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Heidi Schwarzenbach*

Diagnostic relevance of circulating cell-free and exosomal microRNAs and long non-coding RNAs in blood of cancer patients

Diagnostische Relevanz von zirkulierenden zellfreien und exosomalen mikroRNAs und langen nicht-kodierenden RNAs im Blut von Krebspatienten

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Abstract: MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) belong to a large group of non-coding RNA (ncRNA) molecules that possess diverse functions in epigenetic and chromatin structure modifications. In particular, they post-transcriptionally inhibit the protein expression of their target mRNAs and therefore, are involved in different tumor-associated signaling pathways. As the expression of both ncRNAs is frequently dysregulated in cancer and shows unique signatures, they may play a crucial role in cancer pathogenesis. They are passively and actively released into the blood circulation, and accordingly, their plasma/serum and exosomal profiles reflect disease development, tumor load and malignant progression towards metastatic relapse. Considering the biology and nature of ncRNAs in the regulation of different cellular processes, they may have potential clinical utility as blood-based tumor markers and future therapeutic targets in the treatment of cancer patients. The present review focuses on the diagnostic value of cell-free and exosomal ncRNAs in plasma/serum of cancer patients and the challenges to establish them as future tumor markers.

Keywords: cell-free miRNAs; cell-free lncRNAs; exosomes; liquid biopsy.

Zusammenfassung: MikroRNAs (miRNAs) und lange nicht-kodierende RNAs (lncRNAs) gehören zu einer großen Gruppe von nicht-kodierenden RNA- (ncRNA) Molekülen, die Funktionen bei epigenetischen und Chromatinstruktur-Modifikationen besitzen. Insbesondere inhibiieren sie post-transkriptionell die Proteinexpression ihrer Ziel-RNAs und sind deshalb in verschiedene Tumor-assoziierte Signalwege involviert. Da die Expression der beiden ncRNAs häufig bei Krebs dereguliert ist und einzigartige Signaturen aufweist, spielen sie eine wichtige Rolle in der Pathogenese von Krebs. Sie werden passiv und aktiv in den Blutkreislauf befreit und folglich reflektieren ihre Plasma/Serum- und exosomale Profile die Entwicklung der Krankheit, die Tumorlast und die maligne Progression bis hin zum metastatischen Relaps. Betreffend der Biologie und Natur der ncRNAs in der Regulation der verschiedenen zellulären Prozesse, sind sie als Blut-basierte Marker und zukünftige therapeutische Zielemoleküle bei der Behandlung von Krebspatienten für die Klinik potenziell wichtig. Der vorliegende Reviewartikel befasst sich mit dem diagnostischen Wert von zellfreien und exosomalen ncRNAs im Plasma/Serum von Krebspatienten und den Herausforderungen sie als zukünftige Tumormarker zu etablieren.

Schlüsselwörter: Exosomen; flüssige Biopsie; zellfreie miRNAs; zellfreie lncRNAs.

Introduction

Worldwide, cancer figures among the leading causes of morbidity and mortality, with approximately 8.2 million cancer related deaths and 14 million new cases in 2012. Over the next 2 decades, the number of new cases is

***Correspondence: Heidi Schwarzenbach,** PhD, Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany, Phone: +49 40 7410 57494, Fax: +49 40 7410 56546, E-Mail: hschwarz@uke.uni-hamburg.de

expected to rise by about 70% (World Cancer Report, 2014). Cancer is a multifactorial, multistep and intricate disease that evolves from the perturbation of cellular homeostasis. Early diagnosis of cancer is the most effective method to prevent cancer progression and to achieve higher survival rates for patients. Traditional diagnostic tools (e.g. protein markers) for cancer detection are still not satisfactory for early and more convenient diagnosis, and usually, cannot distinguish between malignant and benign tumors. In this respect, significant efforts have been made in finding more informative blood-based biomarkers. Due to their particular characteristics and functions, circulating non-coding RNAs (ncRNAs), assumed to have high levels of disease-associated sensitivity and specificity, may be promising non-invasive candidate tumor markers. Their clusters are often expressed in a tissue-, development- or disease-specific manner, and they modify the expression of numerous tumor-associated genes [1].

Whereas there are about 20,000 protein-coding genes, representing <2% of the whole genome sequence, at least 90% of the genome can be transcribed into ncRNAs, implicating ncRNAs as having a significant relevance in cancer. According to their length, ncRNAs are grouped into two main classes. Those transcripts of <200 nucleotides (nt) in length are referred to as small ncRNAs, including Piwi-interacting RNAs, small-interfering RNAs, microRNAs (miRNAs) and some bacterial regulatory RNAs. The other group contains long ncRNAs (lncRNAs) ranging from 200 to 100,000 nt in length [2]. The current review refers to miRNAs and lncRNAs.

MiRNAs and lncRNAs are protected against RNase digestion, and therefore, they are highly stably when circulating in the human bloodstream. They are integrated in various secreted extracellular microvesicles or complexed with specific RNA-binding proteins. MiRNAs are released into the blood circulation by various cell physiological events, such as apoptosis, necrosis and active secretion. RNA transcripts released by apoptotic and necrotic cells form complexes with AGO2 and HDL proteins, or are located in apoptotic bodies. MiRNAs are also incorporated in exosomes. These small membrane vesicles which are between 30 and 100 nm in size are actively released from multiple cell types, including dendritic cells, lymphocytes and tumor cells, by exocytosis [3, 4]. So far, the mechanism underlying the release of lncRNAs and their occurrence as protein-associated RNAs have still not been systematically investigated in blood. However, lncRNAs may be secreted in the same manner as miRNAs. Recently, it was reported that lncRNAs may also be integrated in exosomes, where they are protected from RNase activity [5–7].

The presence of these ncRNAs in blood allows their quantification by a minimally-invasive procedure of blood withdrawal, suggesting that aberrantly expressed ncRNAs discovered in blood may be attractive candidates for potential blood-based biomarkers. Hence, a better understanding of their expression profiles may help to identify them as biomarkers capable of detecting cancers early. Their screening may, additionally, provide information on target molecules and aberrant signaling pathways that can be blocked by a chosen targeted therapy. Growing evidence indicates that there is an interaction between miRNAs and lncRNAs. This crosstalk may modulate gene expression patterns that drive cellular processes involved in physiologic and pathologic functions [8]. Investigation of this regulatory network could broaden our knowledge on its implication in cancer.

Numerous ncRNAs have been quantified in plasma and serum as well as in other liquid biopsies (e.g. urine, saliva, pleural effusion and cerebrospinal fluid) and functionally characterized in cell lines and mouse models. Their genomic profile is associated with different cancer types, prognosis, response to treatment and emergence of resistance [9–11]. However, based on the huge extent of studies, the present review article is preferably focusing on the diagnostic relevance of circulating cell-free and exosomal miRNAs and lncRNAs in plasma and serum of cancer patients.

Characteristics of miRNAs

MiRNAs consist of 19–25 nt, are single-stranded, and are derived from hairpin precursor molecules of 70–120 nt. They predominantly act as translational repressors that bind to complementary sequences in the 3′ untranslated region (UTR) of their target mRNAs. In this manner, protein expression is repressed either by post-transcriptional inhibition of translation or by degradation of the target mRNAs. For mRNA binding, a core sequence of only 2–7 nt is necessary. In most cases, miRNAs only partly bind to their complementary target mRNA sequence [12]. An incomplete complementary binding results in translational repression or deadenylation of their target mRNAs, whereas a complete complementary binding leads to degradation of their targets. In this process, miRNAs bind to two proteins (GW182 protein and a member of the Argonaute family) and form a complex called the miRNA-induced silencing complex (miRISC). This complex then serves as an inhibitor of translation [13]. In contrast, miRNAs can also serve as activators of gene expression by targeting gene regulatory sequences. Target sites of miRNAs have been found in gene promoters, and these complementary sequences are as common as those within the 3′UTR of mRNAs [14].

Characteristics of lncRNAs

The majority of lncRNAs is transcribed by RNA polymerase II from strands opposite to those of protein-coding genes, ultra-conserved regions, or introns, and then, undergo polyadenylation and pre-RNA splicing. They have been associated with a spectrum of biological processes including modification of gene expression through recruitment of histone modification complexes, alternative splicing and nuclear import, and been described as structural components, precursors to small RNAs and even as regulators of mRNA decay. They are enriched in cell nucleus, suggesting that they possess gene regulatory functions. Similar as miRNAs, lncRNAs are able to repress and reduce expression of specific genes, especially adjacent protein-encoding genes. Their dysregulated expression levels in patients with different cancer types are associated with tumiorigenesis, cancer progression and metastases [2, 15].

Techniques applied for the quantification of ncRNAs

Apart from low amounts of starting material, the procedure of quantification of circulating miRNAs and lncRNAs in liquid biopsies, such as plasma or serum, is essentially the same as that of cellular ncRNAs from tissues or cell lines. Figure 1 summarizes the pre-analytical and analytical factors that have to be considered for the quantification of ncRNAs. So far, plasma and serum are the bodily fluids that have been most commonly used for ncRNA profiling in cancer patients. MiRNAs and lncRNAs are usually extracted from tissues and body fluids using commercial products that enrich RNA by phenol/guanidinium (TRIzol, Life Technologies, Darmstadt, Germany) followed by isopropanol precipitation, and column-based techniques (e.g. miRNeasy kit, Qiagen, Hilden, Germany or mirVana kit, Life Technologies). In comparison to these methods, higher plasma miRNA amounts can be obtained from small inputs of body fluids by using superparamagnetic Dynabeads covalently bound to two sets of 377 anti-miRNA oligonucleotides (TaqMan miRNA ABC Purification Kit, Life Technologies). The miRNA isolation relies on hybridization of endogenous miRNAs to the corresponding antimiRNA oligonucleotides attached to these beads. However, limitation of this technique is that only a limited number of miRNAs can be quantified. Current methods of cDNA synthesis are universal poly-A tailing and stem-loop primer extension. The specificity and sensitivity of stem-loop primers seem to be better than those of conventional linear ones, because base stacking and spatial constraint of the stem-loop structure may improve the thermal stability and prevent it from binding double-strand genomic DNA molecules [16]. Quantitative reverse transcription PCR (qRT-PCR) is the gold standard to quantify ncRNAs. This method has high sensitivity and specificity, and is suitable for quantification with a highly dynamic range. To obtain large-scale profiles of the extracted circulating miRNAs and lncRNAs, microarrays or next-generation sequencing (NGS) are recommended. These methods detect much broader profiles, but are labor-intensive and expensive compared with real-time/Taqman PCR that usually quantifies a single miRNA or lncRNA. Microarray-based technologies, relying on hybridization to specific probes, can cover nearly 2000 mature human miRNAs sequences listed in the miR database (Sanger miRBase). Due to sequence specific bias in enzymatic labeling, lack of an absolute quantification ability, and relatively lowly dynamic range, this technique is relatively limited in terms of sensitivity and specificity [17]. In contrast to microarrays, NGS enables profiling of all expressed miRNAs. It is largely sequence-independent and does not rely on the design of primers or probes specific to each miRNA. NGS is based on adapter-ligated sample RNA and cDNA libraries which are amplified by PCR and sequenced. The output

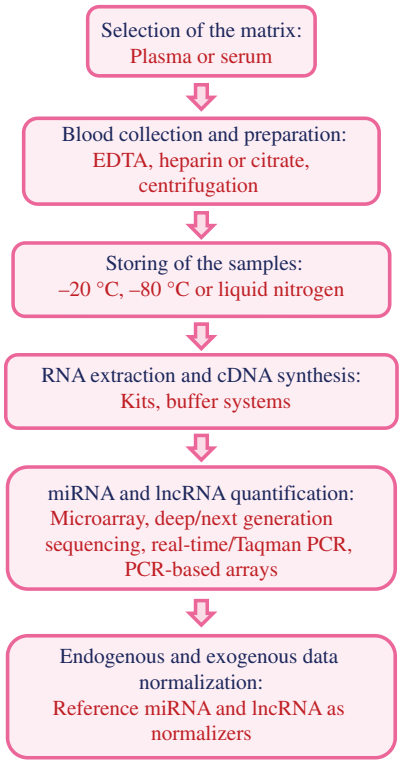

Figure 1: Summary of the pre-analytical and analytical factors that have to be considered for the quantification of ncRNAs.

delivers sequencing reads of varying lengths corresponding to miRNAs, which are then aligned to the reference sequence of choice. Limitations of this technique include sequence-specific biases due to enzymatic ligation, relatively high cost and the need for trained computer scientists to analyze the abundant data output [18]. A relatively new method uses real-time PCR-based arrays that are available in different sizes (e.g. mounted with 48, 96 or 384 different miRNAs or lncRNAs). These arrays are easier in performance and give a good overview of the deregulation of known cancer-specific ncRNAs.

However, all these different technical platforms significantly vary in sensitivity and specificity, and make a comparison among the studies difficult. Moreover, no current consensus exists with respect to a reference RNA for data normalization of miRNAs and lncRNAs. To avoid a false interpretation of data, the choice of endogenous and exogenous reference controls is crucial. RNU6, miR-93 and miR-16 are the most frequently used endogenous controls, to quantify miRNAs [19]. Four lncRNAs (BC200, Zfhx2as, H19 upstream conserved 1 and 2, and HOXA6as) have been shown to be suitable as normalizers for lncRNA levels [20]. For the inter-individual variability of an efficient handling, exogenous cel-miR-39 is usually spiked in the plasma/serum samples, prior to the quantification of ncRNAs [19].

Diagnostic value of cell-free miRNAs in plasma and serum

In cancer, miRNA expression is frequently dysregulated because half of their genes are localized in fragile chromosomal regions exhibiting DNA amplifications, deletions or translocations during tumor development and progression [21]. To date, numerous studies have reported that miRNAs are involved in the regulation of different cellular processes, such as apoptosis, cell proliferation, epithelial to mesenchymal transition (EMT), cell migration, cell invasion and metastases, in different cancer types [22]. Therefore, investigations on their dysregulated profiles are particularly attractive to establish new candidate markers for cancer diagnostics and to provide information on the aberrant signaling pathways associated with the pathology of cancer. As there is a huge amount of publications on miRNAs, the current overview is based on recent studies on the diagnostic potential of circulating plasma/serum miRNAs, and on my personal view of what have been important clinical translational events, and cannot therefore, be comprehensive.

In 2008, the presence of circulating miRNAs in serum was first described in patients with diffuse large B cell lymphoma, demonstrating a significant association of high expression levels of miR-21 with relapse-free survival [23]. Since then, miR-21, that targets the tumor suppressor gene *PTEN* and Programmed Cell Death 4 (*PDCD4*) is one of the most investigated circulating miRNAs [24]. Its upregulation is of diagnostic relevance in a variety of human malignancies [25]. High serum levels of this miRNA are associated with tumor progression, lymph node metastasis, advanced clinical disease and a poor survival [9, 26]. For example, higher serum levels of miR-21 were found in patients with hormone-refractory prostate cancer than in men with androgen-dependent and localized prostate cancer. Androgen-dependent prostate cancer patients with low serum prostate-specific antigen (PSA) levels had similar serum levels of miR-21 to those of patients with localized prostate cancer or benign prostatic hyperplasia (BPH) [27]. In respect to breast cancer, patients with non-metastatic Human Epidermal growth factor Receptor 2 (HER2)-positive breast cancer had higher serum levels of miR-21 than patients with non-metastatic HER2-negative disease [28]. MiR-10b is one of the first miRNAs to be identified to have a function in the metastasis of breast cancer. The afore-mentioned laboratory showed that patients with metastatic HER2-positive breast cancer had higher serum levels of miR-10b than patients with metastatic HER2-negative disease [28]. Ma et al. reported that in metastatic cell lines miR-10b was highly expressed and could affect cell migration and invasion [29]. Gee et al. studied miR-10b expression in breast cancer patients with and without nodal metastases, but could not confirm a significant association between miR-10b levels and metastasis or prognosis [30]. Moreover, my laboratory could also not observe any association of miR-10b with metastasis of breast cancer [31]. Another miRNA widely studied in different cancer entities is miR-16. Inconsistent results referring to its diagnostic value have also been reported for this miRNA [32–37]. Since its first discovery in chronic lymphocytic leukemia where miR-16 is commonly deleted, miR-16 has been reported to act as a tumor suppressor by inhibiting a number of oncogenic mRNA targets and various aspects of cancer progression in vitro and in vivo [38]. However, other studies have implicated this miRNA in oncogenic pathways of different cancer types [39, 40]. For example, Ell et al. detected that the expression of miR-16 was elevated in osteoclast differentiation and correlated with bone metastasis burden [35]. My laboratory showed that the plasma levels of miR-16 were increased in breast cancer patients [41], whereas the serum levels of miR-16 were neither up- nor downregulated in ovarian

cancer patients compared with healthy women [37]. These findings show that the expression of miR-16 may be cancer- and stage-specifically regulated. On the other hand, the use of miR-16 as a normalizer may be a standard approach for most analyses, because it has been described as being highly expressed in plasma or serum of diverse cancer types, and relatively invariant across diverse blood samples [19].

MiR-21, miR-10b, miR-34a, miR-145, miR-155 and miR-373 were suggested to be selectively released into the human blood circulation. They are differentially expressed in breast cancer tumors and associated with a particular biology of the tumors favoring progression and metastatic spread [31, 42]. For example, breast cancer patients with advanced tumor stages had significantly more miR-34a in their bloodstream than patients at early tumor stages. Changes in serum levels of miR-34a together with miR-10b and miR-155 correlated with the presence of metastases [43]. The receiver operating characteristic curve (ROC) with an area under the curve (AUC) of 0.801 (sensitivity, 65.0%; specificity, 81.8%) revealed that serum miR-155 had the capability of distinguishing patients with primary breast cancer from healthy subjects [44]. The levels of miR-155 were also quantified in patients with colorectal cancer (CRC). Its increased serum levels could discriminate CRC patients from healthy controls with an AUC of 0.776 [45].

In the pathology of epithelial ovarian cancer (EOC), the role of the miR-200 family consisting of miR-200a, miR-200b, miR-200c, miR-141 and miR-429 has been extensively studied. The family members are involved in the suppression of EMT [46] and have an impact on progression, metastasis and chemo-resistance in EOC [47–49]. Serum miR-221 has been described as a diagnostic biomarker for malignant melanomas and to correlate with staging of the disease [50]. Finally, in patients with HER2-positive breast cancer a specific influence of neoadjuvant therapy with trastuzumab and lapatinib could be observed on the serum levels of miR-210, miR-21 and miR-373 [51].

Diagnostic value of cell-free lncRNAs in plasma and serum

Besides miRNAs, numerous studies have also documented the clinical relevance of circulating lncRNAs as a diagnostic marker for cancer. Tumor-associated expression profiles of lncRNAs have been described, and their signatures are related to tumor development, disease progression and metastases. Moreover, the developmental lineage and differentiation state of various tumor types are also reflected in the lncRNA signature [15].

One of the most studied and the first lncRNAs that has been demonstrated to have an implication in cancer is Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1). This lncRNA promotes tumor growth by regulating the cell cycle of cell lines derived from different cancer types and in tumor xenografts of nude mice [52]. To further support its potential role during cancer progression, MALAT1 has been shown to induce EMT and to regulate angiogenesis [52]. Its diagnostic efficiency as a cell-free serum/plasma molecule has been demonstrated in small-cell lung cancer (NSCLC) and pancreas cancer patients [53, 54]. Another lncRNA that is often quantified in studies is HOX Transcript Antisense RNA (HOTAIR). HOTAIR is also involved in the stimulation of cellular proliferation, and most importantly, it has an impact on cellular migration and invasion, and EMT [55]. Deregulated plasma levels of HOTAIR have been reported in different cancer types [54, 56]. However, aberrant expression of lncRNAs may also be specific to a certain tumor type, such as the upregulation of Highly Upregulated in Liver Cancer (HULC) in hepatocellular cancer [57]. In contrast to oncogenic characteristics, lncRNAs may also possess tumor suppressor features. For example, Growth Arrest-Specific 5 (GAS5), is usually downregulated in multiple cancer types, and induces apoptosis and impedes cell proliferation [58].

Zhang et al. reported that MALAT-1 Derived (MD) miniRNA may be an attractive candidate biomarker for prostate cancer. The current strategy for diagnosing prostate cancer is mainly based on the serum PSA test. However, PSA has low specificity and has led to unnecessary biopsies. In addition, PCA3 has been described to be a well-established urine biomarker for the detection of prostate cancer. The commercial Progensa PCA3 urine test has considerably reduced the number of biopsies, but its application is only suitable for early diagnosis of prostate cancer, and not for locally advanced or metastatic disease [59]. Accordingly, plasma MD miniRNA could be qualified as a further diagnostic marker for prostate cancer, because this lncRNA was able to discriminate prostate cancer patients from BPH controls with an AUC of 0.79, and displayed a better diagnostic performance than PSA, but the combination of both biomarkers revealed the best diagnostic performance, compared with either single biomarker [60].

A 5-lncRNA signature, including lncRNA-LET, PVT1, PANDAR, PTENP1 and linc00963, was identified in the serum of renal cell carcinoma patients and could distinguish malignant from benign tumors [61]. In the plasma of NSCLC patients, SPRY4-IT1, ANRIL, and NEAT1 were significantly increased. ROC analysis revealed an AUC of

0.876 (sensitivity, 82.8%; specificity, 92.3%) for this panel of lncRNAs [62]. Moreover, the combination of serum XIST and HIF1A-AS1 could also distinguish NSCLC patients from controls (AUC = 0.931), and their occurrence in serum could be confirmed with their expression in tumor tissues from the same individuals [63]. In comparison with cancer-free controls, three lncRNAs (XLOC_006844, LOC152578 and XLOC_000303) were up-regulated in the plasma of CRC patients with an AUC of 0.919 and 0.975 [64]. The plasma levels of H19 were significantly higher in gastric cancer patients compared with normal controls with an AUC of 0.838 (sensitivity, 82.9%; specificity, 72.9%) [65]. Also, the plasma levels of UCA1 provided a better diagnostic performance for detection of gastric cancer (AUC = 0.928), and there was a significantly positive correlation of UCA1 expression levels between tumor tissues and plasma samples (r = 0.931) [66]. The plasma levels of POU3F3, HNF1A-AS1 and SPRY4-IT1 were significantly higher in esophageal squamous cell carcinoma (ESCC) patients than normal controls. The use of POU3F3 in combination with the serum biomarker squamous cell carcinoma antigen (SCCA) was most effective in diagnosis with an AUC of 0.926, (sensitivity, 85.7%; specificity, 81.4%), to detect ESCC at an early stage [56].

Diagnostic value of exosomal miRNAs and lncRNAs

Exosomal miRNAs and lncRNAs have been implicated in exosome-mediated biological functions, demonstrating their clinical utility and their diagnostic potential [67].

Exosomes, small membrane vesicles of between 30 and 100 nm, are actively released from multiple cell types, including dendritic cells, lymphocytes and tumor cells by exocytosis [68]. The amount of secreted exosomes has been associated with tumor invasiveness both in vitro and in vivo, and to promote migration and proliferation of tumor cells [69]. They can mediate cell-to-cell communication by transferring their oncogenic cargo, such as proteins, lipids and nucleic acids, among cells. In their new location these molecules may be functional and influence the recipient cell phenotype. This molecular shuttle may result in the transformation from wild type cells into malignant cells [70, 71]. In contrast, when the exosomal cargo harbors tumor suppressive activity, then, it may exert inhibition of tumor formation, complementing cancer immune surveillance [72].

Taylor and Gercel-Taylor demonstrated that exosomes derived from epithelial tumors express anti-epithelial cell adhesion molecules (EpCAMs) on their surface and used them for their selective exosome isolation by a modified magnetic activated cell sorting (MACS) procedure. They found that the levels of exosomal miR-200a, miR-200b and miR-200c were higher in EOC patients with FIGO stage I, II or III than in patients with benign ovarian diseases, and showed, for the first time, the diagnostic value of exosomal miRNAs [73]. A systematic review on the potential role of exosomal miR-21 as a general biomarker for cancer was performed by Shi. This meta-analysis included a total of 10 pooled studies covering 318 patients with 10 different cancer types and 215 healthy controls. His findings showed the widespread dysregulation of exosomal miR-21 in different cancer types with a strong potential to be used as a universal biomarker to identify cancers (AUC = 0.93; sensitivity, 75%; specificity, 85%) [24]. Our data showed a prevalence of miR-101, miR-372 and miR-373 in exosomes over the cell-free ones in the serum of both cohorts of breast cancer patients and healthy women [74]. Similarly, Li et al. showed that the expression of miR-141 was higher in exosomes compared with that of cell-free miR-141 in the serum of prostate cancer patients, BPH patients and healthy men. Moreover, the levels of exosomal miR-141 were higher in prostate cancer patients than BPH patients and healthy men, and higher in metastatic than localized prostate cancer (AUC = 0.869; sensitivity, 80%; specificity, 87.1%) [75]. Pfeffer et al. reported that exosomal miR-17, miR-19a, miR-21, miR-126, and miR-149 were expressed at higher levels in the plasma of patients with metastatic sporadic melanoma than patients with familial melanoma patients or unaffected control subjects [76]. Matsumura et al. correlated the expression levels of the exosomal miR-17-92a cluster with the recurrence of CRC. The serum levels of exosomal miR-19a were upregulated by gene amplification in CRC patients as compared with healthy individuals [77]. Moreover, we observed a cancer-specific upregulation of exosomal miR-200a, miR-200b and miR-200c and their ability to distinguish between malignant and benign ovarian tumors. Exosomal miR-200b and miR-200c were associated with lymph node-positive status, advanced tumor (FIGO) stages, values of the tumor marker CA125 and poor overall survival in EOC patients [78].

Apart from exosomal miRNAs, exosomal lncRNAs in plasma/serum of cancer patient were also quantified. However, their investigations are only in their infancy. Li et al. demonstrated that the levels of cell-free and exosomal LINC00152 in the plasma of gastric cancer patients were similar, and suggested that this lncRNA is protected by exosomes in human bloodstream [7]. Wang et al. showed that the expression of exosomal HOTAIR together with miR-21 was higher in the serum of patients with laryngeal

squamous cell carcinoma (LSCC) than in patients with vocal cord polyps (AUC = 87.6; sensitivity, 94.2; specificity, 73.5%). There were also significant differences in exosomal HOTAIR and miR-21 expressions between advanced and early tumor stages, as well as lymph node metastasis status [6].

Conclusions

The present outline on cell-free and exosomal miRNAs and lncRNAs demonstrates the significantly aberrant expression of ncRNAs in blood of cancer patients and their promising features to allow non-invasive cancer diagnosis. Quantification and characterization of dysregulated levels of ncRNAs offers additionally the possibility to provide insights into cancer pathogenesis and aberrant signaling pathways which they are involved in. Thus, further studies are required to more intensely characterize these molecules and their functions in the cancer-associated aberrant signal cascades, in particular with reference to the interaction between lncRNAs and miRNAs, and the regulation of miRNA expression by lncRNAs. However, for the development of sensitive and reliable ncRNA-based assays and a systematic demonstration of their clinical utility as tumor biomarkers for disease diagnosis as well as prognosis, it is necessary to quantify them in independent cohorts and large prospective multicenter studies during different tumor phases and stages, to verify the reproducibility of data and validate the optimal plasma/serum ncRNA cut-off levels. Their fluctuating changes in temporal and local context also have to be taken into contextual evaluation of ncRNA measurements. Particularly, the analysis methods, including the variation in pre-analysis and analysis factors, sample preparation procedures and data normalization have to be standardized, as a lot of discrepancies referring to up- or downregulation of a single ncRNA and its use as a normalizer exist among the studies. It is also important to keep in mind that ncRNAs have heterogeneous origins. Indeed, the vast majority of ncRNAs in plasma/serum and exosomes originate from blood cells, endothelial cells and other organs, suggesting that their cancer-specific expression may be masked by ncRNAs from other sources.

Quantification of ncRNAs in body fluids and exosomes is an attractive and promising field of biomarkers. Regarding the strong correlations that have been established between deregulated ncRNAs expression and cancer development and progression, therapy strategies targeting these transcripts have been validated. To date, miR-34 has become the first cancer-targeted miRNA drug (MRX34, http://www.clinicaltrials.gov; Identifier: NCT01829971), entering phase I clinical trials in patients with advanced hepatocellular carcinoma [1].

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References

1. Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov 2013;12:847–65.
2. Ponting CP, Belgard TG. Transcribed dark matter: meaning or myth? Hum Mol Genet 2010;19:R162–8.
3. Asaga S, Kuo C, Nguyen T, Terpenning M, Giuliano AE, Hoon DS. Direct serum assay for microRNA-21 concentrations in early and advanced breast cancer. Clin Chem 2011;57:84–91.
4. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 2010;101:2087–92.
5. Isin M, Uysaler E, Ozgur E, Koseoglu H, Sanli O, Yucel OB, et al. Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. Front Genet 2015;6:168.
6. Wang J, Zhou Y, Lu J, Sun Y, Xiao H, Liu M, et al. Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. Med Oncol 2014;31:148.
7. Li Q, Shao Y, Zhang X, Zheng T, Miao M, Qin L, et al. Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer. Tumour Biol 2015;36:2007–12.
8. Yoon JH, Abdelmohsen K, Gorospe M. Functional interactions among microRNAs and long noncoding RNAs. Semin Cell Dev Biol 2014;34:9–14.
9. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nature reviews Clin Oncol 2014;11:145–56.
10. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014;20:460–9.
11. Qi P, Zhou XY, Du X. Circulating long non-coding RNAs in cancer: current status and future perspectives. Mol Cancer 2016;15(1):39.
12. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.
13. Höck J, Meister G. The argonaute protein family. Genome Biol 2008;9:210.
14. Portnoy V, Huang V, Place RF, Li LC. Small RNA and transcriptional upregulation. Wiley Interdiscip Rev RNA 2011;2:748–60.

15. Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. Cancer Cell 2016;29:452–63.
16. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
17. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X. MicroRNA: function, detection, and bioanalysis. Chem Rev 2013;113:6207–33.
18. Chugh P, Dittmer DP. Potential pitfalls in microRNA profiling. Wiley Interdiscip Rev RNA 2012;3:601–6.
19. Schwarzenbach H, Machado da Silva A, Calin G, Pantel K. Data normalization strategies for MicroRNA quantification. Clin Chem 2015;61:1333–42.
20. Kraus TF, Greiner A, Guibourt V, Lisec K, Kretzschmar HA. Identification of stably expressed lncRNAs as valid endogenous controls for profiling of human glioma. J Cancer 2015;6:111–9.
21. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704–14.
22. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Kerin MJ. MicroRNAs as novel biomarkers for breast cancer. J Oncol 2009;2009:950201.
23. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 2008;141:672–5.
24. Shi J. Considering exosomal miR-21 as a biomarker for cancer. J Clin Med 2016;5:42.
25. Huang Y, Yang YB, Zhang XH, Yu XL, Wang ZB, Cheng XC. MicroRNA-21 gene and cancer. Med Oncol 2013;30:376.
26. Liu XG, Zhu WY, Huang YY, Ma LN, Zhou SQ, Wang YK, et al. High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer. Med Oncol 2012;29:618–26.
27. Zhang HL, Yang LF, Zhu Y, Yao XD, Zhang SL, Dai B, et al. Serum miRNA-21: elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy. Prostate 2011;71:326–31.
28. Anfossi S, Giordano A, Gao H, Cohen EN, Tin S, Wu Q, et al. High serum miR-19a levels are associated with inflammatory breast cancer and are predictive of favorable clinical outcome in patients with metastatic HER2(+) inflammatory breast cancer. PLoS One 2014;9:e83113.
29. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007;449:682–8.
30. Gee HE, Camps C, Buffa FM, Colella S, Sheldon H, Gleadle JM, et al. MicroRNA-10b and breast cancer metastasis. Nature 2008;455:E8–9; author reply E9.
31. Eichelser C, Flesch-Janys D, Chang-Claude J, Pantel K, Schwarzenbach H. Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression. Clin Chem 2013;59:1489–96.
32. Dwivedi SK, Mustafi SB, Mangala LS, Jiang D, Pradeep S, Rodriguez-Aguayo C, et al. Therapeutic evaluation of microRNA-15a and microRNA-16 in ovarian cancer. Oncotarget 20167:15093–104.
33. Bhattacharya R, Nicoloso M, Arvizo R, Wang E, Cortez A, Rossi S, et al. MiR-15a and MiR-16 control Bmi-1 expression in ovarian cancer. Cancer Res 2009;69:9090–5.
34. Wyman SK, Parkin RK, Mitchell PS, Fritz BR, O'Briant K, Godwin AK, et al. Repertoire of microRNAs in epithelial ovarian cancer as determined by next generation sequencing of small RNA cDNA libraries. PLoS One 2009;4:e5311.
35. Ell B, Mercatali L, Ibrahim T, Campbell N, Schwarzenbach H, Pantel K, et al. Tumor-induced osteoclast mirna changes as regulators and biomarkers of osteolytic bone metastasis. Cancer Cell 2013;24:542–56.
36. Delfino KR, Rodriguez-Zas SL. Transcription factor-microRNA-target gene networks associated with ovarian cancer survival and recurrence. PLoS One 2013;8:e58608.
37. Meng X, Joosse SA, Muller V, Trillsch F, Milde-Langosch K, Mahner S, et al. Diagnostic and prognostic potential of serum miR-7, miR-16, miR-25, miR-93, miR-182, miR-376a and miR-429 in ovarian cancer patients. Br J Cancer 2015;113:1358–66.
38. Calin GA, Croce CM. Genomics of chronic lymphocytic leukemia microRNAs as new players with clinical significance. Semin Oncol 2006;33:167–73.
39. Miles GD, Seiler M, Rodriguez L, Rajagopal G, Bhanot G. Identifying microRNA/mRNA dysregulations in ovarian cancer. BMC Res Notes 2012;5:164.
40. Lavecchia A, Di Giovanni C, Novellino E. Inhibitors of Cdc25 phosphatases as anticancer agents: a patent review. Expert Opin Ther Pat 2010;20:405–25.
41. Stückrath I, Rack B, Janni W, Jäger B, Pantel K, Schwarzenbach H. Aberrant plasma levels of circulating miR-16, miR-107, miR-130a and miR-146a are associated with lymph node metastasis and receptor status of breast cancer patients. Oncotarget 20156:13387–401.
42. Chan M, Liaw CS, Ji SM, Tan HH, Wong CY, Thike AA, et al. Identification of circulating microRNA signatures for breast cancer detection. Clin Cancer Res 2013;19:4477–87.
43. Roth C, Rack B, Muller V, Janni W, Pantel K, Schwarzenbach H. Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. Breast Cancer Res 2010;12:R90.
44. Sun Y, Wang M, Lin G, Sun S, Li X, Qi J, et al. Serum microRNA-155 as a potential biomarker to track disease in breast cancer. PLoS One 2012;7:e47003.
45. Lv ZC, Fan YS, Chen HB, Zhao DW. Investigation of microRNA-155 as a serum diagnostic and prognostic biomarker for colorectal cancer. Tumour Biol 2014;36:1619–25.
46. Koutsaki M, Spandidos DA, Zaravinos A. Epithelial-mesenchymal transition-associated miRNAs in ovarian carcinoma, with highlight on the miR-200 family: prognostic value and prospective role in ovarian cancer therapeutics. Cancer Lett 2014;351:173–81.
47. Muralidhar GG, Barbolina MV. The miR-200 family: versatile players in epithelial ovarian cancer. Int J Mol Sci 2015;16:16833–47.
48. Zuberi M, Mir R, Das J, Ahmad I, Javid J, Yadav P, et al. Expression of serum miR-200a, miR-200b, and miR-200c as candidate biomarkers in epithelial ovarian cancer and their association with clinicopathological features. Clinical Transl Oncol 2015;17:779–87.
49. Brozovic A, Duran GE, Wang YC, Francisco EB, Sikic BI. The miR-200 family differentially regulates sensitivity to paclitaxel and carboplatin in human ovarian carcinoma OVCAR-3 and MES-OV cells. Mol Oncol 2015;9:1678–93.

50. Kanemaru H, Fukushima S, Yamashita J, Honda N, Oyama R, Kakimoto A, et al. The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker. J Dermatol Sci 2011;61:187–93.
51. Muller V, Gade S, Steinbach B, Loibl S, von Minckwitz G, Untch M, et al. Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: a translational research project within the Geparquinto trial. Breast Cancer Res Treat 2014;147:61–8.
52. Yoshimoto R, Mayeda A, Yoshida M, Nakagawa S. MALAT1 long non-coding RNA in cancer. Biochim Biophys Acta 2016;1859:192–9.
53. Peng H, Wang J, Li J, Zhao M, Huang SK, Gu YY, et al. A circulating non-coding RNA panel as an early detection predictor of non-small cell lung cancer. Life Sci 2016;151:235–42.
54. Kishikawa T, Otsuka M, Ohno M, Yoshikawa T, Takata A, Koike K. Circulating RNAs as new biomarkers for detecting pancreatic cancer. World J Gastroenterol 2015;21:8527–40.
55. Bhan A, Mandal SS. LncRNA HOTAIR: a master regulator of chromatin dynamics and cancer. Biochim Biophys Acta 2015;1856:151–64.
56. Tong YS, Wang XW, Zhou XL, Liu ZH, Yang TX, Shi WH, et al. Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. Mol Cancer 2015;14:3.
57. Li C, Chen J, Zhang K, Feng B, Wang R, Chen L. Progress and prospects of long noncoding RNAs (lncRNAs) in hepatocellular carcinoma. Cell Physiol Biochem 2015;36:423–34.
58. Yu X, Li Z. Long non-coding RNA growth arrest-specific transcript 5 in tumor biology. Oncol Lett 2015;10:1953–8.
59. Zhang A, Zhang J, Kaipainen A, Lucas JM, Yang H. Long non-coding RNA: a newly deciphered "code" in prostate cancer. Cancer Lett 2016;375:323–30.
60. Xue D, Zhou CX, Shi YB, Lu H, He XZ. MD-miniRNA could be a more accurate biomarker for prostate cancer screening compared with serum prostate-specific antigen level. Tumour Biol 2015;36:3541–7.
61. Wu Y, Wang YQ, Weng WW, Zhang QY, Yang XQ, Gan HL, et al. A serum-circulating long noncoding RNA signature can discriminate between patients with clear cell renal cell carcinoma and healthy controls. Oncogenesis 2016;5:e192.
62. Hu X, Bao J, Wang Z, Zhang Z, Gu P, Tao F, et al. The plasma lncRNA acting as fingerprint in non-small-cell lung cancer. Tumour Biol 2015;37:3497–504.
63. Tantai J, Hu D, Yang Y, Geng J. Combined identification of long non-coding RNA XIST and HIF1A-AS1 in serum as an effective screening for non-small cell lung cancer. Int J Clin Exp Pathol 2015;8:7887–95.
64. Shi J, Li X, Zhang F, Zhang C, Guan Q, Cao X, et al. Circulating lncRNAs associated with occurrence of colorectal cancer progression. Am J Cancer Res 2015;5:2258–65.
65. Zhou X, Yin C, Dang Y, Ye F, Zhang G. Identification of the long non-coding RNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer. Sci Rep 2015;5:11516.
66. Gao J, Cao R, Mu H. Long non-coding RNA UCA1 may be a novel diagnostic and predictive biomarker in plasma for early gastric cancer. Int J Clin Exp Pathol 2015;8:12936–42.
67. Schwarzenbach H. The clinical relevance of circulating, exosomal miRNAs as biomarkers for cancer. Expert Rev Mol Diagn 2015;15:1159–69.
68. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics 2009;6:267–83.
69. Azmi AS, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. Cancer Metastasis Rev 2013;32:623–42.
70. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.
71. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. Protein Cell 2012;3:28–37.
72. Igaz I, Igaz P. Tumor surveillance by circulating microRNAs: a hypothesis. Cell Mol Life Sci 2014;71:4081–7.
73. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 2008;110:13–21.
74. Eichelser C, Stuckrath I, Muller V, Milde-Langosch K, Wikman H, Pantel K, et al. Increased serum levels of circulating exosomal microRNA-373 in receptor-negative breast cancer patients. Oncotarget 2014;5:9650–63.
75. Li Z, Ma YY, Wang J, Zeng XF, Li R, Kang W, et al. Exosomal microRNA-141 is upregulated in the serum of prostate cancer patients. Onco Targets Ther 2016;9:139–48.
76. Pfeffer SR, Grossmann KF, Cassidy PB, Yang CH, Fan M, Kopelovich L, et al. Detection of exosomal miRNAs in the plasma of melanoma patients. J Clin Med 2015;4:2012–27.
77. Matsumura T, Sugimachi K, Iinuma H, Takahashi Y, Kurashige J, Sawada G, et al. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. Br J Cancer 2015;113:275–81.
78. Meng X, Muller V, Milde-Langosch K, Trillsch F, Pantel K, Schwarzenbach H. Diagnostic and prognostic relevance of circulating exosomal miR-373, miR-200a, miR-200b and miR-200c in patients with epithelial ovarian cancer. Oncotarget 2016;7:16923–35.