Extracellular Vesicles and Circulating Nucleic Acids

Review

Open Access

Defining the landscape of circRNAs in non-small cell lung cancer and their potential as liquid biopsy biomarkers: a complete review including current methods

Carlos Pedraz-Valdunciel[1,2], Rafael Rosell[1,3]

[1]Cancer Biology and Precision Medicine Department, Germans Trias i Pujol Research Institute and Hospital, Badalona, 08916, Spain
[2]Biochemistry, Molecular Biology and Biomedicine Department, Universitat Autónoma de Barcelona, Bellaterra, Barcelona, 08193, Spain.
[3]Universitat Autónoma de Barcelona, Bellaterra, Barcelona, 08193, Spain.

Correspondence to: Dr. Carlos Pedraz-Valdunciel, Cancer Biology and Precision Medicine Department, Germans Trias i Pujol Research Institute and Hospital, Camí de les Escoles 08916, Spain. E-mail: carlospedraz@icloud.com

How to cite this article: Pedraz-Valdunciel C, Rosell R. Defining the landscape of circRNAs in non-small cell lung cancer and their potential as liquid biopsy biomarkers: a complete review including current methods. *Extracell Vesicles Circ Nucleic Acids* 2021;7:[Accept]. http://dx.doi.org/10.20517/evcna.2020.07

Received: 22 Dec 2020 **Revised:** 22 Mar 2021 **Accepted:** 2 Jun 2021 **First online:** 6 Jun 2021

Abstract

Despite the significant decrease in population-level mortality of lung cancer patients as reflected in the Surveillance Epidemiology and End Results (SEER) Program national database, lung cancer, with non-small cell lung cancer (NSCLC) in the lead, continues to be the most commonly diagnosed cancer and foremost cause of cancer-related death worldwide, primarily due to late-stage diagnosis and ineffective treatment regimens. Although innovative single therapies and their combinations are constantly being tested in clinical trials, the five-year survival rate of late-stage lung cancer remains only 5% (Cancer Research, UK). Henceforth, investigation in the early diagnosis of lung cancer and prediction of treatment response is critical for improving the overall survival of these patients. Circular RNAs (circRNAs) are a re-discovered type of RNAs featuring stable structure and high tissue-specific expression. Evidence has revealed that aberrant circRNA expression plays an important role in carcinogenesis and tumor progression. This further indicates the potential of circRNAs as diagnostic and predictive cancer biomarkers. Extracellular vesicles (EVs) and platelets have been found enriched in circRNAs. Further investigation is warranted to assess the value of EVs and platelets as liquid biopsy-based readouts for lung cancer detection. This review discusses the origin and biology of circRNAs, and analyzes their present landscape in NSCLC, focusing on liquid biopsies to illustrate the different methodological trends currently available in research. The possible limitations that could be holding back the clinical implementation of circRNAs are also analyzed.

Keywords: circRNA, extracellular vesicles, lung cancer, NSCLC, liquid biopsies, biomarkers

INTRODUCTION

Lung cancer is the most commonly diagnosed cancer, leading to cancer incidence and cancer-related deaths worldwide[1]. With non-small cell lung cancer (NSCLC) accounting for 85% of the cases, the development of the disease is attributed to multileveled and elusive complex interactions between genetic liabilities, sex, environmental toxins, and imbalanced signaling processes.

Although the mortality rate of NSCLC has decreased in previous years, presumably due to the approval and routinization of targeted – and immunotherapies[2], the prognosis in late-stage lung cancer remains dismal. While the 5-year overall survival (OS) of early-stage lung cancer is 85% (stage IA), these numbers fall to only 5% in late-stage cases (stage IV). In addition to tumor tissue characterization, liquid biopsies have been introduced to overcome, or complement, invasive tissue biopsies.

Not only are they instrumental in achieving early detection of the tumor, but they can also be exploited to monitor therapy resistance and provide a more heterogeneous readout of the tumor burden[3]. This allows the identification of resistance mechanisms and can guide second-line therapy selection.

Different body fluids can be used as liquid biopsies, including blood, urine and saliva. Circulating molecules, such as cell-free DNA (cfDNA), RNA or proteins, can either be freely present within these media or can be extracted and analyzed from circulating extracellular vesicles (EVs) or tumor-educated platelets (TEPs)[4].

Lung cancer involves massive changes in RNA metabolism, both in the tumor and circulating EVs and TEPs. Traditional RNA biomarker discovery research for either lung cancer detection or monitoring of treatment response has mainly focused on the expression of mRNA and miRNA[5-7].

Circular RNAs (circRNAs) are a recently re-discovered type of RNA generated by coupling the 5′and 3′ends in a non-canonical process known as back-splicing[8]. This circular structure lacking a poly(A) tail makes most of them resistant to the exonuclease RNase R and, therefore, robustly stable molecules compared to lineal mRNA. While thousands of circRNAs have been described thanks to the technological burst of deep sequencing[9], only the function of a fraction has been elucidated.

Recent investigations have unveiled the role of circRNAs as important players in NCLSC, positioning them as valuable biomarkers for early detection and promising candidates for seeking therapeutic and prevention strategies.[10].

This review analyzes the current state of circRNA research, starting from their biology to their different functions and implications in NSCLC, with a special focus on their not yet fully exploited potential as liquid biopsy biomarkers. We also review the most recently discovered circRNAs, both in solid and liquid specimens.

In addition, we provide a practical and complete guide on the current methodology available for their study, stressing the current limitations that may be preventing their implementation in the clinical setting.

CIRCULAR RNA EXPRESSION IN HUMANS

Although circRNAs have been acknowledged for many years as abnormally spliced "scrambled" transcripts[11], only recently have they been re-defined as biologically active molecules with a significant role in human homeostasis, having a tissue-specific expression profile during the different stages of development[12].

More than 60% of human genes can express circRNAs[13]. However, their expression levels in tissue remain rather low, accounting for only 5-10% of the canonical (linear) mRNA expression[14, 15].

CircRNAs originated by an alternative process called "back-splicing", where the 5´splice donor can stick to the 3´splice acceptor of an upstream exon. This process results in forming a circular structure that can include one or different exonic/intronic regions, depending on the specific mechanism that was inferred during this non-canonical process[16].

They have arisen as key post-transcriptional regulators throughout different functions (Figure 1), with micro-RNA (miRNA) sponging being the most studied. During this process, the circRNA binds to the Argonaute (AGO)- miRNA complex, and either via miRNA degradation or inhibition of the miRNA-mRNA interaction, triggers an expression increase of the later[17].

Recent studies have also revealed that circRNAs could associate with ribosomes and be translated into functional short peptides, in a cap-independent manner [18].

Alternatively, they can also associate with proteins acting as scaffolding for enzymatic reactions.

The process of circRNA synthesis generates an imbalance of the canonical splicing; hence, the back-splicing process itself stands as a direct regulator of the circRNA precursor gene at the transcriptional level.

Biosynthesis and regulation of circRNAs

Different back-splicing mechanisms have been reported in the nucleus, including RNA binding protein (RBP)-mediated circularization, circRNA synthesis by intron pairing, or circularization by intron-lariat formation[16] (Figure 1). The first mechanism is normally executed by associating two adjacent exons and skipping the intronic region during an RBP-assisted circularization process, resulting in an exonic-circRNA (EcircRNA). Numerous RBPs have been described to regulate this mechanism, such is the case of the adenosine deaminase RNA specific-1 protein (ADAR1)[19], NF90/NF110 immune factors[20], muscleblind transcription factor (MBL)[21], heterogeneous nuclear ribonucleoprotein L (HNRNPL)[22], FUS protein[23], Quaking binding protein (QKI)[24], RNA helicase DHX9[25] , and the RNA-binding motif protein 20 (RBM20)[26].

Exon-intron circRNAs (EIcircRNAs) are the result of 2 or more exons circularized along with their corresponding introns via intron-lariat formation.

Intron pairing back-splicing is usually the common process in conserved RNAs with high frequency of *Alu* repeats in flanking sequences. These *Alu* elements complement each other, promoting the hairpin formation and further back-splicing, creating mono-exonic circRNAs (EcircRNAs) as a result[27].

Intronic circRNAs (IcircRNAs) are another type of such a class; however, the mechanism of generation of these molecules remains yet unclear.

After synthesis in the nucleus, circRNAs are exported into the cytoplasm. Recent studies have shown the active role of the UAP56/URH49 helicases in this

size-mediated process. UAP55 is required to transfer molecules longer than 1300 nucleotides, while URH49 intervenes only in short transcript exporting[28] .

Once in the cytoplasm, circRNAs accumulate and exert their function by regulating transcription, normally via sponging targeted miRNAs.

How circRNA gets degraded still remains unclear; however, recent investigation has shed light on this conundrum, unveiling some intriguing mechanisms that underpin circRNA decay. Hansen. et al. describe an Ago2-miR-671-mediated degradation of the circRNA CDR1as (aka ciRS-7)[29]. In another study by Park et al., a cleavage mechanism induced by RNase P/MRP was elucidated in N6-methyladenosine (m6A)-enriched circRNAs[30]. More recently, a study by Liu et al. demonstrated that some circRNAs tend to form intricate duplexes which makes them susceptible to degradation by RNase L upon viral infection[31].

A different mechanism was described by Leung et al. revealing an alternative structure-mediated circRNA regulation process that selectively degrades circRNAs based on 3′-UTR structure complexity via the UPF1/G3BP1 protein complex[32].

CIRCULAR RNAs IN NSCLC

The implication of circRNAs in cancer metabolism has been studied in recent years. Their contribution to mutant glycolysis (via transporter, enzyme, and/or transcription factor regulation), lipogenesis and lipolysis, glutaminolysis, and oxidative respiration has been widely demonstrated[33].

CircRNAs are becoming a new area of interest within cancer research, including NSCLC, where several authors are contributing by investigating the effect that dysregulated circRNA expression can have on the different cancer stages. Although their implication in NSCLC has not been as intensively investigated as other types of non-coding RNAs, circRNAs have been shown to have a significant role in tumorigenesis, tumor development, proliferation, migration, invasion, and sensitivity to NSCLC therapy[34]. In light of these aforementioned findings, recent publications

highlight the potential of these circular transcripts as plausible biomarkers to assess disease status.

CircRNAs as biomarkers of NSCLC

The number of studies on circRNA profiling in NSCLC patients has exploded exponentially in the last few years (Table 1).

ciRS-7 was the first and best characterized circRNA in cancer and served as a foundation stone for current research. Its role in carcinogenesis was first described in hepatocellular carcinoma, following breast and cervical cancer, acting as a competing endogenous RNA (ceRNA) for miR-7[35]. A recent study has introduced ciRS-7 as an important player in lung cancer, which expression seems to correlate with tumor size and both lymph and tumor node metastasis (TNM) stages[36].

A study by Wang et al. recently demonstrated the involvement of circSOX4 in lung adenocarcinoma (LUAC) by activating the WNT signaling pathway via sponging miR-1270 and following upregulation of PLAL2. circSOX4 was found overexpressed in all managed LUAC tissue samples, and further validated across different cell-based preclinical experiments[37] .

circular RNA HIPK3 (circHIPK3) is yet another extensively studied circRNA critical in cell proliferation of different types of cancer[38]. Its specific role in NSCLC has been recently discovered by Xie et al. demonstrating impaired cell proliferation, migration, invasion and autophagy induction via the miR124-3p-STAT3-PRKAA/AMPKa axis upon silencing of the cited circular transcript[39]. Authors also demonstrated that overexpression of circHIPK3 correlates to poor survival, especially in advanced stages.
Another well studied circRNA, circSMARCA5, plays a significant role in NSCLC via the miR-19b-3p/HOXA9 axis, setting the grounds for exploring underlying therapeutic targets[40].

On a similar note, a circular RNA from FGFR3 (circFGFR3) was reported in NSCLC, promoting cell invasion and proliferation of tumors by sequestering miR-22-3p, thus promoting galectin-1 (Gal-1), p-AKT, and p-ERK1/2 expression, and activating downstream pathways[41].

The oncogenic CirFOXM1 was first discovered overexpressed in pancreatic tissues upregulating the pancreatic progenitor cell differentiation and proliferation factor (PPDPF) and metastasis-associated in colon cancer 1 (MACC1) proteins via miR-1304-5p sponging. More recently, the same circ-FOXM1/miR-1304-5p/PPDPF/MACC1 axis was found decisive for NSCLC development and progression[42] .

Chromosomal translocations are cancer-associated events that may strike frequently in some genes, like ROS or ALK, leading to activation of downstream signaling pathways upon sustained expression[43]. These events can also generate oncogenic circRNAs, as has been reported with the solute carrier family 34 member 2 (SLC34A2) and ROS proto-oncogene 1 (ROS1), producing two circRNAs (F-circSR1 and F-circSR2) both promoting cell migration in NSCLC[44].

Precursor mRNA of driver mutations, such as MET, can also lead to the generation of circRNAs. circMET was first described in hepatocellular carcinoma driving immunosuppression and anti-programmed cell death 1 (PD-1) therapy resistance via the miR-30-5p/snail/DPP4 axis[45]. Its role in NSCLC was recently discovered promoting tumor proliferation via the miR-145-5p/CXCL3 axis[46].

Although a circRNA from epidermal growth factor receptor (EGFR) has been reported in mouse ovaries during postnatal development with a marked expression profile, the implication of this circRNA in lung cancer has not been studied yet.
There have been no circRNAs derived from the KRAS gene reported either; however, numerous circRNAs have been portrayed as key intermediaries of the classical pathways and may serve as a readout of these foremost altered genes.

CircRNAs as biomarkers of treatment resistance in NSCLC

Although several studies have unveiled the potential role of circRNAs in lung cancer development and progression, not much has been clarified regarding their contribution to therapeutic resistance, and only a few published studies focus on their involvement in this area (Table 2). circRNAs can be classified as promoters, when their high

expression enhances resistance to cancer therapy; or suppressors, when their expression limits the progression of the disease during treatment, thus acting as inhibitors of resistance.

Astrocyte elevated gene-1 (AEG-1) is a key player in development, progression, and metastasis of lung cancer by regulating the Wnt/ β -catenin pathway. In a recent publication, Li and colleagues showed that circMTDH.4 regulates AEG-1 expression by sponging miR-630, leading to chemo and radio-resistance in NSCLC cells. Sensitivity was restored via the knockdown of the cited circRNA or over expression of its target, miR-630[47].

Two different works have recently been published describing circRNAs that regulate the expression of STAT3. Dong et al. reported that upregulation of hsa_circ_0076305 confers DDP-resistance to NSCLC cells via sponging miR-296-5p, positively modulating STAT3[48].

Xu et al. introduced the role of circAKT3 inhibiting cisplatin sensitivity by regulating mir-516b-5p/STAT3 axis[49].

Other important circRNAs described to be involved in chemotherapy resistance are hsa circ_0071799 via miR-141 (taxol resistance)[50], hsa circ 0091931 via miR-34c-5p[10], hsa_circ_0003998 via miR-326[51], hsa_circ_0001946 via miR-7-5p, miR-671-5p, miR-1270 and miR-3156-5p (NER sigaling, cisplatin resistance)[52], CircPVT1 via miR-145-5p (ABCC1, cisplatin and pemetrexed resistance)[53], CircNFIX via miR-132 (TMZ-resistant)[54], and cESRP1. Huang and colleagues recently discovered a suppressor circRNA that, when downregulated, allows major expression of its target miR-93-5p[55]. This process leads to the upregulation of downstream targets, such as Smad7/p21(CDKN1A), enhancing the transforming growth factor-β (TGF-β) pathway. Furthermore, cESRP1 overexpression boosts cisplatin sensitivity by repressing miR-93-5p and TGF-β pathway in SCLC. Related to this pathway, PDPK1, intermediary of the PI3K/AKT/mTOR pathway, has been discovered to be regulated by the hsa_circ_0004015-miR-1183 axis[56]. Overexpression of this circRNA can induce gefitinib resistance in NSCLC cells by sponging the abovementioned miRNA.

Other authors have centered their investigation on the differential expression of circRNAs that confer resistance to this and other tyrosine kinase inhibitor-based therapies.

Fu et al. found hsa_circRNA_012515 increased in gefitinib-resistant NSCLC cell lines. Further investigation in patient tissue indicated that high expression correlated with lower OS and shorter progression free survival (PFS)[57].

Chen et al. found 10 differentially expressed circRNAs in different osimertinib-resistant lung cancer cell lines. Five of them were further validated and proved to correlate with resistance status (hsa_circ_0043632, hsa_circ_0048856, hsa_circ_0043634, hsa_circ_0050581, and hsa_circ_0023302)[58]. Authors made use of specific software to predict possible targeted miRNAs; however, the axis or mechanism of action has not yet been elucidated.

CircRNAs seem to also have a role mediating response to immunotherapy. circFGFR1 has been described by Zhang et al. to promote progression and anti- PD-1 resistance. By sponging miR-381-3p in NSCLC cells, C-X-C motif chemokine receptor 4 (CXCR4) would result upregulated, leading to progression and resistance to therapy[59].

CURRENT LANDSCAPE OF CIRCULAR RNAs IN LIQUID BIOPSIES AS NSCLC BIOMARKERS

Non-coding RNA-enriched exosomes are strategic players in different cancer stages, especially regarding malignant tumor metastasis[60]. The assessment of circRNA expression by RNAseq analysis in extracellular vesicles was first reported by Li et al., finding circRNAs enriched at least 2-fold in exosomes compared to producer cells[61]. Although some authors defend the theory that exosomal circRNA enrichment may be a mechanism of cellular circRNA clearance[62], few investigators have shown that these circRNA are directly involved in cellular communication, henceforth, acting as direct readouts of several human malignancies, including NSCLC[63].

As a result, circRNAs stand as important liquid biopsy-derived biomarkers, holding potential for NSCLC diagnosis and prediction of treatment response[64].

In a recent study, Chen et al. performed high throughput sequence of plasma-EV RNA cargo of LUAC patients, finding 182 circRNA dysregulated when compared to cancer-free donors, including 105 up-regulated and 78 downregulated. Four upregulated circRNAs were successfully validated by qRT-PCR (hsa_circ_0001492, hsa_circ_0001346, hsa_circ_0000690, hsa_circ_0001439)[65]. Although authors elucidated the specific circRNA-miRNA-mRNA interaction, not much information about their biological impact was provided.

Fei et al. also presented in a recent study a novel circRNA, hsa_circRNA_005661, that could be found enriched in plasma EVs from lung adenocarcinoma patients with lymph node metastasis, presenting it as a biomarker of such stage[66].

Not only plasma-EVs, but serum and whole plasma can serve as a good source of circRNAs (Table 3). Xian and colleagues studied the circRNA differential expression profile in serum EVs from NSCLC patients. As a result, 3 circRNA stood out showing suitable biomarker potential (hsa_circ_0047921, hsa_circ_0007761, and hsa_circ_0056285) with the later correlating with clinical stages and lymph node metastasis in all Chinese patients included in the study[67] .

Hang et al. explored the use of circRNA found in total plasma of NSCLC patients in order to find some candidates that could correlate to malignancy status. Not only did they find a notorious circRNA coming from the FARSA gene, circFARSA, but they also found a set of differentially expressed circRNAs (hsa_circ_0001495, hsa_circ_0000566, hsa_circ_0001238, hsa_circ_0007037, circ_c1orf116, hsa_circ_0001083, hsa_circ_0006451, hsa_circ_0004458, and hsa_circ_0000847) based on which they were able to discriminate NSCLC patients from healthy individuals. Additionally, they performed in-silico investigation of possible targets of circFARSA. Consequently, miR-330-5p and miR-326 emerged as direct targes candidates. Both miR-330-5p and miR-326 may interact directly with fatty acid synthase (FASN), which has been described as a notorious oncogene in various types of cancer[68].

Also, directly from plasma Liu et al. found a two circRNA-based signature that could potentially be used to classify lung adenocarcinoma patients[69]. Hsa_circ_0005962 was found upregulated while hsa_circ_0086414 was barely expressed. In addition, they observed that overexpression of the first was correlated to mutant EGFR expression. In vitro experiments suggested that this circRNA could be involved in cancer proliferation.

Moreover, a fusion-gene circRNA has been studied in liquid biopsies. Tan and colleagues started their line of research exploring the existence of a circRNA derived from the fusion gene EML4-ALK (F-circEA) in H2228 cell lines[70]. After verification, they observed that overexpression of this circRNA could trigger cell migration and invasion, contributing to tumor development. They validated the existence of this circRNA in plasma of NSCLC patients with the EML4-ALK translocation, suggesting that screening of plasma F-circEA in this type of patients could be a valuable approach to monitor the EML4-ALK translocation, and provide further guidance on targeted therapy.

Alhasan et al. showed for the first time that platelets are enriched in circRNAs when compared to nucleated tissues[71], and also, that their content is superior to that on mRNA. Preußer et al. demonstrated that not only platelets are a good source of circRNA, but also platelet-derived extracellular vesicles are enriched in these biomolecules, representing yet another source of potential biomarkers that may be involved in different signaling pathways[72].

Platelets change their RNA profile when in contact with the tumor, enabling them to contribute to the systemic and local responses to tumor growth. As a result, tumor educated platelet (TEP)-RNA can be used as a potential biomarker for cancer diagnostics[73]. Although TEPs could also possibly be enriched in circRNAs, and hold potential value for NSCLC diagnosis, nothing yet has been investigated.

Little has been elucidated regarding NSCLC treatment resistance based on liquid biopsy-based circRNAs. A study of Yu-Tao et al. comparing gefitinib responder and

non-responder NSCLC patients found that higher expression of hsa_circ_0109320 in plasma correlated with longer PFS in gefitinib-treated NSCLC patients[74], however, no information on the potentially affected signaling pathway has been provided.

Current available methods for the study of circRNAs in liquid biopsies

Although there are different methods currently available for the study of circRNAs (Table 4), no consensus has been reached on which protocol to follow for either tissue or liquid biopsy-based circRNA expression analysis.

The range of possibilities when selecting a bio-source is rather ample[75]. Whilst plasma or serum can provide a higher yield of total RNA, tumor released EVs stand out by providing a more accurate picture of lung cancer at the transcriptional level[76]. Procedures such as ultracentrifugation, ultrafiltration, or size-exclusion chromatography are examples of the range of methods accepted by the International Society for Extracellular Vesicles (ISEV) for the study and purification of these biomarkers[77].

In the case of EV circRNA investigation, concentration levels may sometimes be the limitation factor that restricts further downstream processes. Therefore, in this case, EV isolation methods should be focused on achieving a higher EV-derived circRNA yield rather than acquiring extra pure EV samples, which are mainly attained by compromising RNA concentration[78].

De-novo discovery of circRNA

Full-length RNA sequencing emerged as the first method proving beneficial for *de-novo* circRNA identification[9]. By processing total RNA, unmatched reads are selected and assembled by remapping to custom databases containing all human intragenic exon-exon junctions. The protocol first introduced by Salzman et al. has since been improved with new procedures including ribosomal RNA depletion and non-polyadenylated RNA exonuclease-mediated enrichment (RNase R)[79]. Further validation of novel identified targets requires use of specific bioinformatic tools that allow junction site identification from deep-sequencing data. The rise of newly developed bioinformatic methods have boosted the discovery and analysis of thousands of circRNA (Table 5). However, sensitivity may be a limitation when using

next-generation sequencing (NGS) for circRNA discovery since library preparation is frequently associated with the loss of low-expressed molecules[80]. Other methodologies such as microarrays or the nCounter platform have emerged to overcome this issue; however, circRNA discovery in these cases gets restricted to the candidates included either in the array or the gene panel.

Microarrays are useful tools for high-throughput analysis and expression studies of circRNAs where probes are designed to bind specifically to the junction site, getting immobilized, incubated and further sequenced[81]. Samples may normally be subject to RNase R to reduce background noise and enhance detection. This systematically expression profiling process is quite sensitive and straight forward. Current methodology developed by Arraystar also provide all necessary tools in order to get detailed annotation specific to circRNA biology, such as miRNA binding sites or conservation status, to reveal all possible functional roles as miRNA sponges.

The nCounter platform allows multiplex analysis of up to 800 circRNA transcripts by direct capturing and counting of individual targets[82]. This qualitative and quantitative process is rather simple and requires minimal hands on, providing results in less than 48h. Although nCounter is routinely used for RNA expression assessment in both FFPE and fresh tissues, only few studies have investigated its potential when it comes to liquid biopsies. EV-DNA[83], and EV-miRNA[84] profiles have been examined with this platform obtaining different success rates; however, investigation with circRNA remains restricted to tumor and cultured cells[85] and in no case this platform has been explored for lung cancer research so far.

CircRNA identification and validation

For circRNA validation, end-point PCR has been established as the most extended practice using divergent primers spanning the junction site and followed by further Sanger sequencing[63].

RNase R treatment is still a debate whether it is beneficial or not to use it in liquid biopsy samples. RNase R has been widely used for the study of circRNAs since it has the property of affect mostly linear RNA, henceforth, enriching our samples with

circRNAs[86]. However, some circRNAs have demonstrated to be sensitive to the effect of this exonuclease[85]. The often-long incubation periods can compromise the quality of our RNA samples. In addition, RNase treatment has been proved to not be 100% effective towards mRNA depletion which could lead to a circRNA overestimation if quantification by qPCR is the next downstream process and convergent primers are used. Xiao et al. proved that standard RNase R protocols result in up to 20% of highly expressed mRNAs being unaffected[87]. Therefore, the correct design of divergent primers is instrumental for the study of circRNAs, regardless of whether RNase R treatment is applied to the samples or not. Authors also described that RNase R protocol could be enhanced by replacing K+ by Li+ in the reaction buffer so enzyme can digest complex structured linear transcripts; however, this is a convoluted process that, even though scientifically relevant, may not result practical in the laboratory routine.

Northern blot analysis has arisen as another common methodology for the study of circRNAs. Following standard protocols, once the RNA is transferred from the gel onto a blotting membrane, circRNAs are then hybridized with short probes normally designed spanning the junction site, hence, allowing circRNA identification. This method also allows studies on size, isoforms, sequence and abundance of these circular transcripts[88]. However, the usual high amounts of RNA required for this method is rather high, so investigations get restricted mostly to RNA from either tissue or cell lines.

Quantification of circRNA

Nowadays, different methodologies are being used for the quantification of circRNAs both in solid and liquid biopsies. qRT-PCR has been broadly established as one of the easiest and predilected mechanisms of quantification[89], however, different aspects may need to be taken into consideration.

Contrary to tissue, circRNAs are enriched in plasma exosomes.[61] In this case, RNase R treatment may not be recommended due to the low overall RNA concentration that is expected in these vesicles, however, sometimes its use is necessary to validate primer specificity or due to the nature of specific experiments. In this respect, it is important to stress the need of designing divergent primers as previously cited, along with a probe

spanning the junction site. Furthermore, throughout this procedure, the expression of classical reference genes, such as beta-actin or GADPH, will result altered*;* hence, ruling out the possibility of performing circRNA expression evaluation by using classical normalization procedures. In this case, the selection of circular RNA housekeeping genes[90] is crucial for the correct assessment of circRNA expression.

CircRNA amplification via reverse transcription PCR (RT-PCR) often leads to extended concatemeric transcript amplification from a single priming of the reverse transcriptase. This process, triggered by the circular architecture of these molecules, is known as rolling circle amplification, and was first described by You et al. while studying circRNA expression in brain tissues[91]. This event is not problematic if de-novo circRNA discovery is intentional and direct comparison with canonical transcripts is not envisioned (in fact, it can be beneficial for the study of circRNA splice variants). However, this does not apply to transcript abundant studies, in which this mechanism can introduce biases leading to an overestimation of circRNA expression.

Conn et al. demonstrate this in a study with synthetic circRNAs, resulting in a five-fold increase of circRNAs compared to the expected expression upon RT-PCR and further qPCR amplification. This is a factor to take into consideration in the experimental design[92].

The same group has developed a cutting-edge tool to avoid the bias introduced by normal qRT-PCR quantification throughout their newly designed SplintQuant method[92]. This technology is based on the inclusion of custom DNA oligonucleotides that complement target circRNAs, and making use of the PBCV-1 DNA ligase, synthesize cDNA skipping reverse transcription. The system is sensitive, specific and reproducible, allowing the identification and quantification of canonical and non-canonical RNA transcripts including gene fusions and alternative splice variants.

nCounter technology stands out as a very effective and sensitive option for circRNA quantification. Its application for the analysis and quantification of circRNAs has been systematically studied by Dahl et al. in different solid biosources (including formalin

fixed paraffin-embedded specimens) for the study of B-cell malignancies[85], becoming the first group to use this technology for the study of circRNA expression.

Bioinformatic and computational tools for the study of circRNA

Identification of circRNAs can be a straight-forward process when using microarray or nCounter data where the exploratory approach gets restricted to a specific panel of genes. However, detection of circRNA can be a much more complex in the case of deep-sequencing data analysis due to the complexity on the computational workflows. For this purpose, different pipelines and computational analysis tools have been created to facilitate this process (Table 5). Different publicly available databases such as circBank[93], circBase[94], or circView[95] have proved useful to simplify the study of circRNA throwing light on specific features such as miRNA binding sites, m6A modifications, mutations, or unveiling protein-coding potential (Table 5). These databases also allow browsing and download of FASTA files based on specific searching criteria.

DISCUSSION

The recent impact of circRNAs in lung cancer research has become undeniable. Since ciRS-7 was introduced as the first circRNA ever described to play a role in hepatocellular carcinoma[36], many others have followed, extending to different types of cancer, henceforth, consolidating their position as active players in cancer development and progression of malignancy. Recently, publications exploring the biomarker potential of these molecules in NSCLC have remarkably increased, with an exponential growth in the last five years. Nevertheless, despite the patent progress in this field, current research is predominantly restricted to expression analysis of circRNA in tumor samples, with very, little information regarding validation in liquid specimens.

Extracellular vesicles (EVs), like exosomes, are released by most cells in the body and can be easily isolated from plasma[96]. Tumor EVs can mediate intercellular communication between tumor cells and tumor microenvironment[97], therefore, the study of these molecules via their molecular identification can offer a valuable spatiotemporal snapshot of the state of the disease. However, while several publications have widely demonstrated that EV cargo is enriched in circRNAs[61], not many investigators have focused on this line of research, delaying the development of novel

liquid biopsy-based tools for NSCLC detection. While the potential value of liquid biopsies in the clinic has been recognized as beneficial[98], in the research context, liquid bio-sources can be rather challenging, including plasma circRNA investigation.

With a superior relative expression and stability in EVs than the canonical mRNA, the extent of circRNA in EVs still remains very low, frequently limiting further downstream analysis. This is unlikely to be an issue in solid tumors; while circRNA overall expression is frequently low (1-10%)[14], RNA concentration is rarely a limitation. Furthermore, very often the study of circRNA expression relies on enzymatic amplification – qPCR. This course fueled by the circular architecture of these molecules can sometimes lead to the not-so-well-known rolling cycle amplification events, resulting in an inaccurate yet overestimated circRNA quantification[92], frequently leading to untruthful and irreproducible results.

On addition to the above exposed, there is not a general consensus about other fundamental matters such as EV isolation method (if we target the study of the EV circRNA cargo), potential use of RNase R, or readout assessment, among others. As a result, standardization of protocols for the study of circRNA has become instrumental for the study and implementation of these novel biomarkers into the liquid biopsy setting.

Some technologies have arisen as incipient alternatives such as the nCounter platform or the newly developed SplintQuant. Both of them rely on very low RNA input and can overcame the deviation issues that enzymatic qPCR may create.

Additionally, platelets, especially tumor educated platelets, hold a great unexplored potential as a source of circRNAs, not only due to their higher concentration in RNA when compared to EVs, but also due to the high enrichment they present towards these circular biomolecules. Whether platelet derived circRNA signatures could be of better, equal or complementary value to the ones from EVs, requires further investigation.

Nowadays, most studies aim to exploit the biomarker potential of lung cancer circRNAs, frequently leaving aside any additional examination of their inherent

biology. Further research elucidating the different molecular functions of these molecules is greatly needed in order to achieve a future circRNA-based liquid biopsy test.

The rediscovered role of circRNAs as lung cancer biomarkers has the potential to reshape the landscape of liquid biopsies. They count on most features needed to be considered a good biomarker: they can be measured in blood[99], including plasma[68], serum[100], and urine[101]; they are reasonably robust and very stable due to their circular architecture[34], and do not require special handling protocols other than those required for the rest of RNA types. Due to the diverse implications in cancer progression and development of resistance[34], circRNAs could provide additional information improving diagnosis and treatment guidance by either generating new signatures, or complimenting existing ones.

Circulating tumor DNA (ctDNA) is the most commonly explored liquid biopsy for NSCLC, counting with few tests already clinically implemented for the detection of classical mutations such as EGFR Del19 and p. L858R mutation[102]. However, many lung cancer cases are not linked to a specific driver mutation; therefore, research on new biomarkers, including circRNAs, and further development of multi-omic signatures of tumor microenvironment could provide additional diagnostic opportunities for these patients.

However, as mentioned above, several circRNA quantification methods have limitations, and a clear protocol needs first to be established in order to develop any clinically applicable assay. In addition, clinical utility should be demonstrated by providing convincing evidence of the new biomarker performance (in comparison to currently accepted cfDNA/mRNA liquid biopsy tests), and so far, no circRNA biomarker has achieved that status, probably due to the difficulty of recruiting large patient cohorts required to prove biomarker utility.

Further studies in biomarker discovery, molecular biology, and protocol standardization are warranted in the upcoming years to achieve the implementation of these novel biomarkers in the clinical setting.

DECLARATIONS

Acknowledgments

We would like to thank Stephanie Davis for language editing assistance, and Dr. Cristina Aguado-Esteban for her deep insights. Figure 1 created with BioRender.com

Authors' contributions

Authors contributed equally to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This project has received funding from a European Union´s Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement ELBA No 765492.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* 2018, 68:394-424, doi:https://doi.org/10.3322/caac.21492.
2. Howlader N, Forjaz G, Mooradian MJ, Meza R, Kong CY, Cronin KA, Mariotto AB, Lowy DR, Feuer EJ. The Effect of Advances in Lung-Cancer Treatment on Population Mortality. *New England Journal of Medicine* 2020, 383:640-649, doi:10.1056/NEJMoa1916623.
3. Bracht JWP, Mayo-de-Las-Casas C, Berenguer J, Karachaliou N, Rosell R. The Present and Future of Liquid Biopsies in Non-Small Cell Lung Cancer: Combining Four Biosources for Diagnosis, Prognosis, Prediction, and Disease Monitoring. doi: 10.1007/s11912-018-0720-z
4. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nature Reviews Clinical Oncology* 2017, 14:531-548, doi:10.1038/nrclinonc.2017.14.
5. He X, Park S, Chen Y, Lee H. Extracellular Vesicle-Associated miRNAs as a Biomarker for Lung Cancer in Liquid Biopsy. *Frontiers in molecular biosciences* 2021, 8:630718-630718, doi:10.3389/fmolb.2021.630718.
6. Müller Bark J, Kulasinghe A, Amenábar JM, Punyadeera C. Chapter One - Exosomes in cancer. In: Makowski GS, ed. Advances in Clinical Chemistry. Vol. 101: Elsevier; 2021, 1-40. doi: 10.1016/bs.acc.2020.06.006
7. Pinzani P, D'Argenio V, Del Re M, Pellegrini C, Cucchiara F, Salvianti F, Galbiati S. Updates on liquid biopsy: current trends and future perspectives for clinical application in solid tumors. *Clinical Chemistry and Laboratory Medicine (CCLM)* 2021, doi:doi:10.1515/cclm-2020-1685.
8. Tang X, Ren H, Guo M, Qian J, Yang Y, Gu C. Review on circular RNAs and new insights into their roles in cancer. *Computational and Structural*

Biotechnology Journal 2021, 19:910-928, doi:https://doi.org/10.1016/j.csbj.2021.01.018.

9. Salzman J, Gawad C, Wang PL, Lacayo N, Brown PO. Circular RNAs Are the Predominant Transcript Isoform from Hundreds of Human Genes in Diverse Cell Types. *PLOS ONE* 2012, 7:e30733, doi:10.1371/journal.pone.0030733.
10. Hua X, Sun Y, Chen J, Wu Y, Sha J, Han S, Zhu X. Circular RNAs in drug resistant tumors. *Biomedicine & Pharmacotherapy* 2019, 118:109233, doi:https://doi.org/10.1016/j.biopha.2019.109233.
11. Nigro JM, Cho Kr Fau - Fearon ER, Fearon Er Fau - Kern SE, Kern Se Fau - Ruppert JM, Ruppert Jm Fau - Oliner JD, Oliner Jd Fau - Kinzler KW, Kinzler Kw Fau - Vogelstein B, Vogelstein B. Scrambled exons. doi: 10.1016/0092-8674(91)90244-s
12. Lee ECS, Elhassan SAM, Lim GPL, Kok WH, Tan SW, Leong EN, Tan SH, Chan EWL, Bhattamisra SK, Rajendran R, et al. The roles of circular RNAs in human development and diseases. *Biomedicine & Pharmacotherapy* 2019, 111:198-208, doi:https://doi.org/10.1016/j.biopha.2018.12.052.
13. Ji P, Wu W, Chen S, Zheng Y, Zhou L, Zhang J, Cheng H, Yan J, Zhang S, Yang P, et al. Expanded Expression Landscape and Prioritization of Circular RNAs in Mammals. doi: 10.1016/j.celrep.2019.02.078
14. Guo JU, Agarwal V, Guo H, Bartel DP. Expanded identification and characterization of mammalian circular RNAs. *Genome Biology* 2014, 15:409, doi:10.1186/s13059-014-0409-z.
15. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-Type Specific Features of Circular RNA Expression. *PLOS Genetics* 2013, 9:e1003777, doi:10.1371/journal.pgen.1003777.
16. Dragomir M, Calin GA. Circular RNAs in Cancer – Lessons Learned From microRNAs. *Frontiers in Oncology* 2018, 8, doi:10.3389/fonc.2018.00179.

17. Xiao M-S, Ai Y, Wilusz JE. Biogenesis and Functions of Circular RNAs Come into Focus. *Trends in Cell Biology* 2020, 30:226-240, doi:10.1016/j.tcb.2019.12.004.

18. Chen CY, Sarnow P. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* 1995, 268:415, doi:10.1126/science.7536344.

19. Rybak-Wolf A, Stottmeister C, Glažar P, Jens M, Pino N, Giusti S, Hanan M, Behm M, Bartok O, Ashwal-Fluss R, et al. Circular RNAs in the Mammalian Brain Are Highly Abundant, Conserved, and Dynamically Expressed. doi: 10.1016/j.molcel.2015.03.027

20. Li X, Liu CX, Xue W, Zhang Y, Jiang S, Yin QF, Wei J, Yao RW, Yang L, Chen LL. Coordinated circRNA Biogenesis and Function with NF90/NF110 in Viral Infection. doi: 10.1016/j.molcel.2017.05.023

21. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S. circRNA biogenesis competes with pre-mRNA splicing. doi: 10.1016/j.molcel.2014.08.019

22. Fei T, Chen Y, Xiao T, Li W, Cato L, Zhang P, Cotter MB, Bowden M, Lis RT, Zhao SG, et al. Genome-wide CRISPR screen identifies HNRNPL as a prostate cancer dependency regulating RNA splicing. *Proceedings of the National Academy of Sciences* 2017, 114:E5207, doi:10.1073/pnas.1617467114.

23. Errichelli L, Dini Modigliani S, Laneve P, Colantoni A, Legnini I, Capauto D, Rosa A, De Santis R, Scarfò R, Peruzzi G, et al. FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons. *Nature Communications* 2017, 8:14741, doi:10.1038/ncomms14741.

24. Conn Simon J, Pillman Katherine A, Toubia J, Conn Vanessa M, Salmanidis M, Phillips Caroline A, Roslan S, Schreiber Andreas W, Gregory Philip A, Goodall Gregory J. The RNA Binding Protein Quaking Regulates Formation of

circRNAs. *Cell (Cambridge)* 2015, 160:1125-1134, doi:10.1016/j.cell.2015.02.014.

25. Aktaş T, Avşar Ilık İ, Maticzka D, Bhardwaj V, Pessoa Rodrigues C, Mittler G, Manke T, Backofen R, Akhtar A. DHX9 suppresses RNA processing defects originating from the Alu invasion of the human genome. doi: 10.1038/nature21715

26. Khan MA, Reckman YJ, Aufiero S, van den Hoogenhof MM, van der Made I, Beqqali A, Koolbergen DR, Rasmussen TB, van der Velden J, Creemers EE, et al. RBM20 Regulates Circular RNA Production From the Titin Gene. doi: 10.1161/CIRCRESAHA.116.309568

27. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA (New York, N.Y.)* 2013, 19:141-157, doi:10.1261/rna.035667.112.

28. Huang C, Liang D, Tatomer DC, Wilusz JA-O. A length-dependent evolutionarily conserved pathway controls nuclear export of circular RNAs. doi: 10.1101/gad.314856.118

29. Hansen TB, Wiklund Ed Fau - Bramsen JB, Bramsen Jb Fau - Villadsen SB, Villadsen Sb Fau - Statham AL, Statham Al Fau - Clark SJ, Clark Sj Fau - Kjems J, Kjems J. miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. doi: 10.1038/emboj.2011.359

30. Park OH, Ha H, Lee Y, Boo SH, Kwon DH, Song HK, Kim YK. Endoribonucleolytic Cleavage of m(6)A-Containing RNAs by RNase P/MRP Complex. doi: 10.1016/j.molcel.2019.02.034

31. Liu C-X, Li X, Nan F, Jiang S, Gao X, Guo S-K, Xue W, Cui Y, Dong K, Ding H, et al. Structure and Degradation of Circular RNAs Regulate PKR Activation

in Innate Immunity. *Cell* 2019, 177:865-880.e821, doi:10.1016/j.cell.2019.03.046.

32. Fischer JW, Busa VF, Shao Y, Leung AKL. Structure-Mediated RNA Decay by UPF1 and G3BP1. doi: 10.1016/j.molcel.2020.01.021

33. Yu T, Wang Y, Fan Y, Fang N, Wang T, Xu T, Shu Y. CircRNAs in cancer metabolism: a review. *Journal of Hematology & Oncology* 2019, 12:90, doi:10.1186/s13045-019-0776-8.

34. Zhou R, Wu Y, Wang W, Su W, Liu Y, Wang Y, Fan C, Li X, Li G, Li Y, et al. Circular RNAs (circRNAs) in cancer. doi: 10.1016/j.canlet.2018.03.035

35. Xu L, Zhang M, Zheng X, Yi P, Lan C, Xu M. The circular RNA ciRS-7 (Cdr1as) acts as a risk factor of hepatic microvascular invasion in hepatocellular carcinoma. *Journal of Cancer Research and Clinical Oncology* 2017, 143:17-27, doi:10.1007/s00432-016-2256-7.

36. B. Yan WZ, X.-W. Mao, L.-Y. Jiang. Circular RNA ciRS-7 correlates with advance disease and poor prognosis, and its down-regulation inhibits cells proliferation while induces cells apoptosis in non-small cell lung cancer. 2018. Vol. 22 - N. 24 Pages 8712-8721. doi: 10.26355/eurrev_201812_16636

37. Wang L, Zheng C, Wu X, Zhang Y, Yan S, Ruan L, Dai H. Circ-SOX4 promotes non-small cell lung cancer progression by activating the Wnt/β-catenin pathway. *Molecular Oncology* 2020, n/a, doi:10.1002/1878-0261.12656.

38. Chen X, Mao R, Su W, Yang X, Geng Q, Guo C, Wang Z, Wang J, Kresty LA, Beer DG, et al. Circular RNA circHIPK3 modulates autophagy via MIR124-3p-STAT3-PRKAA/AMPKα signaling in STK11 mutant lung cancer. *Autophagy* 2020, 16:659-671, doi:10.1080/15548627.2019.1634945.

39. Xie Y, Yuan X, Zhou W, Kosiba AA, Shi H, Gu J, Qin Z. The circular RNA HIPK3 (circHIPK3) and its regulation in cancer progression: Review. *Life Sciences* 2020:117252, doi:https://doi.org/10.1016/j.lfs.2019.117252.

40. Wang Y, Li H, Lu H, Qin Y. Circular RNA SMARCA5 inhibits the proliferation, migration, and invasion of non-small cell lung cancer by miR-19b-3p/HOXA9 axis. *OncoTargets and therapy* 2019, 12:7055-7065, doi:10.2147/OTT.S216320.

41. Qiu B-Q, Zhang P-F, Xiong D, Xu J-J, Long X, Zhu S-Q, Ye X-D, Wu Y, Pei X, Zhang X-M, et al. CircRNA fibroblast growth factor receptor 3 promotes tumor progression in non-small cell lung cancer by regulating Galectin-1-AKT/ERK1/2 signaling. *Journal of Cellular Physiology* 2019, 234:11256-11264, doi:10.1002/jcp.27783.

42. Liu G, Shi H, Deng L, Zheng H, Kong W, Wen X, Bi H. Circular RNA circ-FOXM1 facilitates cell progression as ceRNA to target PPDPF and MACC1 by sponging miR-1304-5p in non-small cell lung cancer. *Biochemical and Biophysical Research Communications* 2019, 513:207-212, doi:https://doi.org/10.1016/j.bbrc.2019.03.213.

43. Varella-Garcia M. Chromosomal and genomic changes in lung cancer. *Cell adhesion & migration* 2010, 4:100-106, doi:10.4161/cam.4.1.10884.

44. Wu K, Liao X, Gong Y, He J, Zhou J-K, Tan S, Pu W, Huang C, Wei Y-Q, Peng Y. Circular RNA F-circSR derived from SLC34A2-ROS1 fusion gene promotes cell migration in non-small cell lung cancer. *Molecular Cancer* 2019, 18:98, doi:10.1186/s12943-019-1028-9.

45. Huang X-Y, Zhang P-F, Wei C-Y, Peng R, Lu J-C, Gao C, Cai J-B, Yang X, Fan J, Ke A-W, et al. Circular RNA circMET drives immunosuppression and anti-PD1 therapy resistance in hepatocellular carcinoma via the miR-30-5p/snail/DPP4 axis. *Molecular cancer* 2020, 19:92-92, doi:10.1186/s12943-020-01213-6.

46. Pei X, Chen S-W, Long X, Zhu S-Q, Qiu B-Q, Lin K, Lu F, Xu J-J, Zhang P-F, Wu Y-B. circMET promotes NSCLC cell proliferation, metastasis, and immune

evasion by regulating the miR-145-5p/CXCL3 axis. *Aging* 2020, 12:13038-13058, doi:10.18632/aging.103392.

47. Li Y-H, Xu C-L, He C-J, Pu H-H, Liu J-L, Wang Y. circMTDH.4/miR-630/AEG-1 axis participates in the regulation of proliferation, migration, invasion, chemoresistance, and radioresistance of NSCLC. *Molecular Carcinogenesis* 2020, 59:141-153, doi:10.1002/mc.23135.

48. Dong Y, Xu T, Zhong S, Wang B, Zhang H, Wang X, Wang P, Li G, Yang S. Circ_0076305 regulates cisplatin resistance of non-small cell lung cancer via positively modulating STAT3 by sponging miR-296–5p. *Life Sciences* 2019, 239:116984, doi:https://doi.org/10.1016/j.lfs.2019.116984.

49. Xu Y, Jiang T, Wu C, Zhang Y. CircAKT3 inhibits glycolysis balance in lung cancer cells by regulating miR-516b-5p/STAT3 to inhibit cisplatin sensitivity. *Biotechnology Letters* 2020, doi:10.1007/s10529-020-02846-9.

50. Xu N, Chen S, Liu Y, Li W, Liu Z, Bian X, Ling C, Jiang M. Profiles and Bioinformatics Analysis of Differentially Expressed Circrnas in Taxol-Resistant Non-Small Cell Lung Cancer Cells. *Cellular Physiology and Biochemistry* 2018, 48:2046-2060, doi:10.1159/000492543.

51. Yu W, Peng W, Sha H, Li J. Hsa_circ_0003998 Promotes Chemoresistance via Modulation of miR-326 in Lung Adenocarcinoma Cells. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics 2019, 27:623-628. doi: 10.3727/096504018X15420734828058

52. Huang M-S, Liu J-Y, Xia X-B, Liu Y-Z, Li X, Yin J-Y, Peng J-B, Wu L, Zhang W, Zhou H-H, et al. Hsa_circ_0001946 Inhibits Lung Cancer Progression and Mediates Cisplatin Sensitivity in Non-small Cell Lung Cancer via the Nucleotide Excision Repair Signaling Pathway. *Frontiers in oncology* 2019, 9:508-508, doi:10.3389/fonc.2019.00508.

53. Zheng F, Xu R. CircPVT1 contributes to chemotherapy resistance of lung adenocarcinoma through miR-145-5p/ABCC1 axis. *Biomedicine &*

Pharmacotherapy 2020, 124:109828, doi:https://doi.org/10.1016/j.biopha.2020.109828.

54. Ding C, Yi X, Wu X, Bu X, Wang D, Wu Z, Zhang G, Gu J, Kang D. Exosome-mediated transfer of circRNA CircNFIX enhances temozolomide resistance in glioma. *Cancer Letters* 2020, 479:1-12, doi:https://doi.org/10.1016/j.canlet.2020.03.002.

55. Huang W, Yang Y, Wu J, Niu Y, Yao Y, Zhang J, Huang X, Liang S, Chen R, Chen S, et al. Circular RNA cESRP1 sensitises small cell lung cancer cells to chemotherapy by sponging miR-93-5p to inhibit TGF-β signalling. *Cell Death & Differentiation* 2020, 27:1709-1727, doi:10.1038/s41418-019-0455-x.

56. Zhou Y, Zheng X, Xu B, Chen L, Wang Q, Deng H, Jiang J. Circular RNA hsa_circ_0004015 regulates the proliferation, invasion, and TKI drug resistance of non-small cell lung cancer by miR-1183/PDPK1 signaling pathway. *Biochemical and Biophysical Research Communications* 2019, 508:527-535, doi:https://doi.org/10.1016/j.bbrc.2018.11.157.

57. Fu Y, Huang L, Tang H, Huang R. hsa_circRNA_012515 Is Highly Expressed in NSCLC Patients and Affects Its Prognosis. *Cancer management and research* 2020, 12:1877-1886, doi:10.2147/CMAR.S245525.

58. Chen T, Luo J, Gu Y, Huang J, Luo Q, Yang Y. Comprehensive analysis of circular RNA profiling in AZD9291-resistant non-small cell lung cancer cell lines. *Thoracic Cancer* 2019, 10:930-941, doi:10.1111/1759-7714.13032.

59. Zhang P-F, Pei X, Li K-S, Jin L-N, Wang F, Wu J, Zhang X-M. Circular RNA circFGFR1 promotes progression and anti-PD-1 resistance by sponging miR-381-3p in non-small cell lung cancer cells. *Molecular Cancer* 2019, 18:179, doi:10.1186/s12943-019-1111-2.

60. Yang H, Zhang H, Yang Y, Wang X, Deng T, Liu R, Ning T, Bai M, Li H, Zhu K, et al. Hypoxia induced exosomal circRNA promotes metastasis of Colorectal

Cancer via targeting GEF-H1/RhoA axis. *Theranostics* 2020, 10:8211-8226, doi:10.7150/thno.44419.

61. Li Y, Zheng Q, Bao C, Li S, Guo W, Zhao J, Chen D, Gu J, He X, Huang S. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Research* 2015, 25:981-984, doi:10.1038/cr.2015.82.

62. Lasda E, Parker R. Circular RNAs Co-Precipitate with Extracellular Vesicles: A Possible Mechanism for circRNA Clearance. *PLOS ONE* 2016, 11:e0148407, doi:10.1371/journal.pone.0148407.

63. Wang M, Yu F, Li P, Wang K. Emerging Function and Clinical Significance of Exosomal circRNAs in Cancer. *Molecular Therapy - Nucleic Acids* 2020, 21:367-383, doi:https://doi.org/10.1016/j.omtn.2020.06.008.

64. Shang B-Q, Li M-L, Quan H-y, Hou P-F, Li Z-W, Chu S-F, Zheng J-N, Bai J. Functional roles of circular RNAs during epithelial-to-mesenchymal transition. *Molecular Cancer* 2019, 18:138, doi:10.1186/s12943-019-1071-6.

65. Chen F, Huang C, Wu Q, Jiang L, Chen S, Chen L. Circular RNAs expression profiles in plasma exosomes from early-stage lung adenocarcinoma and the potential biomarkers. *Journal of Cellular Biochemistry* 2020, 121:2525-2533, doi:10.1002/jcb.29475.

66. He F, Zhong X, Lin Z, Lin J, Qiu M, Li X, Hu Z. Plasma exo-hsa_circRNA_0056616: A potential biomarker for lymph node metastasis in lung adenocarcinoma. *Journal of Cancer* 2020, 11:4037-4046, doi:10.7150/jca.30360.

67. Xian J, Su W, Liu L, Rao B, Lin M, Feng Y, Qiu F, Chen J, Zhou Q, Zhao Z, et al. Identification of Three Circular RNA Cargoes in Serum Exosomes as Diagnostic Biomarkers of Non–Small-Cell Lung Cancer in the Chinese Population. *The Journal of Molecular Diagnostics* 2020, 22:1096-1108, doi:10.1016/j.jmoldx.2020.05.011.

68. Hang D, Zhou J, Qin N, Zhou W, Ma H, Jin G, Hu Z, Dai J, Shen H. A novel plasma circular RNA circFARSA is a potential biomarker for non-small cell lung cancer. *Cancer medicine* 2018, 7:2783-2791, doi:10.1002/cam4.1514.

69. Liu X-X, Yang Y-E, Liu X, Zhang M-Y, Li R, Yin Y-H, Qu Y-Q. A two-circular RNA signature as a noninvasive diagnostic biomarker for lung adenocarcinoma. *Journal of Translational Medicine* 2019, 17:50, doi:10.1186/s12967-019-1800-z.

70. Tan S, Gou Q, Pu W, Guo C, Yang Y, Wu K, Liu Y, Liu L, Wei Y-Q, Peng Y. Circular RNA F-circEA produced from EML4-ALK fusion gene as a novel liquid biopsy biomarker for non-small cell lung cancer. *Cell research* 2018, 28:693-695, doi:10.1038/s41422-018-0033-7.

71. Alhasan AA, Izuogu OG, Al-Balool HH, Steyn JS, Evans A, Colzani M, Ghevaert C, Mountford JC, Marenah L, Elliott DJ, et al. Circular RNA enrichment in platelets is a signature of transcriptome degradation. *Blood* 2016, 127:e1-e11, doi:10.1182/blood-2015-06-649434.

72. Preußer C, Hung L-H, Schneider T, Schreiner S, Hardt M, Moebus A, Santoso S, Bindereif A. Selective release of circRNAs in platelet-derived extracellular vesicles. *Journal of extracellular vesicles* 2018, 7:1424473-1424473, doi:10.1080/20013078.2018.1424473.

73. Best MG, Sol N, In 't Veld SGJG, Vancura A, Muller M, Niemeijer A-LN, Fejes AV, Tjon Kon Fat L-A, Huis In 't Veld AE, Leurs C, et al. Swarm Intelligence-Enhanced Detection of Non-Small-Cell Lung Cancer Using Tumor-Educated Platelets. *Cancer cell* 2017, 32:238-252.e239, doi:10.1016/j.ccell.2017.07.004.

74. Liu Y-T, Han X-H, Xing P-Y, Hu X-S, Hao X-Z, Wang Y, Li J-L, Zhang Z-S, Yang Z-H, Shi Y-K. Circular RNA profiling identified as a biomarker for predicting the efficacy of Gefitinib therapy for non-small cell lung cancer. *Journal of thoracic disease* 2019, 11:1779-1787, doi:10.21037/jtd.2019.05.22.

75. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Research International* 2018, 2018:8545347, doi:10.1155/2018/8545347.

76. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer — implications for future improvements in cancer care. *Nature Reviews Clinical Oncology* 2018, 15:617-638, doi:10.1038/s41571-018-0036-9.

77. Théry CWitwer KWAikawa EAlcaraz MJAnderson JDAndriantsitohaina RAntoniou AArab TArcher FAtkin-Smith GK, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* 2018, 7:1535750, doi:10.1080/20013078.2018.1535750.

78. Tang Y-T, Huang Y-Y, Zheng L, Qin S-H, Xu X-P, An T-X, Xu Y, Wu Y-S, Hu X-M, Ping B-H, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. *Int J Mol Med* 2017, 40:834-844, doi:10.3892/ijmm.2017.3080.

79. Guria A, Velayudha Vimala Kumar K, Srikakulam N, Krishnamma A, Chanda S, Sharma S, Fan X, Pandi G. Circular RNA Profiling by Illumina Sequencing via Template-Dependent Multiple Displacement Amplification. *BioMed research international* 2019, 2019:2756516-2756516, doi:10.1155/2019/2756516.

80. Hert DG, Fredlake CP, Barron AE. Advantages and limitations of next-generation sequencing technologies: A comparison of electrophoresis and non-electrophoresis methods. *ELECTROPHORESIS* 2008, 29:4618-4626, doi:https://doi.org/10.1002/elps.200800456.

81. Qu S, Song W, Yang X, Wang J, Zhang R, Zhang Z, Zhang H, Li H. Microarray expression profile of circular RNAs in human pancreatic ductal

adenocarcinoma. *Genomics Data* 2015, 5:385-387, doi:https://doi.org/10.1016/j.gdata.2015.07.017.

82. Kulkarni MM. Digital Multiplexed Gene Expression Analysis Using the NanoString nCounter System. *Current Protocols in Molecular Biology* 2011, 94:25B.10.21-25B.10.17, doi:https://doi.org/10.1002/0471142727.mb25b10s94.

83. Kamyabi N, Abbasgholizadeh R, Maitra A, Ardekani A, Biswal SL, Grande-Allen KJ. Isolation and mutational assessment of pancreatic cancer extracellular vesicles using a microfluidic platform. *Biomedical Microdevices* 2020, 22:23, doi:10.1007/s10544-020-00483-7.

84. Garcia-Contreras M, Shah SH, Tamayo A, Robbins PD, Golberg RB, Mendez AJ, Ricordi C. Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes. *Scientific Reports* 2017, 7:5998, doi:10.1038/s41598-017-05787-y.

85. Dahl M, Daugaard I, Andersen MS, Hansen TB, Grønbæk K, Kjems J, Kristensen LS. Enzyme-free digital counting of endogenous circular RNA molecules in B-cell malignancies. *Laboratory Investigation* 2018, 98:1657-1669, doi:10.1038/s41374-018-0108-6.

86. Vincent HA, Deutscher MP. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *Journal of molecular biology* 2009, 387:570-583, doi:10.1016/j.jmb.2009.01.068.

87. Xiao M-S, Wilusz JE. An improved method for circular RNA purification using RNase R that efficiently removes linear RNAs containing G-quadruplexes or structured 3' ends. *Nucleic acids research* 2019, 47:8755-8769, doi:10.1093/nar/gkz576.

88. Koch L. Translated circular RNAs. *Nature Reviews Genetics* 2017, 18:272-273, doi:10.1038/nrg.2017.27.

89. Panda AC, Gorospe M. Detection and Analysis of Circular RNAs by RT-PCR. *Bio-protocol* 2018, 8:e2775, doi:10.21769/BioProtoc.2775.

90. Zhong S, Zhou S, Yang S, Yu X, Xu H, Wang J, Zhang Q, Lv M, Feng J. Identification of internal control genes for circular RNAs. *Biotechnology Letters* 2019, 41:1111-1119, doi:10.1007/s10529-019-02723-0.

91. You X, Vlatkovic I, Babic A, Will T, Epstein I, Tushev G, Akbalik G, Wang M, Glock C, Quedenau C, et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nature neuroscience* 2015, 18:603-610, doi:10.1038/nn.3975.

92. Conn V, Conn SJ. SplintQuant: a method for accurately quantifying circular RNA transcript abundance without reverse transcription bias. *RNA (New York, N.Y.)* 2019, 25:1202-1210, doi:10.1261/rna.070953.119.

93. Liu M, Wang Q, Shen J, Yang BB, Ding X. Circbank: a comprehensive database for circRNA with standard nomenclature. *RNA Biology* 2019, 16:899-905, doi:10.1080/15476286.2019.1600395.

94. Glažar P, Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. *RNA (New York, N.Y.)* 2014, 20:1666-1670, doi:10.1261/rna.043687.113.

95. Feng J, Xiang Y, Xia S, Liu H, Wang J, Ozguc FM, Lei L, Kong R, Diao L, He C, et al. CircView: a visualization and exploration tool for circular RNAs. *Briefings in Bioinformatics* 2019, 20:745-751, doi:10.1093/bib/bbx070.

96. Liangsupree T, Multia E, Riekkola M-L. Modern isolation and separation techniques for extracellular vesicles. *Journal of Chromatography A* 2021, 1636:461773, doi:https://doi.org/10.1016/j.chroma.2020.461773.

97. Han L, Xu J, Xu Q, Zhang B, Lam EWF, Sun Y. Extracellular vesicles in the tumor microenvironment: Therapeutic resistance, clinical biomarkers, and targeting strategies. *Medicinal Research Reviews* 2017, 37:1318-1349, doi:https://doi.org/10.1002/med.21453.

98. Saarenheimo J, Eigeliene N, Andersen H, Tiirola M, Jekunen A. The Value of Liquid Biopsies for Guiding Therapy Decisions in Non-small Cell Lung Cancer. *Frontiers in oncology* 2019, 9:129-129, doi:10.3389/fonc.2019.00129.

99. Wen G, Zhou T, Gu W. The potential of using blood circular RNA as liquid biopsy biomarker for human diseases. *Protein & Cell* 2020, doi:10.1007/s13238-020-00799-3.

100. Fan C-M, Wang J-P, Tang Y-Y, Zhao J, He S-Y, Xiong F, Guo C, Xiang B, Zhou M, Li X-L, et al. circMAN1A2 could serve as a novel serum biomarker for malignant tumors. *Cancer Science* 2019, 110:2180-2188, doi:10.1111/cas.14034.

101. Jacky Lam WK, Dennis Lo YM. Circular RNAs as Urinary Biomarkers. *Clinical Chemistry* 2019, 65:1196-1198, doi:10.1373/clinchem.2019.309773.

102. Akhoundova D, Mosquera Martinez J, Musmann LE, Britschgi C, Rütsche C, Rechsteiner M, Nadal E, Garcia Campelo MR, Curioni-Fontecedro A. The Role of the Liquid Biopsy in Decision-Making for Patients with Non-Small Cell Lung Cancer. *Journal of clinical medicine* 2020, 9:3674, doi:10.3390/jcm9113674.

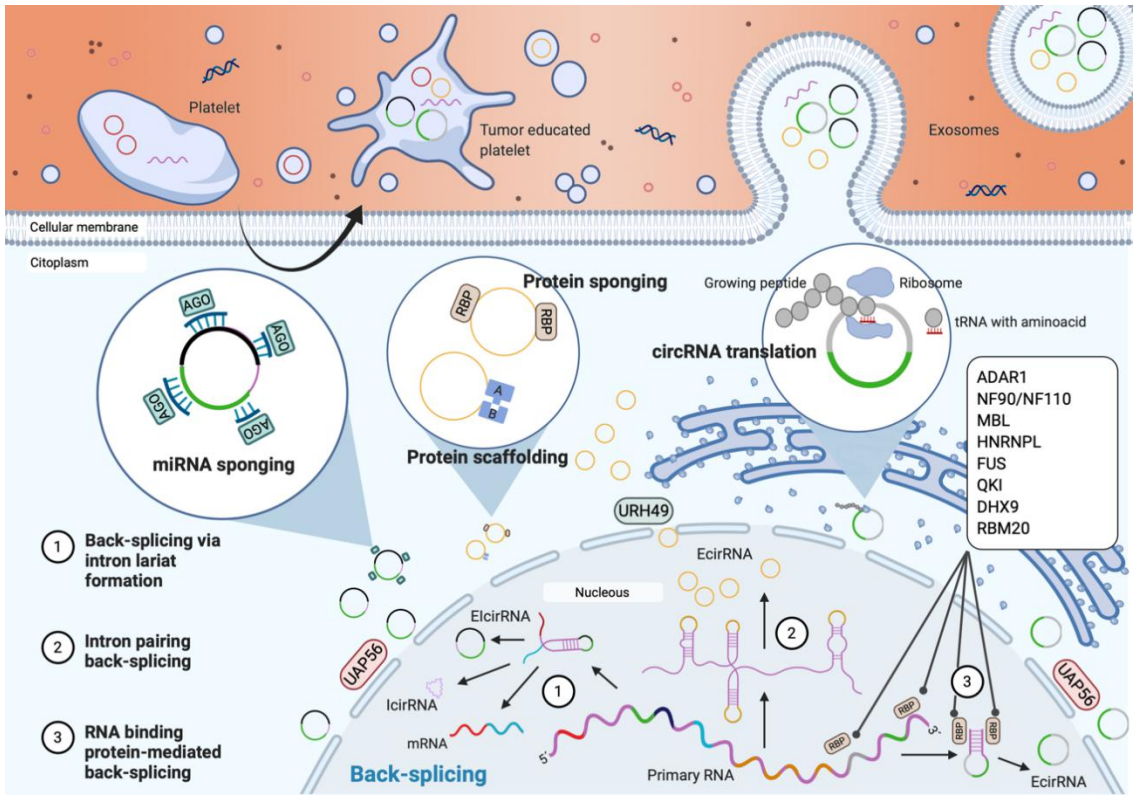
Platelet
Tumor educated platelet
Exosomes
Cellular membrane
Citoplasm
AGO
miRNA sponging
RBP
Protein sponging
Protein scaffolding
Growing peptide
Ribosome
tRNA with aminoacid
circRNA translation
ADAR1
NF90/NF110
MBL
HNRNPL
FUS
QKI
DHX9
RBM20
URH49
EcirRNA
Nucleous
ElcirRNA
IcirRNA
mRNA
UAP56
Back-splicing
Primary RNA
1 Back-splicing via intron lariat formation
2 Intron pairing back-splicing
3 RNA binding protein-mediated back-splicing

Figure 1. Biosynthesis and molecular functions of circRNAs. CircRNAs are generated by three different mechanisms of back-splicing (via lariat formation, intron pairing or RNA binding proteins). Resultant circRNAs can be formed by only exonic regions (EcircRNAs), intronic regions (IcircRNAs) or both (EIcircRNAs). circRNAs are exported into the cytoplasm in a size-mediated manner by URH49 and UAP56. Once in the cytoplasm, circRNAs will perform their functions including miRNA and protein sponging, protein scaffolding, or even translate into small functional peptides. circRNAs will be released into the blood stream inside exosomes mediating cellular communication. Most cellular types, including tumor cells, will secrete circRNA-containing EVs. Platelets can modify its content when in contact with the tumor, including their circRNA expression profile.