

The Algorithms Cruz Rodriguez (CR) are Proposing A Novel Vaccine RNA-Peptide against Breast, Ovarian, and Lung Cancers Disease: Exosomes as Carrier in Cancer Progression and Metastasis

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ABSTRACT

Exosomes are a novel horizon in modern therapy and open exciting new opportunities for advanced drug transport and targeted release. Exosomes are biological nanoparticles with dimensions ranging from 30 to 100 nm and are generated by all cell types in the human body. Exosomes-which are extracellular vesicles that function in intercellular communication-may play a key role in the dissemination of vaccine as well as host-derived molecules during cancer disease. We highlight the composition and function of exosomes produced during cancer disease in our work, how these vesicles could function as carriers of the RNA-peptide molecule. Finally, according to the algorithms CRUZ RODRIGUEZ (CR) we are proposing various methods to develop a novel vaccine against breast, ovarian, and lung cancers. Therefore, six RNA-peptides from our previous study were chosen based on the potential of inducing strong fusion stability (FS=58.50 cruz) and exosome affinity (EA=59.80 ro), Biological Action (BA= 1.0223 ro/cruz). This result may lead to the development of promising new therapeutic approaches in cancer management by using exosome-mediated miRNA-peptide vaccine therapy. Due to, according to the algorithm CR the Optimal Biological Action (OBA) value for antitumoral RNA-peptide with Exosome as carrier are: $0.8 < OBA < 1.3$. These results suggest that the designed vaccine can elicit specific immune responses against three types of cancer; however, these results need experimental studies to confirm the efficacy and safety profile of the proposed vaccine structure.

Keywords

Exosomes, RNA-peptide, Fusion stability (FS), Exosome affinity (EA), Biological action (BA), Optimal Biological Action (OBA), Diagnosis, Vaccine, Drug carrier, Cancer therapy, Algorithms CRUZ RODRIGUEZ (CR)

Abbreviations

AFM: Atomic force microscopy; ALIX: ALG-2-interacting protein X; BBB: Blood brain barrier; CNS: Central nervous system; ddPCR: Droplet digital real time PCR; DGCR8: DiGeorge Syndrome Critical Region gene 8; DNA: Deoxyribonucleic acid;

dRT-PCR: Digital real time PCR; ESCRT: Endosomal sorting complexes required for transport; EVs: Extracellular vesicles; FGF2: Fibroblast growth factor 2; HIV: Human immunodeficiency virus; miRISC: miRNA-induced silencing complex; miRNA: Micro ribonucleic acid; mRNA: Messenger ribonucleic acid; MVBs: Multivesicular bodies; NTA: Nanoparticle tracking analysis; PCR: Polymerase chain reaction; PEG: Polyethylene glycol; Pre-miRN: Primary miRNA; Pri-miRNA: Primary miRNA; RNA: Ribonucleic acid; Onco-miRNAs: Oncogenic microRNAs; qRT-PCR: Quantitative real time PCR; PABP: Poly (A) binding protein; SEM: Scanning electron microscopy; STAT3: Signal transducers and activators of transcription 3; TEM: Transmission electron microscopy; TRBP2: Transactivating response RNA binding protein-2; TS: Tumor suppressor; TS-miRNAs: Tumor suppressive miRNAs; TSG101: Tumor susceptibility gene 101; VEGF: Vascular endothelial growth factor; UTR: Untranslated region; WB: Western blotting; XPO5: Exportin-5.

Introduction

Cancer is the world's second leading cause of death and a global health concern, with about 19.3 million new cases of cancer and almost 10.0 million deaths annually; according to the GLOBOCAN 2020 database, this is 17.0 percent of human deaths (lung:%18.0, breast:%7.0 and ovary: 2.1 of total cancer deaths) that occurred in 2020 [1].

It is estimated that about half of the worldwide population will develop invasive cancer at some stage during their lives [2]. Early stage of cancer diagnosis is important for a better prognosis, which can extend life expectancy and decrease the number of deaths from cancer [3,4]. Many forms of cancer can have an incubation period of about 20 years of before it is detectable by ultrasound, x-ray-based computed tomography (CT), endoscopy or other methods of detection, including tissue biopsy. However, rather than the traditional tissue biopsy, the novel highly sensitive detection technique, liquid biopsy-based exosome analysis, provides a promising platform for the early diagnosis, therapeutic and prognostic process of a disease. Due to their minimal invasiveness, painlessness, ease of use, lower sample volume, quick, cost-effective, more precision and high throughput for personalized cancer therapy, sampling of biological fluids (liquid biopsies), a new star in early cancer detection, is becoming increasingly common [5-7]. In this respect, it is important to realize that if they are detected at an early stage, most cancers can be treated more effectively.

Circulating exosomes released into all body fluids and carrying a unique collection of DNA fragments (originated from nucleus and mitochondria), a variety of RNA species, lipids and specific protein biomarkers and can function as cargo to transfer this information from donor cells to target cells, contributing to the recipient cells being reprogrammed.

In tumorigenesis, cell-to-cell communication and transformation are essential: single tumor cells have to communicate with each other and with host nontumor cells in order to promote the growth, survival, progress, enhance angiogenesis and metastasis of the

tumor [8]. It is becoming increasingly clear that exosomes derived from tumor cells play a crucial role in this phase of communication by transporting various biomolecules, including proteins, lipids and nucleic acids, which can be transferred from source cells to target cells in active form [9]. They are highly stable and resistant to degradation enzymes such as blood-derived ribonucleases and can retain their contents intact for a longer period of time than other materials such as liposomes and cells, this may allow the exosomes to exert their function at distant sites. Almost all tumor cells contain aberrant microRNAs (miRNAs) expression, that a main component of the small non-protein coding RNA family. Upregulated or downregulated small non-coding miRNAs specific to tumor cells are highly critical for development of cancer by altering oncogenes, tumor suppressor (TS) genes and hence cancer-related signal transduction pathways. In cancer cells, oncogenic microRNAs (onco-miRNAs) are aberrantly over-expressed and target the degradation of key tumor suppressor messenger RNA (TS-mRNA) to promote cell proliferation and tumor development. In contrast to those onco-miRNAs, the elevated tumor suppressive miRNAs (TS-miRNAs) expressions inhibit tumor development by inactivating oncogene expressions and hence are thus usually downregulated in the growth of cancer cells [11].

The biogenesis of exosomes

Almost all of the cells in our body release a various types of nano-sized lipid bilayer membrane vesicles into the extracellular environment, which are collectively called as extracellular vesicles (EVs) [1,12]. These EVs have been identified in different types of biological fluid, including breast milk, saliva, semen, nasal secretion, cerebrospinal fluid (CSF), lymph, tear, aqueous humor, amniotic fluid, urine and blood (Figure 1). The blood plasma and serum are the most accessible biological fluid along with urine and saliva for early detection, screening and monitoring treatment response in cancer [13-15].

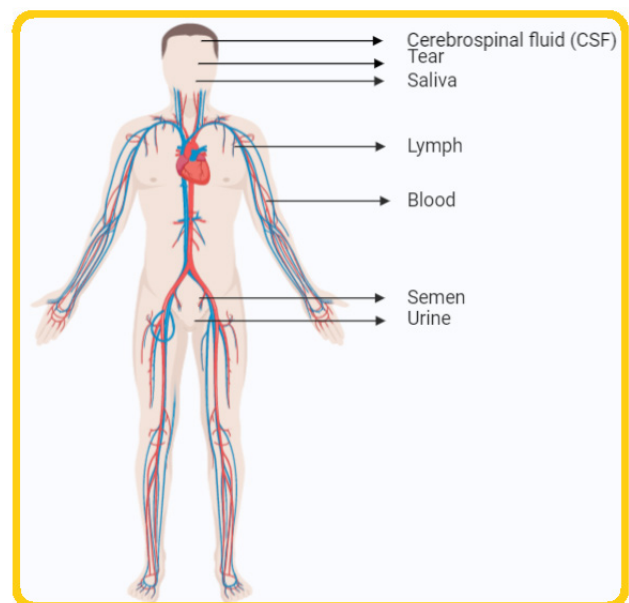


Figure 1: The various biological fluids.

The liquid biopsy has been recognized as a powerful real-time approach for the molecular monitoring of diseases. This method utilizes the detection of biomarkers in blood for prognostic and predictive purposes, which in the near future will represent a change in the paradigm of molecular biology understanding and the heterogeneity of diseases. It could establish a molecular profile that opens the door to a significant number of clinical applications that are the target of precision medicine, in addition to enabling it to be repeated as many times as necessary. It is important to know that most diseases can be more efficiently treated if they are detected in early stages. The key point is that specific analytical methods (explained in the analytical validation section) and cut-off values (explained in the clinical validation section) should be determined for each type of specimen (e.g. blood, urine, breast milk).

Based on their cellular origin, size and dimensions, mode of release, their contents, and functions, these cell membrane-derived EVs can be broadly divided into three distinct classes. They are referred to as exosomes (~30–100 nm), microvesicles (~100–1000 nm), and apoptotic bodies (~500–3000 nm) [10].

Exosomes were discovered initially by Pan and Johnstone in 1983 from developing mammalian reticulocytes [16]. Exosomes are of endosomal origin, unlike other cellular vesicles, such as apoptotic bodies (highly heterogeneous in size and composition) produced at the final stages of apoptosis from the plasma membrane of

programmed cell death, and microvesicles (ectosomes, shedding vesicles) are formed by the direct outward budding of the cell plasma membrane.

Exosome is a member of extracellular vesicle and it is a recent popular research object in tumor biology study. Structurally, exosomes are the smallest EVs, exhibit a cup-shaped structure when examined by cryoelectron microscopy (CEM) and are more homogeneous in shape than the other EVs (Figure 2). Exosomes are formed by a process involving the endocytic pathway (endocytosis) through the inward budding of intraluminal vesicles (ILVs) from the plasma membrane and are secreted by fusion of late endosomes or multivesicular bodies (MVBs) with the plasma membrane and released into the extracellular space (exocytosis) [17-24].

Exosome formation can be classified into three distinct stages: the formation of early plasma membrane endocytic vesicles (early-sorting endosome, ESE), the inward budding of the endosomal membrane forming intraluminal vesicles resulting in MVBs consisting of several ILVs, and the fusion of these MVBs with either cellular lysosome in which they are degraded (autophagosomes) mainly by hydrolysis, or the plasma membrane, that releases the vesicular contents, known as exosomes (late endosome) into the extracellular space (Figure 3) [25].

Exosomes are produced by virtually all normal and pathological cells and are found in all body fluids and *in vitro* grown cell lines.

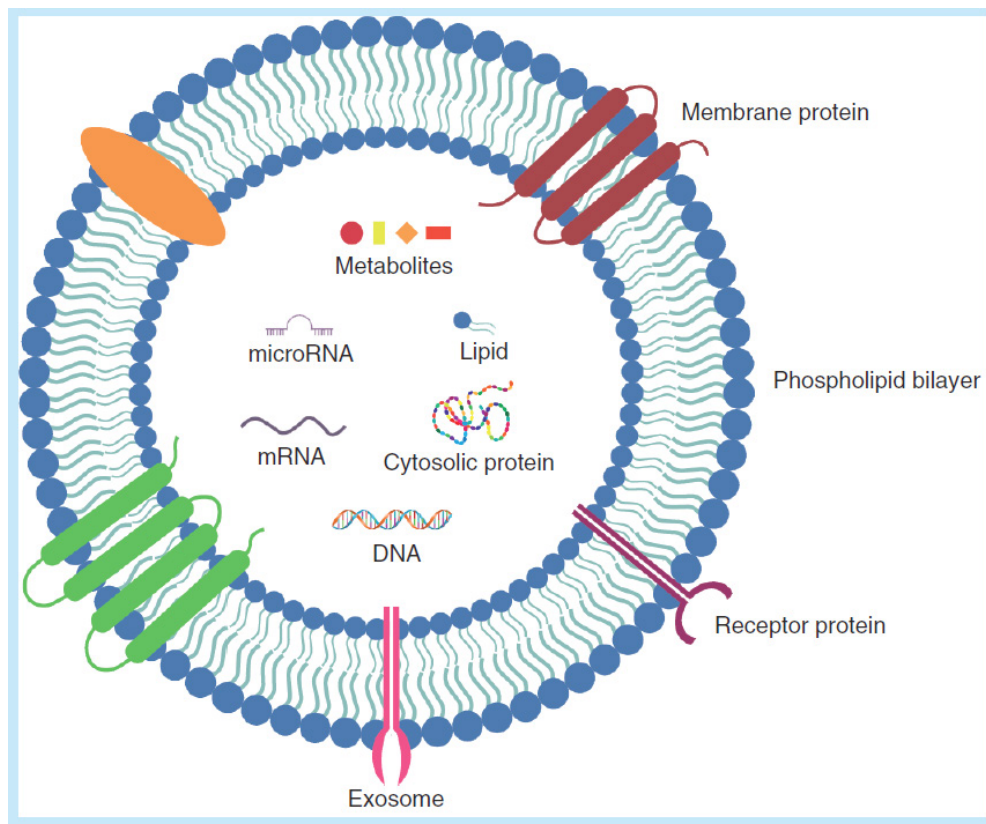


Figure 2: The structure of exosome. Exosomes contain various proteins, nucleic acids, lipids and metabolites [10].

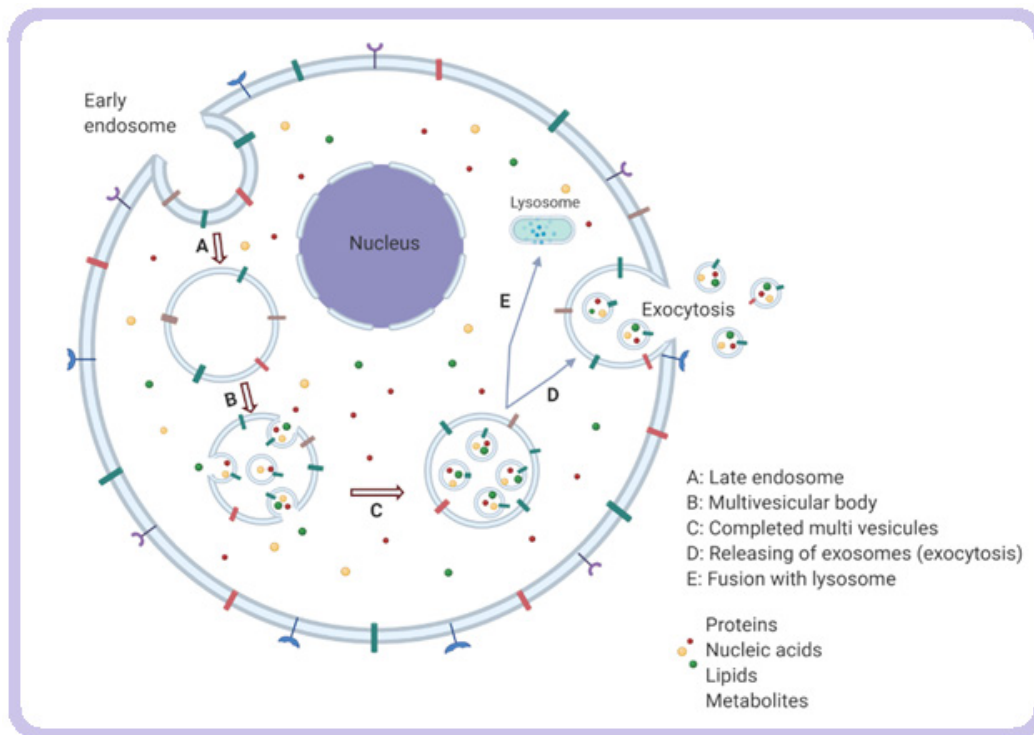


Figure 3: The formation of exosomes and release pathway. (A) The exosome is derived from early plasma membrane-formed endosomes. (B) Early endosome becomes late endosomes. (C) Then forms early multivesicular bodies. (D) Late multivesicular bodies. (E) In order to release exosomes, late multivesicular bodies may either be degraded by lysosomes or fuse with the membrane.

Intercellular communication between tumor cells and with their neighboring non-tumor cells and distant organs is crucial for the survival pathway, proliferation, metastasis and drug resistance. A growing body of evidence indicates that exosomes play a critical role in this cell-cell communication process [10].

Exosomes are biological nanoparticles formed by almost all cell types in the human body with an average diameter of between 30 and 100 nm in size. [26,27]. They are released into all kinds of body fluids contain DNA, RNA species and specific protein biomarkers that are important as biomarkers to anticancer therapy.

The thousands of exosomes are released by a single parent cell in a day. While in response to pathological conditions, exosomes are found to be secreted in high numbers by tumor cells compared to normal cells. In fact, exosomes are admirably equipped to serve as communication vehicles and their surface is decorated by the parent cell-derived signaling molecules [28-30].

Cytoplasmic biomolecules, including proteins, nucleic acids and lipids, are trapped within lumens during exosome biogenesis. The cargo molecules of exosomes are sorted and loaded either through endosomal sorting complexes required for the transport system (**ESCRT**)-dependent in cooperation with apoptosis-linked gene 2-interacting protein X (ALIX) and tumor susceptibility gene 101 protein (TSG101) or through **ESCRT-independent** (with tetraspanins proteins and lipids particularly sphingosine-1-phosphate and ceramide dependent) mechanisms [31-33].

Tetraspanins, which are transmembrane proteins that can directly induce the membrane curvatures enabling exosome formation. The lipid-modifying enzymes such as sphingomyelinase, which generates ceramides at the cell surface that promote endocytic pathway of exosome formation [34]. In addition to regulating exosome release and exchange, ESCRT proteins at the membrane of MVBs are thought to be involved in packaging and sorting of biomolecules into exosomes [35]. Exosomes are heterogen structures with their size range, content, biomarkers, functional impact on recipient cells (inducing cell survival, apoptosis or immunomodulation), and cellular origin based on the tissue or organ.

Exosomes, membrane-bound vesicles are released by both healthy and cancerous cells into the extracellular space and all body fluids. However, cancer cells can produce about tenfold more exosomes than healthy cells. Typically, exosome uptake with recipient cells usually is made up the processes called endocytosis that can be assessed by using advanced equipment such as flow cytometry or confocal microscopy. Endocytosis is an umbrella term for a variety of molecular internalization pathways for the uptake of cell-derived exosomes [36]. Tumor cells-derived exosomes are released into their surrounding extracellular space, and may contribute the initiation of tumor progression, immune response suppression, neovascularization, metastasis and drug resistance [37].

Exosomes are valuable sources of tumor biomarkers in researches, as their contents are a wealth of information on the state of the

cells which they originated and function in biological processes and they are released in all biological fluids, such as blood, tear, amniotic fluid, urine and saliva (Figure 4). Exosomes are also recognized as critical regulators of intercellular communication by transferring their contents (Figure 5).

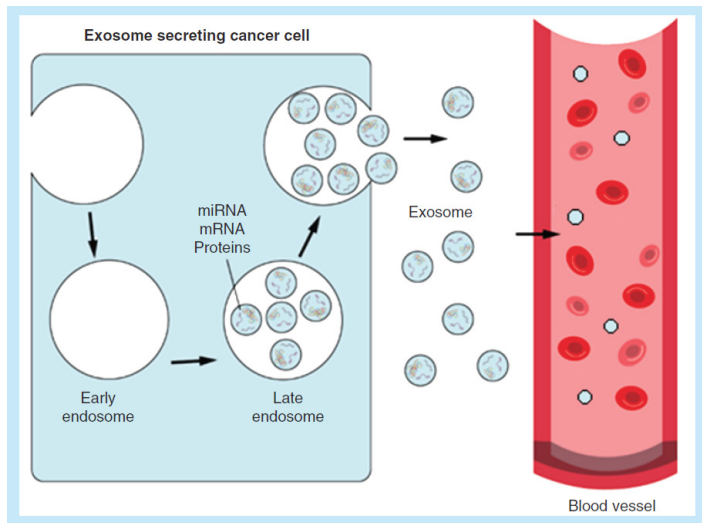


Figure 4: Biogenesis of exosomes and sorting into the blood. Exosomes may either be fused for degradation with lysosomes or with the plasma membrane for releasing exosomes into the extracellular space. Exosomes that are released from cells will then be taken up by neighboring recipient cells or travelled through biological fluids such as blood, urine or saliva [10].

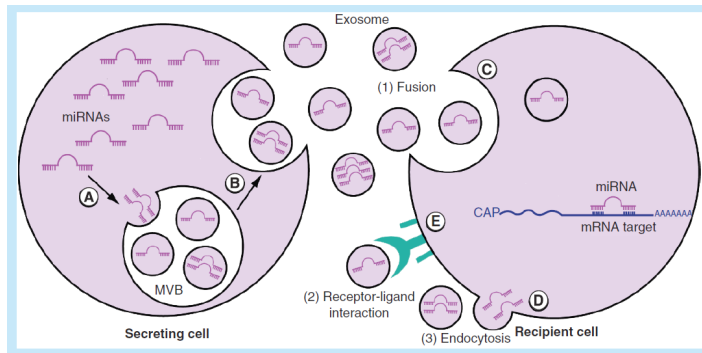


Figure 5: Transfer of exosomal miRNA from the donor cell into the recipient cells. (A) During MVB formation, miRNAs are sorted to exosomes. (B) Exosomes are then released into the extracellular space. (C) Exosomal miRNAs can be delivered to recipient cells with endocytosis. (D) Receptor-mediated endocytosis is primarily used to fuse exosomes with the plasma membrane using soluble N-ethylmaleimide sensitive fusion into cells. (E) Exosomes may also bind to a receptor and activate specific signaling pathways. MVB: Multivesicular body [10].

Exosomes represent a hot area of research with many promising results in fighting disease with agents derived from abnormal cells. Indeed, many studies demonstrated that exosomal contents, including DNA (both originated from nucleus and mitochondria), various RNA species (coding RNAs and non-coding RNAs) and cytosolic and cell surface proteins that are important as cancer predictive biomarkers for early cancer diagnosis and determining

the prognosis of cancer patients. Exosome contents not only mirror the composition of the donor cell but also reflect a regulated sorting mechanism. These bioactive molecules are transferred from donor cells to target cells by exosome transport system, leading to reprogramming of the recipient cells. Therefore, the specific exosomes secreted by tumor cells that contain the biomarkers can be used to predict the existence of a *tumor* in patients.

They are an excellent delivery system for anti-disease miRNAs in therapeutic instruments because of their small scale, natural products of the body cells (non-immunogenic), non-toxic characteristics and crossing the various biological barriers [10,14,27,38]. A total of 40,000 exosome-related papers were reported using Google Scholar in the study period from 2000 to 2020.

Biological role of exosomes in cancer progression and metastasis

Exosomes play a fundamental biological function in cell-to-cell communication locally and between organs, causing physiological changes in recipient cells by transferring their cargo and have been involved as key mediators in many diseases such as cancer, neurological diseases, cardiovascular diseases, autoimmune diseases among many others [2,39]. Tumor cell-released exosomes cause changes in their recipient cells, thereby playing a key role in promoting primary tumor formation, angiogenesis and metastasis induction, stromal fibroblast activation, cancer extracellular matrix adhesion sculpting, host immune response suppression, cell death resistance and drug-resistance development [20,33,40]. Studies have shown that, chemotherapy and radiotherapy induce the secretion of exosomes from cancer cells which have effects on surrounding cells [35,41-42].

Exosomes are composed of various proteins, such as ESCRT (ESCRT 0, I, II and III; which are required for transport), tetraspanins (transmembrane proteins induce vesicle formation), Rab GTPases (RAS-related RAB family proteins: Rab2/Rab7/Rab9/Rab11/Rab27/Rab35; which are essential for the release of exosomes, tumor growth and metastasis), heatshock proteins (HSP20/HSP60/HSP70/HSP90) and transformation growth factor beta (TGF β). On the other hand, selecting, binding and uptake of exosomes to the surface of recipient cells is mediated by different proteins such as tetraspanin family proteins (proteins with four transmembrane domains, TSPAN: CD9, CD37, CD49, CD53, CD63, CD81 and CD82), immunoglobulins, proteoglycans, lectins and intercellular adhesion molecules (e.g., integrins with alpha subunits [ITGA], and with beta subunits [ITGB]) on the exosome and the target cell surface. These attachments can then mediate transferring of exosomal cargo molecules into the recipient cell at new locations, and subsequently altering their metabolism (Table 1) [11,20,43-45]. Exosomes may also involve in transporting of infectious agents such as bacteria, viruses, fungi and parasites from infected cells to the healthy cells.

Exosomes may serve as carriers to transport oncogenic proteins and nucleic acids mainly onco-miRNAs from the donor tumor cell

to normal target recipient cells at a distance from the originating cell. These horizontal molecular transfers of exosomal factors can modulate specific cell signaling pathways in recipient cells to regulate processes such as gene expression, development, immune response and pathological state of the body. The creation of new capillary blood vessel formation (angiogenesis) to provide blood supply is an essential part of preparing a site for future colonization by cancer cells (Figure 6). In order to do this, cancer cells secrete exosomes that function in different ways to induce neoangiogenesis in their pre-metastatic niche, and facilitate tumor cell migration [11]. Exosomal oncogenic miRNAs have been implicated in the angiogenic and extracellular matrix remodeling of the tumor microenvironment, such as miR-105 from breast cancer cell-derived exosomes suppresses endothelial tight junction zonular occludens 1 (ZO-1) expression, contributing to increased metastasis by impairing blood vessel integrity and enhancing vascular permeability [33,46].

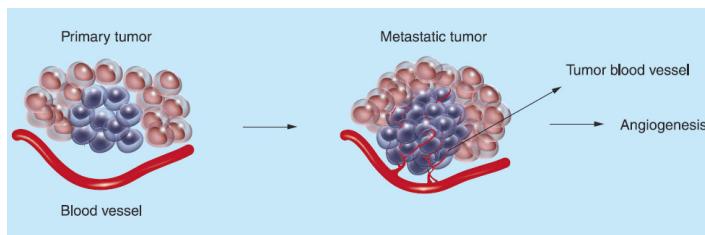


Figure 6: Primary tumor formation and angiogenesis. (A) Cell proliferate to form primary tumor formation (without blood vessels). (B) Tumor mass increases and generates angiogenic factors that promote the development of new blood vessels from the main blood vessel to the cells of the tumor. [10].

Exosomal miRNAs such as miRNAs such as miR-9, miR-23a, miR-92a, miR-103, miR-105, miR-126, miR-132 miR-135b, miR-155, miR-210, miR-221 and cytokines [(e.g., interleukins: IL-6 and IL-8, TNF- α , transforming growth factor β , fibroblast growth factor 2 (FGF2), and vascular endothelial growth factor (VEGF)] have been shown to be proangiogenic factors to promote neovascularization and metastasis. [46-53]. For instance, exosomal miR-9 secreted by tumor cells promotes the pathway of Janus kinase/signal transducers and transcription activators by cytokine signaling 5 suppressor levels to promote tumor angiogenesis [54]. Additional work showed that overexpression of miR-23a and miR-105 induces vascular leakiness and promoting metastasis [47, 55]. In cancer patients, metastasis is the primary cause of mortality, responsible for more than 90% of all cancer-associated deaths [2,56]. Metastasis is an incredibly complicated process in which cancer cells originating from a primary malignant tumor and colonize in distant organs in the body, producing secondary tumors at the new tissue sites. Various studies have indicated that tumor cell-derived exosomes play a critical role in tumor metastases pathology by utilizing tumor-signaling pathways such as caveolin-1, HIF-1 α , miR-21, miR-105, miR-148b, miR-210, β -catenin and oncogenic kinases (e.g., mutated EGFR, RAS and MAP kinases) [2,57-61].

Exosome isolation methods

High-purity of exosome is necessary for the successful use of exosomes as a source of biomarker detection in liquid biopsies [37]. Therefore, the choice of the appropriate technique for separation and isolation is important. Despite significant effort into this relatively new field of research, exosomes are extremely difficult to identify, separate and purify from other components in the blood plasma due to their nano-sized with diameters between 30 and 100 nm and low density, and require considerable time and effort. In order to achieve high quality in exosome preparations, two crucial points need to be controlled: the adequate collection/storage of body fluid samples (such as fluid source, preparation conditions and storage temperature) and the high-purity/high-yield of the isolated exosomes.

Blood is an obvious source for the collection of miRNAs as its collection is minimally invasive and can be taken routinely in the clinics for monitoring and treatment of cancer. For exosomal microRNAs (exo-miRNAs) study, plasma is preferred in blood because the preparation of plasma is less complicated and slightly simpler than that of serum, because serum contains a high number of platelet-released vesicles in response to coagulation [62,63]. In a recent study of exo-miRNAs the expression profile of 312 human blood plasma and serum samples obtained from 13 healthy volunteers indicated that exo-miRNAs were 78% of the total plasma RNAs and 53% of the total serum RNAs [64].

Plasma is a yellow liquid portion of blood consists of water (~ 92%) with many substances dissolved in it such as: ions, salts, gases, proteins, nutrients and metabolites, and acts as the extracellular matrix (ECM) of blood cells. Blood plasma constitutes approximately 55% of the body's total blood volume. For exosome isolation, blood samples (~3.0 ml) of patients with cancer and healthy controls collected in potassium EDTA-coated anti-coagulant tubes should be processed within 30 min after collection. Heparin-coated tubes should be avoided for blood collection as it is associated with false-negative PCR amplifications, due to inhibition of the polymerase enzyme in the reaction. Whole blood samples are coolly centrifuged at $1500 \times g$ for about 10 minutes to remove dead cells and then at $10,000 \times g$ for about 10 minutes to remove the cellular debris and nonexosomal vesicles (such as apoptotic bodies and microvesicles). Separated plasma sample aliquots should be used immediately, or stored at approximately -80°C until use.

Different conventional protocols have been developed and implemented in recent years to isolate and purify exosomes from various types of body fluids and *in vitro* cell culture media: ultracentrifugation-based technique at $100,000 \times g$ [42,65], nanomembrane concentrator-based approach [66], immunoaffinity-based capture using monoclonal antibody-coupled nanobeads [67,68], density gradient separation using sucrose or Percoll [69], alternating current electrokinetic microarray chip technology (ACE) [3,70], nanowire-anchored microfluidic platforms (immune-isolation in microfluidics, filtration in microfluidics

and contact-free mode) [71-73] and utilizing a commercially available synthetic polymer-based precipitation reagents (Figure 7) [74,75]. Each of these various techniques has their own benefits and limitations for isolation and purification of exosome from various biological liquid samples. Ultracentrifugation is currently the most widely used (> 80%) method for exosome isolation. Another commonly used method is polymer-based precipitation [such as ExoQuick (System Biosciences), Exo-Spin (Cell Guidance Systems) and Total Exosome Isolation kits (Invitrogen)] that is a relatively simple, rapid and cost-effective to separate exosomes with a high yield from liquid biopsy or cell culture medium. In this method, the polymer such as polyethylene glycol (PEG) solution precipitate the exosomes at low speed centrifugation with a high recovery efficiency.

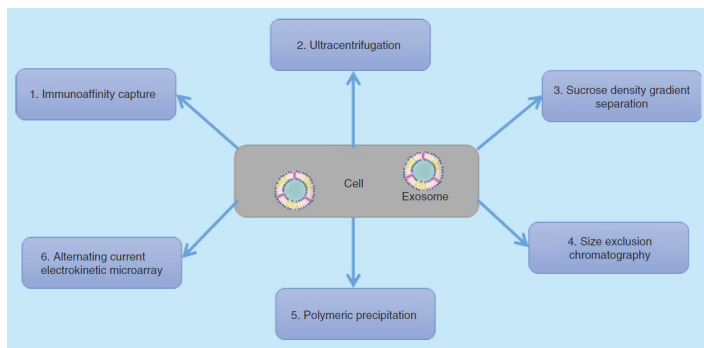


Figure 7: Overview of the various methods of exosome isolation and purification [10].

Overall, the preanalytical steps such as sample collection, storage temperature, freeze-thaw cycles, exosome purity, concentration and processing time are important for the efficient and reliable method for the analysis of exosomes. Meanwhile, patient factors such as sex, age, smoking habits, diet, different medical treatments and physical activity may influence the miRNAs expression level in sample of selected patient and control groups.

While significant advances have been used to isolate and analyze exo-miRNA to date, a fast, sensitive and cost-effective gold standard technique remains required to generate effective, pure isolation, identification, high yield extraction and accurate quantification for research of exo-miRNA from body fluids (<0.01%) [76].

Physical characterization and molecular analysis techniques for exosome

Because of their small size with an average diameter of ~70 nanometers, the accurate characterization and quantification of exosomes is still technically challenging. Many innovative approaches have been developed and applied in recent years to overcome these challenges [77]. One of the best methods used for exosome size estimation and quantification is nanoparticle tracking analysis (NTA, NanoSight). The most widely used physical characterization methods are microscopy based methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), cryoelectron microscopy (cryo-

EM) and atomic force microscopy (AFM) [78,79]; dynamic light scattering (DLS) [80,81]; nanoparticle tracking analysis (NTA) [80-82]; tunable resistive pulse sensing (TRPS) [83]; and single EVs analysis [84].

The molecular methods used to analyze the concentration, quantity and profile of the exosome content are quantitative real time PCR (qRT-PCR) [61], digital PCR (chip-based dPCR, droplet digital PCR, ddPCR) [77,85], western blotting (WB), whole genome sequencing (next-generation sequencing) [86], exome-targeted sequencing (next-generation sequencing) [86], microarray profile [87] and ELISA [88]. For the quantification of miRNA expression housekeeping genes such as RNU6 or RNU48 which are highly abundant and essential can be used as control in qRT-PCR analysis (Figure 8).

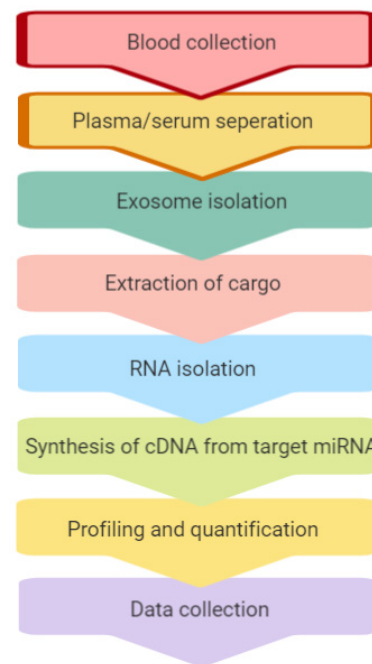


Figure 8: A schematic diagram depicting a viable workflow for the use of blood exosome-based biomarkers.

Exosome-derived miRNA biogenesis and function

A highly sensitive biomarker assay based on miRNA in biological fluids could detect the presence of the tumor at the earliest possible stage (0-1). Study on circulating miRNA is a novel expanding field of molecular tumor biomarker research in addition to conventional tissue biopsy-based current studies. Because they possess all characteristics (miRNA profiling, diagnosis, prognosis, therapy response and predictive biomarkers), are detectable in liquid biopsy. Body fluid such as blood sample, enables physicians and researchers to detect the development of cancer in the very earliest stage.

miRNAs encapsulated by exosomes are remarkably stable compared to free RNAs in circulation because exosomes can protect miRNA against RNase-mediated degradation or under

nonphysiological conditions (multiple freeze-thaw cycles, long-term storage and extreme pH) [89,90]. It has been reported that exosomally derived miRNA has been demonstrated to remain stable at -20 °C for up to 5 years and is resistant to freeze-thaw cycles [20,60,91-93]. Compared to non-tumor-derived liquid biopsy, tumor-derived liquid biopsy contained a higher level of exosome related miRNA, indicating potentially altered molecule profiles of exosomal contents in tumor cells that may be involved in tumor-associated biological processes. For example, the expression of onco-miRNA-21 and onco-miRNA-155 are upregulated up to 100 fold in some types of cancer when compared to the normal tissues. Defects in the miRNA biogenesis pathway could be related to tumorigenesis. miRNAs have been extensively involved in the pathogenesis of many human diseases including cancer, and have also been shown to be taken up as cargo in exosomes by either distal or neighboring recipient cells as a method of cell-cell communication to potentially influence the tumor pathogenesis [77,94-98].

miRNA holds great promise as a novel nucleic acid-based therapeutic against a wide variety of diseases, including cancer, infectious diseases and genetic disorders. miRNAs are a kind of small non-coding RNA molecules with an average of 20 nucleotides in length, regulates most of protein-coding genes' expression at the post-translational level [10]. miRNAs inhibit downstream targets' expression through RNA interference and silencing. In animal cells, 60% of the protein synthesis is controlled by miRNA (miRNA inhibiting target mRNA) [99]. Due to the specificity of various cells, different groups of miRNAs are expressed in different cells types and tissues in order to produce specific sets of proteins. Nowadays, 2,838 of human mature miRNAs have been identified, each of which are estimated to control hundreds of genes [100,101, ExoCarta: www.exocarta.org, 2021]. The classical mechanism of miRNAs to regulate their target gene is by binding mainly to the 3' untranslated region (3' UTR) of target mRNA to exert negative regulatory effects (silence) on gene expression.

Furthermore, dysregulation of miRNAs contributes to the development and progression of human malignancies, including lung and breast cancer. miRNAs are promising candidate biomarkers of liquid biopsy of clinical samples due to their cancer-specific expression profiles and essential roles in cancer initiation and progression. In addition, miRNA expression profiles offer molecular signatures for the tumor classification, early diagnosis, prognosis and progression of cancer, and thus could be developed as cancer biomarkers [102].

Currently, miRNAs are known as fundamental regulator of gene expression at the post-transcriptional level particularly in cancer cell, and play an essential role in tumorigenesis, metastasis and resistance to chemo- or radio-therapies. Using PubMed, over 80,000 articles were found relevant to miRNAs in the title or keyword [100]. It has been reported that there are about 100,000 endogenous miRNA molecules per cell in a mammalian cell [103,104]. It has also been estimated that a single exosome can carry up to approximately 500 copies of miRNAs [63]. In normal

human blood, the amount of exosomes has been reported as around 10^9 exosomes/ml in cancer patients [105,106].

miRNAs are a major class of small, conserved and single-stranded non-coding RNA molecules with a length of 18-22 nucleotides (NTs) in their mature form, which play significant roles in virtually all biological pathways including the controlling of gene expression, cell growth, proliferation, differentiation, immunity response, apoptosis, metabolism, tumorigenesis and progression. miRNA genes can be located in varying genomic contexts, which include intergenic (located in between protein coding genes) and intragenic (located in protein coding genes) as short noncoding RNA regions where they are transcribed by the promoter of the host gene. miRNAs are transcribed from the corresponding miRNA genes containing their own promoters (transcribed independently) or intragenically from spliced portions of protein-coding genes (transcribed dependently to the promoter of a host gene) in human [107].

The transcription process is made by RNA polymerase II (Pol II), generating long noncoding primary miRNAs (pri-miRNAs, 1-3 kilobases) in the nucleus. The pri-miRNAs contain one or more miRNAs and are 5'-end methyl capped (7MGpppG) and 3-polyadenylated (~ 200 Adenines) tail. These pri-miRNAs are further processed by the nuclear RNase III endonuclease (DROSHA) together with the double-stranded RNA-binding proteins, such as DiGeorge Syndrome Critical Region gene 8 (DGCR8), then, leading to primary miRNAs (pre-miRNA, around ≈ 70 NTs in length), which fold into imperfect stem-loop structures. Then the pre-miRNAs are exported from nucleus to the cytoplasm through the nuclear pore complex by nuclear export factor, Exportin-5 (XPO5), together with the guanosine-5'-triphosphate Ras-related GTP-binding nuclear protein (RAN). There, a complex of DICER-PACT-TRBP2 formed by ribonuclease (RNase) III enzyme DICER, protein activator of the interferon induced protein kinase (PACT) and human immunodeficiency virus (HIV) transactivating response RNA binding protein-2 (TRBP2), cleaves the stem-loop of the double-stranded pre-miRNA. Then, this double-stranded miRNA referred to as the miRNA: miRNA duplex is separated into the mature single stranded miRNAs (18-22 NTs) prior to their loading into Argonaute complex. Next, a miRNA-induced silencing complex (miRISC) is formed with a number of proteins; Argonaute 2 (AGO2) protein, glycine (G)-tryptophan (W) repeat containing protein of 182 kDa (GW182), poly (A) binding protein (PABP), CCR4-NOT and poly(A) nucleases 2 and 3 (PAN2-PAN3) proteins [10,108]. Then, the miRISC complex helps to navigate the mature single miRNAs (18-22 NTs) to the target mRNA with base pairing, consequently resulting by translational repression or by degradation of the target mRNA. miRNAs can also serve as positive activators of target gene expression after binding to the promoter region. Generally, one of the miRNA: miRNA duplex take action on the target mRNA, but occasionally both strands may take action (Figure 9).

At the post-transcriptional level, intracellular miRNAs are involved in the gene expression regulation, functioning as negative regulators of mRNA translation by binding to its complementary base pair

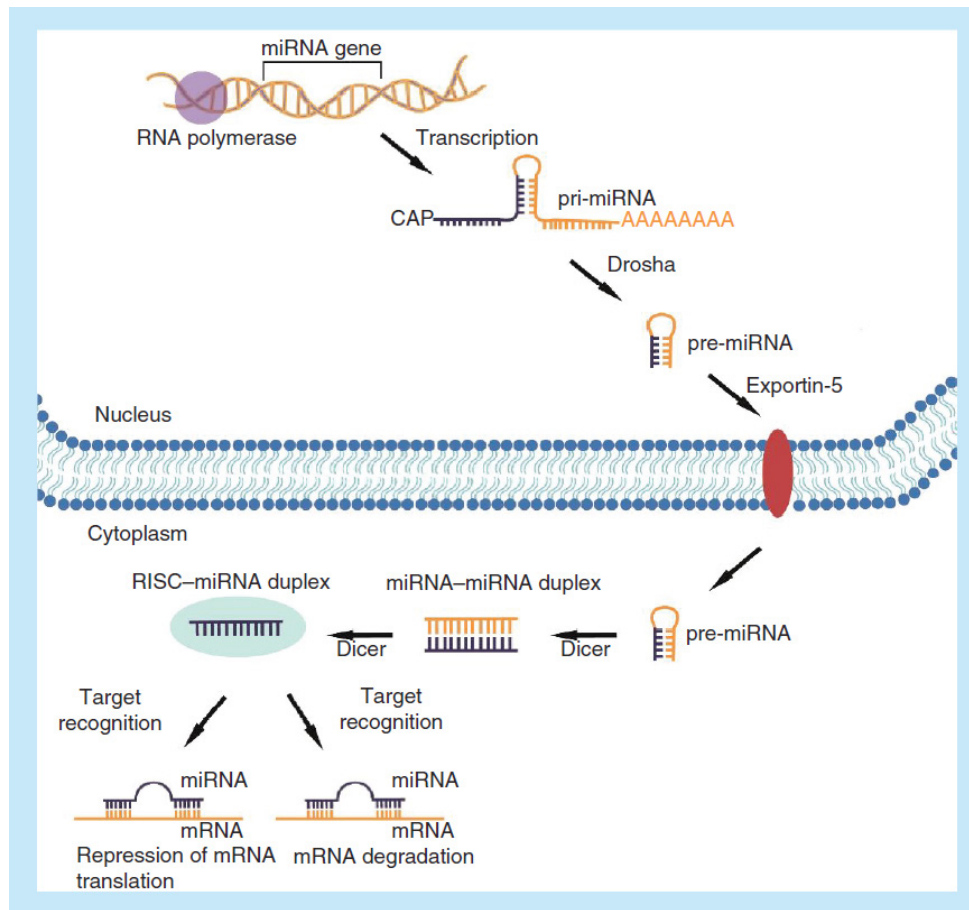


Figure 9: The biogenesis of microRNA. In the nucleus, noncoding genes of miRNAs are transcribed into primary miRNAs that are further processed into primary miRNA and then exported to the cytoplasm where they are finally converted into their mature forms. Mature miRNA then binds with base pairing to its target mRNA, acting as negative regulators of mRNA translation (either mRNA degradation or inhibition of protein expression from mRNA) [10].

sequences (usually around 6–8 NTs), mainly through binding 3'-untranslated (UTR) region or occasionally 5'-untranslated regions of their target mRNA molecules [10,25,109-112]. The binding of miRNAs-miRISC complex to their target mRNAs mainly leads to the mRNA degradation as Argonaute protein is an RNase it catalyzes the cleavage of the mRNA or GW182 blocks translation initiation by preventing ribosomal complex formation that cause translational repression of that mRNA at the post-transcriptional level [10,96,108,113].

It is estimated that miRNAs are involved in regulating about 60% of all protein-coding genes expression in human, primarily by inhibiting the translation of a considerable number of mRNAs. Many distinct miRNAs are often required among the miRNA-mRNA regulatory relationships to act cooperatively to target a single mRNA. On the other hand, the computational and experimental data indicate that a single miRNA can also affect the expression level of more than 100 mRNAs by targeting a transcription factor [114-116]. Recent evidence has supported that miRNAs play crucial roles in the pathophysiology of many perhaps all human cancers. Depending on the target mRNA, miRNAs can function either as tumor-suppressor miRNAs or as onco miRNAs and play a key role in tumor development, progression, drug resistance

and metastasis. Many oncogenic miRNAs are responsible for maintaining a high cell proliferation rate, metastasis and activating oncogenic transcription factors, which have been reported to be aberrantly expressed in different cancer cells [117,118].

Cell-released exosomes can be regarded as delivery vehicles for transferring miRNAs to target recipient cells. Exosomes protect miRNAs from shear stress enzymes and degradation, enabling them to be more stable within exosome than free miRNAs and to be efficiently integrated by individual recipient cells [119]. A growing body of evidence shows that exosomes derived from tumor cells have become a key candidate for promoting tumor cell proliferation, invasion, angiogenesis, distant metastasis and remodeling of the tumor microenvironment through transmitting onco-miRNAs [120]. New growth in the vascular networks (angiogenesis) is essential for malignant tumor growth and metastasis because new blood vessels provide extra oxygen and nutrients to promote tumor growth and also to remove waste products [121].

Cancer cell-released exosomal-miR-21, exo-miR-23; exo-miR-29; exo-miR-103 and exo-miR-210 have been shown facilitate proliferation, angiogenesis and migration of tumors

[115,116,120,122-126]. Increasing evidence reveals that exo-miR-21 may be a promising biomarker for many types cancer including lung, breast, bladder and prostate cancer. In recipient cells, exosomal onco-miR-21 can lead to activation of signal transducer and activators of transcription 3 (STAT3), which increases cellular vascular endothelial growth factor (VEGF) receptor levels, indicating that miR-21 plays a critical role in angiogenesis and malignant lung cancer transformation. The overexpressed onco-miR-21 exerts its promoting effect on the anti-apoptotic protein Bcl-2 and tumor suppressor protein p53, and its increased concentration is positively associated with metastasis and poor prognosis of breast and lung cancer patients [127]. Pakravan *et al.* reported that overexpression of exo-miR-10 and exo-miR-100 promote suppression of angiogenesis and downregulation of VEGF in human breast cancer cell model [128,129]. Cancer cells secreted exosomes could promote chemotherapy resistance in a wide range of human tumors [130].

Consequently, tumor-derived exosomes play a pivotal role in the progression cancer; exosomal miRNAs in particular, help regulate the transcriptome pool of recipient cells. It seems that discovery in the field of exosomal miRNAs biology could uncover the underlying mechanisms promoting the aggressive feature of tumors [120]. However, despite our increasing awareness about their role, further studies are needed to gain a better understanding of the role of exosomal miRNAs as biomarkers in carcinogenesis and cancer progression.

Clinical applications of exosomes in cancer

Cancer is one of the leading causes of worldwide death and morbidity. Many types of cancer therapies, such as chemotherapy, surgery, radiotherapy, and immunotherapy, have been developed. These treatments, however, can also kill healthy cells and contribute to serious side effects. Therefore, there is an urgent need to establish new therapeutic approaches to precisely eliminate cancerous cells [131].

Due to their important natural roles in various cellular processes, combined with high stability, tissue cell specific expression pattern and secretion into all biological fluids, numerous studies over the past ten years have shown that exosomal miRNAs represent a very promising new therapeutic approach to human cancer treatment [8,20,96,132]. Considering these findings, during the transformation of normal cells into malignant tumors, exo-miRNAs play a very important role [96]. Recent studies have indicated circulating exo-miRNAs as potential diagnostic and prognostic biomarkers in therapeutic monitoring for recurrence of cancers [133]. Inhibition of the expression of onco-miRNAs and their activity by delivery of antagonist tumor-suppressive miRNAs for the treatment of cancer is one of the novel exosome therapeutic strategies. Exosomes loaded with therapeutic anti-miRNA oligonucleotides complementary to the sequence of the targeted mature oncogenic miRNAs can be delivered either systemically or directly by local injection into the tumor tissue. Blocking the fusion or uptake of exosomes with target cells via receptors to inhibit tumorigenesis is another therapeutic strategy. Exosome can

be isolated from a patient's fluids and after modification; it can be transferred back to the same patient with its cargo for targeted tumor therapy [52,134-135]. The native exosomes purified from cell media can be loaded with therapeutic cargos (miRNAs or drugs) and then delivered by the free diffusion, sonication, incubation or electroporation technique [136].

More studies have demonstrated that TS-miRNA-loaded exosomes can be used against proangiogenic mRNAs to prevent tumor angiogenesis. The exosomal miRNAs effectively engage target mRNA and suppress gene expression in recipient cells, engineering of exosomes to deliver a specific miRNA payload has been developed for the central nervous system (CNS) diseases and cancer [33]. Exosomes can also be used in genetic therapy, which allows the delivery of desired therapeutic genetic materials to target cells in for the purpose of clinical gene therapies [11,25]. Furthermore, exosome-delivered some miRNAs can be considered perfect candidates in using specific gene knockdown, enabling it to play a silencing role in gene expression to inhibit tumor growth.

Exosomes can not only represent potential biomarkers in medicine, but also a very valuable and effective 'nanovector' as transport vehicles for the delivering of the anticancer drugs to the target tissues with low immunogenicity and toxicity in cancer therapy relative to other drug delivery vehicles [7,137]. Since exosomes are small, non-toxic, non-immunogenic and native to human since their membrane composition is similar to the body's cells with a long life span in circulating system and it can be used as therapeutic drug delivery vehicle to the target cells.

In addition, targeting efficient drug-loaded exosome-based vehicles can cross various biological barriers, such as the blood-brain barrier (BBB), enabling targeted delivery of neuropharmacological agents into the brain effectively. For this purpose, bovine milk-isolated exosomes have been loaded with anti-cancer agent withaferin A and used against breast and lung cancer in mice models. In contrast to the free drug, the study reported a significant higher efficacy of the drug loaded into the exosome [138]. Engineering designs allow the loading of exosomes with miRNAs, siRNAs, genes, antisense oligonucleotides, chemotherapeutic agents, immune modulators, peptides, antioxidants, and ligands, among other strategies to target delivery in cancer [139]. Enrichment of exosomes on the basis of their surface ligand presentation may also enable the development of receptor proteins that enable binding of exosomes to target tissue cells. Ligand enrichment on engineered exosomes may also be used to induce or inhibit signaling events in recipient cells or to target exosomes to specific cell types [140].

Several clinical trials are currently underway around the world using engineered exosome-based cancer therapy. However, to use exosomes clinically, further studies and sufficient validation are required to resolve a number of contentious issues such as; purification, characterization and application of exosomes in cancer treatment [20,141]. For exosome analysis methods to be useful in clinical settings, they should use small sample volumes, be rapid, sensitive, specific, high yield, high purity, low cost and

clinical validation. It would be extremely valuable to have generic biological standards.

Current challenges of exosomal miRNA using liquid biopsy in cancer

Although interesting exosome biology is being unraveled largely using cell-culture systems, there is a need for experiments using *in vivo* animal models to study biogenesis, trafficking, and cellular entry of exosomes [33]. The important limitation in using exosomal miRNA as clinical markers in liquid biopsy is purification, concentration, source clarity and validation of the samples. It is clear that exosomes provide novel and important applications for the treatment of diseases, although there are still challenges ahead. Together, the scope of using exosome is currently limited, likely, utilization of these biomolecules and their biomarkers will soon be clinically in position.

Methodology

In previous study, we identified 16 cloning DNA (cDNA) – peptides from Her2 and PARP-1 antigens. These cloning were codified as RNA-peptides had high exosome affinity and stability against breast, ovarian, and lung cancers. In this study, we designed *in silico* three vaccine composed of high efficacy RNA-peptides from this collection. This vaccine may induce immunity against breast, ovarian, and lung cancers. Also, we used suitable endogenous exosome as adjuvants in the structure of the vaccine to potentiate the immunogenicity of the antigens. The three RNA-peptide were produced by caspases 3/7 action [142-144].

Results

1. RNA-PEPTIDE sequence:

AAAAAAAAAACUCCUAGAACUAGCAUACAGAUG—
CTSPKANKEILDEVDGVDEVAKKKSK

2. miRNA: AAAAAAAAAA—CUC CUA GAA CUA GCA
UUA CAG AUG

Size= 34 nt; MW=10993 daltons

3. CTSPKANKEILDEVDGVDEVAKKKSK

Size= 26 amino acids; MW= 2832.22 daltons

Theoretical pI: 6.29

Fusion Stability (FS)

FS = a*b*c*d = 10*3.881407*0.7647*1.971 = 58.50 cruz

a = Size poly A / Size poly Cys = 10 / 1 = 10

b = MW miRNA / MW peptide = 10 993 / 2832.22 = 3.881407

c = Size peptide / Size miRNA = 26 / 34 = 0.764705882

d = [(2*(A+ U) + 3*(C+G)) / (PI peptide²)] = [(2*(18+ 6) + 3*(6+4)) / 6.29*6.29] = [(2*(24) + 3*(10)) / 39.5641] = (48 + 30) / 39.5641 = 78 / 39.5641 = 1.971484

FS=58.50 cruz

Exosome Affinity (EA)

EA = FS* [(MW peptide / MW miRNA) + (Size peptide / Size primer)]

If [(MW peptide / MW miRNA) + (Size peptide / Size primer)] = 1

[(2832.22 / 10 993) + (26 / 34)] = [(0.257638) + (0.7647)] = 1.0223

Then

EA = FS*1.0223 = 59.80 ro

EA=59.80 ro

and

Biological Action (BA)

BA = EA / FS

BA = 59.80/58.50

BA = 1.0223 ro/cruz

Optimal Biological Action (OBA) value for antitumoral RNA-peptide with exosome as carrier are:

1.8 < OBA < 1.3

This result may lead to the development of promising new therapeutic approaches in cancer management by using exosome-mediated miRNA-peptide vaccine therapy.

Conclusion and future perspective

The investigating of exosomes as multicomponent signaling complexes mediating cell to cell communication between both nearby and distant tissue cells is an emerging area as a novel form of communication, as well as a delivery vehicle to carry their cargo containing proteins, nucleic acids, drugs, and vaccines. Employing exosomes as a vehicle for vaccine delivery is demonstrated to be an efficient strategy since of the natural characteristics of exosomes, having low immunogenicity, in addition to their ability to reach particular target cells at any site within the body, including lung, breast and ovarian cancer cells.

Exosomes are an excellent delivery system for anti-oncogenic miRNAs and anticancer drugs in therapeutic applications due to their small size, natural origin of the body cells, nontoxic characteristics and crossing the many biological barriers [25,135,145,146,153]. Tumor cell-derived exosomal miRNA research is extremely dynamic and promises innovative approaches in many fields, including cancer prevention, early diagnosis, monitoring progression and personalized therapy [20]. In general, relative to their healthy cell-derived exosomes, there are distinct variations in the tumor-derived exosomal miRNA expression profiles. It has also been shown that differences in the expression profiles of non-coding miRNAs associate with different tumor characteristics, such as development of the tumor, angiogenesis, invasion and metastasis.

It may be possible to inactivate exosome transport proteins such as ESCRT, ALIX and tetraspanins that reduced exosome secretion from cancer cell to normal neighboring cells and inhibiting of exosome-target cell interaction, this may become a novel approach for anticancer therapies to inhibit metastasis. Engineered cell-derived exosomes may be used as novel and efficient personalized cancer therapy to deliver functional molecules and therapeutic agents, including anticancer drugs and functional antitumor miRNAs, to targeted cancer cells or tissues. Another potential

new therapeutic strategy, mature onco-miRNA formation can be inhibited by using inhibitor molecular against to Droscha, Exportin-5 and DICER proteins as anticancer therapy. Additionally, another possible example is also the inhibition for exosome secretory pathway by promoting lysosomal degradation in tumor cell.

As a result, exosomes are excellent biocompatible, reducing toxicity and immunogenicity, exhibiting great stability in body fluids, the ability to pass through biological barriers and can be loaded with specific molecules to targeted cells in treatment of various diseases including neurodegeneration and cancer. To conclude, exosomes are small secreted particles with broad cancer functions.

Although rapid advances in exo-miRNA detection methods have been made, further efforts are still required to obtain more sensitive, rapid, sufficient yield and cost-effective methods to find more accurate characterization and functions of body fluid exosomal miRNAs, providing a new strategy for better prevention, early and accurate diagnosis and personalized cancer treatment. This will provide great opportunities for the clinical translation of engineered exosome delivery for *in vivo* tracking, prognosis monitoring, and cancer therapy.

Finally, according to the algorithms CRUZ RODRIGUEZ (CR) we are proposing a novel and effective vaccine treatment against breast, ovarian, and lung cancers. Our further *in vivo* analysis will enable exosomal miRNA-peptide vaccine to be used in lung, breast, and ovarian cancer treatment.

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