# Importance of Extracellular Vesicle Derived RNAs as Critical Colorectal Cancer Biomarkers

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**ABSTRACT:** Colorectal cancer typically begins from a nonmalignant polyp formation in the large intestine that, over time, develops into colorectal cancer. The growth of benign polyps can be checked if detected in the early stages of the disease. Doctors usually recommend colonoscopy to average and high-risk individuals for colorectal cancer screening. Elevated carcinoembryonic antigen (CEA) is a broadly used biomarker for colorectal cancer. The genetic and epigenetic alteration of genes such as p53, BRAF, APC, and PIK3CA is also correlated with colorectal cancer in various clinical studies. In general, tissue biopsy is most frequently used for colorectal cancer diagnosis, but the whole tumor heterogeneity cannot be accessed by this technique. Furthermore, such a highly invasive technique is not suitable for repeated testing. Recently, extracellular vesicles (EVs), lipid bilayer enclosed sacs secreted from colorectal cancer cells, are emerging as a diagnostic tool for colon cancer detection. The major advantages of using EVs for colon cancer diagnosis are (i) EVs can be isolated in a noninvasive manner from the body fluid and (ii) EV incorporated cargoes (mostly RNAs) reveal various aspects of colorectal cancer. EV-RNAs are also implicated in tumor invasion and influence the immune system for the further spread of tumors. However, due to the lack of standardized EV detection strategies, diagnostic applicability is limited. Herein, we review the recent literature on the pathobiological dependence of colorectal cancer on EV-RNAs. Further, we present the advantages of identification and characterization of EV-RNAs to explore the connection between differential expression of extracellular vesicle incorporated RNAs and colorectal cancer. How this approach may potentially translate into point of care colorectal cancer diagnostics is also discussed.

**KEYWORDS:** extracellular vesicle, noncoding RNA, miRNA, biomarker, colorectal cancer diagnosis
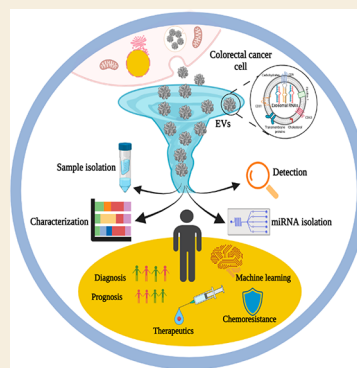
## 1. INTRODUCTION

Colorectal cancer is the second most deadly cancer in women and the third most prevalent form of cancer in men. According to global cancer statistics, almost 576,858 people died in 2020 worldwide due to colorectal cancer (www.miskawaanhealth.com). In a consensus report published by the Indian Council of Medical Research (ICMR), per 100,000 patients, the annual incidence rate (AAR) of colon cancer is 4.4 in men and 3.9 in women.[1] Though it is a dangerous form of cancer, it is treatable to check further progression if detected early. So, colorectal cancer staging is very significant if overall survival (OS) is concerned. Traditionally, the colonoscopy screening technique is the gold standard suggested for high-risk individuals. One of the advantages of colonoscopy is the ability to perform a biopsy and subsequent pathological inspection of the tissue. Although this is an established detection method, due to high tissue invasiveness, such a recurring biopsy may not be feasible.[2] Generally, it is not recommended for older people due to complications. The carcinoembryonic antigen (CEA) is extensively used as a molecular biomarker for colorectal cancer but lacks enough sensitivity and specificity to be used as a prognostic biomarker

for colorectal cancer.[3] Therefore, there exists an unmet need for alternative noninvasive detection methodologies to be developed for colorectal cancer diagnosis.

Extracellular vesicles (EVs) are double-layer bound lipid sacs of size 30−10 000 nm, excreted from almost all cells in different biological fluids, and potentially take part in cell−cell communication.[4] EVs are majorly classified into exosomes (30−100 nm), microvesicles (100−1000 nm), apoptotic bodies (50−5000 nm), and large oncosomes (1000−10000 nm) (Figure 1). EVs can carry the pathophysiological signature of the parent cell. The incorporated EV moieties have earned much attention as critical biomarkers for disease diagnosis. Moreover, the intrinsic properties of EVs to cross immunological barriers and high stability in blood circulation make them promising drug delivery vehicles.[5,6] Rose Johnstone first
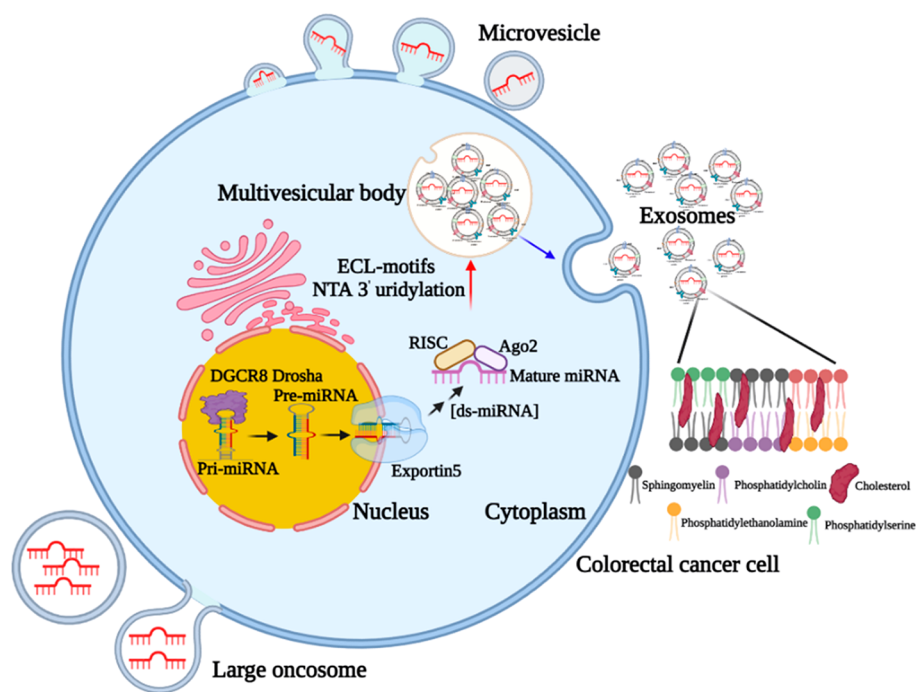
**Figure 1.** Biogenesis and sorting process of miRNA inside the EVs (exosomes, macrovesicles, and large oncosomes are shown). miRNA biogenesis comes with a series of molecular events.[6] At first, pri-miRNAs are trapped by RNase III enzyme Drosha and the DiGeorge Critical Region 8 (DGCR8) RNA binding protein, and the pre-miRNA display a stem-loop structure and are exported to the cytoplasm by Exportin-5. After that, dsRNA is formed by cleaving the pre-miRNA loop and 5′/3′ nucleotide. One of the loops is then attached with RNA-induced Silencing Complex (RISC), which have members of the Argonaute (Ago) protein family.[6] Specific Exo motifs allow protein recognition and exosome sorting.

introduced the term "exosome" to describe the small-sized lipid membrane-bound vesicles secreted in the extracellular space. However, the existence of EVs was first reported in two different reports published in 1983.[7] The creation of several societies (ISEV, ANZSEV, MISEV, etc.) with the emergence of various dedicated journals (EJEV, JEV, EVNCA, etc.) in this field and the publication of over 14,000 research articles have recognized the potential of EVs in the almost four decades since EVs were discovered. It is well established that EVs mostly incorporate various proteins, sugars, lipids, and various genetic materials (DNA and RNAs) protected from extracellular nucleases and proteases. Whole-genome sequencing of EVs revealed that they carry genome sequences for all chromosomes.[8,9] Solexa sequencing found more than 16 434 DNA fragments in the EVs isolated from human plasma.[10] Studies divulged that EVs also contain high concentrations of mitochondrial DNA as well.[4,10] Apart from EV surface protein biomarkers, recent studies have explored the presence of RNAs incorporated in the EV inner cavity as an emerging biomarker for colorectal cancer.[11−13] These EV-RNAs are engaged in altering the normal biochemical pathway to promote cancer formation, and they are involved in epithelial to mesenchymal transformation (EMT) in colorectal cancer.[12] According to many independent studies, intercellular transport of EV incorporated RNAs imparts the functional aspect of recipient cells.[4] Such as EV-loaded let7a, let7b, miR-148, and miR-99a support mammalian reproduction and embryo development in those cells that take up EVs.[14] The proteogenomic profile of EVs plays an important part in altering the cancer-immune microenvironment. Accordingly, cancer cell-derived EVs facilitate immune escape and cancer metastasis. Moreover, EV incorporated RNA subtype and mutational status exhibit the pathological condition of parent cells.[15]

Although differential ultracentrifugation is regarded as the gold standard for EV isolation, unfortunately, there is no standard scalable EV isolation technique available to date.[16] The quantity and quality of extracted RNAs are a concern for researchers during EV incorporated RNA isolation.[17] Although the exact mechanism for the stability of "prone to degradation" RNAs in the nuclease-rich blood is still unknown, it is believed bilayer EV membrane protects incorporated RNAs from degradation.[17] Till now, the critical roles of several EV incorporated RNAs have been discovered for colorectal cancer.[15] It has been apparent that the EV-RNAs are not restricted to cellular pathophysiological function. Any specific alternation in the sequence information can aid cancer progression.[18] Such alterations can be instigated as hallmarks of colorectal cancer. The subtype and heterogeneity of RNAs depend on the pathophysiological condition of the EV secreting parent cell.[4] A range of mechanisms explains the strategy of EV-RNA to escape endosomal degradation pathways. During the specific and nonspecific interaction of EV between different cells, the cellular endocytic pathway makes the EVs release incorporated RNAs along with other cargoes to recipient cells.[19] In this manner, EV-RNA alteration not only helps in local cancer progression but acts as a transporter for distant cancer metastasis.[19]

In this Review, the key mechanism of RNA packaging inside EV and the role of EV incorporated RNAs in colorectal cancer immune regulation are discussed. In addition, EV-RNA content, characterization methods are critically analyzed. Further, the prospect of EV-RNA as a potential diagnostic and prognostic biomarker for colorectal cancer is discussed in detail.

## 2. INTRA-EV-RNA SORTING

Cell-free nucleic acids secreted from the colorectal tumor cells can represent the metastatic potential of the colorectal tumor. The genetic and epigenetic changes in different circulating nucleic acids are examined as a potential noninvasive colorectal cancer biomarker. The statistically significant increased concentration of CA 19-9, TIMP-3, and CA72-4 cell-free nucleic acids appear to be a sensitive colorectal cancer biomarker.[20] Further, the epigenetic alteration of EY4A, TAC1, and SEPT9 circulating nucleic acids represents the premalignant metastatic potential of colorectal cancer.[21,22] A significant increase in circulating mRNA concentrations of CEA, E-CAD, and CD45 is observed in high-risk colorectal cancer.[20] Therefore, these altered circulating nucleic acids can be used as diagnostic and prognostic biomarkers for the early detection of colorectal cancer. However, the expression level of these circulating nucleic acids varies from one patient to other, which restricts the standardization of a particular detection method. Furthermore, bloodborne circulating exonuclease can also degrade the freely floating nucleic acids. For these reasons, double membrane-bound EV incorporated nucleic acids are an ideal selective biomarker in terms of increased stability of the biomarker of interest.

The biogenesis of EVs involves the formation of a double invagination in the plasma membrane forming intraluminal vesicles within multivesicular bodies (MVB).[4] The fusion of MVB with plasma membrane ultimately releases the EV into the extracellular space.[4,20] EVs are broadly classified into ectosomes and exosomes.[21] Here we mentioned all the subtypes as EV. During the biogenesis, the cell's endosomal sorting complexes package different pools of RNA based on the microenvironments and pathophysiological conditions of the cells. Other than carrying mRNA, rRNA, and tRNA, EV also contains different types of noncoding RNAs like miRNA, lncRNA (long noncoding RNA), circular RNA, piRNA (PIWI Interfering RNA), etc.[22,23] The bilayer lipid membrane of EVs protects the incorporated RNAs from ribonuclease-mediated degradation. That makes EVs a better biomarker than circulating nucleic acids in early cancer detection.[4] However, sometimes RNA degradation fragments have also been detected in EVs.[22−24]

Following transcription in the nucleus, RNA molecules associated with various RNA binding proteins (RBP) follow the cytoskeleton and microtubular networks to be incorporated inside the EVs.[25,26] Furthermore, interfering RNA species like miRNA, siRNAs, etc. are processed by endogenous enzyme machinery components like Dicer and Drosha.[27−30] The miRNA-induced silencing complex (RISC) was also found to contribute to miRNA sorting (Figure 1). RNA sequence-specific RBPs like ALIX, AGO2, MVP, etc. play an important role in RNA loading,[27,31,32] RBPs like heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1) and scaffold-attachment factor B1 (SAFB) control the incorporation of noncoding interfering RNAs.[33−35] According to Kosaka et al., sphingomyelinase plays an important role in miRNA sorting in EVs.[36] The exact function of several noncoding RNAs (ncRNA) is still not fully understood yet.[37] Nevertheless, few studies indicated the contribution of specific RNA binding proteins in ncRNA sorting inside EVs. For example, Shurtleff et al. reported that YBX1 RBP protein regulates the incorporation of different ncRNAs inside human embryonic HEK293T derived EV.[37] Further, they showed the overexpression of YBX1 in the case of cancer.[37]

Though several mechanisms have been proposed based on the RNA incorporation inside the EV, the proper mechanism is still poorly understood.[27−32] Different post-translational modifications in RBPs may initiate miRNA import machinery inside EVs.[38] Diaz et al. reported the function of La protein modification in the incorporation of miR-122 inside breast cancer MDB-MB231 derived EVs.[39] In another report, Mukherjee et al. reported the role of Hu protein in the extracellular export of miR-122 through EVs. The EV mediated export of miR-122 depends on the post-translational ubiquitinoylation level of Hu protein in starved human Huh7 hepatic cells.[40] It has been reported that the base sequence information in RNA also determines the incorporation based on hydrophobic modification and lipid components of the EV membrane. Bolukbasi et al. proposed that the specific sequence at the 3′ UTR (Untranslated region) of mRNA decides the loading into EV.[41] Molecular modification of RBPs like phosphorylation, ubiquitylation, etc. also regulates the incorporation of RNAs inside EVs.[38−40] Other than that, the cell type, cell origin, and pathophysiological condition of cell also determine the heterogeneity of EV RNA content.[4]

The translational regulation by EV incorporated miRNAs mainly depends on the base sequence information on the miRNA.[42−44] A complete complementarity with the target sequence leads to activation of AGO-dependent translational silencing and target mRNA decay. However, partial complementarity with the target sequence, mainly at the 3′ untranslated region leads to blockage of target mRNA without degrading it.[42−44]

## 3. EV-RNA MEDIATED INTERCELLULAR COMMUNICATION

Cell communication is an essential step in transferring information to coordinate the homeostasis and development within an individual.[42] EVs are mainly engaged in the contact-independent manner of cell communication.[43] According to reports, EV enclosed RNAs facilitate the exchange between cells to participate in efficient cell communication.[4,33]

To improve the understanding of EV-RNA-based communication, The Extracellular RNA Communication Consortium (ERCC) was initiated in 2013 (https://commonfund.nih.gov/exrna). Since then, the ERCC has contributed significantly to understanding the biology of EV-RNA, biomarker identification, proper isolation, and characterization in the advancement in the EV field. EV incorporated RNAs are found to play an important role in immune regulation.[44] Yi et al. reported that the unidirectional transfer of EV-miRNA from activated T cells to APCs (antigen presenting cells) regulates the cell-mediated immunity to a certain extent.[45] According to reports, changes in the cellular microenvironments such as pH, oxygen level, and inflammation guide EV-RNA trafficking between cells.[14] Seminal plasma secreted EV incorporated noncoding RNAs like let7-a, miR-148a, miR-375, and miR-99a have an important part in the genital immune response.[14] Not only the immune response but also EV-associated RNAs are also associated with human reproduction and embryonic development. Besides transferring infectious RNA molecules from disease cells to healthy cells to manifest disease progression, EV incorporated RNAs also function to prevent diseases like cardiovascular and atherosclerosis.[4,46] Murine cardiac progenitor cell derived EVs contain miR-21-5p and miR-22a that
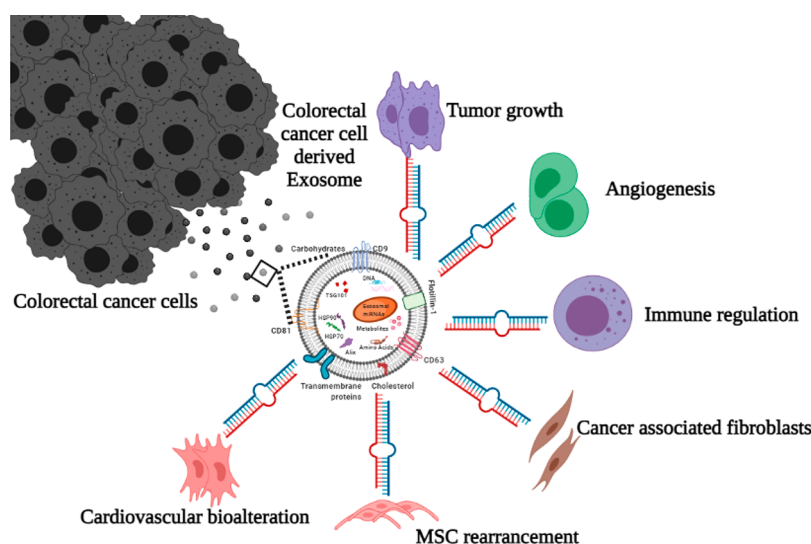
**Figure 2.** Crucial role of exosomal miRNAs in tumor initiation and development are presented. Colorectal cancer cells can transfer miRNAs through the exosomes to the nearby cells. These exosomal RNAs can control cell proliferation, invasion, and metastasis by orchestrating their downstream targets.[29,73]

facilitate cardiac fitness.[47,48] In addition, EV-RNAs actively participate in cancer development and cell apoptosis by controlling the gene expression pattern.[46] Not only that, the horizontal transport of EV incorporated miRNAs promotes chemoresistance by regaining stem cell properties.[49] For example, horizontal transfer of miR-21-5p from the cancer microenvironment to the cancer cell incites the phosphorylation of growth-inducing factors such as Akt and Bad, and leads to increased resistance against the chemotherapeutic drug oxaliplatin.[50] EV-miR21 and EV-miR155 are capable of targeting the cell cycle regulator BRG1 to increase the metastatic potential of colorectal cancer.[50] The development of a vascular network through angiogenesis is critical to cancer cells for growth and nutrition.[29] Concerning the explicit role of angiogenesis in cancer, parent cell-secreted RNA containing EVs has an important role to play. In a complex angiogenic network, KLF2 is a transcription regulator for angiogenesis.[29,50] EV-miR25-3p targets and repress KLF2 activity to mediate angiogenic function throughout the colorectal cancer microenvironment.[50]

Collective shreds of evidence showed the cross-talk of biological information through EV-RNAs, enabling cellular coordination. As a result, EV not only provides a fertile medium for cancer development but facilitates other essential pathophysiological functions (Figure 2).[4] The cell-selective biochemical properties of EVs make them an ideal candidate to validate for a specific disease. Other than cell-specific EV membrane composition, the EV incorporated transcriptome reflects the pathophysiological properties of the parent cell. Mainly, based on deep sequencing of the EV transcriptome and characterization of EV membrane tetraspanins, the organ specificity of EVs is inferred.[4] Considering all the facts, EV incorporated RNAs can be considered as an efficient natural messaging "app", which may not be the most popular in the current time but can be in the future, as research in this particular field grows.

## 4. CHARACTERIZING EV-RNA CONTENT

The structural chemistry of free RNAs makes them unstable and susceptible to nucleophilic attack; hence, scientists find EV bound RNAs, free from breakage, to be more attractive as disease diagnosis biomarkers.[37] Though the RNA content of EVs mainly depends on the subpopulation and pathophysiological condition, recent studies revealed that storage, isolation, and characterization strategies also lead to bias in the reproducibility of EV-RNA content characterization.[51] Nevertheless, both serum and plasma are excellent bioliquid sources for EV incorporated RNA detection. However, the exact reason for the content difference in these two, is still unknown.[52] In the current scenario, mainly RT-PCR and microarray-based assays are utilized to identify the presence of a particular RNA inside EVs.[53] Yet, unknown RNA molecules usually remain undetectable. In addition to that, due to high sequence similarities among the EV-RNAs, there is a high chance of cross-hybridization.[54] Furthermore, due to a deficiency in total control over huge EV-RNA pools, the microarray method offers major disadvantages in EV-RNA characterization.[54] Next-generation sequencing approaches make it possible to overcome such limitations.[55] This method is not restricted to any prior understanding of the genome framework. In a short turnaround time, it offers high sensitivity.[53,56] Many public databases are available based on EV incorporated RNA sequencing. ExoRBase hosts EV incorporated RNA sequence information for both healthy and unhealthy individuals (www.exorbase.org). Presently, information on thousands of sequences for EV-RNA is processed in EV-RNA Atlas under the ERC program. Further, EV-RNA Atlas analyze recently published data using various software and sort into online databases to serve the research community (www.exrna-atlas.org). ExoCarta has an extensive EV-RNA molecular signature, is generally used in understanding the contribution of parent cells to EV-RNA type (www.exocarta.org).[51]

The immense advantages of RNA sequencing over traditional hybridization-based assays motivate researchers in EV-RNA library construction for extensive deep sequencing.[51,56] The library evaluation and cluster analysis value the evaluation of cytological characteristics of EV-mRNA profiles.[57] For instance, EV-RNA expressing inflammatory proteins can direct different functional pathways in the immune system. Generally,

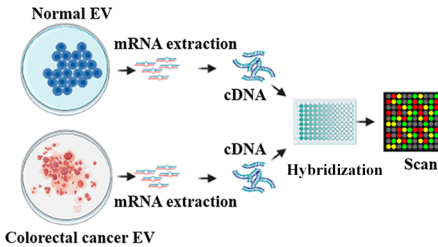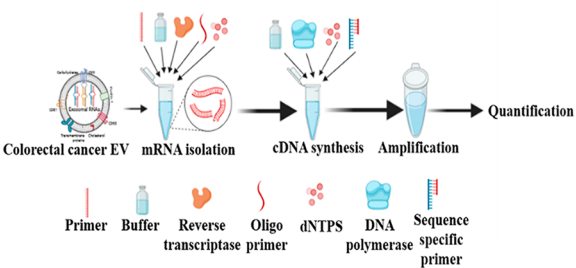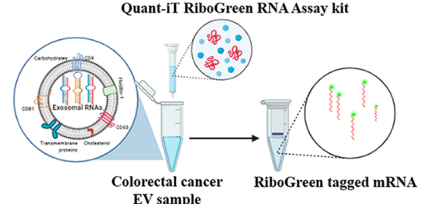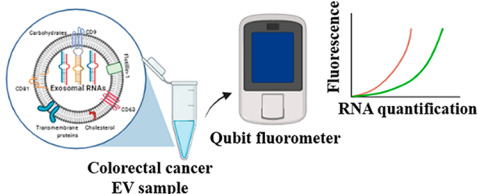**Table 1. Summarized List of EV-RNA Characterization Methods**
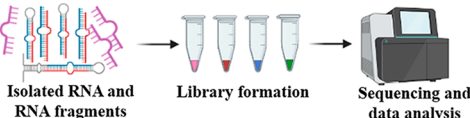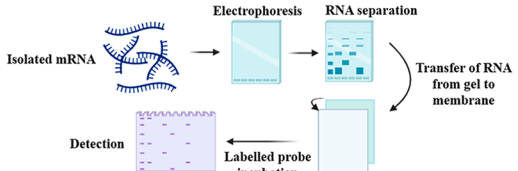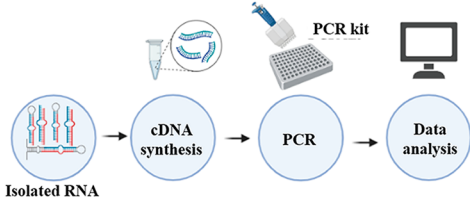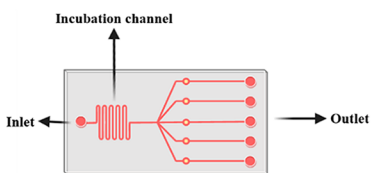
| Characterization method | Advantages and Disadvantages |
|---|---|
| **Microarray**  Normal EV → mRNA extraction → cDNA; Colorectal cancer EV → mRNA extraction → cDNA → Hybridization → Scan | 1. At a time, quantification of multiple transcriptomes<br>2. Fast and simple<br>1. Narrow limit of detection<br>2. Not suitable for unknown sequence targets<br>3. Chance of probe cross-hybridization<br>References: [60,61] |
| **Quantitative reverse transcription-polymerase chain reaction(qRT-PCR)**  Colorectal cancer EV → mRNA isolation → cDNA synthesis → Amplification → Quantification; Primer, Buffer, Reverse transcriptase, Oligo primer, dNTPS, DNA polymerase, Sequence specific primer | 1. Very sensitive detection<br>2. Reduce the contamination during the measurement<br>1. Multiplex detection is limited<br>2. High cost<br>3. Amplicon size cannot be monitored<br>References: [62,63] |
| **Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific)**  Quant-iT RiboGreen RNA Assay kit; Colorectal cancer EV sample → RiboGreen tagged mRNA | 1. Less sample volume needed<br>2. Highly sensitive toward low concentration<br>1. Sensitivity decreases upon medium contamination<br>References: [64,65] |
| **Qubit RNA HS (high sensitivity) assay (Thermo Fisher Scientific)**  Colorectal cancer EV sample → Qubit fluorometer → RNA quantification (Fluorescence) | 1. Accurate<br>2. Can detect low RNA concentration<br>1. Large sample volumes needed<br>2. Sensitive towards the presence of DNA contamination<br>References: [66,67] |

**Table 1. continued**

| Characterization method | Advantages and Disadvantages |
|---|---|
| **Bioanalyzer Pico chip (Agilent Technologies)**<br><br>https://www.agilent.com | 1. Very sensitive<br>2. Requires low sample volume<br>1. Not suitable for small RNA fragment<br>References: [53,68] |
| **Next-generation sequencing**<br><br>Isolated RNA and RNA fragments   Library formation   Sequencing and data analysis | 1. Generates a large number of reads<br>2. Very sensitive<br>1. Expensive<br>2. Takes huge run time<br>References: [53,69] |
| **Northern blotting**<br> | 1. Very specific<br>2. Can be stored for a long time<br>1. Multiple detections is a problem<br>2. Time-consuming<br>References: [53,70] |
| **miRCURY™ LNA array**<br> | 1. High Specificity<br>2. High Sensitivity<br>3. Less sample volume needed<br>1. Target sequence information should be known<br>References: [53,71] |
| **Microfluidics**<br> | 1. Integration of experimental setups on a single chip<br>2. Low sample volume<br>3. Multiple analyses can run at a time<br>4. Low LOD<br>1. Complex to pattern<br>2. Flow rate control is very crucial<br>References: [72, 82] |

Illumina (www.illumina.com) and Ion torrent Hiseq platforms are used in analyzing EV-RNA transcriptomics.[53] In limited read numbers and differential expression of EV, next-generation sequencing is a real game-changer in characterizing EV-incorporated RNA pools. Many individual reports have described the multiomic study of EVs with a computational approach.[58,59] The pairwise alignment of an EV incorporated transcriptome with existing RNA sequence information has

exhibited pathobiological insights and differential RNA expression of EVs.[51,59] Although a few articles have already been published in this area, more data sets have yet to be generated with computational analysis of EV composition.

Notwithstanding, the cellular RNA pool is quite different from the EV pool, mainly in terms absence of ribosomal machinery.[40] So, in terms of quality and quantity, there is a significant difference in cellular and EV incorporated RNA content.[40] Traditional RNA detection strategies might not be suitable for EV-RNAs. Here, we will discuss different methods of EV-RNA characterization in terms of quality, quantity, and stability in Table 1.

Despite the advantages of various methods, EV research is still in the early stages and needs further advancement.[73−77] Though "Bioanalyzer" is a popular choice for EV-RNA length profile characterization, it is not specific for RNA; contaminating DNA samples can hamper the quantification.[78] Pico chip employs sensitiveness toward medium salt concentration, while Quant-iT RiboGreen RNA Assay kit (Thermo Fisher Scientific) is not sensitive toward bioliquids with salt. However, this assay displays sensitivity for phenol-chloroform.[78] For these reasons, sample pretreatment is essential before any characterization.[79] At the same time, from a characterization point of view, it is very crucial to differentiate EV incorporated RNAs from cellular RNAs.[4] That being the case, RNases can be applied before the isolation of EV-RNA.[4,80] Several extraction kits are commercially available to solve the needs. However, extraction of EV-RNA with such commercial kits imposes difficulty in moderate size deep sequencing of EV incorporated lncRNAs.[22] Moreover, most assays apply chemical treatment, which substantially increases the purification steps.[78] In this scenario, precise control over isolation and nanoscale characterization with microfluidics offers several advantages.[81] Ramshani et al. developed a charge inversion-directed microfluidic device.[82] The platform contains two nanocompartments, one for lysing and another for sensing. In the lysing chip, they applied surface acoustic waves to EVs to release incorporated miRNA. Further, they detected released miR-21 on the sensing chip based on the interaction with the membrane fabricated complementary oligonucleotide.[82] The acoustic pressure-controlled microfluidic achieves a LOD score of 1 pM within a turnaround time of 30 min.[82] This way, acoustic wave-based isolation and characterization of EV-RNA push all the boundaries in the "separation on chip" detection system. Richards et al. utilized a similar approach for extraction but on an ion-exchange membrane to quantify the EV-miRNA.[83] They fabricated a complementary miRNA sequence ion exchange membrane for the sequence-specific hybridization.[83] The application of current through this membrane becomes hampered as more miRNA gets hybridized. This quantifiable alteration of voltage was correlated with miRNA concentration.[83] One of the major advantages of this biosensor is the reusability after dehybridizing the bound miRNAs.

Collectively, current knowledge about EVs is limited. Greater scrutiny and much more extensive research are highly needed. Proper validation and standardization of characterization techniques will assuredly support EV transcriptomic research. Emerging CRISPR technology can also be a game-changer in EV research.[84] The dCas protein not only can empower the CRISPR-based gene regulation but also can enable EV-RNA characterization. Though there are many ifs and buts with CRISPR, with proper imagination, there might be a chance to characterize EV-RNA differently.

## 5. EV-RNA: A SECRET PLAYER IN CANCER IMMUNE RESPONSE

The human immune system has several mechanisms to counter against foreign substances. Cancer mutated cells generate different abnormal tumor-associated antigens that are encountered by the immune surveillance system as "nonself".[85] Nonetheless, cancer cells possess certain mechanisms to escape the surveillance system, and when the immune system loses its functionality, it leads to malignancy.[4,85] Tumor cell-derived EVs play a vital role in evading immune surveillance and transmitting the immune-suppressive message across the tumor microenvironment.[86] Emerging evidence suggests that EV membrane incorporated RNAs can avert such immune responses.[87,88] Furthermore, cancer-derived EVs can convey death signals to immune cells to bypass the recognition.[89]

According to Takano et al., colorectal cancer cell-derived, EV-miR203 is involved in cellular immunosuppression based on activation of M2-tumor-associated macrophages.[90] EV incorporated miR-203 mainly functions as a communication messenger between tumor and healthy immune cells to increase the proliferation efficiency of cancer cells by evading the cytokine signaling 3 of colorectal cancer and distant organs like the liver.[90] Further, miR889 triggers the expression level of DAB2IP that downregulates the immune infiltration of B cells and CD8+ T cells in colorectal cancer.[91,92] Upregulation of miR-21 in the colorectal exosome downregulates endothelial progenitor cells (ECP) by directly targeting IL-6 R, which results in dysregulation of the thrombus repair process, a prognostic indicator of cancer.[93] Cross-talk between cancer and healthy cells is very common in the development of the tumor microenvironment. Mutant TP53 in colorectal cancer was found to influence macrophages to change into cancer-supportive macrophages via exosomal miRNA. Colorectal cancer mutant TP53 secretes miR-1246 incorporated exosomes that interact with nearby macrophages to convert them into "tumor-associated macrophages" (TAM).[94] Eventually, these mutated macrophages result in a phenotypic switch to promote epithelial to mesenchymal transformation (EMT) and further secrete tumor responsive factors such as IL-10 and TGF$\beta$, contributing to cancer.[94] In the context of colorectal cancer, miR 21 and miR155 are also hypothesized to play a major role in TAM formation.[95] The uptake of exosomal miR-141 can lead to the overexpression of wnt/$\beta$ catenin signaling pathways to promote EMT.[95]

Exosomal-miRNA-mediated immune regulation does not always influence cancer formation but impacts tumor suppression associated with immune response regulation in some instances.[96] Specific colorectal cancer cell-derived EV-miR1255b-5p has been found to repress the wnt/$\beta$ catenin pathway and contribute to tumor suppression.[96] A certain colorectal cancer cell-derived exosomal miR-486-5p acts as a negative regulator in some transcription factors, associated with growth-promoting receptors like insulin-like growth factors that induce tumor suppression.[97] In line with that, miR-125a-5p downregulates one of the important cellular factors, VEGF, and has been found to diminish the proliferation efficiency in colorectal cells.[98,99] Furthermore, EV-RNA can trigger an innate immune response by activating toll-like receptors (TLRs).[100] The activation of TLRs is independent of base sequence information. miRNA and other
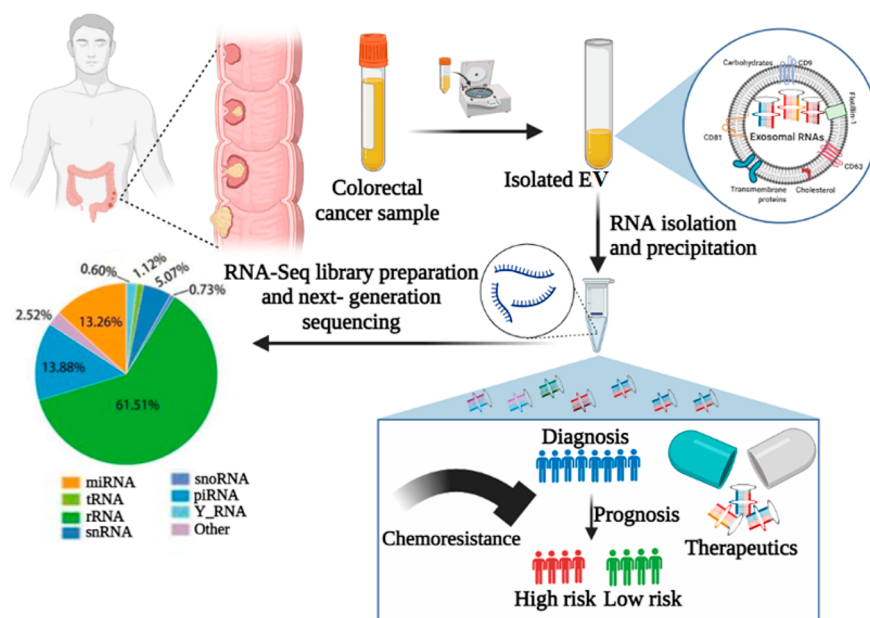
**Figure 3.** Colorectal cancer cell derived exosome secretion and isolation using the gold standard ultracentrifugation approach[16] and subsequent RNA isolation and RNA-seq data acquisition and analysis[74,75] for exosomal RNA based clinical applications. Colorectal cancer cell derived EVs are enriched with RNAs (shown in the pie chart, reproduced from ref 77 under the terms of the Creative Commons Attribution License, copyright 2021, publisher name: BMJ Journals) which offers next generation therapeutics and new biomarker for diagnosis and prognosis of cancer.

**Table 2. Summary of EV RNAs as a Promising Candidate for Colorectal Cancer Diagnosis**

| serial no. | bioliquid source | overexpressed miRNA type | function in colorectal cancer | AUC value[a] | ref |
|---|---|---|---|---|---|
| 1 | serum | miR-23a | metastasis | 0.95 | 101 |
| 2 | | miR-1246 | cytoskeleton remodelling | 0.94 | 101 |
| 3 | | miR-25-3p | metastasis | 0.68 | 119 |
| 4 | | miR-106b-3p | ECM degradation | 0.94 | 95 |
| 5 | | miR-301a | cell growth | 0.84 | 120 |
| 6 | | miR-125a-3p | chemoresistance | 0.68 | 121 |
| 7 | | CRNDE-h | chemoresistance | 0.89 | 113 |
| 8 | | miR18a | dual function | 0.80 | 13 |
| 9 | plasma | miR-21 | cell-proliferation | 0.91 | 123 |
| 10 | | LNCV6-98390 | drug-resistance | 0.77 | 115 |
| 11 | | miR-27a | cell cycle regulation | 0.89 | 124 |
| 12 | | miR92a-3p | metastasis | 0.85 | 125 |
| 13 | | miR-6803-5p | tissue invasion | 0.74 | 126 |
| 14 | | LNCV6_84003 | EMT | 0.73 | 127 |
| 15 | | GAS5 | cell proliferation | 0.87 | 116 |
| 16 | | miR-29a | ECM remodeling | 0.82 | 128, 129 |
| 17 | cell lines | miR-1268 | metastasis | 0.92 | 102 |

[a]AUC (Area under the curve) represents the efficacy of a diagnostic marker. The value can go from 0 to 1. The 0 represent zero percentage accuracy while 1 exhibit 100% perfection as a promising biomarker. A perfect biomarker must have an AUC value close to 1.

non-miRNAs are able to mediate TLR and set the innate immune response system in motion.[100] The alteration of RNA expression in EVs can substantially influence the cancer immune response.[45] By understanding the exact dysregulation profile, it is possible to construct a proper comprehensive framework for analyzing the immune state of an individual.

## 6. CLINICAL SIGNIFICANCE OF EV-RNA AS A PROMISING BIOMARKER FOR COLORECTAL CANCER

Recent progress in the EV field revealed that colorectal cancer EV cargoes, namely, RNA, miRNA, circular RNA, and lncRNA, are expressed more unusually than a normal cell.

Incorporated cargoes are free from degradation and can potentially be used as a potential biomarker for colorectal cancer (Figure 3).

### 6.1. EV-miRNA as a Diagnostic Marker

The differential expression pattern of EV-miRNA holds immense potential in colorectal cancer early diagnosis.[101] miRNA profiling of colorectal cancer EVs is done either with colorectal tissue or by using blood plasma.[101] To mention a few examples, EV isolated from containing miRNAs such as miR-23a, miR-1246, let-7a, miR-1229, miR-150, miR-223, and miR-21, which are highly overexpressed in colorectal cancer patients when compared with a healthy individual.[101] Based on measurements of the area under the curve (AUC) value, it was

found that the diagnostic capability of miR-23a, miR-1246, and miR-21 is much more accurate than that of other miRNAs having high AUC values (close to 1) (Table 2). Recently, Zhou et al. demonstrated the downregulation of miR150-5p, a promising diagnosis and prognosis candidate for colorectal cancer with an AUC value of 0.870.[102] Further, the colorectal cancer exosome overexpressing miR-106b-3p promotes epithelial to mesenchymal transition (EMT) and can be used as a potential biomarker for colorectal cancer.[95]

In the serum sample, overexpression patterns of different EV-miRNAs were also explored in cancer cell lines of different colorectal cancer stages. In one study, Cheng et al. found that the expression level of several miRNAs inside EV is more unique than that of cell lysate.[19] Moshammer et al. revealed that miR-21 and miR 200c-3p were upregulated in all colorectal cancer cell lines, but other miR-200 family RNAs hardly get transferred to EV.[50] Furthermore, depending on the cancer stages and the unique mutational status of each cell line, the EV-miRNA upregulation−downregulation pattern changes.[103−105] In one experiment, Bjørnetrø validated the expression level of six EV miRNAs in different colorectal cancer cell lines, HCT 116, HCC2998, KM20L2, RKO, and LoVo, that were cultured either at normal oxygen levels (21%) or in low oxygen levels (0.2%).[106] They found that the expression levels of EV miR-30d-5p, miR-30e-5p, miR-31−5p, and miR-194-5p were dysregulated to a large extent, depending on the culture oxygen concentration.[106] Due to uncontrollable cell proliferation, hypoxia is common in cancer, and thereby, hypoxic condition specific EV-miRNAs can be regarded as promising early colorectal cancer biomarkers.[96] Not only the oxygen level but also the mutational condition of specific genes influence the changes in EV-miRNA expression in different colorectal cancers.[94] For instance, dominant P53 mutation in colorectal cancer enhanced the expression level of EV-miR1246, leading to extracellular matrix (ECM) degradation, one of the critical criteria for cancer malignancy.[94]

## 6.2. EV-miRNA as a Chemotherapy Resistance Biomarker

In addition to cancer diagnosis, analysis of this natural delivery vehicle for cancer treatment prognosis has been widely pursued in recent years.[6] Takano et al. found the upregulation of EV-miR203 associated with poor colorectal cancer prognosis.[90] In continuation to that finding, Matsumura noted that exosomal miR19a is also related to poor prognosis.[107] One of the significant concerns with cancer therapy is chemotherapy resistance. Though the puzzle has not been solved yet, it is believed that cancer cells acquire chemoresistance by genetic and epigenetic alternation.[95,108] To improve the treatment outcome, it is imperative to access whether the chemotherapy is effective. For that reason, researchers are studying the possible relation between exosome dysregulated miRNA expression and colorectal cancer chemoresistance.[109] Jin et al. found overexpression of serum-derived EV incorporated miR-1246, miR-21-5p, miR-96-5p, and miR-1229-5p in 5-fluorouracil resistant colorectal cancer.[110] Further, Yagi et al. explored the higher expression level of EV-miR125-5b in 5-fluorouracil, leucovorin, and oxaliplatin resistant cancer cells. Continual monitoring of chemotherapeutic efficacy at very early stages of treatment highly demands a minimally invasive approach.[111] Although some work based on the detection of EV-miRNA biomarkers to solve this purpose has been reported, further research is necessary to provide certainty to oncologists to determine whether to change the treatment.

## 6.3. EV-lncRNAs: Possible Role as a Colorectal Cancer Biomarker

Long noncoding RNAs, the most significant class of RNA molecules, though they never express proteins, play a crucial role in gene regulation at both transcriptional and post-transcriptional levels.[111,112] Understanding the mutational status and exact role of lncRNA in cancer is a growing area of focus. Ren et al. found the overexpression of lncRNA H19 incorporated in exosomes derived colorectal cancer-associated fibroblasts.[113] Another report investigated the upregulation of EV incorporated colorectal neoplasia differentially expressed-h (CRNDE-h) as a promising biomarker with an AUC value of 0.892.[114] They also found CRNDE-h and CRNDE-p inside exosomes as a potential diagnostic−prognostic biomarker for colorectal cancer.[114] It is also reported that colorectal cancer patient plasma-derived exosomes containing LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003, and LNCV6_98602 are overexpressed based on cancer stage; this was found to be a promising biomarker with AUC value close to 0.7.[115] Another fascinating study explored serum-derived exosome lncRNA biomarker growth arrest-specific 5 (GAS5) as a promising avenue for colorectal cancer diagnosis with an AUC value of 0.964.[116] Further, exosome colorectal cancer-associated transcript 2 (CCAT2) was proposed as an excellent candidate for colorectal cancer. The exact mechanism of biogenesis and function is not been fully understood until now.[115] Collectively, recent experiments proved that there is a need for further study to use lncRNA as a promising, reliable, and accurate early liquid biopsy biomarker. Nonetheless, the population size of the study needs to be increased to establish if a biomarker is statistically significant.

## 6.4. Other Biological Information-Rich EV-RNAs from Colorectal Cancer

Other exosomal RNA species may also play an essential role as colorectal cancer biomarkers. Recent studies confirm that not only linear but circular RNAs can also be transferred horizontally through EVs.[117] Circular RNAs are mainly generated by back alternative splicing. Being circular without any terminal ends makes it more stable against nuclease degradation.[119] Though the exact reason for circular RNA sorting inside EVs is still unknown, according to reports, the circular RNAs could be incorporated through binding with RNA binding proteins (RBPs).[118,119] Reports also indicated that EV-incorporated circular RNAs are six times higher than in cells.[117,118] The abnormal expression level of these circular RNAs influences the immune response with cancer formation even in colorectal cancer.[117−123] For instance, Li et al. suggested circular RNA KLDHC10 overexpression in colorectal cancer cell-derived exosomes compared to those from a healthy individual.[117] The studies mentioned have indicated the diagnostic efficacy of different RNAs as a colorectal cancer marker. The markers with high AUC values are capable of distinguishing cancer patients from healthy individuals.[117] Though few studies targeted early diagnosis, further in-depth research in terms of health condition, sex, geographical location, and age is needed. A list of colorectal cancer cell-derived exosomal RNAs and their role in the cancer progression cascade with diagnostic accuracy is summarized in Table 2. Large-scale clinical trials impart a strong, robust scientific foundation in guiding research in the right direction. This can reveal the actual efficacy of a treatment or diagnosis process in physiological conditions to a large population, based

on the distribution of a wide variety of samples by gender, age, geographical location, etc. Although EVs were initially believed to be a random occurrence of the cell, various independent research studies have shown the efficacy of EVs in cancer diagnosis.[4] Currently, several EV-based diagnoses have been registered for clinical trials. Utilizing high throughput sequencing and other sensitive methods, the expression pattern of EV-loaded dysregulated RNAs with colorectal cancer is reported, and few studies are ongoing (www.clinicaltrials.gov). The dysregulated RNA characteristics are shown in Table 3.

**Table 3. Characteristics of EV RNAs in Colorectal Cancer Clinical Trials**

| serial no. | biomarker | no. of patients | specificity (%)[a] | sensitivity (%)[b] | ref |
|---|---|---|---|---|---|
| 1 | miR-1246 | 88 | 84 | 95.5 | 102 |
| 2 | miR-23a | 88 | 81 | 92 | 102 |
| 3 | miR-17-3p | 170 | 70 | 64 | 130 |
| 4 | miR-92a | 196 | 71.2 | 84 | 130 |
| 5 | Let-7a | 88 | 44 | 50 | 131 |
| 6 | miR-135 | 315 | 95 | 46.2 | 101 |
| 7 | miR-223 | 100 | 41 | 46.6 | 131 |
| 8 | miR-29a | 196 | 89.1 | 69 | 101 |
| 9 | miR-1224-5p | 100 | 28 | 31.8 | 101 |
| 10 | miR-17-92 | 316 | 81.5 | 69.5 | 122 |
| 11 | miR-1229 | 100 | 20 | 22.7 | 101 |

[a]Specificity = true negative/(true negative + false positive).
[b]Sensitivity = True positive/(true positive + false negative).

## 7. FUTURE OUTLOOK

There is no accurate diagnostic test that can detect early colorectal cancer with high sensitivity. For this reason, standardization of an effective method is in high demand in the current scenario. In this Review, we have indicated the potential of EVs in human physiology and further discussed the mechanism behind specific RNA incorporation inside EVs with a perspective on immune escape and colorectal cancer outcome.[129−133] Besides the base sequence information on specific RNAs, a recent investigation detected the glycosylation modification on RNA surfaces. Bertozzi's lab proposed that the glycosyl groups decorate on RNA surfaces in a similar way to proteins.[134] There is a high possibility of variation in glycosylation patterns between diseased patients and healthy controls.[134] In this slightly offbeat yet exciting area of modern biology, it will be very interesting to investigate the presence of glycosylated RNA inside/on the surface of EVs, if any. The new inputs and scientific insights in this particular field will be very valuable in the diagnosis of glioblastoma, mesothelioma, and other aggressive cancer subtypes.[77,135] Though different opinions exist, the ability of EV to cross the blood-brain barrier (BBB) makes it a desirable source of cancer biomarkers in general.[136−138] The progress of technology has advanced the detection of the analyte of interest in many aspects, such as mRNA detection in cells by the modification of complementary DNA/synthetic xenonucleic acids molecules on the atomic force microscopy tip surface followed by RT-PCR noninvasively.[137] The same strategy can also be realized for cancer EVs for temporal RNA analysis.[138] With more research and advanced technology, post-transcriptional modifications of EV incorporated DNA and RNAs can also be sensed at a single nucleotide level. In addition to measuring the proteogenomic

profile of cancerous EVs using conventional biochemical methods, various biophysical characterization techniques have been customized for EV detection such as nanoparticle tracking assay, dynamic light scattering, flow cytometry, etc.[6] An excellent initiative called EV-TRACK (www.evtrack.org) has been initiated, aiming to standardize EV characterization. The identification of specific EV subpopulations to incorporated nucleic acid analysis with the help of spectroscopic methods, like SERS, surface plasmon resonance, electrochemical detection, FT-IR spectroscopy, etc., can be conducted from bench to bedside.

The pathobiological heterogeneity of tumor cells offers the biggest challenge in the effective diagnosis of cancer.[138] Simultaneous identification and characterization of EV incorporated cargoes can overcome such limitations. Detection of a single analyte is not sufficient, at least for cancer diagnosis. Further, disease monitoring and management with point of care (POC) diagnosis have emerged as an excellent platform that is rapid, simple, patient-friendly, and cost-effective in recent times. The global POC market is expected to reach $36 billion by 2028 with a CAGR (compound annual growth rate) of 3.25%.[132] With the unprecedented pressure that has been placed on the medical sector after the current COVID-19 pandemic, it is very relevant to advance technologies that can facilitate rapid medical decisions and are easy to use for nontechnical personnel. To meet the expectations, scientists are constantly developing multiplex EV sensing medical devices by applying different machine learning algorithms. The scientific curiosity behind mimicking the pathophysiological condition of cancer cells with tiny EVs has advanced EV research over the past decade. However, currently, there is a lack of available standard isolation and characterization techniques for EVs.[53] In addition, the physiological condition of EVs, storage conditions, and understanding of vesicle stability are still unclear.[53] That being the case, it is essential to recognize the unmet needs of existing technology to prioritize EV research with every novel idea and advancement in science.

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