REVIEW

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Circulating RNAs in medical diagnostics and as disease-relevant biomarkers

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The development of malignant tumors from healthy tissues is associated with profound changes in expression profiles of a large number of mRNAs, miRNAs and lncRNAs. These changes on the one hand permit insights into the biology of individual tumors; on the other hand, tumor-derived RNAs can also be detected in circulating blood and serve as specific markers for differential diagnosis and patient prognosis.

1. Introduction

First reports on the presence of cell-free nucleic acids in human blood circulation date back as far as the year 1948 (Mandel et al. 1948). Subsequently, different RNA species were identified in a multitude of human secretions, including urine, saliva, liquor, and even breast milk (overview in Schwarzenbach et al. 2011). The majority of studies, however, focuses on the analysis of RNA in the blood stream.

Since blood samples are already widely used for different diagnostic purposes in clinical routine and can be obtained with minimal detriment to patients and control subjects, blood is an attractive material for the development of diagnostic tests. *In vitro* and in the analytical laboratory, blood can be separated into different fractions using simple centrifugation steps: an acellular fraction (plasma or serum), a "buffy coat" containing the blood leukocytes, and a fraction with red blood cells and platelets. For the preparation of plasma, anti-coagulants (EDTA, citrate or heparin) are added to the sample to inhibit the clotting process. In contrast, to obtain serum, the blood sample is first allowed to go through the coagulation process before centrifugation and harvesting of the cell-free supernatant which is then devoid of any coagulation factors. Circulating nucleic acids can be detected in both, plasma and serum, and there is currently no general consensus concerning the question as to which material is more suitable for RNA-based diagnostic approaches.

Analyses of the biological and diagnostic significance of the presence of RNAs in blood circulation have been performed in the context of many different diseases, including for example myocardial infarction (overview in Schwarzenbach et al. 2011; Wang et al. 2014). As the vast majority of studies deal with the relevance of circulating RNAs in the context of malignant diseases, this review article will focus on the role(s) of circulating RNAs in cancer.

2. Circulating mRNAs

Following first reports from the 1970's describing generally elevated RNA levels in the plasma of patients suffering from multiple myeloma as compared to normal controls (Hamilton et al. 1979), it was first demonstrated in 1999 that specific tumor-derived mRNAs are present in the plasma of patients with malignant melanoma (Kopreski et al. 1999). Both observations were surprising given that active RNases can already be detected at relatively high levels in plasma and serum from healthy subjects, and are often significantly increased in samples from tumor patients (Reddi and Holland 1976). Thus, RNAs in circulation must be somehow protected from enzymatic degradation. Biochemical and ultramicroscopic analyses subsequently demonstrated the presence of extracellular vesicles in the blood of leukemia patients as well as an active secretion of such vesicles from cultured human colon carcinoma cells (Rosi et al. 1988). Moreover, it was established that these vesicles indeed contain RNA as "cargo" (Rosi et al. 1988). Another potential source of vesicle-associated circulating RNAs are so-called "apoptotic bodies", i.e. vesicular structures (Fig. 1) that form during the desintegration of cells that undergo programmed cell death (= apoptosis). Experiments involving cultured human leukemia and melanoma cells demonstrated that cellular mRNAs are actively sequestered into apoptotic bodies separate from cellular DNA (Halicka et al. 2000). It remains unclear, however, if circulating mRNAs can also be protected from degradation outside of vesicular structures by formation of complexes with other macromolecules, as is already well-established for microRNAs (miRNAs) (see below).

Tumor-associated mRNAs have been detected in the blood of patients suffering from a wide variety of malignant diseases (Fleischhacker 2006). Some of these analyses employed qualitative rather than quantitative methods, hampering a directly comparative interpretation of the results. Yet an increasing number of publications proves that the quantitative analysis of circulating mRNAs in the blood of tumor patients is in principle suitable to identify potent diagnostic, prognostic and/or predictive biomarkers. As an example, the quantitative detection of CCND1 mRNA (coding for the cell cycle regulator cyclin D1) enabled the distinction between breast cancer patients with good and bad response to tamoxifen treatment, respectively (Garcia et al. 2008). In melanoma patients, elevated SOCS1 and SOCS3

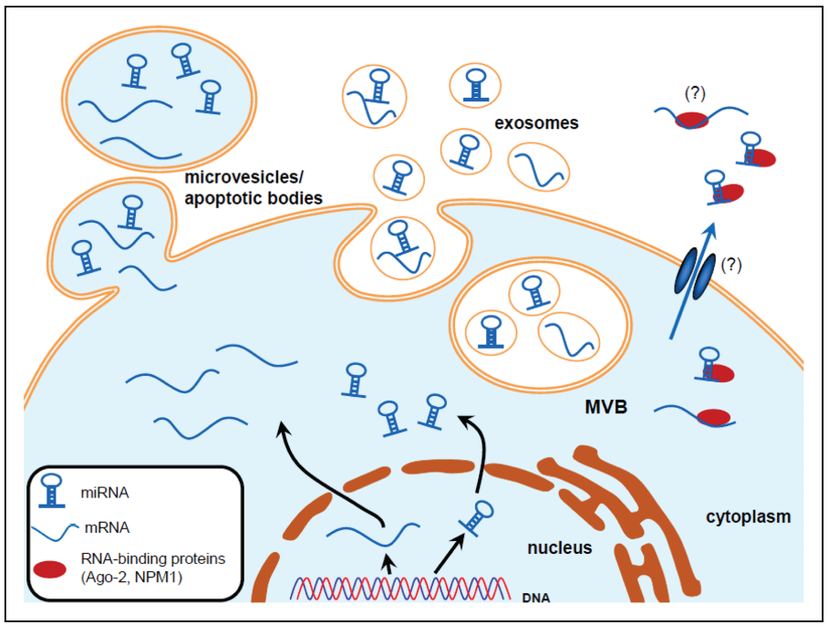

Fig. 1: Tumor-derived RNAs can be detected in circulating blood and serve as specific markers for differential diagnosis and patient prognosis. Extracellular vesicles containing RNA as "cargo" form by different mechanisms, either by direct budding from the plasma membrane (microvesicles, apoptotic bodies) or by generation of specialized "exosomes" which are formed intracellularly within so-called multivesicular bodies (MVB).

mRNA levels were associated with reduced survival, and down-regulation of ISG15 mRNA under therapy with PEG-IFNα-2b was correlated with poorer response to therapy (Busse et al. 2013). Likewise, elevated levels of MUC1 and VEGF mRNA in the blood of non-small cell lung carcinoma (NSCLC) patients was associated with reduced overall survival as well as poor response to therapy with gefitinib (Li et al. 2014).

The interpretation of these results generally requires caution since the number of patients analyzed in each individual study was often very limited and the results will first have to be validated in larger trials. An example of contradictory results is the detection of hTERT mRNA, coding for the human telomerase, which is well-known to be overexpressed in many human malignancies. While Miura et al. (2006) reported that analyses of serum samples from lung carcinoma patients showed a significant correlation of hTERT mRNA levels with tumor size, metastasis, tumor recurrence and smoking habits of the patients, a number of other studies found no significant association with any clinical parameters (Fleischhacker and Schmidt 2007).

3. Circulating miRNAs

The first report on disease-specific patterns of circulating miRNAs stems from 2008: elevated levels of miR-21 in serum from patients with diffuse B-cell lymphoma correlated with better recurrence-free survival (Lawrie et al. 2008). Since then, the number of publications dealing with the identification and characterization of circulating miRNAs as biomarkers in different malignant diseases has risen exponentially and has long surpassed the number of reports on circulating mRNAs.

There are several factors that are responsible for the fact that miRNAs are being regarded as especially promising candidates for circulating biomarkers. On the one hand, it is now well-established that miRNAs act as regulatory molecules by modulating the expression of protein-coding genes to control a multitude of physiological and pathophysiologycal processes. Characteristic changes in miRNA levels are therefore often correlated with clinical parameters such as tumor stage, patient prognosis or response to therapy (Di and Croce 2013). On the other hand, miRNAs have proven to be exceptionally stable analytes which can be reliably and quantitatively detected in different biological specimens collected and archived following a wide variety of different protocols and procedures. For example, while larger nucleic acid molecules like mRNAs and genomic DNA are often found to be strongly fragmented and chemically modified in formalin-fixed paraffin-embedded (FFPE) tissue samples, which hampers their analysis, miRNAs can be detected and quantified in such sample material with good precision and reproducibility (Nuovo et al. 2009). This opens up the possibility to use the vast collections of FFPE specimens from pathological archives, which often contain large numbers of histopathologically and clinically well-characterized specimens, in the search for novel clinically relevant biomarkers. Moreover, using *in situ* hybridization techniques, specific miRNA expression patterns can sometimes even be correlated with different subentities within individual tumors, e.g. well-differentiated vs. undifferentiated areas or invasive front *vs*. tumor center etc.

A number of studies proved that miRNAs from tumors regularly enter the blood circulation *via* different mechanisms and can thus serve as specific markers of disease (Nair et al. 2014). As described above for mRNAs, miRNAs are protected from enzymatic degradation in circulation, and it is now well-established that miRNAs are actively secreted in microvesicles as well as packaged in apoptotic bodies that enter circulation (Nair et al. 2014; Quackenbush et al. 2014). In addition, miRNAs are also found in plasma or serum independently of vesicular structures in the form of complexes with RNA-binding proteins such as nucleophosmin 1 or argonaute 2, and in this form are protected from degradation as well (Vickers et al. 2011; Arroyo et al. 2011) (Fig. 1).

For the detection and quantification of circulating miRNAs, different methods are available ranging from microarray applications to next-generation sequencing techniques (NGS) and single- or multiplex RealTime-PCR (qRT-PCR)- methods; qRT-PCR has become the most widely used technique in this area, owing to the inherent principle of amplifying target RNAs from source materials which results in a particularly high detection

sensitivity. Independently of the method used, though, normalization of the detected signals remains an unsolved challenge. Normalization of raw signals is required in order to correct for differences in measured intensities which stem from technical variations, such as differences in the efficiency of RNA extraction, quality of the source material, pipetting errors etc., rather than from true differences in marker abundancy. In qRT-PCR analyses of miRNAs from tissue samples, raw signals are often normalized to signals from small "housekeeping" RNAs, such as U6 snRNA, RNU48, 5S RNA or 18S RNA, which are assumed to be evenly expressed under most circumstances. However, the suitability of these RNAs for normalization of miRNAs extracted from serum or plasma is not validated to date, so that some authors favor alternative concepts such as the addition of exogeneous miRNAs of known concentration as reference for normalization ("spike in control") (Nair et al. 2014).

In spite of the remaining methodological challenges, which in part hamper the comparison of results from different studies, a multitude of studies have already established circulating miRNAs as sensitive and specific markers for diagnostic, prognostic and predictive purposes. For example, an NSCLC-associated serum miRNA signature has been identified which predicts the presence of early stage tumors in asymptomatic patients (Bianchi et al. 2011), while another study described nine miRNAs detected in plasma from NSCLC patients which correlate with patient survival (Boeri et al. 2011). Several publications have reported on the detection of different combinations of miR-145, miR-155, miR-382, miR-451, miR-148b, miR-409-3p and miR-801 expression which were found to be suitable for distinguishing breast cancer patients from healthy control individuals (Schwarzenbach 2013). In addition, circulating miR-10b and/or circulating miR-215, miR-299-5p and miR-41 were able to aid in the identification of breast cancer patients with increased risk for lymph node or bone metastasis (Wang et al. 2014).

Across different types of tumors, elevated levels of miR-21 were detected in the plasma or serum of patients with NSCLC, pancreatic cancer, gastric carcinoma, ovarian cancer, hepatocellular carcinoma and prostate cancer, and were described as independent prognostic factors in breast and lung cancer (Wang et al. 2011, 2014). Similarly, elevated levels of miR-155 were detected in the blood from patients suffering from diffuse large B-cell lymphoma (DLBCL), breast cancer or lung cancer, as well as in urine from bladder cancer patients and in pancreatic secretions from pancreatic cancer patients (Wang et al. 2014).

As described above for mRNA studies, many of the investigations cited here were also conducted with very limited numbers of patients and controls, thus requiring validation of the results in larger clinical trials. In spite of these limitations and the remaining technical challenges, circulating miRNAs are firmly established as promising biomarkers in the diagnostic workup of tumor patients.

4. LncRNAs and circular RNAs: an outlook

While mRNAs and miRNAs are well-characterized as functional molecules and potential biomarkers in different physiological and pathophysiological contexts, the diagnostic relevance of other RNA classes remains to be shown. These include, among others, the long non-coding RNAs (lncRNAs) as well as the only recently discovered large class of circular RNAs (circRNAs).

LncRNAs were initially regarded as unspecific by-products of cellular transcription. Yet, more recent studies have shown that lncRNAs can actively influence processes such as cell differentiation, growth, apoptosis and even tumor progression by influencing translational, posttranslational and epigenetic functions (Yang and Deng 2014). The lncRNA HOTAIR, for example, mediates epigenetic silencing of a specific HOX gene cluster in tumor cells (Prensner and Chinnaiyan 2011), and increased expression of HOTAIR correlates with poor prognosis and increased metastasis in breast cancer patients (Gupta et al. 2010; Ernst and Morton 2013).

In contrast, little information is so far available on circRNAs, whose astonishingly wide-spread abundance has been realized in 2013 (Memczak et al. 2013). The biological functions of circRNAs are still largely unknown. However, bioinformatic analyses as well as first experimental data demonstrate that at least some circRNAs can act as "miRNA sponges", i.e. they contain multiple binding sites for the same miRNA. By sequestering such miRNAs, miRNA sponges may be involved in the regulation of a wide variety of biological processes (Memczak et al. 2013). The high number and inherently high stability as well as the tissue- and stage-specific expression pattern of circ-RNAs suggest that they may indeed be promising biomarker candidates.

Further work will have to be conducted to establish if members of one or both of these classes of RNAs can be stably detected in the blood stream and, similar to mRNAs and miRNAs, interrogate important clinical parameters. These questions are currently in the focus of intense studies by several clinical and basic science research groups worldwide.

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