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Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer

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Breast cancer is a highly prevalent disease, accounting for 29% of invasive cancers in women. Survival from this disease depends on the stage at diagnosis, with patients who are detected earlier having more favourable outcomes. It is because of this that research groups are focusing on the development of a blood-based biomarker for breast cancer. Such biomarkers may facilitate the detection of breast cancer in its infancy before it has spread beyond the primary site. MicroRNAs (miRNAs) have shown immense potential in this setting. These short, non-coding RNA sequences have been shown to be dysregulated in breast cancer. Despite showing immense promise, miRNAs have not been successfully implemented in the clinical setting due to a lack of a standardised approach which has resulted in conflicting results. These challenges may be addressed at least in part through the study of exosomes. The biomarker potential for exosomes holds huge promise and may revolutionise the way in which we diagnose and manage breast cancer. These nanovesicles may be isolated from a variety of bodily fluids, including serum, and their miRNA content has been shown to reflect that of the parent breast cancer cell. This review will highlight the nomenclature and defining characteristics of exosomes, and current methods of isolation of serum-derived exosomes. Initial promising reports on the potential utility of exosomal miRNAs to be used as breast cancer biomarkers will also be addressed.

Breast cancer is a highly prevalent disease, accounting for an estimated 29% of all new female invasive cancers diagnosed in 2015.[1] According to figures published by Siegel *et al*., this disease was responsible for more than 40,000 cancer-related deaths in women in the United States in 2015. Superior survival rates have been identified in cases where breast cancer is detected at an early stage.[1]

This disease represents a complex heterogeneous group of tumours that display significant diversity with respect to histopathological features and therapeutic response.[2,3] The use of gene expression profiling has led to the general acceptance of a molecular classification of breast cancer, which groups tumours into four distinct subtypes namely Luminal A, Luminal B, human epithelial growth factor receptor-2 (HER2) amplified and basal-like breast cancer (BLBC).[4,5]

The recognition of breast cancer as a diverse group of diseases has led to the general acceptance that targeted therapy based on cancer subtype represents the optimal therapeutic strategy. On a similar vein, the diagnostic approaches to breast cancer should be subtype oriented. Traditional diagnostic methods, such as mammography, although very effective, are limited in that they require a minimum tumour size for detection. This can lead to the situation where locoregional or possibly distant metastasis may have already taken place before breast cancer diagnosis. In addition to this, not all breast tumours are detectable mammographically at an early stage when they are amenable to treatment. It is as a result of this, and the influence that stage at diagnosis has on survival, that multiple research groups have focused on blood-based biomarkers which will facilitate detection of breast cancer in its infancy before it has spread beyond the primary site. MicroRNAs (miRNAs) have shown immense potential in this setting. These short, non-coding sequences of RNA have been shown to be dysregulated in a variety of cancers,[6,7] including breast cancer.[8] Although these changes are detectable in the malignant tissue of patients with breast cancer, dysregulated miRNA levels can also be measured in the blood of patients.[8] This area is, however, fraught with challenges and despite immense promise, has not been successfully implemented in the clinical setting. One reason for this is that it is unclear as to which fraction of blood is the ideal source for the detection of cancer-related miRNAs, with whole blood, serum and plasma as starting materials all being reported in the literature.[9–11] The lack of a standardised approach has resulted in conflicting results, with miR10b, for example, being observed at significantly higher levels in the serum of breast cancer patients when compared with healthy controls in one study, while a separate study reported no

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significant difference in miR10b levels in the whole blood of breast cancer patients *versus* healthy individuals.[9,10] In addition to this, investigators are focusing efforts on the discovery of circulating miRNAs which may allow breast cancer subtypes to be distinguished from each other.[12–14] These challenges may be addressed at least in part through the study of a specific fraction of blood known as exosomes.

Exosomes are membrane-derived vesicles that are actively secreted by cells."[15] Although previously thought of as a means by which cells could dispose of unwanted biomolecules, they are now known to be capable of the transfer of genetic material including messenger RNA (mRNA), miRNA and small regulatory RNAs (sRNAs).[16–19] Numerous studies have shown that miRNA-containing exosomes are secreted into the circulation.[16,20] As such, exosome-encapsulated miRNAs may represent an ideal biomarker for diseases at an early stage.[21] Exosomes have been shown to confer the phenotypic traits of parent cells on recipient cells.[22,23] They are released by a variety of cell types including reticulocytes, epithelial cells, B cells, T cells and cancer cells.[24–28] The functions of exosomes are diverse, with exciting studies demonstrating their ability to have a functional impact on recipient cells and therefore highlighting the role that they play in intercellular communication in the tumour microenvironment.[22,29] Additionally, the therapeutic potential for these microvesicles has been explored, with many groups researching a means by which exosomes may be manipulated to deliver gene therapy into cancer cells.[30,31] The biomarker potential for exosomes is another facet of this vast field of research that holds huge promise and may revolutionise the way in which we diagnose and manage breast cancer in the near future. These nanovesicles may be isolated from a variety of bodily fluids, including serum,[32,33] and their miRNA content has been shown to reflect that of parent breast cancer cells.[23,34,35] This may allow researchers both to identify a breast tumour and to stratify the subtype to which it belongs. This review will examine the defining characteristics of exosomes and current methods of isolation of serum-derived exosomes. Initial promising reports on the potential utility of exosomal miRNAs to be used as biomarkers for breast cancer will also be addressed.

Exosomes: Definition and Synthesis

Exosomes have been defined as microscopic vesicles that have a "saucer-like" morphology and are composed of a lipid bilayer.[36] They arise from intraluminal vesicles (ILVs), which are formed within cellular multivesicular bodies (MVBs) and are released into the extracellular compartment upon fusion of the MVB with the plasma membrane.[15] ILVs accumulate during endosome maturation (giving a multivesicular appearance), during which transmembrane and peripheral membrane proteins are incorporated into their membrane. Sorting of proteins and lipids at the limiting membrane of endosomes during ILV formation leads to the encapsulation of a specific set of molecules within these nanovesicles. This cargo sorting process is thought to be mediated by ESCRT components, lipids and/or tetraspanins-enriched microdomains. Exosomes from different cellular types contain a common set of molecules but also display parent cell-specific components.[37] The mechanism of MVB fusion with the plasma membrane and subsequent exosomal release, although not fully elucidated, is thought to be dependent on the Rab 11 GTPase and others.[15] Once released into the extracellular milieu, ILVs are termed exosomes.[15] Exosomes typically measure 50–100 nm in diameter as a result of the restrictive size of the corresponding ILVs.[15] The definition of what constitutes an exosome has come under intense scrutiny in recent years due to a lack of consensus in terms of terminology and size. These issues were discussed at the inaugural meeting of the *International Society for Extracellular Vesicles* (ISEV) in Gothenberg, Sweden in 2012, relevant points from which were published by Gould and Raposo in the *Journal of Extracellular Vesicles* the following year.[38] The first issue that was debated was that of nomenclature of extracellular vesicles, including exosomes. In terms of exosomes, three definitions of these microvesicles were identified: the first describes vesicles that are formed within endosomes and are released upon fusion of the MVB with the plasma membrane of the cell[26,39]; the second was broader, encompassing vesicles that "may serve a physiologic function"[40,41]; and the final included vesicles that are pelleted following centrifugation at ~70,000–100,000*g*.[39] Owing to this lack of consensus on the definition of an exosome, Gould and Raposo[38] advocated that authors provide a clear, consistent definition when applying the term exosomes. Furthermore, the isolation protocol should be clearly outlined in all publications. The use of the term "extracellular vesicle" to describe all secreted vesicles was also encouraged.[38,41]

The matter of vesicle size and morphology was also addressed, with a broader detected size of 50–100 nm agreed upon.[39] The authors stated that investigators, reviewers and editors should remain cognisant of this broader size definition going forward.

Isolation, Characterization and Quantification of Breast Cancer Exosomes

A variety of exosome isolation protocols, which are suitable for use in the setting of human or animal serum, have been described in the literature.[22,23,42–44] The vast majority of these report successful isolation of exosomes through the use of differential centrifugation (*e.g.*, 800*g* for 10 min, then 2,000*g* for 10 min[42,43]) followed by microfiltration using a 0.2–0.45 μm sterile filter and a final ultracentrifugation step (*e.g.*, 100,000*g* for 2 hr[22,23,42,43]). Successful isolation has also been reported using a combination of differential centrifugation and ultracentrifugation in the absence of microfiltration.[43,45,46] Pelleted exosomes are typically resuspended in phosphate-buffered saline (PBS) following the final ultracentrifugation step. An alternative, although more costly, exosome isolation method is available for use in the absence of

an ultracentrifuge. This involves the use of commercially available kits that use precipitation reactions to isolate exosomes from serum. An example of this type of kit is the Total Exosome Isolation™ solution (Invitrogen, California, United States) which is added to the exosome source following centrifugation at 2,000*g*.[47] The exosomal source is then refrigerated overnight at 4°C and centrifuged at 10,000*g* for 1 hr in order to pellet out the exosomes which are then resuspended in PBS.[46] Similarly, the ExoQuick exosome precipitation solution™ (BioCat, Heidelberg, Germany) has been successfully used to isolate exosomes from human serum samples.[33,46,48] This involves the addition of a specified volume of ExoQuick solution to the serum sample and refrigerating for 30 min. Next, the sample is centrifuged at 1,500*g* in order to pellet the exosomes. The pellet is then resuspended in sterile or nuclease-free water following the removal of supernatant. The exoEasy Maxi Kit (Qiagen, Hilden, Germany) involves the addition of buffer solution to the samples, inverting and then standing at room temperature before adding to an exoEasy spin column and centrifuging at 500*g* for 1 min.[49] The flow-through is then discarded and 10 ml of buffer XWP is added to the column and centrifuged at 5,000*g* for 5 min. Next, 400 µl–1 ml of buffer XE is added to the membrane and incubated for 1 min before centrifuging at 500*g* for 5 min. The final step involves the reapplication of the eluate to the spin column membrane, incubating for 1 min and then centrifuging at 5,000*g* for 5 min before collecting the exosome-containing eluate. Additional exosome isolation methods, including size-exclusion liquid chromatography[50,51] and microfluidic devices,[52] have also been reported in the literature, although they are less frequently used.

Following successful isolation of exosomes from serum, researchers proceed to characterization of the pellet to verify the presence of exosomes. For the most part, a combination of morphological examination and Western blot analysis targeting exosome-associated proteins are used to confirm the presence of exosomes. Proteins that are commonly targeted include CD 63[23,46] and CD9,[53] which are members of the tetraspanin family, FASN[54] and VCP.[54] Morphological examination is typically carried out using transmission electron microscopy (TEM), which allows investigators to appreciate the vesicular shape of exosomes in addition to obtaining an estimated measure of their diameter.[23,53,55] This technique can involve the addition of an optimal concentration of fixed exosomes onto glow-discharged carbon formvar 400 mesh copper grids. The samples are allowed to absorb to the formvar before rinsing in water, staining with uranyl acetate and air drying. Exosome samples are then imaged using TEM.[55] Variations on this procedure have been described by other research groups.[22,45,56] An alternative method involves fixation of exosomes in formalin, followed by secondary fixation in oxmium tetroxide. The exosomes are then dehydrated in graded ethanol before embedding in a resin, sectioning and viewing using TEM.[57] In cases where immunogold (IG) labelling is required the grids are placed in a blocking buffer and then incubated with the primary antibody of interest. Next, the grids are floated on drops of secondary antibody labelled with 10-nm gold particles. Finally, the exosomes are fixed and stained before viewing using TEM.[53] IG TEM was used in some cases to confirm the presence of the exosomal markers CD9, flotillin 1 and CD81.[53] In addition, IG-TEM has been used to distinguish exosomes derived from cancerous sources from those derived from non-cancer sources through the detection of Glypican-1, which is reportedly exclusive to cancer cell exosomes.[53] Fluorescence-activated cell sorting (FACS) has been utilised to detect cell surface proteoglycans (*e.g.*, Glypican-1) that are thought to be enriched on exosomes from cancer cells.[53] Flow cytometry methods are, however, limited by the small size of exosomes. As a result of this, exosomes must first be coupled to a larger substance, *e.g.*, aldehyde/sulphate latex beads. This has been successfully used to quantify exosomes by FACS.[53]

Exosome quantification can be carried out directly or indirectly. Standardised protein assays allow indirect quantification of exosomes through the analysis of protein content and this is the most widely reported approach used.[46,58] Alternatively, Exosome ELISA kits (System Biosciences) allow investigators to quantify the amount of exosomes that have been isolated based on the level of the exosome-associated proteins including CD9, CD63 and "CD 81."[46,58] An alternative exosome quantification technique involves the use of Nanosight™ nanoparticle tracking analysis which uses light diffraction patterns to measure the size and the concentration of exosomes.[59,60] Similarly, direct quantification of exosomes may be performed using the qNano Gold (Izon Science) which measures nanoparticles using the tunable resistive pulse sensing (TRPS) principle, reporting concentration as a function of a defined size range.[61,62] One challenge to most current exosome quantification methods is the possibility of samples being contaminated with non-exosomal particles, which in the case of serum-derived exosomes may include albumin and immunoglobulin G.[59]

Exosomal miRNAs

Our understanding of exosomes is continuously evolving as a result of robust laboratory-based research. The elegant work performed by Melo *et al.*[42] underscored the fact that cancer cell-derived exosomes differ greatly in content and functional effect to exosomes derived from non-tumourigenic cell lines. The authors first demonstrated how metastatic breast cancer cell-derived (MDA-MB-231 and 4T1) exosomes were enriched in miRNAs when compared with non-metastatic breast cancer exosomes (MCF7). In addition, profiling of purified cancer cell exosomes after 72-hr culture revealed enrichment of miRNAs compared to 24-hr incubation, an effect that was not observed in the case of control cell (MCF10A and NMuMG) exosomes (normosomes). Downregulation of pre-miRNAs was detected in cancer exosomes over time with levels being inversely proportional to the

corresponding miRNAs. No variation in normosome pre-miRNA levels was observed under the same conditions. Exosomes derived from cancer cells and from the serum of patients with breast cancer were found to contain the RNA-induced silencing complex (RISC)-loading complex (RLC) proteins, Dicer, TRBP and AGO2, which are involved in miRNA biogenesis. The accumulation of Dicer specifically in cancer exosomes is reportedly mediated through CD43. These data demonstrated the ability of cancer cell exosomes to convert pre-miRNAs to mature miRNAs. Subsequent examination of the effect of transfer of cancer cell exosomes revealed increased survival and proliferation in a non-tumourigenic population of cells.[42] This effect was examined further by injecting non-tumour cells with exosomes derived from a metastatic breast cancer cell line in a murine model, which resulted in tumour formation. A key finding in this study was that Dicer blockade inhibited tumour formation, suggesting the pivotal downstream effect that it plays in the transformation of non-tumourigenic cells to malignant cells. Similarly, serum exosomes from patients with breast cancer coinjected with MCF-10A cells resulted in tumour formation in mice, while no tumours were formed when cancer exosomes were replaced with exosomes from healthy volunteers.[42] Serum from patients with breast cancer was also noted to contain higher levels of exosomes than that which was isolated from healthy donors.[42]

Work carried out by Tosar *et al.*[19] involved sequencing of the intracellular sRNA content of breast epithelial cell lines (MCF-7 and MCF-10A) and comparison with extracellular fractions enriched in microvesicles, exosomes and ribonucleoprotein complexes. The results demonstrated a non-selective secretion model for the majority of miRNAs, with a few showing preferential secretion patterns. The authors proposed that this model might explain, at least in part, some of the conflicting published data regarding miRNA secretion. In contrast, 5′ tRNA halves and 5′ RNA Y4-derived fragments of 31–33 nt showed preferential secretion patterns. These data demonstrate that different sRNA families have characteristic secretion patterns and highlight their potential role in the extracellular space.

There has been a huge surge of interest in the use of circulating exosome-encapsulated miRNAs as biomarkers for breast cancer in recent years. An important factor that must be taken into consideration with any biomarker is that of the robustness of the source material, in this case, exosomes. Studies have demonstrated that exosomal-protein levels in samples derived from biofluids are well preserved when stored at −80°C when compared with samples stored at 4°C and processed within 1 hr.[63] In addition, levels of exosomal markers were similar in samples that were stored at 4°C for 24 hr and then at −80°C. This finding has enhanced the clinical applicability of exosomes.[32]

A variety of detection methods for circulating exosome-encapsulated miRNA detection have been described in the literature, most of which centre around the use of real-time polymerase chain reaction (PCR) analysis.[23,33] Lee *et al.*[46] used molecular beacon (MB), a nano-sized oligonucleotide probe to carry out *in situ* miRNA detection. This involved quantitative detection of miRNAs in exosomes derived from breast cancer cell lines. Permeabilization of the MCF-7-secreted exosomes using Streptolysin O bacterial toxin enhanced the delivery of MB into exosomes and increased miRNA hybridization. The investigators then detected significantly higher miR-21 levels in MCF-7 exosomes when compared with normal cell-derived exosomes, thus highlighting its potential as a biomarker for breast cancer. Next, exosomes from MCF-7 cells were spiked into human serum samples in order to determine if MB had the capacity to detect serum exosomal miRNAs without pretreatment of the serum. Here, the investigators found that MB could be successfully used for the detection of exosome-encapsulated miRNAs spiked into serum without the need for additional treatment. This study clearly outlined a technique that may be valuable in the field of breast cancer.

Exosome-Encapsulated miRNAs as Breast Cancer Biomarkers

A recent paper by Eichelser *et al.*[33] investigated the ability to detect specific miRNAs in the serum exosomes of patients with breast cancer and to determine if molecular subtype identification could be achieved using this modality. This method of subtype discrimination may be of particular value in the clinical setting given that survival from breast cancer differs significantly between subtypes: patients with Luminal A breast cancer have superior survival to those with either HER2 or basal-like subtypes.[33] Molecular subtype also influences the treatment modalities that will be effective in the management of breast cancer. The researchers isolated the exosome fraction from the serum of 50 patients with breast cancer and 12 healthy controls. Cell-free and exosomal miR-101, miR-372 and miR-373 levels were compared between cohorts. Higher relative values of the aforementioned miRNAs were found in serum exosomes when compared with cell-free circulating levels.[33] Furthermore, exosomal miR-101 and miR-372 were found to be significantly higher in the serum exosomes of patients with breast cancer than in healthy controls. This finding was not observed in the case of miR-373, but within the breast cancer cohort, it was found to be significantly elevated in triple-negative patients when compared with luminal cancers or healthy controls. Similarly, it was higher in ER/PR-negative cases compared to receptor-positive patients. The results of this study indicate that higher exosomal levels of expression of miR-373 in breast cancer are indicative of a triple-negative phenotype. These data highlight the potential for serum-specific exosomal miR-373 to serve as a biomarker for aggressive, triple-negative and hormone receptor-negative breast cancers. A key point that must be noted in relation to this study is that a wide range of values for miR373 was detected in both ER/PR-negative cases and

receptor-positive patients. This level of variation may present challenges incorporating miRNAs into the clinical setting.

The use of exosome-encapsulated miRNAs as a prognostic marker for metastatic progression in breast cancer was reported by Zhou *et al.*[45] In this study, the authors measured serum exosomal miRNA levels in breast tumour-bearing animals compared to tumour-free controls, and found that miR-105 was significantly higher in animals in the pre- and metastatic stages. Next they detected significantly higher miR-105 levels in the serum-derived exosomes of patients who went on to develop distant metastases ($n = 16$) compared to those who did not go on to develop metastatic disease ($n = 22$) (mean follow-up of 4.2 years). The results of this study suggest that serum-exosomal miR-105 may have the potential to predict, or diagnose at an early stage, patients who may develop or have already developed breast cancer metastasis.

Future Directions

The field of exosome-encapsulated miRNAs as a serum-based biomarker is evolving rapidly and this facet of cancer research offers great potential. First, our understanding of exosomes in terms of nomenclature and size is becoming more refined as a result of international collaboration. Furthermore, exosomes have been shown to be robust and can be stored for extended periods without significantly affecting the integrity of encapsulated miRNAs. This feature of exosomes increases their potential applicability in the laboratory/clinical interface. The miRNAs that are contained within breast cancer cell-derived exosomes are reflective of their parent cells and therefore may offer a tumour-related profile that is more specific than the miRNA profile of whole blood or even serum.

The potential applications of exosome-encapsulated miRNAs are vast: they have the potential to be used for breast cancer diagnosis, subtype specification and for prediction and monitoring of response to therapy. Recent exciting work demonstrated how tumour-derived exosomes that are taken up by organ-specific cells prime the pre-metastatic niche through the expression of differing integrin profiles.[29] Such data on exosomal integrins may allow researchers and clinicians to predict the metastatic site of breast tumours[29] and compliment exosomal-miRNA research in order to give a patient-specific tumour profile and prognostication information. As plasma/serum is routinely collected in multiple cancer centres worldwide there exists a wealth of samples from patients with breast cancer on which validation of findings from smaller studies may be carried out in a relatively short period of time. Such collaboration is the key to moving this field of research forward and examining its true applicability in the clinical setting.

One challenge that exosome-based research currently faces is that of the multistep process that is required from serum collection to miRNA quantification. This represents a disadvantage when compared with developing breast cancer biomarker detection methods, some of which entail the detection of miRNAs or other markers directly in whole blood or serum, without the need for further processing, and therefore may be more readily integrated into the clinical setting. A plethora of kits are currently available, however, which allow rapid exosome isolation and RNA extraction. As this field of research continues to grow, platforms for analysis, with high-throughput capabilities will undoubtedly be developed. An additional potential limitation to the use of exosomal miRNAs as biomarkers for breast cancer is the strong variation that has been noted in exosomal miRNA levels as evidenced by wide ranging cycle threshold values on RQ-PCR.[33,45] Such variation may impact on the clinical applicability of exosomal miRNAs as breast cancer biomarkers. Furthermore, patient co-morbidities may also impact on miRNA levels, and may lead to a lack of specificity for breast cancer. This difficulty may be addressed to some degree by ensuring that appropriate statistical tests are used when analysing exosomal miRNA data. Additionally, when exosomes are isolated from a small volume of serum/plasma (e.g., 250 μl) the resulting RNA yields may be low.[64] This may lead to the difficulty in the downstream detection of miRNAs using RQ-PCR. Understanding exosome source, factors impacting their abundance and content and the identification of endogenous controls that are not impacted by disease status will be key to this.

Despite these challenges, it is essential that researchers continue to advance this field in order to determine the clinical utility of exosomal miRNAs as breast cancer biomarkers so that it may have a meaningful impact on patient outcomes.

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