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Circulating microRNAs: emerging biomarkers for diagnosis and prognosis in patients with gastrointestinal cancers

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Summary

To identify novel non-invasive biomarkers for improved detection, risk assessment and prognostic evaluation of cancer, expression profiles of circulating microRNAs are currently under evaluation. Circulating microRNAs are highly promising candidates in this context, as they present some key characteristics for cancer biomarkers: they are tissue-specific with reproducible expression and consistency among individuals from the same species, they are potentially derived directly from the tumor and therefore might correlate with tumor progression and recurrence, and they are bound to proteins or contained in sub-cellular particles such as microvesicles or exosomes, making them highly stable and resistant to degradation. This review highlights the origin of circulating microRNAs, their stability in blood samples, and techniques to isolate exosomal microRNAs, and then addresses the current evidence supporting potential clinical applications for circulating miRNAs for diagnostic and prognostic purposes.

Main Text

Introduction

The identification and validation of biomarkers that can be measured routinely in easily accessible samples, and that are able to diagnose cancer, predict treatment efficacy, and the risk of progression or relapse, is a major challenge in cancer research. In recent years, much work has focussed on a novel class of molecules that are promising candidates in this context: MicroRNAs (miRNAs, miRs). MiRNAs are a well-known class of regulatory molecules, which control translation and stability of messenger RNA (mRNA) post-transcriptionally. It is postulated that one single miRNA can regulate the expression of mRNA from up to hunderds of gene [1]. Since the first release of miRBase (a database that overviews all known miRNAs) in 2002, the number of miRNA loci has increased constantly, with 24521 miRNAs identified in 206 species in 2013 [2]. Interestingly, a considerable number of these miRNAs map to regions of the human genome that are known to be altered in cancer [3]. Esquela-Kerschner et al. established the term „oncomirs“ for miRNAs with oncogenic function, implying that abnormalities in miRNA expression might either directly result in de-differentiation of cells allowing tumor formation to occur [4], or that on the other hand alterations of miRNA expression could be used for tumor classification, diagnosis and prognosis in cancer.

Most early studies investigated miRNA expression patterns in fresh frozen samples such as tumor biopsies or resection specimens, or in paraffin embedded samples. However, miRNAs can also present as “circulating miRNA” and be found in a variety of human body fluids such as urine, saliva, amniotic fluid and pleural fluid [5-9]. Since the first detection of circulating placenta-specific miRNAs in plasma reported by Chim et al. in 2008 [10], numerous studies have investigated miRNAs in blood samples. In 2012 Russo et al. presented a database called miRandola [11]. This collated information from 89 studies, and contained 2132 entries with 581 circulating miRNAs identified in 21 sample types.

It has been shown that circulating miRNAs present tissue-specific, reproducible and consistent expression among individuals in the same species [5], derive potentially directly from tumor tissue, thereby possibly correlating with tumor progression and recurrence [12,13], are highly stable in the circulation due to RNA binding proteins [14-17] or sub-cellular particles such as microvesicles or exosomes [17-20] and are resistant to unfavourable physiological conditions such as repeated freeze-thawing cycles or low / high pH [12,21]. Moreover, Weber et al. examined the expression of miRNAs in plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, and seminal fluid from normal individuals and found miR-335, miR-509-5p, miR-515-3p, miR-873 and miR-616 to be among the most abundant miRNAs present in all or most of the fluid types. Several of the highly abundant miRNAs in these fluids were common among multiple fluid types, however some of the miRNAs were enriched in specific fluids. The authors concluded that fluid type-specific miRNAs may have functional roles associated with the surrounding tissues [6]. Pigati et al. reported that the release of miRNAs into blood, milk and ductal fluids is selective and differs between normal and malignant mammary epithelial cells implicating a potential correlation of miRNA release and malignancy [22]. It therefore seems very likely, that circulating miRNAs might be valuable biomarkers for cancer diagnostics and prognostic evaluation. This

paper summarises the origin of circulating miRNAs, their stability in blood samples, and techniques to isolate exosomal miRNAs. It also highlights potential clinical applications for circulating miRNAs as biomarkers for diagnostic and prognostic evaluation. In particular this review focuses on applications relevant to gastrointestinal malignancies. Of the 15 most frequent malignancies worldwide, 5 are cancers of the gastro-intestinal tract (colorectum, stomach, liver, oesophagus, pancreas), and circulating microRNAs show potential as clinically relevant biomarkers in these cancers.

Circulating miRNAs in blood samples

Origin of circulating miRNAs

There is increasing evidence that circulating miRNAs can be detected in blood samples from healthy individuals and patients with cancer, and several different mechanisms of origin for these circulating miRNAs have been established recently. Circulating miRNAs can originate from 1) active secretion via microversicles (MV) including exosomes [23-25], 2) active secretion in a protein / miRNA complex [15,24,25], 3) circulating miRNAs originating from blood cells such as immunocytes [25], and 4) passive secretion by tumor apoptosis and necrosis [24-26]. Consequently, when actively secreted, circulating miRNAs can indicate certain characteristics of the underlying tumor such as metastatic potential [27-29] or chemo-sensitivity [30-33]. Furthermore, when passively secreted, they might be used as biomarkers of cell destruction, for example, in the context of myocardial infarction [26,34] or liver cirrhosis [35]. Figure 1 presents an overview about different origins of circulating miRNAs.

Circulating exosomal miRNA

Cells can release three types of vesicles: apoptotic bodies (500 nm – 3 um in diameter; released by cells undergoing apoptosis), microvesicles (100 nm – 1 um in diameter; released directly via plasma budding) and nanovesicles including exosomes (30 nm – 100 nm respectively 40 - 100 nm in diameter; released by exocytosis from endosome multivesicular bodies) [36,37]. Exosomes were first discovered in the 1970s [38]. After initial “mis-interpretation” as cell debris [39], exosomes were found in recent years to play a key role in cell transport, immune function and cancer. Exosomes are released into the extracellular environment via fusion of multivesicular bodies (MVB) with the plasma membrane [40]. Many cells, including reticulocytes, dendritic cells, B and T cells, mast cells, epithelial cells and tumor cells have the capacity to release exosomes [40]. Exosomes are present in nearly all body fluids [41] such as blood [42], urine [43], ascites [44], amniotic fluid [41] and saliva [45]. Exosomes share a lot of common (structural, adhesion and transportation) proteins, but they also contain cell-specific proteins such as major histocompatibility complex (MHC) class II or intestinal A33 when derived from B lymphocytes respectively colorectal cancer cells [46-48]. Valadi et al. demonstrated that exosomes carry mRNA and miRNA when released from mast cells. Interestingly, many of these RNA species seem to be specifically packaged exclusively into exosomes. Additionally, the mRNAs in the exosomes are translatable and encode proteins that are often exosome specific [40,49,50]. However, exosomal RNA profiles also differ between exosomes from different parental cells [51,52] and tumor-derived

exosomes may contain some “tumor specific” or “tumor enriched” miRNAs [42]. The release of exosomes might be regulated by tumor suppressor- or onco-genes, as exosomes are found more abundantly in the blood stream of patients with cancer compared to healthy individuals [42,53]. In addition, release of exosomes appears to be regulated by a ceramide-dependent secretory machinery [23].

Circulating miRNA / protein complex

Turchinovich et al. investigated miRNA expression in plasma samples from healthy controls, and showed that expression of miR-16, miR-21 and miR-24 did not differ significantly in the samples, compared to the results obtained by simply centrifuging and filtering (0.22 µm filters) samples (i.e. with exosomes) with ultracentrifugated samples (i.e. without exosomes). They concluded that > 97% of the miRNA amount was “exosome free”. These extracellular miRNAs were demonstrated to bind to Argonaute (AGO) proteins (AGO2 in particular - a part of the RNA-inducing silencing complex), and to be extremely stable. The authors suggested that some miRNA / AGO2 complexes might be directly released by cells either as a by-product of cell death, or in a paracrine manner [15]. Another study investigating all four Argonaute proteins showed that only 14 out of 43 miRNAs presented similar binding patterns to either AGO1 or AGO2 in blood plasma, and that AGO-specific miRNA profiles in blood cells were markedly different from those in plasma. From these findings, the authors concluded that many cell types contribute to circulating miRNA and the majority of these are likely to be non-blood cells [16]. Other researchers demonstrated that high-density lipoproteins (HDL) and low-density lipoproteins (LDL), both involved in the transport of triglycerides and cholesterol, have the capability to carry miRNAs in their core [54,55].

Circulating miRNAs originating from blood cells

Recent studies revealed that, at least in part, cell-free circulating miRNAs could originate from blood cells [16,56,57]. Pritchard et al. compared the expression of 10 miRNAs in plasma with blood cell counts. MiRNA expression presented cell type and cell count specific patterns for red blood cells (miR-451, miR-92a, miR-16, and miR-486-5p) and myeloid blood cells (miR-223, let-7a, miR-197, and miR-574-3p; Pritchard 2012). However, this has to be considered in the context of blood cell derived miRNAs, as expression of miRNAs such as miR-16 and miR-451 can be affected by hemolysis [16,56-58]. The contribution of blood cells to cell-free circulating miRNAs has been raised as a consideration in related cancer biomarker studies, and it has been suggested that some reported circulating cancer associated miRNAs might be due to a secondary effect on blood cells, and not originate from tumor cells.

Stability of circulating miRNAs

Circulating miRNAs are protected from endogenous RNase activity by either being bound to proteins or by being packed into microparticles such as exosomes or microvesicles [17]. Li et al. analysed the expression of selected miRNAs (miR-16, miR-223, miR-30a, miR-320b, let-7b, miR-92a, miR-423-5p and miR-21) [14] and demonstrated that the vesicular structure of microvesicles provides general protection for stored miRNAs in healthy individuals. In addition, Tayler et al. investigated the stability of exosomal miRNAs under various storage conditions

(short time storage up to 96 h at 4C versus long time storage up to 28 days at -70C), and they found no significant differences in the stability of miRNAs between these groups [42]. Finally, Köberle and co-workers compared the stability of miRNAs packed in microvesicles versus those bound to proteins after incubation with RNase A or RNase inhibitor. They showed that vesicle-associated miRNAs appeared to be more stable and more resistant towards RNase A treatment compared to protein-bound miRNAs [59].

Techniques for isolation of exosomal miRNAs

Today, various approaches have been established for the isolation of exosomal miRNAs. Of these, ultracentrifugation is by far the most widely accepted approach with potential for clinical application [60,93]. An alternative technique for miRNA isolation from exosomes is the immuno-isolation method. This uses magnetic beads coated with monoclonal antibodies (anti-HLA DP, DQ, DR) to couple exosomes through the proteins expressed on their surface [61,62]. A further way to isolate exosomal miRNAs is the use of the commercially available ExoQuick™ Kit. ExoQuick™ is a precipitation reagent that pellets exosomes simply by adding the reagent into pre-processed samples [63]. In addition a density gradient technique can also be used. For example, this is applied in the OptiPrep™ technique which separates vesicles based on the iodixanol gradient [65]. Finally, a fairly easy technique and potentially useful step for reducing contamination with non-exososmal miRNA in the context of exosome isolation protocols might be treatment of serum samples with RNase, as this might allow vesicle-associated (i.e. better protected from RNase treatment) and non-vesicle associated (i.e. less protected from RNAse treatment) miRNAs to be distinguished [59].

All aforementined methods for exosome isolation have distinct advantages and disadvantages. Momen-Heravi and co-workers recently compared several of the isolation techniques for extracellular vesicles (EVs) including exosomes [93]. Ultracentrifugation for example, which is considered to be the current gold standard, is lengthy (4–5 h), requires an ultracentrifuge and recovers a relatively small proportion of the EVs. Furthermore, the sedimentation efficiency of EVs and sedimentation stability depend on the rotors being used, and viscosity also plays a role in the recovery of EVs [93]. On the other hand, immuno-isolation is a promising method for specific isolation and characterization of EVs and has been shown to be the most effective strategy for isolation of EVs when compared to differential centrifugation and density gradient separation, and this technique offers the advantage of flow cytometric, immune-blot, and electron microscopy analysis of bead-EVs complexes. However, this approach is not suited for large sample volumes, and captured EVs may not retain functionality [93].

It has been shown previously that ExoQuick™ produces the largest amount and highest quality of exosomal RNA and proteins when compared to ultracentrifugation, immuno-isolation or size exclusion chromatography techniques. However, this technique isolates exosomes in general, and does not exhibit specificity for the originating cell [64,93]. Kalra et al. investigated the density gradient technique compared to two other EV isolation techniques (differential centrifugation coupled with ultracentrifugation and epithelial cell adhesion molecule immunoaffinity pull-down), and showed that OptiPrep™ density gradient separation was superior to the two other approaches for isolating pure populations of exosomes [65]. Finally,

regarding the additional purification step of using RNAse treatment, this technique is the least expensive approach, easiest to perform, and can be performed using even smaller sample volumes [59]. However, it should be noted that surface proteins on vesicles can protect RNA from degradation, so in order for RNAse treatment to be effective it should be coupled with protease treatment [66].

General considerations about technical aspects of miRNA analysis in blood samples

As outlined in a recent review by Moldovan and colleagues [92], developing circulating miRNA profiles as biomarkers is still in the early stages. There are many aspects that contribute to variability or potential inconsistency of research results, and these must be overcome before clinical translation can occur.

With regards to sample acquisition and miRNA extraction, several issues have to be considered when planning experiments and interpreting data. It has been shown that factors such selection of the appropriate sample (e.g. plasma or serum), collection tubes (EDTA, citrate, heparin), extraction methods (phenol/chloroform, silica: distinct differences in required fluid volume, yield, procedural contaminants etc.), quality and quantity control, fasting or blood draw timing, all potentially impact on results of miRNA analyses in blood samples.

With regards to miRNA profiling methods, there are a number of different technical approaches available, all of which present distinct advantages and disadvantages. These techniques include qRT-PCR, microarrays, sequence specific hybridization in solution followed by miRNA molecule counting based on reporter probes, and direct sequencing. While qRT-PCR approaches are fairly easy to perform and provide the best sensitivity and specificity in combination with very high absolute quantification/accuracy and flexibitiy, this technique is somewhat limited by its moderate to low throughput. Microarray techniqes provide very high throughput, but sensitivity / specificity / absolute quantification/accuracy and flexibitiy are lower compared to qRT-PCR approaches. In-solution hybridization yields moderate quality in all these mentioned aspects.

The most promising technology for miRNA profiling might be the Next-Generation Sequencing, as it is the only technique that allows identification of novel miRNAs and is not limited to known miRNAs. In addition, this technique provides better specificity and flexibility (both very high) compared to microarray approaches, yields moderate sensitivity, but absolute quantification/accuracy. However, costs of this approach are very high, and significant computational infrastructure and bioinformatics support is required [92].

Circulating miRNAs: a surrogate for underlying malignat diseases?

Apart from the need to optimise and standardise methodology for circulating miRNA research, before clinical translation can be considered it is essential to confirm whether sensitive and specific circulating miRNAs profiles can be clearly linked to specific tumor types and tumor stages. As a starting point towards this, it was first demonstrated that the amount of circulating EVs containing miRNA cargo is elevated in certain disease states, including various types of cancer [93,96]. However, since this time there is strong debate as to whether profiles of circulating miRNAs reflect

the expression pattern in the underlying tumors. Some authors have reported that circulating miRNAs in breast cancer or testicular germ cell tumor patients might not exhibit the same expression pattern as the underlying tumors [94,95]. However, others showed that the expression pattern of miRNAs in circulating exosomes in lung cancer and ovarian cancer patients for example were similar to the expression profile in the underlying tumor [96,97,99], or that selected miRNAs were generally lower expressed in microvesicles in glioma patients but correlated well with the tumor [98,99]. Chan and colleagues studied miRNA profiles in paired breast cancer tissue and serum samples, and suggested that their data might indicate selective release of miRNAs from breast cancer tissue into the blood stream [95]. Other studies have clearly demonstrated that specific miRNAs are selectively packaged into exosomes for export from tumor cells, and this results in a significant difference between the originating cancer cell miRNA profile compared to the cancer exosome miRNA profile [100,101]. Therefore it may not be reasonable to expect good concordance between tumor tissue miRNA profiles and tumor exosome miRNA profiles. Moreover, the issue of concordance between tissue and exosome miRNA profiles is probably less important than the question of whether miRNAs in the peripheral circulation correlate with tumor diagnosis and prognosis in cancer patients with sufficient sensitivity and specificity. This question will be examined in the following paragraphs.

Clinical applications of circulating miRNAs as diagnostic / prognostic biomarkers in cancer

Given the fact that circulating miRNAs are on the one hand very stable in the peripheral blood stream, and on the other hand - even more importantly – mostly actively secreted into the blood, they might yield excellent biomarkers for cancer detection and prognosis. Hence, analysis of expression profiles of circulating miRNAs might provide a new tool for cancer diagnostics and screening, or for prognostic evaluation in individual patients.

Circulating miRNAs as diagnostic markers

Molecular biomarkers that detect cancer should exhibit significant and reproducible differences in expression between patients with cancer compared to healthy or other non-cancer controls. If these criteria are met, then these biomarkers might be useful for cancer screening, diagnosis or surveillance. A number of studies have analyzed expression profiles of circulating miRNAs in a variety of cancers, and investigated whether miRNA profiles differ between patients with compared to without cancer. There is increasing evidence demonstrating significant associations between the expression of certain miRNAs and different cancer types, highlighting their potential use as diagnostic biomarkers.

For esophageal squamous cell carcinoma (ESCC), for example, Hirajima and co-workers reported a significantly higher concentraton of miR-18a in plasma from patients with cancer, compared to healthy volunteers (sensitivity / specificity: 86.8% / 100%; AUC = 0.9449) [30]. Another group examined selected miRNAs and showed that relative expression levels of circulating miR-155 and miR-183 were significantly reduced in patients with cancer, compared to controls. For miR-155, the authors determined an AUC of 0.66 [67]. A further study examining selected miRNAs revealed significantly higher plasma levels of miR-21 in patients with cancer compared to healthy volunteers, whereas the levels of miR-375 and miR-184 were significantly lower. In addition, the miR-21/miR-375 ratio was significant higher in patients with cancer, and the authors calculated an AUC value of 0.816 for miR-21/miR-375 ratio. Interestingly, the authors found a reduction of miR-21 level after tumor removal. However, these results should be interpreted with caution as miR-184 could not be detected in 50% of the patients with cancer due to its low expression [68]. Finally, Tanaka and colleagues showed that serum levels of miR-21, miR-145, miR-200c and let-7c were significantly higher in patients with cancer compared to healthy volunteers [69]. Sheinerman et al. compared miRNA expression pattern in plasma samples of patients with gastrointestinal diseases (including esophageal cancer, gastric cancer, colon cancer and Crohn’ s disease) and in patients with pulmonary diseases such as asthma, pneumonia and non-small cell lung cancer. By using pairs of specific miRNAs, the authors could distinguish between patients with gastrointestinal disease and healthy controls with an accuracy of 95% (miRNA pairs: miR-215/miR-30e-3p, miR-215/miR-145, miR-203/miR-30e-3p, miR-203/miR-145) or patients with pulmonary disease with an accuracy of 94% (miRNA pairs: miR-145/miR-155, miR-486-5p/miR-155, miR-145/miR-30e-3p, miR-192/miR-31). Unfortunately, the authors failed to provide details about the subtype of esophageal cancer included in this study [70].

In gastric cancer, several authors reported similar deregulation of circulating miRNAs in patients with cancer compared to healthy controls. Li et al., for example, demonstrated upregulation of miR-21 and miR-223, and downregulation of miR-218 in patients with cancer compared to controls (miR-21: sensitivity / specificity: 74.29% / 75.71%, AUC = 0.7432; miR-218: sensitivity / specificity: 94.29% / 44.29%, AUC = 0.7432; miR-223: sensitivity / specificity: 84.29% / 88.57%, AUC = 0.9089) [71]. Another group confirmed significant miR-21 upregulation in patients with gastric cancer compared to other malignancies (sensitivity / specificity: 56.7% / 94.9 %, AUC = 0.81) [72]. Tsujiura et al. showed plasma concentrations of miR-17-5p, miR-106a, miR-106b and again of miR-21 to be significantly higher in patients with cancer compared to controls, whereas the levels of let-7a were significantly reduced. By using expression ratios between up- compared to downregulated miRNAs, the authors further demonstrated an improvement in sensitivity and specificity respectively in AUC (up to 0.879). Interestingly, the authors further investigated whether the miRNA deregulation observed in the plasma samples could also be seen in the miRNAs from the corresponding cancer tissue specimens. They showed that miR-106b presented a higher expression in primary gastric cancer tissue samples compared to adjacent normal mucosa from seven out of the eight patients (87.5%), whereas let-7a showed lower expression in seven of these patients. The authors concluded, that the miRNA levels from the primary cancer tissue and the plasma samples showed similar tendencies in most patients, and suggested that the levels of plasma miRNAs might reflect the expression level of the underlying tumor [73,74]. Zhu and collegueas recently published a detailed review and meta-analysis, which addressed deregulation of circulating miRNAs in patients with gastric cancer, and included 18 articles from 2010 to 2013. The review highlighted alterations in expression levels of miR-21, miR-27a and miR-106b, and emphasised that five cited studies reported upregulation of miR-21 in patients with cancer [74].

Regarding colorectal cancer, there have been only a few publications that address the potential for circulating miRNAs to be used for diagnosis in this cancer type. Giraldez et al., for example, performed a genome-wide profiling study of circulating miRNAs in a large number of plasma samples from patients with colorectal cancer, pre-malignant neoplastic lesions such as advanced adenomas and healthy controls. They found 6 miRNAs (miR-15b, miR-18a, miR-19a, miR-19b, miR-29a and miR-335) were significantly upregulated in patients with cancer compared to controls (AUC between 0.80 and 0.70). Interestingly, in patients with colonic adenomas, only miR-18a was significantly upregulated (AUC = 0.64) [75]. Another study screened using microfluidic array technology the expression of 380 miRNAs in plasma samples from healthy controls compared to patients with adenomas or colorectal cancer. The authors showed that a panel of 8 miRNAs (namely miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532, miR-532-3p and miR-652) accurately identified patients with adenomas (AUC = 0.868). Furthermore, a 5-miRNA panel (miR-15b, miR-21, miR-142-3p, miR-331 and miR-339-3p) distinguished patients with advanced adenomas from patients with cancer (AUC = 0.856) [76].

For pancreatic cancer, again a fairly large body of evidence has confirmed deregulation of circulating miRNAs in blood samples from patients with cancer compared to controls. Morimura et al. demonstrated significant upregulation of miR-18a levels in plasma from patients with pancreatic cancer compared to healthy controls (AUC = 0.9369) [77]. Further, Wang et al. reported upregulation of miR-21, miR-210, miR-155 and miR-196a in patients with ductal adenocarcinoma compared

to healthy controls (sensitivity / specificity for this panel: 64% / 89%; AUC 0.82) [78]. Similarly, Ho and co-workers showed a four-fold upregulation of miR-210 (normalized to miR-54) in plasma samples from patients with pancreatic cancer compared to controls [79]. Another study investigated miRNA expression in serum from patients with ductal adenocarcinoma, chronic pancreatitis and healthy controls. They investigated the expression of selected 7 miRNAs (miR-21, miR-155, miR-181a, miR-181b, miR-196a, miR-221 and miR-222), which were validated in previous studies and shown to be highly expressed in pancreatic ductal adenocarcinoma tissue. Interestingly, upregulation of miR-21 distinguished patients with cancer from healthy controls or patients with pancreatitis, whereas miR-155 and miR-196a were upregulated in patients with either cancer or pancreatitis compared to healthy controls [80]. Liu et al. demonstrated upregulation of miR-26 and miR-196a in patients with cancer compared to controls. Furthermore, these authors showed that a combination of miR-26, miR-196a and the standard tumor marker CA 19-9 achieved an extraordinarily high sensitivity and specificity of 92.0% respectively 95.6% (AUC = 0.979) for cancer compared to controls [81]. Another research group reported that the expression of a panel of 7 miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191) was significantly upregulated in patients with ductal adenocarcinoma compared to controls (accuracy 86.8%, AUC = 0.993). Most interestingly, the accuracy of this panel was far better than established tumor markers such as CA 19-9 and CEA [82]. Furthermore, Li et al. detected significantly elevated levels of miR-1290 in patients with pancreatic cancer in comparison to healthy controls or patients with chronic pancreatitis (AUC = 0.72), patients with pancreatic neuroendocrine tumors, and patients with intraductal papillary mucinous neoplasm (AUC = 0.76) [83].

For hepatocellular carcinoma (HCC), a number of studies reported as well deregulations of circulating miRNAs to correlate to tumors. Shen et al., for example, found in a first set of plasma samples from patients with HCC compared to healthy controls seven miRNAs (miR-19b-1, miR-24, miR-29c, miR-92a, miR-376a, miR-378 and miR-520c-3p) to be significantly differentially expressed in patients with cancer. A validation cohort confirmed only miR-483-5p to be associated with increased risk of hepatocellular carcinoma (sensitivity / specificity: 55.1% / 85.7%) [84]. Luo and co-workers found miR-122a levels were significantly lower in serum samples from patients with HCC compared to controls (sensitivity / specificity: 70.6% / 67.1%; AUC = 0.707). In combination with Alpha-Feto-Protein (AFP), the detection rate of HCC could be improved further (sensitivity / specificity: 87.1% / 98.8%) [85]. However, these data have to be interpreted with caution as miR-122a might also be involved in viral replication in hepatitic C (HCV)- and hepatitis B (HBC)-related liver cancer [86,87]. In addition, another study reported that five plasma miRNAs (miR-23a, miR-23b, miR-342-3p, miR-375 and miR-423,) were upregulated in HBV-related HCC patients, thereby providing a diagnostic miRNA signature to discriminate HBV-related HCC from controls (sensitivity / specificity: 99.9% / 99.4%; AUC = 0.999). Interestingly, the authors further reported a signature of four upregulated miRNAs (miR-10a, miR-223 miR-375 and miR-423), which differentiate patients with hepatitis B from healthy controls (sensitivity / specificity: 99.3% / 98.8%; AUC = 0.999) [88]. Finally, Li et al. found three miRNAs (miR-21, miR-222 and miR-224) were significantly overexpressed in serum samples of patients with HCC compared healthy volunteers [89].

To summarize, there is increasing evidence that levels of certain circulating miRNAs in patients with gastro-intestinal malignancies (Upper GI, Lower GI and Hepato-Pancreato-Biliary) differ from those in healthy or non-malignant controls. Table 1 summarise the data described above. This demonstrates the emerging potential for circulating miRNAs to be potent novel biomarkers for screening, diagnosis or surveillance of cancer.

Circulating miRNAs as prognostic markers

Another important aspect of clinical decision making for patients with cancer is the consideration of clinically relevant tumor characteristics, such as prognosis, metastatic potential or resistance towards conventional chemotherapies, and the abilty to reliably predict these opens the possibility of tailored or individualized cancer treatment. Molecular biomarkers that predict cancer prognosis need to present significant and reproducible expression differences between different prognosistic subgroups. For example, patients with locally advanced malignancy, lymph node involvement or metastases have a poorer prognosis, and if biomarkers could be used as surrogate markers that predict these factors and prognosis or likely success of treatment, then treatments might be tailored to the individual patient and tumor. Indeed, some recent early studies have addressed the question whether or not circulating miRNAs might be potential prognostic biomarkers in different cancer types. Whilst only a few studies have addressed this, initial evidence highlights the potential for these markers.

For example, in esophageal squamous cell carcinoma (ESCC) expression levels of miR-18a in plasma have been shown to be significantly higher in patients with early stage tumors; i.e. pTis or stage I tumors [30]. nother research group has demonstrated a significantly increased risk for ESCC to be associated with reduced expression of circulating miR-155 and miR-183 (even after adjusting their data for potential confounders, such as smoking status and alcohol consumption; Liu 2012). Komatsu and co-workers found that patients ESCC and high plasma miR-21 levels showed a tendency to a shorter long term survival, while patients with elevated miR-375 levels survived longer. Interestingly, patients with both high miR-21 and low miR-375 levels, had a significantly worse prognosis than patients with the opposite expression pattern for these miRNAs (3-year survival rate: 48.4 versus 83.1%). Multivariate analysis confirmed the combination of high miR-21 and low miR-375 levels in plasma to be an independent prognostic factor (hazard ratio 3.8 [1.14-12.5]) [68]. Currently, only one study has investigated the potential for circulating serum miRNAs to predict treatment response in ESCC. Tanaka et al. found that low levels of miR-200c correlated with a good clinical response to 5-fluorouracil (5-FU) and cisplatin plus adriamycin chemotherapy regimens (evaluated via computer tomography, endoscopy and PET/CT) but not with a 5-FU and cisplatin plus docetaxel regimen ($p = 0.7167$). High expression of miR-200c was also associated with shortened progression-free survival. The authors found no significant relationship between expression levels of miR-200c in pretreatment biopsies of the primary tumor and response to chemotherapy [69].

In gastric cancer, Zheng and co-workers demonstrated a significant association between elevated miR-21 levels and increasing TNM stage and tumor size (AUC = up to 0.853) [90]. And Zhu et al. evaluated in their aforementioned meta-analysis the prognostic value of circulating miRNAs, and found that elevated levels of circulating

miR-17-5p, miR-20a, miR-21 and miR-200c were significantly correlated with overall survival, and predicted poor prognosis [74].

For patients with colorectal cancer, similar deregulations of certain miRNAs in different outcome-relevant subgroups have been described. Toiyama et al. identified miR-200c as the serum miRNA being best associated with metastasis. These authors found significantly higher serum miR-200c levels with stage IV cancer compared to stage I-III, and high expression miR-200c was also significantly associated with lymph node metastasis, liver metastasis and the development of distant metastases. High miR-200c levels, furthermore, represented an independent predictor of lymph node metastasis in these patients [hazard ratio = 4.81, 95% confidence interval = 1.98–11.7) [27]. Another two studies investigated the potential for circulating miRNAs to predict treatment response: Zhang et al. used TaqMan low-density arrays to screen miRNA expression in serum from a large patient cohort that underwent chemotherapy and compared the expression pattern to treatment response. They found 17 serum miRNAs correlated with chemosensitivity, and finally selected a panel of five miRNAs (miR-20a, miR-130, miR-145, miR-216 and miR-372), which were significantly downregulated in the responder group. This panel reached an AUC of up to 0.918 for its ability to distinguish between responders and non-responders (AUC for CEA = 0.689 and CA19-9 =0.746) [31]. Finally, another research group compared the expression of 742 miRNAs in plasma samples from cancer patients before commencement and after four cycles of 5-FU/oxaliplatin chemotherapy. They identified three miRNAs (miR-106a, miR-130b and miR-484), which were significantly upregulated before treatment in non-responders. Moreover, high expression of miR-27b, miR-148a, and miR-326 was associated with decreased progression-free survival, and high miR-326 expression correlated with decreased overall survival (HR 1.5, 95% CI 1.1-2.0) [32].

In patients with pancreatic cancer high levels of miR-196a in serum have been found to be significantly associated with unresectable disease, the TNM staging and median survival (high versus low expression of miR-196a: median survival of 6.1 months versus 12 months [80]. Similarily, another research group reported a panel of 7 upregulated miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191), which improved the detection of early stage pancreatic cancer, compared to CA 19-9 or CEA (detection rate of 46.2% / 62.5% respectively 30.8% / 62.5% in stage I / stage II cancer). Furthermore, patients with high levels of miR-21 had poorer survival [82]. In addition, Wang et al. demonstrated, that elevated serum miR-21 levels were independent prognostic factors for both, lower time-to-progression and overall survival. As miR-21 expression in cancer tissue has been reported to be associated with clinical outcome and the success of gemcitabine treatment in patients with pancreatic cancer, the authors suggested that miR-21 serum levels might serve as potential predictors of chemosensitivity [91]. Finally, serum miR-1290 levels have been shown to distinguish between patients with early-stage pancreatic cancer compared to controls better than CA19-9 levels, and miR-1290 has been identified to be independent predictor of pancreatic cancer prognosis [83].

For hepatocellular carcinoma (HCC), comparable results have been published with correlations between the expression of circulating miRNAs and factors affecting outcome also reported. Koeberle et al., for example, compared selected serum miRNA levels in patients with HCC compared to liver cirrhosis. While serum miR-1 and miR-122 levels did not differ significantly between patients with cancer compared

to liver cirrhosis without HCC, the authors found that patients with HCC with higher miR-1 and miR-122 serum levels survived longer than individuals with low expression. Furthermore, the authors demonstrated miR-1 serum levels to be independently associated with overall survival. Serum miR-122 levels correlated negatively with MELD score, supporting the hypothesis that patients with high miR-122 serum levels do have a better liver function and survive longer [59]. In addition, Luo et al. found miRNA-122a expression to be significantly lower in males, and there was a trend towards lower expression in patients who smoked cigarettes, consumed alcohol, had a family history of HCC or were positive for Hepatitis B virus infection. Li and colleagues showed that high levels of miR-221 correlated significantly with existence of cirrhosis, tumor size and tumor stage in patients with HCC. In addition, these authors reported a trend towards increased miR-221 expression with progression of TNM stage (stage I < stage II < stage III–IV) [89].

These initial results suggest that circulating miRNAs in patients with cancer might be useful new biomarkers which might aid assessment of prognosis, response to treatment and outcome in gastro-intestinal malignant tumors in the Upper and Lower GI tract, and also in Hepato-Pancreato-Biliary tumors. Table 2 summarises the data in the paragraphs above.

Conclusion

Since the first reported discovery of miRNAs several years ago, it has become very clear that these components of the epigenetic machinery play a key role in cancer initiation, development and progression. In 2008 came the first report of miRNAs detection in peripheral blood samples. Since then, a number of research groups examined these so called “circulating miRNAs”, and found that there are different types of origin for these molecules in the blood stream, and that these circulating miRNAs are quite stable in the circulation. These characteristics suggest that circulating miRNAs might be useful blood-based biomarkers in patients with cancer. In this context, two aspects are of particular interest for the clinician: on the one hand, ideal biomarkers should present significant and reproducible differences in expression between patients with compared to without cancer in order to allow their use for screeing purposes, diagostics and surveillance of cancer. On the other hand, molecular biomarkers that impact on prognosis should present significant and reproducible differences in expression between different patient subgroups, thereby contributing to the prognostic assessment of patients with cancer. The early data available for gasto-intestinal malignant tumors demonstrates a promising picture. A large number of miRNAs have been reported to show different expression patterns between patients with cancer compared to controls, and between subgroups of patients with better compared to worse prognosis. A number of these miRNA candidates have been detected in more than just one study, and their expression also correlates with the presence of tumors or cancer prognosis in different tumor types, highlighting their multi-facted potential clinical utility in cancer. Table 3 presents in this context an overview about the most promising circulating miRNA candidates for diagnostic or prognostic evaluation.

However, it is still to early to determine whether circulating miRNAs will influence daily clinical practice and decision making in the future. Data so far have been derived from early studies that have several limitations. Most of these studies have included only small groups of patients, and there have been no prospective evaluations of circulating miRNAs as potential biomarkers reported so far. Furthermore, there are a wide range of experimental techniques currently in use for the assessment of circulating miRNAs, and no standardisation of approach to this area. As all of these approaches (including sample aquisation, preparation, isolation of miRNAs or EVs, miRNA profiling and interpretation of data amongst others) are varied, a definitive comparison of the results of different studies is not possible. Moreover, the question about the origin of circulating miRNAs yet to be fully answered, although this will impact on our understanding of biological aspects of tumor initiation and progression, and the meaning of circulating miRNAs in the context of cell-to-cell interactions. Finally, as this review has focussed on cancers of the gastro-intestinal tract, caution should be applied when extrapolating to other cancer types. Each tumor type (or even subtype) will need to be evaluated separately, if circulating miRNAs are to be defined as a new source of molecular biomarkers in the respective cancers. These considerations clearly show that further research efforts is required to definitively establish circulating miRNAs as clinically useful biomarkers that will impact on the assessment and treatment of patients in the clinical setting.

However, despite the early stage of research on circulating miRNAs to date, the initial data in this field appear positive, and support the contention that circulating

miRNAs have great potential to improve diagnostic and prognostic evaluation in patients with cancer in the near future.

Author contribution:

Kirsten Lindner, Richard Hummel, Joerg Haier, David I Watson, and Damian J Hussey developed the idea for this review article and designed the manuscript. Kirsten Lindner, Richard Hummel and Zhe Wang reviewed the literature and collected data. Kirsten Lindner and Richard Hummel wrote the manuscript with support of the other authors. All authors approved the final manuscript.

Conflicts of interests:
None

FIGURES:

FIGURE 1. Different sources of circulating miRNAs
In the cell nucleus, primary miRNAS (pri-miRNAs) were transcripted and cleaved by the microprocessor complex Drosha/DGCR8 into shorter miRNA precursors (pre-miRNA), and finally transported in the cytoplasm. Mature miRNAs were then released via different ways into the circulation:

A. Released in microvesicles after for example fusion of multivesicular bodies (MVBs) with the plasma membrane (exosomes) or the release by endocytic membrane transport pathway (endosomes)
B. Released in a miRNA-protein complex, as for example bound to Ago2, HDL and LDL.
C. Encapsulated into apoptotic bodies by (tumor) apoptosis and tumor necrosis
D. Derivated from blood cells

TABLES

TABLE 1. Circulating miRNA as diagnostic marker
The table presents an overview about deregulations of specific miRNAs in gasto-intestinal malignant tumors from the Upper to the Lower GI tract including Hepato-Pancreato-Biliary tumors, compared to healthy or non-malignant controls. ESCC = esophageal squamous cell carcinoma, CA = cancer, GA = gastric cancer, CP = chronic pancreatitis, IPMN = intraductal papillary mucinous neoplasms, BPD = benign pancreatic disease, HBV-rel. = HBV related.

TABLE 2. Circulating miRNA as prognostic marker
The table presents an overview about deregulations of specific miRNAs in prognostic relevant subgroups of patients with gasto-intestinal malignant tumors from the Upper to the Lower GI tract including Hepato-Pancreato-Biliary tumors. ESCC = esophageal squamous cell carcinoma, CTX=chemotherapy.

TABLE 3. Most promising circulating miRNA candidates for diagnostic or prognostic evaluation
The table presents a selection of circulating microRNAs that were found in more than one study to be deregulated between cancer patients and controls (diagnostic) or between different prognostic relevant subgroups of cancer patients (prognostic). In total, 46 miRNAs were described in more than one study to be deregulated. However, we selected miRNAs that were named more than once.
Red cell: up-regulation of miRNA; green cell: down-regulation of miRNA.
ESCC = esophageal squamous cell carcinoma, CA = cancer, miR-19 family: includes miRNA19a and miRNA19b; miR-29 family includes miRNA29a and miRNA-29c; miR-106 family includes miRNA106a and miRNA106b; let-7 family includes let-7a and let-7c.

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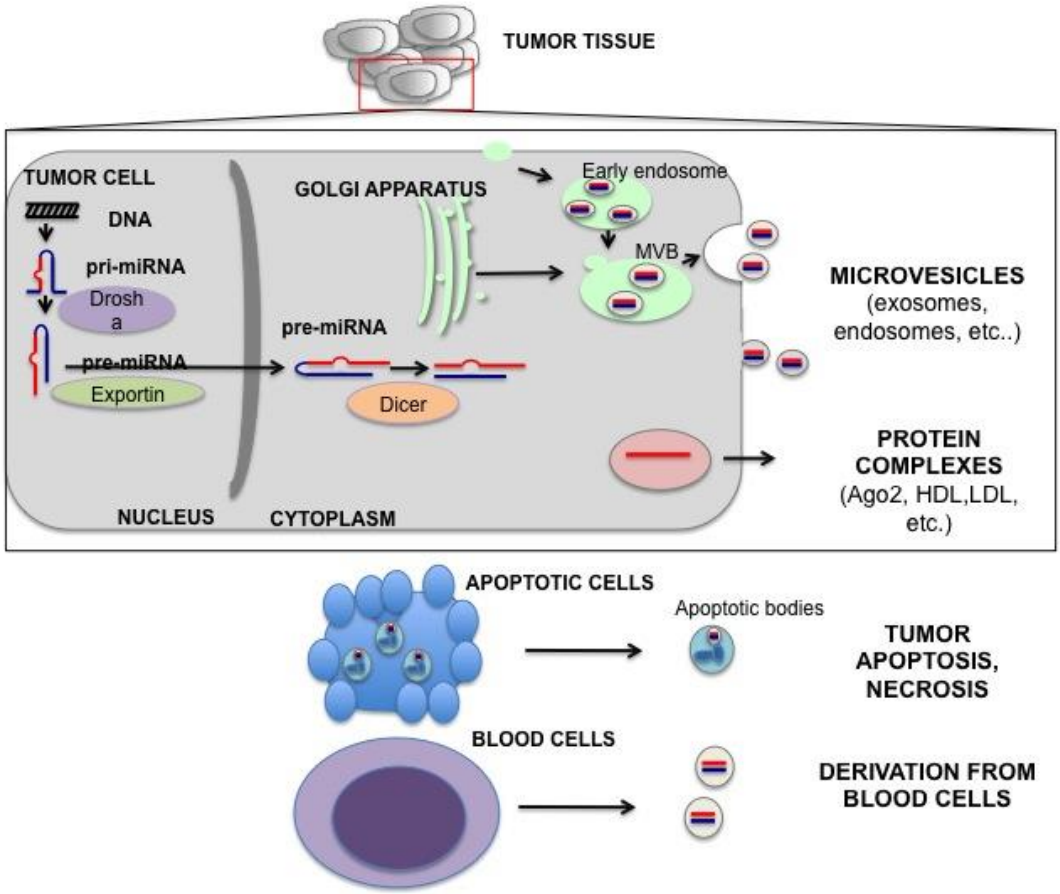
FIGURE 1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MICROVESICLES
(exosomes,
endosomes, etc..)
PROTEIN
COMPLEXES
(Ago2, HDL,LDL,
etc.)
NUCLEUS
CYTOPLASM
APOPTOTIC CELLS
Apoptotic bodies
TUMOR
APOPTOSIS,
NECROSIS
BLOOD CELLS
DERIVATION FROM
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