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Exosomal MiRNAs as Biomarkers for Radiation Toxicity in Breast Cancer Patients

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EXOSOMAL MIRNAS AS BIOMARKERS FOR RADIATION TOXICITY IN BREAST CANCER PATIENTS

A thesis submitted in partial fulfillment of the requirements for the degree of Biochemistry at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

BCP	breast cancer patients
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
Exo-miRNA	exosomal microRNA
K2EDTA	potassium ethylene diamine tetraacetic acid
miRNA	micro RNA
mRNA	messenger RNA
NP	nanoparticle
NTA	nanoparticle tracking analysis
ROX	carboxy-X-rhodamine
RT	radiotherapy
TEM	transmission electron microscopy
WB	western blot

ABSTRACT

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Radiotherapy (RT) is a standard treatment for most breast cancer patients (BCPs), but is often accompanied by acute and late toxic effects in normal tissue. Exosomes are nano vesicles about 30-150nm in size that originate from the endosomal network and are found in most body fluids. Exosomes are a fundamental driver of intercellular communication by transferring proteins, lipids and microRNA (miRNA). Exosomal miRNA (Exo-miRNA) signatures may serve as non-invasive prediction biomarkers of post-radiation toxicities of BCPs.

Eighty six BCPs treated in the Radiation Oncology Department were enrolled in an IRB

approved study. BCPs were evaluated weekly during RT and at prescribed intervals following completion of RT for the development of toxicity LENT-SOMA scale. Acute toxicity effects were assessed using physician reported toxicity scale CTCAE v4. Blood samples were collected one day before RT. The PureExo® Exosome Isolation Kit was used to isolate exosomes from the plasma. Exo-miRNAs were isolated and cDNA was synthesized for all samples.

Exo-miRNAs were analyzed from the plasma of BCPs divided into four groups: (1) Low toxicity (n=9), (2) Moderate toxicity (n=45), (3) High acute toxicity (n=7), and (4) High late toxicity (n=25). For preliminary analysis, cDNA samples in each group were pooled together and the four groups were analyzed for the expression of 179 miRNAs commonly found in human serum/plasma. Twenty-four out of 179 tested exo-miRNAs demonstrated a high potential in the prediction of post-RT toxicity of BCPs.

CHAPTER 1

INTRODUCTION

Breast cancer affects 1 in 8 women worldwide and is the second leading cause of death due to cancer for women in the United States.[1,2] Breast cancer arises from dysregulated growth of mutated cells that begins predominantly in the luminal epithelial cells of the milk-producing lobules and ducts of the breast.[1] Continued proliferation of these abnormal cells has the potential to metastasize to secondary sites via the blood and lymphatic vessels, further invading normal tissues and significantly worsening patient outcomes.[1] Despite the overall decrease in breast cancer mortality over the last several decades due to improvements in diagnosis and treatment methods, breast cancer remains the most commonly diagnosed cancer in women worldwide.[2] Furthermore, the current standard treatments for cancer often take a significant physical toll on the body despite not always being effective in improving patient prognosis.[3] Thus, there is a clear need for optimizing cancer treatment based on the genetic differences that exist between each cancer diagnosis.

Radiotherapy is the most crucial non-surgical treatment of cancer. This treatment is administered in over half of all breast cancer cases.[3] Radiotherapy induces cellular damage by exploiting the tumor cells' unstable genome. Because cancerous cells divide and proliferate rapidly, they accrue more genetic mutations which are ineffectively repaired or completely bypassed by normal DNA damage repair mechanisms.[4] Therefore, tumor cells are more susceptible to the damaging effects of ionizing radiation compared to normal tissue cells.

However, healthy tissue does not often go unaffected by the effects of radiation therapy.[3]

Acute and late effects are often observed as a consequence of irradiation. Acute effects are those that normally occur within weeks of treatment and may persist for up to several months upon the completion of therapy. These short-term effects are characterized by damage to tissues that are rapidly proliferating, such as hair follicles or epidermal cells. As a result, conditions such dermatitis, breast pain, and hyperpigmentation are often observed as side effects of radiotherapy.[3,5] Late effects usually arise between six months to several years after treatment. These effects are often chronic, persisting for many years after undergoing treatment, and often irreversible. These effects may include fibrosis and collagen reabsorption, causing hardening or reduction of the irradiated breast tissue and nearby organs.[3] Additionally, vascular damage can significantly impact skin integrity and appearance.[3,5] Because of the severe impact that radiotherapy may have on healthy tissue, this limits the admissible treatment dosage for all patients despite the fact that response to treatment varies between individuals.[3] Although several candidate genes have been identified that may contribute to differences in patient response, there are currently no reliable biomarkers to predict responses to radiotherapy.[6,7]

MicroRNAs (miRNAs) have become a great area of interest in research, particularly in the context of cancer. MiRNAs are short stretches of single-stranded, non-coding RNA that are 18-25 nucleotides in length and influence post-transcriptional gene expression by binding to and destabilizing messenger RNAs (mRNAs).[8,9] The effect that miRNAs have on cell activity lends to their vast potential in applications such as risk factor assessment, diagnosis, progression

monitoring, and treatment of breast cancer.[8] MiRNA is produced in the nuclei of virtually all eukaryotic cells. It is first transcribed from DNA into pri-miRNA which is cleaved to produce pre-miRNA. This pre-miRNA is then transported to the cytoplasm and modified to produce mature miRNA. The mature miRNA targets the 3'-UTR of its specific target mRNAs to alter the transcription.[9] MiRNA can affect mRNA transcription not only within its cell of origin but within nearby cells as well as distant cells of different origins and cell types.[10] MiRNA may be secreted freely into the circulation bound to the protein Ago2 and may be found both intravascularly in the plasma or serum and also extravascularly, such as in the saliva, breastmilk, urine, or amniotic fluid.[8,9,11] Over 2,000 mature miRNAs have been identified in humans. Many of these miRNAs have been identified as being differentially expressed in various cancers. Several studies have shown that tumor cells secrete miRNA in order to maintain the tumor microenvironment and therefore enhance tumor growth and metastasis.[10] Interest in research regarding miRNAs that are transported and delivered specifically via extracellular vesicles (EVs) has increased dramatically within the last decade.[12,13]

Exosomes are small, membrane-bound EVs 30-150 nm in size. They are released by nearly all eukaryotic cells and are found in various biological fluids. Exosomes are similar to other EVs such as microvesicles in that they are membrane-derived particles that are secreted by cells into the extracellular space in order to mediate cell-to-cell communication.[11,14] Compared to microvesicles, they may also contain similar cargo; such as miRNA, mRNA, proteins, and lipids. However, exosomes differ from other EVs in terms of their biogenesis, membrane composition, size, trafficking mechanisms, and mechanism of content release.[14] Exosome formation begins with the endocytic pathway where clathrin-coated domains bud inward from the cell membrane

to produce the early endosome. As the endosome matures it gives rise to a multivesicular body containing intraluminal vesicles whose formation is directed by the ESCRT (endosomal sorting complex required for transport) pathway.[14,15] ESCRT proteins are responsible for the identification and wrapping of cargo within intraluminal vesicles as well as vesicle scission.[16] There appears to be a few mechanisms by which specific miRNAs are selected for packaging into exosomes. Passive loading of exosomes is based primarily on which miRNAs are most highly concentrated in the cell. Active mechanisms may depend on particular sequence motifs at the 3' end of the miRNA, which are recognized by specific proteins that assist their loading into the exosomes.[10] After exosome secretion via RAB and SNARE proteins, there appears to be multiple mechanisms by which exosomes are recognized and subsequently taken up by their target cells.[16,17] This includes opsonization, chemokine release, and cell adhesion.[15] Furthermore, exosomes produced by immune cells express MHC class I and II, which may be recognized by T cells.[15,17] Uptake of exosomes into their appropriate target cells is thought to occur via receptor-ligand interactions, fusion of the exosome and cell membranes, and phagocytosis.[15] Exosomal miRNAs and other cargo may then be released into the cell where they alter cell activity.

The potential clinical applications of exosomal miRNAs continues to expand as exosome biogenesis, cellular targeting, and miRNA functioning become more clearly understood. Most notably, analysis of exosomal miRNA expression has gained notoriety as a potential method for detecting tumor growth, metastasis, and examining the genetic profile of a tumor.[18] Identification of particular miRNAs that are associated with cancer development and metastasis have highlighted the therapeutic possibility of targeting and therefore inhibiting the action of specific tumorigenic miRNAs.[9,18] Similarly, miRNAs that maintain homeostasis and

promote normal physiological signaling may provide another therapeutic strategy whereby these miRNAs can be delivered to specific target cells.[9,10]

MiRNA expression analysis as an indicator of tumor radiosensitivity may yield valuable information with the potential to improve radiation treatment, but has thus far been less extensively explored compared to the aforementioned areas of miRNA research. Heterogeneity across tumors lends to their varying sensitivity to radiotherapy, but predicting and characterizing the exact nature of this heterogeneity remains a challenge.[18] Studies focused on the topic of miRNA expression in response to radiotherapy have identified particular miRNAs that may be involved in tumor cell response to ionizing radiation. Griñán‐Lisón et al. identified eight miRNAs linked to the variations in response of cancerous stem cells to radiotherapy.[19] Additionally, Pajic et al. showed that miR-139-5p is associated with increased oxidative stress, decreased DNA damage repair, and increased apoptosis in tumor cells.[20] MiRNAs associated with the toxicity response in normal tissue have received less investigative attention, however a few studies have identified miRNAs that appear to promote tissue toxicity. In a 2019 study, Esplugas et al. demonstrate that radiotherapy increases the expression of miRNA-155, -221, -146, and -222 in breast cancer patients, which have been linked to the development of cardiovascular toxicity.[21] In a separate study, miR-215 was also associated with cardiac toxicity induced by radiotherapy in breast cancer patients.[22] Additionally, research on glioblastoma patients who received radiotherapy revealed that elevated expression of miR-10b and miR-21 is associated with higher toxicity grade, while miR-34a appears to serve as a dosimeter for radiation exposure.[6] This study is similar to those previously mentioned, but investigates miRNA

expression in patients with tumors of the central nervous system rather than breast cancer. Furthermore, these studies do not investigate miRNA isolated from EVs. This highlights the need for more studies that examine the effects of radiotherapy specifically on exosomal miRNA expression in breast cancer patients.

The present study aims to identify exosomal miRNAs whose differential expression prior to radiotherapy may serve as a predictor of the degree to which individual breast cancer patients exhibit radiotoxicity in normal tissues. Such data is critical in order to identify reliable biomarkers that can predict radiotoxicity. These biomarkers are necessary to minimize treatment risk and maximize the benefit to each patient. This study utilizes qPCR as a high throughput method to screen for hundreds of known miRNAs that have been purified from plasma samples of patients whose toxicity responses range from minimal to severe. The miRNAs identified in this study may not only serve as a biomarkers for post-radiation toxicity, but may help identify the genetic processes that influence toxicity response. Therefore, the presented data may contribute to the potential use of miRNA as a tool to improve personalized patient therapy.

MATERIALS AND METHODS

Patient Samples:

Blood samples drawn from 86 women diagnosed with breast cancer were used in the present study. These blood samples were collected 1 day before the start of radiation treatment. Patient samples were delegated to one of four groups depending on the patient's reaction to radiation. Samples delegated to Group 1 (n=9) were obtained from patients who exhibit minimal toxicity in response to radiation. Group 2 (n=45) describes patients with moderate response to radiation, while Group 3 (n=7) describes patients with severe acute reaction and Group 4 (n=25) describes patients with severe long-term reaction. The blood samples were collected in tubes coated with K2EDTA (Potassium Ethylene Diamine Tetra Acetic acid). This material acts as an anticoagulant to prevent blood clotting. On the same day of collection, blood samples were centrifuged at 3,000x g for 15 minutes at 4°C to obtain the plasma fraction, which was aliquoted and saved in vapor phase of liquid nitrogen.

Extracellular Vesicles (EVs) Extraction:

EVs were isolated from patient plasma samples using the PureExo® Exosome Isolation Kit for Serum and Plasma (101Bio, Palo Alto, CA, USA) according to the manufacturer's instructions. The concentration and size of EVs were measured using ZetaView® Nanoparticle Tracking Analysis. All samples were diluted in 1xPBS to a final volume of two milliliters. Ideal measurement concentrations were found by pre-testing the ideal particle per frame value (140–200 particles/frame). The manufacturer's default software settings for EVs were selected accordingly. For each measurement, three cycles were performed by scanning 11 cell positions

each and capturing 80 frames per position under the following settings: Focus: autofocus; Camera sensitivity for all samples: 78; Shutter: 100; Scattering Intensity: detected automatically; Cell temperature: 25°C. After capture, the videos were analyzed by the built-in ZetaView Software 8.04.02 SP2 with specific analysis parameters: Maximum area: 1000; Minimum area: 5; Minimum brightness: 25; Hardware: embedded laser: 40 mW at 488 nm; Camera: CMOS. The number of completed tracks in NTA measurements was always greater than the proposed minimum of 1000 in order to minimize data skewing based on single large particles.

Transmission Electron Microscopy (TEM):

Isolated EVs were fixed and prepared for TEM. TEM analysis was carried out by the Microscopy Core of VCU.

Western blotting (WB) and Antibodies:

Equal amounts of total protein from each sample were loaded and separated by SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were exposed to the following primary antibodies at specific dilutions: anti-CD9 (dilution 1:1000, Novus Biologicals), anti-CD63 (dilution 1:1000, ThermoFisher), anti-TSG101 (dilution 1:500, Cell Signaling), anti-Albumin (dilution 1:1000, Cell Signaling). Specific protein bands were detected using the following infrared-emitting conjugated secondary antibody: anti-rabbit DyLight™ 800 4X PEG Conjugate (dilution 1:10,000, Cell Signaling). WB images were generated and analyzed using the ChemiDoc Infrared Imaging System (Bio-Rad).

RNA Purification:

MicroRNA was purified from isolated exosome samples using the MiRNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with a few modifications to maximize the recovery of miRNA from EVs. Synthetic Spike-in UniSp-2-4-5 miRNA controls (Qiagen, Hilden, Germany) were added to 200 µL of EV sample and homogenized with 750 µL of TRIzol™ LS reagent (Invitrogen, Carlsbad, CA, USA), followed by 200 µL of chloroform. Each sample was vortexed for sixty seconds and incubated at room temperature for five minutes. Phase separation was performed by centrifuging the samples at 12,000×*g* for fifteen minutes at 4°C. Three hundred microliters of the upper aqueous phase was transferred to a new tube. Glycogen (5 mg/mL; Invitrogen, San Diego, CA, USA) for miRNA precipitation was added to this aqueous phase before being mixed with 1000 µL of cold 100% molecular grade ethanol. The tube was vortexed for thirty seconds and incubated at − 80 °C for one hour to allow miRNA precipitation. After precipitation, samples were transferred to a Qiagen RNeasy® Mini spin column in a collection tube followed by centrifugation at 15,000 × g for thirty seconds at room temperature. The Qiagen RNeasy® Mini spin column was rinsed with 700 µL Qiagen RWT buffer and centrifuged at 15,000 × g for thirty seconds at room temperature. This was followed by another rinse with 500 µL Qiagen RPE buffer and centrifugation at 15,000 × g for thirty seconds at room temperature. Eighty percent ethanol was then added to the column and centrifuged at 15,000 x g for one minute at room temperature. The column was then transferred to a new microcentrifuge tube and and centrifuged according to the manufacturer's instructions in order to dry the membrane. The column was again transferred to a new microcentrifuge tube with the lid left uncapped for one minute to allow the column to dry further. Eighteen microliters

of RNase-free pre-warmed (+65°C) water was then added to the dry column. After one minute of incubation, total EV-miRNA was eluted by centrifugation at 15,000 × g for one minute. The samples with total EV-miRNA were stored in a freezer at −80°C. The concentration and purity of the EV-miRNA samples were measured using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE).

RT and qPCR reactions:

EV-miRNA samples were diluted with RNase-free water to a concentration of 5 ng/μL as instructed by the miRCURY® LNA® RT Kit protocol (Qiagen, Hilden, Germany). 5x miRCURY RT Reaction Buffer, 10x miRCURY RT Enzyme Mix, and UniSp6 synthetic spike-in control were added to each template RNA in accordance with the manufacturer's instructions. Ten microliters of reaction mixture were loaded into the Biometra TRIO PCR Thermal Cycler. Samples were incubated at 42°C for 90 minutes, then incubated at 95°C for five minutes and then immediately cooled at 4°C.

For qPCR analysis of differential miRNA expression between the four toxicity groups, the miRCURY LNA™ miRNA Serum/Plasma PCR Panel (Qiagen, Hilden, Germany) was used. The panel contains 2x179 LNA miRNA primer sets commonly found in human serum and plasma. Each panel also contains sets of negative controls (H_2O), and five sets of the following RNA Spike-in controls: UniSp-2-4-5 (concentration ratio 10000:100:1) – for control of RNA purification; UniSp6 – for control of cDNA synthesis; UniSp3 – for inter-plate calibration. The amplification was performed in a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA) in 384 well plates. The plates are produced in ready-to-use format: primer sets are dispensed and lyophilized in the wells in amounts sufficient for one 10 μL reaction per

well. The Serum/Plasma Focus miRNA PCR Panels also contain seven potential reference genes: a) miR-103-3p, miR-191-5p and miR-423-5p; b) miR-93-5p and miR-425-5p: these potential reference genes are chosen because they are usually stably expressed in serum/plasma; c) miR-451a and miR-23a-3p: these can be used as a control for hemolysis. If ΔCt(miR-23a-3p – miR-451a) is >7, it may be an indication of excessive hemolysis.

All cDNA samples of each group were pooled together. The miRCURY LNA™ miRNA Serum/Plasma PCR Panel was run with equal amounts of the pooled cDNA samples for each group according to the manufacturer's instructions. The real-time PCR data was normalized by ROX (carboxy-X-rhodamine) passive reference.

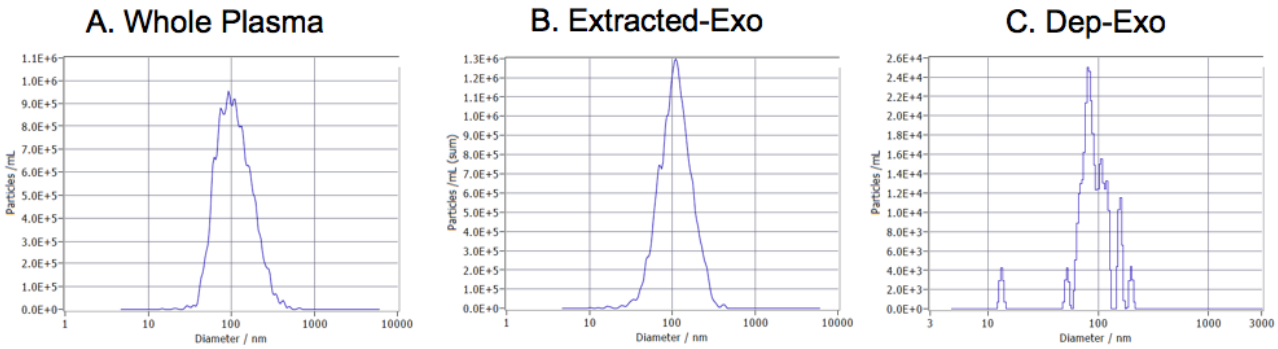
Data Analysis:

The amplification curves were analyzed using the QuantStudio™ Design & Analysis Software v1.4.2, both for determination of Ct values and for melting curve analysis. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for each particular assay.

RESULTS:

EVs were precipitated from 0.5 mL plasma samples and reconstituted in 0.2 mL of 1xPBS buffer. The Nanoparticle Tracking Analysis demonstrated that EV concentration in the native plasma before exosome precipitation was equal to $1.9x10^{11}$ particles/mL, and the concentration of EVs after precipitation and resubmission in 0.2 mL of 1xPBS buffer was equal to $3.5x10^{11}$ particles/mL (Figure 1). Analysis of the plasma sample after EV extraction showed a significant decrease in the concentration of EVs; the concentration of this EV-depleted plasma was $3.7x10^{5}$ particles/mL (Figure 1). This means that >99% of the EVs were effectively precipitated from the plasma samples. If 100% of EVs had been collected from 0.5 mL of plasma into 0.2 mL of 1xPBS then the concentration factor would be 2.5, and the EV concentration in 1xPBS buffer would be equal to $4.75x10^{11}$ particles/mL. However, the final concentration of the extracted EVs was $3.5x10^{11}$ particles/mL, indicating that ~74% of precipitated EVs were released from the precipitation pellet, and ~26% remained trapped. These results are comparable to the published literature results for EV isolation from plasma using the PureExo® Exosome Isolation Kit.[25] Based on these results and those from previously published literature, the EVs isolated are thought to primarily consist of exosomes.[4,11]

Figure 1. Nanoparticle tracking analysis (ZetaView®) exosome concentration and size representation in the plasma sample: (A) whole plasma before exosome isolation; (B) fraction of the extracted exosomes (extracted-Exo); (C) exosome-depleted plasma (dep-Exo).



Original Concentration: 1.9E+11 Particles / mL

X Values (all sizes are given in nm)

	Number	Concentration	Volume
X10	59.2	59.2	117.3
X50	103.4	103.4	227.4
X90	200.7	200.7	427.6
Span	1.4	1.4	1.4
Mean	122.1	122.1	262.4
StdDev	65.2	65.2	134.3

Original Concentration: 3.5E+11 Particles / mL

X Values (all sizes are given in nm)

	Number	Concentration	Volume
X10	60.7	60.7	107.7
X50	107.3	107.3	188.6
X90	189.3	189.3	296.7
Span	1.2	1.2	1.0
Mean	119.7	119.7	202.8
StdDev	54.4	54.4	80.7

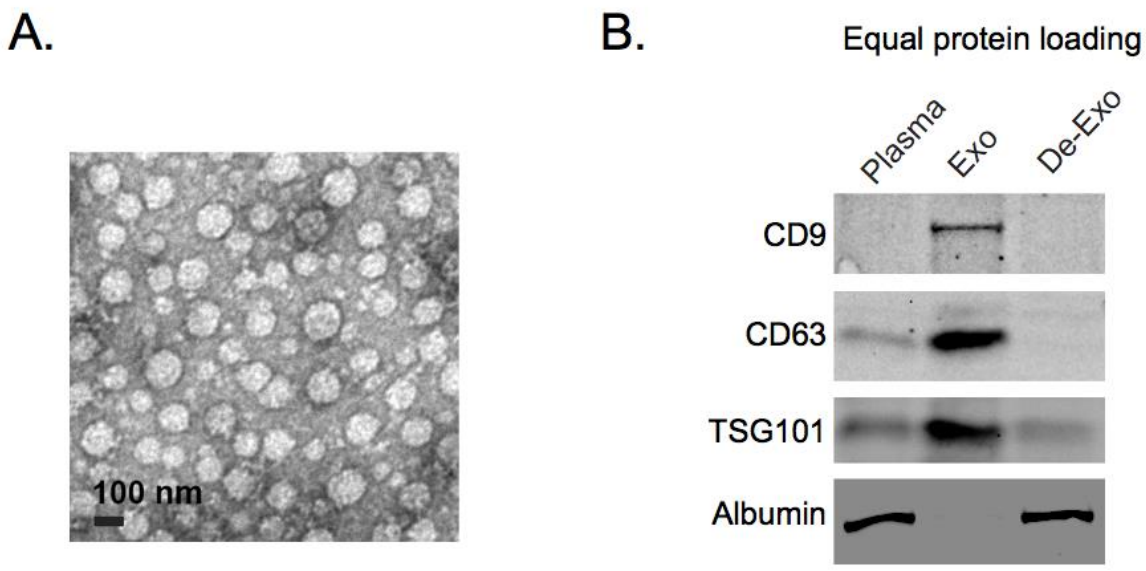
Original Concentration: 3.7E+5 Particles / mL

X Values (all sizes are given in nm)

	Number	Concentration	Volume
X10	65.3	65.3	79.2
X50	85.8	85.8	119.2
X90	150.8	150.8	193.6
Span	1.0	1.0	1.0
Mean	95.3	95.3	132.7
StdDev	34.2	33.7	41.4

Isolated EVs were additionally characterized by transmission electron microscopy (TEM) and Western blot (WB) analysis of the specific EV markers. The TEM image of the isolated EVs demonstrated multiple spherical particles with the size range close to 100 nm (Figure 2A). WB analysis was performed for the equal amounts of total protein form the native plasma, isolated EVs, and exosome-depleted plasma. There was significant accumulation of the EV markers CD9, CD63, and TSG101 in the isolated EV sample compared with the native plasma and EV-depleted plasma samples (Figure 2B). Isolated EVs also showed significantly lower levels of the plasma protein albumin in comparison with whole plasma and EV-depleted plasma, which indicates a low contamination of isolated EVs with plasma proteins (Figure 2B). MiRNA purification from EV samples produced an average concentration of 18.51±5.29 ng/μL. Each miRNA sample was eluted in 18μL, thus an average of 333.22±90.13 ng of miRNA was purified from each EV sample. The 260/280nm absorbance ratio for each miRNA sample was between 1.7-2.0. Previous studies which have also used the MiRNeasy Micro Kit system have shown similar effectiveness for RNA purification.[27,28]

Figure 2. Analysis of the extracted exosomes. (A) Transmission electron microscopy image of the extracted exosomes; bar = 100nm. (B) The expression of CD9, CD63 and TSG101, as well as albumin in the isolated particles was determined by western blotting. Lanes from left to right are whole plasma (before exosome extraction), fraction of the extracted exosomes (Exo), and fraction of the exosome-depleted plasma (de-Exo) respectively.



After cDNA synthesis, equal amounts of each sample from the same toxicity group were pooled together for each panel. The pooled cDNA samples for each group were analyzed for expression of 179 miRNAs commonly found in serum/plasma samples with SYBR® Green Master Mix. Melting curve analysis demonstrated single distinct peaks in the plots for the negative derivative of fluorescence vs. temperature for each probe, including Spike-in controls (Figures 3, 4A). Results from RT-PCR revealed a total of 24 miRNAs that were differentially expressed between the previously described toxicity groups (Table 1). Additionally, five miRNAs were identified as potential normalization controls, as they demonstrated very close expression levels for all groups. Some of these miRNAs (e.g. miRNA-16-5p) have been previously reported as reliable internal controls for extravesicular miRNA analysis[29]. Spike-in controls 2-4-5 showed uniform expression levels across all four toxicity groups, which exhibit ratios close to the ratios in which they were added to the EV samples (Figures 4B, 4C). The ideal ratio for the Unisp-2-4-5 is 10000:100:1. The ratio obtained for the four pooled samples was 10000:68.46±2.32:0.5±0.03 (Figure 4).

Figure 3. Melting curves for 24 miRNAs demonstrated a high potential in the prediction of

post-RT toxicity of breast cancer patients and five miRNAs as potential normalization controls.

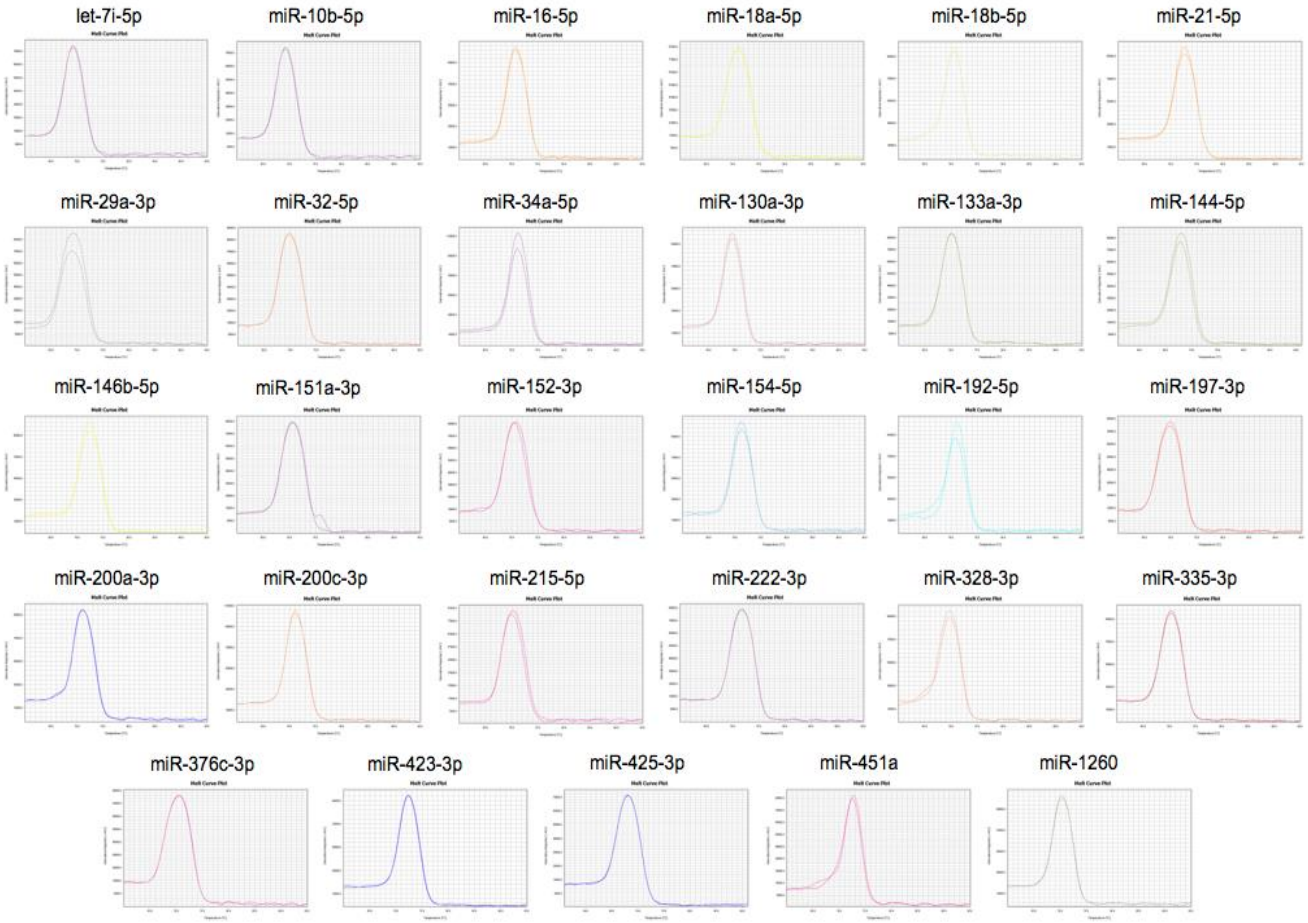


Table 1. **Potential miRNA candidates for prediction of post-RT normal tissue toxicity and**

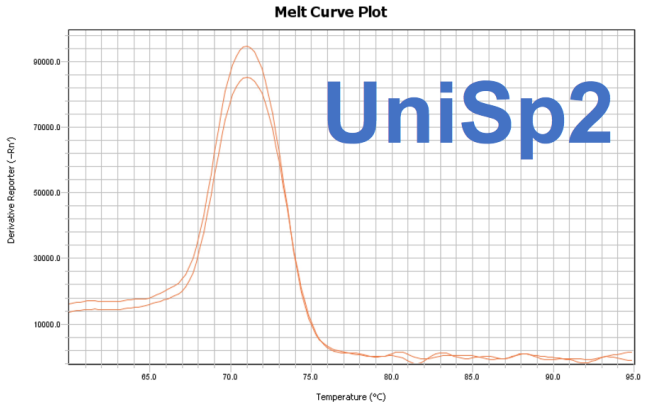
potential normalization controls. MiRNA-165p was used as a normalization control to calculate ΔCt values.

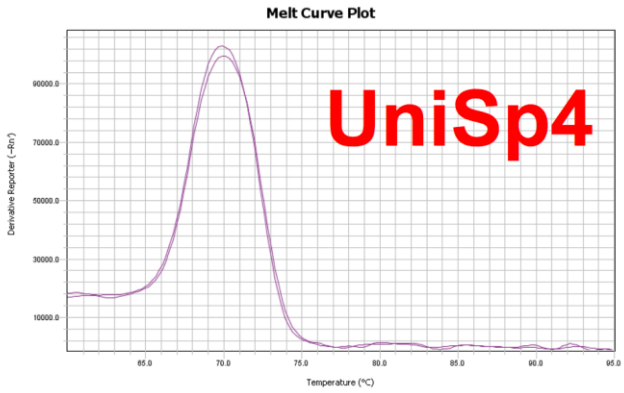
Target	Normalized by miR-16-5p				
	Group 1	Group 2	Group 3	Group 4	
	ΔCt-value	ΔCt-value	ΔCt-value	ΔCt-value	
hsa-miR-10b-5p	Undetermined	7.462	5.471	4.397	Group 1 prediction
hsa-miR-133a-3p	Undetermined	7.258	5.389	4.554	
hsa-miR-144-5p	Undetermined	8.878	6.848	7.697	
hsa-miR-152-3p	Undetermined	8.646	5.736	5.742	
hsa-miR-18a-5p	Undetermined	7.106	5.320	6.666	
hsa-miR-197-3p	Undetermined	7.351	6.345	7.401	
hsa-miR-200c-3p	Undetermined	8.933	5.113	5.669	
hsa-miR-222-3p	Undetermined	7.209	6.338	4.071	
hsa-miR-29a-3p	13.318	5.531	4.362	3.545	
hsa-miR-32-5p	Undetermined	5.319	5.789	5.946	
hsa-miR-130a-3p	3.676	5.448	17.410	4.745	Group 3 prediction
hsa-miR-151a-3p	4.819	8.215	Undetermined	6.534	
hsa-miR-192-5p	3.462	7.222	Undetermined	6.032	
hsa-miR-200a-3p	Undetermined	Undetermined	6.551	Undetermined	
hsa-miR-215-5p	3.648	7.874	Undetermined	6.031	
hsa-miR-34a-5p	4.508	6.778	Undetermined	4.582	
hsa-miR-423-3p	5.120	6.043	Undetermined	5.737	
hsa-miR-425-3p	5.618	8.072	Undetermined	5.585	
hsa-miR-146b-5p	10.520	11.249	20.097	Undetermined	Groups 3 and 4 prediction
hsa-miR-154-5p	12.010	8.527	Undetermined	Undetermined	
hsa-miR-18b-5p	8.213	8.243	16.523	Undetermined	
hsa-miR-328-3p	4.937	6.627	13.441	21.470	
hsa-miR-335-3p	8.761	8.399	18.274	19.023	
hsa-miR-376c-3p	Undetermined	Undetermined	18.647	5.776	
hsa-miR-16-5p	0.000	0.000	0.000	0.000	Normalization controls
hsa-miR-451a	-1.093	-0.880	-1.767	-1.676	
hsa-let-7i-5p	4.405	5.870	4.041	5.506	
hsa-miR-21-5p	1.086	2.024	0.900	0.878	
hsa-miR-1260a	1.144	2.173	0.946	0.949	

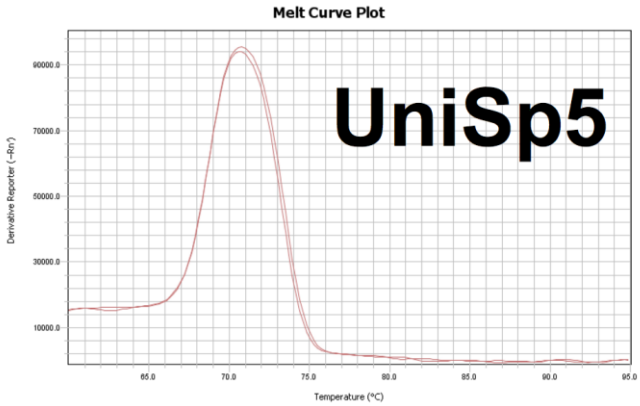
Figure 4. UniSp2-4-5 expression analysis: (A) Melting curves for UniSp2-4-5 spike-in control

RNAs. (B) Amplification plots of UniSp2-4-5 for all four groups. (C) Relative expression of UniSp2-4-5 for Groups 1-4.

A.







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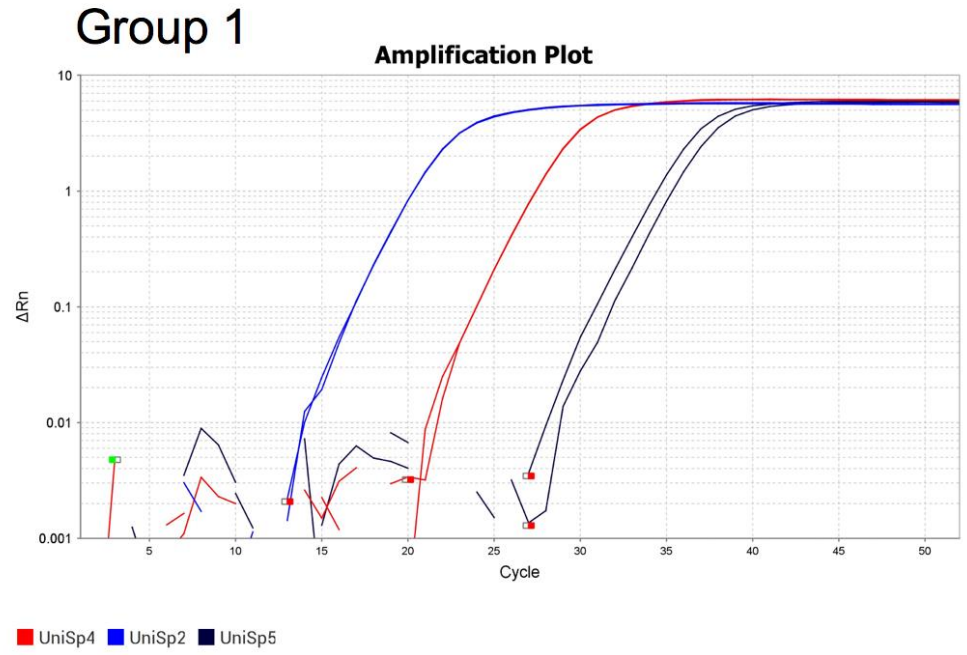
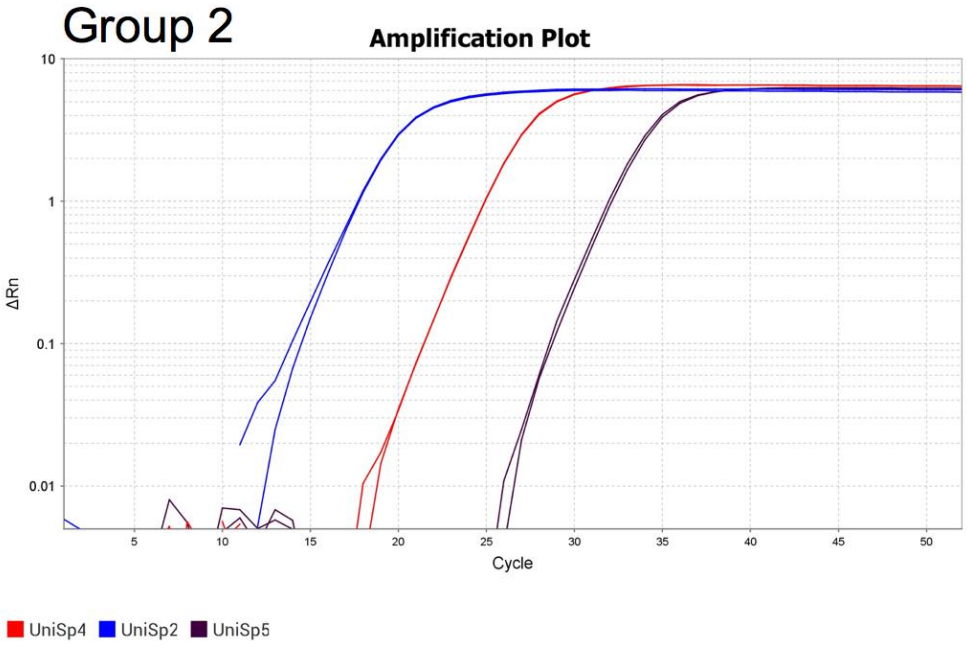
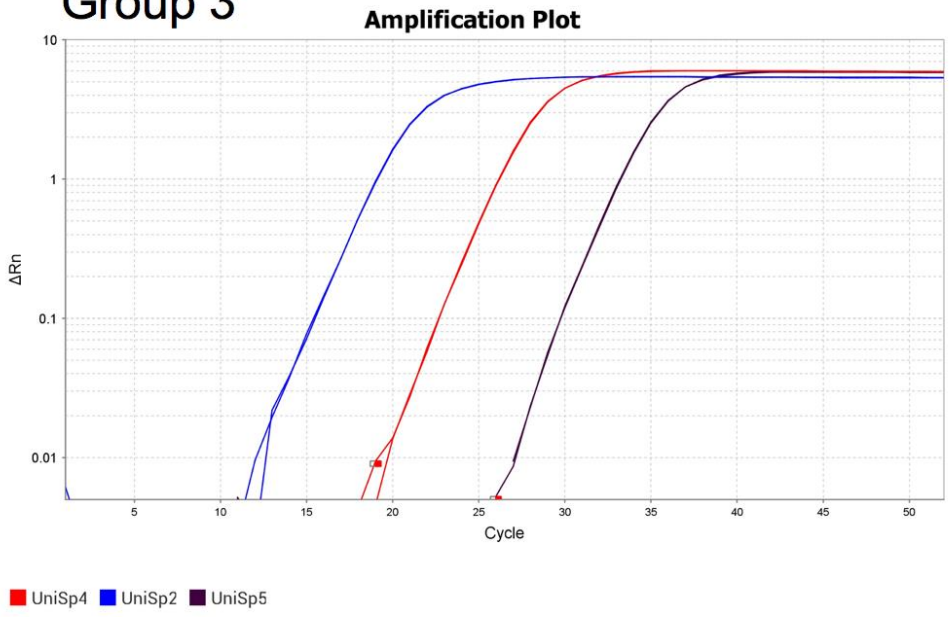
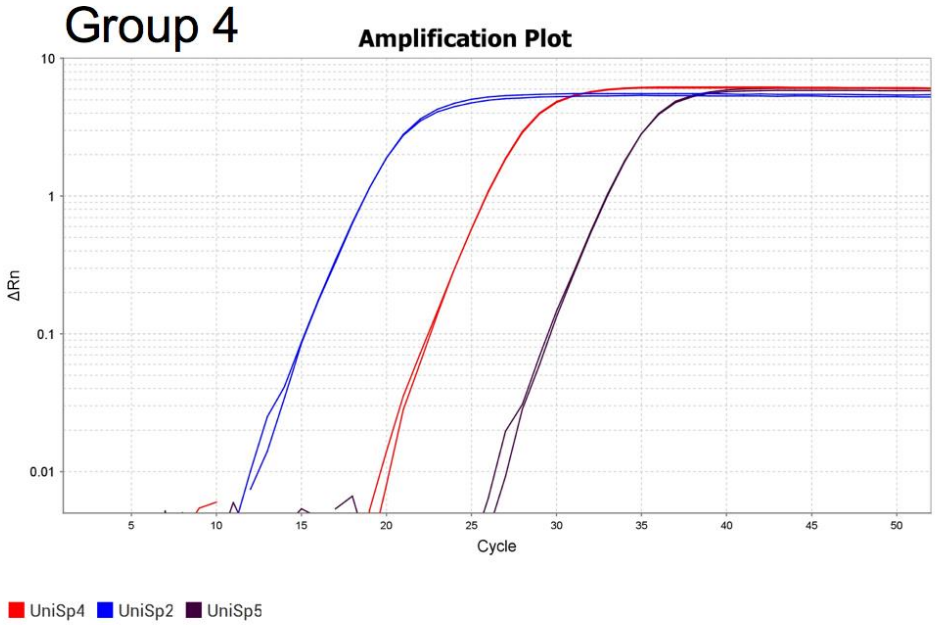

Group 1
Amplification Plot
ΔRn
Cycle
UniSp4
UniSp2
UniSp5


Group 2
Amplification Plot
ΔRn
Cycle
UniSp4
UniSp2
UniSp5

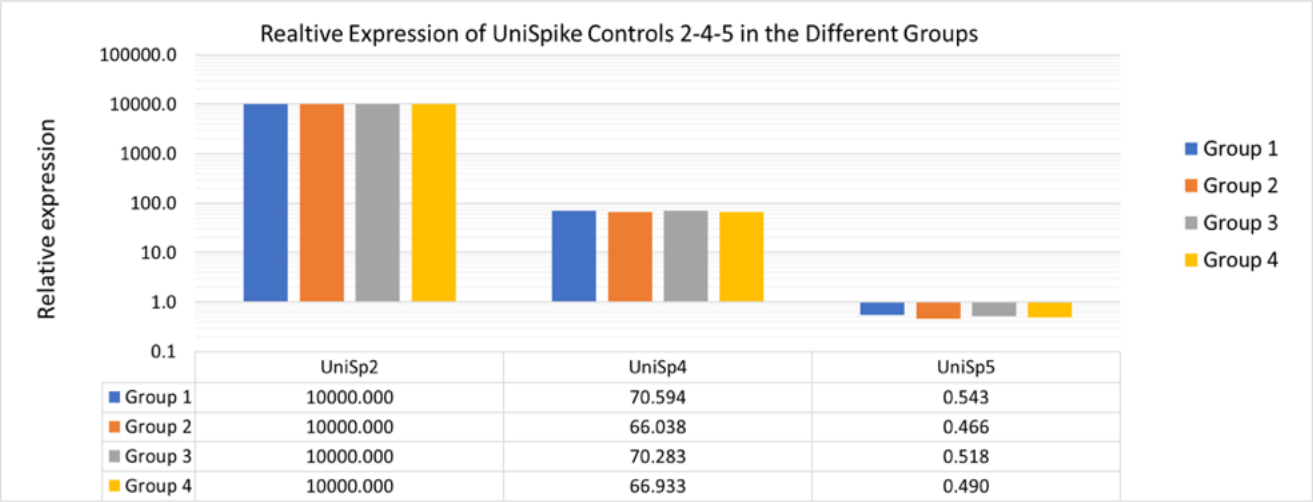
Group 3



Group 4



C.



	UniSp2	UniSp4	UniSp5
Group 1	10000.000	70.594	0.543
Group 2	10000.000	66.038	0.466
Group 3	10000.000	70.283	0.518
Group 4	10000.000	66.933	0.490

Although the relative expression ratios for Unisp2-4-5 indicates that less concentrated miRNAs were purified with somewhat less efficiency than more highly concentrated miRNAs, the very small deviation between the pooled samples demonstrates a very high accuracy and reproducibility of this miRNA purification method. Expression of EV-miRNAs for all toxicity groups were normalized by Spike-in controls, and ΔCt values were calculated by using miRNA-16-5p as an internal control. MiRNAs that demonstrated significant expression differences between the pooled samples were divided into four different groups (Table 1). As potential predictors for Group 1, nine miRNAs (miR-10b-5p, miR-133a-3p, miR144-5p, miR-152-3p, miR-18a-5p, miR-197-3p, miR-200c-3p, miR-222-3p, miR32-5p) showed negligible expression, whereas Groups 2, 3, and 4 showed relatively high and similar expression for each of these miRNAs. The same tendency in expression was demonstrated for miR-29-5p. The ΔCt value for this miRNA was 13.31 for Group 1, 5.53 for Group 2, 4.36 for Group 3, and 3.55 for Group 4.

Eight miRNAs were identified as prediction candidates for Group 3. Six miRNAs (miR-151a-3p, miR-192-5p, miR-215-5p, miR-34a-5p, miR-423-3p, miR-425-3p) showed no expression, whereas Groups 1, 2, and 4 showed relatively high expression for all these miRNAs. Expression of miR-130a-3p was significantly lower in Group 3 (ΔCt value = 17.41) compared to Group 1 (ΔCt value = 3.68), Group 2 (ΔCt value = 5.45), and Group 4 (ΔCt value = 4.75). MiR-200a-3p demonstrated a relatively high level of expression (ΔCt value = 6.55) whereas expression of the same miRNA in Groups 1, 2 , and 4 was undetermined.

Additionally, six EV miRNAs were identified as potential biomarkers for both Groups 3 and 4. MiR-146b-5p, miR-154-5p, miRNA-18b-5p, and miR-335-3p demonstrated significantly lower or negligible expression levels in Groups 3 and 4 compared to Groups 1 and 2. Mir-376c-3p showed no expression in Groups 1 and 2 with moderate expression in Group 3 (ΔCt value = 18.65) and a high expression level in Group 4 (ΔCt value = 5.78). Interestingly, only mir-328-3p demonstrated a significant expression difference for all four groups with a steady increase in ΔCt from Group 1 to Group 4: 4.94, 6.63, 13.44, and 21.47, respectively.

Finally, five miRNAs were identified as potential normalization controls: miR-16-5p, miR-451a, let-7i-5p, miR-21-5p, and miR-1260a.

DISCUSSION

The ZetaView® Nanoparticle Tracking Analysis (NTA) results show isolated particles with an average diameter characteristic of extracellular vesicles. Based on adherence to the PureExo® kit protocol and the size distribution of the particles, it is suspected that the isolated particles are primarily exosomes, but this cannot be determined from NTA results alone. Taken together with our western blot results, the identity of the isolated EVs can more clearly be elucidated. The concentration of classical exosome markers CD9, CD63, and TSG101 in the isolated EV fraction appeared much higher compared to the whole plasma and exosome-depleted fractions. It should, however, be noted that these proteins are present to some degree in other subpopulations of EVs and can also be found freely circulating in plasma.[17] Furthermore, although our observed particle size and concentration of exosomes are similar to some existing publications, varying results have also been published. While some research has claimed exosome concentration to range from $0.88x10^8$ to 13.38×10^8 exosomes/mL of plasma, others have reported that between $8.35x10^9$ and $10.9x10^9$ exosomes/mL are found in breast cancer patients, with particle size averaging 95nM for patients with localized tumors and 125nM for those with metastasis.[26,31] One likely explanation for the differences in reported concentrations is the use of different isolation methods.[32,33] In a study that utilized the PureExo® system with serum from healthy donors, only 7.1×10^7 particles per 0.5mL were obtained.[25] This highlights other factors that may cause differences in the concentrations and morphology of isolated EVs, such as extraction from serum versus plasma, differences in the pathological state of the patients from which the samples are obtained, differences in the particle analysis methods utilized, and

variations in adherence to isolation protocol.[34,35] In the present study, the PureExo® isolation kit was chosen as a polymer precipitation method for isolating exosomes with high enough purity suitable for miRNA purification and PCR analysis.[25,36]

According to the qPCR results, miRNA purification of EV samples was performed efficiently, with qPCR curves for UniSp-2-4-5 showing an expression ratio close to the expected 10000:100:1 ratio with little variation between samples. Furthermore, the 260/280 absorbance spectra for each purified sample was within the range accepted as pure for RNA isolation.[37] The MiRNeasy Micro Kit protocol is a phenol/chloroform-based extraction method that is followed by purification of small RNAs by filtration of remaining contaminants through a silica membrane spin column.[27] MiRNAs are then eluted from the column using RNase-free water. This system has been shown in previous publications to isolate miRNA of higher quality compared to other kits, which is important for downstream PCR applications.[27] Furthermore, the miRCURY LNA™ miRNA Serum/Plasma PCR Panel was chosen for miRNA expression analysis as it screens for 179 miRNAs of which are known to be expressed in plasma and are of particular interest based on previously published research.[38]

The 24 miRNAs that have been identified in this study as being differentially expressed between the four radiotoxicity groups may serve as candidates for predictors of radiotoxicity in breast cancer patients, while miR-16-5p, miR-451a, let-7i-5p, miR-21-5p, or miR-1260a may serve as normalization controls. Of these 29 total miRNAs, several have been previously studied in the context of breast cancer. A few have been explored in relation to chemotherapy resistance and even fewer have been mentioned regarding irradiation response. For example, miR-18-a has been determined to cause radiosensitizing effects in lung cancer stem-like cells, however expression was analyzed from whole plasma rather than EVs.[39] Perhaps this miRNA is also

responsible for sensitizing healthy tissue to irradiation in breast cancer, as our data showed that this miRNA was not expressed in patients with minimal reaction to radiation compared to those who showed normal to severe effects. The presented data also determined that miR-222-3p is downregulated in Group 1. This miRNA has been previously described as a "double-edged sword" because low expression of this miRNA in serum was associated with higher response to Trastuzumab and therefore better survival rates in HER-2 positive patients, but simultaneously promotes cardiotoxicity.[40] Perhaps this miRNA plays different roles in response to chemotherapy compared to radiation, as it has also been noted by previous publications that the exact role of miR-222-3p requires further clarification.

The only miRNA expressed at detectible levels in patients with minimal toxicity was miR-29a-3p, although it was still expressed at lower levels compared to Groups 2-4. In the context of breast cancer, this miRNA is thought to be associated with poor patient prognosis by acting as an upstream regulator of SETDB1 in the BRCA gene.[41] Its response to irradiation has also been examined in oral squamous cell carcinoma cell lines, whereby it appears to enhance tumor cell sensitivity to radiotherapy.[42] These studies do not examine miRNA expression in vivo, but it is possible that the increased expression of this miRNA in Groups 2-4 reflects its function in enhancing healthy tissue sensitivity to radiotherapy.

Of the miRNAs that differentiate Group 3 from the other three groups, miR-34a-5p has been recognized by previous publications regarding its response to irradiation in breast cancer patients. It was found that the concentration of this miRNA from isolated leukocytes increases in response to radiation and appears to play a role in tumor growth suppression by inhibiting double-stranded break repair.[43] MiR-34a-5p was least expressed in Group 2 patients, followed by Groups 1 and 4, and was not expressed in Group 3. Further research is necessary in order to

better understand the role of miR-34a-5p that is causing this differential expression.

Six miRNAs showed similar expression in Groups 3 and 4 compared to patients who experience minimal or moderate toxicity. For example, miR-328-3p has been associated with the suppression of breast cancer progression as well as the sensitization of non-small cell lung cancer and osteosarcoma to radiotherapy.[44,45] Perhaps the mechanisms by which this miRNA sensitizes tumor tissue to irradiation contrast to those that sensitize healthy tissue to radiotoxicity since this miRNA was expressed significantly less in Groups 3 and 4 compared to Groups 1 and 2. These studies, however, analyze miRNA expression from tumor tissue and nearby non-tumor tissues rather than circulating EVs. This averts the focus away from miRNAs that are transported in the blood to mediate communication between cells, and instead limits the scope of study to miRNAs which may only be expressed within their cell of origin as a consequence of exposure to radiation. This, therefore, does not contribute to data that is needed in order to establish a reference for liquid biopsy.

The exact causes for higher or lower expression of particular miRNAs in certain patients compared to others prior to irradiation are largely unknown, but it does appear that patients who exhibit minimal tissue response to irradiation naturally produce lower levels of EV miRNA that influence irradiation responses such as acute inflammation, tissue fibrosis and vascular damage. The exact mechanisms by which these miRNAs influence tissue response also requires further research. It is has been demonstrated that miR-222, for example, influences inflammation-mediated neovessel formation.[21] Its upregulation has been associated with antiproliferation and apoptosis in endothelial cells, while promoting the opposite effects in smooth muscle cells.[21] Esplugas et al. determined that miR-222, as well as miR-146a, -155 and -221 were increased in association with cardiotoxicity in breast cancer patients, supporting previous findings that these

miRNAs promote inflammation, oxidative stress, and atherosclerosis.[21] In our presented data, EV miR-222-3p was not detected in Group 1, but was expressed at comparable levels for Groups 2-4. Together with previously published literature, this suggests that the miR-222 cluster of miRNAs targets mRNAs which influence protective effects in normal tissue. Identifying the respective mRNA targets of particular miRNAs would provide insight as to their mechanism of action and perhaps why expression levels vary between individuals before or after radiotherapy.

Several previous studies have described particular miRNAs as endogenous control candidates for data normalization. In particular, let-7a-5p, miR-21-5p, and miR-16-5p have shown stable expression in healthy, benign, and malignant tissue.[29] Our data determined miR-16-5p to be the most stably expressed, which has been deemed the best endogenous control particularly for the study of metastatic breast cancer.[29] These miRNAs are deemed "housekeepers," meaning that they are responsible for basic cellular functioning and therefore highly conserved and equally expressed in most cells.[46] This provides a standard by which to adjust expression data for biological and analytic variations.[46]

The importance of the data obtained from this study is the identification of new potential biomarkers for radiotoxicity response. This may ultimately help predict patient response to radiotherapy and therefore help optimize patient treatment plans accordingly. As pointed out by studies that address tumor response to radiation, it may be futile to administer radiotherapy to patients whose tumors express miRNAs associated with tumor radioresistance.[3] Our findings take this notion a step further by considering that perhaps patients with particular miRNAs associated with severe levels of toxicity in addition to those associated with tumor resistance might avoid or at least limit radiotherapy as a primary form of treatment.

There is also a potential future in the use of miRNA as a therapeutic agent for sensitizing tumor tissues to radiation while protecting surrounding tissues. Administration of miRNAs associated with minimal adverse reaction or perhaps inhibiting those that promote toxicity are possible avenues for therapy.[47] Electroporation and transfection are two mechanisms by which specific miRNAs or other therapeutic drugs may be loaded into exosomes.[48] In addition to exosomes, bioengineered non-viral nanoparticles (NPs) offer a biocompatible and easily produced method for nucleic acid delivery. These include inorganic NPs such as gold nanoparticles as well as organic NPs such as micelles and lipid NPs.[49] However, there are several challenges that must be overcome before the widespread implementation of vesicle-delivered, miRNA-based therapy. Namely, the exact mechanism of exosome recognition and uptake by its intended cell targets is not well known.[12] Furthermore, miRNAs have multiple mRNA targets, and their roles in various tissues are not always well known. Therefore, it is possible that delivering or inhibiting specific miRNAs for therapeutic benefit could also have unintended negative side effects.[50]

In order for any application of the presented data to be implemented, further studies must be conducted. Performing PCR for each individual patient sample is needed to verify the presented results due to the possibility of outlier samples skewing the data. After ruling out particular miRNAs based on this information, miRNAs that remain statistically significant require further study to determine their role and significance in radiotoxicity response. Furthermore, the data must be normalized for variables such as patient age, race, cancer stage, adjuvant treatment, and other health conditions. Subsequent experiments following the same protocol but with plasma samples obtained during and after treatment should also be performed. This may provide valuable information regarding the physiological processes that occur in

response to radiation treatment. This would likely yield interesting results especially considering that Stepanović et al. determined that the most significant alteration in miRNA expression in response to radiotherapy occurs halfway through radiation treatment in glioblastoma patients.[7] Their study found that baseline levels of non-EV miR-21 in plasma were higher in patients who exhibited toxicity response in normal tissue, with expression levels significantly increased halfway through radiation treatment. Patients with lower circulating levels of miR-21 before radiotherapy had better outcomes in their normal tissue response to radiotherapy, whereas patients with higher circulating levels prior to treatment appeared to experience toxic effects as a result of the increase in miR-21 that occurs in response to treatment. Furthermore, higher baseline levels of miR-10b were observed in patients exhibiting toxicity compared to patients without toxicity. MiR-10b levels for patients with toxicity slightly decreased halfway through irradiation treatment. Interestingly, levels of expression in patients without toxicity response were decreased at this time point, but were increased after the last treatment dosage to levels very close to that of baseline for toxicity patients.[7] Considering that the ΔCt values for miR-10b-5p were much higher for Groups 2-4 compared to Group 1, it would be interesting to analyze BC patient samples obtained after radiation to see if the same trend is observed.

As of present, data that is closely comparable to that which is presented in this paper has not been published. Studies which have investigated miRNA biomarkers indicative of radiotoxicity in breast cancer patients use different methods for purification and analysis or do not evaluate miRNAs isolated from EVs. Furthermore, existing studies evaluate patients who receive different radiotherapy dosages and schedules, and do not evaluate toxicity on the same basis. For example, Esplugas et al. focus strictly on cardiotoxicity associated with circulating miRNAs that are not contained within EVs.[21] Other publications may evaluate patients who do

or do not exhibit radiotoxicity in general, but they do not differentiate between patients who experience acute versus chronic effects.[7] In turn, this has likely decreased the number of comparable publications available. Reproducibility is a frequent challenge in exosomal miRNA research given the plethora of isolation methods and materials available.[51] Additionally, there are no universally accepted miRNAs for use as normalization controls.[51] These challenges as well as many others must be met before the possibility of clinical applications.

The data presented in this study provides the initial findings necessary for further examination in this area of exosomal miRNA research. Twenty-four miRNAs that are differentially expressed between groups of breast cancer patients based on the response of their normal tissue to radiotherapy have been identified, as well as five miRNAs that are stably expressed and may serve as normalization controls. It would be particularly interesting to further examine some of the miRNAs discussed above which have also been shown in previous publications to play a role in radiotherapy response. Future studies using the same protocol implemented in this research would contribute significantly to the lack of comparable studies and may therefore expand upon and affirm the results of the presented data.

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VITA

Mina McGinn was born in Westerly, Rhode Island on February 10, 1994. She has lived in the greater Richmond, Virginia area since 2005. After completing her Bachelor of Science Degree at Virginia Commonwealth University in 2016, Mina began research in the lab of Dr. Martin Safo in the Department of Medicinal Chemistry at VCU where she assisted with the identification of antisickling molecules for the treatment of sickle cell disease. Her contributions focused on the modification of hemoglobin structure in response to the binding of allosteric effectors. Her experience also concentrated on the interaction between hemoglobin and 5-HMF, as well as its further clinical applications.

Mina also has seven years of clinical background in dentistry. She worked passionately as a dental assistant for Biggers Family Dentistry in Midlothian, Virginia from 2016 - 2020 and has continued to volunteer at the Hanover Interfaith Free Dental Clinic since 2018. In 2021, Mina earned her Premedical Graduate Sciences Certificate at VCU with the intent of pursuing a degree in dentistry following the completion of her Master of Science in Biochemistry. She aims to ultimately integrate her research and clinical experiences into her professional career in the dental field.