Combination of Exosomal MiRNA Markers with BI-RADS for Accurate Diagnosis of Breast Tumor

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Abstract

Background

Early and accurate identification of breast masses is of vital importance in clinic. Ultrasound imaging is an efficient strategy for breast mass screening, while the accuracy needs further improvement. Plasma exosomes have recently been suggested as promising candidate as disease markers. In this study, we aimed to explore the potential of plasma exosomal miRNAs in differentiation of breast masses.

Methods

Plasma exosomes were collected from patients with benign or malignant breast masses using a novel, facile, low-cost, and effective method of sequential salting-out and polyethylene glycol (PEG)-based approaches. Plasma exosomal miRNAs were extracted and profiled by RNA sequencing. Differential exosomal miRNAs were then tested by qPCR for screening of potential biomarkers. The diagnostic efficiencies of exosomal miRNAs alone or in combination with breast imaging reporting and data system (BI-RADS) in differentiation of breast masses were evaluated on a validation cohort via analysis of receiver operating characteristic (ROC) curve.

Results

Prior salting-out strategy yielded exosomes with higher purity, thus guaranteeing the accuracy of exosome-based diagnosis. miRNA sequencing showed different exosomal miRNA expressions between patients with malignant or benign breast masses, among which, hsa-miR-423-5p, hsa-miR-103a-3p and hsa-let-7i-5p were chosen and tested for diagnostic ability. ROC curve showed that these three miRNAs could serve as promising biomarkers, with hsa-miR-423-5p showing the highest diagnostic efficiency. Combination of exosomal miRNAs with BI-RADS further improved the diagnostic efficacy.

Conclusions

Plasma exosomal miRNAs could serve as excellent biomarkers for breast mass diagnosis and could significantly improve diagnostic accuracy when combined with BI-RADS.

Background

Breast cancer has become a serious threat to women's health, and the average age of onset of the disease is becoming younger and younger. Accurate differentiation between breast cancer and benign mass is of vital importance for improving clinical prognosis [1, 2]. Developing new diagnostic methods or improving existing diagnostic techniques are the main ways to further improve the diagnosis efficiency between benign and malignant breast masses.

At present, medical imaging is the main method for early detection and diagnosis of breast diseases [3, 4]. Mammography is one of the main technologies for breast cancer screening, but it has a vital disadvantage of radiation [5, 6]. With the sustained development of ultrasound technology, ultrasound imaging has become the most important method for diagnosis of breast masses [7, 8]. Currently, breast imaging reporting and data system (BI-RADS) is being used as a standard method for assessing breast lesions by ultrasound imaging [9]. Although with the application of BI-RADS classification, standardization and consistency of breast mass diagnosis have been largely improved, misdiagnoses might occur in some atypical breast masses, especially in regard with category 4 lesions, due to the observer variability among physicians and objective errors [10–13]. Improvement of diagnostic accuracy could save benign cases from unnecessary biopsy and ensure that malignant lesions wouldn't escape diagnosis [14, 15].

As our understanding of molecular mechanisms underlying cancer biological behaviors progresses, biomolecules have been explored for accurate disease diagnosis, especially in combination with imaging evaluation. Numerous studies have shown that microRNA (miRNA) expression is dysregulated in various stages of breast cancer [16]. MiRNAs have been reported to be involved in regulation of cell cycle, apoptosis, angiogenesis, epithelial mesenchymal transformation, metastasis and drug resistance, as well as differentiation and self-renewal of breast cancer stem cells [17–19]. Several studies have proposed miRNA as biomarker for early detection of breast cancer to distinguish healthy subjects from breast cancer patients [20, 21]. However, the free miRNAs in circulation are unstable and difficult to detect. Exosomal miRNAs have attracted much attention as biomarkers for disease diagnosis in recent years. Exosomes are nanoscale vesicles that play vital roles in cellular communication [22–24]. Due to its low molecular weight, miRNAs can be easily assembled into exosomes, constituting the major biologically active molecules of exosomes. Compared with free miRNA in the circulation, exosomal miRNAs are ideal and stable biomarkers since they are enriched in exosomes and are protected from degradation by RNase [25–27].

In the present study, we first designed prior salting-out strategy for exosome isolation and then isolated plasma exosomes from patients with benign or malignant breast masses and analyzed their exosomal miRNA expression by miRNA sequencing. Three miRNAs that were mostly up-regulated in breast cancers were then evaluated for potential application as biomarker for breast mass diagnosis. Together, the results showed that plasma exosomal miRNAs could significantly improve diagnostic accuracy of breast masses, especially in combination with BI-RADS.

Materials And Methods

Clinical samples

Six patients who underwent ultrasound-guided biopsy of breast masses in Tangdu Hospital during March 2019 were randomly chosen for sequencing of plasma exosomal miRNAs. A validation cohort for analysis of diagnostic power of candidate miRNAs was also recruited from 53 patients with breast

masses who underwent ultrasound imaging and ultrasound-guided biopsy from the Department of Ultrasound Diagnostics, Tangdu Hospital from July to August, 2019. The malignancy of breast masses was confirmed by histopathology and tumor stage was defined according to TNM staging. The present study was approved by the institutional review board of Tangdu Hospital and informed consent was obtained from each patient.

Breast Ultrasonography

Ultrasound imaging for breast masses was performed by experienced physicians using the routine scanning protocol on Resona 7 (Mindray, China) with a 10 MHz linear transducer. Both longitudinal and transverse sections were recorded for size measurement, acoustic characteristics and further imaging evaluation. After ultrasound examination, the breast masses were evaluated and categorized according to the fifth edition of BI-RADS.

Isolation and characterization of plasma exosomes

Blood samples from patients were collected in anticoagulant tubes and sit at room temperature for 10 min, then centrifugated at 3000 r/min for 5 min for isolation of plasma. For traditional isolation of plasma exosomes, the samples were filtrated with 0.22 μm filters and then processed by Exo Quick^{TM} according to the manufacturer's protocol. For isolation of exosomes by prior salting-out strategy, 100 μl $(NH_4)_2SO_4$ was added to 1 ml plasma sample before subsequent exosome isolation.

Isolated exosomes were characterized by transmission electron microscopy (TEM) for morphology analysis, particle size analysis for size distribution and western blot for protein content analysis. Specific protocols were the same as previously described [28].

Exosomal RNA isolation and RT-qPCR

Total RNA was extracted from the yielded pellets of exosomes using TRIzol reagent (Invitrogen, USA) according to the manufacture's instruction. The purity and concentration of RNA was assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The isolated RNA was reversely transcribed by miRcute Plus miRNA First-strand cDNA Synthesis Kit (Tiangen, China). For miRNA sequencing, the samples obtained from test cohort were assessed and analyzed by authorized institute RiboBio (Guangzhou, China). Target genes of differential miRNA (|log2(Fold Change) |>1, $P<0.05$) were predicted using TargetScan, miRDB, miRTarBase and miRWalk. Candidate target genes were then analyzed by annotation and enrichment in Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) biological pathways to explore major functions of the candidate target genes.

Real-time quantitative polymerase chain reaction for miRNA expression was performed using FastStart Essential DNA Green Master (Roche, USA) following instructions provided by the manufacturer. U6 was used as internal control.

Statistical analysis

Data are represented as mean ± SEM or as indicated. The diagnostic power for differentiating patients with benign and malignant breast masses was assessed by calculating the area under the receiver operator characteristic (ROC) curve (AUC). P values of <0.05 was considered as statistically significant.

To evaluate the combined diagnostic ability of BI-RADS-US and/or multiple candidate miRNAs in the diagnosis of breast benign and malignant masses, BI-RADS-US or miRNAs were first divided into benign and malignant variables through the cut-off point, and then logistic regression analysis was carried out to calculate the weight of each index. Then, the number of each index was put into the regression equation according to their respective weights and the diagnostic value of the combined indexes was analyzed by ROC curve analysis.

Results

Prior salting-out strategy improves the efficiency and purity of plasma exosome isolation

Efficient isolation of highly pure exosomes from the blood plasma is essential for exosome-based diagnosis and the large amounts of plasma proteins increase the difficulty for exosome isolation. To solve the problem, the proteins were removed by prior salting-out strategy (Fig. 1A). As expected, abundant proteins were salted out for plasma after treated with saturated $(NH_4)_2SO_4$ (Fig. 1B). The results from TEM demonstrated that compared to conventional exosome isolation, the addition of $(NH_4)_2SO_4$ for salting out of protein minimized impurity contamination in isolated exosomes (Fig. 1C). Particle size analysis showed that the size distribution was more concentrated in exosomes isolated after protein salting out (Fig. 1D). Western blot for analysis of protein content of isolated exosomes revealed a higher proportion of membrane proteins in exosome isolated after protein salting out, indicating higher purity of the isolated exosomes (Fig. 1E). Together, these results demonstrate that protein salting out prior to exosome isolation improved the purity of isolated exosomes.

Differential exosomal miRNA expressions in patients with benign or malignant breast masses

Fifteen patients who received breast ultrasound imaging and ultrasound-guided breast biopsy for pathological diagnoses in Tangdu Hospital during March 2019 were randomly selected. After exclusion of cases with rare pathological diagnoses or cases that didn't fall into BI-RADS category 4, there were 5 cases in the malignant group and 5 in the benign group. Among them, 3 cases of benign and 3 cases of malignant breast mass were randomly enrolled for plasma exosomal miRNA sequencing (Fig. 2). The average age of recruited subjects were 49±5.5 years (ranging from 38-58). General characteristics and pathology diagnoses of enrolled subjects were shown in Table S1.

Deep sequencing was performed on exosomal miRNAs from blood samples of the patients. A total of 1226 miRNAs were detected in malignant breast mass group and 1278 in benign group. Among them, 1107 miRNAs were expressed in both groups, 119 miRNAs were detected only in malignant group and 171 were detected only in benign group (Fig. 3A). With the use of |log2(Fold Change) |≥1, P□0.05 as threshold, 47 miRNAs were found significantly up-regulated and 57 miRNAs were found down-regulated

in malignant group compared to benign group (Fig. 3B). Further analysis of the exosomal miRNAs showed distinct expression between the two groups (Fig. 3C).

To explore the relationship between the differential exo-miRNA and malignancy of breast masses, KEGG pathway enrichment analysis was performed. The top 30 enriched signaling pathways shown in Fig. S1 included focal adhesion, Wnt signaling pathway, PI3K-Akt signaling pathway, pathways in cancer, etc., which indicated that these differential miRNAs might be implicated in cancers.

Exosomal miRNAs as potential biomarkers for discriminating benign and malignant breast masses

A total of 53 patients with breast masses were enrolled as validation cohort, including 36 cases of malignant breast mass and 17 cases of benign breast mass (Fig. 4). General characteristics of subjects were shown in Table S2 and the pathological diagnoses of the 53 breast masses were shown in Table S3. First, qPCR was performed to evaluate the expression level of candidate miRNAs in patients with malignant or benign breast mass. According to the results, three miRNAs were found most significantly up-regulated in malignant group, hsa-miR-423-5p, hsa-miR-103a-3p and hsa-let-7i-5p (Fig. 5A). Then, we explored the potential diagnostic ability of these miRNAs in differentiating patients with benign and malignant breast masses. Prior to the test of diagnostic performance of exosomal miRNAs, we tested the diagnostic power of BI-RADS in differentiating patients in the validation cohort. The results yielded an AUC of 0.807 (95% CI 0.683-0.931) with 78% sensitivity and 77% specificity (Fig. 5B). BI-RADS showed the optimal diagnostic performance when 4A/4B was used as cutoff value (Table S4). As for the diagnostic potential of the candidate exosomal miRNAs, ROC curve analyses showed that each individual miRNA alone could differentiate malignant cases from benign cases. The AUC of miR-423-5p was 0.807 (95% CI 0.684-0.930), with 94% sensitivity and 53% specificity. The AUC of miR-103a-3p was 0.759 (95% CI 0.624-0.895) with 94% sensitivity and 47% specificity. The AUC of let-7i-5p was 0.707 (95% CI 0.561-0.853) with 61% sensitivity and 82% specificity (Fig. 5C-5E).

To further improve the diagnostic accuracy, miRNAs were combined for discrimination between malignant and benign breast masses. The results showed that the combination of miR-423-5p and miR-103a-3p exhibited the highest diagnostic power with an AUC of 0.821, sensitivity of 72.0% and specificity of 82.3% (Fig. S2), which might serve as diagnostic markers for breast cancer.

Combination of exosomal miRNAs with BI-RADS significantly improved diagnostic accuracy of breast masses

Next, we tested the diagnostic accuracy of exosomal miRNAs in combination with BI-RADS. The diagnostic efficacy of BI-RADS in combination with single miRNA or multiple miRNAs were evaluated by the ROC curve analysis. The results demonstrated that BI-RADS-US in combination with triple RNAs (miR-423-5p, miR-103a-3p and let-7i-5p) had the highest diagnostic value with an AUC of 0.867, 83% sensitivity and 82% specificity (Fig. 6).

Exosomal miRNAs could help diagnosis of malignancy of BI-RADS category 4 breast masses

In the diagnosis of breast mass based on BI-RADS-US classification, the key challenge lies in the identification of category 4 masses. Therefore, we exclude category 3 breast masses which possess malignancy ≤ 2% and category 5 breast masses which embrace malignancy ≥95% and focused on the diagnosis of malignancy of category 4 breast masses. According to the results, diagnostic performance of BI-RADS-US in differentiating category 4 breast masses (4a, 4b, 4c) showed an AUC of 0.754 with 76% sensitivity and 74% specificity (Fig. 7A). Then, we analyzed the potential diagnostic ability of candidate miRNAs alone or the combination of multiple miRNAs for discrimination of category 4 breast masses. the results showed that for single miRNA diagnosis, exosomal miR-423-5p had the highest diagnostic value (AUC 0.820) (Fig. 7B) and the combination of miR-423-5p, miR-103a-3p and let-7i-5p further improved the diagnostic efficiency, with the area under the curve of 0.827, 81% sensitivity and 80% specificity (Fig. S3).

We further analyzed the diagnostic value of BI-RADS-US in combination with candidate miRNAs for diagnosis of category 4 breast masses. The results showed compared to BI-RADS-US alone, combination of BI-RADS-US with exosomal miRNAs significantly improved diagnostic accuracy and the highest diagnostic value occurred at the combination of BI-RADS with miR-423-5p (Fig. 7C, Fig. S4).

Discussion

In the present study, we first designed a novel exosomes extraction method using prior salting-out strategy to increase the purity of isolated exosomes. Then, we isolated plasma exosomes from patients with benign or malignant breast masses using the above strategy. Differential plasma exosomal miRNA expressions between the groups were analyzed by deep sequencing and then verified by RT-PCR. By a cohort study, we further demonstrated that exosomal miRNAs, miR-423-5p, miR-103a-3p and let-7i-5p were useful in differential diagnosis of breast masses with miR-423-5p showing the highest diagnostic value. The diagnostic ability of exosomal miRNAs further improved when used in combination with BI-RADS.

With the ever-increasing incidences, breast cancer seriously threatens the public health. Early and accurate diagnosis of breast mass is of vital importance for improving disease prognosis. The present study targeted the bottleneck of ultrasound in diagnosis of breast masses and explored the potential diagnostic ability of exosomal miRNAs in differentiating benign and malignant breast masses. The results showed that plasma exosomal miRNAs, including miR-423-5p, miR-103a-3p and let-7i-5p were useful in diagnosis of breast masses. ROC curve analysis showed that a combination of the three miRNAs with BI-RADS exhibited highest diagnostic value with an AUC of 0.867 (83% sensitivity and 82% specificity). As for the diagnosis of category 4 breast masses, which are the most difficult ones to diagnose, combination of BI-RADS-US with exosomal miRNAs also showed excellent performance in determining the malignancy of the masses with an AUC of 0.850 (90% sensitivity and 73% specificity).

At present, imaging examination is the main method for early detection and diagnosis of breast diseases. The clinical treatment of suspected benign and malignant masses by imaging is still controversial [14, 29, 30]. On one hand, unnecessary biopsies may lead to excessive medical treatment and increased

physical and psychological burden of patients. On the other hand, the accuracy of biopsy greatly relies on the collected tissues and misdiagnosis may occur when diseased tissue escaped biopsy.

Peripheral bloods contain various substances from primary tumors as well as metastatic sites and can comprehensively reflect body status, making it ideal target for disease screening and diagnosis. Traditional blood-based measurement for cancer diagnosis include protein markers such as carcinoembryonic antigen (CEA) or prostate-specific antigen (PSA), circulating tumor cells (CTC) and cell-free circulating tumor DNA (ctDNA). Recently, exosomal miRNAs have been suggested as novel tumor markers in several cancers [31–34].

Although there are a few studies exploring plasma/serum exosomal miRNAs as potential markers in diagnosis of diseases, the results varied from study to study [21]. The main reason may be that the complexity and variability of blood contents interfered the stability of the results. Different isolation methods of exosomes might also affect the results. Larger samples and multicenter studies might be needed in the future to further confirm the diagnostic efficiency of exosomal miRNAs and establish unified diagnostic standards.

It should be noted that let-7i-5p was traditionally considered as tumor-suppressing gene [35]. However, our study found that the expression of let-7i-5p increased in cancer patients. The seemingly contradictory result might be explained by that cancer cells specifically sorted out the tumor-suppressing gene by mechanism of exosomes so that they can better maintain their proliferation.

There are several limitations concerning the present study. First, the sample size is small, thus it's important to increase the sample size to further verify the reliability of the exosomal miRNAs for diagnosis of the benign and malignant breast masses. Also,

the sensitivity of the existing combined diagnosis still needs improvement since in the differential diagnosis of benign and malignant breast masses, the consequence of missed diagnosis is more serious. How to establish diagnostic criteria and find reasonable cutoff value still warrants large sample study and clinical verification.

Abbreviations

PEG
polyethylene glycol
BI-RADS
breast imaging reporting and data system
ROC curve
receiver operating characteristic curve
miRNA
microRNA
AUC

area under curve
CEA
carcinoembryonic antigen
PSA
prostate-specific antigen
CTC circulating tumor cells
ctDNA
cell-free circulating tumor DNA.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee at Tangdu Hospital, Fourth Military Medical University. The ethical board approval number is TDLL2018-03-49. All methods were carried out in accordance with relevant guidelines and regulations. The authors declared that they have obtained ethical approval and informed consent was obtained from all subjects and/or their legal guardian(s).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files please upload the file in respective section.

Competing interests

All the authors declare no conflicts of interest.

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Author contributions

YD and LY contributed to the conception of the study, YZ, XZ and ZL performed the experiments, and they were major contributors in writing the manuscript, JJ and WS helped perform the analysis with constructive discussions. CX contributed statistical analysis All authors read and approved the final manuscript.

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References

1. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J: Cancer statistics in China, 2015. CA Cancer J Clin 2016, 66(2):115–132.
2. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, Jemal A, Kramer JL, Siegel RL: Cancer treatment and survivorship statistics, 2019. CA: a cancer journal for clinicians 2019, 69(5):363–385.
3. Cho N, Han W, Han BK, Bae MS, Ko ES, Nam SJ, Chae EY, Lee JW, Kim SH, Kang BJ *et al*: Breast Cancer Screening With Mammography Plus Ultrasonography or Magnetic Resonance Imaging in Women 50 Years or Younger at Diagnosis and Treated With Breast Conservation Therapy. JAMA Oncol 2017, 3(11):1495–1502.
4. Kerlikowske K, Sprague BL, Tosteson ANA, Wernli KJ, Rauscher GH, Johnson D, Buist DSM, Onega T, Henderson LM, O'Meara ES *et al*: Strategies to Identify Women at High Risk of Advanced Breast Cancer During Routine Screening for Discussion of Supplemental Imaging. Jama Intern Med 2019, 179(9):1230–1239.
5. Miglioretti DL, Lange J, van den Broek JJ, Lee CI, van Ravesteyn NT, Ritley D, Kerlikowske K, Fenton JJ, Melnikow J, de Koning HJ *et al*: Radiation-Induced Breast Cancer Incidence and Mortality From Digital Mammography Screening: A Modeling Study. Ann Intern Med 2016, 164(4):205–214.
6. Yaffe MJ, Mainprize JG: Risk of radiation-induced breast cancer from mammographic screening. Radiology 2011, 258(1):98–105.
7. Berg WA, Bandos AI, Mendelson EB, Lehrer D, Jong RA, Pisano ED: Ultrasound as the Primary Screening Test for Breast Cancer: Analysis From ACRIN 6666. Jnci-J Natl Cancer I 2016, 108(4).
8. Ciurea A, Gersak M, Onoe R, Ivan O, Ciortea C: The role of ultrasound in the imaging assessment of the augmented breast. A pictorial review. Med Ultrason 2014, 16(3):256–261.
9. Rao AA, Feneis J, Lalonde C, Ojeda-Fournier H: A Pictorial Review of Changes in the BI-RADS Fifth Edition. Radiographics 2016, 36(3):623–639.
10. Lee HJ, Kim EK, Kim MJ, Youk JH, Lee JY, Kang DR, Oh KK: Observer variability of Breast Imaging Reporting and Data System (BI-RADS) for breast ultrasound. Eur J Radiol 2008, 65(2):293–298.
11. Lee YJ, Choi SY, Kim KS, Yang PS: Variability in Observer Performance Between Faculty Members and Residents Using Breast Imaging Reporting and Data System (BI-RADS)-Ultrasound, Fifth Edition (2013). Iran J Radiol 2016, 13(3):e28281.
12. Lazarus E, Mainiero MB, Schepps B, Koelliker SL, Livingston LS: BI-RADS lexicon for US and mammography: interobserver variability and positive predictive value. Radiology 2006, 239(2):385–391.

13. Kim EJ, Kim SH, Kang BJ, Kim YJ: Interobserver agreement on the interpretation of automated whole breast ultrasonography. Ultrasonography 2014, 33(4):252–258.
14. Gaffney S, Harston C: To Biopsy or Not To Biopsy: A Review of 558 Circumscribed Breast Masses Demonstrated by Ultrasound. Am J Roentgenol 2012, 198(5).
15. Zhou C, Thomson T, Switzer P: Diagnosis of breast carcinoma by ultrasound-guided fine needle aspiration and by simultaneous breast core biopsy: A review of 680 cases. Acta Cytol 2007, 51(2):291–292.
16. Goh JN, Loo SY, Datta A, Siveen KS, Yap WN, Cai W, Shin EM, Wang C, Kim JE, Chan M *et al*: microRNAs in breast cancer: regulatory roles governing the hallmarks of cancer. Biol Rev Camb Philos Soc 2016, 91(2):409–428.
17. Cai WL, Huang WD, Li B, Chen TR, Li ZX, Zhao CL, Li HY, Wu YM, Yan WJ, Xiao JR: microRNA-124 inhibits bone metastasis of breast cancer by repressing Interleukin-11. Mol Cancer 2018, 17(1):9.
18. Cantini L, Bertoli G, Cava C, Dubois T, Zinovyev A, Caselle M, Castiglioni I, Barillot E, Martignetti L: Identification of microRNA clusters cooperatively acting on epithelial to mesenchymal transition in triple negative breast cancer. Nucleic Acids Res 2019, 47(5):2205–2215.
19. Eastlack SC, Dong S, Ivan C, Alahari SK: Suppression of PDHX by microRNA-27b deregulates cell metabolism and promotes growth in breast cancer. Mol Cancer 2018, 17(1):100.
20. Nassar FJ, Nasr R, Talhouk R: MicroRNAs as biomarkers for early breast cancer diagnosis, prognosis and therapy prediction. Pharmacol Therapeut 2017, 172:34–49.
21. Andorfer CA, Necela BM, Thompson EA, Perez EA: MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer. Trends Mol Med 2011, 17(6):313–319.
22. Colombo M, Raposo G, Thery C: Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual review of cell and developmental biology 2014, 30:255–289.
23. Théry C, Zitvogel L, Amigorena S: Exosomes: composition, biogenesis and function. Nature Reviews Immunology 2002, 2(8):569–579.
24. Wortzel I, Dror S, Kenific CM, Lyden D: Exosome-Mediated Metastasis: Communication from a Distance. Developmental cell 2019, 49(3):347–360.
25. Hannafon BN, Trigoso YD, Calloway CL, Zhao YD, Lum DH, Welm AL, Zhao ZZJ, Blick KE, Dooley WC, Ding WQ: Plasma exosome microRNAs are indicative of breast cancer. Breast Cancer Res 2016, 18.
26. Joyce DP, Kerin MJ, Dwyer RM: Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer. Int J Cancer 2016, 139(7):1443–1448.
27. Sun ZQ, Shi K, Yang SX, Liu JB, Zhou QB, Wang GX, Song JM, Li Z, Zhang ZY, Yuan WT: Effect of exosomal miRNA on cancer biology and clinical applications. Molecular Cancer 2018, 17.
28. Li Z, Zhou X, Wei M, Gao X, Zhao L, Shi R, Sun W, Duan Y, Yang G, Yuan L: In Vitro and in Vivo RNA Inhibition by CD9-HuR Functionalized Exosomes Encapsulated with miRNA or CRISPR/dCas9. Nano letters 2019, 19(1):19–28.

29. Yu YH, Wei W, Liu JL: Diagnostic value of fine-needle aspiration biopsy for breast mass: a systematic review and meta-analysis. Bmc Cancer 2012, 12.
30. Raza S, Goldkamp AL, Chikarmane SA, Birdwell RL: US of Breast Masses Categorized as BI-RADS 3, 4, and 5: Pictorial Review of Factors Influencing Clinical Management. Radiographics 2010, 30(5):1199–1213.
31. Rodriguez-Martinez A, de Miguel-Perez D, Ortega FG, Garcia-Puche JL, Robles-Fernandez I, Exposito J, Martorell-Marugan J, Carmona-Saez P, Garrido-Navas MD, Rolfo C *et al*: Exosomal miRNA profile as complementary tool in the diagnostic and prediction of treatment response in localized breast cancer under neoadjuvant chemotherapy. Breast Cancer Res 2019, 21.
32. Li XD, Wang XP: The emerging roles and therapeutic potential of exosomes in epithelial ovarian cancer. Molecular Cancer 2017, 16.
33. Mrowczynski OD, Zacharia BE, Connor JR: Exosomes and their implications in central nervous system tumor biology. Prog Neurobiol 2019, 172:71–83.
34. Yang FM, Ning ZQ, Ma L, Liu WT, Shao CC, Shu YQ, Shen H: Exosomal miRNAs and miRNA dysregulation in cancer-associated fibroblasts. Molecular Cancer 2017, 16.
35. Chirshev E, Oberg KC, Ioffe YJ, Unternaehrer JJ: Let-7 as biomarker, prognostic indicator, and therapy for precision medicine in cancer. Clin Transl Med 2019, 8(1).

Figures

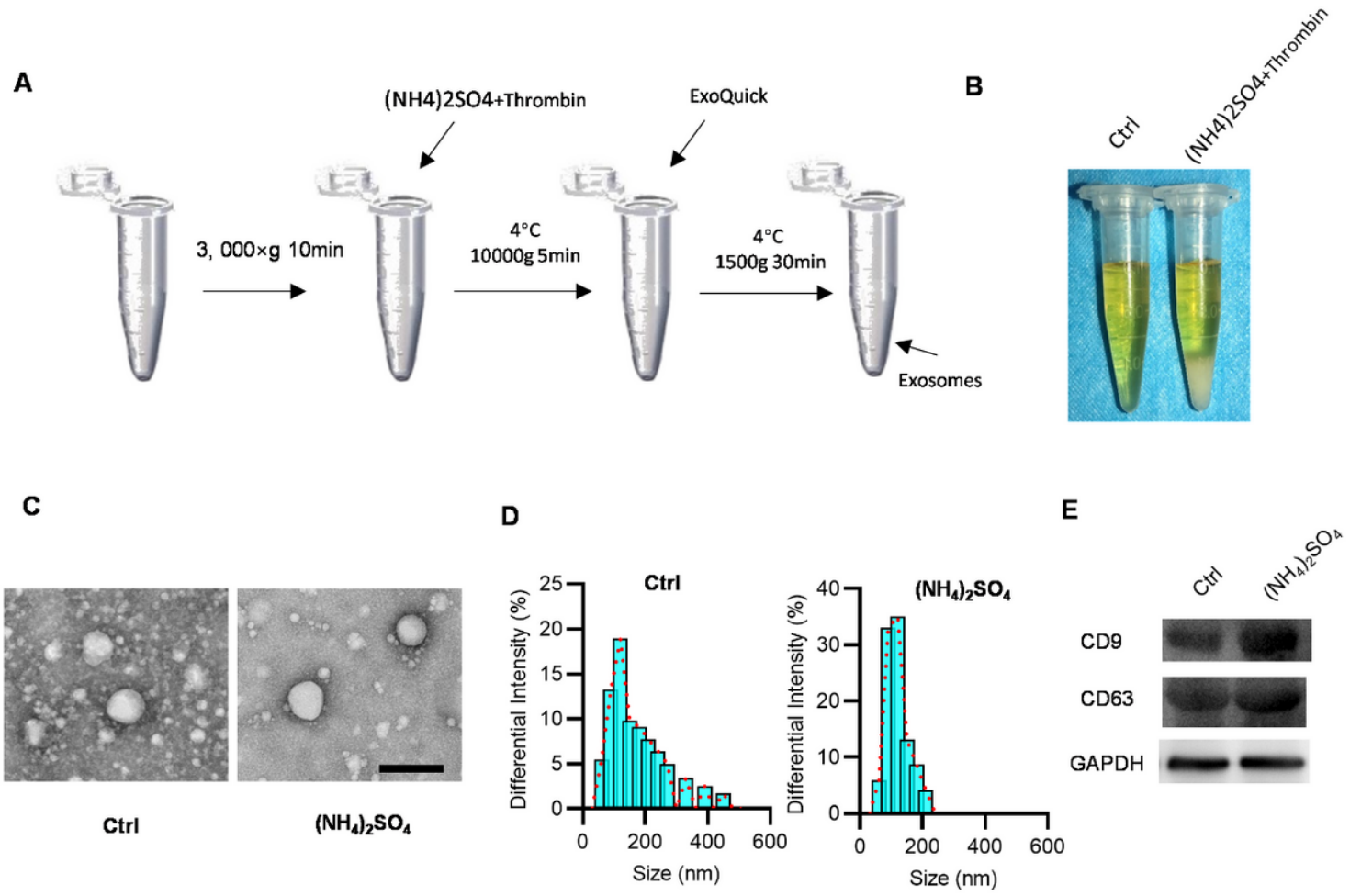


Figure 1

Plasma exosomes isolated by different methods. **A.** Schematic procedures for exosome isolation by prior salting-out strategy. **B.** Salting-out of plasma proteins after addition of (NH4)2SO4+Thrombin. **C.** Representative transmission electron microscopy images of plasma exosomes isolated by indicated methods. **D.** Size distribution of exosomes isolated by different methods. **E.** Western blot analysis of protein contents of exosomes isolated by indicated methods. Scale bar=100 nm.

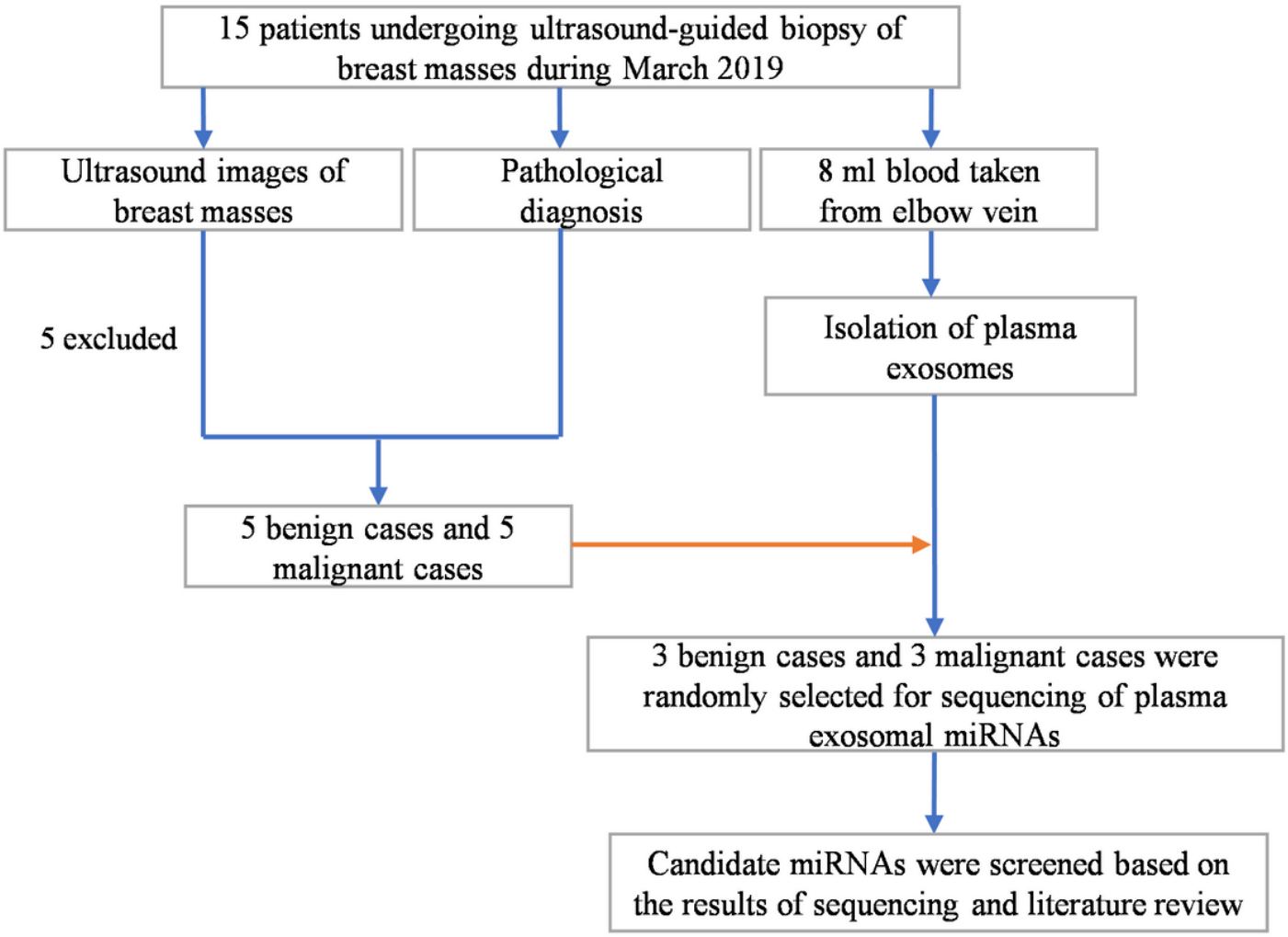


Figure 2

Subject enrollment and flow chart for analysis of exosomal miRNAs expression in patients with breast masses.

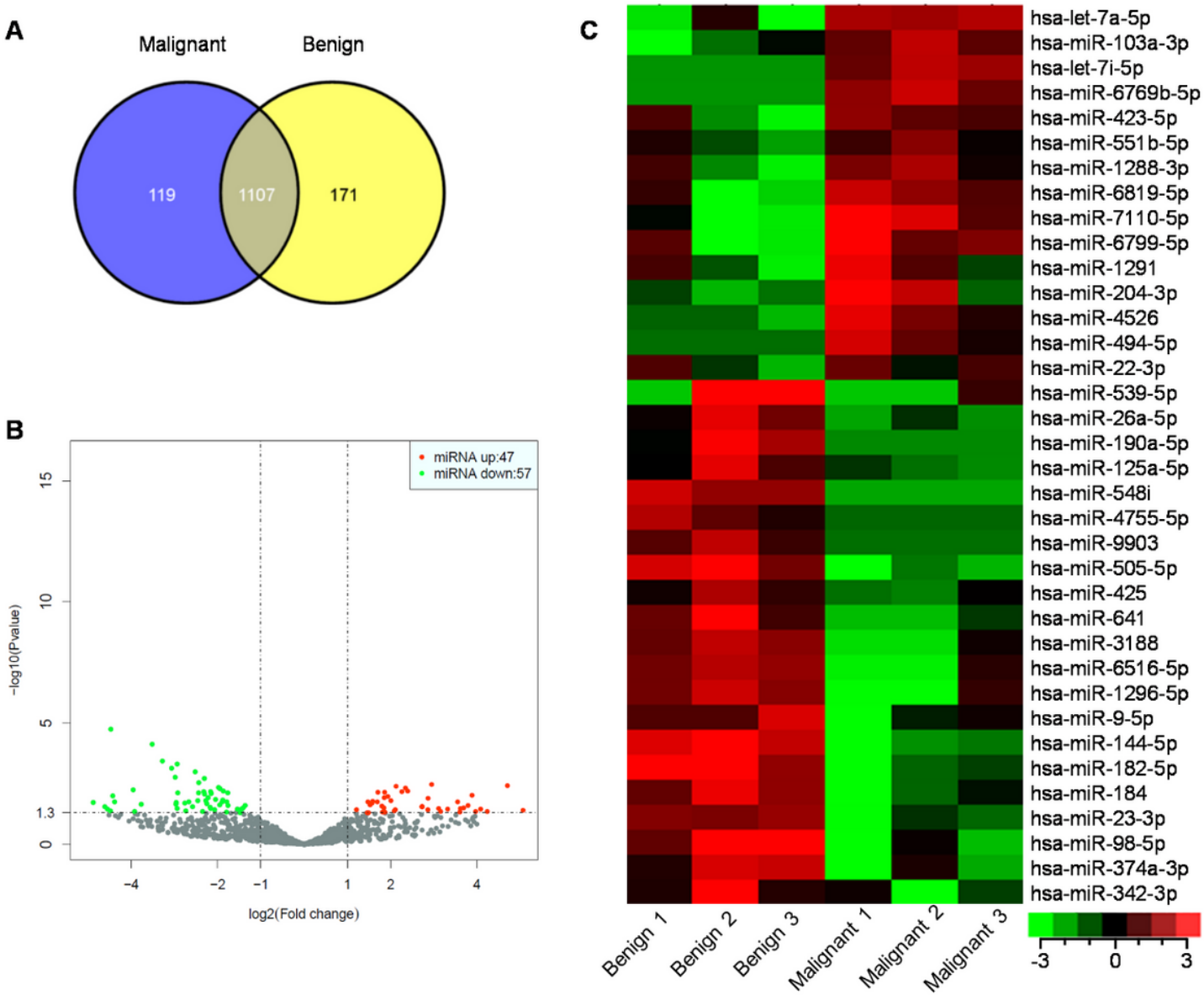


Figure 3

Differential exosomal miRNA expression in patients with benign or malignant breast masses. **A.** Venn chart showing co-expression and specific expression of exosomal miRNAs in patients with malignant or benign breast masses. **B.** Volcano plot showing dysregulation of exosomal miRNAs in different patients. The results were shown as compared to benign masses. **C.** Heatmap showing the differential expression of specific miRNAs in malignant or benign patients.

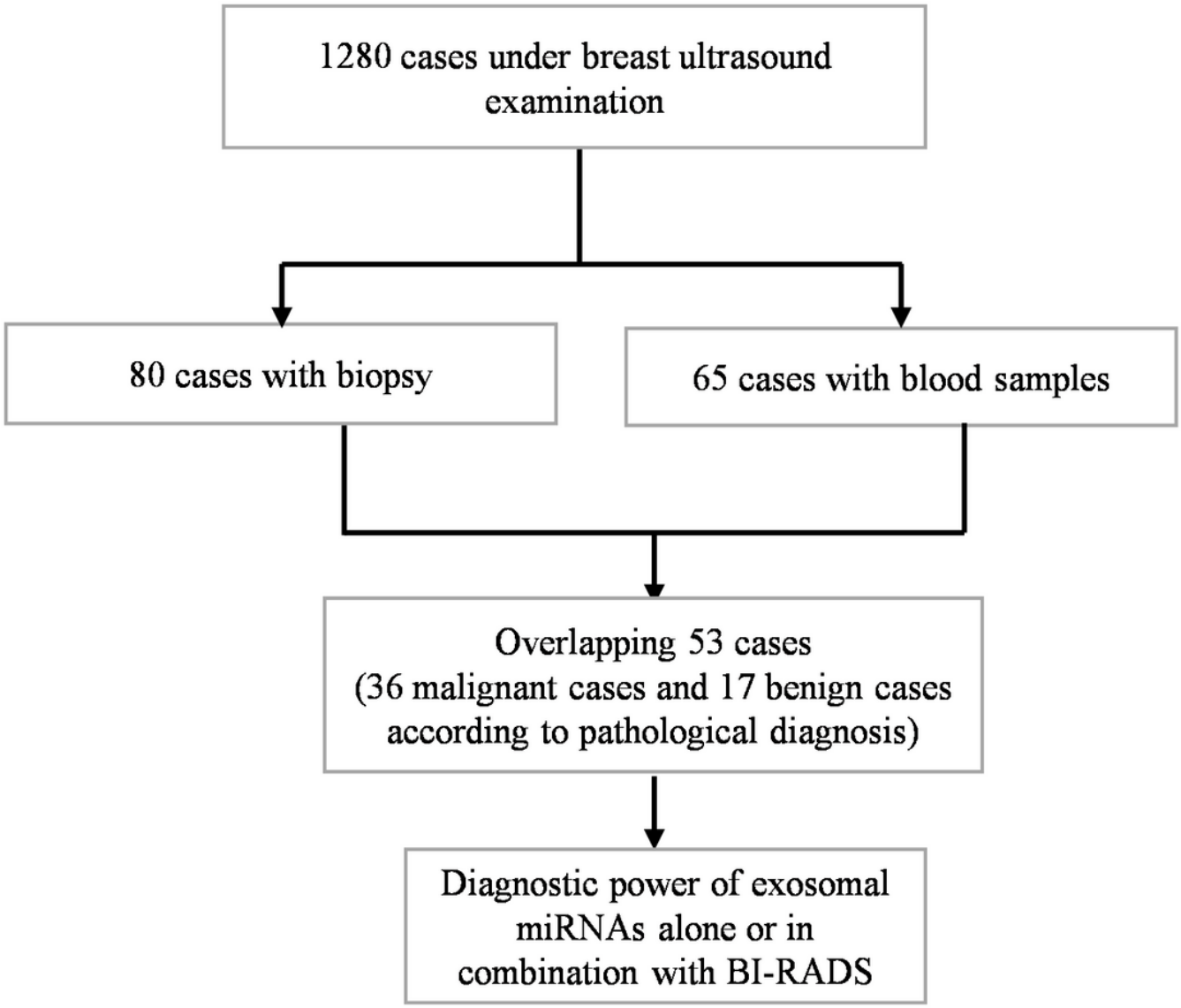


Figure 4

Enrollment and analyses of validation cohort

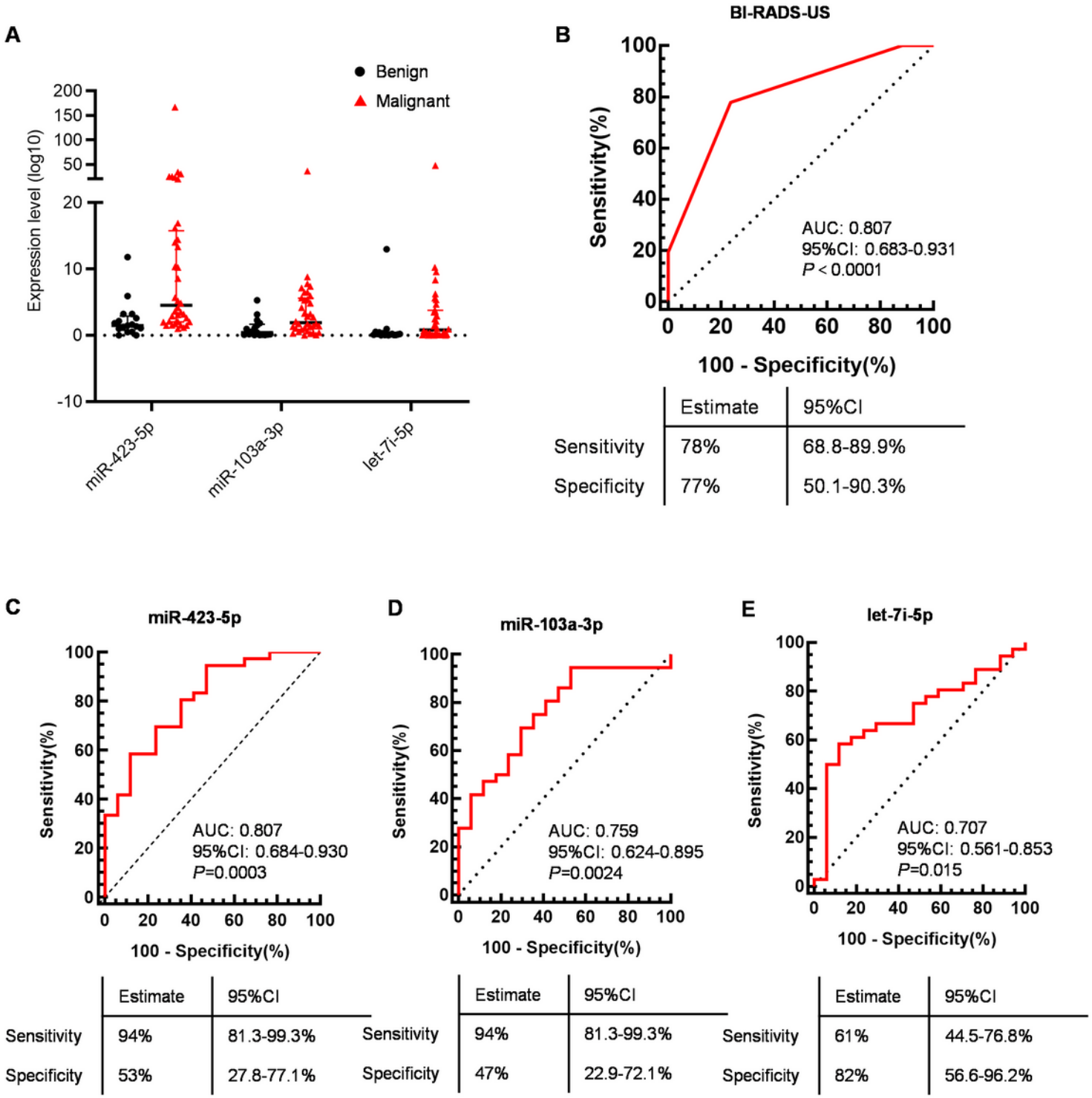


Figure 5

Diagnostic potential of exosomal miRNAs in differentiating benign and malignant breast masses**. A.** Expression of candidate miRNAs in recruited patients with benign or malignant breast masses. **B.** Diagnostic power of BI-RADS-US in differentiating benign and malignant breast masses. **C-E.** Diagnostic power of candidate miRNAs including miR-423-5p (C), miR-103a-3p (D) and let-7i-5p (E) in differentiating benign and malignant breast masses.

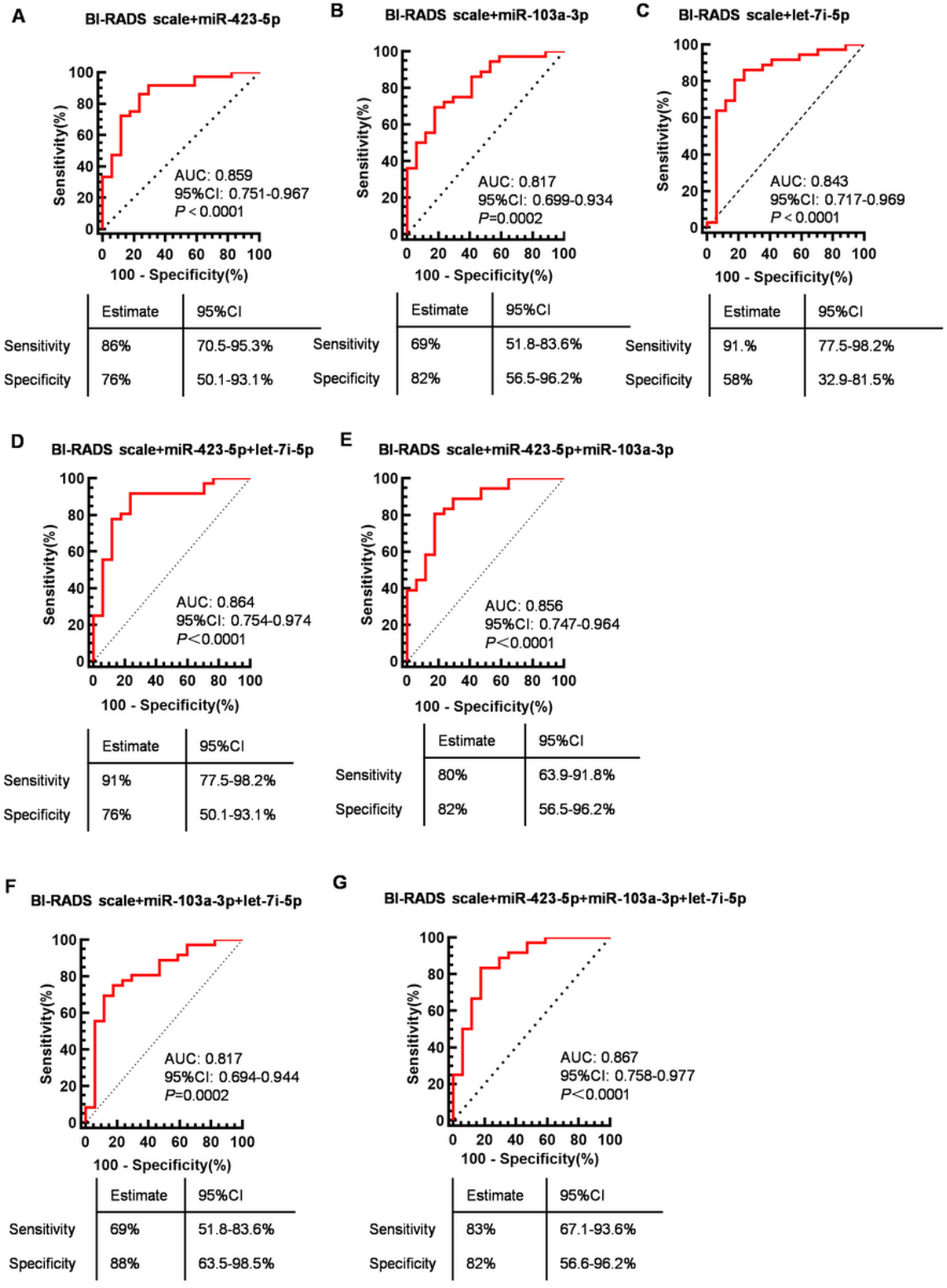
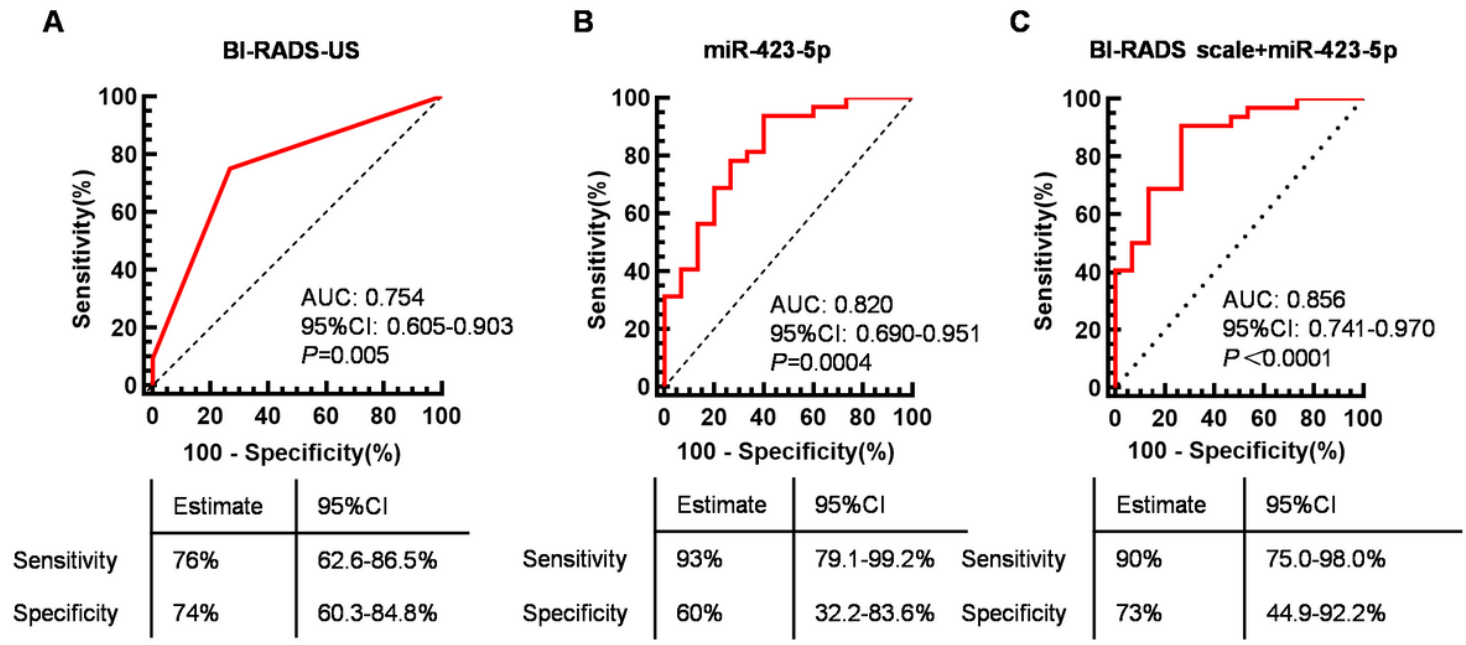


Figure 6

Diagnostic potential of exosomal miRNAs in combination with BI-RADS-US. Single candidate miRNAs or multiple candidate miRNAs were combined with BI-RADS-US for analysis of malignancy of breast masses. **A-G.** Diagnostic power of BI-RADS plus candidate miRNAs including miR-423-5p (A), miR-103a-

3p (B), let-7i-5p (C), miR-423-5p+let-7i-5p (D), miR-423-5p+miR-103a-3p (E), miR-103a-3p+let-7i-5p (F) and miR-423-5p+miR-103a-3p+let-7i-5p (G) in differentiating benign and malignant breast masses.



	Estimate	95%CI
Sensitivity	76%	62.6-86.5%
Specificity	74%	60.3-84.8%

	Estimate	95%CI
Sensitivity	93%	79.1-99.2%
Specificity	60%	32.2-83.6%

	Estimate	95%CI
Sensitivity	90%	75.0-98.0%
Specificity	73%	44.9-92.2%

Figure 7

Diagnostic ability of BI-RADS-US in combination with exosomal miRNAs in discrimination of category 4 breast masses. **A.** Diagnostic ability of BI-RADS-US alone in discrimination of category 4 breast masses. **B.** Diagnostic ability of miR-423-5p alone in discrimination of category 4 breast masses. **C.** Diagnostic ability of BI-RADS-US in combination with miR-423-5p for discrimination of category 4 breast masses.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.doc