Exploring miRNA-target gene profiles associated with drug resistance in patients with breast cancer receiving neoadjuvant chemotherapy

MIN WOO KIM*, SOL MOON*, SUJI LEE, HYOJUNG LEE, YOUNG KIM, JOON YE KIM, JEE YE KIM and SEUNG IL KIM

Department of Surgery, Yonsei University College of Medicine, Seoul 03722, Republic of Korea

Received November 28, 2023; Accepted January 24, 2024

DOI: 10.3892/ol.2024.14291

Abstract. Exosomal microRNAs (miRNAs) are closely related to drug resistance in patients with breast cancer (BC); however, only a few roles of the exosomal miRNA-target gene networks have been clinically implicated in drug resistance in BC. Therefore, the present study aimed to identify the differential expression of exosomal miRNAs associated with drug resistance and their target mRNAs. *In vitro* microarray analysis was used to verify differentially expressed miRNAs (DEMs) in drug-resistant BC. Next, tumor-derived exosomes (TDEs) were isolated. Furthermore, it was determined whether the candidate drug-resistant miRNAs were also significant in TDEs, and then putative miRNAs in TDEs were validated in plasma samples from 35 patients with BC (20 patients with BC showing no response and 15 patients with BC showing a complete response). It was confirmed that the combination of five exosomal miRNAs, including miR-125b-5p, miR-146a-5p, miR-484, miR-1246-5p and miR-1260b, was effective for predicting therapeutic response to neoadjuvant chemotherapy, with an area under the curve value of 0.95, sensitivity of 75%, and specificity of 95%. Public datasets were analyzed to identify differentially expressed genes (DEGs) related to drug resistance and it was revealed that *BAK1*, *NOVA1*, *PTGER4*, *RTKN2*, *AGO1*, *CAP1*, and *ETS1* were the target genes of exosomal miRNAs. Networks between DEMs and DEGs were highly correlated with mitosis, metabolism, drug transport, and immune responses. Consequently, these targets could be used as predictive markers and therapeutic targets for clinical applications to enhance treatment outcomes for patients with BC.

Correspondence to: Professor Jee Ye Kim or Professor Seung Il Kim, Department of Surgery, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea
E-mail: jeeye0531@yuhs.ac
E-mail: skim@yuhs.ac

*Contributed equally

Key words: miRNA, exosomes, gene profile, drug resistance, breast cancer, neoadjuvant chemotherapy

Introduction

Pathological complete response (pCR) refers to the complete absence of cancer cells from both primary and metastatic tumors after neoadjuvant chemotherapy (NAC). pCR is the optimal outcome for patients with breast cancer (BC). However, numerous patients with BC show resistance to NAC, with only a minority of the patients (20-25%) attaining pCR (1,2). Identifying prognostic indicators of drug resistance is essential for predicting tumor response and clinical outcomes (3). Despite reports of drug resistance mechanisms, the mechanisms underlying NAC resistance remain to be fully elucidated (4-6). Additionally, reliable biomarkers for predicting pCR are lacking, which complicates the effective assessment of treatment outcomes.

Numerous mechanisms regarding drug resistance have been proposed, including alterations in drug transport, metabolism, DNA repair, and cell signaling pathways (7,8). However, the mechanisms underlying NAC resistance remain unclear and are likely to be multifactorial. Recently, several microRNAs (miRNAs) were discovered to be significantly upregulated in drug-resistant cancer cells, emerging as potential biomarkers for evaluating tumor responses to treatment (9,10). MiRNAs regulate gene expression by targeting multiple mRNAs for degradation or translational inhibition (11). They are the most abundant biomarker sources found in exosomes, for example, as cell-to-cell signal transporters; they play a critical role in the pathogenesis of BC by regulating target gene networks involved in key processes, such as cell proliferation, apoptosis, and metastasis (12). Dysregulated exosomal miRNAs that reach adjacent and distant cancer cells and regulate drug resistance-related genes can potentially serve as predictive indicators of tumor responses to treatment.

Numerous studies have demonstrated the involvement of miRNAs in the development and progression of drug resistance in cancer (13,14). However, a number of unidentified miRNAs, target genes and molecular mechanisms remain associated with drug resistance. Therefore, the construction of a miRNA-mRNA network that uncovers the mechanisms

underlying drug resistance could help identify potential novel biomarkers for improving the prediction accuracy of tumor response to treatment. In the present study, tumor-derived exosomes (TDEs) were profiled in the plasma using liquid biopsy to identify the specific molecules or signatures responsible for transferring drug resistance. The rationale of the authors of the present study was based on the understanding that exosomes transport diverse molecules, including proteins and nucleic acids, which influence the behavior of recipient cells (15). Given the well-documented cooperativity of exosomal miRNAs that regulate tumorigenesis and patient survival in numerous cancers (16), miRNAs and their target gene networks could potentially serve as predictive markers of metastasis, relapse, and drug resistance. Previous studies by the authors identified specific miRNA signatures in exosomes that are significantly elevated in BC, thereby not only confirming the presence of cancer cells but also allowing the evaluation of molecular subtypes and metastatic potential in patients with BC (17,18). Therefore, the role of exosomal miRNAs was investigated, as a clinical tool to elucidate diagnostic, prognostic, and treatment response assessments by performing an integrated analysis. To ensure a more accurate and precise analysis, the authors focused specifically on investigating the changes in miRNA expression within TDEs because their clinical value is relatively unexplored.

Investigating the role of exosomal miRNAs in BC drug resistance can elucidate molecular mechanisms underlying BC drug resistance and aid in the development of novel diagnostic and therapeutic strategies. Furthermore, investigating epigenetic alterations in drug-resistant tumors offers a deeper understanding of how exosomal miRNAs affect the molecular landscape and therapeutic responses of tumors. Analysis of such epigenetic changes regulated in drug-resistant tumors can provide crucial information regarding the complex interactions between exosomal miRNAs and tumor-associated processes. In the present study, five drug resistance-related miRNA combinations were identified, particularly in TDEs and their predicted target genes, via network analysis using public datasets. The findings of the present study may aid in the development of targeted therapies that modulate miRNA expression and regulate associated target gene expression. Consequently, this may lead to improved treatment outcomes in patients with BC.

Materials and methods

Patient selection. Informed consent for the use of plasma samples for research purposes was obtained from all participants. Clinical samples were obtained from subjects who visited Severance Hospital (Seoul, South Korea), according to the guidelines of the independent Ethics Committee of Yonsei University College of Medicine (IRB approval no. 4-2020-1292; approval date January 4, 2021; Seoul, South Korea). A total of 35 patients with BC (20 with BC exhibiting no pCR after NAC and 15 patients showing pCR after NAC) who visited Severance Hospital between May 2015 and August 2020 were retrospectively registered in the present study. Preoperative plasma samples were collected from the participants before anesthesia. The criteria for inclusion in the analysis were as follows: i) NAC prior to plasma specimen acquisition; ii) confirmed pathological diagnosis of BC; and iii) hemolysis assessed before the isolation of exosomes to evaluate plasma sample quality. Moreover, the study excluded participants with breast cancer types other than invasive ductal carcinoma and those with a history of other malignancies or existing medical conditions. The clinical characteristics of participants enrolled in the present study are summarized in Table SI and the research design is illustrated in Fig. 1.

Cell lines and chemo-drugs. Doxorubicin hydrochloride (brand name Adriamycin; cat. no. D4000) and docetaxel (brand name Taxotere; cat. no. D1000) were purchased from LC Laboratories. Cyclophosphamide (brand name Cytoxan; cat. no. S1217) was purchased from Selleck Chemicals. Adriamycin (5 mg/ml) and Cytoxan (20 mg/ml) were dissolved in saline (JW Pharmaceutical Co., Ltd.). Taxotere (5 mg/ml) was dissolved in dimethyl sulfoxide (Duchefa Biochemie; cat. no. D1370). Aliquots were stored at -20˚C for a maximum of 6 months and thawed immediately before use. Human BC cell lines MDA-MB-231 (MM231; HTB-26), MDA-MB-468 (MM468; HTB-132), and HCC1395 (CRL-2324) were purchased from the American Type Culture Collection. All cell lines were grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640; cat. no. 22400-089) supplemented with 10% fetal bovine serum (FBS; cat. no. 12483-020) and 1% penicillin-streptomycin (cat. no. 15140-122) (all from Gibco; Thermo Fisher Scientific, Inc.). All cells were grown as monolayer cultures and maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Generating drug-resistant BC cell lines. Drug-resistant BC cells were established by consistently increasing the concentrations of clinically used regimens, including Adriamycin, Cytoxan, and Taxotere, in the culture medium (19). Adriamycin and Cytoxan (1:10 weight ratio) followed by Taxotere were alternately added to the cells at specific concentrations [1/120, 1/90, 1/60, 1/30, 1/10, and 1 time of the half-maximal inhibitory concentration (IC_{50}) values of each drug] when they reached 60-70% confluency as per the protocol reported in the previous study by the authors (20). After continuous exposure to chemo-drugs for 6 months, the parental BC cells (herein referred to as '$MM231_{wild\text{-}type}$', '$MM468_{wild\text{-}type}$' and '$HCC1395_{wild\text{-}type}$') were allowed to grow in fresh medium for an additional month until the surviving cells recovered favorably. After 7 months, the stabilized drug-resistant BC cells (herein referred to as '$MM231_{resistant\text{-}type}$', '$MM468_{resistant\text{-}type}$' and '$HCC1395_{resistant\text{-}type}$') were generated and stored in a deep freezer (-70 to -80˚C) for further investigation. The drug resistance characteristics were confirmed by comparison of the IC_{50} values between the cells (Fig. S1 and Table SII).

Microarray analysis. Small RNA was isolated from cultured cells using a mirVana™ miRNA Isolation kit (Thermo Fisher Scientific, Inc.; cat. no. AM1560) according to the manufacturer's instructions, using TRIzol reagent (Thermo Fisher Scientific, Inc.; cat. no. 15596026). The extracted small RNA was quantified using UV absorption at a wavelength of 260 and 280 nm with a spectrophotometer (NanoDrop 3000; Thermo Fisher Scientific, Inc.) and then stored at -80˚C until further analysis. Affymetrix GeneChip microarray (Thermo

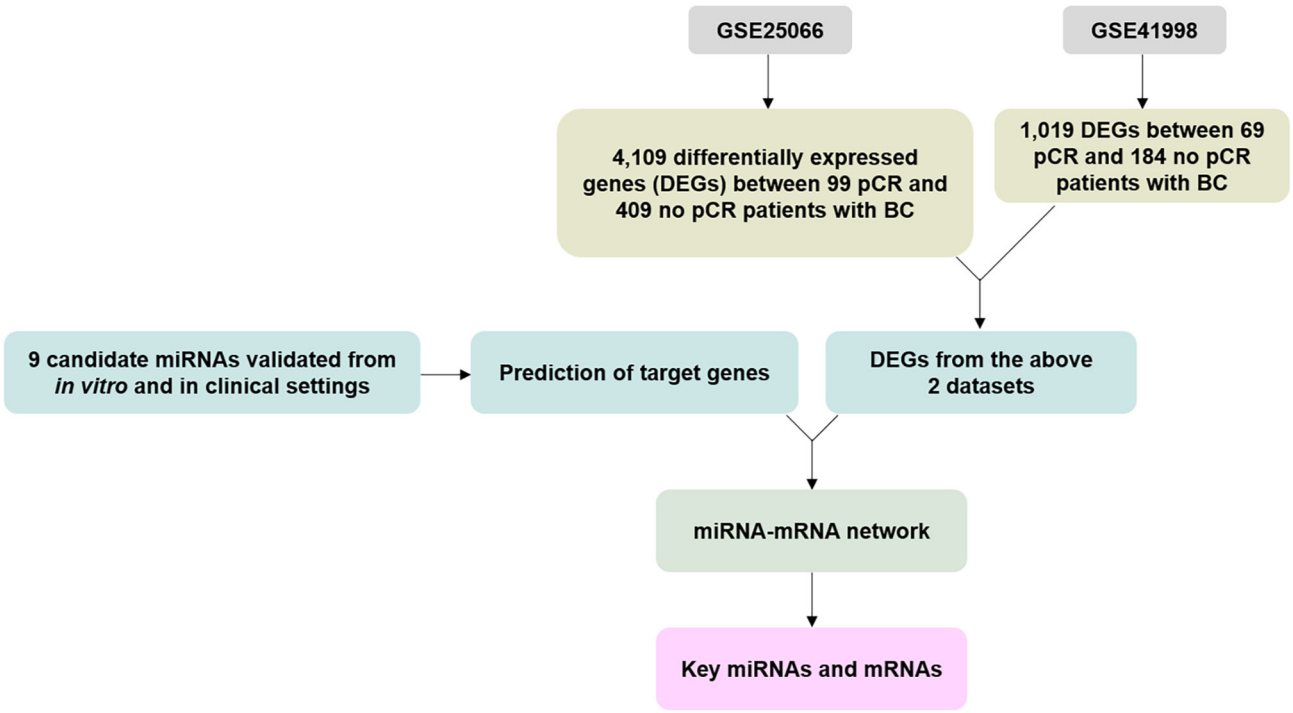

Figure 1. Schematic of the research design for determining the associations between exosomal miRNAs and their target genes. DEGs, differentially expressed genes; pCR, pathological complete response; BC, breast cancer; miRNA, microRNA.

Fisher Scientific, Inc.) runs were performed on the RNA eluates. Intensity values of the CEL files were normalized to remove bias between the arrays using the Robust Multiarray Average and Detection Above BackGround algorithms implemented using the Affymetrix Expression Console software (version 1.4.1; Thermo Fisher Scientific, Inc.). Overall signal distributions of each array were plotted and compared using tools available from the Bioconductor Project (https://www.bioconductor.org/) to verify for normalization. After confirming that the data were properly normalized, differentially expressed miRNAs (DEMs) that exhibited >2-fold difference between the average signal values of the control and treatment groups were selected manually. In addition, the normalized data of selected DEMs were imported into R software (version 4.1.2; R Core Team) for t-test. Genes with a $P<0.05$ were extracted as significant DEMs for further study.

Characterization of tumor-derived exosomes. Microbeads attached to TDEs were fixed for 24 h in Karnovsky's fixative consisting of 2% glutaraldehyde (Merck KGaA; cat. no. 354400) and 2% paraformaldehyde (Merck KGaA; cat. no. 818715) dissolved in 0.1 M phosphate buffer (pH 7.4). Next, the fixed samples were washed twice for 30 min with 0.1 M phosphate buffer (Merck KGaA; cat. nos. S7907 and S9638). The beads were post-fixed with 1% OsO4 for 2 h and dehydrated using a gradually ascending ethanol series (50-100%) with a Critical Point Dryer (Leica Microsystems; cat. no. CPD300). They were then coated with platinum using an ion sputter (Leica Microsystems; cat. no. ACE600) and observed under a field-emission scanning electron microscope at x20,000 magnification (SEM; MERLIN; Carl Zeiss AG). Confocal microscopy measurements were obtained to confirm the presence of TDEs using 3-μm microbeads. The captured exosomes were detected using a primary PE-Cy7-labeled antibody (BD Biosciences; cat. no. 561982) against the general exosome marker CD63. Fluorescence images were obtained using a Zeiss LSM 780 confocal microscope (Carl Zeiss AG). All data were obtained from at least three independent experimental replicates. The concentrations and size distributions of the TDEs resuspended in phosphate-buffered saline (PBS) were measured using a Nanoparticle Tracking Analyzer (NTA; NanoSight NS300 system; Malvern Panalytical Ltd.) Analysis was performed using NTA 3.1 software (Malvern Panalytical Ltd.) with default settings according to the manufacturer's software manual. The camera focus was adjusted to distinctly visualize TDEs that did not exceed a particle signal.

Tumor-derived exosomal miRNA analysis. To validate the miRNA profile of exosomes, TDEs were isolated following a previously reported method (18,21). miRNAs were extracted from three pairs of TDEs originating from wild-type and resistant-type BC cell lines using a mirVana™ miRNA Isolation kit (Thermo Fisher Scientific, Inc.). RNA concentration was measured using a Qubit™ microRNA Assay kit (Thermo Fisher Scientific, Inc.; cat. no. Q32880) with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.; cat. no. Q32866). The extracted RNA was reverse transcribed using the TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.; cat. no. 4366597). Candidate miRNAs were selected from the cellular microarray data (Table SIII), public datasets (GSE71142; Fig. S2) (22), and other reference studies on drug-resistant exosomal miRNAs (23-27). Differential expression levels of 12 miRNAs (miR-21-5p, miR-122-5p, miR-125b, miR-146a-5p, miR-148-5p, miR-155, miR-210-3p, miR-222-5p, miR-484, miR-501-5p, miR-1246-5p and miR-1260b) were measured via cDNA amplification reactions

with a TaqMan Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher Scientific, Inc.; cat. no. 4324018) and TaqMan microRNA Assay kit (Thermo Fisher Scientific, Inc.; cat. no. 4440887) in a CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc.; cat. no. 3600037). Individual miRNAs were reverse-transcribed with the following conditions: 30 min at 16˚C to anneal primers, 30 min at 42˚C for the extension, and 5 min at 85˚C to stop the reaction. qPCR was run using cDNA with the following conditions: 10 min at 95˚C for enzyme activation, followed by 40 cycles consisting of denaturing at 95˚C for 15 sec, and annealing and elongation at 60˚C for 10 min. miRNA expression levels were normalized using miR-16 as a normalization control for exosomal miRNAs. All experiments were performed according to the manufacturer's protocol and repeated in duplicate. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression of exosomal miRNAs (28).

Exosome education. To compare miRNA signature profiles of wild-type, educated-type (BC cells treated with drug-resistant exosomes), and resistant-type BC cells using quantitative PCR in BC cells, $2x10^5$ wild-type BC cells were seeded in 6-well plates and incubated at 37˚C for 24 h, followed by treatment with $1x10^{10}$ of drug-resistant exosomes re-suspended in a complete culture medium and incubated at 37˚C for 24 h. Subsequently, the exosome-treated cells were harvested for real-time PCR to evaluate miRNA expression levels. To verify the drug tolerance of wild-type, educated-type, and resistant-type BC cells, $5x10^3$ of both wild-type and resistant-type BC cells were seeded in 96-well plates. After a 24-h exosome education using $1x10^9$ of drug-resistant exosomes, the cells were incubated with chemo-drugs for 48 h and their cell viabilities were measured using MTT assay. The purple formazan was then dissolved in methanol at room temperature for 30 min on an orbital shaker and the absorbance was recorded at 570 nm with a correction wavelength of 690 nm with a spectrophotometer (NanoDrop 3000; Thermo Fisher Scientific, Inc.).

Receiver operating characteristic (ROC) analysis. ROC analysis of drug-resistant exosomal miRNA markers was performed on data from plasma samples of 35 patients with BC using MedCalc (version 20.014; MedCalc Software Ltd.). Univariate ROC analysis was utilized for each miRNA target to obtain the ROC curve, area under the curve (AUC), AUC standard error (SE), and 95% confidence interval (CI) for evaluating the diagnostic power of the drug-resistant miRNA marker combinations. After performing a univariate ROC analysis on each combination of drug-resistant miRNA targets, the 'best' combination with the highest AUC was selected and also the lowest SE of AUC.

Identification of DEGs from the dataset and network analysis. The present study included the gene expression profiles corresponding to the GSE25066 (29) and GSE41998 (30) datasets from the Gene Expression Omnibus (GEO) database. GSE25066 (508 samples) included 409 and 99 patients with BC exhibiting no response and complete response after NAC, respectively. GSE41998 (253 samples) included 184 and 69 patients with BC showing no response and complete response after NAC, respectively. DEGs were identified using DEGSeq (version 1.48.0) (31) with $P<0.05$, whereas log2FC ≥1 and log2FC ≤-1 cutoffs were used to denote upregulated and downregulated DEGs, respectively. Volcano plots were generated using ggplot2 (32) (version 3.3.5). Gene ontology (GO) was analyzed using ClusterProfiler (33). Representations of GO were generated by the DAVID tool (http://www.geneontology.org/) for functionally annotated molecular functions, biological processes, and biological pathways. To predict the miRNA-mRNA targets, TargetScan software (version 6.0) (https://www.targetscan.org/vert_60/) was used, an open platform for the prediction of miRNA targets. Gene function was annotated based on the biological process of GO gene set (34) (C5; MSigDB collections, BROAD Institute) and gene reference into function (GeneRIF; http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene) database.

cBioportal for survival analysis. The cBioPortal for Cancer genomics is an open-access resource (http://www.cbioportal.org/), providing survival analysis for >10,000 tumor samples from 23 breast cancer studies in TCGA pipeline. This database was applied to predict the patient's survival using miRNA target gene sets (*BAK1*, *NOVA1*, *PTGER4*, *RTKN2*, *AGO1*, *CAP1* and *ETS1*) that are highly related to drug resistance ($miRNA^{high\ R}$) and miRNA target gene sets (*E2F2*, *ITGA3*, *SKP2*, *RIPK2* and *STAT3*) that are moderately related to drug resistance ($miRNA^{moderate\ R}$) as queries. The generated results were displayed as Kaplan-Meier curves with P-values from the log-rank test. The search parameters included alterations (missense mutations, splice mutations, truncating mutation, structural variant, deep deletion and copy number alterations) from whole genome/exome sequencing and targeted sequencing data with the default setting. OS and DFS were calculated on the basis of cBioPortal's online instruction.

Statistical analysis. Each experiment was repeated at least three times independently. Data are presented as the mean ± standard deviation. All statistical analyses were performed with either unpaired Student's t-test, or multiple comparison tests following one-way analysis of variance (ANOVA), using R and R studio (version 4.1.2; R Core Team) and GraphPad Prism software (version 9.0.0; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of drug resistance-associated miRNAs in BC. Microarrays were used to identify DEMs with drug resistance-associated properties. MiRNA expression levels were compared with BC cells that were untreated and continuously treated with drugs. After evaluating miRNA expression using 2,578 human mature miRNA probe sets, 144 DEMs were identified in resistant-type BC (compared with wild-type controls) using the following cut-off criteria: $P<0.05$; fold change ≥2 (Fig. 2A). To visualize the expression patterns of the identified DEMs, hierarchical clustering was performed, and heatmaps were generated for each cell line. The results showed that MM231, MM468, and HCC1395 cells contained 42, 31 and 88 DEMs, respectively (Fig. 2B). Among the 144 DEMs identified, only 64 miRNAs were significantly upregulated, indicating that these were high-confidence candidates involved in drug resistance. When the common DEMs were

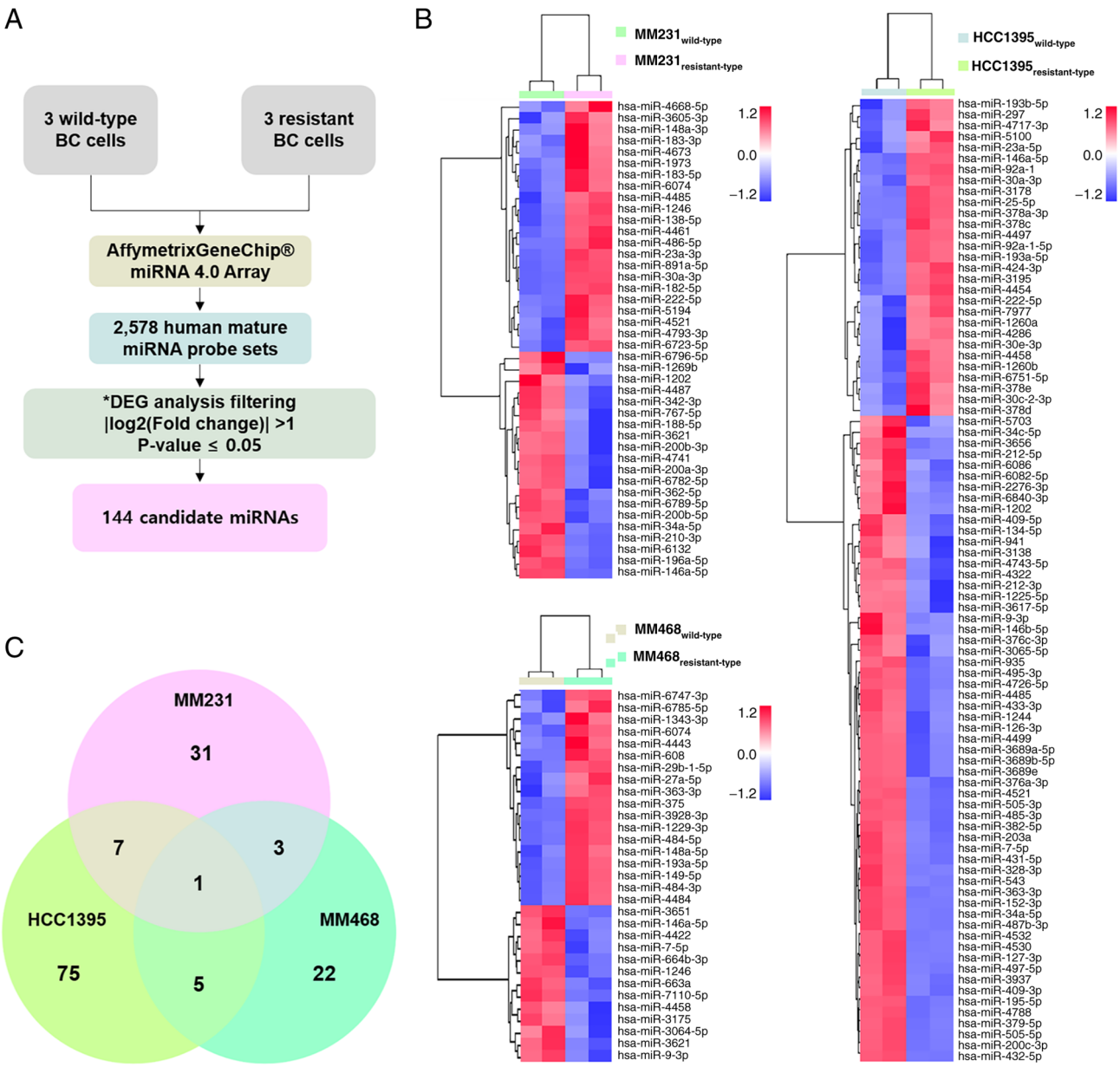

Figure 2. (A) Flow chart illustrating the steps for miRNA expression microarray analysis using AffymetrixGeneChip®. (B) Heat maps with hierarchical clustering showing the differential expression of miRNAs in $MM231_{wild\text{-}type}$, $MM231_{resistant\text{-}type}$, $MM468_{wild\text{-}type}$, $MM468_{resistant\text{-}type}$, $HCC1395_{wild\text{-}type}$, and $HCC1395_{resistant\text{-}type}$ BC cell lines. (C) Venn diagrams illustrating the number of all upregulated and downregulated DEMs in three pairs of BC cell lines. The intersection in the center represents the common DEMs among the three groups. miRNA or miR, microRNA; BC, breast cancer; DEMs, differentially expressed miRNAs.

assessed, 16 were common between at least two cell lines, and 1 was found to be common across all three cell lines (Fig. 2C). These observations highlighted the heterogeneity of miRNA expression patterns across different BC cell lines. The upregulated miRNAs could be further investigated as potential targets or biomarkers for drug resistance, considering both the common and unique regulatory mechanisms in BC.

Evaluation of exosomal miRNAs for predicting drug resistance. A panel of 12 candidate exosomal miRNAs was selected for analysis. These miRNAs (miR-21-5p, miR-122-5p, miR-125b, miR-146a-5p, miR-148a-5p, miR-155, miR-210-3p, miR-222-5p, miR-484, miR-501-5p, miR-1246-5p, and miR-1260b) were selected based on a comprehensive assessment that incorporated microarray data, public datasets, and relevant studies on drug-resistant exosomal miRNAs (Fig. 3A). This crosschecking approach provided a basis for selecting specific miRNAs as potential candidates for drug-resistant exosomal miRNAs and strengthened the reliability of the selection process. These miRNAs were further analyzed in exosomes isolated from the three BC cell lines (MM231, MM468, and HCC1395) and their expression patterns between the wild-type and resistant-type (Fig. 3B-D) were compared. Although the overall miRNA expression patterns varied among exosomes derived from BC cells, a significant finding emerged. Notably, miR-21-5p, miR-122-5p, miR-125b, miR-146a-5p, miR-148a-5p, miR-155, miR-484, miR-1246-5p and miR-1260b exhibited a marked increase in drug-resistant exosomes compared with the wild-type counterparts. This intriguing result suggested that these confirmed miRNAs

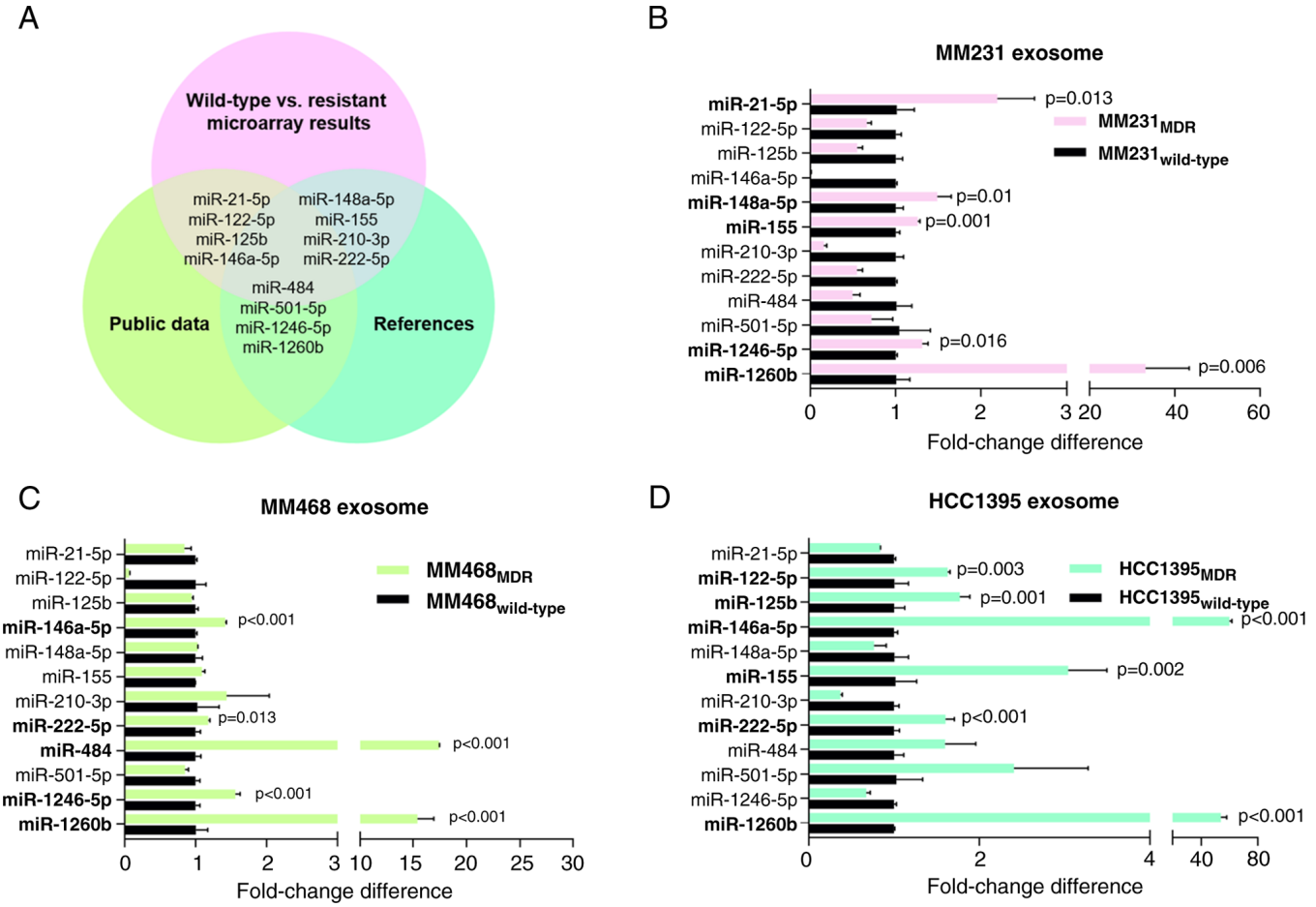

Figure 3. (A) Venn diagram showing candidate exosomal miRNAs related to drug resistance. Bar charts showing fold-change in exosomal miRNAs from wild-type and resistant-type cells. A total of 12 selected candidate exosomal miRNAs were analyzed in the three different breast cancer cell lines (B) MM231, (C) MM468, and (D) HCC1395, using quantitative PCR. Significant miRNAs are indicated in bold text. miRNA or miR, microRNA.

exhibited consistent expression patterns in both exosomes and parental cells and were potentially involved in the drug resistance mechanisms of BC through exosomal communication.

Exosome education for acquisition of drug resistance. To investigate the impact of drug-resistant exosomal miRNAs on drug tolerance, wild-type BC cells were exposed to resistant-type exosomes and the relationship between miRNA expression levels and cell viability was analyzed. The radar plots revealed significant differences in miRNA expression patterns between wild-type and resistant-type BC cells (Fig. 4A). Notably, the miRNA expression patterns of wild-type BC cells became nearly identical to those of resistant-type BC cells after exposure to resistant-type exosomes. Furthermore, examination of the cell viability of wild-type BC cells following treatment with exosomes derived from resistant-type BC cells revealed a substantial increase in chemoresistance to both Adriamycin and Taxotere (Fig. 4B). Specifically, after Adriamycin treatment at a drug concentration equivalent to the IC_{50} value of wild-type BC cells, a survival increase of 36.9, 10.1 and 9.6% was noted for $MM231_{wild\text{-}type}$, $MM468_{wild\text{-}type}$, and $HCC1395_{wild\text{-}type}$ cells, respectively. Under the same conditions, treatment with Taxotere resulted in a survival increase of 27.9, 28.3 and 21.8% for BC cells. These findings indicated that exosomal miRNAs from resistant-type BC cells have the capacity to reshape miRNA expression patterns in wild-type BC cells and can confer the wild-type BC cells with an increased resistance to chemotherapy drugs, causing their behavior to more closely resemble that of drug-resistant BC cells.

Validation of selected exosomal miRNAs in patient samples. Exosomes were isolated from the plasma of patients with BC. These plasma exosomes exhibited a particle size distribution of 30-350 nm in diameter, with an average of 155.3 nm (Fig. 5A). TDEs were specifically targeted using the immunoaffinity method, and then a specific population of exosomes of interest released from the tumor cells was isolated. This specificity allowed for focused analysis of exosomes relevant to research on drug-resistant miRNAs. Furthermore, SEM was employed to confirm whether the isolated TDEs possessed the characteristic features of exosomes. Microscopy revealed that the isolated TDEs expressed CD63, a positive tetraspanin marker associated with exosomes (35), and possessed the expected morphological and structural characteristics of exosomes (Fig. 5B). The expression of selected 12 drug-resistant miRNAs in 35 patients with BC were analyzed, including 20 and 15 patients showing no response and complete response following NAC, respectively, using real-time PCR. The raw Ct values of the 12 miRNAs are listed in Table SIV. The results showed that nine miRNAs (miR-21-5p, miR-125b,

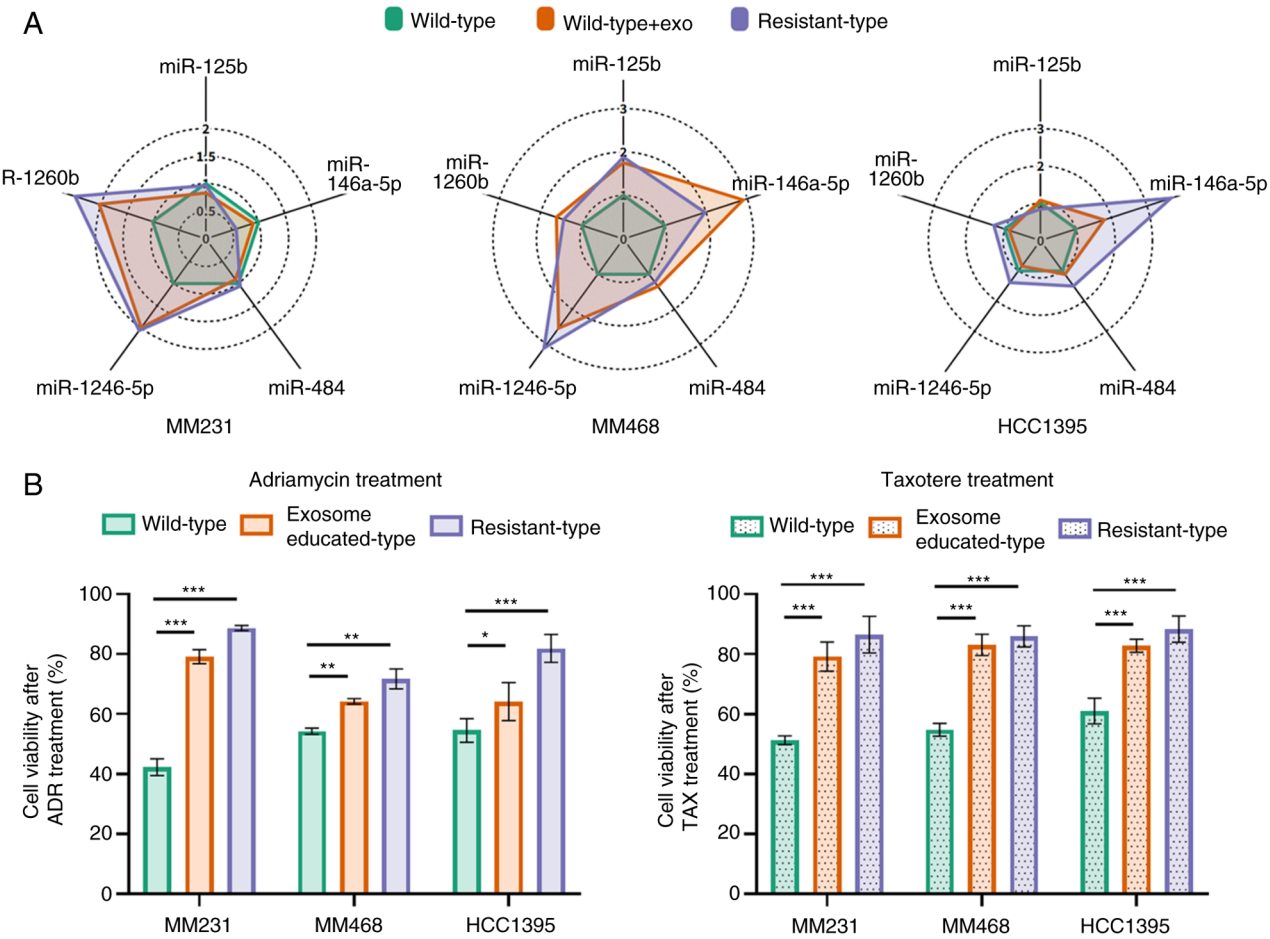

Figure 4. (A) Radar plots of miRNA signature profiles from quantitative PCR in BC cells, including MDA-MB-231 (MM231), MDA-MB-468 (MM468), and HCC1395. The relative expression levels of miRNAs in wild-type BC cells, drug-resistant exosome 24 h-treated (educated-type) BC cells, and resistant-type BC cells were compared. The line labels represent the drug-resistant miRNAs, and the ring labels represent the fold change calculated from each group/wild-type difference. (B) The cell viabilities of wild-type, educated-type, and resistant-type BC cells were compared after 48 h of incubation with Adriamycin and Taxotere. miRNA or miR, microRNA; BC, breast cancer; ADR, Adriamycin; TAX, Taxotere.

miR-146a-5p, miR-155, miR-222-5p, miR-484, miR-501, miR-1246-5p, and miR-1260b) were significantly upregulated in patients with BC who showed no response following NAC (Fig. 5C). These findings suggested that the upregulated miRNAs may be strongly associated with drug resistance in BC. No significant differences were observed in the other three miRNAs (miR-122-5p, miR-148a-5p and miR-210) between the groups in the patient cohort.

ROC curve analysis of validated exosomal miRNAs as prognosis markers. Considering the elevated levels of several miRNAs in patients with BC who exhibited no response to NAC, their roles as prognostic markers were determined using ROC curve analysis. A total of five miRNAs (miR-125b-5p, miR-146a-5p, miR-484, miR-1246-5p and miR-1260b) demonstrated relatively high discriminatory abilities, as indicated by their AUC values ranging from 0.817 to 0.898 (Fig. 6A). These miRNAs had the potential to serve as prognostic markers for identifying patients with BC likely to develop drug resistance following NAC. Conversely, miR-21-5p, miR-155-5p, miR-222-5p, and miR-501-5p showed moderate outcomes, with AUC values <0.8 and miR-122, miR-148a-5p, and miR-210 exhibited poor outcomes, with AUC values <0.6 (Table SV). The integrated model consisting of the five miRNAs showed even greater discriminatory ability, with an AUC of 0.950 (95% CI: 0.819-0.995; P<0.001), 75% sensitivity and 95% specificity (Fig. 6B). These results suggested that the combined 5-miRNA signature, among various combinations, could provide enhanced predictive outcomes for identifying patients with BC showing drug resistance (Fig. S3; Tables SVI and SVII). Therefore, compared with standalone miRNA markers, more effective combinations are proprosed, to maximize AUC and achieve improved diagnostic/prognostic accuracy (Table SVIII).

Meta-analysis of drug-resistant gene expression patterns in BC with NAC case studies. To reinforce the limited scale of clinical validation, a meta-analysis of datasets containing gene expression profiles in patients who received sequential NAC, such as doxorubicin, cyclophosphamide and paclitaxel, after histologically confirming primary invasive breast adenocarcinoma was performed to identify DEGs between the response and no-response groups. Analysis of GSE25066 identified 2,266 downregulated and 1,843 upregulated DEGs. Analysis of GSE41988 identified 455 downregulated and 564 upregulated DEGs (Fig. 7A). Subsequently, cutoffs of adjusted P-values

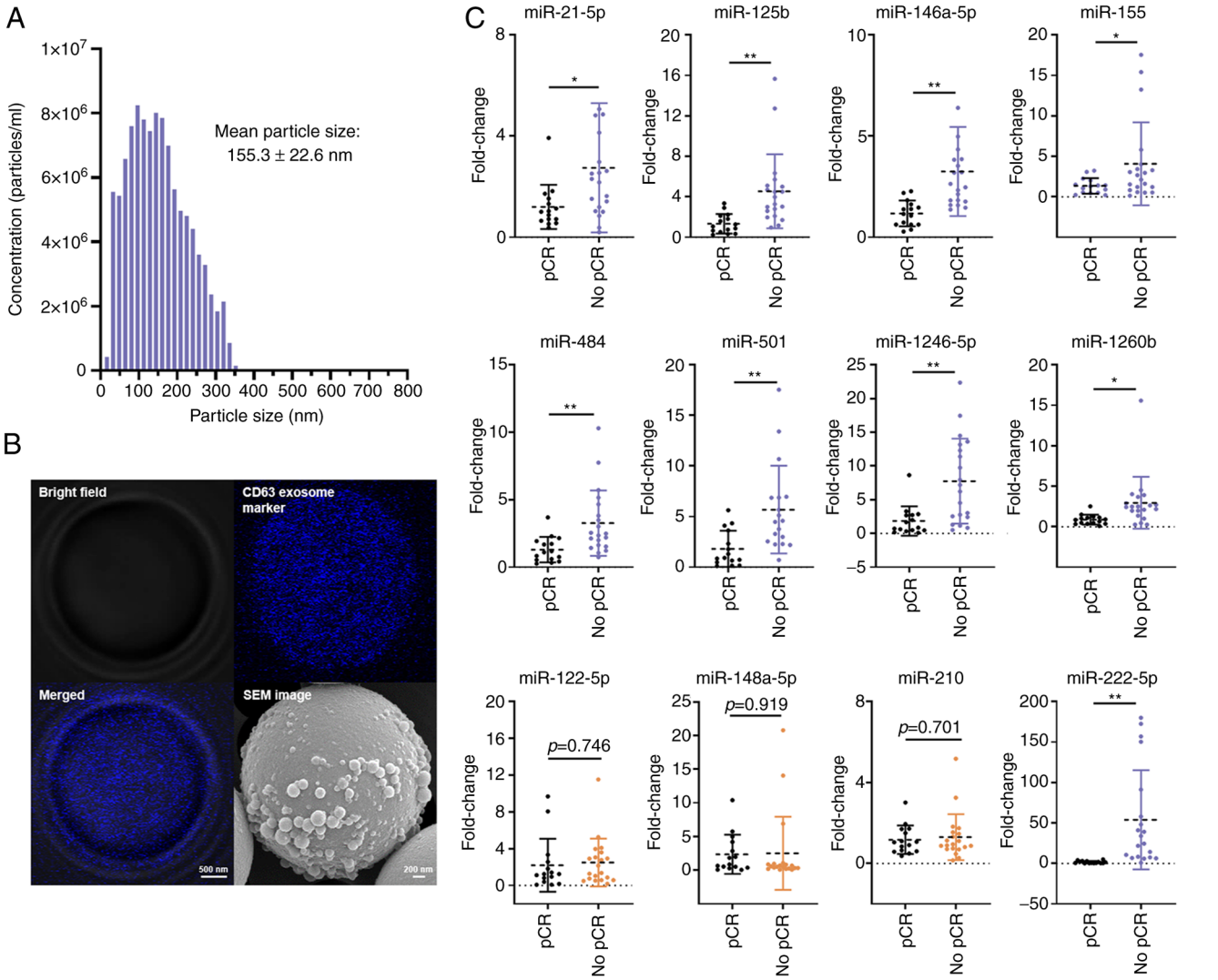

Figure 5. (A) Characterization of tumor-derived exosomes isolated from plasma of patients with BC via nanoparticle tracking analysis. (B) Confocal and SEM images of exosomes bound to microbead surfaces after immuno-affinity capture. Microbeads were functionalized with antibodies against the BC-targeting markers ITGAV, ITGA2 and EpCAM. Scale bars represent 500 and 200 nm, respectively. The size distribution and exosome imaging were obtained from representative patient 1. (C) Exosomal miRNAs related to drug resistance were validated in plasma samples from 35 patients with BC. Statistical analyses were performed using an unpaired Student's t-test between two groups. *P<0.05 and **P<0.01. The statistically significant miRNA candidates are indicated using blue dots and non-significant miRNA candidates are indicated using red dots. BC, breast cancer; SEM, scanning electron microscopy; miRNA or miR, microRNA.

<0.05 and |log2FC|>1 were used for each analysis. Moreover, GO analysis was performed for each significantly dysregulated DEG to identify GO terms representing the biological function of the genes. Clustering of GO terms based on their semantic similarity suggested two and seven GO clusters for the upregulated and downregulated genes, respectively (Fig. 7B). Notably, the analysis demonstrated that the upregulated GO clusters were enriched in the 'regulation of cellular response to growth factor stimulus', 'intracellular transport' and 'cytokine-mediated signaling pathway', strongly suggesting their involvement in drug resistance mechanisms. Conversely, the downregulated GO clusters were associated with the regulation of 'DNA recombination', 'DNA replication', 'RNA splicing', 'mRNA processing', and 'regulation of mitotic cell cycle', suggesting a potential impairment of the G2/M checkpoint and DNA repair pathways. These findings aligned with the documented effects of drug resistance mechanisms in solid tumors, as elucidated by several pivotal studies (36-38).

Network analysis of drug-resistant genes. By integrating miRNAs, their respective target genes, and GO data, the authors successfully confirmed a drug resistance regulatory network. TargetScan predictions were utilized for identifying the target genes of the nine candidate miRNAs. To ensure reliability, the authors focused on high-confidence interactions with binding scores ≥90 (Table SIX). Subsequently, the miRNA-target gene interactions were subjected to GO analysis, with specific emphasis on the GO terms showing an association with drug-resistant genes. This narrowed the analysis to the molecular functions, biological processes, and biological pathways most relevant to drug resistance in BC (Fig. 8A). The significant drug-related categories of molecular function were 'transcription regulator activity', 'transporter activity' and 'catalytic activity'. GO biological processes included 'regulation of nucleic acid metabolism', 'signal transduction', 'apoptosis', 'cell communication', and 'cell growth and maintenance'. Moreover, the GO terms for target mRNAs of the nine candidate miRNAs

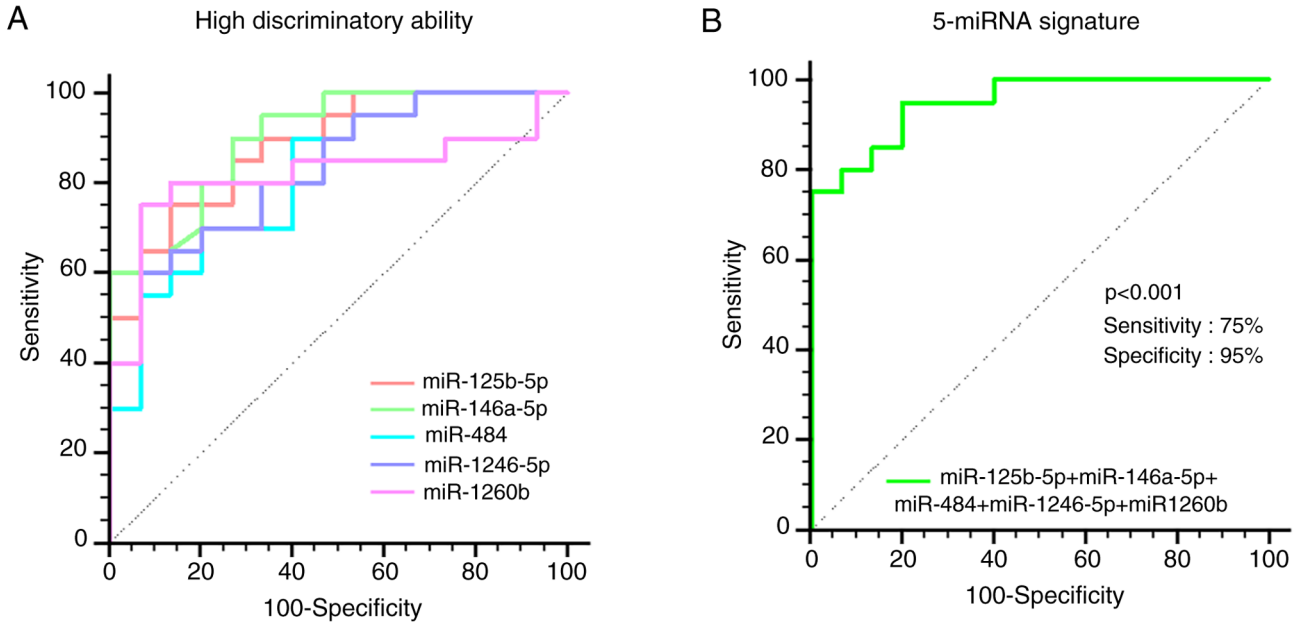

Figure 6. Discriminatory effect of candidate exosomal miRNAs that were significantly upregulated in patients with breast cancer exhibiting no pathological complete response assessed using receiver operating characteristic curves and area under the curve values. (A) Five exosomal miRNAs were validated as targets with high discriminatory ability and (B) four exosomal miRNAs were validated as targets with poor discriminatory ability. miRNA or miR, microRNA.

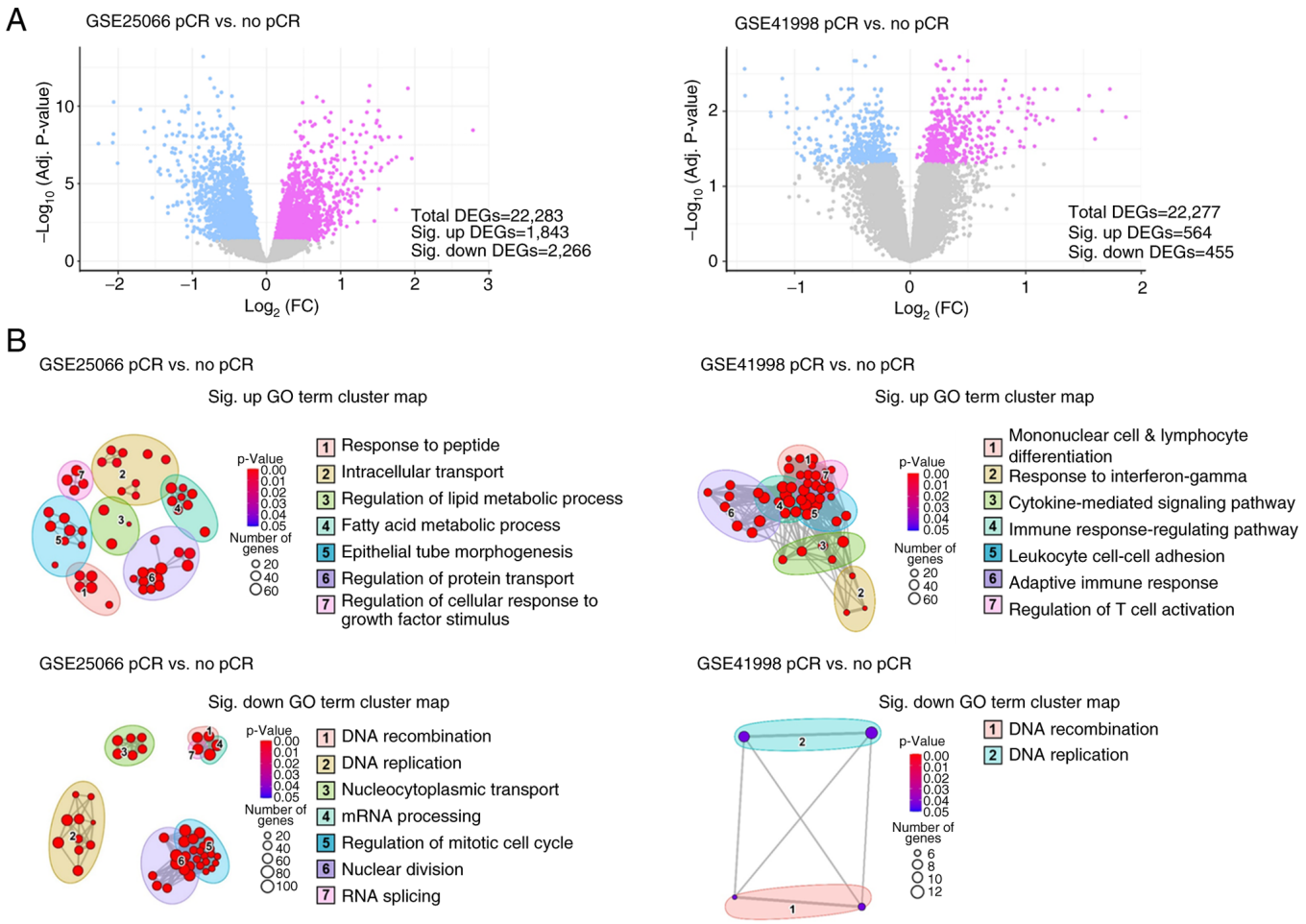

Figure 7. Transcriptome analysis in drug-resistant and drug-sensitive breast cancer tissues. (A) Volcano plot of DEGs in drug-resistant tissue samples compared with those in drug-sensitive tissue samples as identified by GSE25066 and GSE41988 public data analyses. (B) GO cluster map of significantly upregulated and downregulated DEGs in drug-resistant tissue samples compared with those in drug-sensitive tissue samples. Top-ranked terms consisted of interesting GO clusters that were selectively labeled. DEGs, differentially expressed genes; GO, gene ontology; pCR, pathological complete response.

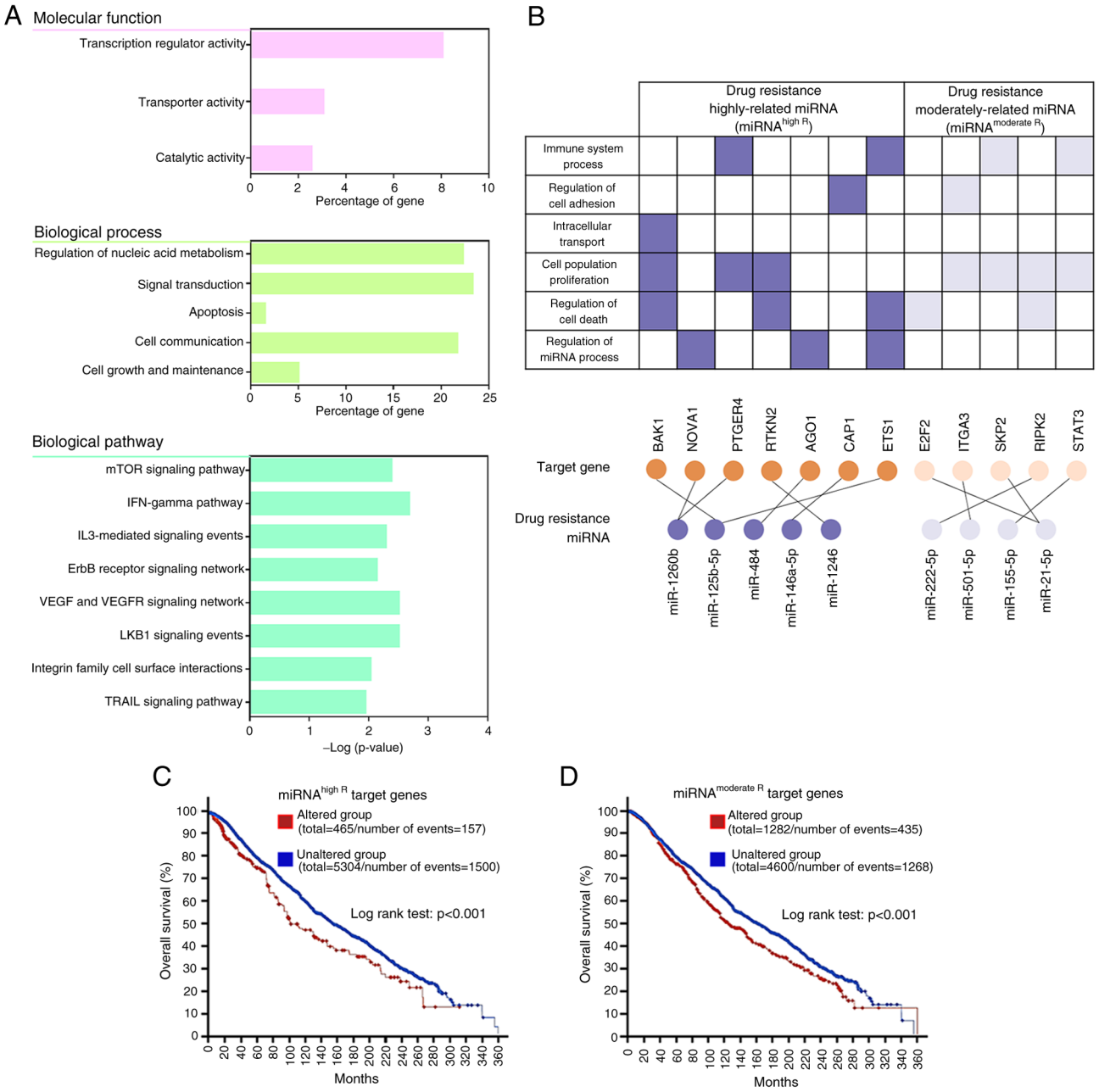

Figure 8. (A) GO analysis of target genes of the candidate drug-resistant exosomal miRNAs. (B) miRNA-mRNA interaction network. The network illustrates the putative miRNAs and their predicted target mRNAs associated with drug resistance in BC after neoadjuvant chemotherapy. The candidate miRNAs were classified into two groups of drug resistance: Highly-related miRNAs (miRNA$^{high\ R}$) and moderately-related miRNAs (miRNA$^{moderate\ R}$) based on their discriminatory power. (C) The survival analysis in BC patients with target genes of miRNA$^{high\ R}$ and (D) miRNA$^{moderate\ R}$ using public databases. GO, gene ontology; miRNA or miR, microRNA; BC, breast cancer.

were associated with multiple biological pathways participating in 'mTOR signaling pathway', 'IFN-gamma pathway', 'IL3-mediated signaling events', 'ErbB receptor signaling network', 'VEGF and VEGFR signaling network', 'LKB1 signaling events', 'integrin family cell surface interactions' and 'TRAIL signaling pathway'. Notably, these results were similar to the findings in Fig. 7, presenting the analysis of the metadata for drug resistance genes, implying that the nine miRNAs are related to drug resistance and potentially regulate target genes to influence tumor response to chemotherapy. Furthermore, miRNA$^{high\ R}$ target gene sets (*BAK1*, *NOVA1*, *PTGER4*, *RTKN2*, *AGO1*, *CAP1* and *ETS1*) and miRNA$^{moderate\ R}$ target gene sets (*E2F2*, *ITGA3*, *SKP2*, *RIPK2* and *STAT3*) that are related to multiple biological activities related to drug resistance were revealed (Fig. 8B). Notably, *NOVA1*, *ITGA3*, *SKP2* and *RIPK2* were common after cross-validation of metadata analysis, DEMs and target DEGs, suggesting that these four genes are more importantly involved in drug resistance.

Survival analyses of drug-resistant genes. To evaluate the clinical significance of the identified drug-resistant miRNAs and their target genes, survival analyses was performed using data from a comprehensive collection of cancer studies, accessed through cBioPortal, encompassing thousands of

patient samples filtered by BC classification. Kaplan-Meier curves and log-rank tests were performed for individual genes identified in the present study, followed by the analysis of combined gene-altered signatures involving miRNA$^{high\ R}$ or miRNA$^{moderate\ R}$, which showed significant results (Fig. S4). The gene-altered group exhibited a decreased lifespan than the unaltered group, indicating a high correlation with patient prognosis. For example, the median overall survival in the miRNA$^{high\ R}$ target gene signature-altered and unaltered groups was determined to be 110.77 and 154.50 months, respectively (Fig. 8C). Similarly, the median overall survival rates in the miRNA$^{moderate\ R}$ target gene signature-altered and unaltered groups were 124.20 and 161.13 months, respectively (Fig. 8D). In addition, both gene signatures showed significant differences in progression-free survival and relapse-free survival (Fig. S5). These findings highlighted the prognostic significance of the drug resistance target genes, including *BAK1*, *NOVA1*, *PTGER4*, *RTKN2*, *AGO1*, *CAP1*, *ETS1*, *E2F2*, *ITGA3*, *SKP2*, *RIPK2* and *STAT3*, in patients with BC. Taken together, these results provided compelling evidence for a strong association between the altered expression of drug-resistant genes induced by the regulation of drug-resistant miRNAs and poor prognosis among patients with BC.

Discussion

In the present study, it was identified that exosomal miR-125b-5p, miR-146a-5p, miR-484, miR-1246-5p and miR-1260b were highly enriched in TDEs from patients with BC who displayed tolerance to chemotherapy. To the best of the authors' knowledge, only a few studies have reported the potential roles of these miRNAs in tumorigenesis and cancer treatment (39). Furthermore, research on the functional aspects of exosomal miRNAs remains relatively rare. One of the key challenges in exosome research is the uncertainty regarding whether exosomal miRNAs accurately reflect the miRNA expression patterns in the originating tissue. This ambiguity can sometimes lead to conflicting findings, and it is partly attributed to the inherent heterogeneity of both tumors and exosomes. Additionally, the isolation and analysis of tumor-derived exosomes have not yet reached an optimized standard. The present study aimed to mitigate these challenges by isolating a comprehensive set of tumor-derived exosomes, focusing on those associated with drug resistance. The comparative analysis in the present study, linking these candidate exosomal miRNAs with their target genes, elucidated a clear and significant involvement of these five aforementioned exosomal miRNAs in the response of the tumor to chemotherapy and, subsequently, in the clinical outcomes of the patients.

When investigating the individual functions of each miRNA in drug resistance, the previous research of the authors (40) on the function of miRNAs in tumor tissue, provided valuable insights. It was reported in that study that high miR-1260b expression was markedly associated with bulky tumor size, advanced stage, lymph node invasion, and a shorter period of overall survival. In addition to the oncogenic function of miR-1260b, it was identified that its target is *CASP8*, a key gene in the p53 tumor suppressor pathway, implicating its involvement in drug resistance (40). In the present study, a significant association between exosomal miR-1260b and regulation of the key drug-resistance genes, *NOVA1* and *PTGER4*, was revealed. As an RNA-binding protein, *NOVA1* is known to exert influence over miRNA activity and the regulation of RNA splicing (41,42). Its versatile role in post-transcriptional gene regulation renders it a highly promising candidate in the context of drug resistance. In addition, *PTGER4* is a critical target in the transduction pathways essential for cancer cell survival and tumor progression, including the AKT and ERK pathways implicated in numerous other cancers (43). Further investigations are required to elucidate the precise role of exosomal miR-1260b in BC drug resistance.

Zheng *et al* (44) reported that increased miR-125b-5p expression in tumor tissue was associated with a lack of pCR after anthracycline-taxane-based chemotherapy in BC. Similarly, Zhou *et al* (45) revealed that the downregulation of the pro-apoptotic gene *BAK1*, which is a direct target of miR-125-5p, could suppress Taxol-induced apoptosis and result in increased resistance to Taxol. In the present study it was revealed that *ETS1* is another possible target of exosomal miR-125-5p. *ETS1* has shown ambiguous functions as an oncogene and a tumor suppressor gene in numerous types of cancers, but its function as a tumor suppressor has been clearly reported recently in BC (46). Another drug-resistant miRNA candidate, miR-146a-5p, has been reported to be overexpressed in cisplatin-resistant BC cells, affecting the cell levels of the tumor suppressor *BRCA1*, *HOXD10* homeobox family, tumor suppressor *CDKN1B*, and *ESR1* gene (47). Moreover, Dai *et al* (48) demonstrated that miR-1246 affected cell migration, invasion and doxorubicin resistance in BC by targeting the transcription factor *NFE2L3*. According to the analysis in the present study, *CAP1* and *RTKN2* are potent targets of miR-146a-5p and miR-1246, respectively. These genes are known to be involved in tumor invasion, apoptosis, and immune response in lung cancer, but the molecular mechanisms underlying drug resistance in BC remain unclear (49-51). Notably, the previously reported roles of miR-484 in regulating drug resistance in BC are in contrast with the findings of the present study. For example, Ye *et al* (52) demonstrated that the upregulation of miR-484 reduced cell proliferation and reversed chemo-resistance to gemcitabine in BC. Jia *et al* (53) argued that miR-484 is typically described as a tumor suppressor; however, this claim could be simplistic and one-sided. Existing evidence primarily addresses miR-484 expression in tumor tissues rather than exosomal miR-484 expression. Considering the potential discrepancies among circulating cell-free, tumor tissues, and exosomal miRNAs, the function of miR-484 in TDEs requires careful investigation.

Contrary to the initial expectations, limited consistency was observed between the target genes identified using TargetScan and those suppressed in drug-resistant tumors. This suggested that the regulatory relationship between them is not straightforward, indicating the presence of additional regulatory mechanisms or complexities in the context of drug resistance. The regulatory roles of miRNAs are complex and involve various mechanisms that mediate gene regulation. To fully understand the regulatory networks of the exosomal miRNAs involved in drug resistance, it is crucial to understand the specifics of these mechanisms. Drug-resistant miRNAs present in the blood of patients undergoing treatment may not exclusively target or suppress tumor suppressor

genes or oncogenes. The involvement of miRNA-mediated feedback and feed-forward loops, which are regulatory circuits involving reciprocal regulation between miRNAs and their target genes, should also be considered. These loops add an additional layer of complexity to the control of gene expression and could contribute to the observed inconsistencies (54). Moreover, although the expression of miRNAs and their targets is often highly correlated, it is possible that additional factors, such as epigenetic modifications, transcriptional factors, or protein-protein interactions, influence the observed inconsistencies. A recent study has emphasized the significance of these factors in miRNA-mediated gene regulation (55). Therefore, it is essential to explore these alternative mechanisms and conduct further experimental validation to gain a more comprehensive understanding of the regulatory networks of exosomal miRNAs involved in drug resistance. By investigating the interplay between miRNAs, their targets, and other regulatory elements, the complexities underlying miRNA-mediated gene regulation and mechanisms that contribute to drug resistance can be better understood.

In summary, the present study provided valuable insights regarding the molecular mechanisms underlying drug resistance in BC. By constructing a miRNA-mRNA network, key exosomal miRNAs were identified (miR-125b-5p, miR-146a-5p, miR-484, miR-1246-5p and miR-1260b) and their target genes (*BAK1*, *NOVA1*, *PTGER4*, *RTKN2*, *AGO1*, *CAP1* and *ETS1*) that may be involved in the development of drug resistance in patients with BC. These miRNAs and their target genes are promising candidate biomarkers for predicting tumor response to treatment and may serve as potential therapeutic targets for patients with BC. An interesting finding, in the present study, is based on how the response of cells sensitive to chemotherapy drugs can change due to transference of drug-resistant exosomes (a process called exosome education). Overall, the present study highlights the importance of early diagnosis with liquid biopsy because it provides valuable information for predicting patient prognosis by extracting and analyzing drug-resistant exosomes, which can affect tumor response to treatment. Given the heterogeneity of exosomes and their dynamic nature during treatment, it is crucial to explore changes in exosomal miRNA profiles to enhance the understanding of treatment responses for improving patient outcomes. Further investigations are warranted for establishing exosomal miRNA signatures derived from liquid biopsies as reliable indicators of tumor response in BC.

However, there are still a few limitations to acknowledge. First, circulating biomarkers, including exosomes, ctDNAs, and miRNAs, possess other contaminants that exist in the blood, and their origin is difficult to define. Despite the efforts of the authors to analyze tumor-derived exosomes using the immuno-affinity isolation method, it is not considered that all the exosomes isolated in the present study reflect tumor information. An innovative exosome isolation technology is essential for a more accurate analysis. Second, the results obtained in the present study were based on a relatively small sample size, which may limit the generalizability of the findings, thus further large-scale validation is required. Finally, experimental validation through gain/loss-of-function approaches has not been carried out in the present study. It is expected that future investigations will address the impact of these miRNAs and target genes in cancer treatment and provide directions for overcoming drug resistance.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Severance Hospital Research Fund for Clinical Excellence (grant no. C-2023-0006) and the National Research Foundation of Korea Grants (grant nos. 2021R1I1A1A01051594 and 2022R1F1A1074605).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The microarray data generated in the present study are available in the GEO database under accession code GSE237873.

Authors' contributions

MWK, JeYK and SIK conceived the study. SL, SM, YK, JoYK and HL were responsible for the collection, curation and analysis of the experimental data. YK, JoYK and HL prepared the methodology and software. MWK and SM validated the analysis, and confirm the authenticity of all the raw data. SM wrote the original draft and MWK revised the manuscript. SIK and JeYK were responsible for study supervision. All authors contributed to writing of the manuscript and have read and approved the final manuscript.

Ethics approval and consent to participate

Clinical samples were obtained from subjects who visited Severance Hospital in South Korea, according to the guidelines of the independent Ethics Committee of Yonsei University College of Medicine (IRB approval no. 4-2020-1292, approved on January 4, 2021; Seoul, South Korea). The study was performed following the principles of the Declaration of Helsinki. Informed consent for the use of plasma samples for research purposes was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bianchini G, De Angelis C, Licata L and Gianni L: Treatment landscape of triple-negative breast cancer-expanded options, evolving needs. Nat Rev Clin Oncol 19: 91-113, 2022.
2. Spring LM, Fell G, Arfe A, Sharma C, Greenup R, Reynolds KL, Smith BL, Alexander B, Moy B, Isakoff SJ, *et al*: Pathologic complete response after neoadjuvant chemotherapy and impact on breast cancer recurrence and survival: A comprehensive meta-analysis. Clin Cancer Res 26: 2838-2848, 2020.

3. Echeverria GV, Ge Z, Seth S, Seth S, Zhang X, Jeter-Jones S, Zhou X, Cai S, Tu Y, McCoy A, *et al*: Resistance to neoadjuvant chemotherapy in triple-negative breast cancer mediated by a reversible drug-tolerant state. Sci Transl Med 11: eaav0936, 2019.
4. Hoogstraat M, Lips EH, Mayayo-Peralta I, Mulder L, Kristel P, van der Heijden I, Annunziato S, van Seijen M, Nederlof PM, Sonke GS, *et al*: Comprehensive characterization of pre- and post-treatment samples of breast cancer reveal potential mechanisms of chemotherapy resistance. NPJ Breast Cancer 8: 60, 2022.
5. Lips EH, Michaut M, Hoogstraat M, Mulder L, Besselink NJ, Koudijs MJ, Cuppen E, Voest EE, Bernards R, Nederlof PM, *et al*: Next generation sequencing of triple negative breast cancer to find predictors for chemotherapy response. Breast Cancer Res 17: 134, 2015.
6. Zhao Y, Schaafsma E and Cheng C: Gene signature-based prediction of triple-negative breast cancer patient response to Neoadjuvant chemotherapy. Cancer Med 9: 6281-6295, 2020.
7. Nedeljkovic M and Damjanovic A: Mechanisms of chemotherapy resistance in triple-negative breast cancer-how we can rise to the challenge. Cells 8: 957, 2019.
8. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE and Gottesman MM: Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat Rev Cancer 18: 452-464, 2018.
9. Fu Y, Yang Q, Yang H and Zhang X: New progress in the role of microRNAs in the diagnosis and prognosis of triple negative breast cancer. Front Mol Biosci 10: 1162463, 2023.
10. Sueta A, Fujiki Y, Goto-Yamaguchi L, Tomiguchi M, Yamamoto-Ibusuki M, Iwase H and Yamamoto Y: Exosomal miRNA profiles of triple-negative breast cancer in neoadjuvant treatment. Oncol Lett 22: 819, 2021.
11. O'Brien J, Hayder H, Zayed Y and Peng C: Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 9: 402, 2018.
12. O'Brien K, Breyne K, Ughetto S, Laurent LC and Breakefield XO: RNA delivery by extracellular vesicles in mammalian cells and its applications. Nat Rev Mol Cell Biol 21: 585-606, 2020.
13. Si W, Shen J, Zheng H and Fan W: The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenetics 11: 25, 2019.
14. An X, Sarmiento C, Tan T and Zhu H: Regulation of multidrug resistance by microRNAs in anti-cancer therapy. Acta Pharm Sin B 7: 38-51, 2017.
15. van Niel G, Carter DRF, Clayton A, Lambert DW, Raposo G and Vader P: Challenges and directions in studying cell-cell communication by extracellular vesicles. Nat Rev Mol Cell Biol 23: 369-382, 2022.
16. Mitra R, Adams CM, Jiang W, Greenawalt E and Eischen CM: Pan-cancer analysis reveals cooperativity of both strands of microRNA that regulate tumorigenesis and patient survival. Nat Commun 11: 968, 2020.
17. Gwak H, Park S, Kim J, Lee JD, Kim IS, Kim SI, Hyun KA and Jung HI: Microfluidic chip for rapid and selective isolation of tumor-derived extracellular vesicles for early diagnosis and metastatic risk evaluation of breast cancer. Biosens Bioelectron 192: 113495, 2021.
18. Kim MW, Park S, Lee H, Gwak H, Hyun KA, Kim JY, Jung HI and Il Kim S: Multi-miRNA panel of tumor-derived extracellular vesicles as promising diagnostic biomarkers of early-stage breast cancer. Cancer Sci 112: 5078-5087, 2021.
19. Gradishar WJ, Anderson BO, Abraham J, Aft R, Agnese D, Allison KH, Blair SL, Burstein HJ, Dang C, Elias AD, *et al*: Breast Cancer, Version 3.2020, NCCN Clinical practice guidelines in oncology. J Natl Compr Canc Netw 18: 452-478, 2020.
20. Kim MW, Niidome T and Lee R: Glycol Chitosan-Docosahexaenoic acid liposomes for drug delivery: Synergistic effect of doxorubicin-rapamycin in drug-resistant breast cancer. Mar Drugs 17: 581, 2019.
21. Kim MW, Koh H, Kim JY, Lee S, Lee H, Kim Y, Hwang HK and Kim SI: Tumor-specific miRNA signatures in combination with CA19-9 for liquid biopsy-based detection of PDAC. Int J Mol Sci 22: 13621, 2021.
22. Chen X, Wang YW, Xing AY, Xiang S, Shi DB, Liu L, Li YX and Gao P: Suppression of SPIN1-mediated PI3K-Akt pathway by miR-489 increases chemosensitivity in breast cancer. J Pathol 239: 459-472, 2016.
23. Zhou M, Liu Z, Zhao Y, Ding Y, Liu H, Xi Y, Xiong W, Li G, Lu J, Fodstad O, *et al*: MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through Suppression of Pro-apoptotic Bcl-2 Antagonist Killer 1 (Bak1) Expression. J Biol Chem 285: 21496-21507, 2010.
24. Tao L, Wu YQ and Zhang SP: MiR-21-5p enhances the progression and paclitaxel resistance in drug-resistant breast cancer cell lines by targeting PDCD4. Neoplasma 66: 746-755, 2019.
25. Yu DD, Lv MM, Chen WX, Zhong SL, Zhang XH, Chen L, Ma TF, Tang JH and Zhao JH: Role of miR-155 in drug resistance of breast cancer. Tumour Biol 36: 1395-1401, 2015.
26. Chen X, Lu P, Wang DD, Yang SJ, Wu Y, Shen HY, Zhong SL, Zhao JH and Tang JH: The role of miRNAs in drug resistance and prognosis of breast cancer formalin-fixed paraffin-embedded tissues. Gene 595: 221-226, 2016.
27. Si Z, Zhong Y, Lao S, Wu Y, Zhong G and Zeng W: The Role of miRNAs in the resistance of anthracyclines in breast cancer: A systematic review. Front Oncol 12: 899145, 2022.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25: 402-408, 2001.
29. Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A, Vidaurre T, Holmes F, Souchon E, Wang H, *et al*: A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. JAMA 305: 1873-1881, 2011.
30. Horak CE, Pusztai L, Xing G, Trifan OC, Saura C, Tseng LM, Chan S, Welcher R and Liu D: Biomarker analysis of neoadjuvant doxorubicin/cyclophosphamide followed by ixabepilone or paclitaxel in early-stage breast cancer. Clin Cancer Res 19: 1587-1595, 2013.
31. Wang LK, Feng ZX, Wang X, Wang XW and Zhang XG: DEGseq: An R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics 26: 136-138, 2010.
32. Ito K and Murphy D: Application of ggplot2 to pharmacometric graphics. CPT Pharmacometrics Syst Pharmacol 2: e79, 2013.
33. Yu GC, Wang LG, Han YY and He QY: clusterProfiler: An R Package for comparing biological themes among gene clusters. Omics 16: 284-287, 2012.
34. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdóttir H, Tamayo P and Mesirov JP: Molecular signatures database (MSigDB) 3.0. Bioinformatics 27: 1739-1740, 2011.
35. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O and Geuze HJ: Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem 273: 20121-20127, 1998.
36. Chen DS and Mellman I: Elements of cancer immunity and the cancer-immune set point. Nature 541: 321-330, 2017.
37. Dong X, Bai X, Ni J, Zhang H, Duan W, Graham P and Li Y: Exosomes and breast cancer drug resistance. Cell Death Dis 11: 987, 2020.
38. Marra A, Trapani D, Viale G, Criscitiello C and Curigliano G: Practical classification of triple-negative breast cancer: Intratumoral heterogeneity, mechanisms of drug resistance, and novel therapies. NPJ Breast Cancer 6: 54, 2020.
39. Wang M, Yu F, Ding H, Wang Y, Li P and Wang K: Emerging function and clinical values of exosomal MicroRNAs in cancer. Mol Ther Nucleic Acids 16: 791-804, 2019.
40. Park S, Kim J, Cho Y, Ahn S, Kim G, Hwang D, Chang Y, Ha S, Choi Y, Lee MH, *et al*: Promotion of tumorigenesis by miR-1260b-targeting CASP8: Potential diagnostic and prognostic marker for breast cancer. Cancer Sci 113: 2097-2108, 2022.
41. Storchel PH, Thummler J, Siegel G, Aksoy-Aksel A, Zampa F, Sumer S and Schratt G: A large-scale functional screen identifies Nova1 and Ncoa3 as regulators of neuronal miRNA function. Embo J 34: 2237-2254, 2015.
42. Zhang YA, Liu HN, Zhu JM, Zhang DY, Shen XZ and Liu TT: RNA binding protein Nova1 promotes tumor growth in vivo and its potential mechanism as an oncogene may due to its interaction with $GABA_A$ Receptor-γ2. J Biomed Sci 23: 71, 2016.
43. Catalano RD, Wilson MR, Boddy SC, McKinlay AT, Sales KJ and Jabbour HN: Hypoxia and prostaglandin E receptor 4 signalling pathways synergise to promote endometrial adenocarcinoma cell proliferation and tumour growth. PLoS One 6: e19209, 2011.
44. Zheng Y, Li S, Boohaker RJ, Liu X, Zhu Y, Zhai L, Li H, Gu F, Fan Y, Lang R, *et al*: A MicroRNA expression signature In Taxane-anthracycline-Based neoadjuvant chemotherapy response. J Cancer 6: 671-677, 2015.
45. Zhou M, Liu Z, Zhao Y, Ding Y, Liu H, Xi Y, Xiong W, Li G, Lu J, Fodstad O, *et al*: MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. J Biol Chem 285: 21496-21507, 2010.

46. Kim GC, Lee CG, Verma R, Rudra D, Kim T, Kang K, Nam JH, Kim Y, Im SH and Kwon HK: ETS1 suppresses tumorigenesis of human breast cancer via trans-activation of canonical tumor suppressor genes. Front Oncol 10: 642, 2020.
47. Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI and Kovalchuk O: Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. Int J Cancer 127: 1785-1794, 2010.
48. Dai YC, Pan Y, Quan MM, Chen Q, Pan Y, Ruan YY and Sun JG: MicroRNA-1246 mediates drug resistance and metastasis in breast cancer by targeting NFE2L3. Front Oncol 11: 677168, 2021.
49. Li LP, Huang YC, Zhang Y, Peng A, Qin J, Lu S and Huang Y: RTKN2 is associated with unfavorable prognosis and promotes progression in non-small-cell lung cancer. Onco Targets Ther 13: 10729-10738, 2020.
50. Chen K, Ye C, Gao Z, Hu J, Chen C, Xiao R, Lu F and Wei K: Immune infiltration patterns and identification of new diagnostic biomarkers GDF10, NCKAP5, and RTKN2 in non-small cell lung cancer. Transl Oncol 29: 101618, 2023.
51. Zeng J, Li X, Liang L, Duan H, Xie S and Wang C: Phosphorylation of CAP1 regulates lung cancer proliferation, migration, and invasion. J Cancer Res Clin Oncol 148: 137-153, 2022.
52. Ye FG, Song CG, Cao ZG, Xia C, Chen DN, Chen L, Li S, Qiao F, Ling H, Yao L, *et al*: Cytidine deaminase axis modulated by miR-484 differentially regulates cell proliferation and chemoresistance in breast cancer. Cancer Res 75: 1504-1515, 2015.
53. Jia YZ, Liu J, Wang GQ and Song ZF: miR-484: A Potential Biomarker in Health and Disease. Front Oncol 12: 830420, 2022.
54. Tsang J, Zhu J and van Oudenaarden A: MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. Mol Cell 26: 753-767, 2007.
55. Nersisyan S, Galatenko A, Galatenko V, Shkurnikov M and Tonevitsky A: miRGTF-net: Integrative miRNA-gene-TF network analysis reveals key drivers of breast cancer recurrence. PLoS One 16: 2021.