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MicroRNAs: The next generation of cancer biomarkers

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Abstract

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that have been shown to be involved in a wide range of biological processes, including cancer. miRNAs are known to regulate the expression of genes, and their dysregulation has been linked to the development of cancer. In recent years a great deal of attention is received by miRNAs due to their potential as biomarkers for cancer. Biomarkers are measurable indicators of a biological state, and they can be used to diagnose, monitor, and treat diseases. miRNAs can be detected in biological fluids such as blood and saliva. This makes them ideal candidates for early cancer detection and monitoring. We herein reviewed current methods for the isolation of circulating miRNAs. Provide the most recent update about clinical trials aiming at using miRNAs as biomarkers for cancer. Additionally, we highlighted some pitfalls that should be realized to take advantage of the massive potential of miRNAs as a cancer biomarker. However, the potential of miRNAs as cancer biomarkers is very promising but advancements in factors such as miRNA isolation methods, and the type of samples are critical to incorporate miRNA-based diagnostic and prognostic markers in modern-day treatment regimens for cancer. This review concludes that miRNAs have enormous clinical significance as cancer biomarkers and recommends carefully selecting methods for the isolation of miRNAs based on the type of sample, and the downstream applications to generate clinically relevant results.

Introduction

MicroRNAs (miRNAs) are short 19 to 25 nucleotides non-coding RNAs [1, 2] and are referred to as master regulators in the cells [3-5]. They were reported for the first time in 1993 [6, 7], in *Caenorhabditis elegans*; however, their role in gene regulation was recognized ten years later in 2001 [8-10]. MiRNAs are the primary post-transcriptional regulators of gene expression in numerous tissues and developmental stages. Research has proved the critical role of miRNAs in the development, differentiation, inflammatory response, and development of cancer. They accomplish this by controlling gene expression through densely woven regulatory networks and extremely specialized interaction types [3]. To date, over 2500, miRNAs have been identified in humans alone [11]. After all the processing, the mature miRNAs are capable of altering the expression of protein-coding genes [12] by binding to and disrupting messenger RNA (mRNA) expression [13] or by curtailing translation [14]. All mRNAs have miRNA binding sites on their 3' untranslated region [15]; therefore, miRNAs can function in regulating the human protein-coding genome [16]. Studies have shown that miRNAs are abundant in serum and plasma as well as extracellular biofluids [17, 18]. The variety of bio-fluids reported in humans are saliva, breast milk, urine, tears, follicular and cerebrospinal fluids that contain miRNAs [19-21].

The in-depth studies of miRNA expression profiles indicated that their release from cells is selective and therefore correlates with different pathophysiological conditions of the body, which can be harnessed for better treatment direction and monitoring of body conditions during specific malignancies such as cancer [19]. All the cellular processes and pathways can be influenced by miRNAs both in normal and pathological conditions [22, 23]. A variety of routes are reported for miRNAs released into the extracellular environment [24]. They can be passively released by damaged, inflamed, apoptotic, or necrotic cells, or maybe from cells like platelets that have a limited half-life [4]. Meanwhile, they can be actively secreted via membrane vesicles produced from cells, such as exosomes, microparticles (MPs), apoptotic bodies, and other shedding vesicles [2]. Similarly, an active secretion by a protein-miRNA complex, wherein either one or both of the lipoproteins (such as high-density lipoprotein: HDL) and the Argonaut (Ago2) protein are associated with miRNAs [16]. The intricate relationship between miRNAs and the genes they target is influenced by several variables, including the location of the miRNAs inside the cell, their quantity, the mRNAs they target, and their affinity for miRNA-mRNA interactions [5]. MiRNAs are released either encapsulated in exosomes or bound to other entities such as Ago2 [25]. Exosomes are believed to encapsulate 10% of circulatory miRNAs [26], while the remaining 90% of miRNAs are in a non-membrane-bound form, attached with Ago2 or some other lipoproteins in the circulation or body fluids [27, 28]. Exosomes are nano-vesicles that circulate in the body and have a role in controlling many biological processes and cell-to-cell communication. Even in the presence of RNase, RNAs enclosed inside exosomes are quite stable [5]. Exosomal miRNAs such miR-9, miR-23a, miR-92a, miR-103, miR-105, miR-126, miR-132, miR-135b, miR210, and miR-221, as well as cytokines (e.g., interleukins: IL-6 and IL-8, TNF-α, transforming growth factor β, FGF2, and VEGF), have been shown to be produced by tumor cells, are neovascularization and metastasis-promoting proangiogenic factors.

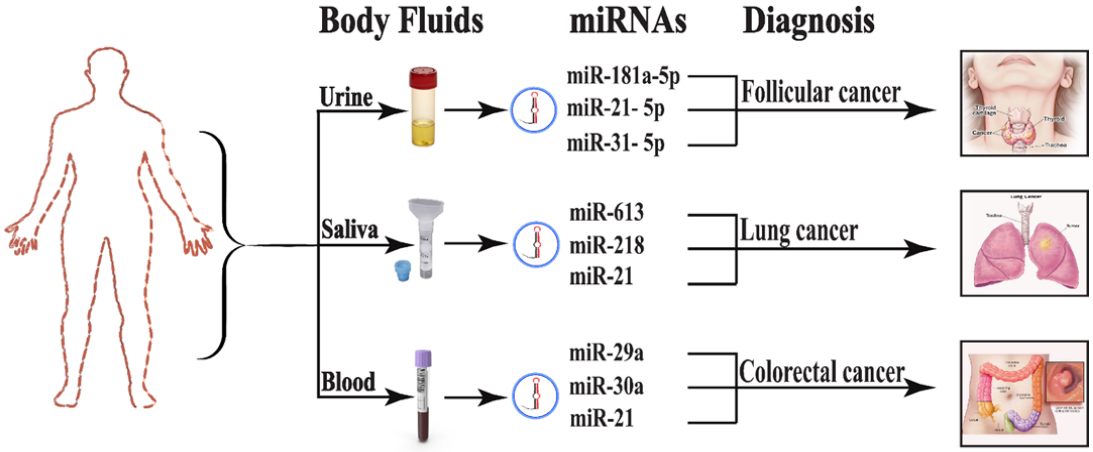

Fig. 1: Graphical abstract

The majority of circulating miRNAs rely on carriers for active secretion to escape degradation, with the exception of the passive release brought on by apoptotic, necrotic, or inflammatory processes [28]. MiRNA expression patterns are typically dysregulated in cancer, which has drawn significant attention to exosomes as potential biomarkers for cancer diagnosis, prognosis, and recurrence [29]. Exosome-translocated miRNAs have the ability to influence tumor growth and take part in a number of processes involved in carcinogenesis and tumor formation. Cancer cells release unique miRNAs in exosomes. These particular miRNAs, which have immunosuppressive qualities, can interact with activated T cells that are specialized for malignancies to stimulate the development of tumors. For instance, miRNA-21 and miRNA-146a may be correlated to cervical cancer. These miRNAs can reduce the ability of 293T cells to identify and destroy cancer cells, and they can even cause apoptosis in 293T cells, which is a hallmark of cervical cancer [31]. MiRNAs are coded by more than 1000 genes that are located on every chromosome in the human genome except the Y chromosome [29].

The biogenesis of miRNA in a cell starts from the nucleus and ends at the cytoplasm [30, 31]. A functional miRNA is derived by multi-step processing of large primary (pri) and precursor (pre) transcripts. Pri-miRNAs are transcribed by RNA polymerase II and later processed by Drosha to pre-miRNA [32]. These pre-miRNAs are moved by Exportin5 from the nucleus to the cytoplasm by Dicer, where they are transformed into mature miRNAs and sorted into exosomes by one of five possible pathways: The following pathways are reliant on different proteins: (1) nSMase2; (2) 3′ miRNA sequence; (3) miRNA motif and sumoylated hnRNPs; and (4) miRNAISC-related pathway; (5) A route associated to ceramide [9]. Mature miRNAs can be selectively incorporated into the exosomes or coupled with Ago2 protein and released into the extracellular milieu. Alternatively, they can be entrapped in microvesicles or attached to some other lipoproteins prior to their release into the extracellular environment [21].

Recent studies have indicated that the ESCRT complex, a group of proteins involved in endosomal sorting, plays a role in the synthesis, uptake, and cargo sorting of exosomes. The highly conserved procedure used by the ESCRT complex to pick the "cargo" protein marked by ubiquitintin, guide it to multivesicular bodies (MVBs), and subsequently split from the peripheral membrane is similar to the process used in cytokinesis and viral budding. Exosomal origin is better understood by research on late endosome components notably Alix, tetraspanins, and tumor susceptibility gene 101 (TSG101) [10].

The biogenesis of mature miRNA, as shown in **Fig. 2** is regulated during transcription as well as a post-transcriptional level [33]. A worth mentioning fact is that miRNAs are remarkably stable even under hostile conditions like high/low temperature, and high/low pH. They can overcome extended storage time and freeze-thaw cycles [34]. Extracellular miRNAs are even more stable than mRNAs [35, 36]. The reason for this stability is that miRNAs are associated with Ago2 proteins or lipoprotein complexes [12, 37, 38] or entrapped inside exosomes [39] or other microvesicles, hence protected from RNases [40].

It has been deduced from bioinformatics processing that a single miRNA can alter /influence 60% of mammalian mRNAs [21, 42]. Deregulation of miRNAs are associated with several types of cancers [22, 43] including breast, prostate, glioma, colorectal cancers, and lymphoma [11]. Chromosome or genomic alterations in cancer-related genes may directly reflect changes in miRNA expression patterns. Aberrant miRNA expression most certainly has significant therapeutic consequences, in addition to the role of cancer-associated miRNAs identified in a range of tumor tissue specimens [12]. Anomalous miRNA levels can also result from variations in the activity of the miRNA-producing enzymes Drosha and Dicer [13]. These enzymes are negatively regulated in ovarian and bladder cancer but are upregulated in cervical squamous and gastrointestinal cell tumors Moreover, pri-miRNA transcriptional defects may induce changes in circulating miRNAs in cancer [14]. Altered expression of miRNAs can be unique factor in a variety of diseases including cancer [15]. To collect detailed information about miRNAs based clinical studies for cancer detection weexploredClinicalTrials.gov database using the terms "tumor" "cancer" and "neoplasm" in the disease field and "miRNA" "circulating" and "blood" in the other terms field [17]. An online database called ***miR2Disease*** offers precise and detailed information about miRNAs deregulation in various human diseases [44]. Comprehensive information about the classification of extracellular circulating miRNAs can be obtained from ***MiRandola****:* an online database [45]. Another valuable computational tool is ***miRDB*** that provides detailed information about miRNAs targets and functionality [46]. Currently, miRNAs are named based on the order of their discovery, except for miRNAs from miRNAs let-7 family [47]. Sample type, collection protocol, and method of extraction

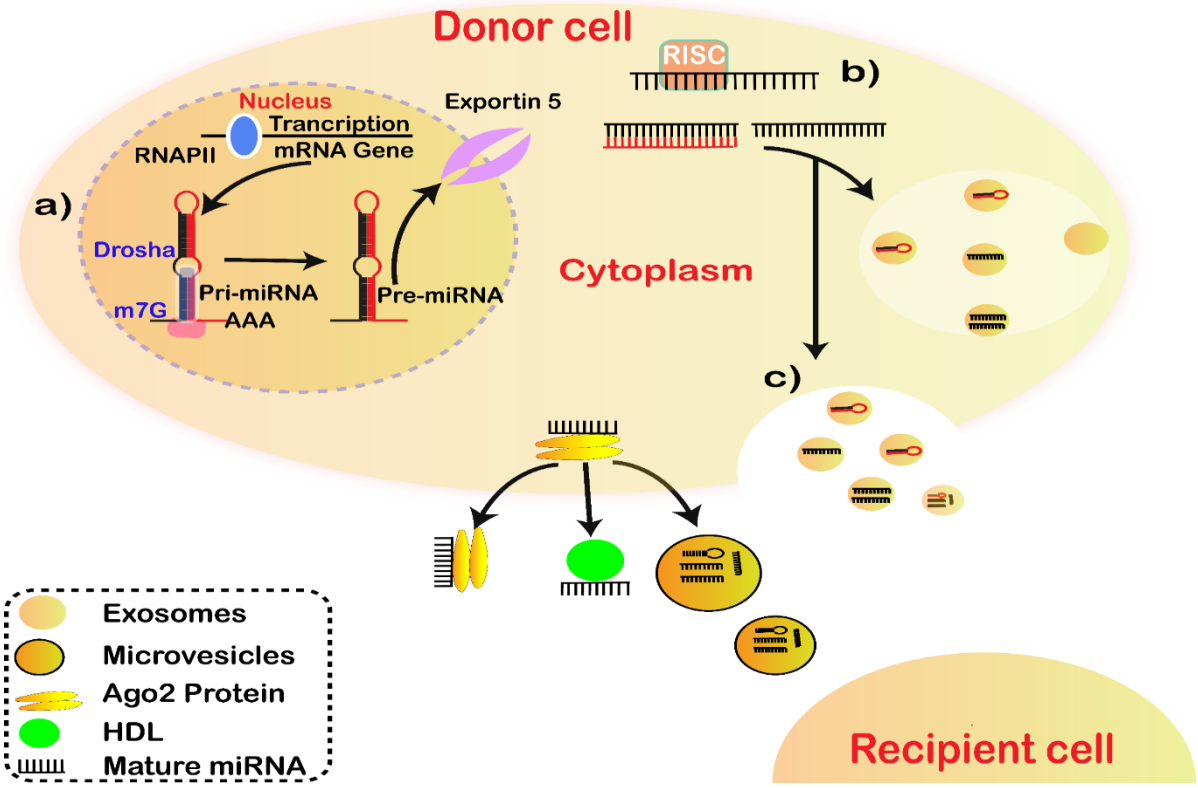

Fig. 2: Biogenesis of miRNAs redrawn from reference [41].

greatly influence the detection of miRNA [48]. The research on miRNA expression in biofluids has rapidly gained a lot of momentum [49] as miRNA expression is indifferent to the type of body fluid, easily detectable, and can be reliably quantitated [50]. A biomarker can be defined as any characteristic that is quantified to assess the pathophysiological condition of the body or response of the body to the treatment regime [51-53]. The pre-requisite for an ideal biomarker is that it must be highly specific, highly sensitive, can be acquired noninvasively as well as its concentration should be indicative of a certain condition of the body [54]. They can be either diagnostic, prognostic or predictive [55]. In 2008, extracellular miRNAs were first reported in maternal plasma followed by serum obtained from cancer patients [56,57].

Clinical significance

1) MiRNAs impact different types of cancers at different levels, from causation to progression [11, 58, 59].
2) Intra-tumour cell communication is facilitated by miRNAs; it may involve target suppression, promote migration and invasion or confer drug resistance in a variety of cancers [60].
3) MiRNAs were considered as non-invasive biomarkers for the first time in the field of cancer biology, minimizing severe invasive procedures for biomarkers isolation in cancer [41, 61].
4) They are stable in healthy people, and their expression is independent of body weight, gender, and age [62-64].
5) Alterations in miRNAs expression pattern can indicate a pathological condition that can be used for monitoring cancer onset and diagnosis [65].

Selection of sample source and miRNAs extraction method

It's a well-established fact that miRNAs exist in a majority of body fluids. Therefore, they can be easily isolated from body fluids mainly serum, plasma, tears, and urine [66]. The miRNA purification procedure depends upon the choice and composition of the

sample source. A new sample type needs to be compared with already established sample types; the data are not necessarily translatable [67]. Studies have already attempted to reduce variations in the isolation efficacy of different procedures/kits [68]. The concentration of circulating miRNAs is even different between blood serum and blood plasma [14, 68, 69]. Urinary miRNAs from urine are more stable and resilient to degradation; they have opened a new area of research for the discovery of non-invasive cancer biomarkers [70]. The concentration of miRNAs in serum, plasma, and other bio-fluids is very less, at the Femtomolar level [71]. It is even less when the samples were collected from model animals of disease [72]. New extraction methodologies are needed to reduce and control inter-sample variability as well as isolate miRNAs even at lower concentrations from different sample types [73]. The wholesome and precise measurement of miRNA depends on its robust isolation [71]. However, the short and variable sequence of miRNAs and their lower concentrations impede the process [74]. The most common detection methods for miRNAs are qRT-PCR and microarray; they are strongly dependent on the precision of the extraction methods [75]. Therefore, it is imperative to optimize and standardize the miRNA isolation methods/ procedure [76]. Many studies reported discrepancies in miRNA measurement and recovery because of the extraction methods [68, 77]. Appropriate extraction methods should be based on factors like sample type, quality, quantity, price, and time [4], as shown in **Table 1.**

Moreover, the extraction methods must be tailored to the specific sample type and intended application [78]. Studies involving circulating miRNAs should mention the details of the collection procedure, sample type, and treatment, the time between collection and further processing, conditions of sample processing, and samples' storage condition [78]. It has been widely recommended that procedures used for isolation in miRNA studies are of vital importance for the precision and accuracy of the results [77]. MiRNAs isolation methods are divided in two main categories. First is guanidine/phenol/chloroform (GPC)- based extraction methods and the second is column/bead/based commercial isolation kits [79, 80].

Guanidine/phenol/chloroform (GPC) based extraction of miRNAs

This isolation method is based on the difference in solubility of cellular components in organic solvents. This protocol harness phenol and guanidinium thiocyanate, marketed as Trizol for miRNAs extraction [81]. Trizol has the ability to denature proteins such as RNases hence the product acquired can be stored for long term [82]. In this method isopropyl alcohol is added after the phase separation step to precipitate miRNAs Due to the small size of miRNAs, ample time is needed for their isolation by this method. Overnight precipitation at -20°C or -80°C [83], longer pelleting time (16,000–21,000 g centrifugation for 1 h at 4°C) is highly recommended. Trizol-based extraction is considered a “gold standard” [16]. The method's core principle has remained unchanged, although significant improvements and modifications have been introduced to get specific results [84]. Although it’s an efficient method, its drawbacks are that it is labor-intensive and employs hazardous chemicals, such as phenol [85]. The procedure consumes time about 40 to 60 min [85] and requires a relatively large sample volume [81]. Although Trizol is a widely employed method, some imperative factors are to be considered before employing it [16]. MiRNAs with low GC content cannot be isolated during phenol-chloroform extraction; therefore, a column-based RNA adsorption method is recommended to isolate them [86]. TRIzol method decreases inter-assay variability, a common drawback of column-based kits [69]. MiRNAs yield improves by using the Trizol method, but the product is also affected by the secondary structure. TRIzol method one drawback is the contamination of miRNAs by organic solvent [37]. To increase the miRNAs’ yield, overnight incubation and a series of centrifugations must be used in association of the alcohol-based precipitation method [83]. Most of the technologically advanced methods for miRNAs isolation are mainly derived from the Trizol method [87].

Silica-based miRNA extraction methods

Among column-based methodologies, mirVana™ PARIS™ (Life Technologies) and miRNeasy kits (Exiqon) are used prolifically in miRNA studies, that utilize silica in the column system for the isolation of miRNAs [57]. Extraction methods are difficult to compare as many studies do not provide information about the actual yield and quality of miRNA [4]. Both nucleic acids and proteins can be separated by mirVana™ PARIS™ [88]. The salient feature of this method is that cells are disrupted non-ionically prior to phenol/chloroform extraction [89].

Table 1. Summary of conventional miRNAs isolation methods.

	mirVana™ PARIS™	miRNeasy	TRIzol
Time	20 min	25 min	> 60 min
Steps	4	4	5
Cost	$$$	$$	$
Sample Type	Cells and tissues	Cells and tissues	Tissues and body fluids.

Moreover, it is suitable to isolate miRNA molecules lower than 200 nucleotides [90]. Other worth mentioning features of this kit are that it can efficiently isolate miRNAs from tissues as well as body fluid and requires small starting fluid volumes [91]. Vigorous analysis of reported human miRNAs, ~68 human miRNAs are reported to be smaller than 18 nt. Out of these, only 9 miRNAs are isolated by mirVana PARIS kit, making it a less attractive choice for studies involving miRNAs smaller than 18 nucleotides [72]. The miRNeasy kit (Exiqon), utilizes silica in its column system for the isolation of miRNAs [92]. It has been reported to be superior to other silica-based kits in terms of miRNA yield [93, 94]. It harnesses a mini-column, silica, and ethanol for miRNAs' adsorption [40, 95]. The miRNeasy kit is better than TRIzol based on high miRNA yield [91, 96]. The major shortcoming of the miRNeasy kit is its alarming inability to isolate miRNA species smaller than 18 nucleotides [72].

Isolation of miRNAs encapsulated in exosomes

Differential ultracentrifugation (dUC)

This method depends on the progressive separation of particles via sedimentation according to their size and density employing a succession of centrifugal forces and duration [97]. dUC is typically regarded as the gold standard method for exosome separation since it can separate reasonably pure populations of exosomes [98]. Several elements work together to determine this extraction capability [99]. Lipoprotein particles of comparable density had a tendency to precipitate with the final pellet, despite the separated pellet from dUC having a low level of contamination from non-exosome-related proteins A series of cleaning spin processes are performed before the dUC operation begins in order to get rid of cells, cellular debris, apoptotic bodies, and microvesicles [100]. This is accomplished by gradually separating the pellet and supernatant at increasing speeds: 300-400 g for 10 min, then 2000 g, and finally 10,000 g, to isolate a supernatant with a comparatively high concentration of exosomes even though it remains polluted with lipoprotein moiety contamination, other protein aggregates and microvesicles [101]. Following this, samples are spun at 100,000–200,000 g for at least 70 min or for 2 h to complete the final exosomes sedimentation process [102]. The pellet produced here can be ultracentrifuged once more after being resuspended in phosphate-buffered solution (PBS), which will boost purity but reduce the yield of the separated exosomes [103]. From 20 to 250 nm in size, the exosomes portion can be obtained that can be verified for the presence of exosomal protein markers: Flotillin-1, TSG101, Alix, CD9, CD63 and CD81 [104, 105].

It has been reported that 81% of research studies employed ultracentrifugation as their method of choice for exosomes separation [106]. The use and popularity of this traditional method, however, declined, most likely as a result of technical developments in exosomes separation that need less time and work. This approach does not require labeling exosomes, which can prevent cross-contamination, but it is time-consuming, expensive, damages structures, aggregates into blocks, and co-separates lipoproteins, making it unsuitable for downstream analysis [107].

Density gradient centrifugation

Based on ultracentrifugation, density gradient centrifugation is an enhanced separation technique [108]. Exosomes are intended to be purified using density gradient centrifugation, which is typically combined with ultracentrifugation to increase exosome purity There are two primary types: iodixanol and sucrose, the latter of which is frequently employed as a research medium. Therefore, due to their significant similarity in size and density, exosomes and retroviruses cannot be efficiently separated by the sucrose density gradient [109]. Top loading and bottom loading are the two methods for loading the samples. In contrast to top loading, which causes soluble proteins to sediment across the gradient during ultracentrifugation, bottom loading keeps soluble proteins at the bottom during the process [110]. Exosomes migrate along a density gradient

medium in this procedure, which involves placing samples at the top (where densities are higher). According to the underlying theory, particles with various sedimentation coefficients settle in discrete strata during centrifugation that can be subsequently gathered. Exosomes float on a sucrose gradient until they reach equilibrium density, ranging around 1.10 to 1.21 g/mL, generating a fraction zone that is simple to retrieve [111]. Its clinical use is constrained, nonetheless, by the prior preparation, laborious operation, and extended centrifugation time (>16 h) [112].

Ultrafiltration

The ultrafiltration technique forces other cellular debris through the membrane into the sample's effluent component while isolating exosomes above the filter level using centrifugal force and a cellulose membrane [113]. Ultrafiltration is a very easy, quick, and affordable procedure that can reliably separate particles depending on their size and molecular weight [114,115]. Filters having pore sizes of 0.8µm and 0.45 µm are used to exclude larger particles first, producing a filtrate that is comparatively exosome-rich. Then, smaller vesicles are removed from the filtrate by passing them through membranes with holes 0.22µm and 0.1 µm smaller than the required exosomes. The first and last pore filtration membranes are used to define the exosomes' maximum and minimum size ranges. This methodology can be employed as a stand-alone technique or in conjunction with ultracentrifugation to separate big microvesicles and exosomes. Cross-flow filtration or tangential-flow filtration is a different technique from nano-ultrafiltration that depends on successive filtrations to extract exosomes [116]. It is quick and easy, needs little equipment, and doesn't interfere with exosomes' ability to function biologically because of a faulty operation. Exosomes are not damaged during ultrafiltration since it is performed at ambient temperature without the use of chemical reagents, which results in excellent exosome purity [117]. Nevertheless, using too much pressure might rupture or distort bigger vesicles, giving false findings [118].

Extraction of exosomes by ExoQuick Kit

ExoQuick allows for the quantitative, high-throughput separation of exosomes from minimal quantities (as little as 1 ml) of tissue culture medium and specific biological fluids, such as urine, saliva, breast milk, and follicular fluid ExoQuick is a reliable and efficient alternative to ultracentrifugation that is compatible with a wide range of downstream applications In this method conserves valuable material, is easily scalable, produces large quantities of functioning, high-quality exosomes, and may be utilized to separate exosomes for a variety of downstream applications, consisting of (exosomal proteomics, exosomal lipidomics/metabolomics, functional studies, such as in cell-to-cell signaling and basic biology, such as role in tumorigenesis, biomarker studies, and exosomal miRNA profiling,) [119]. The unique polymer ExoQuick effectively precipitates exosomes First, simply remove all cells and cellular debris from your samples before adding the necessary amount of ExoQuick to your cleared biofluid, cooling it, and centrifuging it. The pellet containing your exosomes will be prepared for resuspension in the suitable solution. Simply mix 10 mL of tissue culture medium with 2 mL of ExoQuick, incubate for an overnight period at 4°C, then centrifuge at 1500 g for 30 min to separate exosomes. The pellet of the exosome was re-suspended in 1 mL of diluted PBS (1:40), and the NanoSight LM10 device was used to observe it. ExoQuick retrieved 133 nm exosomes at a concentration of 1.74 x 10^9 particles/mL, according to the analysis. Exosomes extracted with ExoQuick also yield superior samples for investigating nucleic acids that are associated with exosomes, including mRNA, siRNAs, and even miRNAs. Nucleic acids recovered from ExoQuick-purified exosomes are compatible with quantitative analytical procedures including qPCR, microarray analyses, and next-generation sequencing [120] (**Fig. 3**).

Latest update on miRNAs based clinical studies for various cancers

miRNAs are valued clinically for the management of a variety of cancers [121]. From the expression level of miRNAs, information about cancer stages, progression, and metastasis can be obtained [122,123]. To detect cancer in its early stages miRNAs dysregulation can be a more potent indicator prior to biopsy or imaging techniques. In patients of non-small cell lung cancer (NSCLC) plasma miR-21-5p, miR-145-5p, miR-20a-5p, miR- 141-3p, miR-155-5p, and miR-223-3p relatively increased at early stages [124,125]. Exosomal miR-382 emerges to be a valid prognostic biomarker for monitoring NSCLC development [43]. Exosomal miR-3913-3P was discovered to be related to platelet count,

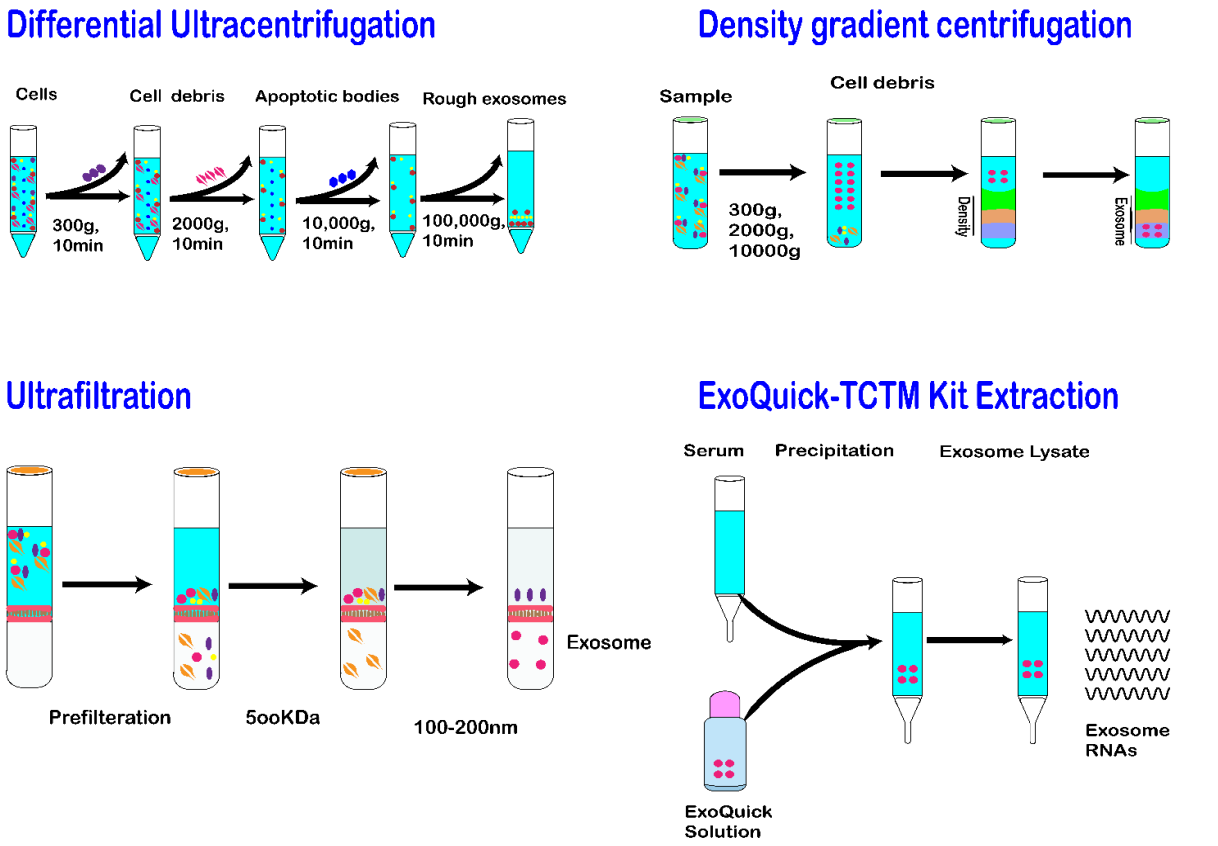

Fig. 3: Various Methods for exosome isolation such as Differential ultracentrifugation, Density gradient centrifugation, ultrafiltration, and Exoquick Kit extraction.

tumor marker carcinoembryonic antigen, tumor, node, and metastasis (TNM) stage, and distant metastasis. Exosomal miR-3913-5p might thus be used as a diagnostic biomarker for resistance in the peripheral blood of NSCLC patients [44].

Many recent studies deciphered the association between miRNAs and lung cancer [126], in particular NSCLC. For example, miRNAs like, miR-26a, miR-210, and miR-212 are mentioned to serve as oncogenes and by contrast, miR-1, miR-126, and miR-149 as tumor suppressors in lung cancer [127]. MiRNAs can assist in differentiating between numerous subtypes of cancer. Cancer subtypes are ranked based on their tissue origin and pathological pathway, for instance, adenocarcinoma (ADC) and squamous cell carcinoma (SCC) are the main subtypes of NSCLSs [128]. Similarly, breast cancer subtypes are decided by the presence of estrogen receptor (ER), progesterone receptor (PR), and HER2/neu receptor [129]. This sub-classification is of huge importance in deciding the treatment direction and drug selection [130]. Serum exosomal miR-148a levels were discovered to be significantly lower in breast cancer patients. Patients with breast cancer who had serum exosomal miR-148a down-regulation had worse clinical outcomes. Consequently, exosomal miR-148a in the serum may serve as a significant biomarker for the prognostication of breast cancer [131]. MiR-138-5p, a protein-coding gene, was transferred by exosomes from breast cancer cells to tumor-associated macrophages, where it reduced the expression of KDM6B. As a result, exosomes were implicated in the transfer of miR-138-5p between cancer cells and macrophages, which raises the possibility that miR-138-5p found in circulating exosomes might be employed as a predictive marker for breast cancer [46]. Plasma exosomal miR-363-5p is a tumor suppressor that is also employed in non-invasive lymph node staging and prognosis prediction in breast cancer [132] Different types of miRNA inhibitors used in cancer are given in **Table 2**.

Expression patterns of miRNAs can help in distinguishing NSCLC from healthy individuals and can then differentiate between ADC and SCC subtypes.

Table 2: Different types of miRNA inhibitors used in cancer along with their phase data

Clinical Trial ID	Cancer Type	miRNA Target	Status
miR-21-5p inhibitor (AZD3819)	Non-small cell lung cancer (NSCLC)	miR-21-5p	Phase II
miR-21-5p inhibitor (MK-0346)	NSCLC	miR-21-5p	Phase I
miR-17-5p inhibitor (PF-06882315)	NSCLC	miR-17-5p	Phase I
miR-205-3p inhibitor (ONO-5338)	NSCLC	miR-205-3p	Phase I
miR-21-5p inhibitor (AMG 510)	Head and neck cancer	miR-21-5p	Phase I
miR-17-5p inhibitor (AZD7413)	Head and neck cancer	miR-17-5p	Phase I
miR-205-3p inhibitor (ONO-5353)	Head and neck cancer	miR-205-3p	Phase I

Two miRNAs, miR-16- 5p and miR-486-5p level were uplifted in ADC and SCC cases relative to healthy individuals. Another miRNA, miR-9-5p expression was normal across NSCLC patients and controls, but declined in ADC patients relative to SCC ones. However, miR-205-5p was upregulated only in SCC patients alone [133]. Alike other cancers classification papillary and follicular cancer can be separated by combined expression of exosomal miR-21- 5p, miR-31-5p, and miR-181a-5p [134]. High expression of serum exosomal miR-106b-3p can distinguish metastatic colorectal cancer (CRC) from nonmetastatic. [135]. Serum exosomal miR-874 was shown to be significantly downregulated in 125 CRC patients compared to 70 healthy controls, 45 benign adenomas, and 125 controls without CRC. It was shown that the expression of serum exosomal miR-874 is a statistically significant independent predictor of overall survival in CRC patients. As a result, exosomal miR-874 expression in serum may be a significant biomarker for the diagnosis and prognosis of CRC [136]. Patients with CRC, especially those who had liver metastasis, had significantly higher blood levels of exosomal miR-122. Serum exosomal miR-122 has been proposed to be CRC prognostic marker using both single- and multiple-variable logistic regression [137]. Moreover, Low miR-193a expression and high let-7g expression were associated with a decreased survival rate and may serve as markers for the diagnosis and prognosis of CRC [138].

Prostate cancer is the second most lethal cancer in men after lung cancer. [139]. Six plasma exosomal miRNAs showed differential expression in 108 treatment-naive prostate cancer patients and 42 castration-resistant prostate cancer (CRPC) patients (miR-423-3p, miR-320d, miR99a-5p, miR-320b, miR-150-5p and miR-320a), in which CRPC was especially associated with exosomal miR-423-3p.

Therefore, to detect and predict castration resistance early, exosomal miR-423-3p may serve as a prognostic biomarker [140]. In contrast to healthy persons, prostate cancer patients had considerably lower levels of urinary exosomal miR-375 expression, whereas, their levels of miR-486-5p, miR-451a, and miR-486-3p expression were much higher. It was successful to discriminate between localized and metastatic prostate cancer using urine exosomal miR-375. [140]. The sixth most prevalent cancer in the world is oral squamous cell carcinoma (OSCC), which can affect the gingiva, hard palate, retromolar trigone, hard palate, mouth floor, tongue, and buccal mucosa [141]. Fascinatingly, salivary exosomal miR-24-3p has demonstrated potential for OSCC diagnosis as a substitute for blood or urine Malignant cells grew more quickly when exosomal miR-24-3p was overexpressed, and OSCC cells multiplied more quickly. When exosomal miR24a-3p was overexpressed by controlling the expression of genes involved in the cell cycle. Additionally, by inhibiting PER1, exosomal miR-24a-3p can prevent OSCC cells from growing. Consequently, exosomal miR-24a-3p may serve as both a diagnostic and therapeutic target for OSCC [142]. miRNAs can substantially contribute in monitoring tumor metastasis. Tumour metastasis negatively affects curative approaches, diminishing the rate of survival, and uplift the risk of recurrence [143]. The lack of a dependable biomarker to monitor the tumour metastasis to different sites is still needed to be eliminated. MiRNAs can be of substantial importance in this regard, as crmiRNAs were found to be associated with tumorigenesis or metastasis [143-144]. miRNAs can be harnessed to predict the sensitivity of tumour to curative strategy in pancreatic ductal adenocarcinoma (PDAC), a major subclass of pancreatic cancer. For instance, miR-155-5p was upregulated in tumour tissues and plasma; information about tumour stage and poor prognosis can be obtained from its expression [145]. In line with these findings, the biggest issue of chemotherapy or radiotherapy can be curtailed by targeting corresponding crmiRNAs and their downstream targets [146]. miRNAs have been reported as prognostic biomarkers of cancers. This fact is

supported by various studies [139-142]. The perks and shortfalls of miRNAs [147]. as a prospective biomarker for cancer are listed in **Table 3**. One of the basic obstacles that have hindered the path of miRNAs toward a potential therapeutic and diagnostic entity in routine clinical practices is the stringency of the simple and standardized isolation protocol.

Both the researchers and clinicians agreed upon the fact that an efficient, effective, and straightforward protocol for the isolation of miRNAs from biofluids will increase the clinical applications of miRNAs as a therapeutic and diagnostic entity [127]. The research on miRNAs used as biomarkers has picked up speed. Many reported miRNAs-based cancer studies are undergoing clinical trials worldwide [144,145]. Up to date, ample manuscripts have tagged miRNAs as an ideal biomarker for various malignancies in humans, including cancer. But compared to this, there is a paucity of clinical trials that have been launched so far to validate the claims [128]; this is depicted clearly by **Fig. 4**. Biomarkers are vastly investigated in cancer research. They have been adopted and tested for screening, diagnosing, and exact staging of cancer as well as treatment outcomes evaluation and prediction. They have been adopted to help in investigating cancer drugs efficacy and the resistance they experience along the pharmacological validation pathway [152], the impact of a drug on its proposed target and biochemical pathways [153]. Biomarker has proven to be highly valuable in beforehand validation of potent drug in the development pipeline, reduces the cost and enhance the market approval for the drug or therapy. But besides the prodigious importance and value [149,150], a limited number of biomarkers have been incorporated successfully in making clinical decisions in oncology [151-153]. In some cases, biomarkers that were initially considered to be promising have been shown to be unreliable or ineffective when used in clinical decision-making [154].

Many portentous biomarkers are confined only to academic literature and have not received clinical application; therefore, there is an utmost need for effective and standardized strategies that can be employed to validate and revalidate the biomarkers for clinical applications [153]. Latest updates about clinical trials can be obtained from the website (http://clinicalTrials.gov). A database that provides information about privately and publicly funded clinical studies conducted across the globe. The search to find the clinical studies were performed by typing "Cancer" in the condition/ disease field and miRNAs in other terms filed. Results showed 277 studies, out of which 69 were shown to be completed, 94 were recruited, 38 were active but not recruiting, 2 were suspended, 13 were terminated, 8 were withdrawn, 17 studies were not yet recruiting, 1 study enrolling by invitation, 35 were with unknown status (**Fig. 5**).

Biomarkers passing through clinical trials are rigorously evaluated for their intervention quality and overall impact on the survival of cancer patients [154]. The paucity of clinical trials and successful implementation can be linked to non-uniformity in miRNA studies, diverse number of sources, methods used for isolation and detection, data analysis, and last but not least, the suitability of rational for which miRNA is employed as biomarker [146]. Contemporary clinicians are advocate of using technologically advance diagnosis and treatment regimens such as the use of miRNAs for cancer. Modern era clinicians aim high to use miRNAs as a tool in precision medicines, diagnosis, and treatment [155]. MRX34, a miRNA mimic, has recently entered clinical trials for the treatment of tumors. This marks the first clinical trial to investigate the therapeutic use of a miRNA mimic in solid and hematological tumors (ClinicalTrials.gov Identifier: NCT01829971). In **Table 4** some of the clinical studies utilizing miRNAs either as a therapeutic or diagnostic entity are shown with their status and the type of cancer they are utilized for. It also highlights the potential intervention that the studies hold.

Conclusion

miRNAs are a promising candidate for cancer diagnosis, prognosis, and monitoring. Many studies reported discrepancies in miRNA measurement and recovery because of the stringency of standardized extraction methods. Therefore, it is pertinent to optimize extraction methodologies as per sample type and future application. It has been widely recommended that the procedure used for isolation in miRNA studies is vital for the results' precision and accuracy. It is vital to understand the diversified origin of miRNAs to harness them for clinically relevant diagnosis and treatment of cancer. From a large number of published papers, it has been clear that miRNAs hold great potential as a biomarker for a variety of cancers, but it has not yet been utilized up to its full potential [156]. Therefore, it is pertinent to scale up clinical studies. Scientists are encouraged to speed up the clinical trials and devise research strategies from the expanding knowledge base of

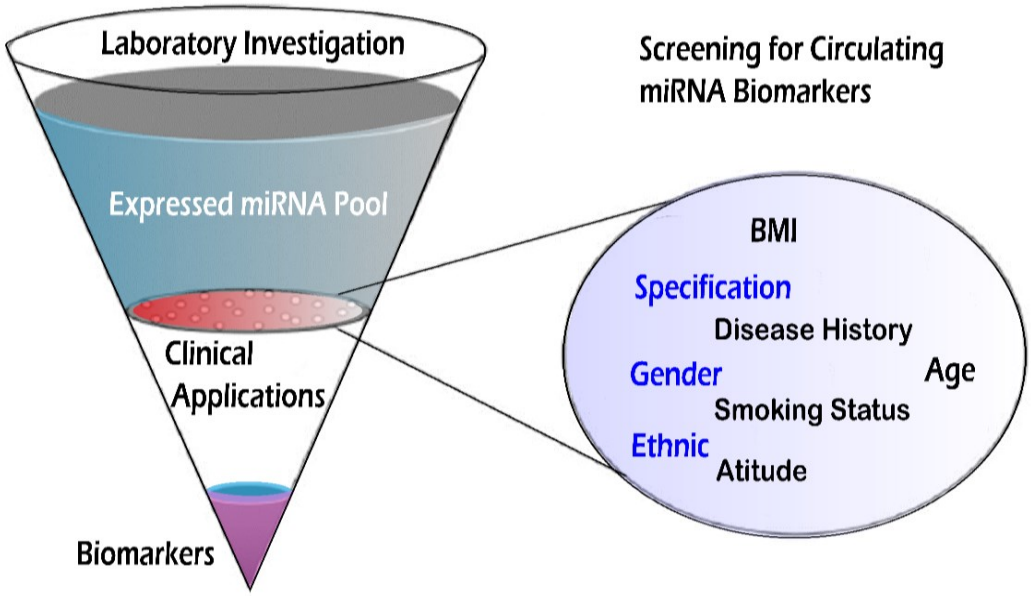

Fig. 4: The canonical process of miRNAs screening for a potential biomarker for cancer. Despite Prodigious laboratory research claims, very few miRNAs biomarkers have progressed to standard clinical application. Redrawn from reference [65].

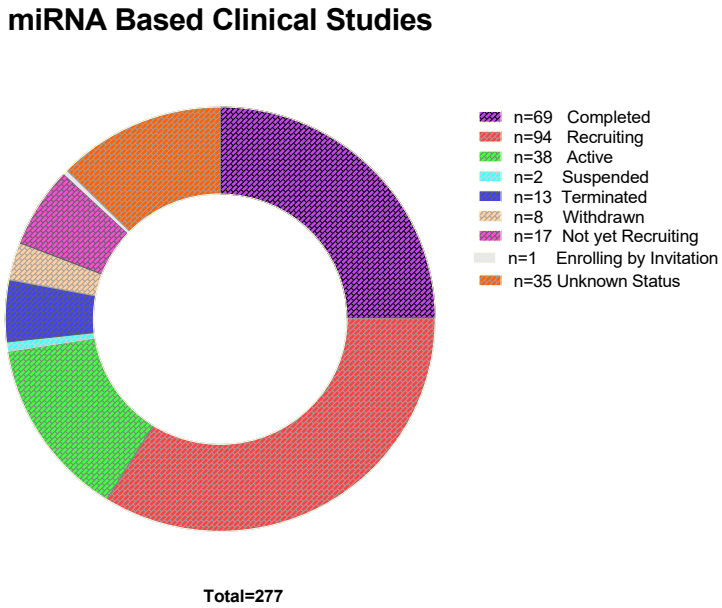

Fig. 4: Updated status of clinical studies of cancer-based on miRNAs (Source https://www.clinicaltrials.gov).

Table 3. The perks and shortfalls of circulatory miRNAs as a prospective cancer biomarker.

Perks	Shortfalls
High stability in circulation [148]	Lack of standardized protocols [148]
Abundance in various body fluids [149]	Variability in sample collection/storage [149]
Specific expression patterns [150]	Presence in healthy individuals [149]
Non-invasive detection [151]	Limited tissue specificity [148]
Potential for early diagnosis [150]	Need for further validation in large cohorts [150]
Correlation with cancer type [151]	Challenges in distinguishing between cancer and other diseases [151]

Table 4. Selected list of miRNAs biomarker used for different diseases.

miRNA Name	Disease Name	Biomarker	References
miR-135a	Gallbladder carcinoma	Yes	[156]
miR-21	Non-small cell lung cancer (NSCLC)	Yes	[157]
miR-17-5p	Head and neck cancer	Yes	[158]
miR-205-3p	Breast cancer	Yes	[159]
miR-221	Glioblastoma	Yes	[160]
miR-222	Glioblastoma	Yes	[161]
miR-124a	Colorectal cancer	Yes	[162]
miR-145	Colorectal cancer	Yes	[163]
miR-210	Hepatocellular carcinoma	Yes	[164]
miR-211	Hepatocellular carcinoma	Yes	[162]

[7]

miRNAs potentiality as cancer biomarkers in the form of publications. The limitations mentioned in the study dents the promising potential of miRNAs as cancer biomarkers but rapid technological advances in miRNA isolation methods from versatile sample types are proving vital. Studies that have successfully completed clinical trials can be of great help in planning of future research focusing on miRNAs biomarkers for cancer. The clinical trials of miRNAs may be limited, but the information available in the form of publications indicates an auspicious role of miRNAs in cancer diagnosis and therapies.

Authors contributions

All the authors have substantial contributions in writing, designing, conceptualization, and completing this review article.

Conflict of interest

The authors declare no conflict of interest.

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