,104], have been reported to be upregulated in various cancer cells and to play potential roles in both oncogenic and tumor-suppression pathways, functioning as epigenetic regulators, guides for alternative splicing, decoys of miRNAs, and a scaffolds for protein complexes[105,106].

Although there is little data on the use of circulating lncRNAs as cancer-detecting markers, they could be used as novel potential biomarkers for diagnostic, prognostic, and therapeutic purposes (Table 2). For example, the secretion of H19 lncRNA in the plasma is increased in gastric cancer patients[107]. In addition, serum MALAT-1 RNA was upregulated in prostate cancer[108] and plasma TUC339 and HULC lncRNA were identified as novel markers for the detection of hepatocellular carcinoma[109,110]. Most recently, Isin *et al*[111] have reported tumor-type specific changes in TUG1 and LincRNA-p21 expression in the plasma in association with B-cell neoplasm.

There are no reports of upregulated lncRNAs in the circulation in pancreatic cancer patients at present. However, some reports have shown significant changes

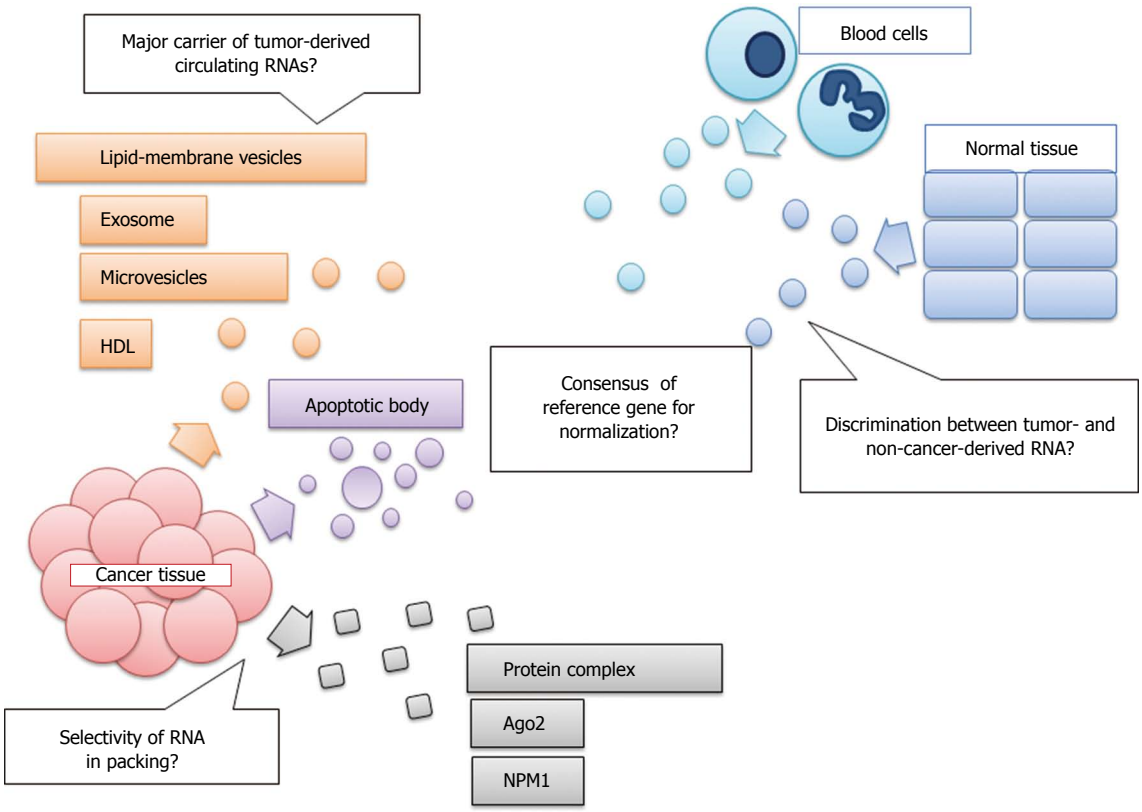

Figure 1 Overview of circulating RNAs from cancerous tissues. The origin of circulating RNAs from cancerous tissues is indicated. Several manners of their encapsulation and possible questions to be addressed are also shown.

in the expression of lncRNA in pancreatic cancer tissues and cell lines, which have been previously reported in other types of cancer[112-117] (Table 3). Recently, novel candidate genes have been found to be diagnostic and prognostic markers of pancreatic cancer using comprehensive microarray or RNA sequence analyses (Table 3)[118-121]. In particular, the expression of HSATII RNA, which is a highly repetitive transcript from centromeric heterochromatic regions, differs significantly in cancer and pre-cancer tissues compared with normal pancreatic tissue. Although it is necessary to examine whether circulating lncRNAs are as stable as miRs, which are resistant to RNases, these tumor-specific lncRNAs might be useful detection markers in the serum/plasma with high sensitivity and specificity.

Origin of circulating RNAs

The origin and manner of release of extracellular cancer-associated RNAs are unclear but could potentially affect the significance of results. It is not clear whether the cancer-associated RNAs detected in the circulation result from tumor cell death and lysis or whether they are actively secreted by tumor cells (Figure 1). While living cells actively release RNA encapsulated in large lipoprotein complexes, such as exosomes or microvesicles (MVs), RNA from dead or dying cells found in blood is associated with apoptotic bodies or protein complexes[122]. An exosome is a 40 to 140-nm-diameter lipoprotein membranous vesicle of endocytic origin that is formed from the fusion of multivesicular bodies (MVB) with the plasma membrane and released into extracellular spaces. MVs are larger than exosomes, with diameters ranging from 100-1000 nm and heterogeneous morphologies, and they originate from the plasma membrane via direct outward budding into the extracellular space. Apoptotic bodies are membrane vesicles that are heterogeneous in shape, range from 50-500 nm in diameter, contain organelles, and are released via outward protrusion of the plasma membrane during the late phase of apoptosis[123-125].

Many studies have shown that tumors specifically secrete exosomes or MVs, which selectively contain specific miRs[126,127]. However, Arroyo *et al*[128] and Turchinovich *et al*[129] have asserted that miRs in circulation are principally found in association with the Ago2 ribonucleoprotein complex and not vesicles, indicating that the detected miRs are derived predominantly via apoptotic and necrotic processes, which occur frequently in tumor cells. Some reports have suggested that high- and low-density lipoproteins (HDLs and LDLs, respectively) contain circulating RNA[130] and that certain miRs are specifically encapsulated within them. At present, it is unclear whether cancer cells choose specific carriers for particular miRs depending on particular biological functions.

Moreover, circulating miRs in cancer patient

are derived from multiple sources, including not only circulating and primary tumor cells, but also immune cells and other blood cells and cancer-adjacent non-cancerous cells. The mechanisms regulating RNA secretion and the determination of selectivity as well as the functional roles of secreted RNAs are poorly understood. Limited studies have been performed to distinguish the efficiencies of antibodies between tumor-derived exosomes and other tissue derived exosomes[131-133]. These studies have distinguished tumor-derived exosomes from other tissue derived exosomes by magnetic-activated cell sorting and flow cytometry using an antibody for tumor-specific exosome surface antigens, such as EpCAM, and CD39/73[131-133]. Precise analyses of these "purified" circulating RNA may reveal a more specific expression signature of primary tumors. On the other hand, cancer-adjacent tissues, such as stromal cells, also release aberrantly expressing RNAs and transmit intercellular signals to cancer cells, such as chemoresistance signals[134], indicating that alternations in RNA expression signatures of cancer-adjacent tissues may also have some potential as cancer diagnostic markers.

TECHNICAL ISSUES

In addition to the stability of miRs in various body fluids, their levels can be easily measured by qRT-PCR, which allows for high-precision signal amplification using the TaqMan PCR method with stem-looped RT primers or oligo-dT primers after the polyadenylation of templates. Nevertheless, there is still significant variability among microarray analysis reports. One of the reasons for this validation is that the total amount of RNA in serum and plasma is small compared to the amount of RNA extracted from tissues or cell lines. Several novel detection methods have been developed to allow for rapid quantification of RNA, improve the sensitivity and specificity of detection and reducing the number of amplification steps required before detection[135]. Detection strategies range from the use of simple molecular beacons[136] and enzymatic luminescence[137] to the use of different nanoparticle-based probes[138-140] and different forms of electrophoresis, such as capillary isotachophoresis[141] and circular exponential amplification[142].

At present, there is no consensus on using endogenous reference RNAs to normalize circulating miRs levels for analyzing qRT-PCR results (Figure 1). It has been suggested that some miRs, such as miR-16, miR-223, and let-7, are highly and constantly expressed in the serum/plasma and are correlated with the number of blood cells[55,70,75]. However, other reports have claimed that miRs that have been recommended as controls are significantly altered in certain pathological states[87,143]. Because gene expression can vary within a population, to identify miR signatures related to diseases, it is important to determine the range of normal variability across demographic populations[144]. As another alternative to small RNAs, 5S rRNA, and U6 snRNA are commonly used as reference genes when examining cell-extracted miR levels[68,80,93]. However, some reports have suggested that they are very unstable because they are degraded by RNases in the plasma/serum, unlike miRs[54]. At present, external control normalization with a spiked mycetogenic gene or normalization to the total amount of input RNA is widely performed (Tables 1 and 2).

Recently, microarray chip analysis of lncRNAs has been exponentially developed in gene number and has become a convenient measuring tool. However, comprehensive RNA sequence analysis by next generation sequencing can yield more information about unannotated genes and alternative spliced transcript signatures that may be possible candidates for novel detecting markers[145]. Several robust RNA sequencing protocols have been developed that are applicable even with a minute amount of templates and at the single cell level[146,147]. In fact, tumor-specific alternative splicing has already been reported in pancreatic cancer[35,148-151]. These alternatively spliced transcripts are considered more specific because they are unaffected by transcripts produced by the same gene in normal cells. A candidate gene with a splicing pattern that is distinct between cancer and normal cells may represent a highly specific marker, in addition to tumor-specific mutations of circulating DNA.

BIOLOGICAL FUNCTIONS OF CIRCULATING RNAS

Despite the rapidly increasing number of reports, the biological significance of circulating RNAs in cancer remains unclear. It believed that cancer cells communicate with surrounding non-cancerous cells, such as stromal cells and immune cells, via extracellular RNAs that are protected by their carriers. This intercellular connection contributes to their uncontrolled proliferation, the malignant transformation of surrounding cells, the recruitment of new blood vessels, and the stimulation or escape from the response to cancer cells[31,152,153]. Understanding the mechanisms by which cellular RNAs are selectively loaded into a specific carriers is important, not only for the more precise quantification of tumor-specific circulating RNAs as powerful detection markers but also to improve novel therapeutic strategies, such as RNA delivery to target tissues[154].

CURRENT ISSUES AND FUTURE DIRECTIONS

It is difficult to detect emerging cancerous lesions of pre-cancerous cystic tumors, such as intraductal papillary mucinous neoplasms (IPMNs), and to

distinguish a focal cancer lesion from mass-forming pancreatitis, even when patients are extensively examined, for example, by endoscopic retrograde cholangiopancreatography (ERCP), and endoscopic ultrasonography (EUS). Prophylactic surgical resection of a pre-cancerous tumor is not always acceptable due to its capacity for invading the pancreas and surrounding organs, in contrast with colon and gastric adenomas, which are easily resectable by endoscopic procedures. Consequently, a specific biomarker is desperately needed to detect pre-cancerous lesions or to discriminate between malignant tumors and inflammatory lesions. In this regard, some studies have indicated that the tissue specificity of miRs and the dramatic changes that occur in their expression profiles over the course of oncogenesis should enable the detection pre-cancerous lesions, such as pancreatic intraepithelial neoplasia (PanIN)[58,62-65,155] and IPMN[156], in pancreatic tissues. In addition, some miRs expression changes in aspirated cystic fluid from IPMN have been shown to predict the presence of cancer[157]. It is difficult to obtain surgically resected pancreatic pre-cancerous lesion specimens. In particular, PanIN lesions are very small and are commonly only identifiable at the microscopic level; therefore, they are normally obtained by laser capture microdissection from resected chronic pancreatitis and mainly pancreatic cancer specimens. Furthermore, it is difficult to collect blood samples from patients with a pre-cancerous lesion because current screening examinations rarely detect high-risk patients in advance.

It is accepted that the progression of pancreatic ductal adenocarcinoma is associated with the accumulation of genetic alternation, as well as the adenoma-carcinoma sequence, in colorectal cancer[12]. As genetic alterations accumulate in normal pancreatic epithelial cells, pre-cancerous lesions called PanINs emerge and advance in stage, eventually progressing to invasive cancer. According to this multistep carcinogenesis hypothesis, several oncogenic mouse models have been established[158]. Mice that express a constitutively active mutant *Kras*^{G12D} protein in a pancreas epithelium-specific manner, demonstrate gradual PanIN progression. The combination of endogenous *Kras*^{G12D} expression and dysfunction of tumor suppressor genes, such as *p16*$^{INK4a/Arf}$ knockout, mutant *p53* expression, *p53* knockout, or transforming growth factor-β receptor 2 (*Tgfbr2*) knockout, result in the progression of invasive pancreatic ductal adenocarcinoma, which is histologically closed to human cancer tissue. These pre-cancer and progressive cancer mouse models may have advantages over human samples in terms of genetic simplicity and the limited influence of environmental factors, such as inflammation, for the evaluation of mutation-induced transcription alternations. In fact, we have performed microarray analysis of miRs from mouse normal pancreata, a pre-cancerous PanIN-like tumor and massive ductal adenocarcinoma and have found that the majority of miRs upregulated in cancer, such as miR-21 and miR-192, are already increased at the pre-cancerous stage in Kras-mutated PanIN tissues (unpublished data). Moreover, collecting blood samples from these genetically engineered mice is important to identify cancer- or pre-cancer-associated circulating RNAs that may be present in humans[159]. The evaluation of these circulating RNAs is also important in distinguishing cancer from inflammation because human pancreatic cancer tissues consistently accompany chronic inflammation.

On the other hand, pancreatic cancer has a high rate of metastasis and dissemination, which cause postoperative recurrence and the dysfunction of other organs, such as gastrointestinal obstruction, and eventually result in poor prognosis. One possible avenue for improving prognosis is to predict the presence of undetectable metastasis by clinical imaging and to select the best-suited treatment under preoperative or postoperative conditions. The levels of several individual miRs, including miR-10b, 21, 31, 126, 335, and 373, have been correlated with metastatic outcome in carcinoma patients[160]. Additionally, other classes of ncRNAs, such as HOTAIR[98], have been proposed as putative biomarkers for metastatic potential in human breast tumors. These results suggest their potential utilities as biomarkers for metastatic propensity. Similar strategies may be applicable for the evaluation of pancreatic carcinoma.

CONCLUSION

Although their release mechanisms and biological significance require further study, circulating RNAs are significantly beneficial to the pancreatic cancer field, representing possible novel diagnostic and/or prognostic markers. Improvements in extraction and amplification procedures and growing availability of next-generation sequencing technologies may allow for the discovery of more specific and sensitive RNA markers in the near future.

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