Clinical Chemistry 61:9
000–000 (2015)

Reviews

Current State of Circulating MicroRNAs as Cancer Biomarkers

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BACKGROUND: Numerous studies have demonstrated the existence of stable regulatory RNAs, microRNAs (miRNAs), in the circulation and have shown that the spectrum of these extracellular miRNAs is affected by various pathologic conditions including cancers.

CONTENT: Circulating miRNAs have been the focus of numerous cancer biomarker discovery efforts over the past few years; however, a considerable number of these studies have yielded inconsistent and irreproducible findings. Here, we have summarized and compared the results of studies covering 8 different cancer types to address key questions, including the possibility of using circulating miRNA to detect cancers and what factors may affect miRNA signatures. Although identifying circulating miRNA signatures to detect specific types of early stage cancers can be challenging, study results suggest that it may be possible to use miRNAs to detect cancers in general.

SUMMARY: Circulating miRNA is a rich source for potential disease biomarkers; however, factors, both intrinsic and extrinsic, that may affect measurement of circulating miRNA have not been fully characterized. Better understanding of intra- and intercellular miRNA trafficking and the fundamental biology of cancer cell–derived lipid vesicles may facilitate the development of circulating miRNA-based biomarkers for cancer detection and classification.

Cancer is a leading cause of death worldwide, primarily owing to the lack of early detection methods; as a result, many cancers are diagnosed at advanced stages with poor prognosis *(1, 2)*. It is crucial, therefore, to develop specific and sensitive biomarkers to identify cancer in the early stages to reduce cancer-related mortality. A clinically useful biomarker needs to fulfill several criteria, including being present in easily accessible samples, translatable between preclinical animal models and clinical practice, able to differentiate different disease types, present in concentrations that reflect the severity of the disease, and able to be measured reliably without influence of other factors *(3)*. Proteins have been the traditional focus for cancer biomarker discovery because most of the existing oncology markers are derived from protein targets. However, despite substantial efforts over the past few decades, very little progress has been made. This is partially a result of the lack of technology for obtaining comprehensive protein profiles in various body fluids. Work to develop protein-based biomarkers is also complicated by the wide variety of yet to be fully characterized posttranslational modifications. Since the discovery of cell-free circulating microRNAs (miRNAs),[6] many studies have shown that specific circulating miRNA concentrations correlated with the development and progression of cancer *(4–12)*. Because miRNAs have important regulatory roles in cells and exhibit stability in the extracellular environment, circulating miRNAs have been a focus of attention as potential disease markers. In addition, miRNAs fulfill several properties of good biomarkers, such as accessibility in various bodily fluids, sequence conservation between human and clinically important animal models, and available sensitive measurement methodologies.

Characteristics of Cell-Free Circulating miRNAs

miRNAs are a class of short, noncoding RNAs *(2, 13, 14)*, which regulate the expression of nearly one-third of all human genes by targeting their 3′ untranslated region (UTR). To date, more than 2500 human miRNAs have been identified (miRBase v. 21) (www.mirbase.org) and they are involved in every aspect of cancer development, including differentiation, prolifera-

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Received March 23, 2015; accepted June 16, 2015.
Previously published online at DOI: 10.1373/clinchem.2015.241190

[6] Nonstandard abbreviations: miRNA, microRNA; UTR, untranslated region; HCC, hepatocellular carcinoma; CRC, colorectal cancer; NSCLC, non–small cell lung cancer; MAPK, mitogen-activated protein kinase; NGS, next generation sequencing; qPCR, quantitative real-time PCR.

tion, apoptosis, and invasion *(2, 14)*. Some miRNAs are highly enriched in specific cell or tissue types, such as miR-122-5p in liver, raising the possibility of using specific miRNA signatures to determine the lineage and state of diseases *(15)*. Although the precise mechanism of the release of miRNAs into the extracellular environment is not completely understood, some miRNAs are probably released as a result of normal or pathology-associated cell death *(16, 17)*. It has been shown that miRNAs released by apoptotic and necrotic cells are associated with proteins, including RNA binding proteins NPM1 (nucleophosmin), AGO2 (Argonaute 2 protein), or HDL, to avoid RNase degradation *(18–20)*. Some extracellular miRNAs are released from cells in lipid vesicles *(21)*. Microvesicles are larger vesicles (100 nm–1 μm) released from the cell through blebbing *(21)*. Exosomes are smaller vesicles (30–100 nm) released when multivesicular bodies fuse with the plasma membrane *(22)*.

Circulating lipid vesicles may have an important role in cell-to-cell communication. It has been shown that cancer cells release vesicles containing angiogenic factors to induce angiogenesis *(23)*. miRNAs in lipid vesicles may be picked up by cells through endocytic uptake, membrane fusion, or binding to specific cell surface receptors and thereby affect mRNA targets in recipient cells *(24)*, leading to widespread consequences *(17)*. Therefore, circulating miRNAs may play an important role in the progression of various diseases. Because the cargos carried by these membrane vesicles reflect their cellular origins, there is great interest in identifying biomarkers from cancer cell–derived vesicles. In addition, analyzing the molecular content of these vesicles may shed light on perturbed molecular processes in cancer cells *(25, 26)*. To date, few studies have used purified lipid vesicles for biomarker discovery, likely a result of the difficulty of quickly and reliably obtaining vesicles from a large number of samples. Although several commercial kits are available for vesicle isolation, most utilize polymer-based precipitation of particles of certain sizes in the sample. Therefore, the vesicles obtained from these kits may contain other molecular complexes in circulation. Traditional ultracentrifugation-based methods provide cleaner vesicle populations, but they are time-consuming and require larger sample volumes. Therefore, this approach is not practical when dealing with a large number of samples in biomarker discovery projects. A better isolation method is clearly needed to facilitate the use of lipid vesicles in biomarker discovery.

Deregulated Circulating miRNAS in Different Cancers

The biomarker discovery and development process contains several key steps, including discovery, verification, validation, and clinical application. Various high-throughput profiling technologies are used in the discovery phase, followed by selection of candidate biomarkers that reflect the disease condition. The candidates are then validated with different populations and diseases conditions so that their concentration variability in healthy populations and clinical performance can be assessed. Most of the biomarkers reported in the literature fail to enter clinical practice because of inadequate performance, use of inappropriate statistical methods, inability to develop a reproducible assay, or high variability among the healthy population.

Recent studies have shown the association of circulating miRNAs with cancers. However, the field of circulating miRNA has shown considerable inconsistency between different studies. To investigate the potential of using circulating miRNAs for cancer diagnosis, we conducted a careful survey of published findings on several prominent cancer types, including lung cancer, hepatocellular carcinoma (HCC), gastric cancer, pancreatic cancer, colorectal cancer (CRC), prostate cancer, breast cancer, and cervical cancer. We wished to address questions like the feasibility of developing circulating miRNAs as biomarkers to detect cancers, the likelihood of finding unique circulating miRNA signatures for specific cancer types, and the impact of sample type used, i.e., serum vs plasma on the miRNA signature. We analyzed results from 148 studies with a total number of 11 135 cases and 7456 healthy controls (See Table 1 and Fig. 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol61/issue9). miRNA nomenclature has evolved quickly in the past few years, and to facilitate comparison of the results from different studies, we updated all miRNA names to the latest naming system based on miRBase v.21.

GASTRIC CANCER

A total of 44 dysregulated circulating miRNAs have been identified from 24 studies (1628 patients and 1432 controls) (see online Supplemental Table 1 and online Supplemental Fig. 1). Among these, 27 were observed in serum, 21 in plasma, and 5 in whole blood. Some miRNAs showed a similar trend of changes in different sample types and different studies. For example, increases in miR-20a-5p and miR-221 concentrations were observed in both serum and plasma samples (Table 1 and online Supplemental Table 2) and changes of miR-21-5p, -27a-3p, -106b-5p, and -191-5p in serum, and miR-18-5p, -21-5p, -106b-5p, -100a-3p, -451a, and -486-5p in plasma have been reported by multiple investigators (Table 2). Circulating miRNAs also provide reliable diagnostic power to detect gastric cancer. For example, in 1 report the changes of miR-221-3p, -376c-3p, and -744-5p concentrations in serum can identify cancer 5 years before any clinical symptoms *(27)*. It is interesting to note that the circulating miR-223-3p concentration correlated to *Helicobacter pylori* infection, which is a key risk factor for gastric

Table 1. List of dysregulated miRNAs in different cancers and sample types.[a]

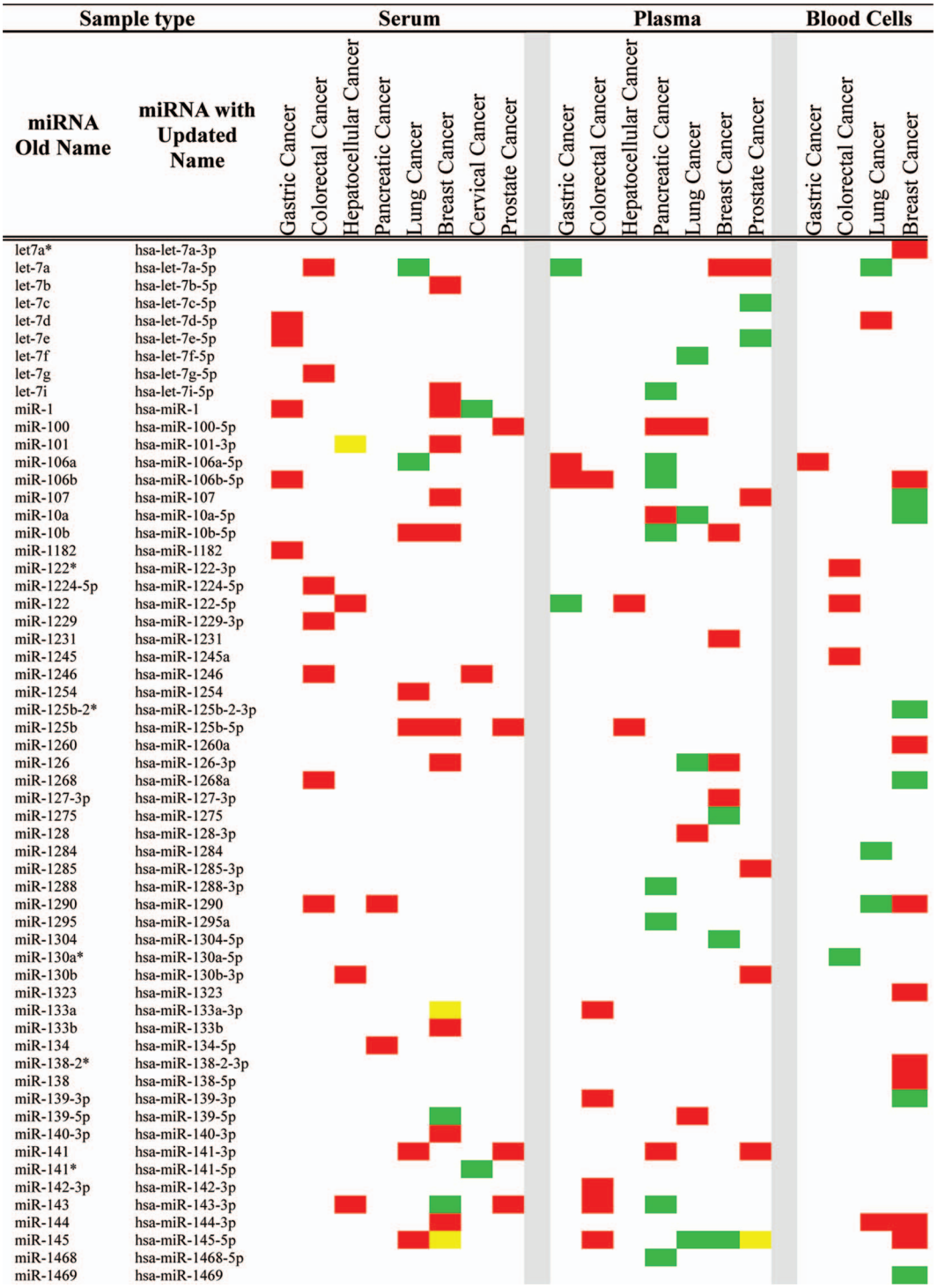

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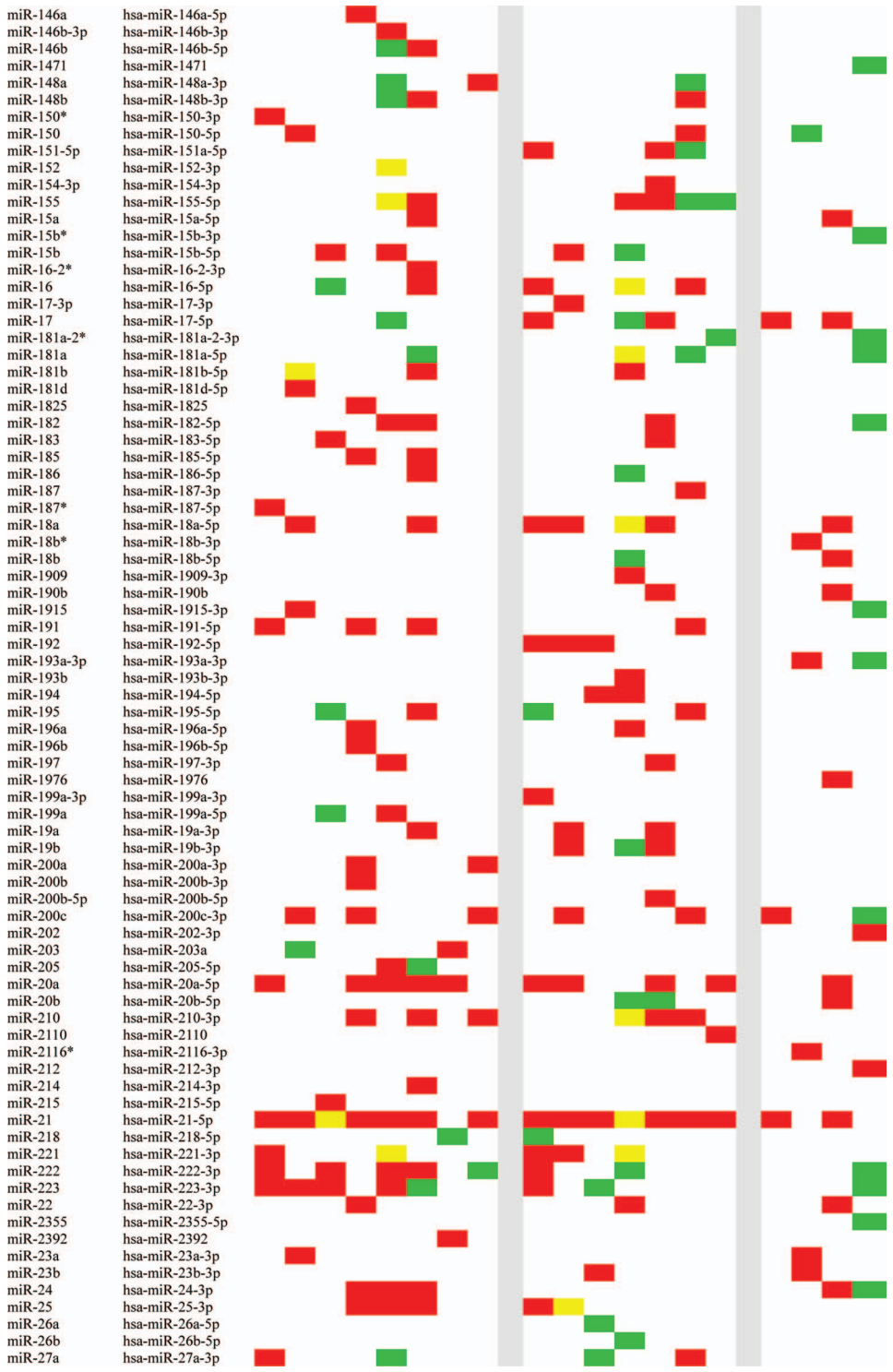
Table 1. List of dysregulated miRNAs in different cancers and sample types.[a] (*Continued from page XXX*)



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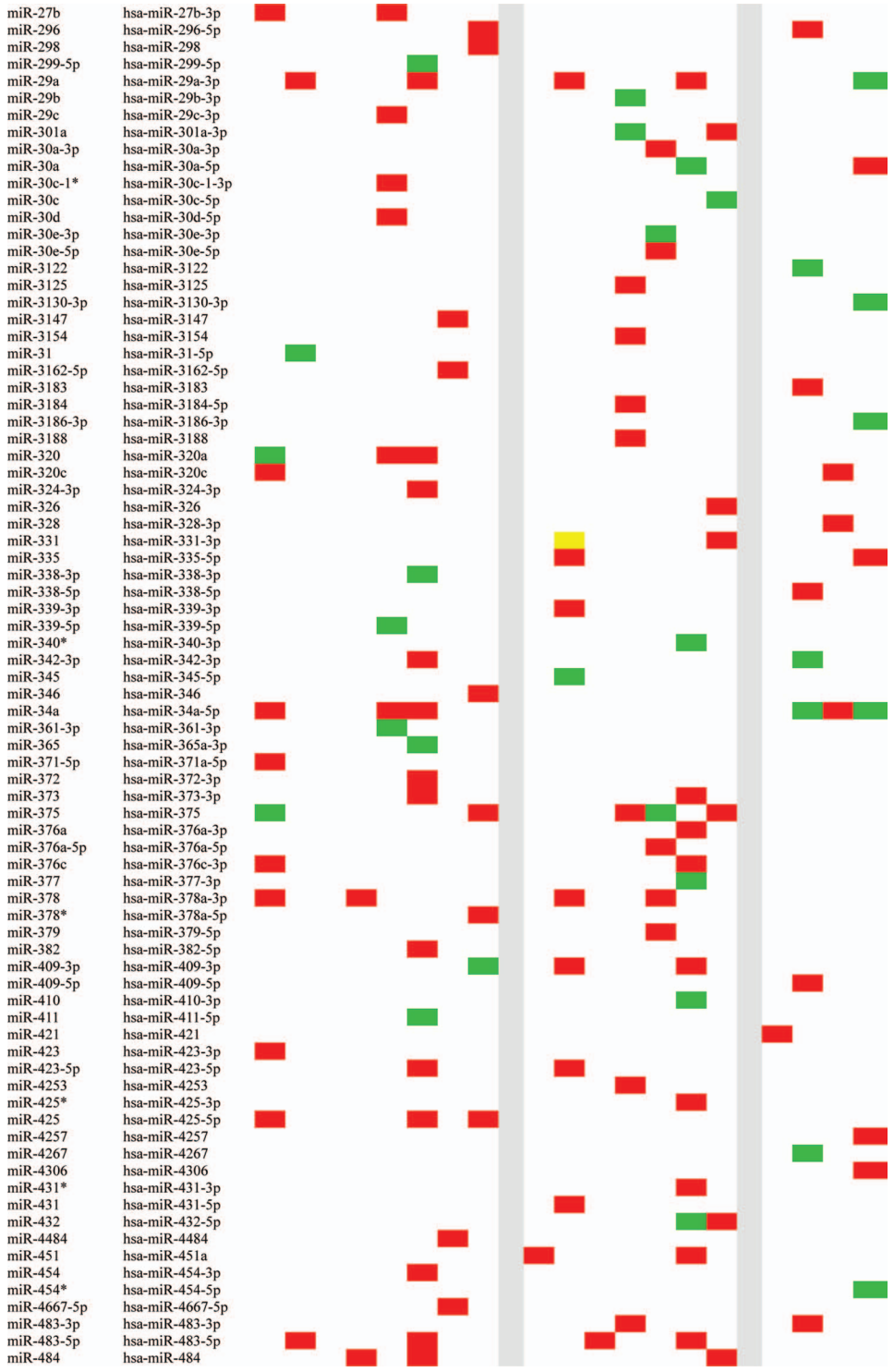

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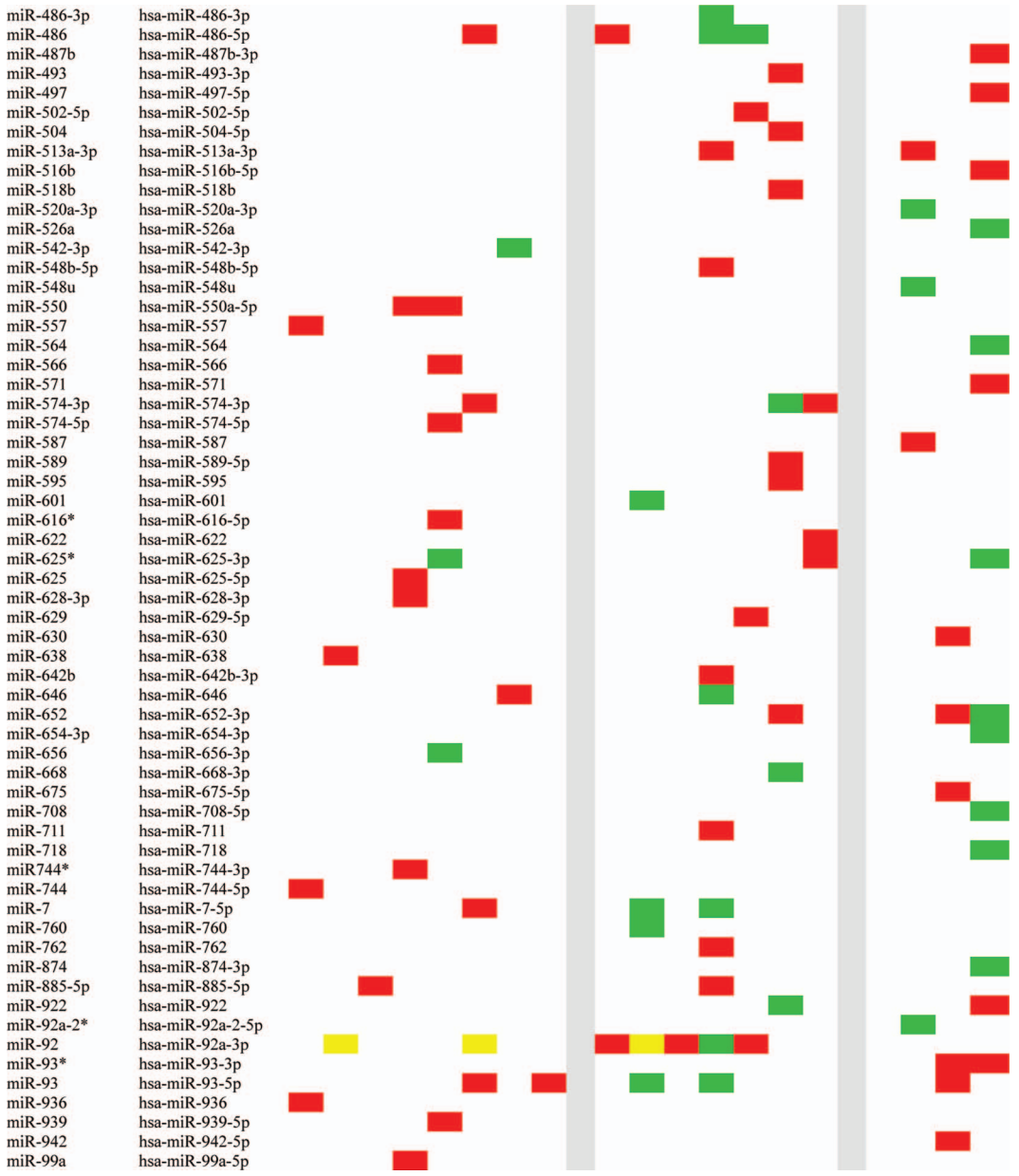

[a] The concentration changes of miRNAs are indicated by color: green represents decreased concentration, red represents increased concentration, and yellow indicates inconsistent concentration changes among different studies.

cancer. miR-21-5p and -199a-3p concentrations were associated with lymph node metastasis and overall survival *(28, 29)*. The plasma concentrations of miR-21-5p, -106b-5p, -451, and -486 were significantly decreased in postoperative paired samples, which suggests that tumor cells either directly release or indirectly effect the release of these circulating miRNAs in plasma *(30, 31)*.

COLORECTAL CANCER

For CRC, we analyzed 25 studies with a total of 2146 CRC patients and 1267 healthy controls and identified 78 dysregulated circulating miRNAs (see online Supplemental Table 1 and online Supplemental Fig. 1). Among these, 22 were observed in serum, 32 in plasma, and 24 in whole blood samples. The concentration changes of

Table 2. List of dysregulated miRNAs reported by multiple studies.[a]

Sample type	Gastric cancer	CRC	HCC	Pancreatic cancer	Lung cancer	Breast cancer	Cervical cancer	Prostate Cancer
Serum								
						hsa-let-7b-5p		
						hsa-miR-10b-5p		
						hsa-miR-125b-5p		
						hsa-miR-155-5p		
						hsa-miR-16-5p		
						hsa-miR-191-5p		
						hsa-miR-20a-5p		
						hsa-miR-214-3p		
						hsa-miR-21-5p		
						hsa-miR-222-3p		
	hsa-miR-106b-5p					hsa-miR-320a		
	hsa-miR-191-5p		hsa-miR-223-3p			hsa-miR-373-3p	hsa-miR-375	
	hsa-miR-21-5p	**hsa-miR-21-5p**	**hsa-miR-122-5p**		**hsa-miR-21-5p**	hsa-miR-425-5p		**hsa-miR-141-3p**
	hsa-miR-27a-3p	**hsa-miR-29a-3p**	hsa-miR-130b-3p	hsa-miR-24-3p	hsa-miR-24-3p	hsa-miR-486-5p	hsa-miR-20a-5p	hsa-miR-100-5p
Plasma								
	hsa-miR-106b-5p	**hsa-miR-21-5p**	hsa-miR-21-5p	hsa-miR-155-5p	**hsa-miR-21-5p**	**hsa-miR-21-5p**		**hsa-miR-141-3p**
	hsa-miR-18a-5p	**hsa-miR-29a-3p**	**hsa-miR-122-5p**	hsa-miR-196a-5p	hsa-miR-155-5p	hsa-miR-210-3p		
	hsa-miR-199a-3p	hsa-miR-15b-5p	hsa-miR-223-3p	hsa-miR-222-3p	hsa-miR-210-3p			
	hsa-miR-21-5p				hsa-miR-486-5p			
	hsa-miR-451a							
	hsa-miR-486-5p							

[a] The dysregulated miRNAs that are in common between serum and plasma in the same cancer type are listed in bold face characters. The shaded miRNAs indicate their concentrations are decreased in cancer samples.

some circulating miRNAs showed discrepancies between studies, such as the concentrations of miR-181b-5p and miR-92a-3p in serum and miR-25-3p, -92a-3p, and 331-3p in plasma (Table 1 and online Supplemental Table 2). On the other hand, independent reports also show consistent concentration changes on some miRNAs, including miR-21-5p and miR-29-3p in patient serum and miR-21-5p, -15b-5p, and -29a-3p in plasma (Table 2).

In 1 study, the miR-221-3p concentration in plasma provided 86% sensitivity and 41% specificity to detect CRC, and high plasma miR-221-3p concentration was associated with poor patient outcome because it correlated with the expression level of tumor protein p53 (*TP53*)[7] in cancer tissue *(32)*. A similar study has demonstrated that the increase of miR-21-5p concentration in circulation correlates with the increase of miR-21-5p in CRC tissue, and the plasma concentration of miR-21-5p can differentiate CRC patients from controls with 90% specificity and sensitivity *(33)*. The circulating miR-29a-3p concentration has been found to be higher in CRC patients with liver metastasis than in patients with local lesions *(34)*. The plasma miR-29a-3p and miR-92a-3p concentrations also appear to provide good diagnostic potential– 83.0% sensitivity and 84.7% specificity in detecting CRC *(35)*.

HEPATOCELLULAR CARCINOMA

Twenty-two dysregulated circulating miRNAs have been reported from 13 studies (a total of 971 HCC patients and 669 healthy controls) in which 14 of these were identified in serum and 11 in plasma (see online Supplemental Table 1 and online Supplemental Fig. 1). The diagnostic potential based on the changed specific circulating miRNA concentrations has been demonstrated by several independent studies, including miR-122-5p, -130-3p, and -223-3p in serum and miR-21-5p, -122-5p, and -223-3p in plasma *(36)*. However, some of the circulating miRNAs identified showed inconsistent changes in different studies. For example, miR-21-5p and -101-3p showed increased concentrations in patient sera in 1 study, but decreased concentrations in other studies *(37)* (Table 1 and online Supplemental Table 2). Furthermore, the use of different types of samples may also affect the results; for example, the miR-223-3p concentration increased in patient serum samples, but decreased in plasma samples (Table 1). On the other hand, miR-122-5p, a hepatocyte-enriched miRNA, showed consistent changes in both patient serum and plasma samples. The change of miR-122-5p in serum provided 81.6% sensitivity and 83.3% specificity to distinguish HCC patients from healthy individuals (see online Supplemental Table 1). Moreover, the increased miR-122-5p concentration in patient serum was significantly reduced in the postoperative samples, which suggests a direct correlation between cancer tissue and circulating miR-122-5p concentrations *(37)*. In cancer tissue, a high miR-125-5p concentration was associated with a favorable overall survival *(38)*. One study indicated an increase in miR-125-5p concentration in the plasma for some HCC patients *(39)*, but whether this increase is a direct reflection of the miR-125-5p concentration in cancer tissue and can be used as a prognostic marker for HCC patients is yet to be determined.

PANCREATIC CANCER

From 15 pancreatic cancer miRNA studies, 77 dysregulated miRNAs have been identified (746 patients and 481 controls), in which 24 were observed in serum, 53 in plasma, 3 in peripheral blood mononuclear cells, and 3 in pancreatic fluid (see online Supplemental Table 1 and online Supplemental Fig. 1). Some studies reported that plasma concentrations of miR-16-5p, -18a-5p, -21-5p, -181a-5p, -210-3p, and -221-3p were increased in pancreatic cancer patients, but others have shown the opposite results (Table 1 and online Supplemental Tables 1 and 2). Conversely, some miRNAs have shown consis-

[7] Human genes: *TP53,* tumor protein p53; *PTEN,* phosphatase and tensin homolog; *TIMP3,* TIMP metallopeptidase inhibitor 3; *RECK,* reversion-inducing-cysteine-rich protein with kazal motifs; *E2F1,* E2F transcription factor 1; *BCL2,* B-cell CLL/lymphoma 2; *RNU6–1,* RNA, U6 small nuclear 1; *ACVR1B,* activin A receptor, type IB; *ACVR1C,* activin A receptor, type IC; *FASLG,* Fas ligand (TNF superfamily, member 6); *TGFB1,* transforming growth factor, beta 1; *TGFBR2,* transforming growth factor, beta receptor II (70/80kDa); *TGFBR1,* transforming growth factor, beta receptor 1; *FAS,* Fas cell surface death receptor; *FH,* fumarate hydratase; *EGLN1,* egl-9 family hypoxia-inducible factor 1; *EGLN3,* egl-9 family hypoxia-inducible factor 3; *VHL,* von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase; *EPAS1,* endothelial PAS domain protein 1; *HIF1A,* hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); *CASP8,* caspase 8, apoptosis-related cysteine peptidase; *MSH2,* mutS homolog 2; *RUNX1,* runt-related transcription factor 1; *BMP2,* bone morphogenetic protein 2; *EP300,* E1A binding protein p300; *WNT5A,* wingless-type MMTV integration site family, member 5A; *FZD4,* frizzled class receptor 4; *FZD6,* frizzled class receptor 6; *FZD7,* frizzled class receptor 7; *VEGFA,* vascular endothelial growth factor A; *GSK3B,* glycogen synthase kinase 3 beta; *CTNNB1,* catenin (cadherin-associated protein), beta 1, 88kDa; *TCF7L1,* transcription factor 7-like 1 (T-cell specific, HMG-box); *TCF7,* transcription factor 7 (T-cell specific, HMG-box); *CDKN1A,* cyclin-dependent kinase inhibitor 1A (p21, Cip1); *NKX3–1,* NK3 homeobox 1; *XIAP,* X-linked inhibitor of apoptosis, E3 ubiquitin protein ligase; *ITGA,* integrin, alpha; *ITGB,* integrin, beta; *RB1,* retinoblastoma 1; *LAMA3,* laminin, alpha 3; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PIK3R1,* phosphoinositide-3-kinase, regulatory subunit 1 (alpha); *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *AKT3,* v-akt murine thymoma viral oncogene homolog 3; *MTOR,* mechanistic target of rapamycin (serine/threonine kinase); *CCND1,* cyclin D1; *IL8,* chemokine (C-X-C motif) ligand 8 (*CXCL8*); *E2F1,* E2F transcription factor 1; *E2F2,* E2F transcription factor 2; *E2F3,* E2F transcription factor 3; *COL4A,* collagen, type IV, alpha; *CRK,* v-crk avian sarcoma virus CT10 oncogene homolog; *CCNE2,* cyclin E2; *LAMC1,* laminin, gamma 1 (formerly LAMB2); *RHOA,* ras homolog family member A; *CDKN1A,* cyclin-dependent kinase inhibitor 1A (p21, Cip1); *JAK1,* Janus kinase 1; *STAT3,* signal transducer and activator of transcription 3 (acute-phase response factor); *FGF1,* fibroblast growth factor 1 (acidic); *FGF4,* fibroblast growth factor 4; *FGF7,* fibroblast growth factor 7; *FGFR,* fibroblast growth factor receptor; *PGDFRA,* platelet-derived growth factor receptor, alpha polypeptide; *RAS* •••; *RAF,* •••; *MAPK1,* mitogen-activated protein kinase 1; *MAPK9,* mitogen-activated protein kinase 9; *MAPK10,* mitogen-activated protein kinase 10; *JUN,* jun proto-oncogene; *FOS,* FBJ murine osteosarcoma viral oncogene homolog; *MYC,* v-myc avian myelocytomatosis viral oncogene homolog; *MMP2,* matrix metallopeptidase 2; *RARB,* retinoic acid receptor, beta; *PPARD,* peroxisome proliferator-activated receptor delta; *RHOA,* ras homolog family member A; *TCF7,* transcription factor 7 (T-cell specific, HMG-box); *TCF7L1,* transcription factor 7-like 1 (T-cell specific, HMG-box); *JUP,* junction plakoglobin.

tent changes in different studies as well as different sample types. For example, the concentrations of miR-22-3p and -196a-5p were increased in both serum and plasma samples from patients (see online Supplemental Tables 1 and 2). Similar concentration changes of miR-24-3p in serum and miR-155-5p, -196a-5p, and -222-3p in plasma were reported in several independent studies (Table 2). Some circulating miRNAs showed potential diagnostic value for pancreatic cancer *(29, 40, 41)*. For example the increased miR-200a-3p and miR-200b-3p concentrations in serum provided sensitivities between 71.1% and 84.4% and specificities between 87.5% and 96.9% for detection of pancreatic cancer *(40)*. Reports also suggested that a high concentration of miR-196a-5p could be used to predict poor survival of patients with pancreatic cancer *(29, 42)* and that the serum miR-196a-5p and -196b-5p concentrations could be used to screen individuals at risk from families with familial pancreatic cancer *(42)*.

LUNG CANCER

Lung cancer is an important health problem worldwide and is the most common cause of cancer-related death in western countries. We analyzed 29 studies and identified 87 dysregulated miRNAs with a total of 1999 patients and 1712 controls (see online Supplemental Table 1 and online Supplemental Fig. 1). Among the affected miRNAs, 42 were identified in sera, 34 in plasma samples, 26 in blood cells, and 4 in pleural effusion fluid, with very little overlap. Only 3 miRNAs, miR-17-5p, -20a-5p, and -21-5p, showed consistent changes in serum, plasma and blood cells (Table 1 and online Supplemental Tables 1 and 2). Several dysregulated miRNAs have been reported by more than 1 study (Table 2). Examples include miR-21-5p and -24-3p in serum and miR-21-5p, -210-3p, and -486-5p in plasma samples from non–small cell lung cancer (NSCLC) patients. A panel of 5 miRNAs, including 3 with increased plasma concentrations (miR-21-5p, -182-5p, and -210-3p) and 2 with decreased concentrations (miR-126-3p and -486-5p) provided good diagnostic power for all stages of NSCLC *(43, 44)*. Additionally, the increased miR-21-5p and -155-5p and decreased miR-145 concentrations in plasma also showed good diagnostic potential in differentiating patients with NSCLC from healthy smokers *(45, 46)*. Although it is not included in our analysis, the change in concentration of specific circulating miRNAs has also been reported as a prognostic marker. For example, in lung cancer patients, lower concentrations of let-7a-5p and -486-5p, and a higher concentration of miR-155-5p, were significantly correlated with a shorter survival and poor prognosis *(47)*.

BREAST CANCERS

For breast cancer, we identified 126 dysregulated circulating miRNAs from 35 studies with a total of 2850 patients and 1479 health control samples (see online Supplemental Table 1 and online Supplemental Fig. 1). Among the 126 affected miRNAs, 61 were observed in serum, 45 in plasma, and 52 in whole blood. Several of these miRNAs in serum or plasma from breast cancer patients have been reported in multiple independent reports (Table 2). Eleven miRNAs have shown the same trend of concentration changes between serum and plasma, including miR-10b-5p, -16-5p, -21-5p, -181a-5p, -195-5p, -210-3p, -223-3p, and others (Table 1 and online Supplemental Tables 1 and 2). Increased miR-21-5p, -29a-3p, -195-5p, -373-3p, and let-7a-3p concentrations in serum correlated well with tumor stage, vascular invasion, and cell proliferation index *(48, 49)*. The miR-16-5p, -25-3p, -222-3p, and -324-3p concentrations in serum provided good discrimination of breast cancer patients from controls with a sensitivity of 91.7% and a specificity of 89.6% *(50)*. Compared to existing protein markers, the concentrations of miR-125b-5p *(51)*, -30a-5p, and -155-5p have shown higher accuracy in monitoring the treatment efficacy of metastatic breast cancer compared to the concentrations of CEA (carcinoembryonic antigen) and CA15–3 *(52, 53)*.

A majority of the commonly affected miRNAs showed opposite concentration changes between blood cells and serum or plasma in breast cancer patients (12 out of 14) (Table 1). For example the concentration of miR-24-5p increased in patient serum samples but decreased in whole blood, whereas miR-30a-5p concentration increased in whole blood from patients but decreased in plasma. This inverse correlation between intra- and extracellular miRNA concentrations has been reported previously *(54)*. Establishing a precise concentration discordance between tissue and cell-free circulating miRNA concentrations may have important clinical application. For example the decreased concentration of miR-126-3p in breast cancer tissue correlates with poor prognosis and shorter overall survival *(55)*, but the concentration of miR-126-3p usually is higher in patient serum samples. Another example is miR-223-3p. Several studies have reported a decrease of circulating miR-223-3p concentration in breast cancer patients and increased miR-223-3p concentration in cancer tissues. The miR-223-3p concentration in tissue correlates with the aggressive behavior of cancer, which probably is mediated through the interactions with phosphatase and tensin homolog (*PTEN*) and TIMP metallopeptidase inhibitor 3 (*TIMP3*)—both of which are key proteins involved in cellular proliferation and migration.

CERVICAL CANCER

Only 14 dysregulated miRNAs have been identified from 5 independent studies of cervical cancer (see online Supplemental Table 1 and online Supplemental Fig. 1). The lower number of cervical cancer studies is likely due to the fact that most localized cervical cancer tissues are

accessible and can be diagnosed with regular cytopathological examination. Among the 14 dysregulated miRNAs in serum, miR-1, -20a-5p, -141-5p, -203a, -218-5p, -542-3p, -646, and -1246 also have been reported as differentially expressed miRNAs in cervical cancer tissues and their concentration changes correlated with vascular invasion and lymph node metastasis *(56–58)*. The increase of serum miR-20a-5p concentration in cervical cancer patients has been reported by independent studies (Table 2 and online Supplemental Table 1).

PROSTATE CANCER

Among the 37 dysregulated circulating miRNAs identified from 11 studies (see online Supplemental Table 1 and online Supplemental Fig. 1), 17 were observed in serum and 23 in plasma. Several miRNAs, including miR-21-5p, -141-3p, and -375, showed concentration increases in both serum and plasma from prostate cancer patients (Table 1 and online Supplemental Table 1). Like other cancer types, changes of some circulating miRNAs have been reported by independent studies including miR-100-5p and miR-375 in serum and miR-141-3p in both serum and plasma (Table 2 and online Supplemental Table 1). On the other hand, some reports showed a discrepancy in the change of miR-145-5p concentration in patient plasma (Table 1 and online Supplemental Tables 1 and 2).

The increase of circulating miR-375 and miR-141-3p concentrations in prostate cancer patient serum samples correlates with the increase of miR-375 and -141-3p concentrations in prostate cancer tissue samples *(59)*. Circulating miRNAs can reflect the aggressiveness of the cancer. For example, the increase of miR-375, -141-3p, and -378a-5p concentrations and decrease of miR-409-3p concentration in serum have been observed in patients with metastatic castration-resistant prostate cancer compared to samples from patients with low-risk localized prostate cancer *(60)*. Apart from diagnostic applications, circulating miRNA can also be used to predict the recurrence of prostate cancer. For example, plasma concentrations of miR-20a-5p, -21-5p, and -145-5p provide good correlation with risk of recurrence following localized treatment of prostate cancer *(61)*.

Comparing the Dysregulated Circulating miRNAs Identified

A total of 279 dysregulated circulating miRNAs have been identified from 148 independent studies with 8 different cancers. Among these, 97 dysregulated miRNAs were observed in blood (blood cells or whole blood), 144 in serum, and 148 in plasma (Fig. 1, Table 1, and online Supplemental Table 1). Because whole blood includes intracellular miRNAs in addition to circulating miRNAs, it is not surprising that the spectrum and concentration of miRNA observed is different from that in serum or plasma. Serum was the most frequently used sample type in 73 studies, followed by 58 studies using plasma and 15 studies using whole blood or blood cells (see online Supplemental Table 1). This is likely because serum is the most common sample processed and archived in clinics.

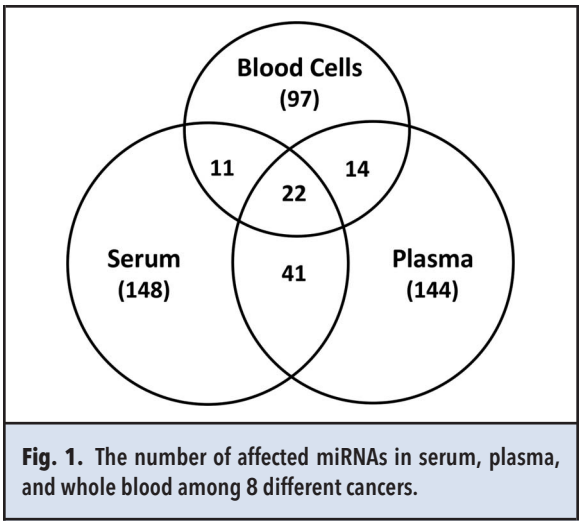

Fig. 1. The number of affected miRNAs in serum, plasma, and whole blood among 8 different cancers.

Very few overlaps were observed in the dysregulated miRNAs either between different types of cancer (Fig. 2) or different types of sample, such as serum vs plasma, within the same cancer type (Table 1 and online Supplemental Tables 1 and 2). For example, of the dysregulated miRNAs identified in HCC patient serum and plasma samples, there are only 3 miRNAs in common (miR-21-5p, -122-5p, and 223-5p). Some dysregulated circulating miRNAs are more frequently observed in serum whereas others are more frequently observed in plasma. For example, changes of miR-222-3p and -223-3p in serum have been observed in 5 out of 8 cancer types examined, whereas miR-92a-3p, -145-5p, -18a-5p, and -155-5p have been seen more frequently in plasma samples (Table 1 and Table 2). This is probably a result of intercellular miRNA trafficking during the coagulation process. A more detailed understanding of the complex coagulation process clearly is needed to determine its impact on the spectrum of cell-free miRNAs between serum and plasma.

One key question for cancer biomarker development is the feasibility of identifying unique miRNA signatures for individual cancer types. The low amount of overlap among the list of dysregulated circulating miRNAs for different cancer types suggests the possibility of having unique miRNA signatures for specific cancer types. There are some concerns regarding the specificity of these signatures; consider for example the concentration changes of circulating miR-122-5p, a highly enriched miRNA in hepatocytes, in patients with liver cancer as well as those with gastric cancer and CRC (Table 1, Table 2, and online Supplemental Table 1). The other

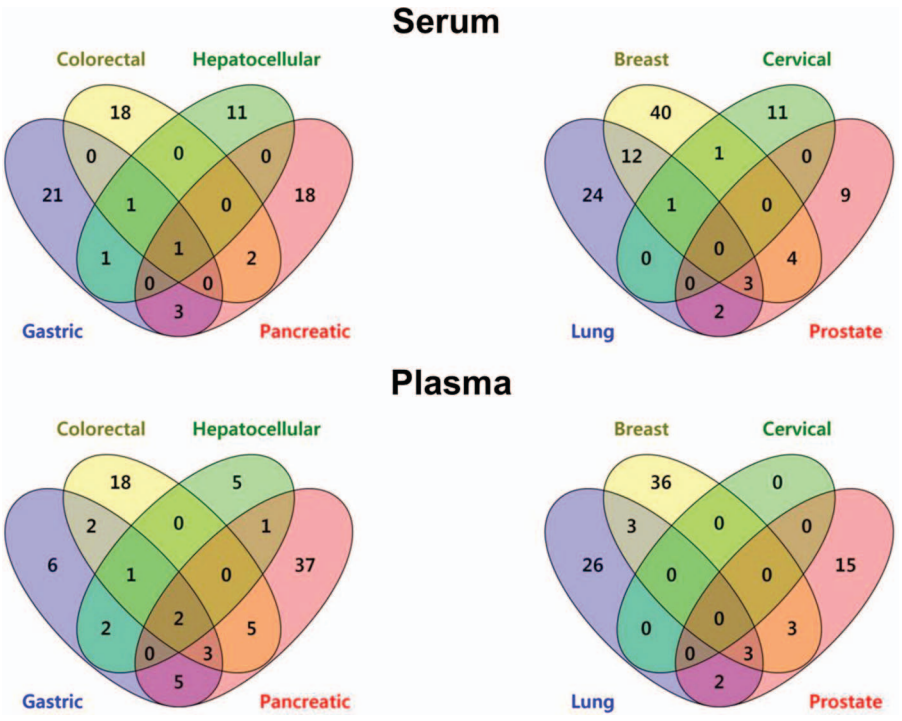

Fig. 2. Venn diagrams illustrate the low degree of overlap of affected circulating miRNAs among different cancer types in serum (upper panel) and plasma (lower panel).
The cancer types are indicated and the numbers represent the number of affected miRNAs. The data are based on miRNAs listed in Table 1.

example is miR-375, which is highly enriched in pancreatic islet cells. The alteration of circulating miR-375 concentration has been observed in patients with lung cancer (plasma), prostate cancer (plasma and serum), and gastric cancer (serum) (Table 1, Table 2, and online Supplemental Tables 1 and 2). This may suggest that most of the concentration changes we observe in specific circulating miRNAs are a result of indirect effects of the body's response to cancer growth, because the number of cancerous cells represents only a small fraction of the cells in the body. This also suggests that it will be difficult to develop unique circulating miRNA signatures to detect specific types of cancer. Instead, it may be more feasible to generate a circulating miRNA-based pan-cancer biomarker panel to detect different types of cancer.

Feasibility of Developing Circulating miRNA-Based Cancer Biomarkers

Although most of the dysregulated miRNAs have been reported in only 1 study, a few of them showed consistent changes in several independent studies (Table 2). It might be fruitful to conduct large-scale validation studies on the basis of these "verified" miRNAs with well-characterized and diverse populations of patient samples. Pathway enrichment analysis based on the predicted/validated targets of these "verified" miRNAs listed in Table 2 revealed the involvement of 2 major categories of biological processes—various signaling pathways, such as mitogen-activated protein kinase (MAPK) signaling pathway, inflammatory responses such as the Toll-like receptor signaling pathway, and various processes involved in immune response and infectious diseases (Table 3). These again may suggest that both the cancer cells and the body's response toward the growth of cancers contribute to the change in the spectrum of circulating miRNAs.

Comparing across the list of dysregulated miRNAs among different cancers (Table 1 and Table 2), miR-20a-5p and miR-21-5p are the 2 most frequently affected circulating miRNAs in cancer in both patient serum and plasma. These 2 miRNAs also are frequently increased in cancer tissues. The higher concentrations of miR-20a-5p and miR-21-5p in the cells increase its invasion and metastatic potential. Whether the increased miR-20a-5p and miR-21-5p concentrations in circulation are a direct result of tumor burden is yet to be determined, because these are not cell type–specific or tissue-enriched miRNAs. On the basis of validated and predicted targets, these 2 miRNAs have been found to target several tran-

Table 3. Biological pathways associated with verified dysregulated circulating miRNAs.[a]

	Serum								Plasma							
KEGG[b] pathway description	Gastric cancer	CRC	HCC	Pancreatic cancer	Lung cancer	Breast cancer	Cervical cancer	Prostate cancer	Gastric cancer	CRC	HCC	Pancreatic cancer	Lung cancer	Breast cancer	Cervical cancer	Prostate cancer
Metabolism																
Cysteine and methionine metabolism		5.66E-03														
Amino and nucleotide sugar metabolism									4.81E-03							
Steroid hormone biosynthesis						2.45E-03										
Genetic information processing																
Protein processing in ER	7.62E-03															
Ubiquitin mediated proteolysis										6.20E-03						
Basal transcription factors												3.53E-03				
Environmental information processing																
ErbB signaling pathway												4.85E-03				9.91E-03
Jak-STAT signaling pathway					4.26E-04					2.71E-03	1.42E-04		2.13E-03			
MAPK signaling pathway	8.26E-04		2.68E-03	2.98E-03	1.75E-03						1.52E-03		7.57E-03			
Cytokine–cytokine receptor interaction		7.52E-03		3.48E-03	1.37E-04					4.23E-04	2.40E-03		3.81E-03	2.32E-03		
ECM-receptor interaction		9.76E-03														
Cellular processes																
Focal adhesion		2.81E-03								2.53E-03						
Apoptosis					1.40E-03								6.22E-04			
p53 signaling pathway					4.59E-03					5.34E-03						
Peroxisome												8.46E-04				
Organism systems																
Vascular smooth muscle contraction	4.96E-03															
Protein digestion and absorption		6.99E-03														
B-cell–receptor signaling pathway												3.53E-03	5.56E-04			
Chemokine signaling pathway					9.78E-03								5.02E-03			
Fc ε RI signaling pathway											7.59E-03		5.54E-03			
Fc γR-mediated phagocytosis													1.29E-03			
Hematopoietic cell lineage					3.59E-03											
NOD-like receptor signaling pathway			6.07E-04								2.02E-04					
RIG-I–like receptor signaling pathway												1.90E-04	9.85E-03			
Toll-like receptor signaling pathway												4.69E-04	1.56E-04			

(*Continued on page XXX*)

Table 3. Biological pathways associated with verified dysregulated circulating miRNAs.[a] (*Continued from page XXX*)

KEGG[b] pathway description	Serum							Plasma						
	Gastric cancer	CRC	HCC	Pancreatic cancer	Lung cancer	Breast cancer	Cervical cancer	Prostate cancer	Gastric cancer	CRC	HCC	Pancreatic cancer	Lung cancer	Breast cancer	Cervical cancer	Prostate cancer
Human diseases																
Pathways in cancer							2.92E-03									
CRC													1.55E-04			
Glioma							6.30E-03									
Melanoma							3.55E-03									
Pancreatic cancer													4.96E-03			
Prostate cancer							5.68E-03									
Small cell lung cancer		4.78E-03								4.53E-03						
Type I diabetes mellitus				2.87E-03	2.92E-04											
Allograft rejection				3.50E-03	1.15E-03											
Graft-vs-host disease					1.20E-03											
Epithelial signaling in *Helicobacter pylori* infection																9.49E-03
Amoebiasis		1.59E-04								2.27E-03						
Chagas disease		1.43E-03			1.21E-03					5.62E-03	1.98E-03	1.52E-04	1.59E-04			
Leishmaniasis					5.27E-04							1.56E-03	1.76E-04			
Malaria		9.52E-03			2.07E-03						6.47E-03		9.52E-03			
Toxoplasmosis		1.64E-04								7.81E-04		4.68E-04	1.64E-04			
Hepatitis C							3.77E-03					6.87E-03				

[a] Values shown are *P* values. The *P* values were derived with the Fisher exact test and represent the degree of enrichment; the lower the number the higher the significance of the enriched terms.

[b] KEGG, Kyoto Encyclopedia of Genes and Genomes; ER, endoplasmic reticulum; Jak-STAT, Janus kinase-signal transducer and activator of transcription; ECM, extracellular matrix; NOD, nucleotide-binding oligomerization domain; RIG-I, retinoic acid–inducible gene 1.

scription factors that facilitate the growth and spread of cancer cells. Several key genes involved in cell proliferation, for example *PTEN*, a tumor suppressor to attenuate cell proliferation, are the target of miR-21-5p (Fig. 3). Furthermore, miR-21-5p could promote invasiveness by targeting inhibitors of matrix metalloproteinases [*RECK* (reversion-inducing-cysteine-rich protein with kazal motifs) and *TIMP3*] in cancer cells *(62)*. miR-20a-5p is a member of the 17–92 miRNA cluster—an important set of miRNAs associated with normal embryo development. It modulates the cell cycle, proliferation, and apoptosis through interactions with key genes like *E2F1* (E2F transcription factor 1) transcription factor and *BCL2* (B-cell CLL/lymphoma 2), an apoptosis regulatory protein (Fig. 3).

Techniques and Obstacles in miRNA Measurement

Despite some interesting and exciting findings, the field of circulating miRNA has been hindered by measurement-associated inconsistency and irreproducibility. Studies have already identified some of the factors contributing to these problems, such as sample type, measurement platform, or normalization strategy, but the contribution of other factors has yet to be determined. The major technologies used in research laboratories to measure the miRNA expression in biological samples include microarray, next generation sequencing (NGS), and quantitative real-time PCR (qPCR) *(63)*. Both microarray and qPCR are widely and routinely used to detect and quantify specific nucleic acid sequences. Microarray-based miRNA measurement platforms can provide overall miRNA expression profiles with reasonable cost and throughput. qPCR is more expensive per sample and has a lower throughput, but the amplification technology offers a much higher sensitivity than microarrays. There are several issues associated with both microarray and qPCR-based miRNA measurement methods: (*a*) they can assess the concentration only of known miRNA sequences, (*b*) it is difficult for both methods to separate signals of closely related sequences, and (*c*) both methods require a substantial amount of starting materials, especially for microarray-based measurements.

In contrast, NGS requires less starting material, can identify new miRNA species, is able to differentiate between closely related sequences, and is not affected by length variation of miRNAs (isomirs). Additionally, with sample multiplexing, the cost per sample for miRNA analysis by NGS platforms can be cheaper than microarray or qPCR. However, NGS sequencing library preparation is tedious and may introduce significant biases into the samples, and the large amount of data generated presents data storage and analysis problems. Moreover, reports have shown that different library preparation methods can introduce variations in miRNA profiling results *(64, 65)*. Both microarray and NGS-based platforms are suitable for screening and discovery purposes, but qPCR is still the top choice for validation and clinical tests with large numbers of samples. When using qPCR, however, proper design of primer and measurement conditions is critical, especially when dealing with individual members from a given miRNA family. For example, measuring a commonly altered circulating miRNA in cancer patients, miR-20-5p, one must consider closely related family member sequences (other members of the miR-17–92 family) when designing assay conditions. Owing to the low concentrations of circulating miRNA, qPCR and NGS platforms are the 2 most commonly used tools. Special care also needs to be taken to avoid possible contamination during sample processing when dealing with circulating RNA.

Regardless of the platform used for miRNA measurement, data normalization is another major issue. Proper normalization with internal control(s) is critical for comparing results from different samples, because it not only ensures the data quality but also reduces the variations introduced during sample collection, processing, and measurement. Global normalization approaches such as median or mean centered methods or quantile normalization have been applied for miRNA analyses. Reference miRNAs that show low concentration variations across different samples have also been suggested. For protein coding transcripts, several housekeeping genes have been successfully used as internal controls; however, similarly invariant miRNAs have not been identified *(66)*. Small RNA transcripts such as miR-16-5p, *RNU6–1* (RNA, U6 small nuclear 1), and 5S RNA have been suggested by different studies, but none of these are satisfactory or widely accepted as a normalization control for miRNA measurement. Spike-in synthetic RNAs have also been used in a number of studies, but they cannot 'remove" variations introduced before RNA isolation. Different normalization approaches may account for some of the inconsistencies among the results from different miRNA profiling studies. Therefore, development of a proper normalization procedure is not only necessary for identifying cancer miRNA biomarkers, but also is a key prerequisite for using miRNAs as biomarkers for diagnosis in the clinic.

Future Perspectives and Challenges of Circulating miRNAs as Cancer Biomarkers

The stability, availability of mature measurement technologies, conserved sequences between human and preclinical animal models, and concentration changes correlated to pathology make circulating miRNAs good biomarker candidates for cancer detection. However, to develop circulating miRNA-based biomarkers for clinical

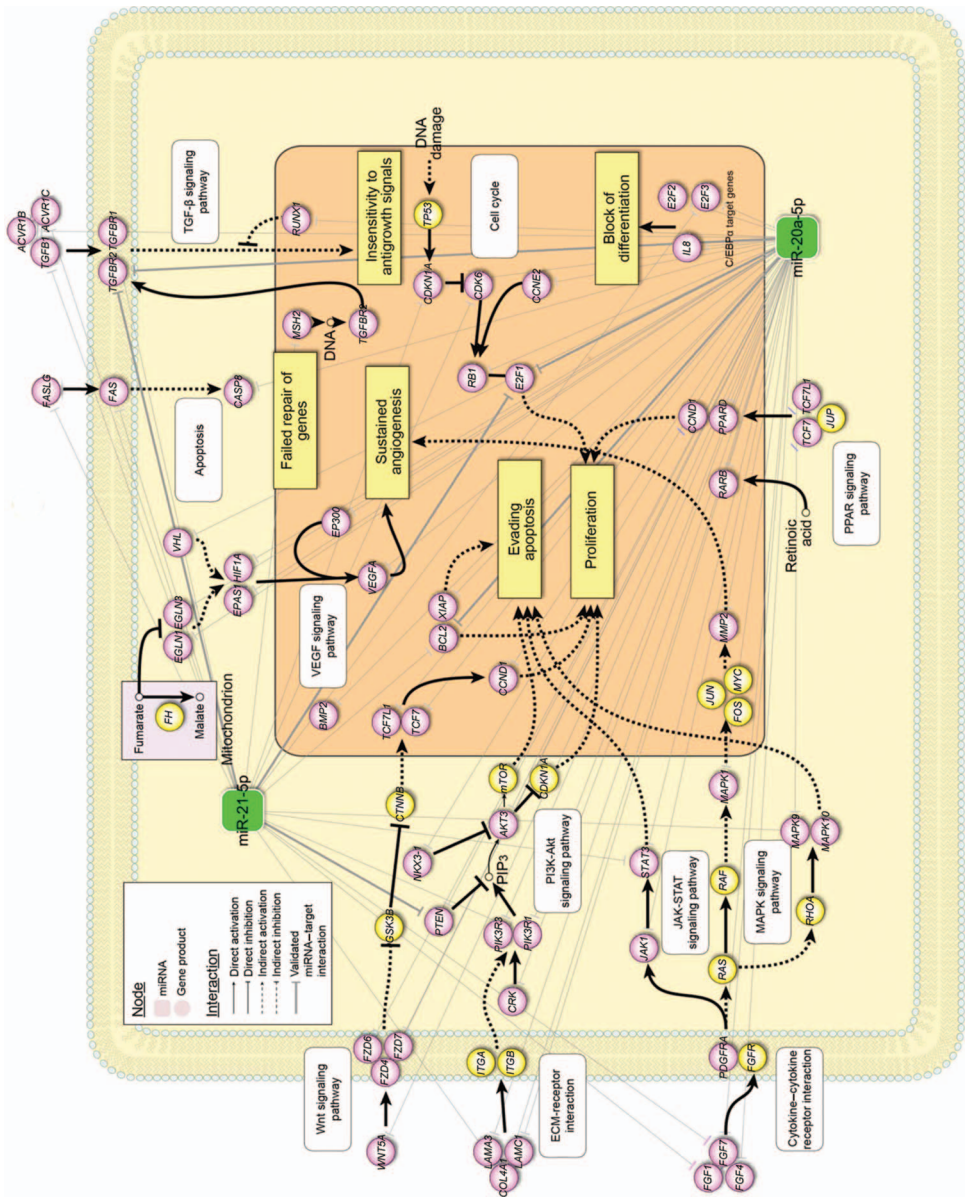

Fig. 3. Involvement of miR-21-5p and miR-20a-5p in the molecular network associated with cancer development.

The network is generated on the basis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (hsa05200). The genes are indicated by circles and miRNAs by squares as indicated in the legend at the upper left corner. The predicted miRNA–mRNA interactions are shown by light gray lines and the thick lines indicate validated miRNA-mRNA interactions. The dotted lines are interactions from KEGG pathway interactions. The identity of genes and miRNAs involved in the process are listed. The yellow circles are some of the important genes in the network that have not been targeted by the 2 miRNAs.

Continued on page xxx

usage there are many challenges that must be addressed. Factors like sex, age, race, sample type, and processing method, as well as RNA isolation and assay methods, may all affect the measurement of circulating miRNAs. Studies on breast cancer circulating miRNA showed differences between African American and European American ethnic groups *(67, 68)*, and several studies have shown the overall miRNA spectrum difference between serum and plasma *(69)*. One study using both NSCLC patient serum and plasma samples found no difference between plasma miRNA spectra in patients and controls but reduced concentrations of miR-17-5p, -27a-3p, -106a-5p, -146b-5p, -155-5p, -221-3p, and let-7a-5p and increased concentrations of miR-29c-3p in patients from the corresponding serum samples *(67)*. In summary, to apply miRNA-based cancer biomarkers clinically, systematic validation studies with randomized trials using diverse populations will be required to validate or assess prior reported clinical performance. So that the concentration changes can better reflect the pathology, the diagnostic panel should have (*a*) a unique miRNA signature with proper specificity and sensitivity for individual cancer types, (*b*) well-defined variations caused by both intrinsic and extrinsic factors, (*c*) proper normalization procedures with reliable internal controls; (*d*) well-characterized reference intervals of specific circulating miRNAs from healthy populations, and (*e*) well-established concentration kinetics of specific miRNA in circulation.

Because exosomes/microvesicles contain proteins and nucleic acids from the cells they originated from, there is considerable interest in exploring the possibility of using the molecular content of lipid vesicles in the circulation to develop cancer type-specific biomarker. Although the idea is reasonable, the difficulty is in isolating or enriching lipid vesicles specifically released by cancer cells. With more efforts in characterizing the content of lipid vesicles and understanding the cellular processes of selecting and packaging molecules into lipid vesicles, it might be possible to identify and use cancer-derived vesicles in circulation to detect specific cancer types and stages.

Conclusion

Extracellular circulating miRNAs offer many attractive features of good biomarkers for cancer detection, such as stability, accessibility, mature measurement technologies, and pathology-associated concentration changes. However, several challenges must be overcome to further develop circulating miRNA-based biomarkers for clinical applications. A comprehensive understanding of factors that may affect miRNA measurement will help to establish a commonly acceptable procedure for sample collection, storage and pro-

TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; PIP3, plasma membrane intrinsic protein 3; PI3K-Akt, phosphoinositide 3-kinase–Akt; JAK-STAT, Janus kinase–signal transducer and activator of transcription; PPAR, peroxisome proliferator-activated receptor; C/EBAα, C/enhancer-binding protein α. Human genes: *ACVR1B,* activin A receptor, type IB; *ACVR1C,* activin A receptor, type IC; *FASLG,* Fas ligand (TNF superfamily, member 6); *TGFB1,* transforming growth factor, beta 1; *TGFBR2,* transforming growth factor, beta receptor II (70/80kDa); *TGFBR1,* transforming growth factor, beta receptor 1; *FAS,* Fas cell surface death receptor; *FH,* fumarate hydratase; *EGLN1,* egl-9 family hypoxia-inducible factor 1; *EGLN3,* egl-9 family hypoxia-inducible factor 3; *VHL,* von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase; *EPAS1,* endothelial PAS domain protein 1; *HIF1A,* hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); *CASP8,* caspase 8, apoptosis-related cysteine peptidase; *MSH2,* mutS homolog 2; *RUNX1,* runt-related transcription factor 1; *BMP2,* bone morphogenetic protein 2; *EP300,* E1A binding protein p300; *WNT5A,* wingless-type MMTV integration site family, member 5A; *FZD4,* frizzled class receptor 4; *FZD6,* frizzled class receptor 6; *FZD7,* frizzled class receptor 7; *VEGFA,* vascular endothelial growth factor A; *GSK3B,* glycogen synthase kinase 3 beta; *CTNNB1,* catenin (cadherin-associated protein), beta 1, 88kDa; *TCF7L1,* transcription factor 7-like 1 (T-cell specific, HMG-box); *TCF7,* transcription factor 7 (T-cell specific, HMG-box); *CDKN1A,* cyclin-dependent kinase inhibitor 1A (p21, Cip1); *NKX3–1,* NK3 homeobox 1; *XIAP,* X-linked inhibitor of apoptosis, E3 ubiquitin protein ligase; *ITGA,* integrin, alpha; *ITGB,* integrin, beta; *RB1,* retinoblastoma 1; *LAMA3,* laminin, alpha 3; *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *PIK3R1,* phosphoinositide-3-kinase, regulatory subunit 1 (alpha); *PIK3R3,* phosphoinositide-3-kinase, regulatory subunit 3 (gamma); *AKT3,* v-akt murine thymoma viral oncogene homolog 3; *MTOR,* mechanistic target of rapamycin (serine/threonine kinase); *CCND1,* cyclin D1; *IL8,* chemokine (C-X-C motif) ligand 8 (*CXCL8*); *E2F1,* E2F transcription factor 1; *E2F2,* E2F transcription factor 2; *E2F3,* E2F transcription factor 3; *COL4A,* collagen, type IV, alpha; *CRK,* v-crk avian sarcoma virus CT10 oncogene homolog; *CCNE2,* cyclin E2; *LAMC1,* laminin, gamma 1 (formerly LAMB2); *RHOA,* ras homolog family member A; *CDKN1A,* cyclin-dependent kinase inhibitor 1A (p21, Cip1); *JAK1,* Janus kinase 1; *STAT3,* signal transducer and activator of transcription 3 (acute-phase response factor); *FGF1,* fibroblast growth factor 1 (acidic); *FGF4,* fibroblast growth factor 4; *FGF7,* fibroblast growth factor 7; *FGFR,* fibroblast growth factor receptor; *PGDFRA,* platelet-derived growth factor receptor, alpha polypeptide; *RAS* ●●●; *RAF,* ●●●; *MAPK1,* mitogen-activated protein kinase 1; *MAPK9,* mitogen-activated protein kinase 9; *MAPK10,* mitogen-activated protein kinase 10; *JUN,* jun proto-oncogene; *FOS,* FBJ murine osteosarcoma viral oncogene homolog; *MYC,* v-myc avian myelocytomatosis viral oncogene homolog; *MMP2,* matrix metallopeptidase 2; *RARB,* retinoic acid receptor, beta; *PPARD,* peroxisome proliferator-activated receptor delta; *RHOA,* ras homolog family member A; *TCF7,* transcription factor 7 (T-cell specific, HMG-box); *TCF7L1,* transcription factor 7-like 1 (T-cell specific, HMG-box); *JUP,* junction plakoglobin.

cessing, and miRNA measurement. Setting up reference sample sets, for both cancer and corresponding normal controls is important to generate consensus and facilitate the validation process. More importantly, understanding the fundamental biology involved in intra- and intercellular miRNA trafficking is critical in interpreting the fundamental biology and pathology reflected by the changes of specific miRNA concentration in circulation. With more understanding of the molecular properties of circulating miRNA, it may be possible to offer a more sensitive and accurate class of biomarker for early cancer detection or improve the performance of existing clinical biomarkers.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Yuqing He, National Science Foundation of China 81201223, International Science and Technology Program of Guangdong 2013B051000080, International Science & Technology Cooperation Program of Dongguan 2013508152005, Science and Technology Program of Dongguan 201450715200325, Guangdong Medical College Integration of Industry, Education and Research C2013004, Innovation Project from the Department of Education of Guangdong Province CX14106G, and Science & Technology Program of Dongguan 2014108101055; Taek-Kyun Kim, DOD research contracts W911NF-10-2–0111 and DOD research contracts HDTRA1-13-C-0055; Yuanlin Ding, National Science Foundation of China 81273166 and International Science & Technology Cooperation Program of Dongguan 20135081520017; Kai Wang (Principal Investigator), DOD research contracts W911NF-10-2–0111 and DOD research contracts HDTRA1-13-C-0055.
Expert Testimony: None declared.
Patents: None declared.

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