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Contacts

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Extracellular Vesicles and Circulating Nucleic Acids

CONTENTS

Volume 2 / Issue 2 / June 2021

Zhang *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:104-26
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Extracellular Vesicles and Circulating Nucleic Acids

Original Article

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Identification of important extracellular vesicle RNA molecules related to sperm motility and prostate cancer

Yu Zhang, Ning Ding, Shenmin Xie, Yaqun Ding, Mengna Huang, Xiangdong Ding, Li Jiang

National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding & Reproduction, Ministry of Agriculture, College of Animal Science & Technology, China Agricultural University, Beijing 100193, China.

Correspondence to: Li Jiang, National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding & Reproduction, Ministry of Agriculture, College of Animal Science & Technology, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, China. E-mail: lijiang@cau.edu.cn

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Abstract

Aim: Many male diseases are associated with sperm quality, such as prostate cancer (PCa), oligospermia, and asthenospermia. Seminal plasma extracellular vesicles (SPEVs) play important roles in sperm function. In this study, we explored the specific RNA molecules in SPEVs that play an important role in sperm motility and found promising biomarkers of PCa in SPEVs.

Methods: Pigs have become an ideal model for human biomedical research. In this study, the whole transcriptome profiles of SPEVs of boars with high or low sperm motility were studied for the first time. Important long non-coding RNAs, microRNAs, and genes were identified through differentially expressed analysis and weighted correlation network analysis (WGCNA). In addition, we established a diagnosis model of PCa by differentially expressed miRNAs homologous with human.

Results: In total, 27 differentially expressed miRNAs, 106 differentially expressed lncRNAs, and 503 differentially expressed genes were detected between the groups. The results of WGCNA show one module was significantly associated with sperm motility ($r = 0.98$, $FDR = 2 \times 10^{-6}$). The value of highly homologous miRNAs for the diagnosis of PCa was assessed and the combination of hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-155-5p, and hsa-miR-378a-3p exhibited the highest sensitivity (AUC = 0.914). Interestingly, mRNA expression of SPEVs was mainly enriched in resting memory CD4 T cells and monocytes, and 33 cell marker genes of monocytes overlapped with

the differentially expressed genes.

Conclusion: These data demonstrate that SPEVs of individuals with high and low sperm motility exhibit distinct transcriptional profiles, which provide valuable information for further research on diagnosis and molecular mechanism of diseases.

Keywords: Seminal plasma extracellular vesicles, transcriptome, sperm motility, prostate cancer, immune cells

INTRODUCTION

In humans, some male diseases, such as prostate cancer (PCa), oligospermia, and asthenospermia, are associated with poor sperm motility and function[1,2]. Sperm motility is one of the important indicators of sperm quality. Due to similarities in the metabolic characteristics, cardiovascular system, and proportional size of organs between pigs and humans, particularly the high similarity between the pig and human genome sequences, pigs have become an ideal model for human biomedical research[3,4]. In addition, the pig model provides the advantages of low genetic variation and alleviation of human-specific confounding factors (such as smoking and drinking)[5]. Therefore, studying complex human diseases using pig models is a good strategy.

Extracellular vesicles (EVs), including exosomes (with an average size of ~100 nm), are double-layered phospholipid membrane vesicles that are released by most cells[6]. Many studies have shown that EVs contain DNA, RNA, metabolites, lipids, and proteins[7]. As carriers of bioactive molecules and important intercellular signaling molecules, EVs can execute many biological functions and are considered to be of great significance[8]. Several studies have been devoted to the identification of effective clinical biomarkers in EVs, particularly microRNAs, lncRNAs, and proteins[9-11].

Semen is composed of sperm and seminal plasma, which contains a large number of EVs. Seminal plasma EVs (SPEVs) are derived from a mixture of fluids secreted by the organs of the testis, epididymis, and accessory glands[12]. A previous study showed that seminal plasma plays an important role in the morphological changes and maturation of sperm[13] and participates in the metabolism, survival, and transportation of sperm in the female reproductive tract[14]. In recent years, some studies have demonstrated that SPEVs play an important role in sperm function, such as sperm motility[15], sperm capacitation, and acrosome reaction[16]. It has been reported that the noncoding RNAs contained in SPEVs are involved in the protection of sperm from the female immune response triggered by contact with sperm in the female reproductive tract[17,18]. Moreover, SPEVs can combine with sperm *in vitro*, promote sperm movement, prolong the effective survival time of sperm, improve the integrity of the sperm plasma membrane, and increase the antioxidant capacity[19]. However, the specific RNA molecules in SPEVs that play an important role in sperm motility remain unclear.

miRNAs are endogenous single-stranded 18-25 nt small noncoding RNAs capable of regulating gene expression at the posttranscriptional level[20]. It has been reported that miRNAs participate in the regulation of many genes involved in reproductive biology, such as germ cell development, maturation, and fertilization[21,22]. miRNAs in human SPEVs have recently been studied by RNA sequencing and microarray technology[23]. For instance, Abu *et al.*[24] (2016) suggested that 7 and 29 miRNAs are expressed at high and low levels, respectively, in seminal exosomes of oligoasthenospermia patients compared with those of normal individuals. In addition, Barcelo *et al.*[25] (2018) compared the miRNA expression profiles of seminal exosomes from patients with different pathological types of azoospermia (obstructive and secretory azoospermia) and identified five miRNAs (miR-182-3p, miR-205-5p, miR-31-5p, miR-539-5p, and miR-

941) as potential biomarkers of patients with secretory azoospermia.

The potential application of miRNAs in EV-based liquid biopsy has attracted extensive attention. Some important miRNAs in EVs that are related to sperm quality and PCa have been identified by comparing patients with healthy controls[26,27]. However, most of these EVs originate from human urine[10,28,29] and blood plasma[30], and only a few studies have found several specific miRNA markers in human semen exosomes[31]. EVs from seminal plasma can better reflect the condition of sperm and prostate tissue. Therefore, highly homologous miRNAs identified in pig SPEVs that are related to sperm quality can provide valuable information for the early diagnosis of patients with PCa.

This study constituted the first study of the whole transcriptome of SPEVs of Yorkshire boars with different sperm motility levels. Differential miRNA, gene, and lncRNA expression profiles were identified between the different groups, and the differentially expressed lncRNA (DEL)-differentially expressed miRNA (DEmi)-differentially expressed gene (DEG) regulatory network was constructed. Several important miRNAs, lncRNAs, and genes affecting sperm motility were identified. In addition, the value of highly homologous miRNAs for the diagnosis of PCa was assessed, and an ROC analysis showed that hsa-miR-24-3p, hsa-miR-27a-3p, hsa-miR-27b-3p, and hsa-miR-23b-3p could be used as promising biomarkers for PCa diagnosis.

METHODS

Ethics statements

All protocols for the collection of semen samples of all animals were approved by the Institutional Animal Care and Use Committee at China Agricultural University (Permit number: DK996). The experiments in this study were conducted according to regulations and guidelines established by this committee.

Animals and samples

The sperm motilities of 230 large white boars were measured using a computer-assisted sperm analysis (CASA) system (IVOS □, France) from a national boar station. The sperm motility performance of three consecutive semen samples from each boar was evaluated. Eleven boars with extremely high or low sperm motility were selected for SPEV extraction. All individuals were sexually mature, with an age between 14 and 36 months. Detailed information of the 11 boars is shown in Supplementary Table 1.

Analysis of sperm motility

One ejaculate from each boar was collected using the gloved-hand method. Specialized professionals obtained the sperm-rich fractions of ejaculates from each individual, and the sperm motilities of these samples were assessed using the CASA system (IVOS □, France). Then, 950 μL of preheated diluent were added to 50 μL fresh semen and mixed gently. After 5 min of incubation at 37 °C, 7 μL of sperm suspension were placed on a prewarmed glass slide and covered with a glass coverslip. The glass slides were examined with a bright field under an optical microscope at a total magnification of 200×. The percentage of motile sperm was estimated in five different microscopic fields for each sample using the CASA system.

Experimental design

According to the sperm motilities of boars, the eleven individuals were divided into two groups: the H group and the L group. The boars in the H group had a higher total sperm motility (> 0.97), whereas the boars in the L group had a lower total sperm motility (< 0.73). Detailed information is shown in Supplementary Table 1.

Isolation of SPEVs

Each semen sample was centrifuged (800× *g*, 20 min at 17 ℃) for the separation of sperm and supernatant. The supernatant was used for the extraction of EVs. SPEVs were isolated by ultracentrifugation. as previously described[32]. Thirty-five milliliters of semen plasma from each sample were centrifuged at 10,000× *g* and 4 ℃ for 30 min to remove cellular debris and smaller pieces of undissolved seminal gel. The supernatant was then transferred to a clean centrifugal tube and centrifuged at 12,000× *g* and 4 °C for 1 h. The supernatant was then transferred to an ultracentrifugation tube (Beckman, USA) and centrifuged at 120,000× *g* and 4 °C for 1.5 h. The sediments were resuspended in DPBS (Gibco, USA), and the previous step was repeated. The sediments were then resuspended in 2 mL of DPBS and filtered through 0.22-μm filters (Millipore, USA).

Transmission electron microscopy

Twenty microliters of SPEVs were placed on a formvar carbon-coated grid for 5 min at room temperature. The grids were washed three times with distilled water, stained with 1.0% uranyl formate (Electron microscope China, China) for 5 min, and dried for 2 min under incandescent light. The grids were observed and photographed under a transmission electron microscopy (HT770, Tokyo, Japan).

Nanoparticle tracking analysis (NTA)

The concentrations of SPEVs were diluted to 1×10^6-1×10^9 particles/mL with PBS. A ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) equipped with a 405-nm laser was used to examine the size and quantity of the isolated particles. A 1-min video shot at a frame rate of 30 frames per second was used to analyze the motion of the particles using NTA software (ZetaView 8.02.28).

Western blot analysis

SPEVs were cleaved with RIPA buffer (Solarbio, Beijing, China) containing 1% protease inhibitor on ice for 30 min. Twenty-five microliters of the protein samples were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). The membrane was blocked with 5% (w/v) skim milk for 2 h, washed five times with 1× TBST, incubated with antibodies against CD9 (sc-13118, Santa Cruz, CA, USA), CD63 (sc-5275, Santa Cruz, CA, USA), Alix (sc-53, 540, Santa Cruz, CA, USA), Calnexin (10,427-2-AP, Promega, Madison, WI, USA), and Tsg101 (sc-13, 611, Santa Cruz, CA, USA) for 12 h at 4 ℃ and then with secondary antibodies for 2 h at 37 ℃, and detected with an ECL system.

RNA extraction and RNA sequencing

Total RNA was isolated from SPEVs using a RNeasy Mini Kit (Qiagen, Germany) according to the instructions. The RNA quality was verified by 1% agarose gel electrophoresis, and the RNA concentration and integrity were measured using the RNA Nano 6000 Assay kit and the Agilent biological analyzer 2100 system (Agilent Technologies, CA, USA).

Whole transcriptome sequencing was performed to obtain insight into the types of RNAs, including miRNAs, mRNAs, and lncRNAs, in SPEVs. Small RNA and long RNA libraries were established. Long RNA libraries were generated using the SMARTer Stranded Total RNA-Seq Kit (Takara Bio Inc.) according to the manufacturer's instructions, and the index code was added to the attribute sequence of each sample. Small RNA libraries were generated using the QIAseq miRNA Library Kit (Qiagen, Frederick, MD, USA) following the manufacturer's recommendations, and the index code was added to the attribute sequence of each sample. The quality of the library was evaluated with an Agilent analyzer 2100 and by qPCR. TruSeq PE cluster kitv3 CBOT HS (Illumina, San Diego, CA, USA) was used to cluster the index coding samples on a cbot cluster generation system. The library of each sample was then sequenced with an Illumina HiSeq 2500 platform (Illumina, USA) to generate paired-end reads.

RNA-sequencing analysis

For the identification of miRNAs, we first used cutadapt to trim adapter sequences of 3' reads (AACTGTAGGCACCATCAAT) and kept the sequences with lengths between 18 and 32 nt after trimming[17]. To filter ncRNAs, such as rRNA, tRNA, scRNA, snRNA, and snoRNA, as well as repeated sequences, the clean data were aligned to the Silva[33], GtRNAdb[34], Rfam[35], and Repbase databases[36]. The remaining sequences were further identified as miRNAs through miRDeep2[37] based on the following steps: (1) alignment to the pig reference genome (ftp://ftp.ensembl.org/pub/release-97/fasta/sus_scrofa) with no mismatch; and (2) further alignment to known mature and precursor miRNA sequences downloaded from the miRbase database (v21) (http://www.mirbase.org/).

For the identification of lncRNAs, we first obtained the clean reads after removing the low-quality reads. The clean reads were mapped to the pig reference genome using Hisat2[38]. Reference genome and gene annotation files were downloaded from Ensembl (ftp://ftp.ensembl.org/pub). The mapped reads of each sample were assembled and merged into transcripts using StringTie[39]. All identified transcripts were guided by the gene models of GffCompare[40]. The novel transcripts were filtered according to the following steps: (1) Among the different class codes, only transcripts annotated by "i", "u", "x", "o", and "e" were retained[41]; (2) Transcripts with a single exon or a length shorter than 200 nt were removed[42]; (3) Transcripts with FPKM ≥ 0.1 were retained; and (4) Four software programs for coding potential analysis, namely CNCI[43], CPC[44], Pfam[45], and CPAT[46], were employed to predict the protein-coding ability, and the transcripts of the overlapping results obtained from these four software programs were considered candidate lncRNAs.

Identification of differentially expressed miRNAs, genes and lncRNAs

DESeq2 was used to identify the DEmis, DEGs, and DELs based on unnormalized read counts[47]. We first filtered the miRNAs, genes, and lncRNAs with low expression levels. The DEmis, DEGs, and DELs were then detected by comparing the H group with the L group. For each comparison, miRNAs, genes, and lncRNAs that satisfied the criteria $P < 0.05$ and |Fold Change| (FC) > 1.5 were considered significantly differentially expressed.

Validation of differentially expressed miRNAs

Ten DEmis (ssc-miR-142-3p, ssc-miR-146a-5p, ssc-miR-155-5p, ssc-miR-184, ssc-miR-223, ssc-miR-23a, ssc-miR-23b, ssc-miR-24-3p, ssc-miR-378, and ssc-miR-378b-3p) were randomly selected to validate the small RNA sequencing results using TaqMan advanced miRNA assays. Total RNA was extracted and purified from SPEVs of the H and L groups using the RNeasy Mini kit (Qiagen, Germany). Two microliters of total RNA from each sample were used for miRNA reverse transcription using an PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Biotechnology, China) according to the manufacturer's recommended protocols. The same amount of *Caenorhabditis elegans* cel-miR-39 miRNA was added to each SPEV sample as an external calibration for RNA extraction, reverse transcription, and miRNA amplification. Real-time quantitative PCR (qPCR) was performed on an ABI7500 qPCR system (Applied Biosystems) with Premix Ex Taq™ (Probe qPCR) (Takara Biotechnology, China) according to the manufacturer's instructions. Specific miRNA TaqMan expression probes (Life Tech, CA, USA) were used for miRNA quantification [Supplementary Table 2]. Each sample was analyzed in triplicate, and all miRNAs were standardized using cel-miR-39 miRNA.

WGCNA of mRNAs and lncRNAs

We constructed DEGs and DELs coexpression networks using WGCNA (v1.12.0) implemented in R. The original RNA-seq count expression matrix containing DEGs and DELs was used as an input file, and the expression matrix was then normalized by the variance stabilizing transformation procedure implemented

in DESeq2[48]. We used the "one step method" to divide the gene expression matrix into different modules based on pairwise Pearson's correlation. In the one-step method, a softpower of 12 was selected as the threshold to identify the coexpressed DEGs and DELs modules. The hub DEGs and DELs within important modules were defined by an absolute value of membership greater than 0.7 and an absolute value of gene significance greater than 0.2[49]. KOBAS software (http://kobas.cbi.pku.edu.cn/) was used for an enrichment analysis of the hub DEGs in important modules[50].

Construction of DEL-DEmi-DEG networks

RNAhybrid was used to predict potential DELs related to DEmis with a predicted energy < -25[51]. miRanda and RNAhybrid were used for the prediction of potential DEGs that interact with DEmis[52]. The parameters used in the miRanda analysis were the following: single residue pair match scores (S) > 150 and Gibbs free energy during double-strand formation (△G) < -20 kcal/mol[52]. Based on the predicted regulatory DEL-DEmi and DEmi-DEG pairs, DEL-DEmi-DEG networks were constructed via shared DEmis. The results were visualized using Cytoscape 3.5.1 software[53].

Establishment of miRNAs for the diagnosis of PCa

We downloaded mature miRNA datasets of PCa from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). In total, 16 homologous DEmis between pigs and humans obtained after filtering DEmis with low expression were used to establish a miRNA diagnosis model for PCa. The efficacy of the candidate miRNAs or their combinations was analyzed by receiver operating characteristic (ROC) curves, and the area under the ROC curve (AUC) was calculated. First, 82 samples were used as the test set (52 cases *vs.* 30 controls) to analyze the diagnostic accuracy of 16 homologous DEmis between pigs and humans through a ROC analysis, and the AUC was also calculated. Homologous DEmis with a high AUC (> 0.7) was used to establish the miRNA diagnosis model of PCa based on the 132 samples validation set, which included 80 cases and 52 controls. The efficacy of the candidate miRNAs or their combinations was analyzed by ROC, and the AUC was calculated. In addition, the SPEV transcriptome data from eight Duroc boars and 11 Yorkshire boars were used to investigate the composition of SPEVs in different immune cell types using the CIBERSORTx tool (https://cibersortx.stanford.edu/runcibersortx.php), and 22 types of immune cells were evaluated in this study[54]. The marker genes of CD4+ T cells and monocytes were obtained from CellMaker websites (http://biocc.hrbmu.edu.cn/CellMarker/).

RESULTS

Characterization of SPEVs of Yorkshire boars

The results of transmission electron microscopy show that most SPEVs appeared intact and had the typical cup shape [Figure 1A]. The mean size of the SPEVs was 108.7 nm, and the particle size ranged from 50 to 200 nm [Figure 1B]. A Western blotting analysis detected extracellular vesicle markers (CD9, CD63, Alix, and Tsg101) in SPEVs isolated from one boar in the high-sperm-motility (H) group and one boar in the low-sperm-motility (L) group. In contrast, Calnexin, a negative marker of EVs, was absent in SPEVs from the two boars [Figure 1C]. Phenotypic analysis showed that significant differences in the total sperm motility were found between the two groups [Figure 1D].

Summary of RNA-seq data

To obtain small RNA libraries, we acquired 333,466,614 raw data , and an average of 30,315,146 raw data was obtained from each sample. After quality control of the raw data, an average of 19,719,374 clean data was obtained from each sample, and, after the annotation of ncRNAs and repeat sequences, the average percentage of reads from each sample that were aligned to the reference genome was 77.53%. The Q30 base percentages of all samples ranged from 94.99% to 96.59%. To obtain long RNA libraries, we acquired 253.24 gigabases (Gb) of clean data, and an average of 23.02 Gb was obtained from each sample. The average Q30

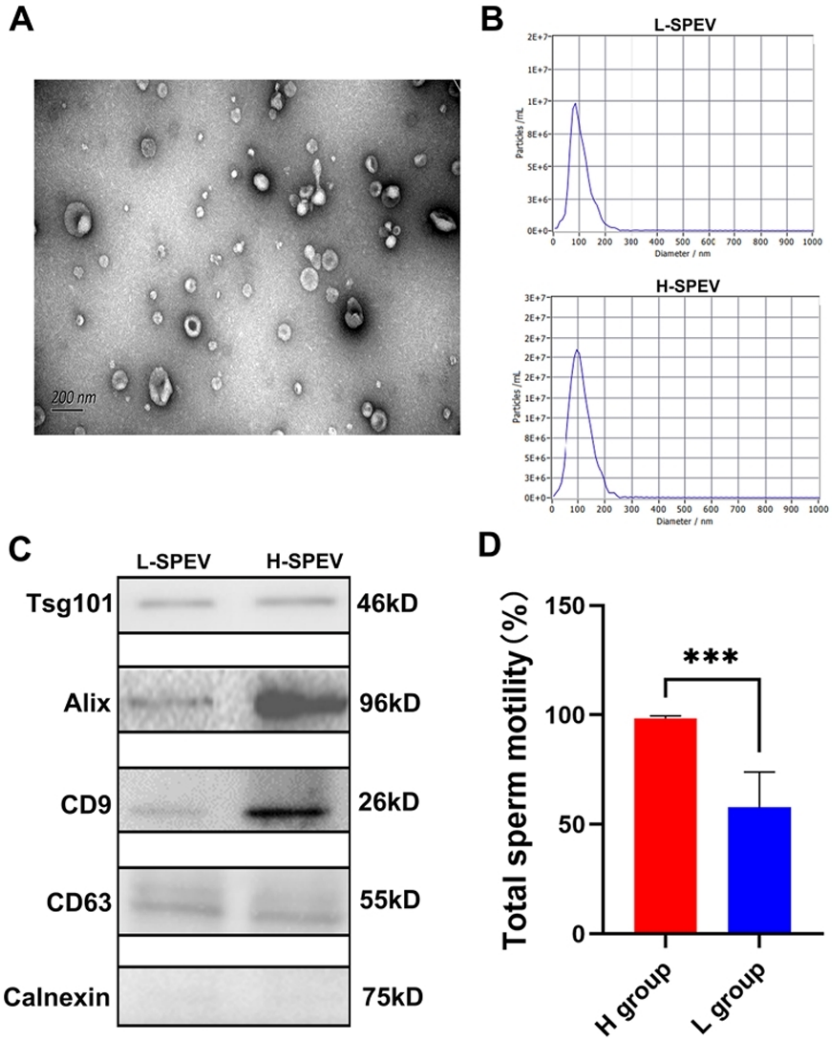

Figure 1. Characterization of SPEVs from Yorkshire boars. (A) TEM image of SPEVs. (B) NTA results showing that the semen-derived EVs were approximately 50-200 nm in diameter. (C) The isolated SPEVs expressed the EV markers Tsg101, Alix, CD9 and CD 63, and the negative marker Calnexin was not found in our isolated SPEV samples. D. Bar plot comparing the total sperm motility between the high-sperm-mobility (H) group and the low-sperm-mobility (L) group.

base percentage of all the samples was 90.97%. Detailed information is shown in Supplementary Table 3. In addition, 203.44 Gb of clean long RNA data were obtained from our previous study of SPEVs of eight Duroc boars.

Overall description of miRNAs of SPEVs

The small RNA libraries read were mapped to the annotated miRNA database (miRBase), and many different types of small RNAs, such as miRNA, ribosomal RNA (rRNA), transporter RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and small cytosol RNA (scRNA), were found in Yorkshire boar SPEVs [Figure 2A]. The average content of miRNAs in all the samples was 47.39%. In total, 626 mature miRNAs were detected in SPEVs, and these included 334 (53.35%) known miRNAs and 292 (46.65%) novel miRNAs [Figure 2B]. The length distribution of miRNAs in all the samples was mainly 21-23 nt [Figure 2C]. As shown in Figure 2D, the vast majority of miRNAs (83.71%) were expressed at low

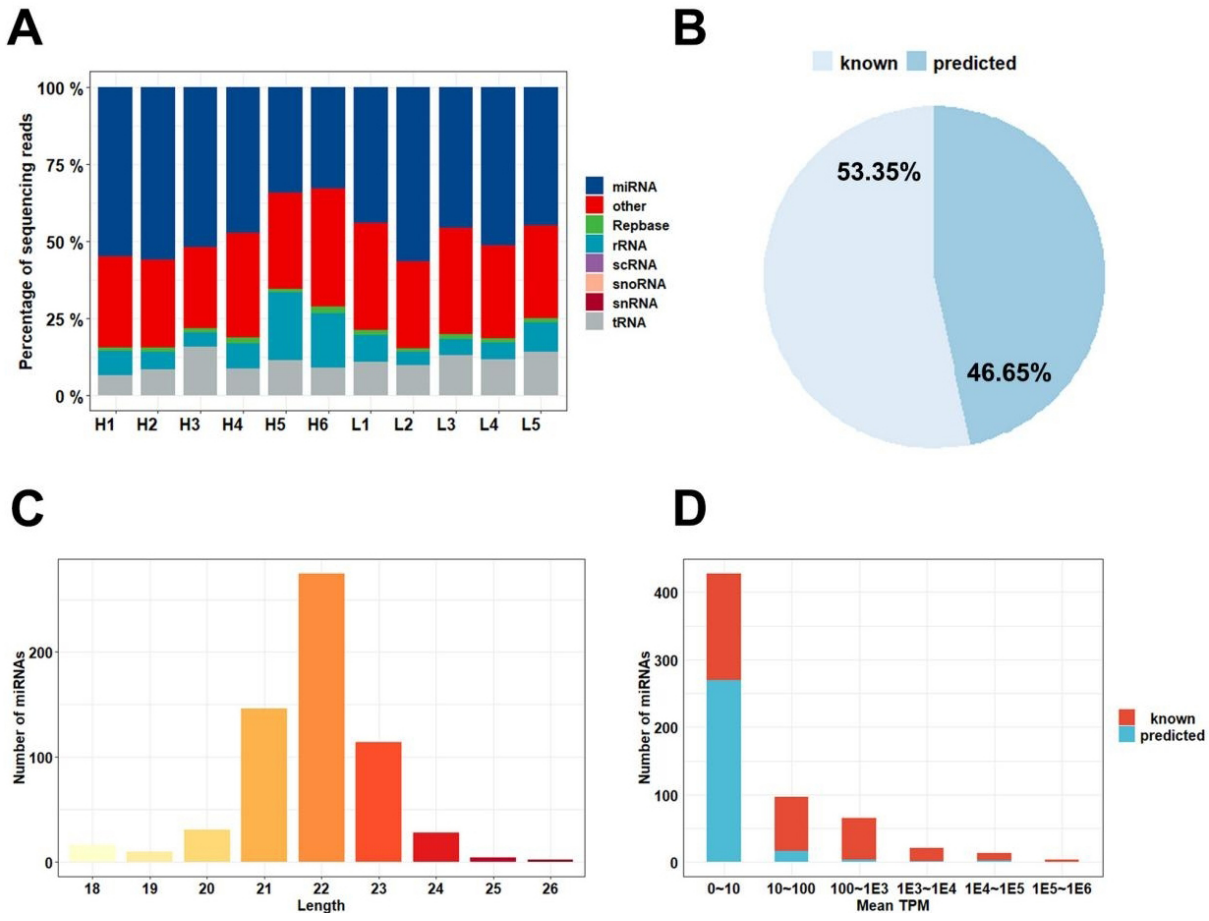

Figure 2. miRNA contents of SPEVs. (A) Read mapping distribution of the short noncoding RNA types in SPEVs. (B) Proportion of known and predicted miRNAs detected in pig SPEVs. (C) Distribution of the miRNA lengths. (D) Number of miRNAs with different expression levels. Most of the miRNAs were expressed at low levels, and 16 miRNAs were expressed at high levels (above 10,000 TPM).

levels (less than 100 TPM on average), and only 16 miRNAs were highly expressed (more than 10,000 TPM on average). The 20 most highly expressed miRNAs accounted for 92.04% of all miRNA-associated expression. The top four miRNAs with the highest expression levels belonged to the let-7 family (ssc-let-7c, ssc-let-7a, ssc-let-7f-5p, and ssc-let-7e), and these accounted for 64.51% of the top 20 miRNAs [Table 1].

Clustered miRNAs and correlation of expression

As recommended by miRBase, 10-kb windows were used to obtain clusters of miRNAs. All miRNAs detected in SPEVs were divided into 58 clusters [Supplementary Table 4]. In total, 163 miRNAs were included in these clusters, 42 of which were new miRNAs. To highlight genomically-clustered miRNAs that could be coexpressed, the correlations among miRNAs contained in the same cluster were calculated. Interestingly, for most clusters (40 out of 58), the miRNAs in the same cluster showed strong expression correlations ($|r| > 0.7$, $P < 0.05$; Supplementary Table 5). In addition, 91 miRNAs within 39 clusters showed a significantly positive correlation [Figure 3].

Identification and validation of differentially expressed miRNAs in SPEVs

In total, 27 DEmis were detected between the H and L groups [Supplementary Table 6]. Most of the DEmis (19 out of 27) showed higher expression in the boars of the L group [Figure 4A], and, among these DEmis, ssc-miR-223 exhibited the highest upregulation ($\log_2$FC = 5.97). To further understand the potential

Table 1. The 20 most expressed miRNAs in SPEVs

miRNA	Mean TPM	% of Top 20	% of miRNA
ssc-let-7c	218093.9	23.69%	21.81%
ssc-let-7a	157823.7	17.15%	15.78%
ssc-let-7f-5p	119953.7	13.03%	12.00%
ssc-let-7e	97927.36	10.64%	9.79%
unconservative_5_246044	77462.69	8.42%	7.75%
ssc-miR-148a-3p	39357.21	4.28%	3.94%
ssc-miR-10a-5p	27201.52	2.96%	2.72%
ssc-miR-10b	27187.77	2.95%	2.72%
ssc-miR-125b	19533.44	2.12%	1.95%
ssc-miR-21-5p	19362.82	2.10%	1.94%
unconservative_6_269105	16654.9	1.81%	1.67%
ssc-miR-200b	15842.16	1.72%	1.58%
ssc-miR-191	14447.83	1.57%	1.44%
ssc-miR-141	13231.56	1.44%	1.32%
ssc-let-7i-5p	11030.17	1.20%	1.10%
ssc-miR-30a-5p	10835.5	1.18%	1.08%
ssc-miR-30d	9248.16	1.00%	0.92%
ssc-miR-125a	9055.415	0.98%	0.91%
ssc-miR-16	8502.95	0.92%	0.85%
ssc-miR-26b-5p	7743.076	0.84%	0.77%

functions of the genes targeted by the DEmis, we performed gene ontology (GO) and KEGG pathway analyses using KOBAS. The target genes of the DEmis were predicted with miRanda and RNAhybrid, and 2579 target genes were predicted by both software programs. The enrichment analyses revealed 107 significant GO terms and 92 significant pathways related to the DEmis obtained from the comparison of the H and L groups (*FDR* < 0.05) [Supplementary Table 7]. The top 20 GO terms and pathways are shown in Figure 4B and C, respectively.

To validate the DEmis identified by small RNA sequencing, 10 DEmis were randomly selected for qPCR verification. The results show that nine miRNAs (ssc-miR-142-3p, ssc-miR-146a-5p, ssc-miR-155-5p, ssc-miR-223, ssc-miR-23a, ssc-miR-23b, ssc-miR-24-3p, ssc-miR-378, and ssc-miR-378b-3p) were significantly upregulated in the SPEVs of the L group compared with those of the H group, and one miRNA (ssc-miR-184) was significantly downregulated in the L group. These results are consistent with the small RNA-seq data [Figure 4D].

lncRNA and mRNA expression profiles in SPEVs

In total, 503 significant DEGs [Supplementary Table 8] and 106 significant DELs were identified from the comparison of SPEVs from the H group and those from the L group [Supplementary Table 9]. The heatmap revealed differences in the expression levels of the significantly dysregulated mRNAs and lncRNAs in the samples with different sperm motility levels [Figure 5A]. Most DEGs (271 out of 503) and DELs (62 out of 106) showed higher expression in the L group than in the H group [Figure 5B], and, among these DEGs, *HSPG2* and *FEN1* exhibited the highest upregulation ($log_2FC = 13.05$) and the highest downregulation ($log_2FC = -8.58$), respectively. Similarly, MSTRG.31099.1 and MSTRG.74876.3 showed the highest upregulation ($log_2FC = 10.94$) and the highest downregulation ($log_2FC = -9.01$), respectively, among the DELs. The gene ontology analysis identified 13 significant biological process categories (*FDR* < 0.05) [Figure 5C, Supplementary Table 10]. The pathway analysis revealed that ubiquitin-mediated proteolysis, pathways in

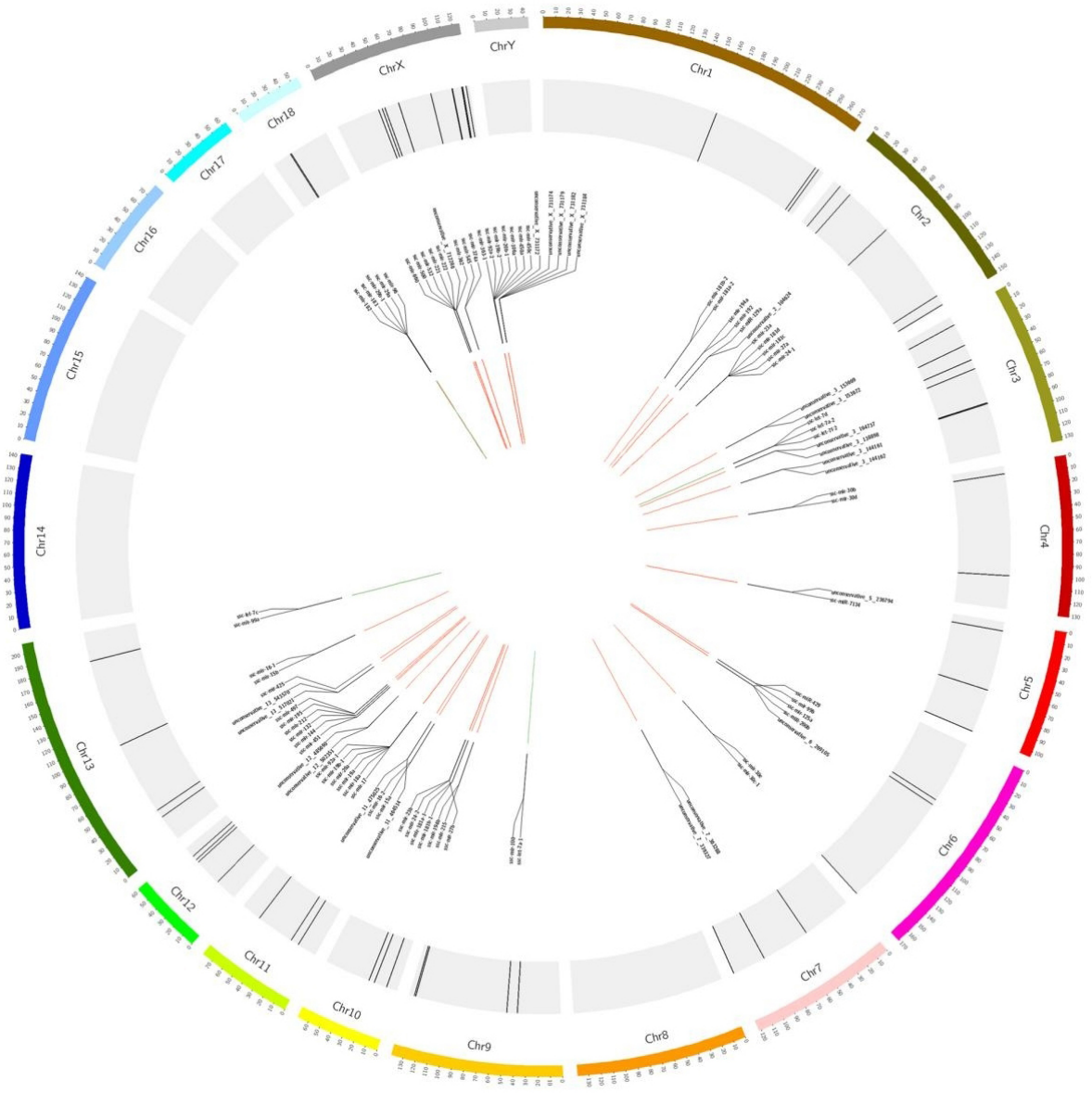

Figure 3. Distribution of miRNA clusters on pig chromosomes. The chromosomes are indicated on the outer circle. The genomic clusters are indicated on the middle circle by black lines. A subset of these miRNA clusters is shown on the inner circle. The correlation between miRNA pairs in the same miRNA cluster is shown in the center of the graph and the positive and negative correlations are indicated by red and green lines, respectively. All significant correlations ($|r| > 0.7$, $P < 0.05$) are visualized.

cancer, glycerolipid metabolism, endocytosis, microRNAs in cancer, and HIF-1 signaling pathway were the predominant biological processes represented [Figure 5D, Supplementary Table 10].

Gene coexpression modules associated with sperm motility

A weighted correlation network analysis (WGCNA) of all long RNA-seq data from SPEVs identified six coexpressed DEGs and DELs modules [Figure 6A], and the heatmap plot of the topological overlap matrix (TOM) is shown in Figure 6B. The DEGs and DELs in the six color modules were then continuously used to calculate their correlation with module traits. Interestingly, we found that the turquoise module, which included 202 DEGs and 54 DELs, was most significantly associated with high or low sperm motility (r =

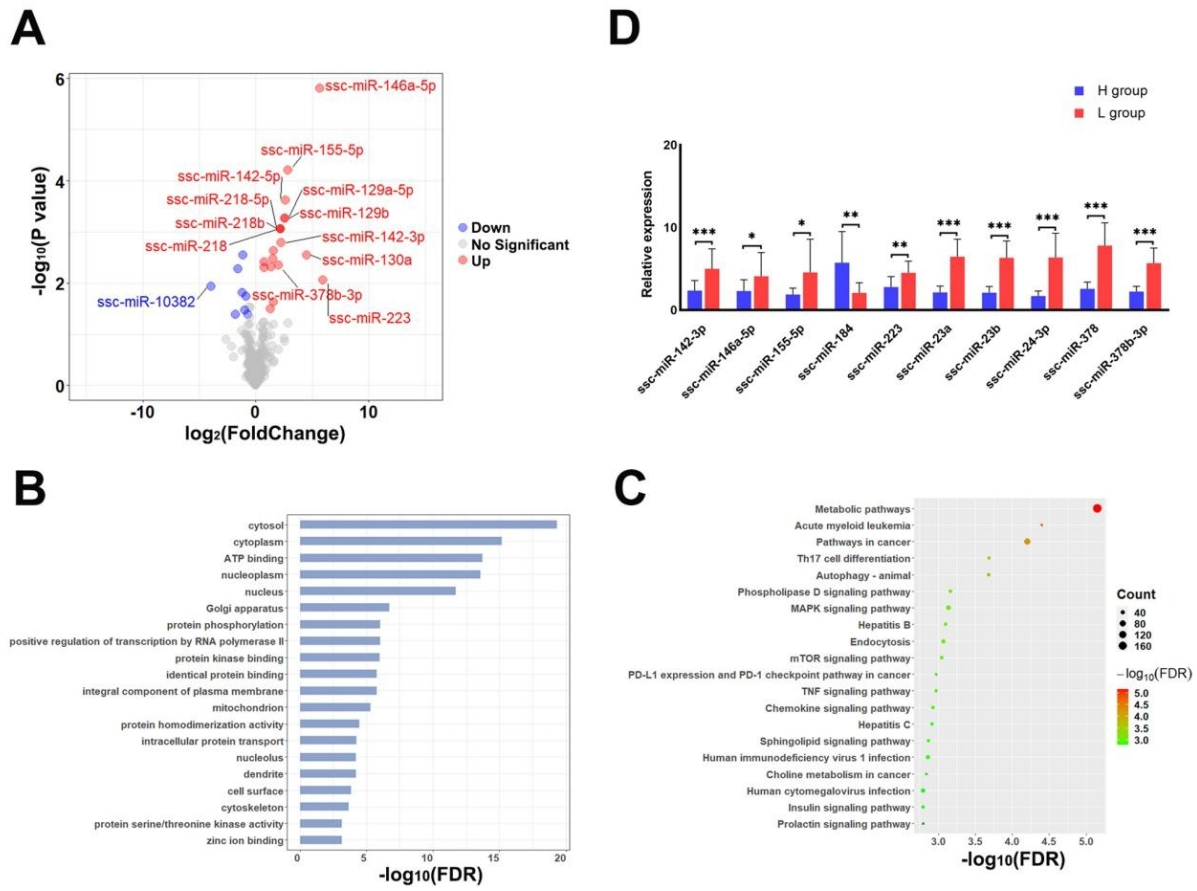

Figure 4. Identification, validation and functional enrichment analysis of DEmis in SPEVs. (A) Volcano plot displaying the DEmis between the SPEVs of the H and L groups. (B) Top 20 biological processes (FDR < 0.05) enriched with mRNAs targeted by DEmis. (C) The top 20 KEGG pathways (FDR < 0.05) enriched with the target genes of DEmis are shown in the bubble diagram. (D) The expression levels of ten DEmis were validated by qPCR.

0.98, *FDR* = 2 × 10^{-6}) [Figure 6C and D]. The second most significant module was the green module (r = 0.73, *FDR* = 0.0175), which included 19 DEGs and 14 DELs [Figure 6C]. We detected hub genes in each significant module and found 374 hub DEGs and 74 hub DELs [Supplementary Table 11].

According to the target relationship among the hub DEGs, DEmis, and DELs, the regulatory networks of SPEVs were constructed [Figure 7A]. In total, 23 pathways, such as microRNAs in cancer, glycerolipid metabolism, PPAR signaling pathway, IL-17 signaling pathway, HIF-1 signaling pathway, Jak-STAT signaling pathway, MAPK signaling pathway, mTOR signaling pathway, calcium signaling pathway, PI3K-Akt signaling pathway, and metabolic pathways, were included in the network [Figure 7A]. In total, 30 hub DEGs were annotated in these important pathways [Figure 7B]. Moreover, five DEmis (ssc-miR-582-5p, ssc-miR-378b-3p, ssc-miR-378, ssc-miR-1296-5p, and ssc-miR-24-3p) were predicted to interact with 29 hub DELs and six hub DEGs (*SLC8A3*, *ECSIT*, *ATP6B0B*, *RPL26L1*, *AKR1A1*, and *MYC*) in the turquoise module, which are involved in 11 signaling pathways [Figure 7C]. Nine DEmis, seven hub DELs, and thirteen hub DEGs in the blue module were involved in endocytosis, mTOR signaling pathway, lysosome, phagosome, metabolic pathways, NF-kappa B signaling pathway, PI3K-Akt signaling pathway, HIF-1 signaling pathway, and MAPK signaling pathway [Figure 7D]. It is worth noting that *MYC* in the turquoise

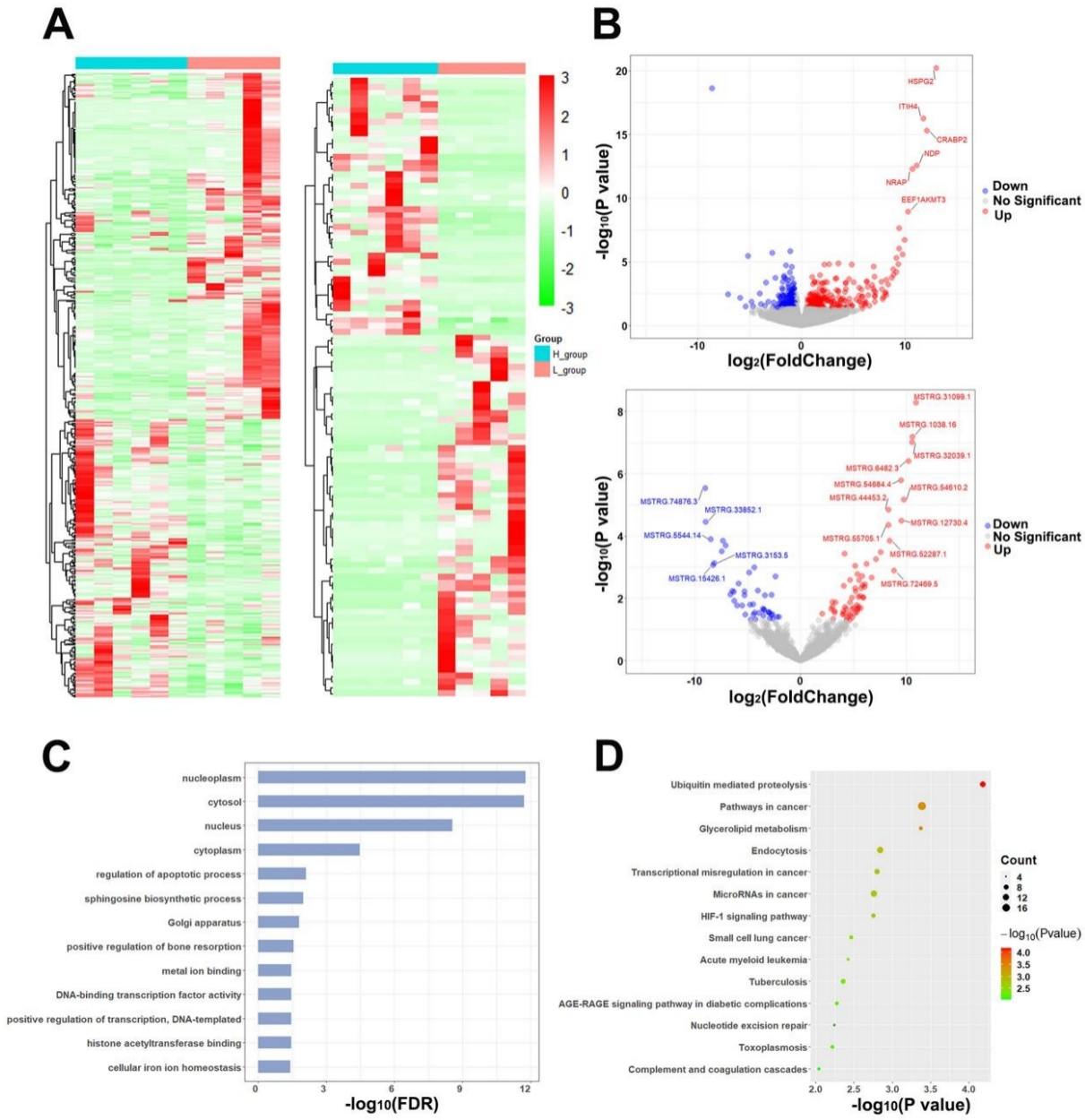

Figure 5. Differential expression and enrichment analysis of genes and lncRNAs in SPEVs. (A) Heatmap plots of DEGs (left) and DELs (right) across all the samples in our SPEV long mRNA dataset. (B) Volcano plot displaying the mRNAs (upper) and lncRNAs (below) showing differential expression between the SPEVs of the H group and those of the L group. (C) Bar plot showing the GO enrichment of DEGs (FDR < 0.05). (D) Bubble plot showing the KEGG enrichment of DEGs ($P < 0.01$).

module and *DDIT4* in the blue module can be targeted by ssc-miR-24-3p, and both of these are involved in the PI3k-Akt signaling pathway [Figure 7C and D].

SPEV-derived miRNAs as biomarkers of PCa

Based on seed sequences of miRNAs, most miRNAs (71.55%) were highly homologous between pigs and humans [Figure 8A]. We compared 27 DEmis detected in this study and found that 22 DEmis were highly conserved between pigs and humans. To avoid bias caused by some miRNAs with low expression levels,

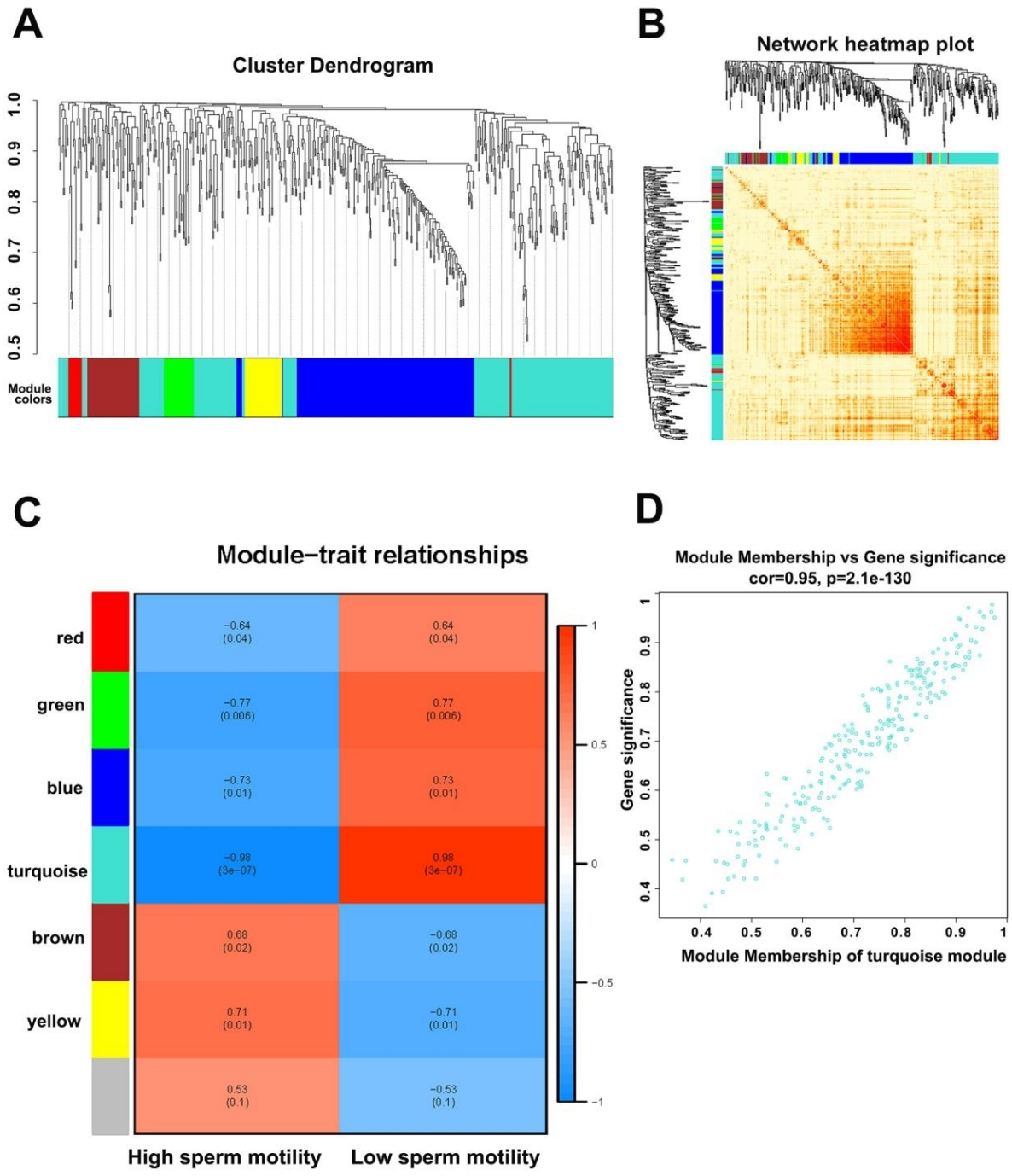

Figure 6. Weighted gene correlation network analysis (WGCNA) of SPEV DEGs and DELs. (A) A cluster dendrogram of the coexpression network module was produced based on the expression of DEGs and DELs. (B) A heatmap plot of DEGs and DELs in the network is shown. (C) The relationship of DEGs and DELs in various modules between the high-sperm-motility (H) and low-sperm-motility (L) group was investigated. (D) The turquoise module exhibited the highest relationship with sperm motility.

only 16 miRNAs (RPM > 1 in all samples) were included in the subsequent analysis. For the screening of potential biomarkers of PCa, we evaluated the specificity and sensitivity of each DEmi in the test set, which included 52 PCa cases and 30 controls. Our results suggest that most candidate DEmis displayed a sensitivity of 0.5-0.9 and a specificity of 0.6-0.9 [Figure 8B]. In addition, six miRNAs (hsa-miR-155-5p, hsa-

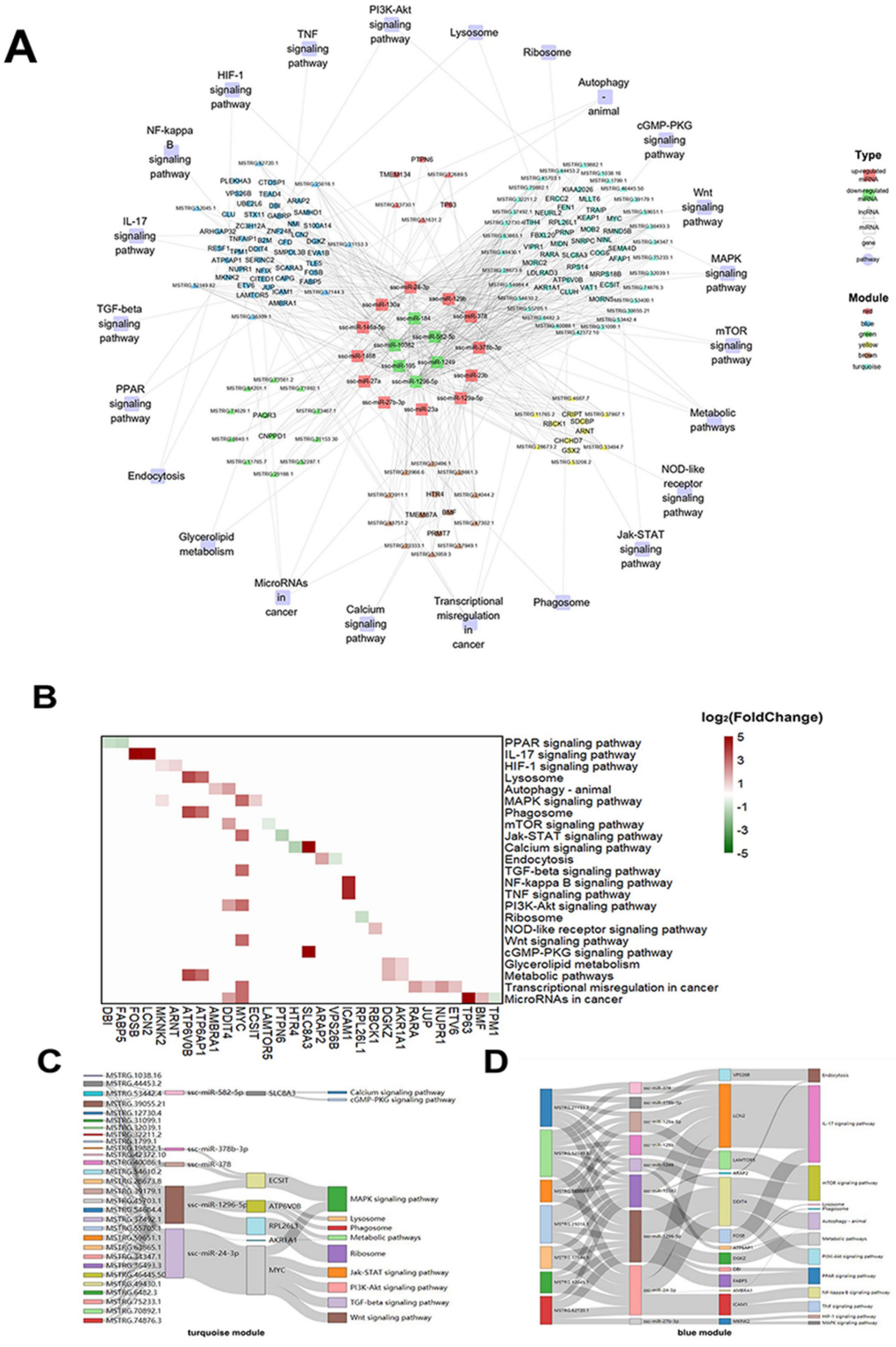

Figure 7. Regulatory DEL-DEmi-DEG network and relationships among DEmis, DELs and DEGs. (A) Regulatory network of DEL-DEmi-DEG pairs in pig SPEVs. The outer circle represents the important pathways related to sperm motility. The middle circle represents the six coexpression modules depicted in different colors. The inner circle displays the 12 upregulated miRNAs (red) and six down regulated miRNAs (green) in the L group. The hub DELs and DEGs are indicated by triangles and circles, respectively. The links show the DEL-DEmi regulatory relationships, DEmi-DEG regulatory relationships and DEG-pathway annotations. (B) Heatmap

demonstratingthe hub DEGs annotatedin important pathways. (green: downregulated in the L group, red: upregulated in the L group). (C) DEL-DEmi-DEG interaction network of the turquoise module. (D) DEL-DEmi-DEG interaction network of the blue module.

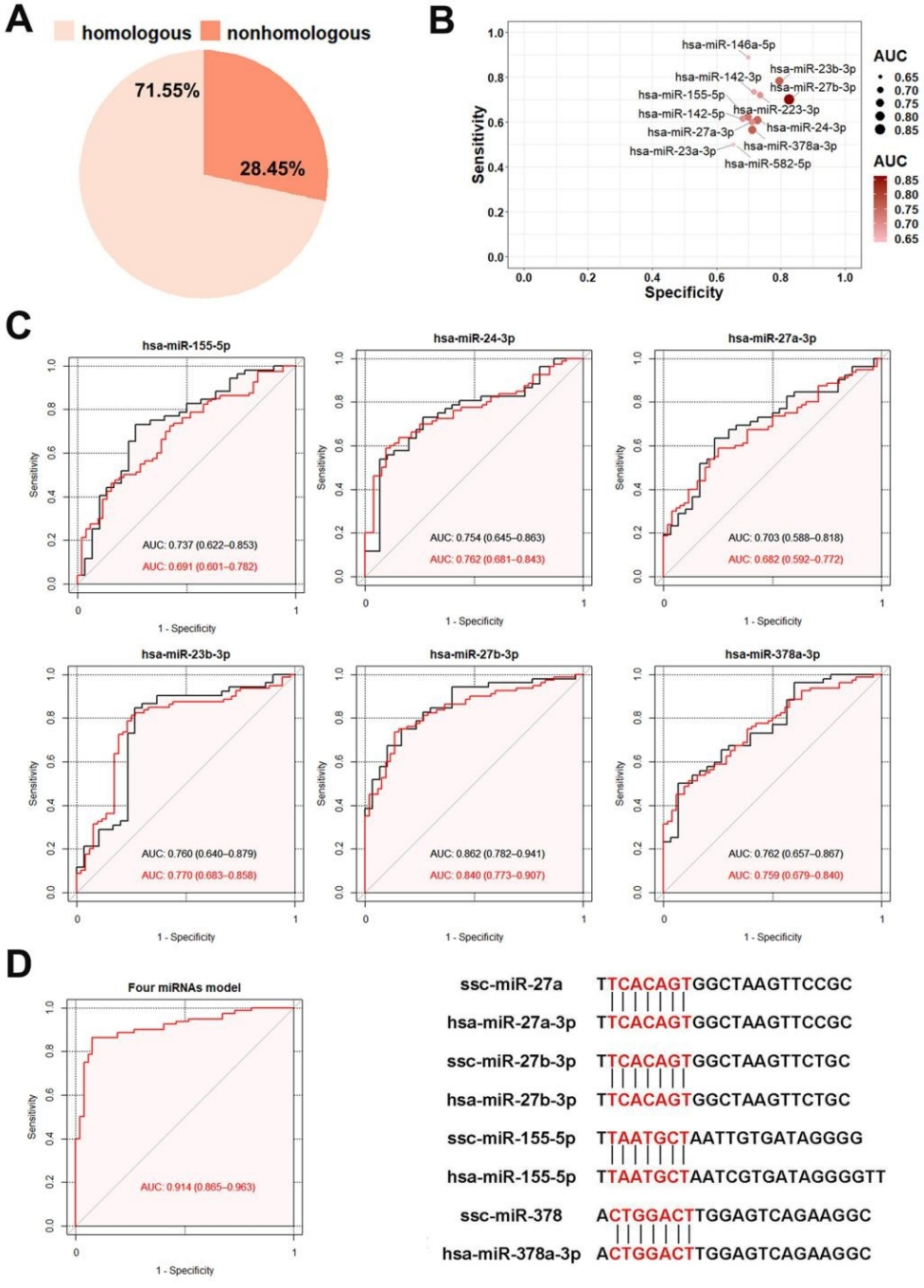

Figure 8. Comparison of miRNAs derived from SPEVs between pigs and humans. (A) Based on seed sequences of miRNAs, 71.55% were homologous between pigs and humans, and 28.45% did not show homology. (B) Twelve DEmis in the logistic module had a sensitivity of 0.5-0.9 and a specificity of 0.6-0.9. (C) ROC analysis of six DEmis in the test and validation sets (black is the test set, and red represents the validation set). (D) ROC analysis of the combination of four DEmis (hsa-miR-155-5p, hsa-miR-27a-3p, hsa-miR-27b-3p and hsa-378a-3p).

miR-24-3p, hsa-miR-27a-3p, hsa-miR-23b-3p, hsa-miR-27b-3p, and hsa-miR-378a-3p) provided high AUC values (> 0.7) for discriminating between patients with PCa and controls. To validate the specificity of these six miRNAs in the test set, logistic regression and ROC analyses were performed using the validation set of 132 individuals, which included 80 PCa cases and 52 controls. hsa-miR-155-5p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-378a-3p, hsa-miR-24-3p, and hsa-miR-23b-3p exhibited AUCs of 0.691, 0.682, 0.840, 0.759, 0.762, and 0.770, respectively, which were close to the AUC values calculated from the test set [Figure 8C]. Additionally, four candidate miRNAs with high AUC values were combined using a logistic model, and better performance was obtained. hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-155-5p, and hsa-miR-378a-3p exhibited an AUC of 0.914 [Figure 8D], which was the highest AUC of all the combinations.

Furthermore, we evaluated the mRNA composition of SPEVs using the CIBERSORTx website to identify the types and proportions of 22 immune cells based on cell markers. Interestingly, the proportions of different types of immune cells varied considerably, and we observed that resting memory CD4 T cells and monocytes were significantly enriched [Figure 9A]. Interestingly, we found that the percentage of resting memory CD4 T cells in the L group (27.9%) was slightly higher than that in the H group (24.1%), and the opposite results were found for monocytes, i.e., the percentage of monocytes in the L group (17.1%) was lower than that in the H group (21.7%). We also found that 33 DEGs detected in this study overlapped with the marker genes of monocytes, and most of these DEGs were upregulated in the L group [Figure 9B].

DISCUSSION

It has been reported that SPEVs can combine sperm *in vitro* and affect sperm function, particularly sperm motility, in mammals[55-57]. Here, we isolated EVs from semen plasma of Yorkshire boars using an ultracentrifugation procedure and performed whole transcriptome sequencing of SPEVs from Yorkshire boars with high or low sperm motility. Some important miRNAs, genes and lncRNAs affecting sperm motility in SPEVs were identified in this study.

Expression of miRNome in SPEVs of Yorkshire boars

A comparison of the small RNA libraries with different noncoding RNA databases revealed that most of the small RNAs in SPEVs were miRNAs. An analysis of the expression levels of these small RNAs showed that 83.71% of the miRNAs were expressed at low levels, and only 16 miRNAs were highly expressed in SPEVs. Among these miRNAs, five miRNAs (ssc-let-7c, ssc-let-7a, ssc-let-7f-5p, ssc-let-7e, and ssc-let-7i-5p) belonged to the let family. Similar results have been obtained in other studies. For example, let-7a, let-7c, let-7f, and let-7i were among the top 10 most abundant miRNAs detected in milk EVs of pigs[58,59], cattle[60], and humans[59,61]. let-7 is one of the earliest miRNAs discovered, and its family members are highly conserved in sequence and function. The let-7 family is reportedly related to human gamete differentiation and PCa[62]. let-7 plays an important role in mammals, such as participating in cell proliferation, differentiation, and apoptosis. Studies have shown that different members of the let-7 family are differentially expressed in the testis, sperm, and seminal plasma of patients with azoospermia, oligospermia, and asthenospermia[25,63]. In addition, previous studies have shown that miR-148a, miR-21-5p, and miR-125b are among the top 10 most abundant miRNAs in milk EVs and blood plasma EVs[64,65]. The high expression of these miRNAs in EVs from different tissues indicates that they might play an important role in the formation of EVs or some basic physiological function. However, we also found that some highly expressed miRNAs in SPEVs are different from those found in EVs derived from other tissues. For example, miR-10a, miR-10b, miR-125a, miR-16, and miR-26b-5p are only highly expressed in seminal plasma, which indicates that these miRNAs might play a particular role in the testis, epididymis, or sperm. It has been reported that miR-10b, miR-16, and miR-26b are related to PCa[66-68]. This finding also suggests the existence of differences in the types and contents of miRNAs in EVs, which might be related to the sorting of

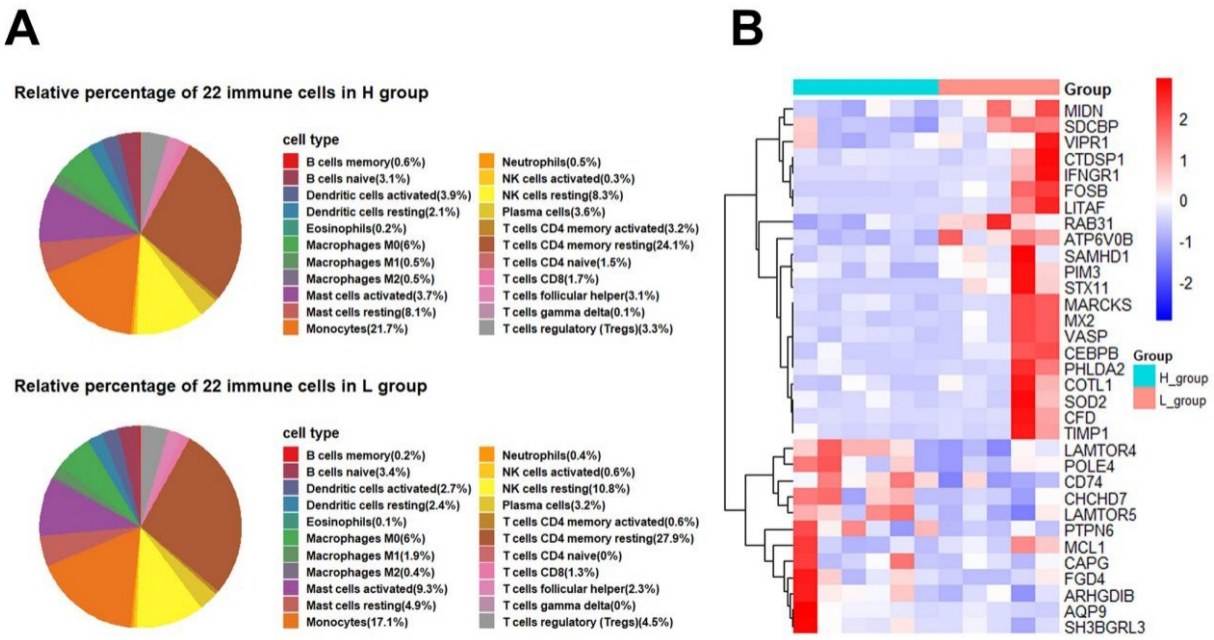

Figure 9. Proportion of different types of immune cells in SPEVs and expression of DEGs that overlap with immunocyte marker genes in the H and L groups. (A) Proportion of 22 immune cell populations in SPEVs of the H and L groups. (B) Heatmap of 30 DEGs that overlapped with monocytes marker genes.

miRNAs during the formation of EVs. Many studies have found that some genes or proteins are related to the sorting of specific miRNAs into exosomes[69]. In this study, we also found the DEG *RAB31*, which was recently reported to control an ESCRT-independent exosome pathway in exosome biogenesis[70]. These results suggest that some DEGs in EVs might affect the sorting of miRNAs in EVs.

Important DEmis, DEGs, and DELs related to sperm motility

In recent years, miRNAs have been recognized as key regulatory factors. It has been reported that miRNAs play an important role in sperm motility, azoospermia, and oligospermia. In the current study, a comparison of the H and L groups identified 27 DEmis, which included 18 important DEmis that were contained in the DEL-DEmi-DEG regulatory network. Interestingly, we observed two miRNA clusters, which included ssc-mir-23b, ssc-mir-24-2, and ssc-mir-27b and ssc-mir-23a, ssc-mir-24-1, and ssc-mir-27a, respectively. The positive correlation between the expression of the miRNAs in these two clusters was greater than 0.97 ($P < 7.74 \times 10^{-7}$ and $P < 6.18 \times 10^{-7}$, respectively). It has been reported that clustered miRNAs can coregulate and participate in many biological processes, such as metabolism, metabolic disorders, and cancer[71,72]. A previous study suggested that the expression of miR-23a, miR-23b, miR-27a, and miR-27b-3p in SPEVs of normal individuals was significantly different from that in SPEVs of individuals with oligozoospermia or asthenospermia[24,73]. Our results indicate that these miRNA clusters might play a synergistic role in regulating sperm motility.

Among the DEGs, ssc-miR-24-3p was predicted to be able to target and regulate 22 DEGs in the turquoise and blue modules, such as *MYC*, *MIDN*, *DDIT4*, *CTDSP1*, *ICAM1*, *CAPG*, and *LCN2*. ssc-miR-27b-3p can target *MKNK2* in the blue module and *PTPN6* in the red module. In addition, ssc-miR-23a and ssc-miR-23b simultaneously target *TMEM87A* in the brown module. Most of these genes were hub genes in the regulatory network and were found to be involved in HIF-1 signaling pathway, autophagy pathway in animals, MAPK signaling pathway, mTOR signaling pathway, Jak-STAT signaling pathway, TGF-beta

signaling pathway, PI3K-Akt signaling pathway, Wnt signaling pathway, transcriptional misregulation in cancer, and microRNAs in cancer. It has been reported that *MYC* is highly expressed in diseased prostate tissues and is a very important gene associated with PCa[74,75]. Some studies have suggested that *MYC* cooperates with the dysregulation of the PI3K/AKT/mTOR pathway to promote PCa cell survival and promote oncogenic signaling in prostate cancer[74]. In addition, previous studies have shown that *DDIT4* is significantly downregulated in prostate cancer cells and that the induction of *DDIT4* expression can regulate *MYC*, which is a downstream target of the mTOR signaling pathway[76,77]. Furthermore, *DDIT4* affects sperm motility through the autophagy pathway. Recent studies have shown that autophagy can degrade long-lived proteins and organelles and thus maintains the stability of spermatogenic cells, ensures sperm meiosis and spermatogenesis, and improves sperm motility[78,79]. However, high autophagy levels can also lead to the excessive consumption of protein and damage to organelles, which results in cell dysfunction and eventually leads to decreases in the number and motility of sperm[80,81]. The results of this study show that ssc-miR-1249, ssc-miR-1296-5p, and ssc-miR-24-3p act on mTOR, autophagy, and PI3K-Akt signaling pathways by targeting the *DDIT4* gene, respectively. Although these results remain to be confirmed, the findings suggest that miRNAs might target not only multiple components of a common pathway but also single components of different regulatory pathways.

lncRNAs can act as sponges of miRNAs to interact with miRNAs[82]. To explore the relationship between DELs and DEmis in SPEVs, we predicted the binding relationship between DELs and DEmis and calculated the correlation between the expression level of DELs and that of DEmis in the turquoise and blue modules. We found that 21 DELs and 5 DELs could target ssc-miR-24-3p in the turquoise and blue modules, respectively. Furthermore, our results show that the expression levels of five DELs (MSTRG.28673.8, MSTRG.39179.1, MSTRG.34347.1, MSTRG.52149.82, and MSTRG.37144.3) were significantly correlated with the expression level of ssc-miR-24-3p (correlation > 0.7, $P < 0.05$). Moreover, these DELs were closely related to *MYC*, *DDIT4*, *LCN2*, and *ICAM1* in the turquoise and blue modules. Some studies have suggested that the RNA in sperm can be derived from SPEVs[83]. Therefore, we believe that these DEmis, DEGs, and DELs in SPEVs play an important role in sperm motility and function.

Diagnosing human PCa by microRNAs derived from SPEVs

Fluid biopsy based on EVs is attracting increasing attention. Previous studies have shown that approximately 40% of semen is derived from prostatic tissue, and its contents are most likely to contain prostate disease-specific derived molecules, which can be potentially used as PCa biomarkers[31]. Due to the anatomical and physiological similarities between pigs and humans, pigs are increasingly regarded as an ideal model of human medicine. In this study, we found 16 DEmis that were highly conserved between pigs and humans by comparing the seed sequences of porcine and human miRNAs. Most of these are reportedly associated with PCa in humans. For example, miR-146a has been well studied in PCa, and several studies have shown that miR-146a inhibits the migration and invasion of PCa cells[84]. Several deregulated miRNAs in different liquid biopsies of PCa, such as mir-130a, mir-24, mir-223, and mir-155, have been reported[31,66,68]. Barcelo *et al.*[31] (2018) found that hsa-miR-142-5p, hsa-miR-142-3p, hsa-miR-130a-3p, and hsa-miR-223-3p are highly expressed in seminal exosomes of patients with PCa.

Based on the human PCa data in the TCGA database, we constructed diagnostic models for the 16 DEmis and found that six DEmis (hsa-miR-155-5p, hsa-miR-24-3p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-23b-3p, and hsa-miR-378a-3p) have good predictive and diagnostic abilities (AUC > 0.7). Using a logistic model, four miRNAs (hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-155-5p, and hsa-miR-378a-3p) were combined, and the AUC value increased to 0.914. Our results suggest that pigs can be used as an ideal animal model for the study of human prostate diseases. Moreover, porcine SPEVs can be used as good research material to obtain valuable information of human male reproductive diseases.

Close relationship between reproductive and immune characteristics

Recent studies have shown that the immune system is significantly related to a variety of reproductive traits[85]. In the present study, we found that some miRNAs in SPEVs are closely related to immunity in mammals. miRNAs are considered a critical regulatory molecule in the immune system[86]. It has been reported that miRNAs play an important role in the development, differentiation, and function of T cells and regulate general cellular biological processes in T cells, such as proliferation and apoptosis[86,87]. miR-155 is upregulated in B and T cells upon activation, and genetic gain- and loss-of-function studies have shown that miR-155 plays an important role in the control of germinal center reactions in vivo[88-90]. miR-146a exhibits a T cell subset-specific expression pattern and is involved in the processes underlying the regulation of specific T cell subsets[91-93]. The miR-17/92 cluster regulates T cell activation[94,95]. miR-17/92-deficient mice show increased pro-B cell apoptosis accompanied by a severe blockage of B cell development at the pro- to pre-B transition[96]. In contrast, the overexpression of miR-17/92 in mice results in the spontaneous activation and pronounced expansion of B and T lymphocytes[97]. In addition, the miRNA cluster consisting of miR-23a, miR-24, and miR-27a plays a critical role in the regulation of immune cell populations through the repression of B lymphopoiesis[98].

To further identify specific types of immune cells associated with sperm, we explored the components of immune cells using the CIBERSORTx website based on the mRNA expression of SPEVs obtained in this study. We found that resting memory CD4 T cells and monocytes were mainly enriched. However, studies on the immune cell components of plasma EVs from normal subjects and patients with hepatocellular carcinoma have shown that neutrophils, M2 macrophages, and other natural killer cells are the most abundant in healthy individuals[99]. The types of immune cells in semen EVs are different from those in plasma, which indicates that the sources and functions of immune cells in different types of EVs might be different. Additionally, we found that 33 DEGs were also marker genes of monocytes, and 30 of these genes, such as *RAB31*, *ATP6V0B*, *CHCHD7*, *LAMTOR5*, and *PTPN6*, were also hub genes in the DEL-DEmi-DEG regulatory network and can be considered important candidate genes for further verification.

In conclusion, the present study provided a comprehensive analysis of the whole transcriptome of Yorkshire boar SPEVs and revealed several important miRNAs, genes, and lncRNAs in SPEVs associated with sperm motility. hsa-miR-155-5p, hsa-miR-23b-3p, hsa-miR-27a-3p, hsa-miR-27b-3p, hsa-miR-24-3p, and hsa-miR-378a-3p might be used as promising biomarkers for the diagnosis of PCa. In addition, we found that reproductive traits exhibit a close relationship with immune traits and that resting memory CD4 T cells and monocytes are enriched in SPEVs. The results obtained in this study provide a new perspective and better understanding for further study of sperm motility in male mammals.

DECLARATIONS

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Authors' contributions

Conceived and designed the experiments: Jiang L
Analyzed the data: Zhang Y
Performed the experiments: Ding N, Xie S, Ding Y, Huang M
Contributed materials: Ding X
Wrote the paper: Zhang Y

Revised the manuscript: Jiang L
All authors read and approved the final manuscript.

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Not applicable.

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Conflicts of interest
All authors declare that they are bound by confidentiality agreements that prevent them from disclosing their conflicts of interest in this work.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

REFERENCES
1. Eisenberg ML, Betts P, Herder D, Lamb DJ, Lipshultz LI. Increased risk of cancer among azoospermic men. *Fertil Steril* 2013;100:681-5. DOI PubMed PMC
2. Husby A, Wohlfahrt J, Melbye M. Vasectomy and Prostate Cancer Risk: A 38-Year Nationwide Cohort Study. *J Natl Cancer Inst* 2020;112:71-7. DOI PubMed
3. Swindle MM, Makin A, Herron AJ, Clubb FJ Jr, Frazier KS. Swine as models in biomedical research and toxicology testing. *Vet Pathol* 2012;49:344-56. DOI PubMed
4. Schachtschneider KM, Madsen O, Park C, Rund LA, Groenen MA, Schook LB. Adult porcine genome-wide DNA methylation patterns support pigs as a biomedical model. *BMC Genomics* 2015;16:743. DOI PubMed PMC
5. Helke KL, Nelson KN, Sargeant AM, et al. Pigs in Toxicology: Breed Differences in Metabolism and Background Findings. *Toxicol Pathol* 2016;44:575-90. DOI PubMed
6. Kalluri R, LeBleu VS. function**,** and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
7. Mead B, Tomarev S. Extracellular vesicle therapy for retinal diseases. *Prog Retin Eye Res* 2020;79:100849. DOI PubMed
8. Margolis L, Sadovsky Y. The biology of extracellular vesicles: The known unknowns. *PLoS Biol* 2019;17:e3000363. DOI PubMed PMC
9. Sandfeld-Paulsen B, Jakobsen KR, Bæk R, et al. Exosomal Proteins as Diagnostic Biomarkers in Lung Cancer. *J Thorac Oncol* 2016;11:1701-10. DOI PubMed
10. Rodríguez M, Bajo-Santos C, Hessvik NP, et al. Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes. *Mol Cancer* 2017;16:156. DOI PubMed PMC
11. Yu S, Li Y, Liao Z, et al. Plasma extracellular vesicle long RNA profiling identifies a diagnostic signature for the detection of pancreatic ductal adenocarcinoma. *Gut* 2020;69:540-50. DOI PubMed
12. Sullivan R, Saez F. Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction* 2013;146:R21-35. DOI PubMed
13. Candenas L, Chianese R. Exosome Composition and Seminal Plasma Proteome: A Promising Source of Biomarkers of Male Infertility. *Int J Mol Sci* 2020;21:7022. DOI PubMed PMC
14. Drabovich AP, Saraon P, Jarvi K, Diamandis EP. Seminal plasma as a diagnostic fluid for male reproductive system disorders. *Nat Rev Urol* 2014;11:278-88. DOI PubMed
15. Machtinger R, Laurent LC, Baccarelli AA. Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Hum Reprod Update* 2016;22:182-93. DOI PubMed PMC
16. Baskaran S, Panner Selvam MK, Agarwal A. Exosomes of male reproduction. Elsevier; 2020. pp. 149-63. DOI PubMed

17. Vojtech L, Woo S, Hughes S, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res* 2014;42:7290-304. DOI PubMed PMC
18. Aalberts M, Stout TA, Stoorvogel W. Prostasomes: extracellular vesicles from the prostate. *Reproduction* 2014;147:R1-14. DOI PubMed
19. Du J, Shen J, Wang Y, et al. Boar seminal plasma exosomes maintain sperm function by infiltrating into the sperm membrane. *Oncotarget* 2016;7:58832-47. DOI PubMed PMC
20. Kalogianni DP, Kalligosfyri PM, Kyriakou IK, Christopoulos TK. Advances in microRNA analysis. *Anal Bioanal Chem* 2018;410:695-713. DOI PubMed
21. Kotaja N. MicroRNAs and spermatogenesis. *Fertil Steril* 2014;101:1552-62. DOI PubMed
22. Chen X, Li X, Guo J, Zhang P, Zeng W. The roles of microRNAs in regulation of mammalian spermatogenesis. *J Anim Sci Biotechnol* 2017;8:35. DOI PubMed PMC
23. Salas-Huetos A, James ER, Aston KI, Carrell DT, Jenkins TG, Yeste M. The role of miRNAs in male human reproduction: a systematic review. *Andrology* 2020;8:7-26. DOI PubMed
24. Abu-Halima M, Ludwig N, Hart M, et al. Altered micro-ribonucleic acid expression profiles of extracellular microvesicles in the seminal plasma of patients with oligoasthenozoospermia. *Fertil Steril* 2016;106:1061-1069.e3. DOI PubMed
25. Barceló M, Mata A, Bassas L, Larriba S. Exosomal microRNAs in seminal plasma are markers of the origin of azoospermia and can predict the presence of sperm in testicular tissue. *Hum Reprod* 2018;33:1087-98. DOI PubMed PMC
26. Fujita K, Nonomura N. Urinary biomarkers of prostate cancer. *Int J Urol* 2018;25:770-9. DOI PubMed
27. Wang J, Ni J, Beretov J, Thompson J, Graham P, Li Y. Exosomal microRNAs as liquid biopsy biomarkers in prostate cancer. *Crit Rev Oncol Hematol* 2020;145:102860. DOI PubMed
28. Bryzgunova OE, Zaripov MM, Skvortsova TE, et al. Comparative Study of Extracellular Vesicles from the Urine of Healthy Individuals and Prostate Cancer Patients. *PLoS One* 2016;11:e0157566. DOI PubMed PMC
29. Foj L, Ferrer F, Serra M, et al. Exosomal and Non-Exosomal Urinary miRNAs in Prostate Cancer Detection and Prognosis. *Prostate* 2017;77:573-83. DOI PubMed
30. Li Z, Ma YY, Wang J, et al. Exosomal microRNA-141 is upregulated in the serum of prostate cancer patients. *Onco Targets Ther* 2016;9:139-48. DOI PubMed PMC
31. Barceló M, Castells M, Bassas L, Vigués F, Larriba S. Semen miRNAs Contained in Exosomes as Non-Invasive Biomarkers for Prostate Cancer Diagnosis. *Sci Rep* 2019;9:13772. DOI PubMed PMC
32. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* 2017;7:789-804. DOI PubMed PMC
33. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41:D590-6. DOI PubMed PMC
34. Chan PP, Lowe TM. GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. *Nucleic Acids Res* 2016;44:D184-9. DOI PubMed PMC
35. Kalvari I, Argasinska J, Quinones-Olvera N, et al. Rfam 13.0: shifting to a genome-centric resource for non-coding RNA families. *Nucleic Acids Res* 2018;46:D335-42. DOI PubMed PMC
36. Bao W, Kojima KK, Kohany O. Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mob DNA* 2015;6:11. DOI PubMed PMC
37. An J, Lai J, Lehman ML, Nelson CC. miRDeep*: an integrated application tool for miRNA identification from RNA sequencing data. *Nucleic Acids Res* 2013;41:727-37. DOI PubMed PMC
38. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015;12:357-60. DOI PubMed PMC
39. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc* 2016;11:1650-67. DOI PubMed PMC
40. Pertea G, Pertea M. GFF Utilities: GffRead and GffCompare. *F1000Res* 2020;9:304. DOI PubMed PMC
41. Lv J, Cui W, Liu H, et al. Identification and characterization of long non-coding RNAs related to mouse embryonic brain development from available transcriptomic data. *PLoS One* 2013;8:e71152. DOI PubMed PMC
42. Kelley D, Rinn J. Transposable elements reveal a stem cell-specific class of long noncoding RNAs. *Genome Biol* 2012;13:R107. DOI PubMed PMC
43. Sun L, Luo H, Bu D, et al. Utilizing sequence intrinsic composition to classify protein-coding and long non-coding transcripts. *Nucleic Acids Res* 2013;41:e166. DOI PubMed PMC
44. Kong L, Zhang Y, Ye ZQ, et al. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res* 2007;35:W345-9. DOI PubMed PMC
45. Finn RD, Bateman A, Clements J, et al. Pfam: the protein families database. *Nucleic Acids Res* 2014;42:D222-30. DOI PubMed
46. Wang L, Park HJ, Dasari S, Wang S, Kocher JP, Li W. CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. *Nucleic Acids Res* 2013;41:e74. DOI PubMed PMC
47. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550. DOI PubMed PMC
48. Yan Z, Huang H, Freebern E, et al. Integrating RNA-Seq with GWAS reveals novel insights into the molecular mechanism underpinning ketosis in cattle. *BMC Genomics* 2020;21:489. DOI PubMed PMC
49. Wang M, Wang L, Pu L, et al. LncRNAs related key pathways and genes in ischemic stroke by weighted gene co-expression network

analysis (WGCNA). *Genomics* 2020;112:2302-8. DOI PubMed

50. Xie C, Mao X, Huang J, et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* 2011;39:W316-22. DOI PubMed PMC
51. Krüger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 2006;34:W451-4. DOI PubMed PMC
52. Li X, Gui Z, Han Y, et al. Comprehensive analysis of dysregulated exosomal long non-coding RNA networks associated with arteriovenous malformations. *Gene* 2020;738:144482. DOI PubMed
53. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13:2498-504. DOI PubMed PMC
54. Newman AM, Steen CB, Liu CL, et al. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol* 2019;37:773-82. DOI PubMed PMC
55. Carlini E, Palmerini CA, Cosmi EV, Arienti G. Fusion of sperm with prostasomes: effects on membrane fluidity. *Arch Biochem Biophys* 1997;343:6-12. DOI PubMed
56. Arienti G, Carlini E, Palmerini CA. Fusion of human sperm to prostasomes at acidic pH. *J Membr Biol* 1997;155:89-94. DOI PubMed
57. Arienti G. The motility of human spermatozoa as influenced by prostasomes at various pH levels. *Biology of the Cell* 1999;91:51-4. PubMed
58. Chen T, Xi QY, Ye RS, et al. Exploration of microRNAs in porcine milk exosomes. *BMC Genomics* 2014;15:100. DOI PubMed PMC
59. van Herwijnen MJC, Driedonks TAP, Snoek BL, et al. Abundantly Present miRNAs in Milk-Derived Extracellular Vesicles Are Conserved Between Mammals. *Front Nutr* 2018;5:81. DOI PubMed PMC
60. Cai M, He H, Jia X, et al. Genome-wide microRNA profiling of bovine milk-derived exosomes infected with Staphylococcus aureus. *Cell Stress Chaperones* 2018;23:663-72. DOI PubMed PMC
61. Simpson MR, Brede G, Johansen J, et al. Human Breast Milk miRNA, Maternal Probiotic Supplementation and Atopic Dermatitis in Offspring. *PLoS One* 2015;10:e0143496. DOI PubMed PMC
62. Nadiminty N, Tummala R, Lou W, et al. MicroRNA let-7c is downregulated in prostate cancer and suppresses prostate cancer growth. *PLoS One* 2012;7:e32832. DOI PubMed PMC
63. Zhou R, Zhang Y, Du G, et al. Down-regulated let-7b-5p represses glycolysis metabolism by targeting AURKB in asthenozoospermia. *Gene* 2018;663:83-7. DOI PubMed
64. Zhang J, Luo H, Xiong Z, Wan K, Liao Q, He H. High-throughput sequencing reveals biofluid exosomal miRNAs associated with immunity in pigs. *Biosci Biotechnol Biochem* 2020;84:53-62. DOI PubMed
65. Zeng B, Chen T, Luo JY, et al. Biological Characteristics and Roles of Noncoding RNAs in Milk-Derived Extracellular Vesicles. *Adv Nutr* ;2020:nmaa124. DOI PubMed
66. Hessvik NP, Phuyal S, Brech A, Sandvig K, Llorente A. Profiling of microRNAs in exosomes released from PC-3 prostate cancer cells. *Biochim Biophys Acta* 2012;1819:1154-63. DOI PubMed
67. Lodes MJ, Caraballo M, Suciu D, Munro S, Kumar A, Anderson B. Detection of cancer with serum miRNAs on an oligonucleotide microarray. *PLoS One* 2009;4:e6229. DOI PubMed PMC
68. Moltzahn F, Olshen AB, Baehner L, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res* 2011;71:550-60. DOI PubMed PMC
69. McKenzie AJ, Hoshino D, Hong NH, et al. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. *Cell Rep* 2016;15:978-87. DOI PubMed PMC
70. Wei D, Zhan W, Gao Y, et al. RAB31 marks and controls an ESCRT-independent exosome pathway. *Cell Res* 2021;31:157-77. DOI PubMed
71. Kabekkodu SP, Shukla V, Varghese VK, D' Souza J, Chakrabarty S, Satyamoorthy K. Clustered miRNAs and their role in biological functions and diseases. *Biol Rev Camb Philos Soc* 2018;93:1955-86. DOI PubMed
72. Chhabra R, Dubey R, Saini N. Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. *Mol Cancer* 2010;9:232. DOI PubMed PMC
73. Eikmans M, D H Anholts J, Blijleven L, et al. Optimization of microRNA Acquirement from Seminal Plasma and Identification of Diminished Seminal microRNA-34b as Indicator of Low Semen Concentration. *Int J Mol Sci* 2020;21:4089. DOI PubMed PMC
74. Rebello RJ, Pearson RB, Hannan RD, Furic L. Therapeutic Approaches Targeting MYC-Driven Prostate Cancer. *Genes (Basel)* 2017;8:71. DOI PubMed PMC
75. Ellwood-yen K, Graeber TG, Wongvipat J, et al. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer Cell* 2005;8:485. DOI PubMed
76. Tirado-Hurtado I, Fajardo W, Pinto JA. DNA Damage Inducible Transcript 4 Gene: The Switch of the Metabolism as Potential Target in Cancer. *Front Oncol* 2018;8:106. DOI PubMed PMC
77. Zhou Y, Wang Q, Guo Z, Weiss HL, Evers BM. Nuclear factor of activated T-cell c3 inhibition of mammalian target of rapamycin signaling through induction of regulated in development and DNA damage response 1 in human intestinal cells. *Mol Biol Cell* 2012;23:2963-72. DOI PubMed PMC
78. Shang Y, Wang H, Jia P, et al. Autophagy regulates spermatid differentiation via degradation of PDLIM1. *Autophagy* 2016;12:1575-92.
79. Huang Q, Liu Y, Zhang S, et al. Autophagy core protein ATG5 is required for elongating spermatid development, sperm

individualization and normal fertility in male mice. *Autophagy* 2020:1-15. DOI PubMed

80. Zhang J, Zhang X, Liu Y, et al. Leucine mediates autophagosome-lysosome fusion and improves sperm motility by activating the PI3K/Akt pathway. *Oncotarget* 2017;8:111807-18. DOI PubMed PMC
81. Liu S, Huang L, Geng Y, et al. Rapamycin inhibits spermatogenesis by changing the autophagy status through suppressing mechanistic target of rapamycin-p70S6 kinase in male rats. *Mol Med Rep* 2017;16:4029-37. DOI PubMed PMC
82. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. *Nature* 2014;505:344-52. DOI PubMed PMC
83. Jodar M. Sperm and seminal plasma RNAs: what roles do they play beyond fertilization? *Reproduction* 2019;158:R113-23. DOI PubMed
84. Iacona JR, Lutz CS. miR-146a-5p: Expression, regulation, and functions in cancer. *Wiley Interdiscip Rev RNA* 2019;10:e1533. DOI PubMed
85. Fang L, Cai W, Liu S, et al. Comprehensive analyses of 723 transcriptomes enhance genetic and biological interpretations for complex traits in cattle. *Genome Res* 2020;30:790-801. DOI PubMed PMC
86. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell* 2009;136:26-36. DOI PubMed
87. Kroesen BJ, Teteloshvili N, Smigielska-Czepiel K, et al. Immuno-miRs: critical regulators of T-cell development, function and ageing. *Immunology* 2015;144:1-10. DOI PubMed PMC
88. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science* 2007;316:608-11. DOI PubMed PMC
89. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science* 2007;316:604-8. DOI PubMed
90. Vigorito E, Perks KL, Abreu-Goodger C, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* 2007;27:847-59. DOI PubMed PMC
91. Rusca N, Dehò L, Montagner S, et al. MiR-146a and NF-κB1 regulate mast cell survival and T lymphocyte differentiation. *Mol Cell Biol* 2012;32:4432-44. DOI PubMed PMC
92. Yang L, Boldin MP, Yu Y, et al. miR-146a controls the resolution of T cell responses in mice. *J Exp Med* 2012;209:1655-70. DOI PubMed PMC
93. Lu LF, Boldin MP, Chaudhry A, et al. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell* 2010;142:914-29. DOI PubMed PMC
94. Wu T, Wieland A, Araki K, et al. Temporal expression of microRNA cluster miR-17-92 regulates effector and memory CD8+ T-cell differentiation. *Proc Natl Acad Sci U S A* 2012;109:9965-70. DOI PubMed PMC
95. Kouchkovsky D, Esensten JH, Rosenthal WL, Morar MM, Bluestone JA, Jeker LT. microRNA-17-92 regulates IL-10 production by regulatory T cells and control of experimental autoimmune encephalomyelitis. *J Immunol* 2013;191:1594-605. DOI PubMed PMC
96. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* 2008;132:875-86. DOI PubMed PMC
97. Xiao C, Srinivasan L, Calado DP, et al. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat Immunol* 2008;9:405-14. DOI PubMed PMC
98. Kurkewich JL, Bikorimana E, Nguyen T, et al. The mirn23a microRNA cluster antagonizes B cell development. *J Leukoc Biol* 2016;100:665-77. DOI PubMed
99. Li Y, Zhao J, Yu S, et al. Extracellular Vesicles Long RNA Sequencing Reveals Abundant mRNA, circRNA, and lncRNA in Human Blood as Potential Biomarkers for Cancer Diagnosis. *Clin Chem* 2019;65:798-808. DOI PubMed

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Extracellular Vesicles and Circulating Nucleic Acids

Mini-review

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Recent updates on the role of extracellular vesicles in the pathogenesis of allergic asthma

Ashokkumar Srinivasan, Isaac Kirubakaran Sundar

Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, University of Kansas Medical Center, Lawrence, KS 66160, USA.

Correspondence to: Dr. Isaac K. Sundar, PhD, Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, University of Kansas Medical Center, Lawrence, KS 66160, USA. E-mail: isundar@kumc.edu

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Abstract

Asthma is a chronic inflammatory disease of the airway diagnosed with different endotypes and phenotypes, characterized by airway obstruction in response to allergens, bacterial/viral infections, or pollutants. Several cell types such as the airway epithelial cells, mesenchymal stem cells and different immune cells including dendritic cells (DCs), T and B cells and mast cells play an essential role during the pathobiology of asthma. Extracellular vesicles (EVs) are membranous nanovesicles produced by every cell type that facilitates intercellular communications. EVs contain heterogeneous cargos that primarily depend on the composition or cell type of origin and they can alter the physiological state of the target cells. EVs encompass a wide variety of proteins including Tetraspanins, MHC classes I and II, co-stimulatory molecules, nucleic acids such as RNA, miRNA, piRNA, circRNA, and lipids like ceramides and sphingolipids. Recent literature indicates that EVs play a pivotal role in the pathophysiology of allergic asthma and may potentially be used as a novel biomarker to determine endotypes and phenotypes in severe asthmatics. Based on the prior reports, we speculate that regulation of EVs biogenesis and release might be under the control of circadian rhythms. Thus, circadian rhythms may influence the composition of the EVs, which alter the microenvironment that results in the induction of an immune-inflammatory response to various environmental insults or allergens such as air pollutants, ozone, diesel exhaust particles, pollens, outdoor molds, environmental tobacco smoke, etc. In this mini-review, we summarize the recent updates on the novel role of EVs in the pathogenesis of asthma, and highlight the link between circadian rhythms and EVs that may be important to identify molecular mechanisms to target during the pathogenesis of chronic inflammatory lung disease such as asthma.

Keywords: Asthma, extracellular vesicles, biomarkers, miRNAs, chronic lung disease

INTRODUCTION

Asthma is a chronic airway disorder and generally characterized by various symptoms like bronchial hyperreactivity, airway obstruction and inflammation-induced airway remodeling. It was previously described as simple allergic airway inflammation treated with antihistamine and prophylactic agents. Nevertheless, as our understanding of asthma grew deep and wide, it could no longer be considered as a diagnosis term like Parkinson's or Alzheimer's. It is an umbrella term that collectively describes the clinical symptoms such as wheezing, breathlessness, chest tightness and cough followed by etiology of asthma. Asthma may be manifested as different endotypes and phenotypes (young atopic, obese middle-aged and elderly asthma). Asthmatics develop clinical symptoms as a result of exposure to different environmental agents that ultimately affect the pathophysiology of the disease[1,2].

Allergens, air pollutants/particulates, ozone, diesel exhaust particles, pollens, microbial infections both bacterial and viral, outdoor molds, environmental tobacco smoke, obesity, exercise, cold air, humidity, genetic predisposition are among the known factors that lead to asthmatic complication either as a result of acute or chronic exposure. Until recently, all asthmatic patients were treated with the same medication. However, this resulted in varying responses to therapies due to the diverse nature of the disease. Therefore, identifying asthma based on the endotype is clinically important to personalize medication for the patients. Asthma is defined as a T2 and non-T2 endotype that includes a range of phenotypes that have similar symptoms, but varying pathophysiology[3]. T2 high endotype has three phenotypes [atopic, late-onset, Aspirin exacerbated respiratory disease] and non-T2 endotype has four phenotypes including non-atopic, smokers, obesity-related and elderly asthma. Both innate and adaptive immunity directly regulate asthma phenotypes along with the involvement of immune cells that play a crucial role in the pathogenesis of asthma. Epithelial cells and dendritic cells (DCs) play a major role in determining the type of T helper cells differentiation and activation via cytokine secretion and antigen presentation[4,5]. Apart from cytokines, the participation of exosomes in determining the type of immune response elicited against an allergen or environmental insult, is also increasingly getting attention in recent years[6,7]. In this review, we discuss the concept of extracellular vesicles (EVs) in determining the endotypes and phenotypes of asthma following exposure to different environmental insults. Understanding the novel role of EVs will help in finding new therapeutic targets for severe asthma that has a very poor clinical outcome.

Extracellular vesicles

EVs are heterogenous membranous vesicles that cannot replicate (lack of functional nucleus), which includes exosomes, ectosomes and apoptotic bodies. Based on the size, EVs can be classified as exosomes or small EVs (50-200 nm) or Ectosomes (< 200 nm) or apoptotic bodies (200-5000 nm). Exosomes are nano-sized vesicles made up of lipid bilayer membranes enclosing various mixtures of biological molecules, like DNA, RNA, proteins, and lipids[8,9]. Exosomes cannot be defined based on their size, as the size range overlaps with the size of microvesicles. Therefore, exosomes are defined based on the mechanism of biogenesis from the host cell. Exosomes are produced by the inward invagination of endosome membrane forming multivesicular bodies (MVB) and ultimately released out of the cell by fusion of MVB with the plasma membrane (exosomes are smaller than MVB themselves)[8,9]. In contrast, ectosomes (microvesicles or microparticles ~100-1000 nm in diameter) are produced by outward budding of the plasma membrane. The last class of EVs are called apoptotic bodies, which are considered as cellular debris and disregarded to have minimal biological function[9,10]. Depending on their function, exosome serves as a cargo vehicle for biologically active molecules to distant target tissue have been characterized[11,12]. Based on the set of different biomolecules packed into the exosomes, the physiological effect it exerts on the target cells may differ. For example, the exosomes derived from embryonic stem cells are enriched with mRNAs and proteins that are responsible for maintaining pluripotency of the cells and can transfer it to the target cells like hematopoietic stem cells[13]. Similarly, microRNAs (miRNAs) that are found in exosomes were shown to be transferred to the target cell and thus functionally silence the target gene[14,15]. The exact mechanism

behind sorting of specific proteins or molecules inside exosomes are not known. Prior studies have shown that exosomes secreted from the same cells can be packed with different protein profiles thereby proving that exosomes are packed with selective proteins or miRNAs[16,17]. Thus, exosomes represent a novel mode of intercellular communication, which may play a major role in many cellular processes, such as immune response, antigen presentation and signal transduction[18,19]. Exosome secretion provides cells with an advantage to rapidly release selective molecules and change target cellular response to environmental stimuli or phenotype of the cell. The roles of exosomes on lung pathology are being increasingly described and are being appreciated as immunogenic potentiators especially in the context of allergy[20]. Studies to understand the role of EVs in determining the endotypes and phenotypes of asthma (i.e., mediating pro-inflammatory response) following different environmental insults may help in devising novel therapies for asthma.

Plasma or serum EVs

Plasma or serum samples are easy to obtain from patients with non-invasive procedures that cause minimal pain during sample collection. As miRNAs are known to be highly stable in body fluids, EVs containing miRNAs from serum samples are best suited to study EV biomarkers in asthma. In a recent study, an abundance of miR-122-5p and miR-191-5p were increased in plasma-derived exosomes from patients with asthma without any difference in asthma severity (moderate-severe)[21]. They showed levels of miR-122-5p positively correlated with blood eosinophil and neutrophil counts, but not lung function. Similarly, levels of miR-191-5p showed a negative correlation with lung function (FEV1% pred) and percentage of blood lymphocytes. Prior studies showed a strong correlation with blood eosinophils and neutrophils counts/percentages in patients with asthma based on the specific clinical phenotype/endotype of the disease[22,23]. Both miR-122-5p and miR-191-5p identified in plasma-derived exosomes from asthmatics (mild-to-moderate or severe eosinophilic asthma) compared to healthy controls may be used as novel biomarkers in asthma after validating these findings using a larger cohort of samples.

Previously, miR-122-5p was known for its proinflammatory property in myocardial infarction and liver diseases[24,25]. Prior report showed miR-122-5p expression was increased during lipopolysaccharide (LPS)-induced acute lung injury. Treatment with miR-122-5p inhibitor showed protection against inflammation and injury via modulating DUSP4 (dual specific phosphatase-4) and ERK1/2 signaling pathway[26]. Based on the prior report, miR-122-5p may play an important role during lung inflammation/injury and therefore by blocking miR-122-5p expression using a specific inhibitor may offer protection against chronic inflammatory lung disease such as asthma. On the contrary, miR-191-5p promotes inflammation by interacting with key proteins and mRNAs that control Th-2 differentiation and function of APCs[21]. Interestingly, miR-191 was also reported to target Bmal1, which is a core clock component that controls circadian rhythms in mouse liver cells[27]. Therefore, miR-191 may be a possible key mediator that connects the circadian rhythms-exosome-inflammation axis in asthma. Additional evidence from equine model of severe asthma showed differential expression of several miRNAs (eca-miR-128, eca-miR-744, eca-miR-197, eca-miR-103, eca-miR-107a, eca-miR-30d, eca-miR-140-3p, eca-miR-7, eca-miR-361-3p, eca-miR-148b-3p and eca-miR-215) in serum that regulates airway remodeling and $CD4^+$ T cell maturation and differentiation[28]. In another human study, miR-125b and miR-126 were found to be increased in exosomes isolated from the serum of asthmatics[29,30]. Identifying plasma/serum-derived exosome containing miRNAs may shed light on the hidden molecular and cellular signaling mechanism that controls the immune status in asthma. Therefore, understanding the role of plasma/serum-derived EV miRNA signatures will be important for discovering new circulating biomarkers that can differentiate asthma phenotypes and may help develop targeted therapies in the future.

Bronchoalveolar lavage fluid EVs

The presence of exosomes in bronchoalveolar lavage (BAL) fluid was characterized for the presence of MHC II and co-stimulatory molecules like CD86 in normal human subjects[31]. Though exosomes carry

antigen-presenting and co-stimulatory molecules, they do not involve in antigen presentation. Rather they are taken up by professional APCs like dendritic cells, which helps in the activation of naïve T-cells[32]. The impact of secreted exosomes in allergic airways has been assessed using animal models. Mice challenged with ovalbumin (OVA) or house dust mite (HDM) show increased secretion of exosomes with different miRNA profiles compared to control mice. Moreover, another similar study showed that treatment with inhibitors of exosome secretion (GW4869) decreased lung inflammation by reducing proliferation and chemotaxis of monocyte[33,34]. Exosomes isolated from healthy control subjects and patients with mild intermittent asthma showed 24 differentially expressed miRNAs, which were related to airway inflammation. Let-7 and miRNA-200 family were able to discriminate asthmatics from normal subjects. In particular, miRNAs from miR-200 family that regulate epithelial-mesenchymal transition is downregulated in asthma. Pathway analysis revealed MAPK and JAK-STAT signaling pathway as the most significantly affected by a subset of exosomal miRNAs in asthma[35]. A detailed total RNA profiling showed mRNA, miRNAs and other small RNAs (tRNAs, rRNAs, snoRNAs and piRNAs) were significantly altered among healthy controls and severe asthmatics. They showed that several of the miRNAs were downregulated in severe asthmatics (miR-625-3p, miR-202-5p, miR-202-3p, miR-568, and miR-151a-5p) associated with reduced lung function (FEV_1) and asthma endotype/phenotype that involves neutrophilic (miR-224-5p, miR-581, miR-151a-5p, and miR-9-5p) and eosinophilic infiltration (miR-615-3p, miR-10b-5p and miR-151a-3p)[36] [Table 1 and Figure 1]. Isolation and characterization of BAL fluid exosomes at the molecular level (miRNA profiling) from normal *vs.* asthmatics is an emerging area of research. Studying BAL fluid EVs, their function and their effect on bronchial epithelial cells may open a wide range of possibilities to understand the mechanism of disease and develop new therapeutic strategies.

Exosomes are known to carry many biologically active molecules, but recently it has been discovered that exosomes from BAL fluid contain mitochondria. Especially the exosomes isolated from BAL fluid of asthmatic patients contain more mitochondria compared to healthy volunteers[37]. In addition, the study also showed that exosomes from the myeloid-derived regulatory cell ($HLA\text{-}DR^+$) in BAL could deliver mitochondria to $CD4^+$ T cells that induce oxidative stress in T cells to reduce inflammation[37]. So far, many studies have delineated the pro-inflammatory property of exosomes, but a study conducted using *BALB/c* mice has demonstrated the tolerogenic (anti-inflammatory) effect of exosomes *in vivo*. When BAL fluid-derived exosomes from mice tolerated to olive pollen (Ole e1) were administered to *BALB/c* mice prior to allergen challenge inhibited IgE response, Th2 cytokine production and airway inflammation by increasing TGF-β in the lung[38]. A significant portion of exosomes isolated from BAL fluid of patients sensitized to birch pollen contained mucin 1 indicating that they are mostly derived from bronchial epithelial cells. In addition, BAL fluid-derived epithelial exosomes were packed with leukotrienes synthesizing enzymes (LTA4H, LTC4S, FLAP, and 15-LO-1) and are highly capable of synthesizing LTB_4 leukotrienes compared to antigen-presenting cells (APCs)-derived exosomes. It acts as both chemoattractant for subsets of T lymphocytes and activates lymphocytes as well as DCs. Elevated levels of LTB_4 from BAL fluid have been reported to increase migration of DC to regional lymph nodes and cause airway hyperresponsiveness *in vivo* in mice[39-41].

Though most exosomes present in BAL fluid come from airway epithelial cells, a significant proportion of it also comes from resident alveolar macrophage (AM) that is present in the lung cavity. Suppressor of cytokine signaling 3 (SOCS3) plays a significant role in suppressing inflammatory cytokine response and its level were reduced in asthmatics and allergen challenged mice[42]. A recent study showed AM-derived EVs were enriched with SOCS3 blocking both STAT3 and STAT6 in human bronchial epithelial cells (BEAS-2B), when challenged with IL-4/IL-13 and HDM. Interestingly, AMs treated with cytokines such as IL-4, IL-33, TSLP and IL-25 contained a very low amount of SOCS3 in both the EVs and cell lysates[42]. SOCS3 loaded EVs from AMs may play an important role in the pathogenesis of asthma and synthetic SOCS3 encapsulated liposomes treated in cells and mouse model of allergic asthma showed attenuation of

Table 1. Extracellular vesicles secreted by structural and immune cells and their associated cellular and molecular functions

EV secreted cell-types/ sources	EV isolation and characterization methods	EV molecular signatures	Key findings/major outcomes	Ref.
BAL fluid	Differential ultracentrifugation; Flow cytometry; Immuno-electron microscopy	HLA-DR (MHC II), CD54, CD63, CD86 and MHC I	Human BALF-exosomes are enriched with antigen-presenting molecules like MHC II (HLA-DR), MHC I, CD54 and co-stimulatory molecules like CD86 Enrichment of surface markers in BALF exosomes isolated from different subjects varies widely	[31]
BAL fluid	Differential ultracentrifugation; Western blotting and FACS analysis	MHC I, MHC II and CD9; Presence of SP-B; Ole e 1 in BALF exosome from tolerized mice contains glycosylated and nonglycosylated forms of Ole e 1	BALF-exosomes from mice tolerized to Ole e 1 exosome blocks allergen-specific IgE and IgG_1 antibodies and reduced Th2 cytokines when pretreated with tolerogenic exosomes, but induces TGF-β	[38]
BAL fluid	Differential ultracentrifugation; FACS analysis	Tetraspanins (CD81, CD63), CD36, HLA-DR (MHC II), MUC1, LTA_4H, LTC_4S, FLAP and 15-LO-1	BALF-exosomes from asthmatics showed higher levels of CD81, CD63 and CD36 that correlate with HLA-DR BALF exosomes contained functional proteins that are specific to the leukotrienes pathway	[39]
BAL fluid	Differential ultracentrifugation with modification, FACS analysis; Exosomal miRNA by microarray and validated by RT-PCR	MHC II and CD63 (MHC class I, CD54 and CD86 were not detected); miRNAs: let-7c, let7b, miR-141, miR-200b, let-7d, let-7a, miR-21, miR-27a, let-7e, miR-34c-5p, miR-19b, miR-0022, miR-0024, miR-0026a, miR-0099a, miR-0200c, miR-1972, miR-665, miR-658, miR-483-5p, miR-1268, miR-0203, and miR-0130a	Identified 24 miRNAs in asthmatics *vs.* healthy control that showed a high correlation with FEV_1 Downregulated miRNAs: let-7c, let7b, miR-141, miR-200b, let-7d, let-7a, miR-21, miR-27a, let-7e, miR-34c-5p, miR-19b, miR-0022, miR-0024, miR-0026a, miR-0099a, and miR-0200c Upregulated miRNAs: miR-1972, miR-665, miR-658, miR-483-5p, miR-1268, miR-0203, and miR-0130a Validated 8 miRNAs: let-7a, miR-21, miR-658, miR-24, miR-26a, miR-99a, miR-200c, and miR-1268	[35]
BAL fluid	ExoQuick exosome precipitation method; qNano nanoparticle counter; TEM; Affymetrix Gene Chip miRNA 3.0 Array; qRT-PCR Validation	miRNAs: let-7a-5p, miR-702-5p, miR-762, miR-574-3p, miR-574-5p, miR-1827, miR-346, and miR-191-5p	BAL fluid EVs from HDM-treated mice showed an 8.5-fold increase compared to sham control BAL fluid EVs differentially express miRNAs in HDM *vs.* sham control: let-7a-5p, miR-702-5p, miR-762, miR-574-3p, miR-574-5p, miR-1827, miR-346, and miR-191-5p; Validated miRNAs in EVs: (upregulated: miR-346, miR-1827 and miR-574-5p) GW4869 treatment reduced EV release and EV miRNAs: miR-1827, miR-574-5p and miR-346 and HDM-induced allergic airway inflammation (differential cell counts and associated Th2 cytokines)	[34]
BAL fluid	Differential ultracentrifugation; NanoSight NS300; TEM; Image Stream analysis; Flow cytometry; SWATH lipidomic analysis	HLA-DR^+ and $CD54^+$ (ICAM-1); $CD9^+CD63^+CD81^+TSG101^+$ Phosphatidylglycerol 34:2, ceramide-phosphate 28:0, and ceramide 34:2	Increased expression HLA-DR^+ and $CD54^+$ in BAL fluid-derived EVs from asthmatics compared to healthy control Lipidomic analysis reveals reduced levels of phosphatidylglycerol, ceramide-phosphates, and ceramides in exosomes from asthmatics compared to healthy control Sphingomyelin 34:1 was abundant in secondhand-smoke-exposed asthmatics compared to healthy controls	[78]
BAL fluid	Differential ultracentrifugation; NanoSight NS300; TEM; Image Stream analysis; Flow cytometry; CryoEM	CD63, HLA-DR, mitochondrial DNA (mt-DNA) and mitochondria	EVs isolated from BAL fluid of asthmatics and myeloid-derived regulatory cells (MDRC)-derived exosomes contain mitochondria (MitoT-$Green^+$ and mitochondrial DNA) MDRC-derived exosomes contain polarized mitochondria that when transferred to T cells result in the formation of a mitochondrial network.	[37]
BAL fluid	Exo-Mir kit for nanovesicle RNA from 20 ml BAL fluid; Small RNA-seq analysis and Validation by qPCR	miRNAs: miR-625-3p, miR-202-5p, miR-202-3p, miR-568, miR-151a-5p, miR-615-3p, miR-10b-5p, miR-151a-3p, miR-224-5p, miR-581, and miR-9-5p	Specific human miRNAs were downregulated in severe asthmatics correlated with FEV_1 and immune-inflammatory phenotypes (eosinophilic *vs.* neutrophilic inflammation or Atopy)	[36]
NHBE and BAL fluid 	Differential ultracentrifugation; TEM; Immunoblotting	Tissue factor (TF), EGFR, and Annexin V	Compression stress-induced TF-containing exosomes in differentiated normal human bronchial epithelial cells (NHBE) TF levels were elevated in human BALF-derived exosomes from asthmatics	[53]

BAL fluid/ Epithelial cells 	Differential ultracentrifugation; Bead-based assay; ELISA; TEM; Flow cytometry	MHC II, HSP70, CD63	IL-13 treatment induced exosome secretion in BEAS-2B cells to facilitate monocyte proliferation and chemotaxis GW4869-mediated reduction in exosomes reduced monocyte/macrophage infiltration in the lungs of OVA-induced mouse model	[33]
Epithelial cells 	Differential ultracentrifugation with modifications; Nano-LC-ESI MS/MS analysis; Immuno EM and flow cytometry	MUC1, MUC4, MUC16, SNA lectin (α-2,6-linked sialic acid), keratan sulfate (5D4), CD59, CD63, MHC class I and II	Mucin and sialic acid lectin enriched exosome confer innate immune defense against viral infection	[50]
Epithelial cells 	Diffrential ultracentrifugation with modifications; NanoSight NS300; TEM; MS analysis; HTG EdgeSeq miRNA	Mucins (MUC3B, MUC13, MUC5AC and MUC5B) and miRNAs (miR-34/449, miR-223 and miR-29)	MUC4 is unique to human tracheobronchial epithelial cells (HTBE)-derived-exosomes and MUC13 and MUC3B are unique to Calu3-exosomes that confer innate defense and contribute to viscoelastic properties to airways Calu-3 treated exosomes showed increased expression levels of miR-3180 and miR-3180–3p Increased expression of these miRNA targets (miR-18a-5p, miR-19a-3p, miR-141-3p, miR-200a-3p, miR-200c-3p, miR-29a-3p, and miR-29b-1-5p) were observed in Calu-3 exosomes and subsequently when Calu-3 exosomes were treated in HTBE cells in their exosomes	[49]
NHBE 	qEV columns (size-exclusion chromatography); ExoQuick-TC reagent; PMX 110 scanning ZetaView; TEM, SeramiR miRNA prolifing; RT-qPCR	CD63, CD9 and CD81 miRNAs downregulated in IL-13 treated NHBEs: miR-210, miR-125a-5p, miR-34a, miR-92b, miR-210	IL-13 treatment alters the miRNA signatures (majority of the miRNAs were downregulated: e.g., miR-210, miR-125a-5p, miR-34a, miR-92b, miR-210, etc.) in apical and basal epithelial cell-derived EVs (both early *vs.* late) are involved in Th2 differentiation and DC maturation Nasal lavage sEVs showed decreased expression of miR-34a, miR-92b and miR-210 correlated with airway obstruction in children	[54]
Fibroblast 	Differential ultracentrifugation with modification; TEM; flow cytometry	CD63	Bronchial fibroblast-derived exosomes from severe asthmatics compared to normal bronchial fibroblasts (healthy control) showed lower levels of cytokine TGF-β2 and control epithelial proliferation and repair	[7]
Dendritic cells 	Differential ultracentrifugation with modification; Human microRNA microarray from Agilent; Flow cytometry	miRNAs: miR-335 miR-760, miR-632, miR-654-5p, miR-671-5p, miR-92a, miR-32, miR-101 and miR-21	Exosomal miR-335 (from primary DCs) is transferred from T cells to APC in an antigen-specific manner Transferred miRNA regulates gene expression of APC	[70]
Dendritic cells 	Differential ultracentrifugation; NanoSight LM10; Immuno-EM; Flow cytometry	MDDCs exosomes and exosomes rFeld1 contains HLA-DR, CD63 and CD81	Exosomes from monocyte-derived dendritic cells (MDDC) carry rFel d1 (cat allergen) and induce IL-4 production in PBMCs from cat-allergenic individuals Demonstrated distribution of aeroallergens via exosomes derived from DCs	[61]
Dendritic cells 	Differential ultracentrifugation; ELISA; Flow cytometry	CD63 and OX40L	TSLP-activated DCs released exosomes that are enriched in OX40L promote Th2 differentiation	[6]
T cells 	Differential ultracentrifugation; flow cytometry	Microvesicles from Jurkat T cells and human $CD4^+$ T cells and $CD8^+$ T cells clone contain CD3ε/ζ complex	Microvesicles from Jurkat cells and T lymphoblasts expressed CD3ε, TCR and CD63 Microvesicles from CD3-activated Jurkat cells and lymphoblasts showed expression of CD2, CD18, CXCR4, and MHC I and to a lesser extent MHC II	[71]
T cells 	Differential ultracentrifugation; Immunoelectron microscopy; FACS analysis	Microparticles from resting and activated T cells contain LFAα/CD11α	Microparticles-derived from activated T cells induces mast cell degranulation (increase in the release of β-hexosaminidase) Microparticles from activated T cells induce cytokine release (IL-8 and oncostatin M) and activate ERK phosphorylation in human mast cells	[74]
T cells 	Differential ultracentrifugation; Human microRNA microarray (Agilent); Flow cytometry	miRNAs are abundant in Jurkat-derived J77 T cell exosomes (miR-760, miR-632, miR-654-5p and miR-671-5p).	Differentially expressed miRNAs in T cells *vs.* T cell-derived exosomes: (upregulated: miR-760, miR-632, miR-654-5p, miR-671-5p) and (downregulated: miR-32, miR-101, miR-21)	[70]
T cells 	Differential ultracentrifugation; flow cytometry; mass spectrometry analysis	Proteins: RAS, ZAP70, RASGRP1, AKT, CD63 and CD81	Jurkat T cell-derived exosomes transfer proteins of RAS/MAPK signaling pathways (RAS, ZAP70, RASGRP1 and AKT) to mast cells and activate ERK phosphorylation *in vitro*	[73]

Tregs 	Differential ultracentrifugation; TEM; Flow cytometry	CD81, CD63 and CD73	TCR activation leads to the secretion of exosomes from $CD4^+$ $CD25^+$ Tregs enriched in CD73 mediates immune suppression via adenosine production	[81]
B cells 	Differential ultracentrifugation; Flow cytometry	MHC classes I and II, CD40, CD 54, CD63, CD80, CD81, CD86 and CD19	B cell-derived exosomes can bind peptides derived from Bet v 1 (Birch peptides) B cell-derived exosomes loaded with Bet v 1 peptides can induce T cell proliferation as well as increase cytokine production in allergen-specific T cells	[76]
B cells 	Differential ultracentrifugation; Human microRNA microarray from Agilent; Flow cytometry	miRNAs: miR-760, miR-632, miR-654-5p and miR-671-5p and miR-32	miRNAs abundant in Raji B cell exosomes (miR-760, miR-632, miR-654-5p and miR-671-5p) Differentially expressed miRNAs in B cells *vs.* B cell-derived exosomes: miR-760, miR-632, miR-654-5p and miR-671-5p (upregulated) and miR-32 (downregulated)	[70]
Eosinophils 	Differential ultracentrifugation; NanoSight LM10; TEM, flow cytometry, Immunoblot analysis	Eosinophil peroxidase (EPO), Major basic protein (MBP), Eosinophil cationic protein (ECP)	Eosinophil-derived exosomes were enriched with eosinophil granule proteins such as EPO, MBP and ECP without any significant difference among asthmatics *vs.* healthy controls	[85]
Eosinophils 	Differential ultracentrifugation; NanoSight LM10; Exosome proteins by RP-LC-MS/MS	EPO, MBP, ECP and periostin	High number of eosinophil-derived exosomes with basic proteins (EPO, MBP, and ECP) were detected in eosinophils from patients with asthma compared to healthy controls Eosinophil-derived exosomes produce ROS and NO	[84]
Eosinophils 	Differential ultracentrifugation	-	Eosinophil-derived exosomes from asthmatics delay wound healing, induce apoptosis and cytokine secretion (TNF, CCL26 and POSTN) in SAEC via PI3K/AKT and JAK-STAT signaling Eosinophil-derived exosomes from asthmatics increased expression of both angiogenesis and fibrosis markers (*CCR3* and *VEGFA*) in bronchial smooth muscle cells	[86]
Neutrophils 	Differential ultracentrifugation and size-exclusion chromatography; electron microscopy; NICOMP 30 device; Nano-LC-MS/MS	Phosphatidylcholine-sterol acyltransferase, Tenascin, Thrombospondin-1, Annexin A7, Neurogenic locus notch homolog protein 2, Lactotransferrin, Integrin-linked protein kinase, Protein S100A9, Fibrinogen A-α chain, Serpin peptidase inhibitor, clade B, member 1, Lipocalin 2, α-1-acid glycoprotein 2, Complement C3, Profilin-1, Triosephosphate isomerase, Integrin-β2	Identified proteins were differentially expressed between unstimulated *vs.* LPS-stimulated neutrophil-derived exosomes LPS-stimulated neutrophil-derived exosomes showed an increase in smooth muscle proliferation implicating their role in airway remodeling	[88]
Mast cells 	Differential ultracentrifugation	Hsp60 and hsc70	MC-derived exosomes induce DC maturation *in vitro* BMMC exosomes show selective enrichment of hsp60 and hsc70 MC-derived exosomes induce DCs to become efficient APCs	[91]
Mast cells 	Differential ultracentrifugation; FACS analysis; Immuno-EM; ELISA and MALDI-TOF-MS	Hsp60, hsc70, MHC II, CD86, CD40, CD40L, LFA-1 and ICAM-1	BMMC-derived exosome enriched in these surface markers (MHC II, CD86, CD40, CD40L, LFA-1 and ICAM-1) induces dendritic cells BMMC-derived exosomes loaded with BSA when injected in mice stimulate B and T lymphocytes	[92]
Mast cells 	Differential ultracentrifugation; TEM, Immunoblotting; FACS analysis; confocal microscopy	BMMC exosomes contained FcεRI	BMMC-derived exosomes can bind to free IgE via FcεRI BMMC-derived exosomes showed reduced airway inflammation, AHR in the OVA-induced allergic asthma	[94]
Mast cells 	Differential ultracentrifugation	-	MC-derived EVs upregulate epithelial-mesenchymal transition markers (*TGFB1*, *TWIST1*, *MMP9* and *BMP7*) at the transcript level in epithelial cells MC-derived EVs induced a mesenchymal-like phenotype and phosphorylation of several protein targets in epithelial cells	[97]

AHR: Airway hyperresponsiveness; BMMC: bone marrow-derived mast cell.

cytokine release and airway inflammation, respectively[42]. Hence, synthetic liposomes containing SOCS3 is an emerging therapeutic approach that may be used for the treatment of patients with asthma [Table 1 and Figure 1]. Targeting exosome-mediated LTB_4 and BLT1 (receptor) pathways may offer alternative

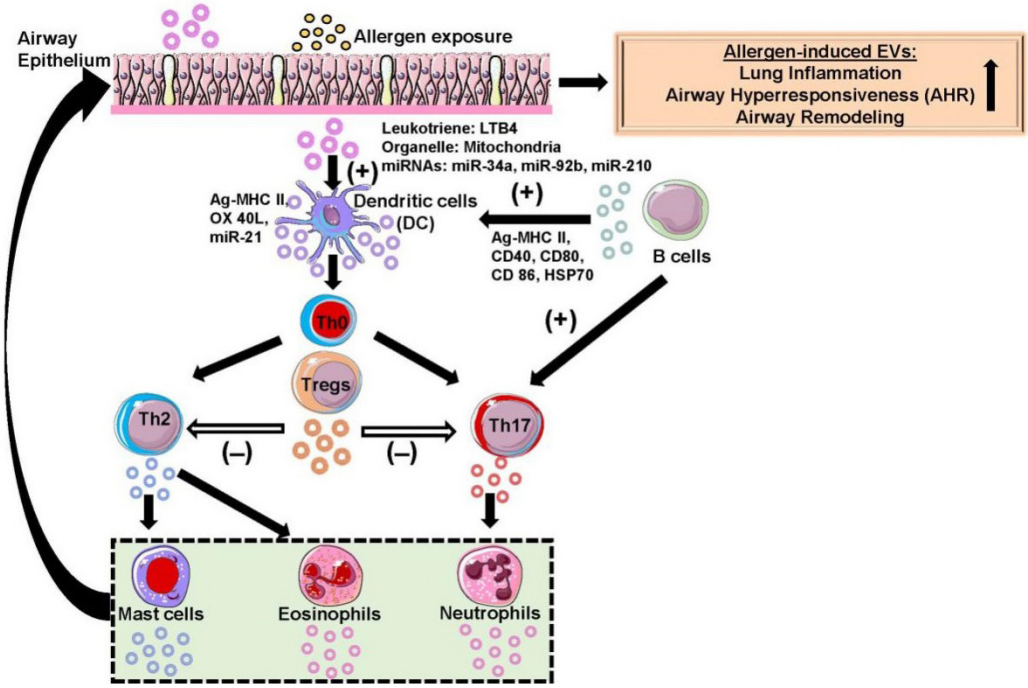

Figure 1. Schematic of extracellular vesicle (EV)-mediated signaling in the lung microenvironment during allergic asthma. A complex interaction occurs between different immune cell-secreted EVs and other target cells (recipient cells) that play a crucial role in the pathophysiology of allergic asthma. Secreted EVs consist of specific biomolecules (e.g., proteins, miRNAs) or organelles (e.g., mitochondria) that cause a phenotypic change in target cells resulting in altered asthmatic lung phenotypes (augmented lung inflammation, airway hyperresponsiveness, and remodeling). EVs have been shown to regulate tissue homeostasis during a normal state and affect target cells leading to the pathobiology of chronic airway disease during a diseased state. EVs released by different immune inflammatory cells (dendritic cells, Th2, Th17, and Tregs, B cells, mast cells, eosinophils, neutrophils, etc.) were represented by an appropriate color of the parent cell and the key biomolecules present in EVs affects the target cells. The directionality of EV-induced changes in recipient cells was indicated using an arrow. EVs that induce cell differentiation or maturation are indicated by a positive symbol (+) and if they inhibit cell maturation or anergy is indicated by a negative symbol (-). This schematic was prepared from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

therapeutic opportunities to patients with asthma that remain uncontrolled despite intensive corticosteroid treatment.

Nasal lavage fluid EVs

Lasser *et al*.[43] first reported the existence of EVs in nasal lavage fluid (NLF). They isolated NLF EVs using ultracentrifugation method and characterized by electron microscope, flow cytometer and Western blot analysis for EV- specific markers[43]. Like BALF, EVs from the nasal cavity are important for studying inflammation as nose is the first line of defense against inhaled particles such as dust, allergens and air pollutant. Initial observation made by the same group showed that even nasal EVs from healthy subjects were able to promote migration of immune cells like neutrophil, monocyte, and NK cells. Mucin-7, Mucin-5B, Immunoglobulin J chain, polymeric immunoglobulin receptor, filaggrin and hornerin were differentially altered in asthma and asthma with chronic rhinosinusitis condition compared to control[43]. Additionally, they also confirmed iNOS activity in isolated NLF EVs from healthy donors[44]. Nasal mucous EVs from allergic rhinitis (AR) and healthy controls were characterized for the presence of CD63 and HLA-DR markers. Additionally, they showed 21 miRNAs upregulated and 14 miRNAs downregulated in nasal EVs from AR group compared to healthy controls. Among them, 4 miRNAs (miR-30-5p, miR-199b-3p, miR-28-3p and miR-874) that are involved in B cell receptor signaling pathway were differentially expressed in nasal EVs from AR[45]. In a recent study, NLF EVs from asthmatics contained higher amounts of tenascin-C (TN-C) an immunomodulatory extracellular matrix protein in response to human rhinovirus

infection. They showed that TLR3, but not TLR7, activation in primary human lung epithelial cells and BEAS-2B cells resulted in TN-C associated EV release. These studies demonstrate TN-C associated EVs are relevant to the biology of viral-induced airway inflammation and exacerbations in asthmatics[46]. Compared to BAL fluid, NLFs are easier to collect and process for EV isolation. NLF has a greater advantage over BAL fluid as the nasal cavity forms the first line of defense against allergen and environmental insults. Therefore, early changes in EV release can be detected in nasal exosomes.

Sputum EVs

Limited studies are available that utilized sputum EVs in asthma. In a study, miRNA signatures from sputum EVs were analyzed in asthmatics. Sputum EVs contained exosome surface markers like CD9, CD63, and HLA-DR as measured by flow cytometry in induced sputum obtained from mild allergic asthma patients before and after allergen challenge. However, there was no significant difference in the surface markers and types of RNA present in sputum exosomes[47]. In another study, increased expression of sputum miRNAs (miR-223-3p, miR-142-3p and miR-629-3p) was associated with neutrophilic airway inflammation in mild to moderate and severe asthmatics. All the three miRNAs were upregulated in patients with severe asthma compared to mild asthma, showing a positive correlation with sputum neutrophil percentage and a negative correlation with sputum macrophage percentage[48]. The later study reported miRNA transcript levels in sputum sample rather than sputum EVs. Since miRNAs are richly packed in EVs from most biological fluids, this may be correlated with sputum EVs. Future studies exploring miRNA signatures from sputum EVs may be used as a potential diagnostic biomarker to differentiate asthma endotype or phenotypes (e.g., neutrophilic *vs.* eosinophilic asthma).

Epithelial cell-derived EVs

Epithelial cells are considered an inert physical barriers that protect against inhaled pathogens or allergens. Apart from providing physical and chemical barriers through mucus production, epithelial cells also modulate the immune response to pathogens and allergens evading the mucosal barrier. *In vitro* experiments revealed that exosome exchange between human tracheobronchial epithelial cells (HTBE) and Calu3 cells elevated mucin production (MUC5B and MUC5AC) in HTBE cells, and they are responsible for viscoelastic properties of airway mucus and airway remodeling *in vivo*. Also miRNAs miR-34/449, miR-223 and miR-29 involved in cilia biogenesis were found to be abundant in HTBE exosomes after exosomal exchange[49]. Epithelial cells are sensitive to external environmental stimuli such as allergens, bacterial and viral infections, or air pollutants and modulate the innate and adaptive immune response against them. HTBE secretions containing exosome-like vesicles rich in MUC1, MUC4, MUC16, and SNA lectin (α-2,6-linked sialic acid) have been shown to provide innate immune defense against lung pathogens[49,50]. Damage to epithelial cells caused by environmental exposure is a common feature in asthma. Ezrin a cytoskeletal protein found on exosomes has been reported to be downregulated in the serum of severe asthmatic patients, with airway epithelial damage caused by IL-13 overexpression[51,52]. Tissue factor is another protein present in epithelial cell-derived exosomes involved in airway inflammation and remodeling[53] [Table 1 and Figure 1]. Therefore, proteomic analysis of epithelial cell-derived EVs or BAL fluid exosomes could lead to the identification of a potential new circulating biomarker and novel therapeutic targets for the treatment and management of asthma.

Most of the immunomodulatory effects of epithelial cells were assigned to their cytokine profile, but later studies have shown that exosomes secreted from epithelial cells induced proliferation and chemotaxis of monocytes. On the other hand, inhibition of exosome secretion using an inhibitor (GW4869) is reported to reduce the proliferation of monocytes[33]. Depending on the miRNA profile of exosomes secreted by epithelial cells can affect the Th2 polarization and DC maturation response to an allergen or cytokine stimulation. IL-13 treatment in normal human bronchial epithelial cells modulated miRNA profile in secreted exosomes. The study showed miR-34a, miR-92b and miR-210 were significantly decreased in

exosomes secreted from epithelial cells[54]. All three miRNAs were involved in the regulation of immune cells, e.g., miR-34a is mainly involved in the differentiation of DCs and DC-mediated T cells activation[54-56]. Similarly, miR-210 and miR-92b are involved in Th1 and Th17 differentiation and epithelial-to-mesenchymal transition, respectively[57,58] [Table 1 and Figure 1]. Furthermore, future studies on molecular characterization of small RNAs using next-generation sequencing approaches will be possible to predict the difference in endotypes or phenotypes of asthma. Thus, it will enhance our understanding to develop alternative approaches that may be used for treating severe asthmatics. The stability of exosomes isolated from different sources and temperature conditions makes it difficult for further analysis of functional exosomes[59].

Dendritic cell-derived EVs

DCs play a key role in detecting and directing immune response at the mucosal surface where they constantly sample antigens. This allows them to determine T cell activation and polarization towards Th1, Th2, Treg, or Th17[60]. Dendritic cells are well known for their antigen-presenting properties, but they can also secrete exosomes bearing allergens on MHC II. A prior study showed that Fel d1 containing exosomes secreted by monocyte-derived dendritic cells (MDDCs) was able to elicit IL-4 cytokine response in PBMCs isolated from patients, allergic to cat allergen and not in healthy volunteers. In addition, those exosomes can also acquire free allergens and cause the production of IL-4 cytokine in PBMCs of allergic patients[61]. They also found that Fel d1 alone caused a similar response in PBMCs, so it is highly doubtful whether the observed effect is due to exosomes or allergen itself. Apart from allergens, exosomes also carry various protein ligand-like OX40L that can specifically help in the proliferation and differentiation of naïve $CD4^+$ T cells to Th2 cells[6]. Not only the exosomes secreted by dendritic cells, but those secreted by other cells can be taken up by dendritic cells also determine the immunoregulatory effect of DCs. Dendritic cells can also inhibit the differentiation of Th1 and promote Th2 through the STAT3 pathway by secreting exosomes packed with miR-21[15,62,63] [Table 1 and Figure 1]. These studies implicate that allergic airway inflammation and immune response during asthma can be reduced or exaggerated depending on the miRNA cargo present in DC-derived exosomes.

Most of the studies on dendritic cell-derived exosomes were primarily conducted either using BMDCs or MDDCs. The specific dendritic cell subsets like conventional types 1 and 2 dendritic cells (cDC1, cDC2s) and plasmacytoid dendritic cells have a very different role in asthma pathogenesis compared to other types. cDC1s are known for promoting $CD8^+$ T cell response, but upon HDM or OVA exposure they induce T cells to differentiate into T reg by producing retinoic acid and activating peroxisome proliferator-activated receptor γ. This helps cDC1s to suppress Th17 and Th2 differentiation upon allergen challenge[64,65]. On the other hand, cDC2s are efficient in taking up allergen and effectively migrate towards myeloid lymph nodes to induce Th2 differentiation after allergen challenge in lung airways in animal models[66-68] [Table 1 and Figure 1]. This emphasizes the need for extensive research that will thoroughly isolate and characterize EVs from specific subsets of dendritic cells to understand the mechanism by which allergens dictate the immune response differentially among individuals in a population. This will help in the clinical diagnosis of the endotypes or phenotypes of asthma and direct patients with better alternative therapies, especially among severe asthmatics.

T and B cell-derived EVs

T cells play a major role in the adaptive immune response during asthma pathogenesis, which are involved in IgE antibody class switching, Th2-related cytokine production, eosinophil recruitment and survival. The mouse model of allergic asthma has established that Th2-mediated immune response is necessary to reproduce features of human asthma[69]. APC-dependent allergen priming of $CD4^+$ T cells decides the endotype and phenotype of asthma. During this process, T cells form an immune synapse with APCs and secrete exosomes in an antigen-driven unidirectional transfer of exosomal miRNAs toward APCs[70]. This

mechanism ensures cellular communication between antigen-presenting cells and T cells for effective activation. Prior research demonstrates that T cell activation induces exosomes enriched in surface markers such as TCR-β, CD3ε, CD2, LFA-1 and CXCR4. The exact role of T cell activation-mediated exosome release remains largely unknown, but it has been speculated that they may interact with APCs complement peptide on MHC II to facilitate their function in target cells[71]. In another *in vitro* study, T cells activated by IL-2 and CD3/CD28 produced EVs enriched in specific tRNAs that repress the activation of $CD4^+$ T cells. It was hypothesized that by utilizing EV biogenesis pathway, T cells get rid of tRNAs that repress its activation as antisense oligonucleotides against the specific tRNA enhance the activation of $CD4^+$ T cells[72].

T cells are also responsible for mast cell activation, which has severe consequences in airway inflammation during asthma. Exosomes secreted from activated T cells have been reported to deliver activated Ras GTPase, ZAP70, RASGRP1 and AKT protein to mast cells thereby enhancing mast cell activation in airways[73,74]. Th2-mediated inflammation is also promoted by B cell exosomes that are carrying allergen peptides on MHC molecules. There has always been a concern about whether B cells can prime naïve $CD4^+$ T cells as they are not professional APCs. In a recent study, B cells have been shown to promote Th2 cytokine response through antigen presentation. Additionally, they showed B cell-restricted MHC II expressing mouse develops Th1 and Th17 immune response when challenged with HDM, but fails to develop Th2 response[75]. The antigen-presenting property of B cell-derived exosomes was shown using *in vitro* experiments. The study showed that birch allergen (Bet v1) loaded B cell exosomes induce T cell proliferation and secretion of IL-5 and IL-13 cytokines, which are key drives of airway inflammation and remodeling in asthma[76] [Table 1 and Figure 1]. The immunostimulatory effect of B cell exosomes may play an important role in driving Th2 response *in vivo*. This indicates that the complex interaction and communication between immune cells and APCs dictate specific phenotypic changes during the pathogenesis of asthma.

In a therapeutic approach, Treg cells hold a special place in asthma research as they can effectively ameliorate airway inflammation. Unique lipid signatures from airway exosomes are also reported to promote Th2 and Th17 polarization by modulating membrane fluidity[77]. Exosomes secreted by Tregs are referred to as tolerosomes that are quantitatively higher compared to other types of T cells and are regulated by intracellular calcium level and synthesis of sphingolipid[78,79]. CircRNA has been isolated and characterized from Treg exosomes, which have been proposed to function as RNA or protein decoy that modulates gene expression[80]. The exact mechanism and function of CircRNA remain unclear. This is another area where more research is needed to better understand the role of Treg-derived EVs in the pathogenesis of asthma. Treg exhibits an anti-inflammatory effect via exosomes expressing CD73 on their surface, which induces anti-inflammatory mediators like adenosine[81] [Table 1 and Figure 1].

Repeated exposure of low-dose allergen results in the development of regulatory T cells in the lung, so Tregs may be used as a potential therapeutic target for severe asthma[82]. Targeting Treg response in asthma may be an indispensable therapeutic approach, as bacterial exosome (*Pseudomonas aeruginosa*) sensitization in OVA challenged mice show reduced serum levels of IgE, Th2 response and increased Tregs in the lungs[83]. Hence, exosomes secreted by Tregs have been implicated to suppress the inflammatory response in different acute and chronic inflammatory diseases. Lack of cell-specific markers to identify the origin of cell-type specific exosome makes it difficult to characterize them in mixed population of EVs from different biofluids. As different types of T cell-specific exosomes carry different sets of miRNAs, they have a wide range of biological functions in asthma. Determining the specific mechanism by which T cell-derived exosomes control other immune cells may be used as a novel biomarker or therapeutic target to treat asthma.

Eosinophil-derived EVs

One of the prominent features of asthma is eosinophilia and eosinophils are linked with T2 asthma endotype. Eosinophils from asthma patients release more exosomes and they are enriched with eosinophilic proteins like eosinophil peroxidase, major basic protein and eosinophil cationic protein[84]. Eosinophil-derived exosomes in healthy and asthmatic conditions share common proteins and are not much different from one another. The only real difference observed was the number of exosomes secreted in asthma patients compared to healthy controls[85]. It also induces apoptosis in epithelial cells impeding wound closure and smooth muscle proliferation[86]. Eosinophil-derived EVs are unlike any other EVs, there is no report regarding eosinophil-derived EVs or exosomes containing miRNAs or other biomolecules. Limited studies are available that demonstrate the role of eosinophil-derived EVs in asthma[84,86] [Table 1 and Figure 1]. Further studies to characterize the exosomal cargo present in eosinophil-derived EVs such as miRNAs and lipid profiles may lead to a better understanding of how eosinophil-derived EVs alter airway remodeling and hyperresponsiveness during asthma.

Neutrophil-derived EVs

A small but significant number of people represent asthma with neutrophilia in airways and they have a poor clinical outcome with steroid treatment, unlike eosinophilic asthma. Although neutrophil infiltration can be seen in most asthma endotypes or phenotypes, it is more common in severe asthma[87]. Neutrophils play an important role in airway remodeling, inducing allergic inflammation by secreting cytokines, MMP9 and exosomes loaded with LTB4 or its synthesizing enzymes. Neutrophil-derived exosome induces smooth muscle cell proliferation as a result of their uptake by airway smooth muscle (ASM) cells[88,89] [Table 1 and Figure 1]. The proliferation of ASM cells can lead to airway remodeling and airway hyperresponsiveness leading to exacerbation of asthma in severe asthmatics. Additional studies to isolate and characterize neutrophil-derived EVs are needed to better understand their role in the pathogenesis of severe asthma where neutrophilic infiltration plays a crucial role.

Mast cell-derived EVs

Mast cells (MC) are known for their IgE-mediated effector function in host defense against parasites and allergens. Prior reports have demonstrated the role of mast cells in both innate as well as an adaptive immune response[90]. Exosomes from mast cells loaded with antigen (BSA, transferrin and OVA), hsp60 and hsc70 promote DC maturation and antigen presentation of DCs to T cells, thus providing additional evidence that antigen-loaded exosomes facilitate priming of naïve T cell[91]. Mast cells treated with IL-4 were able to produce active exosomes that contain immunologically relevant molecules like MHC-II, CD86, LFA-1 and ICAM-1. Therefore, mast cells can activate T and B lymphocytes through exosomes. Antigen-loaded exosomes from mast cells can mount antigen-specific immune response along with DCs[92]. Protein and RNA expression analysis revealed many targets that are involved in asthma pathogenesis. Exosome from bone marrow-derived mast cell (BMMC) carrying FcεRI inhibits IgE-mediated MC activation, whereas exosomes secreted by FcεRI activation in MC can cause pro-inflammatory response[93-95]. MC exosomes also help in shuffling miRNA between $CD34^+$ progenitor cells, controlling maturation processes[96]. Airway remodeling is one of the known features in asthma, and epithelial to mesenchymal transition (EMT) plays a significant role in remodeling. MC exosomes were able to initiate a phosphorylation cascade of proteins involved in EMT and upregulate matrix metalloproteases and TGF-β1[97] [Table 1 and Figure 1]. Therefore, identifying novel ways to selectively inhibit specific protein cargo packaged into mast cell-derived EVs might protect against airway inflammation and remodeling in asthma.

Mesenchymal stem cell-derived EVs

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells capable of differentiating into osteocytes, adipocytes, chondrocytes and hepatocytes. They are found in adipose tissue, bone marrow and umbilical cord. MSCs have been reported to have an immunomodulatory effect in the microenvironment of tissue by

Table 2. Mesenchymal stem cell (MSC)-derived EVs as novel therapeutics for allergic asthma

EV secreted cell-types	EV isolation and characterization methods	EV molecular signatures	Key findings/major outcomes	Ref.
MSCs 	Differential Ultracentrifugation; NanoSight NS300; TEM	-	Administration of either mMSC or hMSC-derived EVs differentially reduced airway hyperresponsiveness, lung inflammation, and Th1, Th2 and Th17 cytokines in *Aspergillus* hyphal extract-induced allergen model	[102]
Human AD-MSCs 	Differential Ultracentrifugation; Zetasizer Nano ZS90 system; SEM	-	Human adipose tissue-derived MSC (AD-MSC)-derived EVs reduced eosinophil counts in the lung tissue and BALF associated with reduced airway inflammation and remodeling in OVA-induced allergic asthma	[104]
Mouse AD-MSCs 	Bead-based isolation method; Dynamic light scattering (DLS); SEM; TEM	CD86, CD40, CD11c	Murine AD- MSC-derived exosomes reduced LPS-induced IL-6, IL-10, and TGF-β cytokine release in DCs Lymphocyte proliferation was reduced in DCs treated with MSC-derived exosomes MSC-derived exosomes suppressed the maturation of BMDCs that are key players in modulating DC-induced immune response	[99]
Human BM-MSC 	Differential Ultracentrifugation; TEM	CD9, CD81	MSC-derived exosomes promote Treg proliferation in PBMCs from asthmatics and healthy controls MSC-derived exosomes significantly increased anti-inflammatory cytokines IL-10 and TGF-β1 production in supernatant from PBMCs compared to control MSC exosomes incubated with PBMCs induced Tregs differentiation	[101]
Human iPSC MSC 	Differential ultracentrifugation; Anion-exchange chromatography; TEM	CD9, CD63, CD81	Human MSC-derived small EV (sEV) blocked ILC2 function in PBMCs from patients with allergic rhinitis in response to IL-2/IL-25/IL-33 Additionally, MSC-sEV reduced IL-9 and IL-13 release in the supernatants of sorted ILC2s following IL-2/IL-25/IL-33 treatment Systemic administration of MSC-sEV attenuated ILC2-dominant allergic airway inflammation caused by IL-33 exposed mice (reduced total cells, eosinophils and neutrophils and IL-5 and IL-13 release in BALF, mucus production including reduced ILC2s in the lung) RNA-seq analysis of MSC-sEV revealed miR-146a-5p transcript level significantly upregulated Finally, miR-146a-5p present in MSC-sEV role in modulating the effects of ILC2 *in vitro* in PBMCs and *in vivo* in mice were proven using miR-146a-5p inhibitor and mimics	[103]
Human BM-MSC 	Differential Ultracentrifugation; TEM; NTA	CD63, CD9, CD81 miR-21-5p, miR-142-3p, miR-126-3p.	Labeled MSC-EVs preferentially targets DCs when co-cultured with T cells as shown by colocalization of MSC-EVs and DCs MSC-EVs treated immature DCs reduced up take of FITC dextran suggesting their role in inhibiting DC maturation and antigen presentation MSC-EVs impairs DC migration to lymph nodes by reducing CCR7 expression MSC-EVs contained miRNAs (miR-21-5p, miR-142-3p, and miR-126-3p) which exerts an effect on DC maturation and function	[107]

secreting cytokines, immune receptors and EVs/exosomes[98]. A recent study demonstrates that exosomes secreted by mouse adipose tissue-derived mesenchymal stromal cells (AD-MSC) decreased interleukin-6 (IL-6), IL-10 and transforming growth factor-β (TGF-β) cytokine release by DCs. It also reduced DCs capacity to induce lymphocyte proliferation and suppressed maturation of bone marrow-derived dendritic cells (BMDCs)[99]. Previous studies showed administration of conditioned media or exosomes secreted from bone marrow-derived MSCs or other sources attenuating chronic pulmonary disease. Bone marrow-derived MSC exosomes rather than MSC themselves were effective in reducing IL-10 and TGF-β release by PBMCs from asthmatics[100]. Moreover, incubation of MSC exosomes with PBMCs increased Treg population and MSC administration in some cases have been reported to cause vascular occlusion and mal differentiation of MSCs. Using exosomes that have the same or better anti-inflammatory effect could help reduce the undesirable outcomes of the disease[101] [Table 2 and Figure 2].

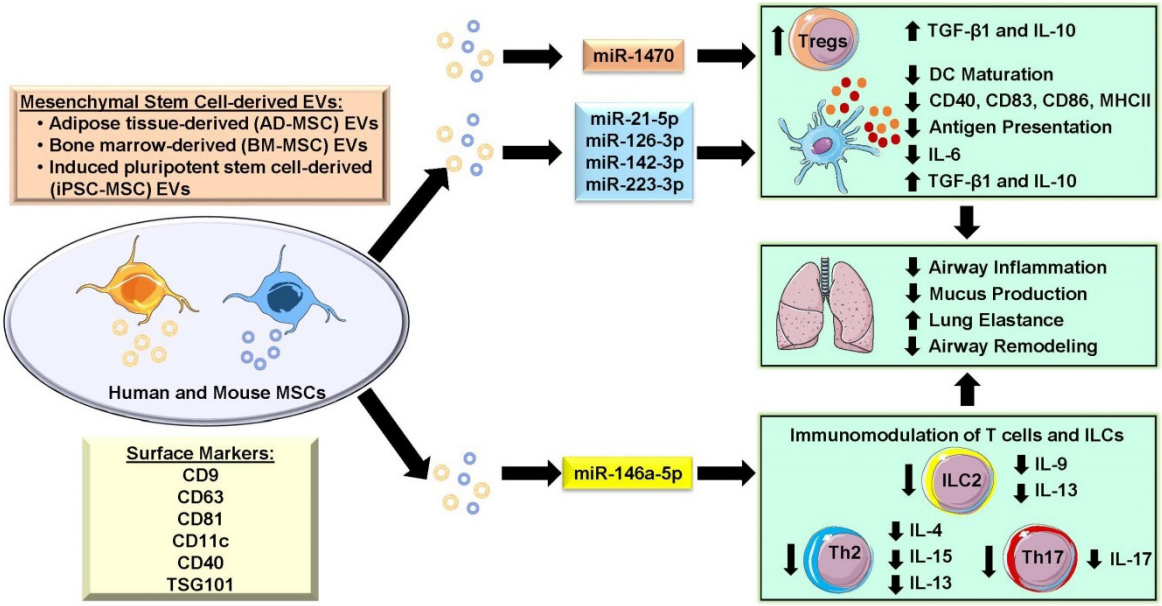

Figure 2. Role of mesenchymal stem cell-derived EVs as novel modulators of lung inflammation and airway remodeling in allergic asthma. EVs isolated from different sources such as bone marrow-MSCs (BM-MSCs), adipose tissues-derived MSCs (AD-MSCs), and induced pluripotent stem cell-derived MSCs (iPSC-MSCs) from human and mouse tissues showed protective response by regulating lung inflammation and remodeling in allergic asthma. MSC-derived EVs contain a wide range of miRNA cargo and other proteins that regulate various aspects of inflammation and immune response *in vitro* and *in vivo*. MSC-derived EVs have been shown to suppress the maturation of dendritic cells (DCs) by downregulating costimulatory molecules, preventing antigen sensitization, and by reducing inflammatory cytokine release in immune cells. Similarly, MSC-derived EVs promote Tregs differentiation, which leads to suppression of Th2 and Th17 immune response in eosinophilic and neutrophilic asthma by producing anti-inflammatory cytokines such as IL-10 and TGF-β1. MSC-derived EVs improve lung function by reducing airway inflammation and remodeling during allergic asthma. This schematic was prepared from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

Studies have also shown that exosomes from human MSCs were effective in reducing Th2 and Th17 cytokines and migration of neutrophil, macrophage and lymphocytes into the lung and BAL fluid in a mouse model of *Aspergillus* hyphal extract-induced allergic airway disease[102]. Innate lymphoid cell type 2 (ILC2) plays a very crucial role in airway inflammation and asthma. ILC2 mainly secrets IL-5 a cytokine that acts as a chemoattractant for eosinophils. MSC EVs were able to reduce IL-9$^+$ and IL-13$^+$ ILC-2, when incubated with PBMCs from patients with allergic rhinitis. In the same study, MSC EVs were able to reduce ILC2 population in the lung, BALF cytokines (IL-5 and IL-13), eosinophil and neutrophil counts in mouse intratracheally administered with recombinant IL-33[103]. In an ovalbumin model, exosomes derived from adipose tissue MSCs were able to reduce eosinophils, IL-4, IL-5, Eotaxin and CD4$^+$ T cells in the lung and thymus. Additionally, they also showed exosomes from MSCs were able to prevent airway remodeling and improve lung mechanics[104]. Anti-inflammatory effects of MSC EVs have been reported in a human study, where the patients affected by graft *vs.* host disease were treated with MSC EVs enriched with anti-inflammatory cytokines like IL-10 and TGF-β1. MSC EVs treatment in patients reduced their steroid dosage significantly[105]. This could be tested in patients with asthma as IL-10 and TGF-β1 were reported to play an important role in reducing airway inflammation[106] [Table 2 and Figure 2].

Another report, human bone marrow MSCs-derived EVs reduced maturation markers like CD83, CD38 and CD80 in dendritic cells. It also reduced the production of pro-inflammatory cytokines such as IL-6 and IL-12p70 and increased anti-inflammatory cytokine TGF-β1. The same study showed that miR-21-5p targeted CCR7 receptor degradation in DCs leading to decrease in response to CCL21 and reduced migration into T cell-rich area in the secondary lymphoid organ. This could lead to reduced T cell response to allergen thereby reducing inflammation and possibly eosinophilia or neutrophilia. Another candidate

miRNA enriched in MSC EVs is miR-126-3p, which abrogates Th2 response by targeting POU domain class 2 associating factor 1, which indirectly downregulates GATA3 via PU.1 in HDM-induced allergic asthma in BALB/C mice[107-109]. On the other hand, miR-142-3p and miR-223-3p were enriched in MSC EVs that augment inflammation by increasing alveolar smooth muscle cell proliferation associated with eosinophilic and neutrophilic infiltration[110,111]. Human bone marrow-derived MSC EVs containing miR-1470 show anti-inflammatory properties by increasing $FOXP3^+$ Tregs in PBMCs from acute asthmatics. miR-1470 in MSC EVs targets c-Jun mRNA in T cells thereby upregulating P27KIP1 which promotes Treg differentiation[112]. Therefore, MSC EVs rather than MSCs themselves may be used as novel therapeutics for the treatment of asthma [Table 2 and Figure 2].

Circadian rhythms and EVs

Evidence from the literature suggests that EVs could be key regulators of circadian rhythms synchronization between the "donor clock" and "recipient clock"[113]. The online EV database such as EVpedia and vesiclepedia provides us with sufficient data that relate to the presence of several proteins/mRNA and miRNAs in EVs[114-116]. The proteins such as PER2, PER3, CK1 families, AMPKβ, AMPKγ, GSK-3α, and GSK-3β and other miRNAs such as miR-27b-3p, miR-132, miR-142, miR-192, miR-194, miR-219a, miR-219b, miR-433 and miR-494 were present in EVs. The presence of these miRNAs in EV cargos could regulate clock function in recipient cells. It is noteworthy to determine how EVs participate in cell-to-cell communication between donor *vs.* recipient cells via clock-related molecules and mediate their function locally in tissues and systemically in the circulation[113]. Here are a few examples: EVs are responsible for modulating posttranslational modification of clock targets such as GSK3 (phosphorylation), thereby regulating core clock molecules BMAL-CLOCK and CRYs[117]. Additional reports suggest that non-coding RNAs in EVs essentially participate in the AMPK-mediated regulation of CRYs[118]. Specific miRNAs such as miR-132 and miR-219 have been shown to regulate PERs in a cell-type/tissue-specific manner[119-121]. A prior report showed a few of the circulating miRNAs such as miR-142-3p, miR-152 and miR-494 demonstrate diurnal oscillation (peaks at mid-day) and participate in the post-transcriptional regulation of Bmal1[122]. In another study, an immune cell type specifically macrophage-derived exosomes containing miR-155 mediates suppressed proliferation and enhanced inflammation in fibroblasts during cardiac injury[123]. However, it is unclear if these miRNAs are present in EVs as well or only present as freely circulating miRNAs in serum and how specific miRNAs target the clock-controlled genes (e.g., *bmal1*, *clock*, *n1d1*, *nr1d2*, *per1-2*, *cry1-2*, etc.) need to be further explored. A study from mouse model that mimics chronic night shift work revealed changes in plasma EVs, expression of clock genes in target tissues associated with altered metabolic function, and increased permeability of the colonic epithelial cell barrier[124].

The first evidence to demonstrate circadian variation exists in the release of EVs locally in lung tissue/bone marrow cells or systemically in the circulation comes from C57BL/6J mice entrained to a regular light-dark cycle (LD:12/12)[125]. EV isolation was performed in lung tissue, whole bone marrow cells and peripheral blood samples collected at 5 different circadian time points (ZT4, ZT8, ZT12, ZT16 and ZT24) revealed time of day-dependent difference in EV concentration[125]. Co-culture experiments showed lung-derived EVs alter transcript level of pulmonary specific mRNA and showed a time-dependent change in the uptake of EVs that modulate transcription (increased uptake at ZT20-ZT24)[125]. Another report shows the diurnal variation in circulating microvesicles (MVs) ($CD41^+$ and Annexin V^+) to the severity of obstructive sleep apnea (OSA) and the effect of continuous positive airway pressure treatment implicating the role of MVs in the pathobiology of OSA[126]. A recent report shows that plasma-derived exosomes from patients with OSA promote endothelial cell senescence (increased p16 and reduced SIRT1 and SIRT6 levels) via oxidative stress-related pathways[127]. Additionally, another report using a rat model showed circadian variation in the release of small EV/exosome marker protein. Tumor susceptibility gene 101 (TSG101) displays a circadian pattern to release urinary small EVs (concentration) and that can be used to normalize circadian variation

while testing for new EV biomarkers[128]. Overall, these data together suggest that circadian rhythms could play a vital role in modulating EV-mediated cell-to-cell communication locally in the lung and systemically in the circulation during normal (healthy) *vs.* diseased state (asthma) that requires thorough investigation in the future. To date there exist no reports that directly link circadian rhythms, EVs, and asthma pathobiology. Furthermore, based on the evidence from the literature there is a strong connection between circadian rhythms and asthma. However, future research will hold accountable to find the missing link and direct role of EVs in circadian rhythms and asthma pathophysiology using novel *in vitro* and *in vivo* approaches.

CONCLUSIONS AND FUTURE DIRECTIONS

EVs have demonstrated their prime role in causing augmented immune-inflammatory response and airway remodeling during asthma. Additionally, EVs/exosomes from specific cell types such as mesenchymal stem cells and T cells have been shown to mediate protection against chronic inflammatory lung disease. Novel drugs or small molecules that can specifically target EV/exosome trafficking[129] like calpeptin, manumycin A and Y27632, and lipid metabolism such as pantethine, imipramine and GW4869 may help reduce EV-mediated activation of target cells in asthma but will require further investigation. Future studies should investigate how EVs themselves or engineered with specific agents/drugs as biological vehicles can be used to reduce airway inflammation and remodeling in asthma. EVs as novel circulating biomarkers that can be used in the diagnosis, prognosis and therapeutics are expanding further due to the available tools and emerging isolation and characterization technologies[130] such as genomics/transcriptomics, lipidomics, proteomics, metabolomics and high-throughput screening to better understand the role and function of EVs in heath and disease of several different chronic inflammatory lung conditions. Overall, there is a greater need to address the knowledge gap to understand the role of EVs relating to chronic lung diseases. Future studies will provide deeper insights into the complex link between different cellular processes such as circadian rhythms, and associated molecular mechanisms that relate to EV biogenesis, trafficking, cargos (e.g., miRNAs, proteins, lipids, *etc.*), their function and EV-mediated signal transduction that occurs during the pathophysiology of allergic asthma.

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Authors' contributions

Conceived and designed the outline of this article: Sundar IK
Prepared the initial draft and compiled all the sections: Srinivasan A, Sundar IK
Prepared the tables and figures: Srinivasan A, Sundar IK
Edited and revised the manuscript: Srinivasan A, Sundar IK
All authors approved the final version of the manuscript.

Availability of data and materials

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Conflict of interest

The authors declare that this research work was conducted in the absence of any commercial or financial

relationships that could be construed as potential conflicts of interest. The updated review of literature and conclusions in this review article are solely those of the authors and do not represent the official views of the National Institute of Health (NIH)/National, Heart, Lung, and Blood Institute.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Chung KF, Wenzel SE, Brozek JL, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343-73.
2. Kaur R, Chupp G. Phenotypes and endotypes of adult asthma: moving toward precision medicine. *J Allergy Clin Immunol* 2019;144:1-12.
3. Kuruvilla ME, Lee FE, Lee GB. Understanding asthma phenotypes, endotypes, and mechanisms of disease. *Clin Rev Allergy Immunol* 2019;56:219-33.
4. Woodruff PG. Subtypes of asthma defined by epithelial cell expression of messenger RNA and microRNA. *Ann Am Thorac Soc* 2013;10:S186-S9.
5. Vroman H, Hendriks RW, Kool M. Dendritic cell subsets in asthma: impaired tolerance or exaggerated inflammation? *Front Immunol* 2017;8:941.
6. Huang L, Zhang X, Wang M, et al. Exosomes from thymic stromal lymphopoietin-activated dendritic cells promote Th2 differentiation through the OX40 ligand. *Pathobiology* 2019;86:111-7.
7. Haj-Salem I, Plante S, Gounni AS, Rouabhia M, Chakir J. Fibroblast-derived exosomes promote epithelial cell proliferation through TGF-β2 signalling pathway in severe asthma. *Allergy* 2018;73:178-86.
8. Bobrie A, Colombo M, Raposo G, Théry C. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* 2011;12:1659-68.
9. Théry C, Witwer KW, Aikawa E, 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. *J Extracell Vesicles* 2018;7:1535750.
10. Kakarla R, Hur J, Kim YJ, Kim J, Chwae Y-J. Apoptotic cell-derived exosomes: messages from dying cells. *Exp Mol Med* 2020;52:1-6.
11. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. *Expert Rev Proteomics* 2009;6:267-83.
12. Waldenström A, Gennebäck N, Hellman U, Ronquist G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS One* 2012;7:e34653.
13. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;20:847-56.
14. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654-9.
15. Montecalvo A, Larregina AT, Shufesky WJ, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 2012;119:756-66.
16. Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ. Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. *Mol Cell Proteomics* 2013;12:587-98.
17. Palma J, Yaddanapudi SC, Pigati L, et al. MicroRNAs are exported from malignant cells in customized particles. *Nucleic Acids Res* 2012;40:9125-38.
18. Barros FM, Carneiro F, Machado JC, Melo SA. Exosomes and immune response in cancer: friends or foes? *Front Immunol* 2018;9:730.
19. Kawahara H, Hanayama R. The role of exosomes/extracellular vesicles in neural signal transduction. *Biol Pharm Bull* 2018;41:1119-25.
20. Admyre C, Telemo E, Almqvist N, et al. Exosomes - nanovesicles with possible roles in allergic inflammation. *Allergy* 2008;63:404-8.
21. Bahmer T, Krauss-Etschmann S, Buschmann D, et al. RNA-seq-based profiling of extracellular vesicles in plasma reveals a potential role of miR-122-5p in asthma. *Allergy* 2021;76:366-71.
22. Zhang XY, Simpson JL, Powell H, et al. Full blood count parameters for the detection of asthma inflammatory phenotypes. *Clinical & experimental allergy* 2014;44:1137-45.
23. Gungen AC, Aydemir Y. The correlation between asthma disease and neutrophil to lymphocyte ratio. *Res J Allergy Immunol* 2017;1:1-4.
24. Cortez-Dias N, Costa MC, Carrilho-Ferreira P, et al. Circulating miR-122-5p/miR-133b ratio is a specific early prognostic biomarker in acute myocardial infarction. *Circulation journal* 2016;80:2183-91.
25. Jin Y, Wong YS, Goh BKP, et al. Circulating microRNAs as potential diagnostic and prognostic biomarkers in hepatocellular carcinoma.

Scientific Reports 2019;9.
26. Lu Z, Feng H, Shen X, et al. MiR-122-5p protects against acute lung injury via regulation of DUSP4/ERK signaling in pulmonary microvascular endothelial cells. *Life Sci* 2020;256:117851.
27. Na YJ, Sung JH, Lee SC, et al. Comprehensive analysis of microRNA-mRNA co-expression in circadian rhythm. *Exp Mol Med* 2009;41:638-47.
28. Pacholewska A, Kraft MF, Gerber V, Jagannathan V. Differential expression of serum microRNAs supports $CD4^+$ T cell differentiation into Th2/Th17 cells in severe equine asthma. *Genes* (*Basel*) 2017;8:383.
29. Zhao M, Juanjuan L, Weijia F, et al. Expression levels of microRNA-125b in serum exosomes of patients with asthma of different severity and its diagnostic significance. *Curr Drug Metab* 2019;20:781-4.
30. Zhao M, Li YP, Geng XR, et al. Expression level of miRNA-126 in serum exosomes of allergic asthma patients and lung tissues of asthmatic mice. *Curr Drug Metab* 2019;20:799-803.
31. Admyre C, Grunewald J, Thyberg J, et al. Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *Eur Respir J* 2003;22:578-83.
32. Mallegol J, Van Niel G, Lebreton C, et al. T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells. *Gastroenterology* 2007;132:1866-76.
33. Kulshreshtha A, Ahmad T, Agrawal A, Ghosh B. Proinflammatory role of epithelial cell–derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol* 2013;131:1194-203, 1203.e1-14.
34. Gon Y, Maruoka S, Inoue T, et al. Selective release of miRNAs via extracellular vesicles is associated with house-dust mite allergen-induced airway inflammation. *Clin Exp Allergy* 2017;47:1586-98.
35. Levänen B, Bhakta NR, Torregrosa Paredes P, et al. Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. *J Allergy Clin Immunol* 2013;131:894-903.
36. Francisco-Garcia AS, Garrido-Martín EM, Rupani H, et al. Small RNA species and microRNA profiles are altered in severe asthma nanovesicles from broncho alveolar lavage and associate with impaired lung function and inflammation. *Noncoding RNA* 2019;5:51.
37. Hough KP, Trevor JL, Strenkowski JG, et al. Exosomal transfer of mitochondria from airway myeloid-derived regulatory cells to T cells. *Redox Biol* 2018;18:54-64.
38. Prado N, Marazuela EG, Segura E, et al. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. *J Immunol* 2008;181:1519-25.
39. Torregrosa Paredes P, Esser J, Admyre C, et al. Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. *Allergy* 2012;67:911-9.
40. Tager AM, Bromley SK, Medoff BD, et al. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat Immunol* 2003;4:982-90.
41. Miyahara N, Ohnishi H, Matsuda H, et al. Leukotriene B4 receptor 1 expression on dendritic cells is required for the development of Th2 responses and allergen-induced airway hyperresponsiveness. *J Immunol* 2008;181:1170-8.
42. Draijer C, Speth JM, Penke LRK, et al. Resident alveolar macrophage-derived vesicular SOCS3 dampens allergic airway inflammation. *FASEB J* 2020;34:4718-31.
43. Lässer C, O'Neil SE, Ekerljung L, Ekström K, Sjöstrand M, Lötvall J. RNA-containing exosomes in human nasal secretions. *Am J Rhinol Allergy* 2011;25:89-93.
44. Lässer C, O'Neil SE, Shelke GV, et al. Exosomes in the nose induce immune cell trafficking and harbour an altered protein cargo in chronic airway inflammation. *J Transl Med* 2016;14:181.
45. Wu G, Yang G, Zhang R, et al. Altered microRNA Expression profiles of extracellular vesicles in nasal mucus from patients with allergic rhinitis. *Allergy Asthma Immunol Res* 2015;7:449-57.
46. Mills JT, Schwenzer A, Marsh EK, et al. Airway epithelial cells generate pro-inflammatory tenascin-C and small extracellular vesicles in response to TLR3 stimuli and rhinovirus infection. *Front Immunol* 2019;10:1987.
47. Sánchez-Vidaurre S, Eldh M, Larssen P, et al. RNA-containing exosomes in induced sputum of asthmatic patients. *J Allergy Clin Immunol* 2017;140:1459-61.e2.
48. Maes T, Cobos FA, Schleich F, et al. Asthma inflammatory phenotypes show differential microRNA expression in sputum. *J Allergy Clin Immunol* 2016;137:1433-46.
49. Gupta R, Radicioni G, Abdelwahab S, et al. Intercellular communication between airway epithelial cells is mediated by exosome-like vesicles. *Am J Respir Cell Mol Biol* 2019;60:209-20.
50. Kesimer M, Scull M, Brighton B, et al. Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. *FASEB J* 2009;23:1858-68.
51. Jia M, Yan X, Jiang X, et al. Ezrin, a membrane cytoskeleton cross-linker protein, as a marker of epithelial damage in asthma. *Am J Respir Crit Care Med* 2019;199:496-507.
52. Wu Q, Eickelberg O. Ezrin in asthma: a first step to early biomarkers of airway epithelial dysfunction. *Am J Respir Crit Care Med* 2019;199:408-10.
53. Park JA, Sharif AS, Tschumperlin DJ, et al. Tissue factor-bearing exosome secretion from human mechanically stimulated bronchial epithelial cells in vitro and in vivo. *J Allergy Clin Immunol* 2012;130:1375-83.
54. Bartel S, La Grutta S, Cilluffo G, et al. Human airway epithelial extracellular vesicle miRNA signature is altered upon asthma development. *Allergy* 2020;75:346-56.
55. Hashimi ST, Fulcher JA, Chang MH, Gov L, Wang S, Lee B. MicroRNA profiling identifies miR-34a and miR-21 and their target genes

JAG1 and WNT1 in the coordinate regulation of dendritic cell differentiation. *Blood* 2009;114:404-14.
56. Huang A, Yang Y, Chen S, et al. MiR-34a promotes DCs development and inhibits their function on T cell activation by targeting WNT1. *Oncotarget* 2017;8:17191-201.
57. Wu R, Zeng J, Yuan J, et al. MicroRNA-210 overexpression promotes psoriasis-like inflammation by inducing Th1 and Th17 cell differentiation. *J Clin Invest* 2018;128:2551-68.
58. Zhao C, Zhao F, Feng H, Xu S, Qin G. MicroRNA-92b inhibits epithelial-mesenchymal transition-induced migration and invasion by targeting Smad3 in nasopharyngeal cancer. *Oncotarget* 2017;8:91603-13.
59. Maroto R, Zhao Y, Jamaluddin M, et al. Effects of storage temperature on airway exosome integrity for diagnostic and functional analyses. *J Extracell Vesicles* 2017;6:1359478.
60. Patente TA, Pelgrom LR, Everts B. Dendritic cells are what they eat: how their metabolism shapes T helper cell polarization. *Curr Opin Immunol* 2019;58:16-23.
61. Vallhov H, Gutzeit C, Hultenby K, Valenta R, Grönlund H, Scheynius A. Dendritic cell-derived exosomes carry the major cat allergen F el d 1 and induce an allergic immune response. *Allergy* 2015;70:1651-5.
62. Sawant DV, Wu H, Kaplan MH, Dent AL. The Bcl6 target gene microRNA-21 promotes Th2 differentiation by a T cell intrinsic pathway. *Mol Immunol* 2013;54:435-42.
63. Stritesky GL, Muthukrishnan R, Sehra S, et al. The transcription factor STAT3 is required for T helper 2 cell development. *Immunity* 2011;34:39-49.
64. Khare A, Chakraborty K, Raundhal M, Ray P, Ray A. Cutting edge: dual function of PPARγ in CD11c+ cells ensures immune tolerance in the airways. *J Immunol* 2015;195:431-5.
65. Nakano H, Free ME, Whitehead GS, et al. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol* 2012;5:53-65.
66. Plantinga M, Guilliams M, Vanheerswynghels M, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013;38:322-35.
67. Furuhashi K, Suda T, Hasegawa H, et al. Mouse lung CD103+ and CD11b high dendritic cells preferentially induce distinct CD4+ T-cell responses. *Am J Respir Cell Mol Biol* 2012;46:165-72.
68. Raymond M, Rubio M, Fortin G, et al. Selective control of SIRP-α-positive airway dendritic cell trafficking through CD47 is critical for the development of TH2-mediated allergic inflammation. *J Allergy Clin Immunol* 2009;124:1333-42.e1.
69. Rosenberg HF, Druey KM. Modeling asthma: pitfalls, promises, and the road ahead. *J Leukoc Biol* 2018;104:41-8.
70. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun* 2011;2:282.
71. Blanchard N, Lankar D, Faure F, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* 2002;168:3235-41.
72. Chiou NT, Kageyama R, Ansel KM. Selective export into extracellular vesicles and function of tRNA fragments during T cell activation. *Cell Rep* 2018;25:3356-3370.e4.
73. Azoulay-Alfaguter I, Mor A. Proteomic analysis of human T cell-derived exosomes reveals differential RAS/MAPK signaling. *Eur J Immunol* 2018;48:1915-7.
74. Shefler I, Salamon P, Reshef T, Mor A, Mekori YA. T cell-induced mast cell activation: a role for microparticles released from activated T cells. *J Immunol* 2010;185:4206-12.
75. Wypych TP, Marzi R, Wu GF, Lanzavecchia A, Sallusto F. Role of B cells in T(H) cell responses in a mouse model of asthma. *J Allergy Clin Immunol* 2018;141:1395-410.
76. Admyre C, Bohle B, Johansson SM, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. *J Allergy Clin Immunol* 2007;120:1418-24.
77. Hough KP, Wilson LS, Trevor JL, et al. Unique lipid signatures of extracellular vesicles from the airways of asthmatics. *Sci Rep* 2018;8:10340.
78. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* 2003;278:20083-90.
79. Trajkovic K, Hsu C, Chiantia S, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008;319:1244-7.
80. Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 2015;25:981-4.
81. Smyth LA, Ratnasothy K, Tsang JY, et al. CD73 expression on extracellular vesicles derived from CD4+ CD25+ Foxp3+ T cells contributes to their regulatory function. *Eur J Immunol* 2013;43:2430-40.
82. Ostroukhova M, Seguin-Devaux C, Oriss TB, et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* 2004;114:28-38.
83. Ding FX, Liu B, Zou WJ, Li QB, Tian DY, Fu Z. Pseudomonas aeruginosa-derived exosomes ameliorates allergic reactions via inducing the Treg response in asthma. *Pediatr Res* 2018;84:125-33.
84. Cañas JA, Sastre B, Mazzeo C, et al. Exosomes from eosinophils autoregulate and promote eosinophil functions. *J Leukoc Biol* 2017;101:1191-9.
85. Mazzeo C, Cañas JA, Zafra MP, et al. Exosome secretion by eosinophils: A possible role in asthma pathogenesis. *J Allergy Clin Immunol* 2015;135:1603-13.

86. Cañas JA, Sastre B, Rodrigo-Muñoz JM, et al. Eosinophil-derived exosomes contribute to asthma remodelling by activating structural lung cells. *Clin Exp Allergy* 2018;48:1173-85.
87. Moore WC, Hastie AT, Li X, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014;133:1557-63. e5.
88. Vargas A, Roux-Dalvai F, Droit A, Lavoie JP. Neutrophil-derived exosomes: a new mechanism contributing to airway smooth muscle remodeling. *Am J Respir Cell Mol Biol* 2016;55:450-61.
89. Majumdar R, Tavakoli Tameh A, Parent CA. Exosomes mediate LTB4 release during neutrophil chemotaxis. *PLoS Biol* 2016;14:e1002336.
90. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135-42.
91. Skokos D, Botros HG, Demeure C, et al. Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J Immunol* 2003;170:3037-45.
92. Skokos D, Le Panse S, Villa I, et al. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J Immunol* 2001;166:868-76.
93. Liang Y, Qiao L, Peng X, et al. The chemokine receptor CCR1 is identified in mast cell-derived exosomes. *Am J Transl Res* 2018;10:352-67.
94. Xie G, Yang H, Peng X, et al. Mast cell exosomes can suppress allergic reactions by binding to IgE. *J Allergy Clin Immunol* 2018;141:788-91.
95. Lecce M, Molfetta R, Milito ND, Santoni A, Paolini R. FcεRI signaling in the modulation of allergic response: role of mast cell-derived exosomes. *Int J Mol Sci* 2020;21:5464.
96. Ekström K, Valadi H, Sjöstrand M, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *J Extracell Vesicles* 2012;1:18389.
97. Yin Y, Shelke GV, Lässer C, Brismar H, Lötvall J. Extracellular vesicles from mast cells induce mesenchymal transition in airway epithelial cells. *Respir Res* 2020;21:1-13.
98. Ullah I, Subbarao RB, Rho GJ. Human mesenchymal stem cells-current trends and future prospective. *Biosci Rep* 2015;35:e00191.
99. Shahir M, Mahmoud Hashemi S, Asadirad A, et al. Effect of mesenchymal stem cell-derived exosomes on the induction of mouse tolerogenic dendritic cells. *J Cell Physiol* 2020;235:7043-55.
100. Mohammadipoor A, Antebi B, Batchinsky AI, Cancio LC. Therapeutic potential of products derived from mesenchymal stem/stromal cells in pulmonary disease. *Respir Res* 2018;19:218.
101. Du YM, Zhuansun YX, Chen R, Lin L, Lin Y, Li JG. Mesenchymal stem cell exosomes promote immunosuppression of regulatory T cells in asthma. *Exp Cell Res* 2018;363:114-20.
102. Cruz FF, Borg ZD, Goodwin M, et al. Systemic administration of human bone marrow-derived mesenchymal stromal cell extracellular vesicles ameliorates aspergillus hyphal extract-induced allergic airway inflammation in immunocompetent mice. *Stem Cells Transl Med* 2015;4:1302-16.
103. Fang SB, Zhang HY, Wang C, et al. Small extracellular vesicles derived from human mesenchymal stromal cells prevent group 2 innate lymphoid cell-dominant allergic airway inflammation through delivery of miR-146a-5p. *J Extracell Vesicles* 2020;9:1723260.
104. de Castro LL, Xisto DG, Kitoko JZ, et al. Human adipose tissue mesenchymal stromal cells and their extracellular vesicles act differentially on lung mechanics and inflammation in experimental allergic asthma. *Stem Cell Res Ther* 2017;8:151.
105. Kordelas L, Rebmann V, Ludwig AK, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014;28:970-3.
106. Fu CL, Ye YL, Lee YL, Chiang BL. Effects of overexpression of IL-10, IL-12, TGF-beta and IL-4 on allergen induced change in bronchial responsiveness. *Respir Res* 2006;7:72.
107. Reis M, Mavin E, Nicholson L, Green K, Dickinson AM, Wang XN. Mesenchymal stromal cell-derived extracellular vesicles attenuate dendritic cell maturation and function. *Front Immunol* 2018;9:2538.
108. Yamashita N, Tashimo H, Matsuo Y, et al. Role of CCL21 and CCL19 in allergic inflammation in the ovalbumin-specific murine asthmatic model. *J Allergy Clin Immunol* 2006;117:1040-6.
109. Mattes J, Collison A, Plank M, Phipps S, Foster PS. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. *Proc Natl Acad Sci U S A* 2009;106:18704-9.
110. Bartel S, Carraro G, Alessandrini F, Krauss-Etschmann S, Ricciardolo FLM, Bellusci S. miR-142-3p is associated with aberrant WNT signaling during airway remodeling in asthma. *Am J Physiol Lung Cell Mol Physiol* 2018;315:L328-33.
111. Roffel MP, Brandsma CA, Van Den Berge M, et al. Unraveling the role of miR-223-3p in the regulation of airway inflammation in asthma and COPD. *ERJ* 2018;52:Suppl 62, PA4998.
112. Zhuansun Y, Du Y, Huang F, et al. MSCs exosomal miR-1470 promotes the differentiation of $CD4^+CD25^+FOXP3^+$ Tregs in asthmatic patients by inducing the expression of P27KIP1. *Int Immunopharmacol* 2019;77:105981.
113. Tao SC, Guo SC. Extracellular vesicles: potential participants in circadian rhythm synchronization. *Int J Biol Sci* 2018;14:1610-20.
114. Kim DK, Kang B, Kim OY, et al. EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *J Extracell Vesicles* 2013;2:20384.
115. Keerthikumar S, Chisanga D, Ariyaratne D, et al. ExoCarta: a web-based compendium of exosomal cargo. *J Mol Biol* 2016;428:688-92.
116. Pathan M, Fonseka P, Chitti SV, et al. Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. *Nucleic Acids Res* 2019;47:D516-9.
117. Arslan F, Lai RC, Smeets MB, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate

PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013;10:301-12.

118. Liu L, Jin X, Hu CF, Li R, Zhou Z, Shen CX. Exosomes derived from mesenchymal stem cells rescue myocardial ischaemia/reperfusion injury by inducing cardiomyocyte autophagy via AMPK and Akt pathways. *Cell Physiol Biochem* 2017;43:52-68.
119. Zhu LL, Huang X, Yu W, Chen H, Chen Y, Dai YT. Transplantation of adipose tissue-derived stem cell-derived exosomes ameliorates erectile function in diabetic rats. *Andrologia* 2018;50:e12871.
120. Xu B, Zhang Y, Du XF, et al. Neurons secrete miR-132-containing exosomes to regulate brain vascular integrity. *Cell Res* 2017;27:882-97.
121. Pusic AD, Kraig RP. Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination. *Glia* 2014;62:284-99.
122. Shende VR, Goldrick MM, Ramani S, Earnest DJ. Expression and rhythmic modulation of circulating microRNAs targeting the clock gene Bmal1 in mice. *PLoS One* 2011;6:e22586.
123. Wang C, Zhang C, Liu L, et al. Macrophage-derived miR-155-containing exosomes suppress fibroblast proliferation and promote fibroblast inflammation during cardiac injury. *Mol Ther* 2017;25:192-204.
124. Khalyfa A, Poroyko VA, Qiao Z, et al. Exosomes and metabolic function in mice exposed to alternating dark-light cycles mimicking night shift work schedules. *Front Physiol* 2017;8:882.
125. Dooner MS, Stewart C, Deng Y, et al. Daily rhythms influence the ability of lung-derived extracellular vesicles to modulate bone marrow cell phenotype. *PLoS One* 2018;13:e0207444.
126. Bikov A, Kunos L, Pállinger É, et al. Diurnal variation of circulating microvesicles is associated with the severity of obstructive sleep apnoea. *Sleep Breath* 2017;21:595-600.
127. Khalyfa A, Marin JM, Qiao Z, Rubio DS, Kheirandish-Gozal L, Gozal D. Plasma exosomes in OSA patients promote endothelial senescence: effect of long-term adherent continuous positive airway pressure. *Sleep* 2020;43:zsz217.
128. Koritzinsky EH, Street JM, Chari RR, et al. Circadian variation in the release of small extracellular vesicles can be normalized by vesicle number or TSG101. *Am J Physiol Renal Physiol* 2019;317:F1098-110.
129. Catalano M, O'Driscoll L. Inhibiting extracellular vesicles formation and release: a review of EV inhibitors. *J Extracell Vesicles* 2020;9:1703244.
130. Zhao Z, Wijerathne H, Godwin AK, Soper SA. Isolation and analysis methods of extracellular vesicles (EVs). *Extracell Vesicles Circ Nucleic Acids* 2021;2:80-103.

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Extracellular Vesicles and Circulating Nucleic Acids

Review

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Extracellular vesicles as a promising biomarker resource in liquid biopsy for cancer

Takaaki Tamura[1,2], Yusuke Yoshioka[1], Shinichi Sakamoto[2], Tomohiko Ichikawa[2], Takahiro Ochiya[1]

[1]Department of Molecular and Cellular Medicine, Tokyo Medical University, Tokyo 160-0023, Japan.
[2]Department of Urology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan.

Correspondence to: Yusuke Yoshioka, Department of Molecular and Cellular Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. E-mail: yyoshiok@tokyo-med.ac.jp

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Abstract

Liquid biopsy is a minimally invasive biopsy method that uses molecules in body fluids as biomarkers, and it has attracted attention as a new cancer therapy tool. Liquid biopsy has considerable clinical application potential, such as in early diagnosis, pathological condition monitoring, and tailored treatment development based on cancer biology and the predicted treatment response of individual patients. Extracellular vesicles (EVs) are lipid membranous vesicles released from almost all cell types, and they represent a novel liquid biopsy resource. EVs carry complex molecular cargoes, such as proteins, RNAs [e.g., mRNA and noncoding RNAs (microRNA, transfer RNA, circular RNA and long noncoding RNA)], and DNA fragments; these cargoes are delivered to recipient cells and serve as a cell-to-cell communication system. The molecular contents of EVs largely reflect the cell of origin and thus show cell-type specificity. In particular, cancer-derived EVs contain cancer-specific molecules expressed in parental cancer cells. Therefore, analysis of cancer-derived EVs might indicate the presence and nature of cancer. High-speed analytical technologies, such as mass spectrometry and high-throughput sequencing, have generated large data sets for EV cargoes that can be used to identify many candidate EV-associated biomarkers. Here, we will discuss the challenges and prospects of EV-based liquid biopsy compared to other biological resources (e.g., circulating tumor cells and cell-free DNA) and summarize the novel studies that have identified the remarkable potential of EVs as a cancer biomarker.

Keywords: Extracellular vesicles, liquid biopsy, cancer biomarker, microRNA

INTRODUCTION

Our body fluids, such as blood, urine, cerebrospinal fluid, saliva, pleural effusion, ascites fluid, breast milk and seminal plasma, contain various biological molecules[1]. Liquid biopsy is a minimally invasive method that uses these molecules as biomarkers, and it is emerging as a new tool in the strategy against cancer[2]. The terms "precision medicine"[3] and "personalized medicine"[4] have recently become popularized in the field of cancer research. Diagnosis and adequate therapy for individual cases of particular cancer types commonly rely on genetic mutation and gene expression analyses or pathological imaging observations of cancer lesions. However, such cancer genomic medicine approaches require collection of cancer tissue by biopsy, which imposes a heavy burden on the patient. In particular, it is difficult to obtain tumor tissue from organs located in the deeper parts of the body. Therefore, the development of minimally invasive methods, such as liquid biopsy, is desired. Liquid biopsy initially emerged only for the purpose of genetic diagnosis; however, it has considerable clinical application potential, such as in early diagnosis, monitoring of pathological conditions, and tailored treatment development according to cancer biology and the predicted treatment response[5]. Importantly, because of its minimally invasive nature, liquid biopsy can be scheduled more often to give more accurate snapshots of the disease at successive time points, which is useful for measuring temporal tumor burden levels and early evidence of recurrence or therapy resistance[6] [Figure 1]. Furthermore, liquid biopsy may reflect the genetic profile of more cancer subclones in a patient than tissue biopsies, which are obtained from only one cancer region[7].

Extracellular vesicles (EVs) have attracted increasing attention as a novel analyte in liquid biopsy[8]. EVs are lipid membranous vesicles that are released from almost all types of cells, including normal cells as well as abnormal cells, such as cancer cells. EVs are reported to be correlated with various biological phenomena and play important roles in cell-to-cell communication via horizontal transfer of cellular cargoes, such as proteins, RNAs [including mRNA and noncoding RNAs (e.g., microRNA; miRNA, transfer RNA; tRNA circular RNA; circRNA and long noncoding RNA; lncRNA)], DNA fragments, and lipids[9,10]. Importantly, the composition of EV cargoes secreted from individual cell types differs greatly depending on the cellular origin, and the characteristic features of EVs derived from various cancer cells have been revealed. Thus, cancer-derived EVs can be analyzed to determine the presence and nature of cancer. Furthermore, EVs have been reported to be found in almost all body fluids[11]. For these reasons, EVs are recognized as a promising liquid biopsy resource for cancer. In this review, we will discuss the feasibility and practicality of EV-based liquid biopsy in clinical settings. In the first half, we will argue the advantages and challenges of EV-based liquid biopsy for clinical application. In the second half, we will summarize recent notable studies investigating cancer-specific EV-related molecules as cancer biomarkers, with a focus on their biological or clinical significance.

CANDIDATE ANALYTES IN LIQUID BIOPSY OTHER THAN EVS

Liquid biopsy can target various cancer-associated analytes in multiple body fluids, and several candidate analytes in liquid biopsy for cancer have been identified. These analytes include circulating tumor cells (CTCs) and circulating nucleic acids, such as cell-free DNA (cfDNA) and some extracellular RNA (exRNA) fragments [Figure 2]. Currently, CTCs and cfDNA are the most widely studied target materials for liquid biopsy[12,13].

CTCs are cancer cells shed by primary or metastatic cancer lesions into the circulation and are considered a crucial determinant of hematogenous metastasis and recurrence. CTCs contain valuable information about

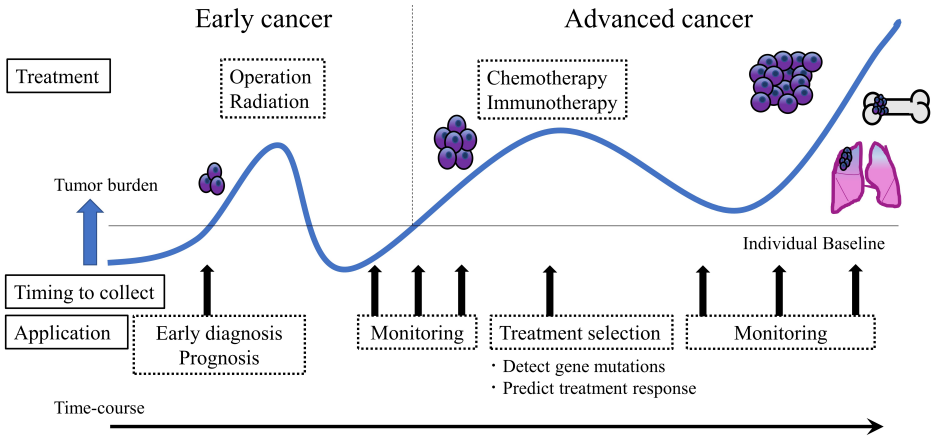

Figure 1. Clinical utility of liquid biopsy in cancer. Liquid biopsy presents a minimally invasive nature and thus has the potential to impact clinical practice at multiple stages of cancer management. This technique can contribute to early diagnosis, pathological condition monitoring, and tailored treatment development according to the cancer biology of individual patients. After cancer treatment, liquid biopsy can support follow-up care by providing early evidence of recurrence or therapy resistance.

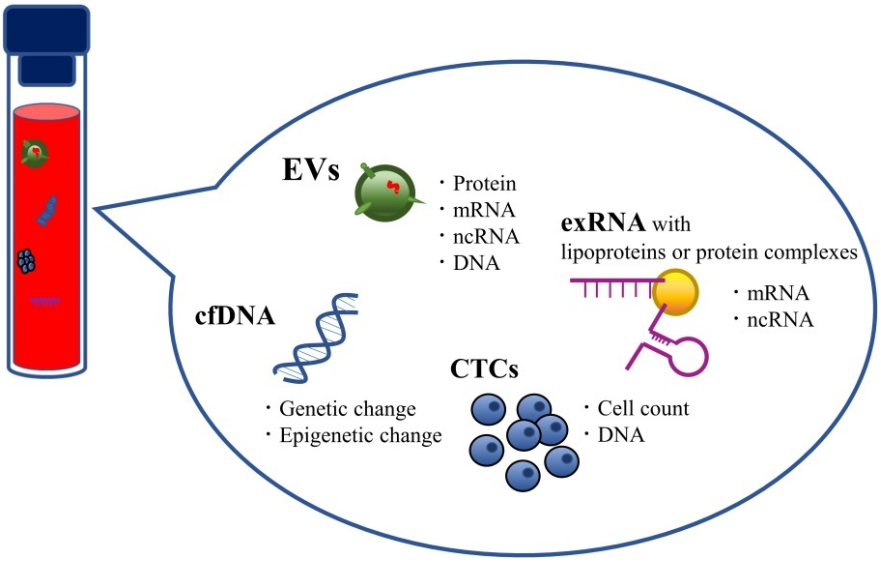

Figure 2. Candidate analytes of liquid biopsy. Body fluids contain several promising biomarkers for cancer. Each candidate analyte can provide considerable information about the cancer biology of individual patients.

the spreading tumor, and early detection of CTCs and treatment of metastatic spread can contribute to improving disease outcomes. Numerous studies in the past decade have shown that CTCs have potential as biomarkers to predict cancer metastasis progression and prognosis[14,15]. A high number of CTCs has been reported to be correlated with clinical outcome[16-19]. However, CTCs are estimated to account for at most one cell among a hundred million circulating cells; thus, there are generally only a small number of CTCs in a few milliliters of blood sample. Furthermore, CTCs must be analyzed as soon as possible after collection because the number of viable cells decreases rapidly. Therefore, analysis of CTCs requires relatively large volumes of fresh blood and advanced technology with extremely high analytical sensitivity and specificity[20-22]. To date, numerous technologies are available that are useful for enrichment and detection of CTCs[23]. Among them, the CellSearch® system has received FDA approval for prognostic clinical evaluation of several cancer types[24]. However, due to the limited number of CTCs in the blood and the current level of

detection technologies, CTCs have not yet been entirely accepted in the clinic. Research groups have recently moved towards analyzing CTC contents (e.g., miRNA[25], mRNA[26], and protein[27,28]) for detection of biomarkers.

cfDNA is a short fragment of nucleic acids found in body fluids, and a component of cfDNA derived from tumor cells is called circulating tumor DNA (ctDNA). The majority of ctDNA originates from apoptotic and necrotic tumor cells. Over the past decade, a large number of studies have reported that measuring ctDNA levels in cancer patients may help in cancer diagnosis and prognosis prediction[29-31]. Interestingly, ctDNA is reported to be horizontally transferred from cancer cells to normal cells via uptake of apoptotic bodies, which is one of the EV subclasses (described below), leading to cancer progression[32,33]. Furthermore, ctDNA is also reported to harbor genetic and epigenetic changes present in the original tumor, and analytical techniques to detect such changes have already been established. By examining these changes, researchers have shown that ctDNA also helps predict treatment response and recurrence[34-38]. The major challenge in ctDNA research is that tumor-specific mutations may only represent 0.01% of the total cfDNA[39,40], thereby increasing the difficulty of detecting rare variants. Moreover, issues limiting cfDNA testing include its relatively short half-life[41,42]. Therefore, sample processing times are critical, and sample preservation requires special precautions, which are also significant barriers to practical use of cfDNA testing in the clinic.

CTC and ctDNA applications are confronted with several challenges, as described above. In short, the significantly low amounts and fragility of CTCs and ctDNA, which show remarkable variations in amount among individuals, increase the difficulty of detection. Moreover, CTC and ctDNA applications may be confined to evaluation of advanced cancer because CTCs are released from cancer tissue that has grown to the point that it causes metastases and ctDNA is primarily released from apoptotic or necrotic cancer tissue[43]. Thus, these entities may not be suitable for early diagnosis and may not reflect the current or near-future state of the disease.

exRNA refers to RNA that is present outside of cells within EVs or associated with platelets, lipoproteins and protein complexes. Similar to cfDNA, almost all non-EV-associated exRNAs are released into circulation by passive secretion. In addition to CTCs and cfDNA, non-EV-associated exRNA has also been well investigated as a candidate analyte in liquid biopsy for cancer. In particular, non-EV-associated miRNAs are recognized as a significant RNA subtype for biomarker discovery because miRNA profiles are associated with cancer-specific conditions. The critical issue is miRNA extraction from biofluid samples; the amounts of miRNA are highly variable among different experiments. Because of the small size of miRNAs and their attachment to other molecules, reproducible extraction remains an inherent issue[44].

WHAT ARE "EVS"?

We will now describe EVs, which are the main focus of this review. "EV" is a collective term that refers to all types of lipid membranous vesicles naturally released from all cell types. From the 1970s to the 1980s, membrane-enclosed vesicle structures were reported in various types of solid tissues, body fluids, and culture supernatants. Depending on their size and origin, these vesicles are called various names, such as prostasomes, exosomes, microvesicles, microparticles, shedding vesicles, ectosomes, apoptotic bodies and oncosomes. Each vesicle type contains slightly different molecular groups due to differences in vesicle biogenesis and secretion pathways. Nevertheless, their designations have been vaguely defined. Therefore, the International Society for Extracellular Vesicles (ISEV), founded in Sweden in 2012, recommends the use of "extracellular vesicle" as a generic term for these vesicles[45,46]. The nomenclature of these vesicles is detailed in the Minimal Information for Studies of Extracellular Vesicles (MISEV), which is the guideline

advocated by the ISEV[47,48].

EVs are classically classified into three main categories: exosomes (approximately 100 nm), microvesicles (approximately 1 μm), and apoptotic bodies (greater than 1 μm)[49]. These three classes of EVs differ in size as well as morphology, content, generation mode, and release mechanism. Exosomes are formed by the inward budding of early endosomes to form multivesicular bodies (MVBs). These MVBs fuse with the limiting plasma membrane to release exosomes into the extracellular space. Microvesicles originate by direct shedding or budding from the plasma membrane. Apoptotic bodies are released from cells undergoing programmed cell death[50]. Recently, Théry *et al*[47,51]. developed a more reasonable classification system for EVs and defined vesicles < 100 nm as small EVs, < 200 nm as medium EVs, and > 200 nm as large EVs. Moreover, they demonstrated that each EV subtype showed different characteristic protein components, suggesting that each EV category is generated and secreted through a specific molecular mechanism[47,51].

EVS AS AN ANALYTE IN LIQUID BIOPSY

In 1983, Pan and Johnstone[52] discovered that cells secrete 100 nm-sized vesicles with a lipid membranous structure and that red blood cells release small vesicles loaded with the transferrin receptor, which is necessary to synthesize hemoglobin during maturation. They regarded EVs as "garbage bags" that are used to expel unused molecules from the cell and reported one of the remarkable EV functions; more importantly, this study demonstrated the presence of proteins in EVs[52,53].

In the 1990s, by analyzing immune cells, Raposo *et al*.[54] found that EVs affect recipient cells. EVs derived from B lymphocytes induced antigen-specific MHC class II-restricted T cell responses, indicating that EVs have functions related to intercellular communication[54].

In 2007, Valadi *et al*.[55] demonstrated that EVs from the human mast cell line HMC-1 and the mouse mast cell line MC/9 contain approximately 1300 mRNAs and 121 miRNAs and thus contribute to the exchange of genetic information between cells. Since that time, miRNAs have been the most widely studied class of short noncoding RNAs (ncRNAs) in EV cargoes[55].

To date, EVs have been reported to contain various proteins, RNAs [including mRNAs and noncoding RNAs (miRNAs, tRNAs, circRNAs and lncRNAs)], and DNA fragments[9]. The past few years have seen extraordinary developments in areas such as mass spectrometry (MS), high-throughput sequencing (HTS) (e.g., DNA sequencing, chromatin immunoprecipitation sequencing, methylation sequencing, and RNA sequencing), and big-data analysis[56,57]. These advanced technologies allow us to select analytes contained in EVs and evaluate them deeply. Significantly, the composition of EV molecular contents reflects the intracellular status of their cellular origin. Moreover, these molecules are stably preserved due to their lipid-layered structure; hence, EVs from body fluids can be analyzed after being stored for a relatively long period of time[58]. A growing body of evidence has suggested that cancer-derived EV cargoes bare a strong resemblance to the intracellular status of their parental cells[59]. Analysis of EV cargoes can help reveal the existence, molecular profile and behavior of cancer. Thus, EV-based liquid biopsy contributes to early cancer diagnosis, monitoring of cancer pathological conditions, and treatment selection according to cancer biology and the predicted treatment response. In addition, EVs are easily accessible in various kinds of body fluids and appropriate for sequential collection. For these reasons, EVs have emerged as a promising biomarker resource and as another kind of liquid biopsy for cancer [Figure 2].

CLASSICAL EV ISOLATION METHODS

Although EVs are a remarkable candidate analyte to detect cancer biomarkers, few EV-associated biomarkers have been implemented in clinical settings, which is partially due to the lack of adequate isolation methods[60]. In principle, EVs must be isolated from various biofluids for analysis. Indeed, in almost all cases, candidate EV-associated biomarkers were detected via isolation processes because target EV-associated molecules should be distinguished from EV-free molecules in the biofluid; however, a unified isolation method is not currently available, which is one of the biggest challenges in EV research.

The most popular technique for EV isolation is ultracentrifugation (UC), which separates EVs based on their size and buoyant density. The UC procedure does not require additional chemicals or pretreatment of the body fluid samples. Moreover, the combination of UC with other procedures, such as ultrafiltration, sucrose cushion, and density gradient centrifugation, can increase the purity of the EV fraction[61-63]. However, UC-based procedures generally require dedicated equipment and an extraordinary amount of time[60]. Moreover, repeated centrifugation can lead to reduced yield due to lost and aggregated EVs[64]. On the other hand, various polymer-based isolation kits, such as ExoQuick™, Total Exosome Isolation™ and miRCURY™, which are commercially available, can save time and labor costs, although issues with high contamination rates and high running costs have been reported[63,65]. An immunoaffinity capture approach using magnetic beads and monoclonal antibodies targeting EV surface antigens is also commonly used to isolate EVs[66,67]. Many reports have demonstrated that this approach achieves a selective and high-purity output; however, there are difficulties in terms of low capacity, low yield and high reagent cost[60]. Size-exclusion chromatography (SEC) is a technique for separating biological molecules based on molecular size in which target molecules are isolated by filtration through a resin-packed column. SEC has been reported to yield highly purified EVs and achieve excellent reproducibility in a relatively short time[60,64]; however, the existence of EVs in multiple fractions results in a low EV concentration in the obtained samples. Consequently, subsequent EV analysis often requires an additional concentration step[68]. Recently, many researchers have studied combined methods integrating multiple isolation techniques together or conducting them sequentially[69]. Among others, the combination of UC and SEC has demonstrated higher purity of EVs and better performance in subsequent analyses than the single-step methods[70-72]; however, in daily clinical settings, combined methods might be avoided due to several factors, such as time consumption, cost, repeatability, and ease of use. As high-quality EV samples are essential for subsequent analyses, there is an urgent need for a simple, cost-effective, and reliable technique for both basic research and clinical practice.

EV-ASSOCIATED PROTEINS

Common EV protein markers

EVs contain abundant proteins that reflect their origin and alterations of their parental cells. Based on the endosome-based biogenesis pathway, EV-specific protein markers include membrane trafficking-associated proteins (e.g., Rab family proteins, annexins), MVB-associated proteins (e.g., Alix, Tsg101 and ESCRT complex), tetraspanins (e.g., CD9, CD63 and CD81) and heat shock proteins (Hsp70 and Hsp90)[73-75]. As these proteins are common to EVs derived from almost all cell types, they can serve as common positive EV markers that can confirm the presence of EVs, and isolated EVs can be assessed via downstream proteomic analyses, such as western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and flow cytometry (FCM), which use common EV proteins as hallmarks. With regard to checking the quality of isolated EV samples, MISEV recommends the use of apolipoproteins A1/2 and B and albumin as negative markers of blood-derived EVs because they are often co-isolated with these molecules[47,76].

EV protein-based platform for cancer diagnosis

Targeting membrane proteins on the surface of EVs is an effective strategy because target cancer-derived proteins can be directly detected without the use of a large sample volume or time-consuming isolation processes for EVs. Recently, great efforts have been devoted to establishing clinically useful detection platforms, including platforms for direct detection of cancer-specific EVs without any isolation or purification procedures. These platforms mainly consist of specific antibody-based technologies that detect EV surface proteins, such as ELISA.

Jørgensen *et al.*[77] established the EV Array, which can detect EVs in unpurified materials in a high-throughput manner. The EV Array is composed of different capture antibodies located on a microarray slide, which capture EVs according to their surface proteins, and the target EVs are detected with a cocktail of biotinylated antibodies against the tetraspanins CD9, CD63, and CD81. The authors validated the performance of the EV Array by comparing plasma from nonsmall cell lung cancer (NSCLC) patients and normal healthy subjects[78]. Shao *et al.*[79] reported that a microfluidic chip platform could distinguish patients with glioblastoma multiforme (GBM) from normal healthy subjects. This microfluidic chip labeled with magnetic nanosensors quantifies an EV-specific protein marker (CD63) and glioblastoma-specific proteins, such as epidermal growth factor receptor (EGFR) and EGFR variant III, on the surface of EVs via micronuclear magnetic resonance (μNMR). In this study, the authors also indicated that GBM EVs reflect gene amplification or mutation and predict the therapy response. Surface plasmon resonance (SPR)-based nanosensors have recently attracted much attention due to their ability to detect a small number of molecules[80]. Im *et al.*[81] developed an SPR-based exosome sensor called nanoplasmonic exosomes (nPLEXs). Each nanohole array of nPLEX is functionalized with antibodies that recognize EV surface proteins. nPLEX was able to differentiate ascites samples from ovarian cancer patients from healthy controls with an accuracy of 97% and identified ovarian cancer cell-derived EVs based on their expression of CD24 and EpCAM. Yoshioka *et al.*[82] also established a highly rapid and sensitive analytical technique called "ExoScreen". This assay consists of two kinds of antibodies against proteins on the surface of EVs that are detectable by photosensitizing beads. A very small sample volume (at least 5 μL) was required to detect EVs in serum from healthy controls without a complicated isolation process. Moreover, the assay could be completed within 2 h. In this study, they identified CD147 as a specific EV-surface protein derived from colorectal cancer cells and revealed that a larger number of CD9/CD147 double-positive EVs could be detected in serum from colorectal cancer patients than in serum from healthy control subjects using this assay. Furthermore, Zhao *et al.*[83] developed a simple microfluidic platform named the "ExoSearch" chip that allows quantitative isolation of EVs using immunomagnetic beads. An "ExoSearch" chip could detect ovarian cancer by measuring three EV cancer protein markers, CA-125, EpCAM and CD24. The development of immune-capturing systems in microchips also provides highly sensitive and reliable detection of cancer markers without requiring a large sample volume or time-consuming EV isolation processes.

Other EV protein biomarkers for cancer diagnosis

EV surface proteins and EV lumen proteins are regarded as candidate cancer biomarkers. Numerous studies have identified cancer-associated EV protein markers using isolation processes, followed by WB, ELISA, and FCM. Promising EV protein markers identified in clinical studies with patient body fluid samples are summarized in Table 1.

Clinically validated traditional molecules have also been identified in EVs, such as prostate-specific antigen (PSA), and they represent novel diagnostic biomarkers. Mitchell *et al.*[84] demonstrated that the expression of PSA and PSMA in urinary EVs can act as treatment response markers in prostate cancer. Additionally, Logozzi *et al.*[85] showed increased PSA expression on EVs in vitro and in the plasma of prostate cancer

Table 1. A list of EV proteins as potential biomarkers for cancer

Cancer types	Biological source	Isolation method	Detection method	Markers	Potential application	Ref.
Urinary cancer						
Prostate cancer	Plasma	UC	ELISA	PSA	Diagnosis/Prognosis	[85]
	Urine	UC + SUC	ELISA/WB	PSA, PSMA	Diagnosis/Monitoring	[84]
	Plasma/Serum	UC/ExoQuick	ELISA/WB	Survivin	Diagnosis/Monitoring	[87]
	Urine	UC	IP/WB	δ-catenin	Diagnosis	[156]
	Serum	UC	WB	MDR-1/P-gp, MDR-3, PABP4	Predict chemoresistance (Docetaxel)	[95,157]
	Plasma	-	FCM	PSMA	Monitoring/Predict chemoresistance	[158]
Bladder cancer	Urine	UC	MS/WB	EH-domain-containing protein 4, EPS8L1, EPS8L2, GTPase NRas, Mucin 4, retinoic acid-induced protein3, resistin, alpha subunit of GsGTP binding protein	Diagnosis	[159]
	Urine	UC	ELISA	TACSTD2	Diagnosis	[160]
	Urine	UC	MS	α-1-anti-trypsin, H2B1K	Diagnosis	[161]
	Urine	UC	WB	HEXB, S100A4, SND1, TALDO1, and EHD4	Diagnosis	[162]
	Urine	UC	WB	Periostin	Diagnosis	[163]
	Urine	UC + SUC	WB	EDIL3	Diagnosis	[164]
	Urine	UC + SUC	FCM	CD36, CD44, 5T4, basigin, CD73, MUC1, α6-integrin	Diagnosis	[165]
Renal cancer	Urine	UC	MS/WB	MMP9, DKK4, EMMPRIN, CP, PODXL, CAIX, CD10, AQP1, dipeptidase 1, syntenin 1	Diagnosis	[166]
Female cancer						
Breast cancer	Serum	ExoQuick	ELISA	Survivin, Survivin2B	Diagnosis/Prognosis	[88]
	Serum/Plasma	ExoQuick	ELISA/WB	CD82	Diagnosis	[167]
	Ascites	UC + SUC	WB	CD24, EpCAM	Diagnosis	[168]
	Plasma	UC	FCM	TRPC5	Prognosis/Predict chemoresistance	[169]
	Serum	UC	FCM	UCH-L1	Predict chemoresistance (Anthracycline/taxan)	[92]
	Serum	UC	FCM/WB	HER2	Predict chemoresistance (Trastuzumab)	[96]
	Plasma	UC	ELISA/FCM	TGFβ1	Predict chemoresistance (Trastuzumab)	[97]
Ovarian cancer	Plasma	-	Exosearch chip	CD24, EpCAM, CA-125	Diagnosis	[83]
	Plasma	UC	WB	TGFβ1, MAGE3/6	Diagnosis	[170]
	Plasma	UC + SUC	WB	Claudin-4	Diagnosis	[171]
	Ascites	UC + SUC	WB	E-cadherin	Diagnosis/Prognosis	[172]
	Ascites	UC	WB	MMP2, MMP9, uPA	Diagnosis	[173]
	Ascites	UC + SUC	WB	CD24, L1CAM, ADAM10, EMMPRIN	Diagnosis/Prognosis	[174]
Digestive cancer						
Pancreatic cancer	Serum	UC	FCM	GPC1	Diagnosis/Prognosis	[89,175]
	Serum	UC + SUC	ELISA	CKAP4	Diagnosis	[176]
	Serum	UC	ELISA	MIF	Diagnosis/Prognosis	[177]

	Plasma	-	ELISA	EpCAM	Prognosis	[91]
Colorectal cancer	Serum	Exoquick	ELISA	CEA	Diagnosis	[178]
	Serum	-	ExoScreen	CD147, CD9	Diagnosis	[82]
	Ascites	UC	WB	claudin-3	Diagnosis	[179]
Gastric cancer	Serum	UC	FCM/WB	HER-2/neu, CCR6, EMMPRIN, MAGE-1, c-MET	Diagnosis	[180]
Others						
Lung cancer	Serum	UC	ELISA	EGFR	Diagnosis	[181]
(NSCLC)	Serum	UC	ELISA/WB	AHSG, ECM1 (with serum CEA)	Diagnosis	[182]
	Serum	-	EV array	30 Proteins	Diagnosis	[78]
	Plasma	-	EV array	CD171, NY-ESO-1, PLAP, Flotilin1	Diagnosis	[183]
	Urine	UC	WB	LRG1	Diagnosis	[184]
Melanoma	Plasma	UC	ELISA (Exo Test)/FCM/WB	Caveolin-1, CD63	Diagnosis/Prognosis	[185]
	Plasma	UC	WB	TYRP2, VLA-4, HSP70, HSP90	Prognosis	[186]
	Plasma	UC/Total Exosome isolation Kit	ELISA/FCM /WB	PDL-1	Predict immunotherapy resistance (Pembrolizumab)	[91]
Glioblastoma	Serum	-	μNMR system	EGFR, EGFRv□, CD63	Prognosis	[79]

NSCLC: Non-small cell lung cancer; LSCC: laryngeal squamous cell carcinoma; UC: ultracentrifugation; SUC: sucrose cushion; ELISA: enzyme-linked immuno-sorbent assay; FCM: flow cytometry; WB: western blotting; IP: immunoprecipitation; MS: mass spectrometry; miRNA: microRNA; tRNA: transfer RNA; circRNA: circular RNA; lncRNA: long non-coding RNA.

patients. The authors emphasized the failure of current PSA testing in discriminating between benign prostatic hypertrophy and prostate cancer in terms of both overdiagnosis and overtreatment, which leads to patient suffering and public and private healthcare expenditures. Moreover, they claimed that EV PSA might resolve the problem associated with differences in PSA cutoff levels based on age, race and individual physiological condition. Similar to PSA, some traditional molecules in EVs have been discussed to have higher relevance to cancer than their total amount in body fluids, such as CEA for colon cancer[86].

Additionally, some EV-associated markers have been reported as diagnostic markers for multiple cancer types. Khan *et al.*[87] showed that Survivin, an inhibitor of apoptosis member, could be detected in plasma-derived EVs from both prostate cancer patients and healthy subjects; however, the relative amount of EV Survivin was remarkably higher in the plasma of prostate cancer patients. Their subsequent study showed that the EV Survivin and its alternative splice variants were also elevated in breast cancer patient plasma[88], which suggests that EV Survivin might be an important diagnostic marker common to several cancer types.

EV proteins can be used to monitor cancer progression and drug resistance

Biomarkers in EVs of several cancer types may be applied for cancer stratification because they change in response to anticancer therapy. Thus, EV proteins may be used as novel biomarkers to monitor cancer progression or to identify patients susceptible to anticancer drugs.

Melo *et al.*[89] reported that glypican-1 (GPC1) could be a specific marker of cancer-derived EVs and that the existence of GPC1-positive EVs in serum could differentiate patients with pancreatic ductal adenocarcinoma (PDAC) from patients with benign pancreas disease or from healthy control subjects with 100% sensitivity and specificity. The levels of GPC1-positive EVs were significantly decreased after surgical resection; moreover, they were related to overall survival (OS) and significantly higher in patients with

distant metastasis than in patients with lymph node metastasis only or no metastases[89]. Although this conclusion is controversial, a replication study was performed to validate this finding, and a subsequent discussion was held in ISEV2017[90]. Similarly, Giampieri *et al.*[91] reported that a higher level of EpCAM-positive EVs before chemotherapy was correlated with shorter progression-free survival (PFS) and OS. In contrast, in this study, an increase in EpCAM-positive EV levels during treatment was correlated with better PFS in PDAC patients[91]. These studies demonstrated that EV-associated proteins could be biomarkers for monitoring tumor burden.

The development of chemoresistance is a persistent problem during cancer treatment. Various studies have reported cell-to-cell transfer of multidrug resistance (MDR) efflux pumps as EV cargoes from chemotherapy-resistant cells to chemotherapy-sensitive cancer cells. EVs from doxorubicin-resistant[92] or docetaxel-resistant[93] breast cancer cell lines transferred chemoresistance to recipient cancer cells through P-glycoprotein (P-gp) loaded onto EVs. Moreover, the same phenomenon in paclitaxel-resistant ovarian cancer cells was also caused by the transfer of functional P-gp mediated by EVs[94]. Docetaxel-resistant prostate cancer cells were also reported to proliferate through cell-to-cell transfer of EV P-gp. In this study, serum EVs from prostate cancer patients who were nonresponders to docetaxel therapy protected prostate cancer cells from the cytotoxicity of docetaxel[95]. These studies indicate that EV-P-gp might be a promising marker to predict chemotherapy resistance in several cancer types. Ubiquitin C-terminal hydrolase L1 (UCH-L1) has also been reported as an EV-based predictive biomarker of chemoresistance in breast cancer. UCH-L1 overexpression has been reported to induce upregulation of P-gp levels through the MAPK/ERK signaling pathway, thereby enhancing an MDR phenotype in breast cancer. Ning *et al.*[92] showed that higher UCH-L1 levels in circulating EVs are correlated with poorer response to adjuvant anthracycline/taxane-based chemotherapy. In addition, they demonstrated that UCH-L1-positive EVs derived from breast cancer cell lines could transfer chemoresistance to recipient cells in vitro, indicating that EVs might be a predictive biomarker of chemoresistance in breast cancer patients.

Interestingly, EV-mediated drug resistance has been reported to be relevant for molecular target drugs as well as chemotherapy drugs. Ciravolo *et al.*[96] reported that 73% of advanced-stage breast cancer patients had HER2-positive EVs in circulation, which hampered the corresponding therapeutic efficacy of trastuzumab monoclonal antibody. Importantly, this study demonstrated that the presence of HER2-positive EVs in the serum of breast cancer patients could be an indicator to predict a patient's response to trastuzumab therapeutic regimens[96]. Martinez *et al.*[97] reported that EVs released from HER2 drug-resistant cells contain larger amounts of the immunosuppressive cytokine TGF-β1. Importantly, a recent neoadjuvant clinical trial by the same group that included trastuzumab and lapatinib further demonstrated that HER2-overexpressing breast cancer patients who were nonresponders to HER2 drug therapy had significantly higher amounts of TGF-β1 in circulating EVs than patients who did not respond to HER2 drug therapy. These results suggest that EV-TGF-β1 can be a biomarker for predictive response to this therapeutic regimen against breast cancer[97].

Recently, with the successful development of immune checkpoint inhibitors (ICIs), cancer immunotherapy has attracted worldwide attention as a new cancer treatment. EV proteins may contribute to identifying patients susceptible to ICIs. Tumor cells avoid immune recognition by upregulating the surface expression of programmed death-ligand 1 (PD-L1), which interacts with the programmed death-1 (PD-1) receptor on T cells to elicit the immune checkpoint response. Indeed, immunotherapy with anti-PD-1 antibodies has shown remarkable therapeutic effects against different tumor types[98]. However, for some patients, the therapeutic response has been reported to be rather poor[99,100]. To address this problem, Chen *et al.*[101] showed that specific EVs reduce the effectiveness of immunotherapy approaches in certain patients with

melanoma. Remarkably, using human melanoma xenografts in nude mice, they showed that metastatic melanoma cell lines release EVs loaded with PD-L1 on their surface and that interferon-γ increases the expression of PD-L1 on these vesicles, which suppresses the function of CD8+ T cells, facilitating tumor growth. Importantly, these authors showed that the level of PD-L1-positive EVs differentiates responders from non-responders to anti-PD-1 therapy. More importantly, this study provides evidence supporting the application of PD-L1-positive EVs as a predictive biomarker for anti-PD-1 therapy in melanoma patients[101].

EV-ASSOCIATED NUCLEIC ACIDS

As described above, high-speed analytical technologies, such as HTS, are powerful tools for identifying candidate nucleic acid biomarkers. For detection and validation of candidate EV nucleic acid biomarkers, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after an isolation process is the most common detection method. Recently, digital polymerase chain reaction (dPCR) has emerged as a novel detection method for EV nucleic acids. dPCR is a qPCR technique that provides a sensitive and reproducible method of measuring the amount of DNA or RNA present in a sample[102]. The tremendous progress in these technologies during the last few decades has allowed us to analyze EV-associated nucleic acids derived from diverse body fluids.

EV RNA biomarkers

Pioneering studies of nucleic acids from isolated EVs have identified various miRNAs and mRNAs as the major components of EVs[55,103]. Subsequently, several important papers reporting on the function of EV-miRNAs were published and showed that the transferred EV-miRNAs can be active in recipient cells and modify the cellular phenotype[104-106]. Over the last decade, studies have revealed that EVs contain other noncoding RNAs, such as tRNAs[107], circRNAs[108] and lncRNAs[109,110]. To date, EV RNAs (as EV cargo) have received the most attention in terms of cancer diagnosis and prognosis because they are easy to quantify using conventional methods, such as qPCR, and stable against RNase-dependent degradation in the circulatory system[111]. Table 2 summarizes the candidate EV RNA markers reported as diagnostic and prognostic tools for cancer.

EV RNAs for cancer diagnosis

Since the discovery of EV-miRNAs by Valadi *et al.*[55] in 2007, numerous studies have been performed to identify diagnostic EV miRNAs for cancer. Taylor *et al.*[112] showed that eight miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214) that were previously characterized as diagnostic markers for ovarian cancer were also upregulated in circulating EVs derived from ovarian cancer patients. Rabinowits *et al.*[113] conducted miRNA-profiling analyses of EV-based liquid biopsy samples and tumor biopsy samples from lung cancer patients and healthy controls. Their study showed a similarity in miRNA patterns between EV-based biopsy samples and tumor biopsy samples from lung cancer patients and demonstrated significant differences between these miRNA patterns and those in EVs from the healthy control group. This result indicates the potential of EV miRNAs as a liquid biopsy source for lung cancer. On the other hand, EV miR-1246 was significantly increased in ESCC patient serum samples but was not elevated in tumor biopsy samples, indicating that the level of EV miRNAs may not necessarily reflect the amounts in parental cells[114].

Regarding EVs in other body fluids, Nilsson *et al.*[115] reported that PCA3 and TMPRSS2:ERG mRNA, which are previously established prostate cancer biomarkers, were also present in urinary EVs from prostate cancer patients. Additionally, Foj *et al.*[116] demonstrated that the levels of miR-21-5p, miR-375, and let-7c-5p were remarkably increased in urinary EVs derived from prostate cancer patients compared with those

Table 2. A list of EV RNAs as potential biomarkers for cancer

Cancer types	Biological source	Isolation methods	Detection methods	RNA types	Markers	Potential application	Ref.
Uninary cancer							
Prostate cancer	Urine	UC + SUC	nestedPCR	mRNA	PSA, PCA-3, TMPRSS:ERG	Diagnosis/Monitoring	[115]
	Urine	-	ExoDx® Prostate (IntelliScore)	mRNA/lncRNA	PCA3, ERG, SPDEF	Diagnosis	[118]
	Urine	UF/UC	RT-qPCR	miRNA	let-7c, miR-21, miR-107, miR-145, miR-196a-5p, miR-204, miR-375, miR-501-3p, miR-574-3p, miR-2909	Diagnosis	[116,187-191]
	Serum/Plasma	UF/SEC/UC	RT-qPCR	miRNA	let-7i, miR-16, miR-21-5p, miR-24, miR-26a, miR-26b, miR-30c-5p, miR-34b, miR-92b, miR-93, miR-103, miR-106a, miR-107, miR-130b, miR-141, miR-181a-2, miR-195, miR-197, miR-200c-3p, miR-210-3p, miR-223, miR-298, miR-301a, miR-326, miR-328, miR-331-3p, miR-346, miR-375, miR-432, miR-574-3p, miR-625, miR-1290, miR-2110	Diagnosis	[187,192-196]
	Plasma	UF/SEC/UC	RT-qPCR	miRNA	miR-17, miR-20a, miR-23a, miR-130b, miR-198, miR-200b, miR-375, miR-379, miR-513a-5p, miR-572, miR-577, miR-582-3p, miR-609, miR-619, miR-624, miR-1236, miR-1290	Prognosis	[119,187]
	Serum	UC	RT-qPCR	miRNA	miR-1246	Prognosis	[197]
	Urine	-	Urine Exosome RNA Isolation Kit / RT-qPCR	lncRNA	p21	Diagnosis	[122]
	Plasma	Total Exosome Isolation Kit	RT-qPCR	lncRNA	SAP30L- AS1, SChLAP1	Diagnosis	[198]
	Plasma / Urine	-	exoRNeasy kit/ddPCR	mRNA	AR-V7	Predict hormone therapy resistance (Abiraterone and/or Enzalutamide)	[132,133]
Bladdar cancer	Urine	UC	ddPCR	miRNA	miR-21, miR-93, miR-200c, miR-940	Diagnosis	[199]
	Urine	-	ddPCR	miRNA	miR-21-5p, miR-4454, miR-720, miR-200c-3p, miR-29b-3p, miR-200b-3p	Diagnosis	[200]
	Urine	UC	RT-qPCR	miRNA	miR-375, miR-146a	Diagnosis	[201]
	Urine	unknown	RT-qPCR	miRNA	miR-146b-5p, miR-155-5p	Diagnosis	[202]
	Urine	UC	RT-qPCR	lncRNA	HOTAIR, HOX- AS-2, MALAT1, SOX2, OCT4	Diagnosis/Prognosis	[203]
	Serum	ExoQuick	RT-qPCR	lncRNA	PCAT1, UBC1, SNHG16	Diagnosis/Prognosis	[204]
	Serum	UC	RT-qPCR	lncRNA	UCA1	Diagnosis	[205]
	Urine / Serum	UC	RT-qPCR	circRNA	PRMT5	Diagnosis/Prognosis	[126]
Renal cancer	Serum	UC + IP	RT-qPCR	miRNA	miR-210, miR-1233	Diagnosis	[206,207]
	Urine	UC	RT-qPCR	miRNA	miR-150-5p, miR-126-3p in combination with miR-34b-5p,	Diagnosis	[208]

					miR-449a and miR-486-5p		
	Serum	Total Exosome Isolation Kit	RT-qPCR	miRNA	miR-224	Prognosis	[209]
	Plasma	ExoQuick	RT-qPCR	miRNA	miR-26a-1-3p, miR- let-7-I, miRN-615-3p	Prognosis	[210]
	Urine	UC	RT-qPCR	lncRNA	GSTA1, CEBPA, PCBD1	Diagnosis	[211]
Female cancer							
Breast cancer	Plasma	FC/UC/ExoQuick	RT-qPCR	miRNA	miR-21, miR-1246	Diagnosis	[212]
	Plasma	UC	RT-qPCR	miRNA	miR-105	Diagnosis	[213]
	Serum	unknown/Total Exosome Isolation Kit	RT-qPCR	miRNA	miRNA-21, miRNA-222, miRNA-155	Prognosis/Predict chemoresistance (miR-21, miR-155: Doxorubicin, Paclitaxel)	[214,215]
	Serum	UC + IP	RT-qPCR	miRNA	miR-200a, miR-200c, miR-205	Diagnosis	[216]
	Bood/Milk/Ductal fluids	UC	RT-qPCR	miRNA	miR-16, miR-1246, miR-451, miR-205	Diagnosis	[217]
	Serum	ExoQuick	RT-qPCR	lncRNA	HOTAIR	Prognossis/Monitoring	[129]
	Serum	ExoQuick	RT-qPCR	lncRNA	SNHG14	Predict chemoresistance (Trastuzumab)	[130]
	Plasma	UC	RT-qPCR	mRNA	TrpC5, mdr1, MUC1 and flotillin2	Predict chemoresistance (Anthracycline/taxan)	[135]
	Serum	Total Exosome Isolation Kit	RT-qPCR	mRNA	GSTP1	Predict chemoresistance (Anthracycline/taxan)	[136]
Ovarian cancer	Serum	UC + IP	RT-qPCR	miRNA	miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, miR-214	Diagnosis	[112]
	Serum	Total Exosome Isolation Kit	RT-qPCR	miRNA	miR-1246	Predict chemoresistance (Paclitaxel)	[218]
Digestive cancer							
Pancreatic cancer	Serum/Urine	UC	RT-qPCR	miRNA	miR-17-5p, miR-21	Diagnosis	[219]
	Plasma	ExoQuick	RT-qPCR	miRNA	miR-155	Predict chemoresistance (Gemcitabine)	[131]
	Saliva	UC	RT-qPCR	mRNA	Apbb1ip, ASPN, Daf2, FoxP1, Bco31781, Gng2	Diagnosis	[220]
Liver cancer (HCC)	Plasma	Total Exosome Isolation Kit	RT-qPCR	tRNA	ValTAC-3, GlyTCC-5, ValAAC-5, GluCTC-5	Diagnosis	[107]
	Serum	Exoquick	RT-qPCR	circRNA	circUHRF1	Diagnosis/immunotherapy resistance (anti-PD-1)	[108]
	Serum	Exoquick	RT-qPCR	miRNA	miR-21, miR-10b	Prognosis	[221]
Gastoric cancer	Serum	unknown	RT-qPCR	miRNA	HOTTIP	Diagnosis/Prognosis	[124]

	Serum	UC	RT-qPCR	lncRNA	lncUEGC	Diagnosis	[123]
	Serum	Exoquick	RT-qPCR	circRNA	circSHKBP1	Diagnosis	[222]
Esophageal cancer (ESCC)	Serum	Exoquick	RT-qPCR	miRNA	miR-21	Diagnosis	[120]
Colorectal cancer	Serum	UC	RT-qPCR	miRNA	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a	Diagnosis	[127]
	Serum	ExoQuick	RT-qPCR	miRNA	miR-4772-3p	Prognosis	[223]
Rectal cancer	Plasma	miRCURY Exosome Isolation Kit	RT-qPCR	miRNA	miR-30d-5p, miR-181a-5p and miR-486-5p	Diagnosis	[224]
Others							
Lung cancer	Plasma	ExoQuick	RT-qPCR	miRNA	miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p	Diagnosis	[225]
Lung cancer (NSCLC)	Plasma	IP	RT-qPCR	miRNA	let-7f, miR-20b, miR-30e-3p, miR-223, miR-301	Diagnosis	[226]
Lung cancer (LSCC)	Plasma	ExoQuick	RT-qPCR	miRNA	miR-205, miR-19a, miR-19b, miR-30b, miR-20a	Monitoring	[227]
Lung cancer (NSCLC)	Serum	UC	RT-qPCR	miRNA	miRNA-222-3p	Prognosis/Predict chemoresistance (Gemcitabine)	[228]
Lung cancer (NSCLC)	Serum	ExoQuick	RT-qPCR	miRNA	miRNA-146a-5p	Predict chemoresistance (Cisplatin)	[229]
Lung cancer (NSCLC)	Plasma	-	exoRNeasy kit/ddPCR	mRNA	PD-L1	Predict immunotherapy resistance (anti-PD-1)	[134]
Melanoma	Plasma	-	exoRNeasy kit/ddPCR	mRNA	PD-L1	Predict immunotherapy resistance (anti-PD-1)	[134]
Glioblastoma	Serum/Cereblospinal fluid	UC	RT-qPCR	miRNA	miR-21	Diagnosis/Prognosis	[230]
	Cereblospinal fluid	UC	RT-qPCR	miRNA	miR-21	Diagnosis	[231]
	Serum	ExoQuick	RT-qPCR	miRNA	RNU6-1, miR-320, miR-574-3p	Diagnosis	[232]
	Serum	-	iMER	mRNA	MGMT, APNG	Predict chimoresistance (Temozolomide)	[137]

HCC: Hepatocellular carcinoma; ESCC: esophageal squamous cell cancer; NSCLC: non-small cell lung cancer; LSCC: laryngeal squamous cell carcinoma; UC: ultracentrifugation; SUC: sucrose cushion; UF: ultrafiltration; IP: immunoprecipitation; RT-qPCR: reverse transcription-quantitative PCR; ddPCR: droplet digital PCR; miRNA: microRNA; tRNA: transfer RNA; circRNA: circular RNA; lncRNA: long non-coding RNA.

from healthy subjects. Analyses of urinary EVs and associated RNAs are difficult to perform because of the low abundance of these molecules and the fact that the urine volume itself varies greatly over time. However, recently, a new technology has been reported in which zinc oxide nanowires are used to catch urinary EVs to increase the yield[117]. Moreover, ExoDx® Prostate (IntelliScore) is a simple, non-DRE, urine-based test for prostate cancer that is commercially available in the United States. This test has been clinically validated for risk stratification of clinically significant prostate cancer (Gleason score ≥ 7) from low-

grade prostate cancer (Gleason score 6) and benign prostate disease, thus avoiding unnecessary prostate biopsy. A patient-specific individual risk score is evaluated based on an original algorithm that combines the expression level of three RNAs (PCA3 noncoding RNA, ERG mRNA, and SPDEF mRNA), which are correlated with clinically significant prostate cancer, detected directly in urinary EV RNAs[118].

To date, the potential of many miRNAs as cancer prognostic markers has been reported. Huang *et al.*[119] showed that increased levels of serum miR-1290 and miR-375 in EVs were correlated with decreased OS in patients with advanced-stage prostate cancer. Tanaka *et al.*[120] revealed that high levels of circulating EV miR-21 could distinguish esophageal squamous cell cancer (ESCC) patients from patients with benign diseases. The levels of EV miR-21 were also correlated with cancer progression and aggressiveness, indicating that EV miR-21 can serve as a diagnostic biomarker for cancer as well as a therapeutic target. In addition to EV miR-21, EV miR-1246 was also identified as a diagnostic and prognostic marker for ESCC[114]. Regarding prognostic markers for digestive cancers, Matsumura *et al.*[121]. reported that serum EV miR-19a-3p might be a prognostic biomarker to predict the recurrence of colorectal cancer. Other noncoding RNAs, such as lncRNAs and the less common family of circRNAs in EVs from cancer cells, have also attracted increasing attention. Urinary EV lncRNA p21 was reported to be elevated in prostate cancer patients and able to distinguish prostate cancer patients from those with benign disease[122]. Lin *et al.*[123] reported that lncRNA upregulated in plasma exosomes from gastric cancer (lncUEGC1) patients could be used to detect early-stage gastric cancer. Serum EV-associated lncRNA HOTTIP was also reported as a diagnostic and prognostic indicator of gastric cancer[124]. Furthermore, Lee *et al.*[125] demonstrated the prognostic significance of circulating EV ncRNAs [miRNA-21 and lncRNA activated by transforming growth factor beta (lncRNA-ATB)] for human hepatocellular carcinoma. In this study, the OS and PFS rates were significantly lower in patients with higher levels of miR-21 and lncRNA-ATB in EVs[125]. Concerning circRNA, Chen *et al.*[126] showed that circRNA PRMT5 was highly enriched in both serum and urinary EVs collected from patients with bladder cancer compared with normal individual cohorts. In this study, they also revealed that EV circPRMT5 levels were significantly correlated with cancer metastasis[126].

EV RNAs to monitor cancer progression and drug resistance

Similar to EV proteins, EV-RNAs have been reported to have potential as biomarkers for monitoring therapeutic effects or resistance to anticancer therapy.

Ogata-Kawata *et al.*[127] reported that the levels of seven miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, miR-23a) in serum EVs were significantly increased in colorectal cancer patients. The serum levels of these miRNAs were significantly decreased after tumor resection, indicating their potential for monitoring tumor burden[127]. Svedman *et al.*[128] investigated EV-associated miRNA levels in patients with metastatic melanoma before, during, and after MAPK inhibitor therapy. They showed that increased levels of let-7g-5p were correlated with lower tumor burden and could clearly differentiate responders from nonresponders; moreover, an increase in the miR-497-5p level during the treatment course was significantly associated with better PFS rates[128]. Several researchers have investigated EV-associated lncRNAs in patients during chemotherapy. HOTAIR is one of the major lncRNAs that is overexpressed in a variety of cancers and promotes cancer cell proliferation, invasion and migration. Tang *et al.*[129] reported that EV HOTAIR was more enriched in serum samples from breast cancer patients than in healthy controls. In addition, they showed that a high pretreatment level of EV HOTAIR was correlated with a poor response to neoadjuvant chemotherapy and tamoxifen hormone therapy. In this study, EV HOTAIR was significantly decreased in all patients after surgery compared with before surgery, indicating that serum EV HOTAIR originates from the tumor tissue, and its level is associated with tumor burden and cancer aggressiveness[129].

Regarding EV lncRNAs, Dong *et al.*[130] have previously shown that isolated EVs from HER2-positive advanced breast cancer patients who were nonresponders to trastuzumab therapy contained more lncRNA SNHG14 than EVs from responders. In this study, the authors showed that EV lncRNA-SNHG14 promoted trastuzumab resistance by activating Bcl-2/apoptosis regulator BAX (Bax) signaling. Similar to this study, other types of EV RNAs have also been reported to be involved in resistance to anticancer therapy. Mikamori *et al.*[131] reported that higher miR-155 expression levels in resected tumor tissue samples from pancreatic ductal adenocarcinoma patients treated with gemcitabine (GEM) were correlated with a poorer prognosis. This study demonstrated that the levels of miR-155 in plasma-derived EVs were consistent with those in pancreatic tissue[131]. The results suggest that circulating EV biomarkers can reflect, to some extent, the tumor burden and possess potential as real-time monitoring biomarkers for anticancer drug resistance. Interestingly, they further demonstrated that high expression of miR-155 in pancreatic cancer cell lines promoted antiapoptotic signaling and EV secretion in vitro. Moreover, they showed that the EVs released by miR-155-overexpressing PDAC cell lines could transfer chemoresistance-associated molecules (including miR-155) to other cancer cells and that the recipient cells subsequently acquired chemoresistance to GEM in vitro. Del Re *et al.*[132] succeeded in detection of androgen receptor splice variant 7 (AR-V7) in EV RNAs from castration-resistant prostate cancer (CRPC) patients. The authors showed that EV AR-V7 mRNA levels were associated with hormonal therapy resistance. OS was significantly shorter among patients with EVs containing AR-V7 mRNA than among those without AR-V7 mRNA present (3 months *vs.* 20 months)[132]. Woo *et al.*[133] reported a practical method for analysis of AR-V7 mRNA in urinary EVs. In this study, the authors adopted a lab-on-a-disc integrated with six independent nanofiltration units, which enabled simultaneous processing of six individual samples. EV mRNA was extracted from each 4 mL urine sample, and AR-V7 and androgen receptor full-length (AR-FL) mRNA levels were quantified by dPCR. The results showed that higher AR-V7 and lower AR-FL expression was detected in urinary EVs derived from patients with CRPC than in those from patients with hormone-sensitive prostate cancer. In addition, they described that AR-V7 transcript levels and the AR-V7/AR-FL ratio in urinary EVs were higher in patients with advanced prostate cancer[133]. These studies indicate that EV AR-V7 mRNA could be a predictive biomarker for hormonal therapy resistance in prostate cancer. Some studies have investigated the relevance between EV RNAs and the response to ICIs. For example, Del Re *et al*[134]. demonstrated that EV-associated PD-L1 mRNA levels could be useful for real-time monitoring of the response to anti-PD-1 antibody therapy. They evaluated PD-L1 mRNA expression levels in plasma EVs from melanoma and NSCLC patients during nivolumab and pembrolizumab therapy. After 2 months of treatment, the EV PD-L1 mRNA copy numbers were significantly decreased in responders but remained unchanged in nonresponders[134]. Regarding the EV-based biomarkers associated with breast cancer chemoresistance, Ma *et al.*[135] performed profiling analyses of mRNA in circulating EVs from breast cancer patients treated with chemotherapy or not. The authors demonstrated that four mRNAs (TrpC5, MDR1, MUC1, and flotillin2) were amplified in EVs from patients with chemotherapy but were not amplified in patients without chemotherapy[135]. Interestingly, considering that TrpC5 was previously shown to regulate P-gp expression in recipient cells, this study suggested that TrpC5-enriched circulating EVs could promote cancer MDR development in these patients. More recently, Yang *et al.*[136] analyzed the levels of glutathione S-transferase P (GSTP1) mRNA in serum EVs from breast cancer patients treated with anthracycline/taxane-based neoadjuvant chemotherapy. GSTP1 is an enzyme that has a critical role in cell detoxification. Importantly, they observed that patients with EVs highly enriched in GSTP1 mRNA showed an inadequate response to chemotherapy[136].

Shao *et al.*[137] established a microfluidic platform termed immunomagnetic exosome RNA (iMER) analysis, which integrates immunomagnetic selection targeting EV-surface proteins and real-time qPCR for collecting EV-associated RNAs into a single microfluidic chip form. Serial measurements of the mRNA levels of MGMT and APNG, two important enzymes involved in repairing DNA damage induced by temozolomide for glioblastoma, demonstrated the feasibility of drug resistance monitoring during

treatment using this integrated platform[137]. Although this kind of novel technology for capturing and analyzing EVs requires further development and validation in a clinical setting, it has the potential to reach the next level of EV utilization to detect cancer biomarkers.

EV DNA biomarkers

In addition to RNA species, DNA fragments have also been identified in EVs. Balaj *et al.*[138] were among the first to show that EVs contain single-stranded DNA. Yokoi *et al.*[139] confirmed the presence of double-stranded DNA in EVs using imaging flow cytometry and described how nuclear content was loaded into EVs. EV DNA is also a promising diagnostic tool due to its ability to carry information regarding cancer-specific mutations[140]. Here, we aim to summarize the growing evidence for EV DNA as a diagnostic marker and to consider its diagnostic advantages compared to cfDNA.

Kahlert *et al.*[141] identified the genomic DNA fragments in EVs from pancreatic cancer cell lines and pancreatic cancer patients. All genomic sequencing revealed mutations in KRAS and p53 in the genomic DNA of EVs derived from pancreatic cancer, suggesting that EV DNA sequencing can be used to determine treatment plans and predict therapy resistance[141]. However, in a subsequent paper, the first author called into question the superiority of EV DNA profiling to cfDNA profiling as an analyte for liquid biopsy[142]. Another remarkable study showed the detection rate of KRAS mutations in circulating EVs in PDAC patients and healthy controls. Mutations were detected in 7.4%, 66.7%, 80%, and 85% of age-matched controls and localized, locally advanced, and metastatic PDAC patients, respectively. In this study, the mutant KRAS detection rate in patients with localized PDAC after surgical resection dropped from 66.7% before surgery to 5% after surgery, indicating that EV-associated KRAS mutations could serve as biomarkers for real-time monitoring of therapy response and tumor burden[143]. A subsequent study demonstrated that the kinetics of EV-associated KRAS mutant allele frequency (MAF) were deeply correlated with neoadjuvant chemotherapy response; 71% of patients with a lack of cancer progression showed decreased KRAS MAF, while 94% of patients with cancer progression showed no decrease in KRAS MAF[144]. Interestingly, whereas the KRAS mutation detection rate in localized and metastatic pancreatic cancer was nearly equivalent for the cfDNA and EV DNA analyses, surgically resected primary tissue samples showed 95.5% concordance with the EV DNA-based assessment and only 68.2% concordance with the cfDNA analysis results. Similarly, agreement between the results for pancreatic patient tissue and liquid biopsy analyses was reported to be 83.3% for EV DNA-based analysis and only 66.8% for cfDNA-based analysis.

Similar to KRAS mutations, several analyses of circulating EVs from NSCLC patients showed the presence of clinically relevant epidermal growth factor receptor (EGFR)-specific mutations[145,146]. Remarkably, these studies highlight the expanded performance associated with combining analysis of EV-associated nucleic acids together with cfDNA *vs.* cfDNA analysis alone. Castellanos-Rizaldos *et al.*[145] reported that the combined use of EV nucleic acids together with cfDNA overcame the limited abundance of the EGFR T790M mutation and other EGFR mutations and contributed to improved sensitivity and specificity compared to cfDNA alone. Krug *et al.*[146] showed that the combined use of EV RNA and cfDNA sequencing improved the detection of EGFR mutations up to 98% *vs.* 84% for cfDNA alone. EV-based liquid biopsy has potential for multiplexing DNA analyses with analyses of other EV cargoes, such as miRNA, lncRNA, and proteins; thus, it can provide highly accurate information about cancer biology.

Other body fluids, such as urine, may also be valuable sources for EV DNA-based liquid biopsies. Lee *et al.*[147] investigated whether genetic alterations in urothelial bladder cancer were reflected in urinary cfDNA or EV DNA and demonstrated concordance between the copy number profiles of tumor tissue and urinary DNA (cfDNA and EV DNA), with allelic frequencies of 56.2% and 65.6%, respectively.

Amplification of MDM2, ERBB2, CCND1, and CCNE1 and deletion of CDKN2A, PTEN, and RB1, whose alterations are all frequently found in bladder cancer, were also identified[147].

In addition to these studies, many studies have indicated that EV DNA may have a significant impact on the origin cells and recipient cells by playing a role in the maintenance of cellular homeostasis[148] and acting as an intercellular messenger[149]. Furthermore, similar to EV RNA, the packaging of DNA into membrane-enclosed vesicles contributes to enhanced stability by protecting it from the external environment and avoiding recognition by the immune system[150]. These findings may demonstrate that EV DNA is superior to cfDNA as a biomaterial in liquid biopsy for cancer; however, current protocols for definitively detecting cancer-derived EV DNA in clinical samples are hampered by high labor costs, high financial costs, and low accuracy. The ctDNA isolation and detection method has already been established and might be used in routine clinical situations in the near future[142]. Although cfDNA was first reported in the 1940s[151], the presence of EV DNA has long been doubted and was only demonstrated in the 2010s[138,140]. The potential of EV DNA as an analyte for liquid biopsies has not been thoroughly investigated but is a promising research area. We must improve strategies to utilize EV DNA and establish more useful methods of applying this molecule for liquid biopsy.

CHALLENGES AND FUTURE PERSPECTIVE

Most potential biomarkers are based on small-scale studies and require longitudinal validation in larger samples. In addition, the consistency and variability in data collected using different technologies are significant problems. The large amounts of data from recently developed detection technologies provide an opportunity to identify key molecules but also represent a challenge to differentiating valuable markers among numerous candidate molecules, and the associated processes need further investigation. Deeper and more rigorous studies are required to accurately correlate these potential markers with clinical practice. Moreover, the basic knowledge regarding the biological characteristics of EVs is still insufficient. The mechanisms that regulate the heterogeneity of cancer EVs have not been fully elucidated, and the factors that affect EV synthesis, secretion and transfer remain poorly understood; overcoming these issues is crucial to improving the accuracy of EV diagnostic outcomes.

The future of EV-based liquid biopsy depends on meeting certain technological issues. Isolation and detection of EVs are undoubtedly the biggest problems mentioned above; however, other technological problems have been observed. For example, RT-qPCR for EV-associated nucleic acids requires more appropriate housekeeping genes or reference genes, and universal genes that meet all the criteria as control genes for EV-associated nucleic acids have not yet been identified[152]. For EV-associated miRNAs, current protocols recommend that samples be processed from aligned volumes and that technical variations should be compensated for using synthetic nonhuman miRNAs, such as *Caenorhabditis elegans* cel-miR-39, as normalization controls. Variations can stem from many sources, such as differences in sample preparation, stabilization, RNA extraction, and target quantification. These differences are not a consequence of the disease state itself. Therefore, optimal genes that are stably expressed, irrespective of the experimental situation or treatment, must be identified to define reference genes for normalizing EV-associated nucleic acid expression. Several housekeeping genes or reference genes have been identified for different native tissues and body fluids, and stable endogenous RNAs have been proposed as internal controls; however, a consensus has not been reached[152]. In 2002, Vandesompele *et al.*[153] demonstrated that the common RT-qPCR procedure of using only one control gene induced relatively large errors. In this study, they claimed that ideal single internal control genes do not exist and recommended the use of at least three adequate control genes for calculating a normalization factor[153]. This issue with normalizers in gene expression analyses continues to this day, and the same issue is found with protein analyses. Quantification of EV-

associated proteins also requires an internal control; however, the most suitable internal control for EV proteins for equivalent amounts of protein has not been identified. Not all EVs contain common EV marker proteins, such as Alix, TSG101, CD9, and CD63[154]. Hence, this problem with internal controls is a major problem associated with EV-based liquid biopsy, and further investigation is needed to develop biomarker research.

Despite these several concerns, EV-based liquid biopsy will provide higher sensitivity and specificity than classical biomarkers due to their stability in body fluids, and new technologies are being developed to solve the current limitations of EV-based liquid biopsy, as mentioned in this manuscript. Translating EV cargo profiles into routine clinical diagnostics would be facilitated by efficient alternatives to EV preparation via ultracentrifugation, such as biofluidic devices for high-throughput analysis. Moreover, extraordinary progress has occurred in analytic technologies, such as MS, HTS, and big-data analysis. These technologies have become irreplaceable and familiar analytical tools for researchers analyzing EV-associated molecules. Indeed, some studies have recommended MS-based methods as an alternative to detect EV protein markers after isolation procedures[155]. Further technological development will advance societal implementation of EV-based liquid biopsy for cancer.

CONCLUSIONS

Useful cancer biomarkers in liquid biopsies are urgently required, and EVs represent a promising resource for cancer biomarkers. The development of technologies is accompanied by novel statistical tools, which can utilize high-dimensional machine learning approaches to analyze big data and provide timely decisions. Thus, the future of EV-based liquid biopsy will be associated with multiple academic fields, such as molecular biology, bioengineering, clinical medicine, machine learning, and statistics. Numerous EV-associated studies will likely enhance the performance of cancer biomarkers in early diagnosis, prognosis, surveillance and treatment. Additionally, recent advances in bioinformatics may demonstrate the biological significance of identified cancer markers, which will provide clues for elucidation of cancer pathophysiology. In conclusion, the development of EV-based liquid biopsy will lead to early diagnosis of fatal cancers and tailor-made treatments for individual patients, and such advancements will extend the healthy human life span and reduce medical costs. Similar to the dramatic changes in our daily lives caused by a microscopic virus, a tiny vesicle may also dramatically advance cancer management.

DECLARATIONS

Authors' contributions

Drafted the manuscript: Tamura T, Yoshioka Y, Ochiya T
Reviewed the manuscript: Yoshioka Y, Sakamoto S, Ichikawa T, Ochiya T
Approved the submitted manuscript: Yoshioka Y
All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* 2019;8:727. DOI PubMed PMC
2. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating Liquid Biopsies into the Management of Cancer. *Nat Rev Clin Oncol* 2017;14:531-48. DOI PubMed
3. Ashley EA. Towards Precision Medicine. *Nat Rev Genet* 2016;17:507-22. DOI PubMed
4. Hamburg MA, Collins FS. The path to personalized medicine. *N Engl J Med* 2010;363:301-4. DOI PubMed
5. Siravegna G, Mussolin B, Venesio T, et al. How liquid biopsies can change clinical practice in oncology. *Ann Oncol* 2019;30:1580-90. DOI PubMed
6. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;6:224ra24. DOI PubMed PMC
7. Cheng F, Su L, Qian C. Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. *Oncotarget* 2016;7:48832-41. DOI PubMed PMC
8. Roy S, Hochberg FH, Jones PS. Extracellular vesicles: the growth as diagnostics and therapeutics; a Survey. *J Extracell Vesicles* 2018;7:1438720. DOI PubMed PMC
9. Yáñez-Mó M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 2015;4:27066. DOI PubMed PMC
10. Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 2015;25:981-4. DOI PubMed PMC
11. Keller S, Ridinger J, Rupp AK, Janssen JW, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. *J Transl Med* 2011;9:86. DOI PubMed PMC
12. Krebs MG, Metcalf RL, Carter L, et al. Molecular analysis of circulating tumour cells - biology and biomarkers. *Nat Rev Clin Oncol* 2014;11:129-44. DOI PubMed
13. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* 2014;32:579-86. DOI PubMed PMC
14. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004;351:781-91. DOI PubMed
15. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 2008;14:6302-9. DOI PubMed
16. Danila DC, Heller G, Gignac GA, et al. Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin Cancer Res* 2007;13:7053-8. DOI PubMed
17. Heller G, McCormack R, Kheoh T, et al. Circulating Tumor Cell Number as a Response Measure of Prolonged Survival for Metastatic Castration-Resistant Prostate Cancer: A Comparison with Prostate-Specific Antigen across Five Randomized Phase III Clinical Trials. *J Clin Oncol* 2018;36:572-80. DOI PubMed PMC
18. Goldkorn A, Ely B, Quinn DI, et al. Circulating tumor cell counts are prognostic of overall survival in SWOG S0421: a phase III trial of docetaxel with or without atrasentan for metastatic castration-resistant prostate cancer. *J Clin Oncol* 2014;32:1136-42. DOI PubMed PMC
19. Scher HI, Heller G, Molina A, et al. Circulating tumor cell biomarker panel as an individual-level surrogate for survival in metastatic castration-resistant prostate cancer. *J Clin Oncol* 2015;33:1348-55. DOI PubMed PMC
20. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004;10:6897-904. DOI PubMed
21. Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem* 2013;59:110-8. DOI PubMed
22. Qin J, Alt JR, Hunsley BA, Williams TL, Fernando MR. Stabilization of circulating tumor cells in blood using a collection device with a preservative reagent. *Cancer Cell Int* 2014;14:23. DOI PubMed PMC
23. Alix-Panabières C, Pantel K. Challenges in circulating tumour cell research. *Nat Rev Cancer* 2014;14:623-31. DOI PubMed
24. Allard WJ, Terstappen LWMM. CCR 20th anniversary commentary: paving the way for circulating tumor cells. *Clin Cancer Res* 2015;21:2883-5. DOI PubMed

25. Markou A, Zavridou M, Sourvinou I, et al. Direct comparison of metastasis-related miRNAs expression levels in circulating tumor cells, corresponding plasma, and primary tumors of breast cancer patients. *Clin Chem* 2016;62:1002-11. DOI PubMed
26. Antonarakis ES, Lu C, Luber B, et al. Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide. *J Clin Oncol* 2017;35:2149-56. DOI PubMed PMC
27. Sinkala E, Sollier-Christen E, Renier C, et al. Profiling protein expression in circulating tumour cells using microfluidic western blotting. *Nat Commun* 2017;8:14622. DOI PubMed PMC
28. Armbrecht L, Rutschmann O, Szczerba BM, Nikoloff J, Aceto N, Dittrich PS. Quantification of Protein Secretion from Circulating Tumor Cells in Microfluidic Chambers. *Adv Sci (Weinh)* 2020;7:1903237. DOI PubMed PMC
29. Zill OA, Banks KC, Fairclough SR, et al. The Landscape of Actionable Genomic Alterations in Cell-Free Circulating Tumor DNA from 21,807 Advanced Cancer Patients. *Clin Cancer Res* 2018;24:3528-38. DOI PubMed
30. Chun FK, Müller I, Lange I, et al. Circulating tumour-associated plasma DNA represents an independent and informative predictor of prostate cancer. *BJU Int* 2006;98:544-8. DOI PubMed
31. Altimari A, Grigioni AD, Benedettini E, et al. Diagnostic role of circulating free plasma DNA detection in patients with localized prostate cancer. *Am J Clin Pathol* 2008;129:756-62. DOI PubMed
32. Bergsmedh A, Szeles A, Henriksson M, et al. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc Natl Acad Sci U S A* 2001;98:6407-11. DOI PubMed PMC
33. Trejo-Becerril C, Pérez-Cárdenas E, Taja-Chayeb L, et al. Cancer progression mediated by horizontal gene transfer in an in vivo model. *PLoS One* 2012;7:e52754. DOI PubMed PMC
34. Mahon KL, Qu W, Devaney J, et al; PRIMe consortium. Methylated Glutathione S-transferase 1 (mGSTP1) is a potential plasma free DNA epigenetic marker of prognosis and response to chemotherapy in castrate-resistant prostate cancer. *Br J Cancer* 2014;111:1802-9. DOI PubMed PMC
35. Azad AA, Volik SV, Wyatt AW, et al. Androgen Receptor Gene Aberrations in Circulating Cell-Free DNA: Biomarkers of Therapeutic Resistance in Castration-Resistant Prostate Cancer. *Clin Cancer Res* 2015;21:2315-24. DOI PubMed
36. Lallous N, Volik SV, Awrey S, et al. Functional analysis of androgen receptor mutations that confer anti-androgen resistance identified in circulating cell-free DNA from prostate cancer patients. *Genome Biol* 2016;17:10. DOI PubMed PMC
37. Salvi S, Casadio V, Conteduca V, et al. Circulating cell-free AR and CYP17A1 copy number variations may associate with outcome of metastatic castration-resistant prostate cancer patients treated with abiraterone. *Br J Cancer* 2015;112:1717-24. DOI PubMed PMC
38. Carreira S, Romanel A, Goodall J, et al. Tumor clone dynamics in lethal prostate cancer. *Sci Transl Med* 2014;6:254ra125. DOI PubMed PMC
39. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med* 2008;14:985-90. DOI PubMed PMC
40. Leung F, Kulasingam V, Diamandis EP, et al. Circulating tumor DNA as a cancer biomarker: fact or fiction? *Clin Chem* 2016;62:1054-60. DOI PubMed PMC
41. Sozzi G, Roz L, Conte D, et al. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *J Natl Cancer Inst* 2005;97:1848-50. DOI PubMed
42. Gormally E, Caboux E, Vineis P, Hainaut P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat Res* 2007;635:105-17. DOI PubMed
43. Cai X, Janku F, Zhan Q, Fan JB. Accessing genetic information with liquid biopsies. *Trends Genet* 2015;31:564-75. DOI PubMed
44. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* 2011;11:426-37. DOI PubMed
45. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J Extracell Vesicles* 2013;2:20389. DOI PubMed PMC
46. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;68:2667-88. DOI PubMed PMC
47. Théry C, Witwer KW, Aikawa E, 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. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC
48. Witwer KW, Théry C. Extracellular vesicles or exosomes? *J Extracell Vesicles* 2019;8:1648167. DOI PubMed PMC
49. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;68:2667-88. DOI PubMed PMC
50. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373-83. DOI PubMed PMC
51. Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016;113:E968-77. DOI PubMed PMC
52. Pan B, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. *Cell* 1983;33:967-78. DOI PubMed
53. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987:262;9412-20. PubMed
54. Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996;183:1161-72. DOI

PubMed PMC

55. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654-9. DOI PubMed
56. Aebersold R, Mann M. Mass-spectrometric exploration of proteome structure and function. *Nature* 2016;537:347-55. DOI PubMed
57. Reuter JA, Spacek DV, Snyder MP. High-throughput sequencing technologies. *Mol Cell* 2015;58:586-97. DOI PubMed PMC
58. Welch JL, Madison MN, Margolick JB, et al. Effect of prolonged freezing of semen on exosome recovery and biologic activity. *Sci Rep* 2017;7:45034. DOI PubMed PMC
59. Tovar-Camargo OA, Toden S, Goel A. Exosomal microRNA biomarkers: emerging frontiers in colorectal and other human cancers. *Expert Rev Mol Diagn* 2016;16:553-67. DOI PubMed PMC
60. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics* 2017;7:789-804. DOI PubMed PMC
61. Nordin JZ, Lee Y, Vader P, et al. Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. *Nanomedicine* 2015;11:879-83. DOI PubMed
62. Gupta S, Rawat S, Arora V, et al. An improvised one-step sucrose cushion ultracentrifugation method for exosome isolation from culture supernatants of mesenchymal stem cells. *Stem Cell Res Ther* 2018;9:180. DOI PubMed PMC
63. Van Deun J, Mestdagh P, Sormunen R, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles* 2014;3:24858. DOI PubMed PMC
64. Coumans FAW, Brisson AR, Buzas EI, et al. Methodological guidelines to study extracellular vesicles. *Circ Res* 2017;120:1632-48. DOI PubMed
65. Helwa I, Cai J, Drewry MD, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* 2017;12:e0170628. DOI PubMed PMC
66. Clayton A, Court J, Navabi H, et al. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J Immunol Methods* 2001;247:163-74. DOI PubMed
67. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006;Chapter 3:Unit 3.22. DOI PubMed
68. Brennan K, Martin K, FitzGerald SP, et al. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci Rep* 2020;10:1039. DOI PubMed PMC
69. Liangsupree T, Multia E, Riekkola ML. Modern isolation and separation techniques for extracellular vesicles. *J Chromatogr A* 2021;1636:461773. DOI PubMed
70. An M, Wu J, Zhu J, Lubman DM. Comparison of an Optimized Ultracentrifugation Method versus Size-Exclusion Chromatography for Isolation of Exosomes from Human Serum. *J Proteome Res* 2018;17:3599-605. DOI PubMed PMC
71. Koh YQ, Almughlliq FB, Vaswani K, Peiris HN, Mitchell MD. Exosome enrichment by ultracentrifugation and size exclusion chromatography. *Front Biosci (Landmark Ed)* 2018;23:865-74. DOI PubMed
72. Wei R, Zhao L, Kong G, et al. Combination of Size-Exclusion Chromatography and Ultracentrifugation Improves the Proteomic Profiling of Plasma-Derived Small Extracellular Vesicles. *Biol Proced Online* 2020;22:12. DOI PubMed PMC
73. Simons M, Raposo G. Exosomes - vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 2009;21:575-81. DOI PubMed
74. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* 2010;73:1907-20. DOI PubMed
75. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* 2014;29:116-25. DOI PubMed
76. Clayton A, Boilard E, Buzas EI, et al. Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles. *J Extracell Vesicles* 2019;8:1647027. DOI PubMed PMC
77. Jørgensen M, Bæk R, Pedersen S, Søndergaard EK, Kristensen SR, Varming K. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. *J Extracell Vesicles* 2013;2:20920. DOI PubMed PMC
78. Jakobsen KR, Paulsen BS, Bæk R, Varming K, Sorensen BS, Jørgensen MM. Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma. *J Extracell Vesicles* 2015;4:26659. DOI PubMed PMC
79. Shao H, Chung J, Balaj L, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med* 2012;18:1835-40. DOI PubMed PMC
80. Kravets VG, Schedin F, Jalil R, et al. Singular phase nano-optics in plasmonic metamaterials for label-free single-molecule detection. *Nat Mater* 2013;12:304-9. DOI PubMed
81. Im H, Shao H, Park YI, et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat Biotechnol* 2014;32:490-5. DOI PubMed PMC
82. Yoshioka Y, Kosaka N, Konishi Y, et al. Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. *Nat Commun* 2014;5:3591. DOI PubMed PMC
83. Zhao Z, Yang Y, Zeng Y, He M. A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis. *Lab Chip* 2016;16:489-96. DOI PubMed PMC
84. Mitchell PJ, Welton J, Staffurth J, et al. Can urinary exosomes act as treatment response markers in prostate cancer? *J Transl Med* 2009;7:4. DOI PubMed PMC
85. Logozzi M, Angelini DF, Iessi E, et al. Increased PSA expression on prostate cancer exosomes in in vitro condition and in cancer patients. *Cancer Lett* 2017;403:318-29. DOI PubMed

86. Huber V, Fais S, Iero M, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* 2005;128:1796-804. DOI PubMed
87. Khan S, Jutzy JM, Valenzuela MM, et al. Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. *PLoS One* 2012;7:e46737. DOI PubMed PMC
88. Khan S, Bennit HF, Turay D, et al. Early diagnostic value of survivin and its alternative splice variants in breast cancer. *BMC Cancer* 2014;14:176. DOI PubMed PMC
89. Melo SA, Luecke LB, Kahlert C, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 2015;523:177-82. DOI PubMed PMC
90. Book: ISEV2017. *J Extracell Vesicles* 2017;6:1310414. DOI PubMed PMC
91. Giampieri R, Piva F, Occhipinti G, et al. Clinical impact of different exosomes' protein expression in pancreatic ductal carcinoma patients treated with standard first line palliative chemotherapy. *PLoS One* 2019;14:e0215990. DOI PubMed PMC
92. Ning K, Wang T, Sun X, et al. UCH-L1-containing exosomes mediate chemotherapeutic resistance transfer in breast cancer. *J Surg Oncol* 2017;115:932-40. DOI PubMed
93. Lv MM, Zhu XY, Chen WX, et al. Exosomes mediate drug resistance transfer in MCF-7 breast cancer cells and a probable mechanism is delivery of P-glycoprotein. *Tumour Biol* 2014;35:10773-9. DOI PubMed
94. Zhang FF, Zhu YF, Zhao QN, et al. Microvesicles mediate transfer of P-glycoprotein to paclitaxel-sensitive A2780 human ovarian cancer cells, conferring paclitaxel-resistance. *Eur J Pharmacol* 2014;738:83-90. DOI PubMed
95. Corcoran C, Rani S, O'Brien K, et al. Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PLoS One* 2012;7:e50999. DOI PubMed PMC
96. Ciravolo V, Huber V, Ghedini GC, et al. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. *J Cell Physiol* 2012;227:658-67. DOI PubMed
97. Martinez VG, O'Neill S, Salimu J, et al. Resistance to HER2-targeted anti-cancer drugs is associated with immune evasion in cancer cells and their derived extracellular vesicles. *Oncoimmunology* 2017;6:e1362530. DOI PubMed PMC
98. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest* 2015;125:3384-91. DOI PubMed PMC
99. Ribas A, Hamid O, Daud A, et al. Association of pembrolizumab with tumor response and survival among patients with advanced melanoma. *JAMA* 2016;315:1600-9. DOI PubMed
100. Zaretsky JM, Garcia-Diaz A, Shin DS, et al. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N Engl J Med* 2016;375:819-29. DOI PubMed PMC
101. Chen G, Huang AC, Zhang W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. *Nature* 2018;560:382-6. DOI PubMed PMC
102. Cao L, Cui X, Hu J, et al. Advances in digital polymerase chain reaction (dPCR) and its emerging biomedical applications. *Biosens Bioelectron* 2017;90:459-74. DOI PubMed
103. Ratajczak J, Miekus K, Kucia M, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;20:847-56. DOI PubMed
104. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010;285:17442-52. DOI PubMed PMC
105. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, et al. Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A* 2010;107:6328-33. DOI PubMed PMC
106. Zhang Y, Liu D, Chen X, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol Cell* 2010;39:133-44. DOI PubMed
107. Zhu L, Li J, Gong Y, et al. Exosomal tRNA-derived small RNA as a promising biomarker for cancer diagnosis. *Mol Cancer* 2019;18:74. DOI PubMed PMC
108. Zhang PF, Gao C, Huang XY, et al. Cancer cell-derived exosomal circUHRF1 induces natural killer cell exhaustion and may cause resistance to anti-PD1 therapy in hepatocellular carcinoma. *Mol Cancer* 2020;19:110. DOI PubMed PMC
109. Gusachenko ON, Zenkova MA, Vlassov VV. Nucleic acids in exosomes: disease markers and intercellular communication molecules. *Biochem* 2013;78:1-7. DOI PubMed
110. Bullock MD, Silva AM, Kanlikilicer-Unaldi P, et al. Exosomal non-coding RNAs: diagnostic, prognostic and therapeutic applications in cancer. *Noncoding RNA* 2015;1:53-68. DOI PubMed PMC
111. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513-8. DOI PubMed PMC
112. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* 2008;110:13-21. DOI PubMed
113. Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer* 2009;10:42-6. DOI PubMed
114. Takeshita N, Hoshino I, Mori M, et al. Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. *Br J Cancer* 2013;108:644-52. DOI PubMed PMC
115. Nilsson J, Skog J, Nordstrand A, et al. Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *Br J Cancer* 2009;100:1603-7. DOI PubMed PMC
116. Foj L, Ferrer F, Serra M, et al. Exosomal and Non-Exosomal Urinary miRNAs in Prostate Cancer Detection and Prognosis. *Prostate* 2017;77:573-83. DOI PubMed

117. Yasui T, Yanagida T, Ito S, et al. Unveiling massive numbers of cancer-related urinary-microRNA candidates via nanowires. *Sci Adv* 2017;3:e1701133. DOI PubMed PMC
118. McKiernan J, Donovan MJ, O'Neill V, et al. A Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer at Initial Biopsy. *JAMA Oncol* 2016;2:882-9. DOI PubMed
119. Huang X, Yuan T, Liang M, et al. Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. *Eur Urol* 2015;67:33-41. DOI PubMed PMC
120. Tanaka Y, Kamohara H, Kinoshita K, et al. Clinical impact of serum exosomal microRNA-21 as a clinical biomarker in human esophageal squamous cell carcinoma. *Cancer* 2013;119:1159-67. DOI PubMed
121. Matsumura T, Sugimachi K, Iinuma H, et al. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *Br J Cancer* 2015;113:275-81. DOI PubMed PMC
122. Işın M, Uysaler E, Özgür E, et al. Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. *Front Genet* 2015;6:168. DOI PubMed PMC
123. Lin LY, Yang L, Zeng Q, et al. Tumor-originated exosomal LncUEGC1 as a circulating biomarker for early-stage gastric cancer. *Mol Cancer* 2018;17:84. DOI PubMed PMC
124. Zhao R, Zhang Y, Zhang X, et al. Exosomal long noncoding RNA HOTTIP as potential novel diagnostic and prognostic biomarker test for gastric cancer. *Mol Cancer* 2018;17:68. DOI PubMed PMC
125. Lee YR, Kim G, Tak WY, et al. Circulating exosomal noncoding RNAs as prognostic biomarkers in human hepatocellular carcinoma. *Int J Cancer* 2019;144:1444-52. DOI PubMed
126. Chen X, Chen RX, Wei WS, et al. PRMT5 Circular RNA Promotes Metastasis of Urothelial Carcinoma of the Bladder through Sponging miR-30c to Induce Epithelial-Mesenchymal Transition. *Clin Cancer Res* 2018;24:6319-30. DOI PubMed
127. Ogata-Kawata H, Izumiya M, Kurioka D, et al. Circulating exosomal microRNAs as biomarkers of colon cancer. *PLoS One* 2014;9:e92921. DOI PubMed PMC
128. Svedman FC, Lohcharoenkal W, Bottai M, et al. Extracellular microvesicle microRNAs as predictive biomarkers for targeted therapy in metastastic cutaneous malignant melanoma. *PLoS One* 2018;13:e0206942. DOI PubMed PMC
129. Tang S, Zheng K, Tang Y, Li Z, Zou T, Liu D. Overexpression of serum exosomal HOTAIR is correlated with poor survival and poor response to chemotherapy in breast cancer patients. *J Biosci* 2019:44. PubMed
130. Dong H, Wang W, Chen R, et al. Exosome-mediated transfer of lncRNASNHG14 promotes trastuzumab chemoresistance in breast cancer. *Int J Oncol* 2018;53:1013-26. DOI PubMed PMC
131. Mikamori M, Yamada D, Eguchi H, et al. MicroRNA-155 Controls Exosome Synthesis and Promotes Gemcitabine Resistance in Pancreatic Ductal Adenocarcinoma. *Sci Rep* 2017;7:42339. DOI PubMed PMC
132. Del Re M, Biasco E, Crucitta S, et al. The Detection of Androgen Receptor Splice Variant 7 in Plasma-derived Exosomal RNA Strongly Predicts Resistance to Hormonal Therapy in Metastatic Prostate Cancer Patients. *Eur Urol* 2017;71:680-7. DOI PubMed
133. Woo HK, Park J, Ku JY, et al. Urine-based liquid biopsy: non-invasive and sensitive AR-V7 detection in urinary EVs from patients with prostate cancer. *Lab Chip* 2018;19:87-97. DOI PubMed
134. Del Re M, Marconcini R, Pasquini G, et al. PD-L1 mRNA expression in plasma-derived exosomes is associated with response to anti-PD-1 antibodies in melanoma and NSCLC. *Br J Cancer* 2018;118:820-4. DOI PubMed PMC
135. Ma X, Chen Z, Hua D, et al. Essential role for TrpC5-containing extracellular vesicles in breast cancer with chemotherapeutic resistance. *Proc Natl Acad Sci U S A* 2014;111:6389-94. DOI PubMed PMC
136. Yang SJ, Wang DD, Li J, et al. Predictive role of GSTP1-containing exosomes in chemotherapy-resistant breast cancer. *Gene* 2017;623:5-14. DOI PubMed
137. Shao H, Chung J, Lee K, et al. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. *Nat Commun* 2015;6:6999. DOI PubMed PMC
138. Balaj L, Lessard R, Dai L, et al. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun* 2011;2:180. DOI PubMed PMC
139. Yokoi A, Villar-Prados A, Oliphint PA, et al. Mechanisms of nuclear content loading to exosomes. *Sci Adv* 2019;5:eaax8849. DOI PubMed PMC
140. Thakur BK, Zhang H, Becker A, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res* 2014;24:766-9. DOI PubMed PMC
141. Kahlert C, Melo SA, Protopopov A, et al. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J Biol Chem* 2014;289:3869-75. DOI PubMed PMC
142. Kahlert C. Liquid Biopsy: Is There an Advantage to Analyzing Circulating Exosomal DNA Compared to cfDNA or Are They the Same? *Cancer Res* 2019;79:2462-5. DOI PubMed
143. Allenson K, Castillo J, San Lucas FA, et al. High prevalence of mutant KRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients. *Ann Oncol* 2017;28:741-7. DOI PubMed PMC
144. Bernard V, Kim DU, San Lucas FA, et al. Circulating Nucleic Acids Are Associated With Outcomes of Patients With Pancreatic Cancer. *Gastroenterology* 2019;156:108-118.e4. DOI PubMed PMC
145. Castellanos-Rizaldos E, Grimm DG, Tadigotla V, et al. Exosome-Based Detection of. *EGFR* ;24:2944-50. DOI PubMed
146. Krug AK, Enderle D, Karlovich C, et al. Improved EGFR mutation detection using combined exosomal RNA and circulating tumor DNA in NSCLC patient plasma. *Ann Oncol* 2018;29:2143. DOI PubMed
147. Lee DH, Yoon H, Park S, et al. Urinary Exosomal and cell-free DNA Detects Somatic Mutation and Copy Number Alteration in

Urothelial Carcinoma of Bladder. *Sci Rep* 2018;8:14707. DOI PubMed PMC
148. Takahashi A, Okada R, Nagao K, et al. Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun* 2017;8:15287. DOI PubMed PMC
149. Fischer S, Cornils K, Speiseder T, et al. Indication of Horizontal DNA Gene Transfer by Extracellular Vesicles. *PLoS One* 2016;11:e0163665. DOI PubMed PMC
150. Jin Y, Chen K, Wang Z, et al. DNA in serum extracellular vesicles is stable under different storage conditions. *BMC Cancer* 2016;16:753. DOI PubMed PMC
151. Mandel P, Métais P. Les Acides Nucleiques Du Plasma Sanguin Chez l' Homme. *C R Seances Soc Biol Fil* 1948;142:241-3. (in French).
152. Schwarzenbach H, da Silva AM, Calin G, Pantel K. Data Normalization Strategies for MicroRNA Quantification. *Clin Chem* 2015;61:1333-42. DOI PubMed PMC
153. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034. DOI PubMed PMC
154. Mathivanan S, Simpson RJ. ExoCarta: A compendium of exosomal proteins and RNA. *Proteomics* 2009;9:4997-5000. DOI PubMed
155. Nguyen HQ, Lee D, Kim Y, et al. Platelet Factor 4 as a Novel Exosome Marker in MALDI-MS Analysis of Exosomes from Human Serum. *Anal Chem* 2019;91:13297-305. DOI PubMed
156. Lu Q, Zhang J, Allison R, et al. Identification of extracellular delta-catenin accumulation for prostate cancer detection. *Prostate* 2009;69:411-8. DOI PubMed PMC
157. Kharaziha P, Chioureas D, Rutishauser D, et al. Molecular profiling of prostate cancer derived exosomes may reveal a predictive signature for response to docetaxel. *Oncotarget* 2015;6:21740-54. DOI PubMed PMC
158. Biggs CN, Siddiqui KM, Al-Zahrani AA, et al. Prostate extracellular vesicles in patient plasma as a liquid biopsy platform for prostate cancer using nanoscale flow cytometry. *Oncotarget* 2016;7:8839-49. DOI PubMed PMC
159. Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. *J Proteome Res* 2008;7:2088-96. DOI PubMed
160. Chen CL, Lai YF, Tang P, et al. Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients. *J Proteome Res* 2012;11:5611-29. DOI PubMed
161. Lin SY, Chang CH, Wu HC, et al. Proteome Profiling of Urinary Exosomes Identifies Alpha 1-Antitrypsin and H2B1K as Diagnostic and Prognostic Biomarkers for Urothelial Carcinoma. *Sci Rep* 2016;6:34446. DOI PubMed PMC
162. Silvers CR, Miyamoto H, Messing EM, Netto GJ, Lee YF. Characterization of urinary extracellular vesicle proteins in muscle-invasive bladder cancer. *Oncotarget* 2017;8:91199-208. DOI PubMed PMC
163. Silvers CR, Liu YR, Wu CH, Miyamoto H, Messing EM, Lee YF. Identification of extracellular vesicle-borne periostin as a feature of muscle-invasive bladder cancer. *Oncotarget* 2016;7:23335-45. DOI PubMed PMC
164. Beckham CJ, Olsen J, Yin PN, et al. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. *J Urol* 2014;192:583-92. DOI PubMed
165. Welton JL, Khanna S, Giles PJ, et al. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics* 2010;9:1324-38. DOI PubMed PMC
166. Raimondo F, Morosi L, Corbetta S, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol Biosyst* 2013;9:1220-33. DOI PubMed
167. Wang X, Zhong W, Bu J, et al. Exosomal protein CD82 as a diagnostic biomarker for precision medicine for breast cancer. *Mol Carcinog* 2019;58:674-85. DOI PubMed
168. Rupp AK, Rupp C, Keller S, et al. Loss of EpCAM expression in breast cancer derived serum exosomes: role of proteolytic cleavage. *Gynecol Oncol* 2011;122:437-46. DOI PubMed
169. Wang T, Ning K, Lu TX, et al. Increasing circulating exosomes-carrying TRPC5 predicts chemoresistance in metastatic breast cancer patients. *Cancer Sci* 2017;108:448-54. DOI PubMed PMC
170. Szajnik M, Derbis M, Lach M, et al. Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy. *Gynecol Obstet (Sunnyvale)* 2013;Suppl 4:3. DOI PubMed PMC
171. Li J, Sherman-Baust CA, Tsai-Turton M, Bristow RE, Roden RB, Morin PJ. Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. *BMC Cancer* 2009;9:244. DOI PubMed PMC
172. Tang MKS, Yue PYK, Ip PP, et al. Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. *Nat Commun* 2018;9:2270. DOI PubMed PMC
173. Graves LE, Ariztia EV, Navari JR, Matzel HJ, Stack MS, Fishman DA. Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. *Cancer Res* 2004;64:7045-9. DOI PubMed
174. Keller S, König AK, Marmé F, et al. Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett* 2009;278:73-81. DOI PubMed
175. Frampton AE, Prado MM, López-Jiménez E, et al. Glypican-1 is enriched in circulating-exosomes in pancreatic cancer and correlates with tumor burden. *Oncotarget* 2018;9:19006-13. DOI PubMed PMC
176. Kimura H, Yamamoto H, Harada T, et al. CKAP4, a DKK1 Receptor, Is a Biomarker in Exosomes Derived from Pancreatic Cancer and a Molecular Target for Therapy. *Clin Cancer Res* 2019;25:1936-47. DOI PubMed
177. Costa-Silva B, Aiello NM, Ocean AJ, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 2015;17:816-26. DOI PubMed PMC
178. Yokoyama S, Takeuchi A, Yamaguchi S, et al. Clinical implications of carcinoembryonic antigen distribution in serum exosomal

fraction-Measurement by ELISA. *PLoS One* 2017;12:e0183337. DOI PubMed PMC
179. Choi DS, Park JO, Jang SC, et al. Proteomic analysis of microvesicles derived from human colorectal cancer ascites. *Proteomics* 2011;11:2745-51. DOI PubMed
180. Baran J, Baj-Krzyworzeka M, Weglarczyk K, et al. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. *Cancer Immunol Immunother* 2010;59:841-50. DOI PubMed
181. Yamashita T, Kamada H, Kanasaki S, et al. Epidermal growth factor receptor localized to exosome membranes as a possible biomarker for lung cancer diagnosis. *Pharmazie* 2013:68;969-73. PubMed
182. Niu L, Song X, Wang N, Xue L, Song X, Xie L. Tumor-derived exosomal proteins as diagnostic biomarkers in non-small cell lung cancer. *Cancer Sci* 2019;110:433-42. DOI PubMed PMC
183. Sandfeld-Paulsen B, Aggerholm-Pedersen N, Bæk R, et al. Exosomal proteins as prognostic biomarkers in non-small cell lung cancer. *Mol Oncol* 2016;10:1595-602. DOI PubMed PMC
184. Li Y, Zhang Y, Qiu F, Qiu Z. Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. *Electrophoresis* 2011;32:1976-83. DOI PubMed
185. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One* 2009;4:e5219. DOI PubMed PMC
186. Peinado H, Alečković M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 2012;18:883-91. DOI PubMed PMC
187. Bryant RJ, Pawlowski T, Catto JW, et al. Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer* 2012;106:768-74. DOI PubMed PMC
188. Xu Y, Qin S, An T, Tang Y, Huang Y, Zheng L. MiR-145 detection in urinary extracellular vesicles increase diagnostic efficiency of prostate cancer based on hydrostatic filtration dialysis method. *Prostate* 2017;77:1167-75. DOI PubMed
189. Koppers-Lalic D, Hackenberg M, de Menezes R, et al. Noninvasive prostate cancer detection by measuring miRNA variants (isomiRs) in urine extracellular vesicles. *Oncotarget* 2016;7:22566-78. DOI PubMed PMC
190. Wani S, Kaul D, Mavuduru RS, Kakkar N, Bhatia A. Urinary-exosomal miR-2909: A novel pathognomonic trait of prostate cancer severity. *J Biotechnol* 2017;259:135-9. DOI PubMed
191. Rodríguez M, Bajo-Santos C, Hessvik NP, et al. Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes. *Mol Cancer* 2017;16:1-6. DOI PubMed PMC
192. Endzeliņš E, Berger A, Melne V, et al. Detection of circulating miRNAs: comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. *BMC Cancer* 2017;17:730. DOI PubMed PMC
193. Li Z, Ma YY, Wang J, et al. Exosomal microRNA-141 is upregulated in the serum of prostate cancer patients. *Onco Targets Ther* 2016;9:139-48. DOI PubMed PMC
194. Hessvik NP, Sandvig K, Llorente A. Exosomal miRNAs as Biomarkers for Prostate Cancer. *Front Genet* 2013;4:36. DOI PubMed PMC
195. Moltzahn F, Olshen AB, Baehner L, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res* 2011;71:550-60. DOI PubMed PMC
196. Lodes MJ, Caraballo M, Suciu D, Munro S, Kumar A, Anderson B. Detection of cancer with serum miRNAs on an oligonucleotide microarray. *PLoS One* 2009;4:e6229. DOI PubMed PMC
197. Bhagirath D, Yang TL, Bucay N, et al. microRNA-1246 Is an Exosomal Biomarker for Aggressive Prostate Cancer. *Cancer Res* 2018;78:1833-44. DOI PubMed PMC
198. Wang YH, Ji J, Wang BC, et al. Tumor-Derived Exosomal Long Noncoding RNAs as Promising Diagnostic Biomarkers for Prostate Cancer. *Cell Physiol Biochem* 2018;46:532-45. DOI PubMed
199. De Long J, Sullivan TB, Humphrey J, et al. A non-invasive miRNA based assay to detect bladder cancer in cell-free urine. *Am J Transl Res* 2015;7:2500-9. PubMed PMC
200. Armstrong DA, Green BB, Seigne JD, Schned AR, Marsit CJ. MicroRNA molecular profiling from matched tumor and bio-fluids in bladder cancer. *Mol Cancer* 2015;14:194. DOI PubMed PMC
201. Andreu Z, Otta Oshiro R, Redruello A, et al. Extracellular vesicles as a source for non-invasive biomarkers in bladder cancer progression. *Eur J Pharm Sci* 2017;98:70-9. DOI PubMed
202. Baumgart S, Meschkat P, Edelmann P, et al. MP78-05 INVASION-ASSOCIATED MIRNAS S AS POSSIBLE DIAGNOSTIC BIOMARKERS OF MUSCLE INVASIVE BLADDER CANCER IN TUMOR TISSUES AND URINARY EXOSOMES. *J Urol* 2018:199.
203. Berrondo C, Flax J, Kucherov V, et al. Expression of the Long Non-Coding RNA HOTAIR Correlates with Disease Progression in Bladder Cancer and Is Contained in Bladder Cancer Patient Urinary Exosomes. *PLoS One* 2016;11:e0147236. DOI PubMed PMC
204. Zhang S, Du L, Wang L, et al. Evaluation of serum exosomal LncRNA-based biomarker panel for diagnosis and recurrence prediction of bladder cancer. *J Cell Mol Med* 2019;23:1396-405. DOI PubMed PMC
205. Xue M, Chen W, Xiang A, et al. Hypoxic exosomes facilitate bladder tumor growth and development through transferring long non-coding RNA-UCA1. *Mol Cancer* 2017;16:143. DOI PubMed PMC
206. Zhang W, Ni M, Su Y, et al. MicroRNAs in Serum Exosomes as Potential Biomarkers in Clear-cell Renal Cell Carcinoma. *Eur Urol Focus* 2018;4:412-9. DOI PubMed
207. Wang X, Wang T, Chen C, et al. Serum exosomal miR-210 as a potential biomarker for clear cell renal cell carcinoma. *J Cell Biochem* ;2018:1492-502. DOI PubMed
208. Butz H, Nofech-Mozes R, Ding Q, et al. Exosomal MicroRNAs Are Diagnostic Biomarkers and Can Mediate Cell-Cell

Communication in Renal Cell Carcinoma. *Eur Urol Focus* 2016;2:210-8. DOI PubMed

209. Fujii N, Hirata H, Ueno K, et al. Extracellular MiR-224 as a prognostic marker for clear cell renal cell carcinoma. *Oncotarget* 2017;8:109877-88. DOI PubMed PMC
210. Du M, Giridhar KV, Tian Y, et al. Plasma exosomal miRNAs-based prognosis in metastatic kidney cancer. *Oncotarget* 2017;8:63703-14. DOI PubMed PMC
211. De Palma G, Sallustio F, Curci C, et al. The Three-Gene Signature in Urinary Extracellular Vesicles from Patients with Clear Cell Renal Cell Carcinoma. *J Cancer* 2016;7:1960-7. DOI PubMed PMC
212. Hannafon BN, Trigoso YD, Calloway CL, et al. Plasma exosome microRNAs are indicative of breast cancer. *Breast Cancer Res* 2016;18:90. DOI PubMed PMC
213. Zhou W, Fong MY, Min Y, et al. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell* 2014;25:501-15. DOI PubMed PMC
214. Rodríguez-Martínez A, de Miguel-Pérez D, Ortega FG, et al. Exosomal miRNA profile as complementary tool in the diagnostic and prediction of treatment response in localized breast cancer under neoadjuvant chemotherapy. *Breast Cancer Res* 2019;21:21. DOI PubMed PMC
215. Santos JC, Lima NDS, Sarian LO, Matheu A, Ribeiro ML, Derchain SFM. Exosome-mediated breast cancer chemoresistance via miR-155 transfer. *Sci Rep* 2018;8:829. DOI PubMed PMC
216. Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008;10:593-601. DOI PubMed
217. Pigati L, Yaddanapudi SC, Iyengar R, et al. Selective release of microRNA species from normal and malignant mammary epithelial cells. *PLoS One* 2010;5:e13515. DOI PubMed PMC
218. Kanlikilicer P, Bayraktar R, Denizli M, et al. Exosomal miRNA confers chemo resistance via targeting Cav1/p-gp/M2-type macrophage axis in ovarian cancer. *EBioMedicine* 2018;38:100-12. DOI PubMed PMC
219. Que R, Ding G, Chen J, Cao L. Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. *World J Surg Oncol* 2013;11:219. DOI PubMed PMC
220. Lau C, Kim Y, Chia D, et al. Role of pancreatic cancer-derived exosomes in salivary biomarker development. *J Biol Chem* 2013;288:26888-97. DOI PubMed PMC
221. Tian XP, Wang CY, Jin XH, et al. Acidic Microenvironment Up-Regulates Exosomal miR-21 and miR-10b in Early-Stage Hepatocellular Carcinoma to Promote Cancer Cell Proliferation and Metastasis. *Theranostics* 2019;9:1965-79. DOI PubMed PMC
222. Xie M, Yu T, Jing X, et al. Exosomal circSHKBP1 promotes gastric cancer progression via regulating the miR-582-3p/HUR/VEGF axis and suppressing HSP90 degradation. *Mol Cancer* 2020;19:112. DOI PubMed PMC
223. Liu C, Eng C, Shen J, et al. Serum exosomal miR-4772-3p is a predictor of tumor recurrence in stage II and III colon cancer. *Oncotarget* 2016;7:76250-60. DOI PubMed PMC
224. Bjørnetrø T, Redalen KR, Meltzer S, et al. An experimental strategy unveiling exosomal microRNAs 486-5p, 181a-5p and 30d-5p from hypoxic tumour cells as circulating indicators of high-risk rectal cancer. *J Extracell Vesicles* 2019;8:1567219. DOI PubMed PMC
225. Cazzoli R, Buttitta F, Di Nicola M, et al. microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. *J Thorac Oncol* 2013;8:1156-62. DOI PubMed PMC
226. Silva J, García V, Zaballos Á, et al. Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival. *Eur Respir J* 2011;37:617-23. DOI PubMed
227. Aushev VN, Zborovskaya IB, Laktionov KK, et al. Comparisons of microRNA patterns in plasma before and after tumor removal reveal new biomarkers of lung squamous cell carcinoma. *PLoS One* 2013;8:e78649. DOI PubMed PMC
228. Wei F, Ma C, Zhou T, et al. Exosomes derived from gemcitabine-resistant cells transfer malignant phenotypic traits via delivery of miRNA-222-3p. *Mol Cancer* 2017;16:132. DOI PubMed PMC
229. Yuwen DL, Sheng BB, Liu J, Wenyu W, Shu YQ. MiR-146a-5p level in serum exosomes predicts therapeutic effect of cisplatin in non-small cell lung cancer. *Eur Rev Med Pharmacol Sci* 2017;21:2650-8. PubMed
230. Shi L, Chen J, Yang J, Pan T, Zhang S, Wang Z. MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. *Brain Res* 2010;1352:255-64. DOI PubMed
231. Akers JC, Ramakrishnan V, Kim R, et al. MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. *PLoS One* 2013;8:e78115. DOI PubMed PMC
232. Manterola L, Guruceaga E, Gállego Pérez-Larraya J, et al. A small noncoding RNA signature found in exosomes of GBM patient serum as a diagnostic tool. *Neuro Oncol* 2014;16:520-7. DOI PubMed PMC

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Extracellular Vesicles and Circulating Nucleic Acids

Commentary

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Bioengineered extracellular vesicle-loaded bioscaffolds for therapeutic applications in regenerative medicine

Sabrina Lazar[1], Sirjan Mor[1], Jianing Chen[1], Dake Hao[1,2], Aijun Wang[1,2,3]

[1]Department of Surgery, University of California, Davis School of Medicine, Sacramento, CA 95817, USA.
[2]Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children, Sacramento, CA 95817, USA.
[3]Department of Biomedical Engineering, UC Davis, Davis, CA 95616, USA.

Correspondence to: Prof. Aijun Wang, Department of Surgery, University of California, Davis School of Medicine, 4625 2nd Avenue, Suite #3005, Sacramento, CA 95817, USA. E-mail: aawang@ucdavis.edu

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Abstract

Extracellular vesicle (EV)-based technologies represent a new advancement for disease treatment. EVs can be administered systemically, injected into the injury site directly, or applied locally in conjunction with bioengineered implantable scaffolds. Matrix-bound vesicles (MBVs), a special class of vesicles localized in association with the extracellular matrix (ECM), have been identified as critical bioactive factors and shown to mediate significant regenerative functions of ECM scaffolds. Loading EVs onto bioscaffolds to mimic the MBV-ECM complex has been shown superior to EV bolus injection in recent *in vivo* studies, such as in providing enhanced tissue regeneration, EV retention rates, and healing efficacy. Different types of natural biomaterials, synthetic polymers, and ceramics have been developed for EV loading, and these EV functionalized biomaterials have been applied in different areas for disease treatment. The EV functionalized scaffolds can be designed to be biodegradable, off-the-shelf biomaterials as a delivery vehicle for EVs. Overall, the bioengineered EV-loaded bioscaffolds represent a promising approach for cell-free treatment in clinical applications.

Keywords: Extracellular vesicles, biomaterials, bioscaffolds, EV scaffolds, EV therapeutics, drug delivery, bioengineering

Extracellular vesicles (EVs) are nanoparticles of various sizes secreted by all cells that can act as cell-cell communicators[1] because they contain RNAs, proteins and lipids to facilitate intercellular communications[2]. Moreover, EVs can make an efficient drug delivery system because they carry the intrinsic ability to cross cellular/tissue barriers such as the blood-brain barrier[3]. Hence, EVs have emerged as a promising strategy in regenerative medicine. The most common mode of EV delivery for tissue repair is systemic intravenous injection of free EVs or local injection directly into sites of injury, which unfortunately may lead to rapid clearance of EVs. Many different approaches have been used to optimize the delivery strategy of EVs, such as increasing the injection dose, surface modification for targeted delivery, or encasing EVs in a biomaterial matrix[1,4,5]. Since EVs arise from cellular paracrine secretions and carry cellular membrane compositions, they often interact with the surrounding extracellular matrix (ECM) environment *in vivo*. In addition, matrix-bound vesicles are identified as an integral and functional component of ECM biomaterial scaffolds mediating significant regenerative functions[6,7]. Therefore, integrating EVs and ECM-mimicking biomaterials to mimic the native EV-ECM complexes provides great potential for preservation and sustained delivery of EVs for regenerative medicine applications [Figure 1][8]. In this commentary, we will outline the recent advances in the administration of EV-functionalized biomaterials and discuss future challenges in this field.

Scaffolds have been widely used to provide physical support for the loading of EVs at injury sites. For example, bioscaffolds can deliver mesenchymal stem/stromal cell derived EVs (MSC-EVs) at specific areas to repair peripheral nerve injury, epidural fibrosis and incisional hernia[1]. EV-loaded scaffolds present an opportunity to advance drug delivery. In addition, EVs can be engineered by genetically modifying EV-producing parent cells, fusing targeting proteins or aptamers to EV surfaces, or altering internal cargo[3,9]. The EV-scaffolds can be directly applied to disease areas, serving as sustained release devices to extend the EVs' retention and prevent mass diffusion away from the site or enzymatic digestion. This approach is more optimal than EV or drug intravenous injection, which could lead to off-target EV accumulation[10]. In addition, EV/scaffold complexes can be developed using a variety of biomaterials and can be optimized for disease-specific or tissue-specific applications.

In order to be safe and effective, bioscaffolds must fulfill criteria such as biocompatibility, degradability and the necessary mechanical properties[1]. Natural biomaterials such as collagen, hyaluronan, and decellularized ECM materials can be used[1]. These materials provide excellent specificity for cell/EV surface receptors, however there is more heterogeneity in natural biomaterials due to intrinsic variations from their biological sources[11,12]. Synthetic materials such as FDA-approved polylactide-*co*-glycolide and beta-tricalcium phosphate[1] can also be used. Synthetic materials are generally cheaper and more homogenous than natural materials in their biological properties, and they can be modified to exert specific biological activities. Natural materials and synthetic materials can also be combined or chemically modified to be used as hybrid biomaterials[1].

To imbue scaffolds with biological activity, EVs can be chemically conjugated to them using targeted proteins or ligands. For example, an integrin α4β1 ligand LLP2A was found to bind strongly to placenta mesenchymal stem cell derived EVs (PMSC-EVs). LLP2A immobilized to a polymer scaffold via Click chemistry[8] can be used to specifically load PMSC-EVs onto the scaffold and the EV-loaded scaffold increased angiogenesis and vascularization in an *ex vivo* aortic ring sprouting assay[8]. Others have leveraged ECM-related proteins to increase EV immobilization by coating scaffolds with fibronectin[13]. On a decellularized bone matrix scaffold coated with fibronectin, bone marrow mesenchymal stem cell derived EVs promoted bone regeneration and angiogenesis *in vivo*[13]. When applied to injury sites, the EVs on the scaffold may be protected and released in a sustained manner from the scaffold and communicate with

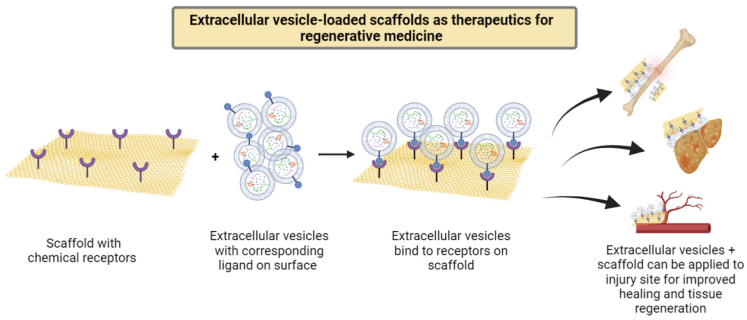

Figure 1. Extracellular vesicles (EVs) and scaffolds can be designed to bind specifically to each other and be applied together onto injury or disease sites. For example, EV bioscaffolds provide localized delivery and sustained therapeutic EV release, and can be applied for wound healing, tissue regeneration, neovascularization, or angiogenesis *in vivo*.

endogenous cells and extracellular components to participate in the remodeling process.

Using bioscaffolds to immobilize and deliver EVs has been shown to be more effective than injecting free EVs when applied *in vivo*. For example, compared to bolus EV injections, EV-functionalized polyethylene glycol hydrogels significantly enhanced liver regeneration by attenuating inflammation and apoptosis in a rat model of chronic liver fibrosis[14]. In a myocardial infarction rat model, EV-loaded peptide hydrogels were superior to EV bolus injection in increasing angiogenesis and reducing inflammation[15]. Bioscaffold-based EV delivery may be more advantageous than traditional EV injections in improving retention and targeted delivery of EVs to the site of injury[10,12,16].

EVs as a special biological component provide more possibilities to functionalize scaffold materials with biological functions. By integrating biochemistry and bioengineering principles, EV bioscaffold products have shown promising therapeutic outcomes in numerous medical studies, such as wound healing, tissue regeneration, vascularization, and angiogenesis [Figure 1][8,13,17]. In addition, appropriate EV delivery systems have shown obvious advantages for further enhancing the function of EV modified bioscaffolds[15,18]. Therefore, future research may focus on further refinement of EV modified scaffolds, such as the loading and release mechanisms, the loading density and release profile, storage stability, and safety must be fully characterized before clinical applications. Scaffold-based EV delivery is becoming a promising cell-free therapeutic approach for tissue regeneration and clinical applications.

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Authors' contributions

Made substantial contributions to conception, design, writing, and editing of the paper, and performed literature searches and interpretation: Lazar S, Mor S, Chen J, Hao D, Wang A

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Not applicable.

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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. Brennan MÁ, Layrolle P, Mooney DJ. Biomaterials functionalized with MSC secreted extracellular vesicles and soluble factors for tissue regeneration. *Adv Funct Mater* 2020;30:1909125. DOI PubMed PMC
2. Yamamoto T, Kosaka N, Ochiya T. Latest advances in extracellular vesicles: from bench to bedside. *Sci Technol Adv Mater* 2019;20:746-57. DOI PubMed PMC
3. Murphy DE, de Jong OG, Brouwer M, et al. Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking. *Exp Mol Med* 2019;51:1-12. DOI PubMed PMC
4. Witwer KW, Wolfram J. Extracellular vesicles versus synthetic nanoparticles for drug delivery. *Nat Rev Mater* 2021;6:103-6. DOI
5. Massaro C, Sgueglia G, Frattolillo V, Baglio SR, Altucci L, Dell'Aversana C. Extracellular vesicle-based nucleic acid delivery: current advances and future perspectives in cancer therapeutic strategies. *Pharmaceutics* 2020;12:980. DOI PubMed PMC
6. van der Merwe Y, Faust AE, Sakalli ET, et al. Matrix-bound nanovesicles prevent ischemia-induced retinal ganglion cell axon degeneration and death and preserve visual function. *Sci Rep* 2019;9:3482. DOI PubMed PMC
7. Huleihel L, Hussey GS, Naranjo JD, et al. Matrix-bound nanovesicles within ECM bioscaffolds. *Sci Adv* 2016;2:e1600502. DOI PubMed PMC
8. Hao D, Swindell HS, Ramasubramanian L, et al. Extracellular matrix mimicking nanofibrous scaffolds modified with mesenchymal stem cell-derived extracellular vesicles for improved vascularization. *Front Bioeng Biotechnol* 2020;8:633. DOI PubMed PMC
9. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;29:341-5. DOI PubMed
10. Yan HC, Yu TT, Li J, et al. The delivery of extracellular vesicles loaded in biomaterial scaffolds for bone regeneration. *Front Bioeng Biotechnol* 2020;8:1015. DOI PubMed PMC
11. Litowczenko J, Woźniak-Budych MJ, Staszak K, Wieszczycka K, Jurga S, Tylkowski B. Milestones and current achievements in development of multifunctional bioscaffolds for medical application. *Bioact Mater* 2021;6:2412-38. DOI PubMed PMC
12. Davison-Kotler E, Marshall WS, García-Gareta E. Sources of collagen for biomaterials in skin wound healing. *Bioengineering (Basel)* 2019;6:56. DOI PubMed PMC
13. Xie H, Wang Z, Zhang L, et al. Extracellular vesicle-functionalized decalcified bone matrix scaffolds with enhanced pro-angiogenic and pro-bone regeneration activities. *Sci Rep* 2017;7:45622. DOI PubMed PMC
14. Mardpour S, Ghanian MH, Sadeghi-Abandansari H, et al. Hydrogel-mediated sustained systemic delivery of mesenchymal stem cell-derived extracellular vesicles improves hepatic regeneration in chronic liver failure. *ACS Appl Mater Interfaces* 2019;11:37421-33. DOI PubMed
15. Han C, Zhou J, Liang C, et al. Human umbilical cord mesenchymal stem cell derived exosomes encapsulated in functional peptide hydrogels promote cardiac repair. *Biomater Sci* 2019;7:2920-33. DOI PubMed
16. Wang C, Wang M, Xia K, et al. A bioactive injectable self-healing anti-inflammatory hydrogel with ultralong extracellular vesicles release synergistically enhances motor functional recovery of spinal cord injury. *Bioact Mater* 2021;6:2523-34. DOI PubMed PMC
17. Xiao S, Xiao C, Miao Y, et al. Human acellular amniotic membrane incorporating exosomes from adipose-derived mesenchymal stem cells promotes diabetic wound healing. *Stem Cell Res Ther* 2021;12:255. DOI PubMed PMC
18. Wasyłeczko M, Sikorska W, Chwojnowski A. Review of synthetic and hybrid scaffolds in cartilage tissue engineering. *Membranes (Basel)* 2020;10:348. DOI PubMed PMC

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Extracellular Vesicles and Circulating Nucleic Acids

Review

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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, s/n, Badalon 08916, Spain. E-mail: carlospedraz@icloud.com

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Abstract

Despite the significant decrease in population-level mortality of lung cancer patients as reflected in the Surveillance Epidemiology and End Results 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. Further investigation is warranted to assess the value of EV- and platelet-derived circRNAs 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, contributing greatly to cancer incidence and cancer-related deaths worldwide[1]. Of those lung cancers, non-small cell lung cancer (NSCLC) accounts 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 therapies 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 lacks a poly(A) tail, making most of them resistant to the exonuclease RNase R and, therefore, making them 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 are 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-miRNA complex, and either via miRNA degradation or inhibition of the miRNA-mRNA interaction, it triggers further mRNA expression[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[22], FUS protein[23], Quaking binding protein (QKI)[24], RNA helicase DHX9[25], and the RNA-binding motif protein 20[26].

Exon-intron circRNAs 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-EcircRNAs as a result[27]. Intronic circRNAs are another type of such a class; however, the mechanism of generation of these molecules remains yet unclear.

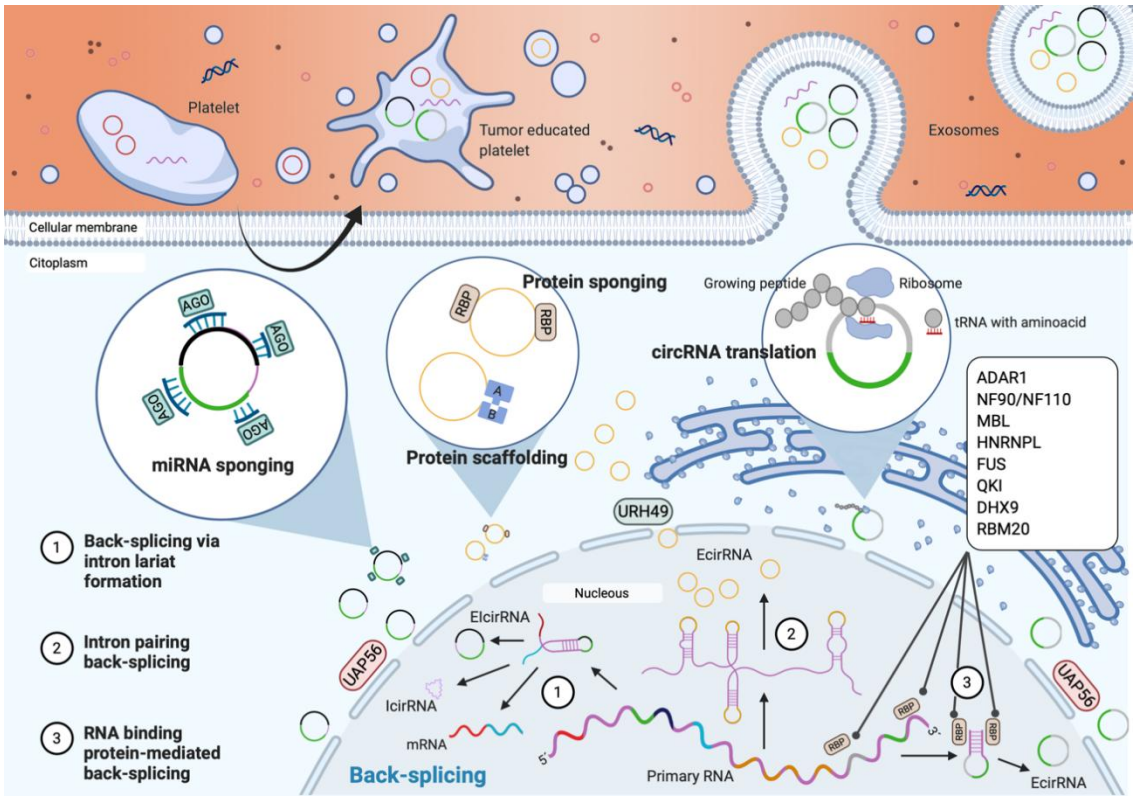

Figure 1. Biosynthesis and molecular functions of circRNAs. CircRNAs are generated by three different mechanisms of back-splicing (via lariat formation, intron pairing or RNA binding proteins). Resultant circRNAs can be formed by only exonic regions (EcircRNAs), intronic regions (IcircRNAs) or both (EIcircRNAs). 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.

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.*[29] describe an Ago2-miR-671-mediated degradation of the circRNA CDR1as (aka ciRS-7). In another study by Park *et al.*[30], a cleavage mechanism induced by RNase P/MRP was elucidated in N6-methyladenosine (m6A)-enriched circRNAs. More recently, a study by Liu *et al.*[31] demonstrated that some circRNAs tend to form intricate duplexes which makes them susceptible to degradation by RNase L upon viral infection.

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

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 for miR-7[35]. A recent study has introduced ciRS-7 as an important player in lung cancer; its expression seems to correlate with tumor size and both lymph and tumor node metastasis stages[36].

A study by Wang *et al.*[37] recently demonstrated the involvement of circSOX4 in lung adenocarcinoma by activating the WNT signaling pathway via sponging miR-1270 and following upregulation of PLAL2. CircSOX4 was found overexpressed in all managed lung adenocarcinoma 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.*[39] demonstrating impaired cell proliferation, migration, invasion and autophagy induction via the miR124-3p-STAT3-PRKAA/AMPKa axis upon silencing of the cited circular transcript. 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 was reported in NSCLC, promoting cell invasion and proliferation of tumors by sequestering miR-22-3p, thus promoting galectin-1, p-AKT, and p-ERK1/2 expression, and activating downstream pathways[41].

The oncogenic circ-FOXM1 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].

Table 1. List of the most relevant recently discovered circRNAs associated with lung cancer

circRNA	Gene	CircBase ID	Source	Regulation	Target	Downstream pathway
circFGFR3	*FGFR3*	-	NSCLC tissues	Upregulated	hsa-miR 22 3p	Galectin 1 AKT/ERK1/2
ircNOL10	*NOL10*	hsa_circ_0000977	LC cells	Downregulated	hsa-miR-7	SCML1
ciRS-7	*CDR1*	-	NSCLC tissues and cell lines	Upregulated	-	-
circABCC4	*ABCC4*	hsa_circ_0030586	LUAC tissues and cell lines	Upregulated	hsa-miR 3186 3p	TNRC6B axis
circCDR1	*CDR1*	hsa_circ_0001946	LUAC tissues and cell lines	Upregulated	hsa-miR-135a-5p	SIRT1/Wnt/β-catenin
circATXN7	*ATXN7*	hsa_circ_0007761	LC tissues and cell lines	Upregulated	-	-
circATAD3B	*ATAD3B*	hsa_circ_0000003	NSCLC tissues and cell lines	Upregulated	hsa-miR-338-3p	IRS2
circP2RX1	*P2RX1*	hsa_circ_0000735	NSCLC tissues and cell lines	Upregulated	hsa-miR-1179, miR-1182	-
circC16orf62	*C16orf62*	hsa_circ_0003645	NSCLC tissues and cell lines	Upregulated	hsa-miR-1179	TMEM14A
circPDZD8	*PDZD8*	hsa_circ_0020123	NSCLC tissues and cell lines	Upregulated	hsa-miR-488e3p	ADAM9
circTUBA1C	*TUBA1C*	hsa_circ_0026134	NSCLC tissues and cell lines	Upregulated	hsa-miR-1256, miR-12	TCTN1 and GAGE1
circCAMK2A	*CAMK2A*	hsa_circ_0128332	LUAD	Upregulated	hsa-miR-615-5p	Fibronectin 1
circFOXM1	*FOXM1*	hsa_circ_0025033)	NSCLC tissues and cell lines	Upregulated	hsa-miR-1304-5p	PPDPF and MACC1
circMTO1	*MTO1*	hsa_circ_0007874	LUAD tissues and cell lines	Downregulated	hsa-miR-17	QKI-5
circPRMT5	*PRMT5*	hsa_circ_0031250	NSCLC tissues and cell lines	Upregulated	hsa-miR-377/382/498	EZH2
circRAD23B	*RAD23B*	hsa_circ_0087855	NSCLC tissues and cell lines	Upregulated	hsa-miR-593e3p, hsa-miR-653e5p	CCND2 and TIAM1
circZKSCAN1	*ZKSCAN1*	hsa_circ_0001727	NSCLC tissues and cell lines	Upregulated	hsa-miR-330-5p	FAM83A (MAP signaling)
circCRIM1	*CRIM1*	hsa_circ_0002346	LUAC cell lines	Downregulated	hsa-miR 182/miR 93	-
circHIPK3	*HIPK3*	hsa_circ_0000284	A549, H838 cell lines	Upregulated	hsa-miR-124-3p, miR-149	STAT3-PRKAA/AMPKα
circPDK1	*PDK1*	hsa_circ_0006006	LUSC tissues	Upregulated	-	-
circPIP5K1A	*PIP5K1A*	hsa_circ_0014130	NSCLC cell lines	Upregulated	hsa-miR 600	HIF-1α
circPRKCI	*PRKCI*	hsa_circ_0067934	NSCLC cell lines	Upregulated	hsa-miR-545, hsa-miR-589	E2F7
circPTPRA	*PTPRA*	hsa_circRNA_0102984	NSCLC tissues and cell lines	Downregulated	hsa-miR-96-5p	RASSF8/E-cadherin
circPVT1	*PVT1*	Hsa_circ_0001821	NSCLC tissues and cell lines	Upregulated	hsa-miR-497	-
circTP63	*TP63*	hsa_circ_0068515	LUSC tissues and cell lines	Upregulated	hsa-miR-873-3p	FOXM1/CENPA-CENPB
circVANGL1	*VANGL1*	-	NSCLC tissues and cell lines	Upregulated	hsa-miR-195	Bcl-2
circZFR	*ZFR*	hsa_circ_0001649	NSCLC tissues and cell lines	Upregulated	hsa-miR-101-3p	CUL4B
circMras	*MRAS*	hsa_circ_0067512	LUAC samples and NSCLC cell lines	Downregulated	hsa-miR 567	PTPRG
F-circSR	*SLC34A2-ROS1*	-	HCC78 cell line	Upregulated	-	ROS
circCDK6	*CDK6*	hsa_circ_000984	NSCLC tissues and cell lines	Upregulated	-	Wnt/β-catenin pathway
circRUNX1	*RUNX1*	hsa_circ_0002360	LUAC tissues	Upregulated	hsa-mir-3620-5p	PHF19

circZNF720	*ZNF720*	hsa_circ_0007059	LC tissues and cell lines	Downregulated	hsa-miR-378	Wnt/β-catenin and ERK1/2
circRNF121	*RNF121*	hsa_circ_0023404	NSCLC tissues and cell lines	Upregulated	hsa-miR-217	ZEB1
circTADA2A	*TADA2A*	hsa_circ_0043278	NSCLC tissues and cell lines	Upregulated	hsa-miR-520f	ROCK1, CDKN1B and AKT3
circLIFR	*LIFR*	hsa_circ_0072309	NSCLC tissues and cell lines	Downregulated	hsa-miR-580-3p	-
circITCH	*ITCH*	N.A.	LC tissues and cell lines	Downregulated	hsa-miR-7 and hsa-miR-214	(PI3K)/AKT
circSMARCA5	*SMARCA5*	hsa_circ_0001445	NSCLC tissues and cell lines	Downregulated	hsa-miR-19b-3p	HOXA9
circRAD23B	*RAD23B*	hsa_circ_0087862	NSCLC tissues and cell lines	Upregulated	hsa-miR-1253	RAB3D
circPIP5K1A	*PIP5K1A*	hsa_circ_0014130	NSCLC tissues and cell lines	Upregulated	hsa-miR-142-5p, hsa-miR-136-5p	IGF-1 and BCL2
circABCB10	*ABCB10*	hsa_circ_0008717	NSCLC tissues and cell lines	Upregulated	-	KISS1
circIGF1R	*IGF1R*	hsa_circ_0005035	NSCLC tissues and cell lines	Downregulated	hsa-miR-1270	VANGL2
circSOX4	*SOX4*	N.A.	LUAD tissues and cell lines	Upregulated	hsa-miR 1270	PLAGL2 (WNT signaling)
circACACA	*ACACA*	hsa_circ_0043256	NSCLC tissues and cell lines	Upregulated	hsa-miR-1183	PI3K/PKB pathway
circBIRC6	*BIRC6*	hsa_circ_0003288	NSCLC tissues and cell lines	Upregulated	hsa-miR-145	FSCN1 and S6K1
circCCDC66	*CCDEC66*	N.A.	NSCLC cell lines	Upregulated	hsa-miR-33a-5p	KPNA4/STAT3
circGFRA1	*GFRA1*	hsa_circ_0005239	NSCLC tissues and cell lines	Upregulated	hsa-miR-188-3p	PI3K/AKT
circLARP4	*LARP4*	N.A.	NSCLC tissues and cell lines	Downregulation	-	SMAD7
circTCONS	*TCONS*	hsa_circ_0000326	NSCLC tissues and cell lines	Upregulated	hsa-miR-338-3p	RAB14
circDHCR24	*DHCR24*	hsa_circ_0012673	LC tissues and cells	Upregulated	hsa-miR-320a	LIMK18521
circMACF1	*MACF1*	hsa_circ_0011780	NSCLC tissues and cells	Downregulated	hsa-miR-544a	FBXW7
circPANX2	*PANX2*	hsa_circ_0012515	NSCLC tissues and cells	Upregulated	hsa-miR-98-5p, hsa-miR-615-5p, hsa-let-7a-5p, hsa-let-7b-5p and hsa-let-7c-5p	-
circMET	*MET*	hsa_circ_0082003	NSCLC tissues and cells	Upregulated	miR-145-5p	CXCL3

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 *et al.*[47] 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.

Two different works have recently been published describing circRNAs that regulate the expression of STAT3. Dong *et al.*[48] reported that upregulation of hsa_circ_0076305 confers DDP-resistance to NSCLC cells via sponging miR-296-5p, positively modulating STAT3. Xu *et al.*[49] introduced the role of circAKT3 inhibiting cisplatin sensitivity by regulating mir-516b-5p/STAT3 axis.

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 signaling, cisplatin resistance)[52], circPVT1 via miR-145-5p (ABCC1, cisplatin, and pemetrexed resistance)[53], circNFIX via miR-132 (TMZ-resistant)[54], and cESRP1. Huang *et al.*[55] recently discovered a suppressor circRNA that, when downregulated, allows major expression of its target miR-93-5p. 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.*[57] 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. Chen *et al.*[58] 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]. The authors made use of specific software to predict possible targeted miRNAs; however, the axis or mechanism of action has not yet

Table 2. List of circRNAs involved in NSCLC treatment resistance

circRNA	Gene	CircBase ID	Source	Regulation	Resistance	Drug	Target	Downstream pathway	Ref.
circSEMA5A	*SEMA5A*	hsa_circ_0071799	NSCLC cells	Upregulated	Chemotherapy	Taxol	hsa-miR-141-5p; also, hsa-miR-1228-5p, hsa-miR-194-3p, hsa-miR-512-5p, hsa-miR-4-5p	-	Xu *et al.*[50], 2018
circFLNA	*FLNA*	hsa_circ_0091931	NSCLC cells	Downregulated	Chemotherapy	Taxol	hsa-miR-34c-5p; also, hsa-miR-105-3p, hsa-miR-1268b, hsa-miR-1226-5p, hsa-miR-1180	-	Xu *et al.*[50], 2018
circMTDH.4	*SNORD115*	-	NSCLC tissue and cell lines	Upregulated	Chemotherapy	5 FU, cisplatin	hsa-miR-630	AEG 1	Li *et al.*[47], 2020
circESRP1	*ESRP1*	hsa_circ_0084927	Lung cancer cells	Downregulated	Chemotherapy	Generic chemotherapy	hsa-miR-93-5p	TGF-β pathway	Huang *et al.*[55], 2020
circARFGEF2	*ARFGEF2*	hsa_circ_0003998	LUAC cells	Upregulated	Chemotherapy	Docetaxel	hsa-miR-326	-	Yu[51], 2019
circCDR1	*CDR1*	hsa_circ_0001946	A549 cell line	Downregulated	Chemotherapy	Cisplatin	hsa-miR-7-5p, hsa-miR-671-5p, hsa-miR-1270, hsa-miR-3156-5p	NER signaling	Huang *et al.*[52], 2019
circPGC	*PGC*	hsa_circ_0076305	NSCLC tissues and cell lines	Upregulated	Chemotherapy	DDP	hsa-miR-296-5p	STAT3	Dong *et al.*[48], 2019
circAKT3	*AKT3*	hsa_circ_0017252	Lung cancer tissues and cell lines	Upregulated	Chemotherapy	DDP, cisplatin	hsa-miR-516b-5p	STAT3	Xu *et al.*[49], 2020
circPVT1	*PVT1*	hsa_circ_0001821	LUAC tissues and cell lines	Upregulated	Chemotherapy	Cisplatin, pemetrexed	hsa-miR-145-5p	ABCC1	Zheng *et al.*[53], 2020
circCDK14	*CDK14*	hsa_circ_0004015	NSCLC cells	Upregulated	Tyrosine Kinase Inhibitors (TKIs)	Gefitinib	hsa-miR-1183	PDPK1 gene	Zhou *et al.*[56], 2019
circKRT17	*KRT17*	hsa_circ_0043632	AZD9291-resistant NSCLCcell lines	Upregulated	Tyrosine Kinase Inhibitors (TKIs)	Osimertinib	hsa-miR-6861-3p, hsa-miR-492, hsa-miR-4743-5p, hsa-miR-6829-3p, hsa-miR-6778-3p	-	Chen *et al.*[58], 2019
circFXYD3	*FXYD3*	hsa_circ_0050581	AZD9291-resistant NSCLCcell lines	Downregulated	Tyrosine Kinase Inhibitors (TKIs)	Osimertinib	hsa-miR-6722-5p, hsa-miR-4641, hsa-miR-4707-3p, hsa-miR-4258, hsa-miR-652-3p	-	Chen *et al.*[58], 2019
circFGFR1	*FGFR1*	hsa_circ_0084003	NSCLC tissues and cells	Upregulated	Immunotherapy	Anti-PD-1 therapy	hsa-miR-381-3p	PD-1	Zhang *et al.*[59], 2019

been elucidated.

CircRNAs seem to also have a role mediating response to immunotherapy. CircFGFR1 has been described by Zhang *et al.*[59] to promote progression and anti-PD-1 resistance. By sponging miR-381-3p in NSCLC cells, C-X-C motif chemokine receptor 4 would result upregulated, leading to progression and resistance

to therapy.

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.*[61], finding circRNAs enriched at least 2-fold in exosomes compared to producer cells. 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.*[65] performed high throughput sequence of plasma-EV RNA cargo of lung adenocarcinoma 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, and 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.*[66] 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 *et al.*[67] 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.*[68] 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 target candidates. Both miR-330-5p and miR-326 may interact directly with fatty acid synthase, which has been described as a notorious oncogene in various types of cancer[68].

Also, directly from plasma Liu *et al.*[69] found a two circRNA-based signature that could potentially be used to classify lung adenocarcinoma patients. Hsa_circ_0005962 was found upregulated while hsa_circ_0086414 was barely expressed. In addition, they observed that overexpression of hsa_circ_0005962 was correlated to mutant *EGFR* expression. *In vitro* experiments suggested that this circRNA could be involved in cancer proliferation.

Table 3. List of the most relevant liquid biopsy-based circRNAs associated with NSCLC

circRNA	Gene	circBase ID	Source	Expression	Target	Ref.
circERBB2IP	*ERBB2IP*	hsa_circ_0001492	LUAD plasma exosomes	Upregulated	hsa-miR-130b-5p, hsa-miR-5195-3p, hsa-miR-4464, hsa-miR1236-3p, hsa-miR-106a-3p	Chen *et al.*[65], 2019
circRNF13	*RNF13*	hsa_circ_0001346	LUAD plasma exosomes	Upregulated	hsa-miR-34B-5P, ha-miR-654-3p, hsa-miR-5683, hsa-miR-4452, hsa-miR-4662b	
circITGAL	*ITGAL*	hsa_circ_0000690	LUAD plasma exosomes	Upregulated	hsa-miR-7161-3p, hsa-miR-9-5p, hsa-miR-6843-3p, hsa-miR-4502, miR-372-5p	
circSCLT1	*SCLT1*	hsa_circ_0001439	LUAD plasma exosomes	Upregulated	hsa-miR-3671, hsa-miR-452-5p, hsa-miR-892c-3p, hsa-miR-223-3p, hsa-miR-4676-3p	
circCD226	*CD226*	hsa_circ_0047921	NSCLC serum exosomes	Downregulated	hsa-miR-let-7g	Xian *et al.*[67], 2020
circATXN7	*ATXN7*	hsa_circ_0007761	NSCLC serum exosomes	Upregulated	-	
circRALB	*RALB*	hsa_circ_0056285	NSCLC serum exosomes	Downregulated	-	
circNPHP4	*NPHP4*	hsa_circ_0005661	LUAD plasma exosomes	Upregulated	-	He *et al.*[66], 2020
circFARSA	*FARSA*	hsa_circ_0000896	NSCLC plasma	Upregulated	hsa-miR-330 5p, hsa-miR-326, hsa-miR-1270	Hang *et al.*[68], 2018
circCCCNB1	*CCCNB1*	hsa_circ_0001495	NSCLC plasma	Upregulated	-	
circVRK1	*VRK1*	hsa_circ_0000566	NSCLC plasma	Upregulated	-	
circCCDC134	*CCDC134*	hsa_circ_0001238	NSCLC plasma	Upregulated	-	
circZCCCJC6	*ZCCCJC6*	hsa_circ_0007037	NSCLC plasma	Upregulated	-	
circ_c1orf116	*C1ORF116*	hsa_circ_0141539	NSCLC plasma	Upregulated	-	
circPMS1	*PMS1*	hsa_circ_0001083	NSCLC plasma	Upregulated	-	
circDNA2	*DNA2*	hsa_circ_0006451	NSCLC plasma	Upregulated	-	
PcircSD3	*SD3*	hsa_circ_0004458	NSCLC plasma	Upregulated	-	
circSMAD2	*SMAD2*	hsa_circ_0000847	NSCLC plasma	Upregulated	-	
circYWHAZ	*YWHAZ*	hsa_circ_0005962	LUAD plasma	Upregulated	hsa-miR-369-5p, hsa-miR-626, hsa-miR-326, hsa-miR-330-5p, hsa-miR-1265, and hsa-miR-622	Liu *et al.*[69], 2019
circBNC2	*BNC2*	hsa_circ_0086414	LUAD plasma	Downregulated	-	
F-circEA	*EMLK4-ALK*		Lung cancer tissues, plasma and cells	Upregulated	-	Tan *et al.*[70], 2018
circZNF91	*ZNF91*	hsa_circ_0109320	NSCLC plasma	Downregulated	-	Liu *et al.*[74], 2019
circZNF117	*ZNF117*	hsa_circ_0134501	NSCLC plasma	Upregulated	-	

Moreover, a fusion-gene *circRNA* has been studied in liquid biopsies. Tan *et al.*[70] started their line of research exploring the existence of a circRNA derived from the fusion gene *EML4-ALK* (*F-circEA*) in the NCI-H2228 cell line. 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.*[71] showed for the first time that platelets are enriched in circRNAs when compared to nucleated tissues, and also, that their content is superior to that on mRNA. Preußer *et al.*[72] demonstrated that platelets are not only 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.

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, 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.*[74] comparing gefitinib responder and non-responder NSCLC patients found that higher expression of hsa_circ_0109320 in plasma correlated with longer progression free survival 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 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. This protocol first introduced by Salzman *et al*[79]. 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 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

Table 4. Current methods for circRNA study

Method	Application	Total RNA input	Advantages	Disadvantages	Ref.
RNAseq	circRNA discovery	Normally ≥ 1µg is needed; however, 1 ng has been used in liquid biopsies showing good results	- Allows whole transcriptome sequence analysis, including rare and low abundant circRNAs	- Time consuming - It involves high quality RNA - Requires expertise for library preparation, sequencing, and Bioinformatics, for data normalization and analysis	Cheng *et al.* [125]
Microarrays	circRNA discovery	2 µg	- Highly sensitive and specific for circRNA profiling - Easy technology, commercial arrays ready to use	- Although it may be possible to work with less RNA, recommended input remains rather high - circRNA discovery gets restricted to the amount of circRNA included in the panel - Requires Bioinformatics expertise for data normalization and analysis	Valladares-Ayerbes *et al.* [126]
nCounter	circRNA discovery and quantification	85 ng	- Allows multiplexed analysis of up to 800 circRNA targets - Does not require amplification (if enough RNA input) - Works well with low quality RNA samples - Very little hands-on time, with results ready within 24 h - User-friendly data analysis software reducing the need for Bioinformatics support	- circRNA discovery gets restricted to the amount of circRNA included in the panel - Technology is costly, and constrained by one company	Zhang *et al.*[127] Dahl *et al.*[85], 2018
qRT-PCR	circRNA quantification	250 ng (3 replicas, 1 gene)	- Well-established technology - Cost-effective	- Does not allow analysis of a large number of genes - Susceptible to template switching and rolling circle amplification bias	
SplintQuant	circRNA quantification	2 nM	- Sensitive and specific approach - Highly reproducibility rates - Eludes the template switching and rolling circle amplification bias found with qRT-PCR	- Novel protocol - No tested in liquid biopsies	Conn[92], 2019
RT-PCR + end-point PCR + Sanger Sequencing	circRNA identification and validation	100 ng	- Well-established technology - Cost-effective - Specific - Gold standard for circRNA validation	- It may require time to test divergent primers - Optimization is required for each pair of primers - Does not allow multiplexing	Panda *et al.*[89], 2018
Northern Blot	circRNA identification and validation	1-50 µg	- Specific circRNA detection - Allows isoform studies - Solves those problems attained to qRT-PCR such as template switching or rolling amplification biases	- Low sensitivity - It requires a big amount of input which makes it incompatible with most liquid biopsy downstream processes	Schneider *et al.*[128]

Table 5. Characteristics of online accessible circRNA resources

Name	Resource	Features	Website	Ref.
circBase	Database	One of the main resources with updated information discovered circRNAs. Provides a useful blat tool for circRNA alignment against the human genome	http://www.circbase.org	Garcia-Contreras *et al.*[84], 2014
circBank	Database	Along with circBase, is one of the most important resources available including a database with most discovered circRNAs along with usegul information	http://www.circbank.cn	Liu *et al.*[93], 2019
circInteratome	Database	Complete database with different features that allow binding site prediction and knock-down experiment designing	https://circinteractome.nia.nih.gov	Dudekula *et al.*[103], 2016
CIRCpedia	Database	Database for the identification of tissue specific circRNAs	http://www.picb.ac.cn/rnomics/circpedia	Dong *et al.*[104], 2018
circRNADb	Database	Searching tool for the identification of EcircRNAs.	http://reprod.njmu.edu.cn/circrnadb	Chen *et al.*[105], 2016
circRNABase	Database	Allows circRNA network prediction	http://www.hzrna.com/circrn-shujuku/circrnabase	circRNABase[106], 2016
circR2Disease	Database	Serves for the identification of circRNA-miRNA interactions associated to different diseases	http://bioinfo.snnu.edu.cn/CircR2Disease/	Fan *et al.*[107], 2018
starBase	Database	Serves for the identification of circRNA-miRNA interactions	http://starbase.sysu.edu.cn/	Li *et al.*[108], 2014
circAtlas	Database	Databased with annotation of circRNAs and with tools that allow identification of circRNA-miRNA interactions	http://circatlas.bols.ac.cn/	Wu *et al.*[109], 2020
circFunBase	Database	A database for functional circRNAs	http://bis.zju.edu.cn/CircFunBase	Meng *et al.*[110], 2019
circad	Database	Serves for the identification of circRNA-miRNA interactions associated to different diseases	http://clingen.igib.res.in/circad/	Rophina *et al.*[111], 2020
circView	Visualization tool	Identification circRNA associated miRNAs and RBPs	http://gb.whu.edu.cn/CircView/	Feng *et al.*[95], 2018
CSCD	Bioinformatic tool	Identification circRNA associated miRNAs and RBPs, with a focus on circRNA with transcription potential	http://gb.whu.edu.cn/CSCD/	Xia *et al.*[112], 2018
cirRNAPL	Bionformatic tool	Identification of circRNA based on extreme learning machine	http://server.malab.cn/CirRNAPL/index.html	Niu *et al.*[113], 2020
nSolver	Program-Bioinformatic tool	Analysis of RNA expression data generated by the nCounter platform	www.nanostring.com	-
circ2Traits	Pipeline	Serves for the identification of circRNA-	http://gyanxetbeta.com/circdb/	Ghosal *et al.*[114], 2013

		miRNA interactions associated to different diseases		
circMeta	Pipeline	Genomic feature annotation and differential expression analysis of circular RNAs	https://github.com/lichenlab/circMeta	Chen *et al.*[115], 2020
circRNAwrap	Pipeline	Pipeline designed for circRNA identification, transcript prediction, and abundance estimation	https://github.com/liaoscience/circRNAwrap	Li *et al.*[116], 2019
SpliceV	Pipeline	Analysis and publication quality printing of linear and circular RNA splicing, expression and regulation	https://github.com/flemingtonlab/SpliceV	Ungerleider *et al.*[117], 2019
CIRCexplorer3	Pipeline	Pipeline for the direct comparison of circular and linear RNA expression	https://github.com/YangLab/CLEAR	Ma *et al.*[118], 2019
circDeep	Pipeline	Permits circular RNA classification from other long non-coding RNA	https://github.com/UofLBioinformatics/circDeep	Chaabane *et al.*[119], 2020
Segemehl	Pipeline	Pipeline for the identification of fusion reads	http://www.bioinf.uni-leipzig.de/Software/segemehl/segemehl_0_2_0.tar.gz	Hoffmann *et al.*[120], 2014
MapSplice	Pipeline	Application for small segment mapping	http://www.netlab.uky.edu/p/bioinfo/MapSpliceDownload	-
DCC	Pipeline	Identification of circRNA from fusion reads	https://github.com/dieterichlab/DCC	Cheng *et al.*[121], 2016
UROBORUS	Pipeline	Allows identification of EcircRNAs	https://github.com/WGLab/uroborus/	Song *et al.*[122], 2016
NCLscan	Pipeline	Identification of non-coding transcripts	https://github.com/TreesLab/NCLscan	Chuang *et al.*[123], 2016
Trcirc	High-throughput Data analysis tool	Allows the prediction of circRNA-transcription factor regulatory networks	http://www.licpathway.net/TRCirc/	Tang *et al.*[124], 2018

detection. This systematically expression profiling process is quite sensitive and straight forward. Current methodology developed by Arraystar includes 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 48 h. 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 affecting 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.*[87] proved that standard RNase R protocols result in up to 20% of highly expressed mRNAs being unaffected. 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.*[91] while studying circRNA expression in brain tissues. 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.*[92] 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.*[85] in different solid biosources (including formalin fixed paraffin-embedded specimens) for the study of B-cell malignancies, 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.

EVs, including 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 overcome 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. To elucidate wheater platelet derived circRNA signatures could be of better, equal, or complementary value of the ones from EVs, additional investigation will be required.

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 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

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Authors' contributions

Authors contributed equally to the article.

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Both authors declared that there are no conflicts of interest.

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Consent for publication

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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 Cancer J Clin* 2018;68:394-424. DOI PubMed
2. Howlader N, Forjaz G, Mooradian MJ, et al. The effect of advances in lung-cancer treatment on population mortality. *N Engl J Med* 2020;383:640-9. DOI PubMed
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. *Curr Oncol Rep* 2018;20:70. DOI PubMed
4. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol* 2017;14:531-48. DOI PubMed
5. He X, Park S, Chen Y, Lee H. Extracellular vesicle-associated miRNAs as a biomarker for lung cancer in liquid biopsy. *Front Mol Biosci* 2021;8:630718. DOI PubMed PMC
6. Müller Bark J, Kulasinghe A, Amenábar JM, Punyadeera C. Exosomes in cancer. Elsevier; 2021. p. 1-40. DOI
7. Pinzani P, D'Argenio V, Del Re M, et al. Updates on liquid biopsy: current trends and future perspectives for clinical application in solid tumors. *Clin Chem Lab Med* 2021. DOI PubMed
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. *Comput Struct Biotechnol J* 2021;19:910-28. DOI PubMed PMC
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 PubMed PMC
10. Hua X, Sun Y, Chen J, et al. Circular RNAs in drug resistant tumors. *Biomed Pharmacother* 2019;118:109233. DOI PubMed
11. Nigro JM, Cho KR, Fearon ER, et al. Scrambled exons. *Cell* 1991;64:607-13. DOI PubMed
12. Lee ECS, Elhassan SAM, Lim GPL, et al. The roles of circular RNAs in human development and diseases. *Biomed Pharmacother* 2019;111:198-208. DOI PubMed
13. Ji P, Wu W, Chen S, et al. Expanded expression landscape and prioritization of circular RNAs in mammals. *Cell Rep* 2019;26:3444-3460.e5. DOI PubMed
14. Guo JU, Agarwal V, Guo H, Bartel DP. Expanded identification and characterization of mammalian circular RNAs. *Genome Biol* 2014;15:409. DOI PubMed PMC
15. Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. *PLoS Genet*

2013;9:e1003777. DOI PubMed PMC
16. Dragomir M, Calin GA. Circular RNAs in cancer - lessons learned from microRNAs. *Front Oncol* 2018;8:179. DOI PubMed PMC
17. Xiao MS, Ai Y, Wilusz JE. Biogenesis and functions of circular RNAs come into focus. *Trends Cell Biol* 2020;30:226-40. DOI PubMed PMC
18. Chen CY, Sarnow P. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* 1995;268:415-7. DOI PubMed
19. Rybak-Wolf A, Stottmeister C, Glažar P, et al. Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol Cell* 2015;58:870-85. DOI PubMed
20. Li X, Liu CX, Xue W, et al. Coordinated circRNA biogenesis and function with NF90/NF110 in viral infection. *Mol Cell* 2017;67:214-227.e7. DOI PubMed
21. Ashwal-Fluss R, Meyer M, Pamudurti NR, et al. circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell* 2014;56:55-66. DOI PubMed
22. Fei T, Chen Y, Xiao T, et al. Genome-wide CRISPR screen identifies HNRNPL as a prostate cancer dependency regulating RNA splicing. *Proc Natl Acad Sci U S A* 2017;114:E5207-15. DOI PubMed PMC
23. Errichelli L, Dini Modigliani S, Laneve P, et al. FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons. *Nat Commun* 2017;8:14741. DOI PubMed PMC
24. Conn SJ, Pillman KA, Toubia J, et al. The RNA binding protein quaking regulates formation of circRNAs. *Cell* 2015;160:1125-34. DOI PubMed
25. Aktaş T, Avşar Ilık İ, Maticzka D, et al. DHX9 suppresses RNA processing defects originating from the Alu invasion of the human genome. *Nature* 2017;544:115-9. DOI PubMed
26. Khan MA, Reckman YJ, Aufiero S, et al. RBM20 regulates circular RNA production from the Titin gene. *Circ Res* 2016;119:996-1003. DOI PubMed
27. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013;19:141-57. DOI PubMed PMC
28. Huang C, Liang D, Tatomer DC, Wilusz JE. A length-dependent evolutionarily conserved pathway controls nuclear export of circular RNAs. *Genes Dev* 2018;32:639-44. DOI PubMed PMC
29. Hansen TB, Wiklund ED, Bramsen JB, et al. miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J* 2011;30:4414-22. DOI PubMed PMC
30. Park OH, Ha H, Lee Y, et al. Endoribonucleolytic Cleavage of m^6A-Containing RNAs by RNase P/MRP Complex. *Mol Cell* 2019;74:494-507.e8. DOI PubMed
31. Liu CX, Li X, Nan F, et al. Structure and degradation of circular RNAs regulate PKR activation in innate immunity. *Cell* 2019;177:865-880.e21. DOI PubMed
32. Fischer JW, Busa VF, Shao Y, Leung AKL. Structure-mediated RNA decay by UPF1 and G3BP1. *Mol Cell* 2020;78:70-84.e6. DOI PubMed PMC
33. Yu T, Wang Y, Fan Y, et al. CircRNAs in cancer metabolism: a review. *J Hematol Oncol* 2019;12:90. DOI PubMed PMC
34. Zhou R, Wu Y, Wang W, et al. Circular RNAs (circRNAs) in cancer. *Cancer Lett* 2018;425:134-42. DOI PubMed
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. *J Cancer Res Clin Oncol* 2017;143:17-27. DOI PubMed
36. Yan B, Zhang W, Mao XW, Jiang LY. 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. *Eur Rev Med Pharmacol Sci* 2018;22:8712-21. DOI PubMed
37. Wang L, Zheng C, Wu X, et al. Circ-SOX4 promotes non-small cell lung cancer progression by activating the Wnt/β-catenin pathway. *Mol Oncol* 2020;14:3253. DOI PubMed PMC
38. Chen X, Mao R, Su W, et al. Circular RNA circHIPK3 modulates autophagy via MIR124-3p-STAT3-PRKAA/AMPKα signaling in STK11 mutant lung cancer. *Autophagy* 2020;16:659-71. DOI PubMed PMC
39. Xie Y, Yuan X, Zhou W, et al. The circular RNA HIPK3 (circHIPK3) and its regulation in cancer progression: review. *Life Sci* 2020;254:117252. DOI PubMed
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. *Onco Targets Ther* 2019;12:7055-65. DOI PubMed PMC
41. Qiu BQ, Zhang PF, Xiong D, 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. *J Cell Physiol* 2019;234:11256-64. DOI PubMed
42. Liu G, Shi H, Deng L, et al. 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. *Biochem Biophys Res Commun* 2019;513:207-12. DOI PubMed
43. Varella-Garcia M. Chromosomal and genomic changes in lung cancer. *Cell Adh Migr* 2010;4:100-6. DOI PubMed PMC
44. Wu K, Liao X, Gong Y, et al. Circular RNA F-circSR derived from SLC34A2-ROS1 fusion gene promotes cell migration in non-small cell lung cancer. *Mol Cancer* 2019;18:98. DOI PubMed PMC
45. Huang XY, Zhang PF, Wei CY, et al. Circular RNA circMET drives immunosuppression and anti-PD1 therapy resistance in hepatocellular carcinoma via the miR-30-5p/snail/DPP4 axis. *Mol Cancer* 2020;19:92. DOI PubMed PMC
46. Pei X, Chen SW, Long X, et al. circMET promotes NSCLC cell proliferation, metastasis, and immune evasion by regulating the miR-145-5p/CXCL3 axis. *Aging (Albany NY)* 2020;12:13038-58. DOI PubMed PMC
47. Li YH, Xu CL, He CJ, Pu HH, Liu JL, Wang Y. circMTDH.4/miR-630/AEG-1 axis participates in the regulation of proliferation,

migration, invasion, chemoresistance, and radioresistance of NSCLC. *Mol Carcinog* 2020;59:141-53. DOI PubMed

48. Dong Y, Xu T, Zhong S, et al. Circ_0076305 regulates cisplatin resistance of non-small cell lung cancer via positively modulating STAT3 by sponging miR-296-5p. *Life Sci* 2019;239:116984. DOI PubMed
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. *Biotechnol Lett* 2020;42:1123-35. DOI PubMed
50. Xu N, Chen S, Liu Y, et al. Profiles and bioinformatics analysis of differentially expressed circrnas in taxol-resistant non-small cell lung cancer cells. *Cell Physiol Biochem* 2018;48:2046-60. DOI PubMed
51. Yu W, Peng W, Sha H, Li J. Hsa_circ_0003998 promotes chemoresistance via modulation of miR-326 in lung adenocarcinoma cells. *Oncol Res* 2019;27:623-8. DOI PubMed PMC
52. Huang MS, Liu JY, Xia XB, 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. *Front Oncol* 2019;9:508. DOI PubMed PMC
53. Zheng F, Xu R. CircPVT1 contributes to chemotherapy resistance of lung adenocarcinoma through miR-145-5p/ABCC1 axis. *Biomed Pharmacother* 2020;124:109828. DOI PubMed
54. Ding C, Yi X, Wu X, et al. Exosome-mediated transfer of circRNA CircNFIX enhances temozolomide resistance in glioma. *Cancer Lett* 2020;479:1-12. DOI PubMed
55. Huang W, Yang Y, Wu J, 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 Differ* 2020;27:1709-27. DOI PubMed PMC
56. Zhou Y, Zheng X, Xu B, et al. 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. *Biochem Biophys Res Commun* 2019;508:527-35. DOI PubMed
57. Fu Y, Huang L, Tang H, Huang R. hsa_circRNA_012515 is highly expressed in NSCLC patients and affects its prognosis. *Cancer Manag Res* 2020;12:1877-86. DOI PubMed PMC
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. *Thorac Cancer* 2019;10:930-41. DOI PubMed PMC
59. Zhang PF, Pei X, Li KS, et al. Circular RNA circFGFR1 promotes progression and anti-PD-1 resistance by sponging miR-381-3p in non-small cell lung cancer cells. *Mol Cancer* 2019;18:179. DOI PubMed PMC
60. Yang H, Zhang H, Yang Y, et al. Hypoxia induced exosomal circRNA promotes metastasis of Colorectal Cancer via targeting GEF-H1/RhoA axis. *Theranostics* 2020;10:8211-26. DOI PubMed PMC
61. Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res* 2015;25:981-4. DOI PubMed PMC
62. Lasda E, Parker R. Circular RNAs Co-precipitate with extracellular vesicles: a possible mechanism for circRNA clearance. *PLoS One* 2016;11:e0148407. DOI PubMed PMC
63. Wang M, Yu F, Li P, Wang K. Emerging function and clinical significance of exosomal circRNAs in cancer. *Mol Ther Nucleic Acids* 2020;21:367-83. DOI PubMed PMC
64. Shang BQ, Li ML, Quan HY, et al. Functional roles of circular RNAs during epithelial-to-mesenchymal transition. *Mol Cancer* 2019;18:138. DOI PubMed PMC
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. *J Cell Biochem* 2020;121:2525-33. DOI PubMed
66. He F, Zhong X, Lin Z, et al. Plasma exo-hsa_circRNA_0056616: a potential biomarker for lymph node metastasis in lung adenocarcinoma. *J Cancer* 2020;11:4037-46. DOI PubMed PMC
67. Xian J, Su W, Liu L, et al. Identification of three circular RNA cargoes in serum exosomes as diagnostic biomarkers of non-small-cell lung cancer in the chinese population. *J Mol Diagn* 2020;22:1096-108. DOI PubMed
68. Hang D, Zhou J, Qin N, et al. A novel plasma circular RNA circFARSA is a potential biomarker for non-small cell lung cancer. *Cancer Med* 2018;7:2783-91. DOI PubMed PMC
69. Liu XX, Yang YE, Liu X, et al. A two-circular RNA signature as a noninvasive diagnostic biomarker for lung adenocarcinoma. *J Transl Med* 2019;17:50. DOI PubMed PMC
70. Tan S, Gou Q, Pu W, et al. Circular RNA F-circEA produced from EML4-ALK fusion gene as a novel liquid biopsy biomarker for non-small cell lung cancer. *Cell Res* 2018;28:693-5. DOI PubMed PMC
71. Alhasan AA, Izuogu OG, Al-Balool HH, et al. Circular RNA enrichment in platelets is a signature of transcriptome degradation. *Blood* 2016;127:e1-e11. DOI PubMed PMC
72. Preußer C, Hung LH, Schneider T, et al. Selective release of circRNAs in platelet-derived extracellular vesicles. *J Extracell Vesicles* 2018;7:1424473. DOI PubMed PMC
73. Best MG, Sol N, In 't Veld SGJG, et al. Swarm intelligence-enhanced detection of non-small-cell lung cancer using tumor-educated platelets. *Cancer Cell* 2017;32:238-252.e9. DOI PubMed PMC
74. Liu YT, Han XH, Xing PY, et al. Circular RNA profiling identified as a biomarker for predicting the efficacy of Gefitinib therapy for non-small cell lung cancer. *J Thorac Dis* 2019;11:1779-87. DOI PubMed PMC
75. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of extracellular vesicles: general methodologies and latest trends. *Biomed Res Int* 2018;2018:8545347. DOI PubMed PMC
76. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer - implications for future improvements in cancer care. *Nat Rev Clin Oncol* 2018;15:617-38. DOI PubMed
77. Théry C, Witwer KW, Aikawa E, 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. *J Extracell Vesicles*

2018;7:1535750. DOI PubMed PMC
78. Tang YT, Huang YY, Zheng L, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. *Int J Mol Med* 2017;40:834-44. DOI PubMed PMC
79. Guria A, Velayudha Vimala Kumar K, Srikakulam N, et al. Circular RNA profiling by illumina sequencing via template-dependent multiple displacement amplification. *Biomed Res Int* 2019;2019:2756516. DOI PubMed PMC
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;29:4618-26. DOI PubMed
81. Qu S, Song W, Yang X, et al. Microarray expression profile of circular RNAs in human pancreatic ductal adenocarcinoma. *Genom Data* 2015;5:385-7. DOI PubMed PMC
82. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Curr Protoc Mol Biol* 2011;Chapter 25:Unit25B.10. DOI PubMed
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. *Biomed Microdevices* 2020;22:23. DOI PubMed
84. Garcia-Contreras M, Shah SH, Tamayo A, et al. Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes. *Sci Rep* 2017;7:5998. DOI PubMed PMC
85. Dahl M, Daugaard I, Andersen MS, et al. Enzyme-free digital counting of endogenous circular RNA molecules in B-cell malignancies. *Lab Invest* 2018;98:1657-69. DOI PubMed PMC
86. Vincent HA, Deutscher MP. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *J Mol Biol* 2009;387:570-83. DOI PubMed PMC
87. Xiao MS, 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 Res* 2019;47:8755-69. DOI PubMed PMC
88. Koch L. RNA: Translated circular RNAs. *Nat Rev Genet* 2017;18:272-3. DOI PubMed
89. Panda AC, Gorospe M. Detection and analysis of circular RNAs by RT-PCR. *Bio Protoc* 2018;8:e2775. DOI PubMed PMC
90. Zhong S, Zhou S, Yang S, et al. Identification of internal control genes for circular RNAs. *Biotechnol Lett* 2019;41:1111-9. DOI PubMed
91. You X, Vlatkovic I, Babic A, et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat Neurosci* 2015;18:603-10. DOI PubMed PMC
92. Conn V, Conn SJ. SplintQuant: a method for accurately quantifying circular RNA transcript abundance without reverse transcription bias. *RNA* 2019;25:1202-10. DOI PubMed PMC
93. Liu M, Wang Q, Shen J, Yang BB, Ding X. Circbank: a comprehensive database for circRNA with standard nomenclature. *RNA Biol* 2019;16:899-905. DOI PubMed PMC
94. Glažar P, Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. *RNA* 2014;20:1666-70. DOI PubMed PMC
95. Feng J, Xiang Y, Xia S, et al. CircView: a visualization and exploration tool for circular RNAs. *Brief Bioinform* 2018;19:1310-6. DOI PubMed
96. Liangsupree T, Multia E, Riekkola ML. Modern isolation and separation techniques for extracellular vesicles. *J Chromatogr A* 2021;1636:461773. DOI PubMed
97. Han L, Xu J, Xu Q, Zhang B, Lam EW, Sun Y. Extracellular vesicles in the tumor microenvironment: therapeutic resistance, clinical biomarkers, and targeting strategies. *Med Res Rev* 2017;37:1318-49. DOI PubMed
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. *Front Oncol* 2019;9:129. DOI PubMed PMC
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 PubMed
100. Fan CM, Wang JP, Tang YY, et al. circMAN1A2 could serve as a novel serum biomarker for malignant tumors. *Cancer Sci* 2019;110:2180-8. DOI PubMed PMC
101. Lam WK, Dennis Lo YM. Circular RNAs as urinary biomarkers. *Clin Chem* 2019;65:1196-8. DOI PubMed
102. Akhoundova D, Mosquera Martinez J, Musmann LE, et al. The role of the liquid biopsy in decision-making for patients with non-small cell lung cancer. *J Clin Med* 2020;9:3674. DOI PubMed PMC
103. Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K, Gorospe M. CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. *RNA Biol* 2016;13:34-42. DOI PubMed PMC
104. Dong R, Ma XK, Li GW, Yang L. CIRCpedia v2: an updated database for comprehensive circular RNA annotation and expression comparison. *Genomics Proteomics Bioinformatics* 2018;16:226-33. DOI PubMed PMC
105. Chen X, Han P, Zhou T. et al. circRNADb: a comprehensive database for human circular RNAs with protein-coding annotations. *Sci Rep* 2016;6:34985. DOI PubMed PMC
106. circRNABase. Available from: http://www.hzrna.com/circrn-shujuku/circrnabase [Last accessed on 30 Jun 2021].
107. Fan C, Lei X, Fang Z, Jiang Q, Wu FX. CircR2Disease: a manually curated database for experimentally supported circular RNAs associated with various diseases. *Database (Oxford)* 2018:2018. DOI PubMed PMC
108. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 2014;42:D92-7. DOI PubMed PMC
109. Wu W, Ji P, Zhao F. CircAtlas: an integrated resource of one million highly accurate circular RNAs from 1070 vertebrate transcriptomes. *Genome Biol* 2020;21:101. DOI PubMed PMC
110. Meng X, Hu D, Zhang P, Chen Q, Chen M. CircFunBase: a database for functional circular RNAs. *Database (Oxford)* 2019:2019.

DOI PubMed PMC

111. Rophina M, Sharma D, Poojary M, Scaria V. Circad: a comprehensive manually curated resource of circular RNA associated with diseases. *Database (Oxford)* 2020;2020:baaa019. DOI PubMed PMC
112. Xia S, Feng J, Chen K, et al. CSCD: a database for cancer-specific circular RNAs. *Nucleic Acids Res* 2018;46:D925-9. DOI PubMed PMC
113. Niu M, Zhang J, Li Y, et al. CirRNAPL: a web server for the identification of circRNA based on extreme learning machine. *Comput Struct Biotechnol J* 2020;18:834-42. DOI PubMed PMC
114. Ghosal S, Das S, Sen R, Basak P, Chakrabarti J. Circ2Traits: a comprehensive database for circular RNA potentially associated with disease and traits. *Front Genet* 2013;4:283. DOI PubMed PMC
115. Chen L, Wang F, Bruggeman EC, Li C, Yao B. circMeta: a unified computational framework for genomic feature annotation and differential expression analysis of circular RNAs. *Bioinformatics* 2020;36:539-45. DOI PubMed PMC
116. Li L, Bu D, Zhao Y. CircRNAwrap - a flexible pipeline for circRNA identification, transcript prediction, and abundance estimation. *FEBS Lett* 2019;593:1179-89. DOI PubMed
117. Ungerleider N, Flemington E. SpliceV: analysis and publication quality printing of linear and circular RNA splicing, expression and regulation. *BMC Bioinformatics* 2019;20:231. DOI PubMed PMC
118. Ma XK, Wang MR, Liu CX, et al. CIRCexplorer3: a CLEAR pipeline for direct comparison of circular and linear RNA expression. *Genomics Proteomics Bioinformatics* 2019;17:511-21. DOI PubMed PMC
119. Chaabane M, Williams RM, Stephens AT, Park JW. circDeep: deep learning approach for circular RNA classification from other long non-coding RNA. *Bioinformatics* 2020;36:73-80. DOI PubMed PMC
120. Hoffmann S, Otto C, Doose G, et al. A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection. *Genome Biol* 2014;15:R34. DOI PubMed PMC
121. Cheng J, Metge F, Dieterich C. Specific identification and quantification of circular RNAs from sequencing data. *Bioinformatics* 2016;32:1094-6. DOI PubMed
122. Song X, Zhang N, Han P, Lai RK, Wang K, Lu W. Circular RNA profile in gliomas revealed by identification tool UROBORUS. *Nucleic Acids Res* 2016;44:e87. DOI PubMed PMC
123. Chuang TJ, Wu CS, Chen CY, Hung LY, Chiang TW, Yang MY. NCLscan: accurate identification of non-co-linear transcripts (fusion, trans-splicing and circular RNA) with a good balance between sensitivity and precision. *Nucleic Acids Res* 2016;44:e29. DOI PubMed PMC
124. Tang Z, Li X, Zhao J, et al. TRCirc: a resource for transcriptional regulation information of circRNAs. *Brief Bioinform* 2019;20:2327-33. DOI PubMed
125. Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine. *Kidney Int* 2014;86:433-44. DOI PubMed
126. Valladares-Ayerbes M, Garrigos C, Taron M, et al. Circular RNAs as biomarkers in liquid biopsy in colorectal cancer. *Clinical Oncol* 2020;38:suppl. DOI
127. Zhang J, Zhang X, Li C, et al. Circular RNA profiling provides insights into their subcellular distribution and molecular characteristics in HepG2 cells. RNA Biol 2019;16:220-32. DOI PubMed PMC
128. Sun L, Vella P, Schnell R, et al. Structural and Functional Characterization of the BcsG Subunit of the Cellulose Synthase in Salmonella typhimurium. *JMB* 2018;18:3170-89. DOI PubMed PMC

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