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Review

cfRNAs as biomarkers in oncology – still experimental or applied tool for personalized medicine already?

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ABSTRACT

Currently, the challenges of contemporary oncology are focused mainly on the development of personalized medicine and precise treatment, which could be achieved through the use of molecular biomarkers. One of the biological molecules with great potential are circulating free RNAs (cfRNAs) which are present in various types of body fluids, such as blood, serum, plasma, and saliva. Also, different types of cfRNA particles can be distinguished depending on their length and function: microRNA (miRNA), PIWI-interacting RNA (piRNA), tRNA-derived RNA fragments (tRFs), circular RNA (circRNA), long non-coding RNA (lncRNA), and messenger RNA (mRNA). Moreover, cfRNAs occur in various forms: as a free molecule alone, in membrane vesicles, such as exosomes, or in complexes with proteins and lipids. One of the modern approaches for monitoring patient's condition is a ‘liquid biopsy’ that provides a non-invasive and easily available source of circulating RNAs. Both the presence of specific cfRNA types as well as their concentration are dependent on many factors including cancer type or even reaction to treatment. Despite the possibility of using circulating free RNAs as biomarkers, there is still a lack of validated diagnostic panels, defined protocols for sampling, storing as well as detection methods.

In this work we examine different types of cfRNAs, evaluate them as possible biomarkers, and analyze methods of their detection. We believe that further research on cfRNA and defining diagnostic panels could lead to better and faster cancer identification and improve treatment monitoring.

1. Introduction

Identification of biomarkers is necessary to help physicians to decide whether conventional or specific targeted therapy or immunotherapy should be used to treat a particular group of patients to improve their survival.[1] Exact treatment strategies should fit molecular features of a specific tumor and be as effective and harmless for patients as possible.[2] This approach leads oncology to personalized medicine, both in chemo- and radiotherapy. For example, the application of the method for drug screening based on induced pluripotent stem cells (iPSCs)[3] or based on patients “slice cultures” are promising systems for assessment of drug susceptibility.[4] For individualization of radiotherapy, the introduction of specific biomarkers of hypoxia, DNA repair or cancer stem cell, checking of EGFR status as well as patient visualisation using PET tracer 18F-FDG are promising approaches.[5]

Monitoring patients' status and deciding whether to continue, stop or change the therapy also require easily available and reliable biomarkers.[6] Another issue is how to diagnose cancer in the early stage of development when the conventional diagnostic techniques, such as tissue immunohistochemical examination, measurement of specific markers in the blood, PET scans or ultrasonography are insufficient.[6,7] All these problems result in searching innovative approaches, such as new diagnostic, prognostic and predictive biomarkers.[6,8]

2. Liquid biopsy

A ‘liquid biopsy’ (also known as fluid or fluid phase biopsy) obtained from non-solid (liquid) biological material, mainly blood or fractions of the blood, is a way of non-invasive biopsy, compared to the traditional tissue biopsy, and an easily available source of biomarkers.[9–13] A liquid biopsy allows collecting the sample at any time during or after the treatment.[12] This feature enables to monitor patients' current status, such as changes in cancer pheno-

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type (changes in the cancer genome, transcriptome or epigenome), response to treatment, changes in immunophenotype or detection of foreign organisms or viruses.[12–16] Several types of liquid biopsies can be distinguished depending on the type of disease and source of molecules or cells: i) in oncology (circulating tumor cells (CTCs), circulating tumor DNAs (ctDNAs), circulating tumor RNAs (ctRNAs)); ii) in cardiology (circulating endothelial cells (CECs)) and iii) in prenatal diagnostics (cell-free fetal DNA (cffDNA) and circulating free RNAs (cfRNAs) from maternal blood or amniotic fluid).[17–19]

It should be noted that CTCs based liquid biopsy for various types of cancer as well as cobas EGFR Mutation Test (Roche Molecular Systems, Inc.) based on cfDNA isolated from plasma of patients with metastatic non-small cell lung cancer (NSCLC) are validated methods approved by the FDA (Food and Drug Administration, USA) as useful prognostic and predictive tools.[20,21] However, the this method is still not commonly implemented.[22]

Nowadays, the use of circulating free RNAs (cfRNAs) based liquid biopsy in diagnostics is in the interest of oncologists. The concept of cfRNAs based liquid biopsy is very simple and could be performed as a parallel diagnostic test and be a supplement to a liquid biopsy based on ctDNAs and/or CTCs. The main steps of this diagnostic method are presented in Fig. 1.

The first report about clinical utility of circulating RNA-proteolipid complexes in the serum of patients with lymphoma and carcinoma (lung, stomach and colon) was published by Wieczorek et al. in 1987, demonstrating their diagnostic and prognostic potential and underlining their features as a cancer biomarker.[23]

cfRNAs potentially have all the features of a classic biomarker[24]: 1) characterize a specific condition of the organism and distinguish groups by that condition; 2) are easily available, stable and can be isolated from a variety of biological fluids such as whole blood, plasma, serum, urine, milk or other fluids[25–28]; 3) the analysis process is fast, easy and lower in cost every year.

However, Laktionov et al. report indicated problems with the adaptation of cfRNAs (and cfDNAs) as a potential biomarker. They concluded that cfRNAs are not detectable in the case of breast cancer and nonmalignant breast tumors, but higher levels of them were measured in plasma of lung malignant and nonmalignant individuals. Moreover, the concentration of circulating RNA and DNA seems to be a reflection of a posttraumatic organ failure.[29] These results show that the changes of circulating RNAs are nonspecific reaction and reflect the current state of a specific organ or whole organism. It is assumed to be the main concern for the use of RNAs as a classical biomarker. However, characteristics of specific response to stress (organ damage, tumor cells apoptosis) could be clinically useful and many different studies indicated the potential of various kinds of circulating RNAs as biomarkers.

3. Circulating free RNAs (cfRNAs)

It is generally assumed, that the ribonucleic acids (RNAs) are unstable molecules and are quite quickly degraded by RNases activity. Products of this process are used to produce new RNAs' molecules, thus maintaining correct cell functions. For example, the mRNA turnover depends on cellular balance between degradation and synthesis as well as on different environmental stimuli and it can be processed via four different pathways.[30] However, some of the mRNAs and non-coding RNAs (shorter and longer) can be found in different body fluids, which contradicts the statement of quick RNA degradation.[31,32] Different types of RNA transcripts: i) short RNA, e.g. miRNA, tRFs and YRNAs, ii) covalently closed, e.g. circRNA, iii) and long, e.g. lncRNA, as well as mRNA, are detectable in whole blood, serum and plasma,[32–34] their main molecular features are characterized in Table 1. Some data from the 70 s suggested that extracellular RNA is heavily methylated, associated with DNA as a part of bigger complexes or single-stranded with low molecular weight. Moreover, it affects DNA synthesis *in vitro* by transferring information from cell-to cell and seems to be resistant to RNases.[35] It is usually thought, that the concentration of RNA in the human plasma is high (to 144 ng RNA per mL of plasma) but some authors report that the actual amount is lower and depends, for example, on individual's state such as health status or type of disease.[36,37] Rubio et al. indicated 40 ng RNA/mL in plasma and about 50-times higher concentration of RNA in human milk measured by QubitTM 2.0 Fluorometer (Thermo Fisher Scientific), but the authors supposed the presence of cellular RNAs in obtained milk.[28] Similarly, circulating RNAs are also detectable; therefore, they are supposed to be protected from the RNases which are highly concentrated in various types of body fluids in healthy and cancer patients.[32] It is known that the quantity assessment of different molecules of circulating RNAs depends on the type of their source. For example, Victoria Martinez et al. using NGS technology identified 3 major classes of small circulating RNAs extracted from the serum of healthy and cancer individuals. The largest amount of circulating RNAs was: miRNA (50.5 %), YRNA (38.5 %), tRFs (10.5 %), rRNA (0.4 %) and other snRNAs and snoRNAs (0.2 %) in the serum of healthy donors and HNSCC cancer cases.[26] Another study also using RNA-seq technology indicated that some RNA classes are highly expressed in serum compared to others, and uniquely-mapped reads showed an abundance of miRNA (45.7 %), mRNA (20.3 %), miscRNA (11.8 %), lncRNA (10.7 %), piRNA (4.3 %), tRFs (1.9 %) and others (5.3 %).[34] These studies underline that the miRNAs are the most abundant kind of cfRNAs in blood circulation.[26,34]

It is known that in blood circulation they occur as a free form, bound to proteins or lipids, or as exosomes protected in various types of membrane microbubbles which provide them with high stability and allow transport.[33,38]

Circulating nucleic acids are probably secreted by cancer cells and/or immune cells, and are the body's specific response to a given physiological/pathological condition.[38] It is known that different types of RNA molecules can penetrate from the extracellular environment to different types of cells, e.g. the immune system or cancer cells, and modulate their activity, or be secreted by these cells into the external environment (blood circulation) in response to obtaining a given phenotypic state.[39,40] This statement is supported by Semenov et al. They proved the role of artificial analogues of human plasma RNAs (C/D box RNAs) in the regulation of processing of HSC8 pre-mRNA based on *in vitro* model of human adenocarcinoma cells (MCF-7). The extracellular analogues modulate the function of ribosomes as well as induce partial splicing impairments, and influence cellular proliferation.[41] Moreover, the authors suggested that Alu-containing circulating RNAs are important regulators of cellular processes.[41]

Some evidence has shown that cancer cells can be the source of circulating cfRNAs. Based on the *in vitro* model of gastric cancer cells Tsujiura et al. proved that miR-18a is released from cells to culture media.[42] Unfortunately, their experiments did not explain whether miR-18a is actively released as free RNA or as molecular complexes with proteins or lipids, or packed into exosomes. In both physiological and pathological conditions RNAs as well as DNAs can be passively and actively secreted during apoptosis or necrosis.[43] However, Tsujiura et al. showed that miRNA in plasma of gastric cancer patients that miR-18a was not released from peripheral blood cells.[42]

3.1. Short RNA transcripts

The first group of RNA acids are short transcripts, including miRNA, tRFs and YRNAs with the length between 22–110 nucleotides. microRNA is a group of well-known approximately 22 nucleotides RNA molecules involved in regulating the expression

Table 1
Characteristics of selected cfRNAs.

Type of RNA	Size	Characteristics	Ref.
miRNA	~22 nt	- non-coding, endogenous, short, single-stranded RNA sequences - miRNA precursors are transcribed from the genome and create immature forms of miRNA called pre-miRNA which are transformed further by Drosha and Dicer enzymes - regulator of gene expression at the post-trancriptional level by targeting the 3′UTR of miRNA reducing expression of the encoded proteins - regulator of processes such as: growth, cell differentiation, immune responses, apoptosis, cell cycle, EMT - easily isolated from cell lines, tissue, formalin-fixed paraffin embedded tissue (FFPET), plasma, serum and other fluids - diagnostic and therapeutic tool in many cancer types - dysregulated expression in many cancer types, associated with tumorigenesis	[139–142]
circRNA	1–5 exons/introns	- non-coding, endogenous, single-stranded RNA with a structure of covalently closed loop without 5′ or 3′ end – resistant to exonuclease degradation by Rnase R - transcribed from pre-mRNA sequences by RNA polymerase II; covalently joing the 5′ and 3′ ends via back-splicing mechanism - classified as: intronic, antisense, sense or exonic, intergenic, intragenic or bidirectional groups - regulator of genes expression at the post-transcription or transcription level; miRNA sponges, protein interaction (sponges, decoys, scaffolding) - involved in human fetal development, myocardial infarction and carcinomas - useful for treatment and diagnostics in many cancer types and other diseases - easily detectable in body fluids (blood, saliva, serum, plasma)	[72,143,144]
YRNA and Y-derived fragments	~80–110 nt	- small, non-coding RNA –4 highly conservative types: YRNA1, YRNA3, YRNA4, YRNA5 - gene clustered at a single chromosomal locus on chromosome 7q36 and transcribed by RNA polymerase III - a stem-loop structure with an internal loop and polyuridine tail and upper stem essential for initiation of DNA replication; lower stem as the Ro60 binding site and controller of nuclear export of YRNA; polyuridine tail as the La protein binding site - easily found in tissue, blood, plasma, serum - dysregulated in many cancer types and diseases - YRNA-derived fragments - 24–34 nt long; processed in apoptotic and lipid-laden macrophages; processed into microRNA-like small RNAs; biological role under evaluation	[26,67–69]
tRFs	10–50 nt	- small, non-coding RNA - some fragments derived from precursor tRNA (pre-tRNA) molecules and others from mature cytoplasmic tRNAs - tRNA halves (tiRNAs) - produced by a specific cleavage in the anticodon loop of mature tRNAs to 30–35 nt tiRNA-5' and 40–50 nt tiRNA-3' –3 types of tRNA-derived small RNA fragments (tRFs): tRF-5 derived from the 5′-ends of mature tRNAs by cleavage of the D loop; tRF-3 formed by a specific cleavage of the 3′-ends of mature tRNAs; and tRF1 produced from the 3′ trailers of pre-mature tRNAs - internal-tRFs (i-tRFs) derived from the internal regions of the mature tRNAs shedeling the anticodon - regulator of gene expression, cell proliferation, invasion and possible function as miRNAs - linked with many cancer types and diseases an possible useful as biomarkers	[63,145]
lncRNA	>200 nt	- long, non-coding RNA - biogenesis dependent on lncRNA gene localization: primary transcripts processed by RNA polymerase II and form lncRNAs, mRNAs, pre-miRNAs, tRNA-like ncRNAs and different unstable forms of RNA molecules by further processes of maturation - in some cases possess ORF and translation of short peptides is possible - mechanism of action dependt on cellular localization; nuclear localization: chromatin, transcriptional and RNA processing; cytoplasmic localization: mRNA stability or translation and influence on signaling cascades - modulators of gene expression in different ways: signaling lncRNA, decoying lncRNA, guiding lncRNA and lncRNA scaffolds - expression of lncRNA strictly dependent on tissue and cell type - easily obtained from tissue, blood, serum and plasma - linked with many cancer types and diseases an possible useful as biomarkers	[77,78,146]
mRNA	~2000 nt	- messenger RNA; proteins coding - transcribed in the nucleus; pre-mRNA is capped, spliced, polyadenylated forming mature mRNA and exported to the cytoplasm - pre-mRNA is source of different kinds of ncRNAs (miRNAs, circRNA, lncRNA etc.) - dysregulation of biogenesis, expression or function of mRNAs play a crucial role in tumor development and other diseases - easily obtained from tissue and some transcripts from blood, serum and plasma but prone to degradation compared to ncRNAs	[147,148]

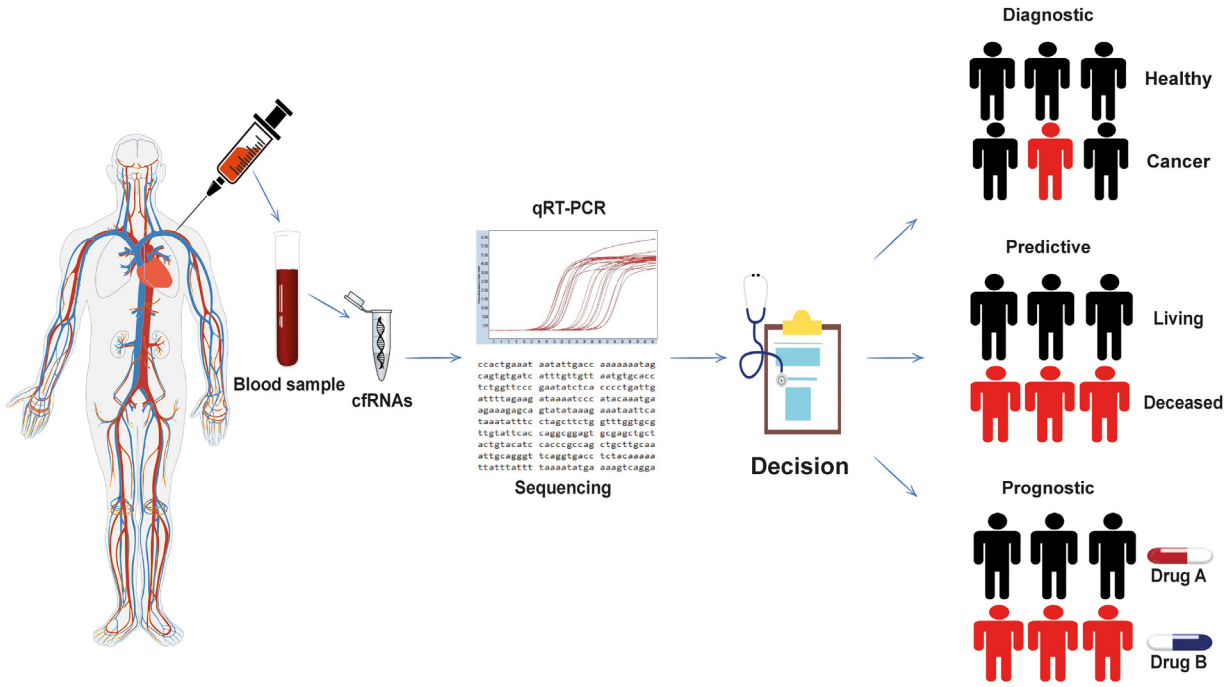

Fig. 1. Schematic illustration of cfRNAs liquid biopsy method and its utility as diagnostic, prognostic and predictive biomarkers.
The first step is taking a sample from the patient any time, but the time point will define the use of obtained information: as diagnostic marker (sampling at the time of diagnosis), as prognostic marker (sampling after finishing course of treatment) or as predictive marker (sampling during specific treatment time points). Next, different types of cfRNAs are isolated from the body fluid and transcribed into cDNAs. After that, cfRNAs are quantified using different available methods such as next-generation sequencing (NGS) (mostly for scientific purpose) or by qRT-PCR method (when the defined cfRNAs panels are available and approved as diagnostic). In the end, the obtained data are translated into specified clinical information followed by therapeutic decision. Figure based on pictures from pixabay.com website.

of genes encoding proteins by binding to the 3′UTR of a particular mRNA transcript. The role of circulating miRNAs in the regulation of the tumor cell phenotype and in the immune system as well as the potential use of these RNAs as biomarkers have been demonstrated.[44,45]

For example, miR-18a as a cfRNA is supposed to be a potential biomarker for colon cancer,[46–48] gastric cancer,[42,49,50] breast cancer,[51,52] esophageal squamous cell carcinoma,[53] retinoblastoma,[54] pancreatic cancer,[55] endometriosis[56] as well as in diffuse large B cell lymphomas.[57,58] Most of these studies indicated that the expression level of miR-18a is up-regulated in patients' plasma or serum and can be useful as a diagnostic, prognostic or predictive biomarker. Liu et al. suggested that a specific panel of circulating miRNAs may have a higher diagnostic value than that of traditional biomarkers.[59] However, other authors, in contrast to Liu et al., have a critical view of liquid biopsy based on miRNAs. They pointed out that these molecules are not reliable biomarkers and their usefulness is only minimal.[60,61]

Short RNA molecules also include transcripts originating from the cleavage of tRNA molecules, previously considered to be involved only in the protein translation process. tRNA-derived RNA fragments (tRFs) by binding to both Argonaute and Piwi proteins regulate gene expression. They can also function as signaling molecules in response to stress.[62] The number of fragments derived from tRNA is often diverse in tumors and some of them were found to participate in cell proliferation, apoptosis and tumor metastasis[63] as well as in infectious, metabolic and neurological diseases.[62] It was shown that 5′tRFs are much more expressed in hematopoietic and lymphoid tissues than in others and they may participate in signaling between cells and be potential immunological signal molecules.[64]

YRNAs are a class of small non-coding RNAs, approximately 80–110 base pairs in length and have characteristics of a stem-loop structure with an internal loop and polyuridine tail. They are components of the Ro60 ribonucleoprotein particle which takes part in DNA replication.[65–68] There are four highly conservative types of YRNAs transcripts in human genome: YRNA1 (RNY1), YRNA3 (RNY3), YRNA4 (RNY4) and YRNA5 (RNY5).[68] The exact function of YRNAs is unknown, but is was indicated that they are involved in the initiation of chromosomal DNA replication,[65–68] cell proliferation, [66] immune system[69] and viral replication.[70] It was noted that YRNAs are involved in the carcinogenesis process of prostate cancer,[65] breast cancer,[66] head and neck squamous cell carcinoma,[26] clear cell renal cell carcinoma and bladder cancer.[67] The YRNAs' transcripts were found in serum, plasma and tissues of healthy and diseased individuals.[66]

3.2. *Covalently closed RNA transcripts*

circRNA are round RNAs that are covalently closed, single-stranded RNA transcripts with 1–5 exons/introns in length. One type of these circular RNAs are molecules that originated from the precursor mRNA (pre-mRNA) during the maturation process (splicing). Various types of circRNAs perform a regulatory function by i) acting as specific m̈olecular spongesëxtracting and temporarily blocking miRNAs, ii) influencing the mRNA maturation process by avoiding excision of a particular intron, iii) enhancing the transcription process or iv) activating an alternative pathway of RNA maturation.[71] circRNAs are tissue specific particles necessary for changes in the cell phenotype. Their expression is altered as a result of specific pathological and disease states, e.g. in the process of tumor cell proliferation.[72] circRNAs were also shown to play a role in regulating the immune system in response to viral or bacterial infections as well as cancer development.[73] circRNAs can contribute to immune regulation through interactions with miRNA; an example is hsa_circ_0020397 which can increase PD-L1 expression by binding to miR-138 (the target miRNA for circRNA) in colorectal cancer cells. The consistent increase of PD-L1 level con-

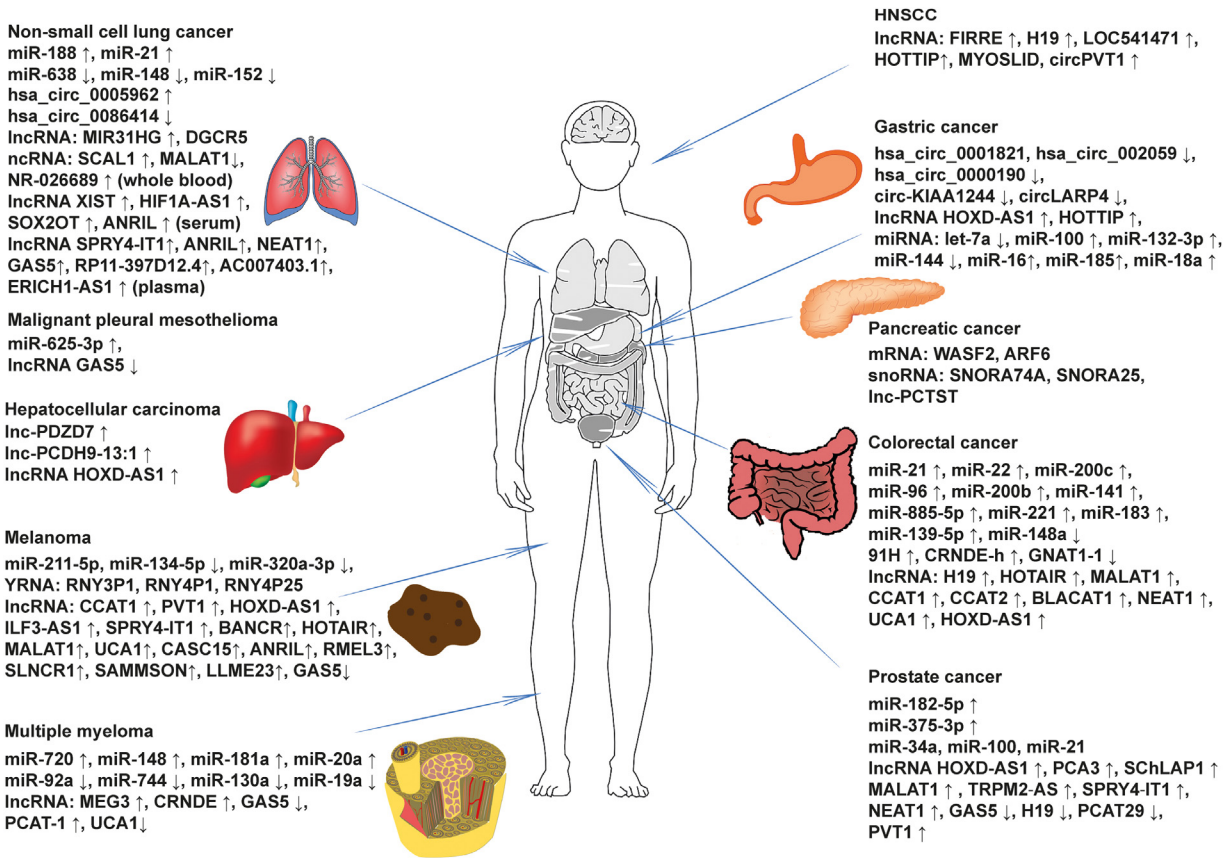

Fig. 2. cfRNAs used in liquid biopsy proposed as diagnostic, prognostic and predictive biomarkers for different kinds of cancers. [149–185] Figure based on pictures from pixabay.com website.

tributes to tumor's escaping from the immune system control.[74,75] Zheng et al. showed that circHIPK3 causes a decrease of miR-124 level and at the same time affects the level of expression of interleukin (IL)-6R which is regulated by this miRNA,[76] suggesting that circHIPK3 may act in tumor immune response.

3.3. *Long RNA transcripts*

Long non-coding RNAs (lncRNAs) are molecules longer than 200 nucleotides. They are actively transcribed, but do not encode proteins. lncRNA molecules have many functional domains, such as RNA, DNA or protein binding sites. They can play a significant physiological role in controlling transcriptional and post-transcriptional processes as well as protein translation or influencing epigenetic modifications. lncRNAs are involved in proliferation, apoptosis, stress response and regulation of cell metabolism or cell phenotype. Some studies indicated that RNAs of that class are candidates for a new class of biomarkers in oncology[77,78] including the circulating lncRNAs as prognostic biomarkers.[31] lncRNAs appear to play an important role as critical regulators of gene expression in the immune system. Studies have shown that lncRNAs' expression is highly specific to the immune cells, affects their differentiation and the functions of innate and adaptive immunity. It is possible that lncRNAs are one of the important elements of the response mechanism to immunotherapy playing a role in the acquisition of specific resistance or suppression.[79]

Some mRNAs, such as GAPDH, tyrosinase, hTERT, mammaglobin or Her-2/neu, can also be detected in the body fluids from healthy and carcinoma patients.[80–84] These transcripts are present in membrane vesicles, lipid complexes, apoptotic bodies, exosomes or as complexes with other molecules.[32] They can be passively as well as actively released from living cells or they are products of cellular death.[32] Moreover, it was observed, that metastatic lymphoma has a higher ability to release plasma membrane vesicles compared to the non-metastatic variant[85] and this process could be responsible for tumor progression.[32] Only few reports about circulating mRNAs have been published up to day, but they have described some other mRNAs as potential biomarkers in different types of cancers.[32] For example, mRNA hTERT perioperative detected using real-time PCR in plasma of laryngeal and hypopharyngeal cancer patients could be used as a biomarker where the presence of circulating hTERT is connected with metastasis and shorter survival.[86]

Currently, cfRNAs expression signatures are created for specific types of cancers and researche is carried out to determine markers that allow rapid diagnosis of cancer, especially the precancerous lesions, prognostic markers and predictive markers.[87] The examples of proposed different types of cfRNAs in oncology are presented in Fig. 2.

4. Analytical problems with cfRNAs

The idea of using circulating RNAs as biomarkers is not new and many studies are focused on different types of cfRNAs in various cancers. However, there are still no validation procedures describing sample processing, RNA extraction methods and quality and quantity assessment, determination of measurement platforms and data normalization. Moreover, the most important issue is the lack of specific cfRNAs panels validated on a large group of patients and healthy individuals that can be used as a diagnostic tool in clinical oncology. These problems need to be solved before widespread use of cfRNAs as biomarkers.

4.1. Sample processing and sample related factors

The most common and the best examined type of body fluid is blood (whole blood, serum and plasma) in research focused on liquid biopsy. The method of taking sample and its storage before the isolation of RNA is a very important issue. First, a sample should be immediately centrifuged to obtain a specific plasma or serum fraction. Thanks to that, contamination of blood cells and other cells as well as hemolysis is reduced. Due to coagulation and hemolysis, blood cells release RNAs into serum and plasma affecting the results.[88–90] Among experimental evidence confirming the importance of proper material processing are results obtained by Cheng et al., where changes in miRNAs levels depend on centrifugation and additional filtration of serum. The main source of miRNAs were platelets, which could be eliminated by centrifugation of fresh or frozen samples stored for six years. Authors recommended estimation of platelets and additional pre- or post-storage centrifugation or filtration.[91] However, the best way to avoid cellular RNAs is to use commercially available blood collection tubes. Qin et al. compared the K3EDTA and Cell-Free RNA BCT^{TM}s (BCTs) tubes and observed that BCTs protect plasma for a long time, also in room temperature, by stabilization of cfRNA and minimization of background RNAs.[92] The appropriate collection tube can also influence the next steps of cfRNAs quantification. Commonly used heparin inhibits the activities of many enzymes such as reverse transcriptase and DNA/RNA polymerases,[93,94] and citrate salts influence on RNAs (e.g. miRNAs) concentration compared to EDTA.[95]

Hemolysis is the next problem observed in sampling. In most cases, the hemolysis process is estimated by macroscopic observation of changes in the serum/plasma color, but this approach is very unreliable. Measuring of oxyhemoglobin using a spectrophotometer (absorbance at λ =414 nm) is supposed to be a better solution.[96] However, it is thought that the best way to detect hemolysis is to measure miR-16, miR-451 or miR-144 whose concentration is higher and more variable in hemolyzed samples.[97–99] This method allows to eliminate samples contaminated with RNAs released by blood cells.

The choice of sample type, serum vs. plasma, is another concern. It is not surprising that various cfRNAs are present in different amounts in body fluids. Wang et al. indicated that miRNA concentration is higher in serum compared to plasma samples.[100] Another example is the observation that not all lncRNAs are present in every type of biological material. Tang et al. showed that out of six studied lncRNAs only HOTAIR and MALAT1 were detectable in the saliva from oral squamous cell carcinoma patients.[101]

The feature of a biomarker is its availability, which is associated with the stability of a particular molecule. Many types of circulating RNAs seem to be stable and can be detectable by RNA sequencing after 40 years of storage.[34] Unfortunately, results presented by Umu et al. slightly differ between the overall RNA contents of the serum and the technical replicates, but the authors noted that these differences were a result of pooling several samples together rather than degradation. However, loss of some data over time is indicated.[34] Furthermore, previous research shows stability of most of analyzed lncRNAs in highly degraded RNA samples compared to intact RNA.[102] Another example of high stability of some lncRNAs is their presence in body fluids such as saliva.[101] lncRNAs' stability in plasma is proved by their resistance to RNase A digestion and overnight incubation at room temperature.[103] In spite of quite a good stability of different types of cfRNAs, it is recommended to handle RNAs' samples as described for mRNAs.[104]

It should be noted that other sample related factors, such as gender,[105,106] rhythmic behavior end exercise[107,108] as well as diet (non-fasting subjects),[109] could have influence on the expression of circulating RNAs.

4.2. RNA extraction methods and quality and quantity assessment

After taking sample and/or its storage cfRNAs need to be extracted. The obtained RNAs should be intact and as pure as possible to allow reverse transcription to be performed efficiently. In the case of serum/plasma, a high concentration of lipids and proteins as well as contaminants from blood (heme and immunoglobulin G (IgG)) could interfere with high quality RNAs and inhibit enzymatic reactions.[110,111] Moreover, some extraction methods could affect RNAs. Li et al. compared seven available and commonly used isolation kits for RNA extraction from plasma and observed kit-dependent biases. Based on the analysis of six artificially added RNAs (length between 200–6000 nucleotides), they observed the highest recovery ($\geq$80%) for Quick-RNA Mini Prep and DirectZol RNA Prep kits (Zymo Research). Moreover, the recovery of RNAs' molecules with specified lengths, DNA contamination, RNA yield and presence of RT-PCR inhibitors depends on the extraction kits.[112] In a study performed by Tanriverdi et al., the differences in quantification of miRNAs depending on RNA isolation kit was observed. Authors noted the best results for In-House RNA isolation kit[113] compared to four commercially available isolation kits dedicated for plasma.[114]

Quality and quantity assessment of cfRNAs should be made using only the methods designed for samples of low RNAs concentration such as Qubit fluorometer[112] or Agilent Bioanalyzer system.[114] NanoDrop spectrophotometer is not recommended in this case.

4.3. Determination of measurement platforms and data normalization

Another problem is the lack of standardized quantification methods. There are three main methods for assessment of RNA transcripts: i) real-time quantitative reverse transcription (qRT-PCR), ii) microarrays platforms and iii) next generation sequencing (NGS). In spite of common application of these techniques, none of them is fully adapted and validated for the analysis of circulating RNAs. Some limitations of these methods could strongly influence obtained results. Detection of cfRNAs' expression in various samples such as urine, peripheral blood, serum, saliva or urine, can be performed using the above mentioned three methods, but the qRT-PCR is the most commonly used. It seems to be the best methodological approach due to its low cost, sensitivity and the ease of use.[78] However, some disadvantages of this method need to be taken into account.

For qRT-PCR the most problematic issues are reverse transcription, choice of reference genes (housekeeping), use of specific primers to detect products during quantification as well as choice of the qRT-PCR chemistry. First, reverse transcription step influences strongly the RNAs' quantification. Commercially available kits for reverse transcription are matched to different transcripts by adding a poly (A) tail[115] or using a stem-loop specific primers[116,117] for short transcripts or for transcripts without poly (A) tails (e.g. some lncRNAs),[100] which improves detection of difficult or rare transcripts.[102] Moreover, the reverse transcriptases are adapted to high temperatures, which allows to amplify difficult sequences with a lot of secondary structures,[118,119] whereas an addition of reaction enhancers such as DTT (dithiothreitol) enables to obtain templates rich in GC fragments.[120] It should be noted that using the references genes to estimate the potential genomic DNA contamination and efficiency of reaction should be the standard element of this step.[121] All of these modifications contribute to reliable qRT-PCR results.[102] Second issue is appropriate reference genes chosen for quantification of cfRNAs. There are no validated genes and different housekeeping genes are used, which makes comparing results difficult.[78,122] The classical housekeeping genes, e.g.

those proposed in commercially available profiling kits, are not suitable for circulating transcripts, and in many situations they are not detectable. This problem was observed in the case of profiling lncRNAs from plasma of melanoma patients, where the most stable lncRNAs were chosen as references due to very high variation or lack of amplification of classical housekeeping genes.[31] However, some housekeeping genes such as GAPDH, seem to be a good reference for circulating RNAs in plasma and serum.[123] that underlines the complexity of the reference problem for circulating transcripts and its dependence on many unknown factors. One of the solutions of this issue is to use the artificial transcript added during RNA isolation such as cel-miR-39 (derived from *Caenorhabditis elegans*) which is supposed to be a better reference than miR-16.[124] Unfortunately, the use of this artificial reference is limited due to its lacking universality and being incapable of adaptation to different kits. Thirdly, using of self-designed primers to detect products during quantification is also problematic. In most cases of self-designed primers, there is no information if they detect one or more isoforms of a specified transcript. Furthermore, there is also lack of knowledge about isoforms for numerous commercially available primers. It should be noted that some transcripts are presented as different kinds of isoforms and can be body fluid specific.[28,34] For example, Umu et al. identified a lot of isomiRs of highly expressed kinds of small RNAs. The identified isomiRs are mostly 3′ isomiRs (78%) or 5′ isomiRs (27%) created by substitution (22%) or canonical forms (8%).[34] Another example, which strongly highlights the relevance of isoforms specification, can be found in studies of estrogen receptor (ER) splice variants[125] or androgen receptor (AR) splice variants in prostate cancer.[126] Authors concluded that different isoforms could have a specified role in cancer biology and be used as potential biomarkers in prostate cancer.[125,126] The advantage of isoforms' specification or using only the commercial primers with validated spectrum of detected transcripts, especially for lncRNAs, will help in comparing different studies in the future.[78] The last important issue in the context of qRT-PCR is the choice of reaction chemistry. The two main methods: TaqMan and dyes incorporating into DNA structure (SYBR green) for qRT-PCR reactions are commonly used.[120] Both of them can be applied for detection of cfRNAs. The TaqMan system seems to be more precise as it eliminates potential genomic DNA contaminations, but its cost is higher compared to the SYBR green system. It should be emphasized that the well-designed primers as well as amplicons' melting curves for all analyses are necessary for high grade qRT-PCR.[31,102]

In spite of being widely used, the qRT-PCR method has limitations including detecting the sequence copies with low abundance.[127] Analysis of both, circulating DNA or RNA, by PCR-based technologies shows very low sensitivity for the detection of rare sequences and leads to an averaged signal.[128] Moreover, the low amount of nucleic acids isolated from liquid biopsy in comparison to those from cells makes this problem worse.[129] However, these limitations can be overcome by using the droplet digital PCR (ddPCR). In this method, each target DNA or RNA molecule is separated into individual compartment prior to PCR amplification.[130] ddPCR is considered to be the most appropriate technique in the application of liquid biopsy, because it has shown superior precision, sensitivity and no need for normalization while detecting low concentration of target DNA or RNA molecules.[131] Because of these advantages ddPCR is the most accurate technique to detect such molecules as miRNAs, lncRNAs and other cfRNAs in liquid biopsy samples.[132]

The microarray platforms and next generation sequencing (NGS) methods are mostly used as the tool for screening in scientific purposes. In spite of this, NGS is used for diagnostics, but for detection of defined genes (defined NGS library) due to a high cost and a lot of difficulty in data interpretation. Microarray platform is a well-established, commercially available method used for RNA profiling enabling to analyze different types of RNAs. It should be noted that thanks to a higher throughput this method is less expensive compared to qRT-PCR. The microarray platforms and next generation sequencing (NGS) methods are mostly used as the tool for screening in scientific purposes. In spite of this, NGS is used for diagnostics, but for detection of defined genes (defined NGS library) due to a high cost and a lot of difficulty in data interpretation. Microarray platform is a well-established, commercially available method used for RNA profiling enabling to analyze different types of RNAs. It should be noted that thanks to a higher throughput this method is less expensive compared to qRT-PCR.[133,134]

Due to a lower cost, higher throughputs and lower RNA input compared to qRT-PCR or microarray platforms, next generation sequencing (NGS) is a better solution for assessment of cfRNAs. The NGS gives an opportunity to detect novel cfRNAs and distinguished RNA isoforms, which is impossible or very difficult using other quantification methods. Moreover, the NGS method in the context of the miRNAs analysis is a more valuable tool due to the possibility of sequencing short transcripts and the possibility of distinguishing sequence similarity between transcripts compared to the microarrays method. However, the NGS technology has some limitations, including difficult bioinformatic data analysis (such as mapping and normalization method),[135] long and multistep processes of library preparation, and a chance of sequencing bias caused on library construction step.[28,34,136,137] Many different methods of library preparation used for examination of cfRNAs by the RNA-seq technology have been already described. It should be noted that sequencing varies depending on RNA molecule characteristics, such as size, sequence, secondary structure and expression level. These features can decrease the biomarker value by influencing reproducibility.[34] However, using adapters for ligation with randomized endonucleotides and computational correction factors, it is possible to reduce sequence-specific bias of sequencing.[138] Because of all these attributes, the next generation sequencing seems to be a promising tool in biomarker research as well as in everyday diagnostics when a panel of defined genes is used.

5. Conclusions and future perspectives

Ongoing research focusing on cfRNAs and their usage as biomarkers' molecules in liquid biopsy are showing their great potential in the development of personalized medicine. There is evidence showing a connection between specific cfRNA levels and the presence of cancer. Moreover, some cfRNAs can specify additional information, such as tumor progression, changes in the molecular profile of cancer as well as information about changes in the immune system as the reaction to disease. This could mean that different types of cfRNAs might be used as diagnostic, prognostic or predictive biomarkers using the liquid biopsy method.

One of the features that makes cfRNAs valuable biomarkers is easy isolation in a stable form from various biological fluids, like whole blood, plasma, serum, urine, milk and others. This means cfRNAs analysis can be performed harmlessly for the patient as a liquid biopsy method as an alternative when some conventional diagnostic techniques cannot be engaged. Currently, there are many different methods used to measure the expression level of cfRNA, the most common one is qRT-PCR. This method is relatively quick and low in cost and, probably, will be the first choice in diagnostics.

However, some problems with cfRNAs based liquid biopsy should be solved. The first one is the use of proper reference genes. The second is creating specific panels for specific types of cancer and specific groups of patients, which would monitor many different cfRNAs, increasing the specificity of the tests. Data indicating that not all types of cancer cause change in cfRNA profile should be taken into account. Furthermore, changes in cfRNAs might be caused by other, non-cancer related factors like a posttraumatic organ

failure. To counter this problem, future research should focus on large groups of patients to create specific panels and, then, validate the selected cfRNAs in various medical centers. Standardization of procedures including sample processing, RNA extraction methods, quality and quantity assessment, determination of measurement platforms and data normalization is the most important issue. The standardized protocols need to be approved and introduced to daily laboratory practice.

We are convinced that cfRNAs based liquid biopsy will play a vital role in future personalized medicine for cancer patients, but there is still need for further research.

Conflict of interest

None.

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