The circRNA and miRNA Biomarkers Identification for Docetaxel Resistance in Breast Cancer

MGI's DNBSEQ sequencing Platform Accelerates the Comprehensive RNA Study in Cancer Research

This application note introduced a research team performed the preparation and sequencing of miRNAs in breast cancer cell lines resistant or sensitive to docetaxel based on the MGI DNBSEQ sequencing platform. The team explored the complex regulatory relationship between circRNA, miRNA, and their controlling genes, and revealedg 3 circRNAs and 8 miRNAs as potential biomarkers for docetaxel resistance in breast cancer research.

The findings were published on the journal *Frontiers in Oncology* in 2021, titled with "A Comprehensive RNA Study to Identify circRNA and miRNA Biomarkers for Docetaxel Resistance in Breast Cancer"[1].

Recommended application: Cancer Genomics (Breast Cancer)

Recommended models: DNBSEQ-G400RS

- ### Suitable for various tumor genomics applications

  MGI offers a product portfolio that meets various practical application needs in cancer research, such as whole-genome sequencing, whole-exome sequencing, targeted sequencing, RNA sequencing, and methylation sequencing.

- ### Offer a complete product combination for experimental procedures

  Based on independently developed automatic solutions and analysis software, MGI provides a complete set of product combination from sample to result output.

- ### Data output is efficient and high-quality

  DNBSEQ sequencing technology has significant features such as high accuracy, low repeat sequence rate, and low index hopping rate, etc.

Background

Breast cancer is the most common and deadly cancer among women worldwide, with its incidence and mortality rates showing a continuous upward trend year by year[2]. The chemotherapy drugs of the Taxane family (Docetaxel and Paclitaxel) have been proven to effectively prolong the survival of breast cancer patients by inducing apoptosis in breast cancer cells. They are currently recommended as important first-line drugs for the treatment of breast cancer[3]. Unfortunately, many breast cancer patients develop resistance to docetaxel due to mutations or transcriptional abnormalities in the docetaxel-resistant gene[4-6]. However, current scientific research has not yet elucidated the regulatory mechanism of docetaxel-resistant genes that produce resistance, nor has it found a biomarker that can be used as a biological marker for breast cancer patients to produce docetaxel resistance.

Reported scientific researches have shown that circRNA is related to the occurrence of cancer and other diseases. The specific mechanism is still under study, but it has been proven that it can affect the expression of mRNA by competing with linear splicing[7]. At the same time, it can also act as a molecular sponge to bind miRNAs and reduce the inhibitory effect of miRNA on its target genes[8]. Therefore, circRNAs and miRNA are potential biomarkers for cancer diagnosis and treatment resistance.

Study Description

This research performed RNA-Seq and miRNA sequencing on docetaxel-resistant and sensitive breast cancer cell lines to inverstigate the correlation between circRNA, miRNA, and docetaxel resistance in breast cancer. Through analysis, this study found that there is a potential interaction relationship between circRNA and miRNA, and it is believed that circRNA may absorb miRNAs related to chemotherapy and regulate the signaling pathway that promotes docetaxel resistance in breast cancer cells.

Materials and Methods

Cell culture and drug treatment

The cell lines used herein are: MDARES and MCF7-RES, both of which are docetaxel-resistant human breast cancer cell lines, as well as their docetaxel-sensitive parental cells MDA-MB-231 and MCF-7.

Cell culture: Cells were cultured in DMEM containing l-glutamine, supplemented with 10% fetal bovine serum for MDA-MB-231, and 5% FCS and 1% non-essential amino acids for MCF-7. The cells were cultured in an incubator containing 5% CO_2 at a temperature of 37℃.

Drug treatment: MCF-RES and MDA-RES cell lines were cultured in medium containing 65 nM and 150 nM docetaxel.

Transcriptome analysis and circRNA detection

Total RNA from MCF-7, MCF7-RES, MDA-MB-231 and MDA-RES cell lines was extracted using RNA purification kits with three replicate samples of each cell line. After purifying RNA and digesting with DNase I, ribosomal RNA (rRNA) was removed and a quality control check was performed before constructing an RNA-Seq library. Sequencing was then carried out using a certain brand of sequencer. The sequencing data were predicted for circRNA using CIRCexplorer software and their relative expression levels were expressed by SRPBM.

miRNA binding site prediction

Three circRNAs were obtained by CIRCexplorer analysis: circABCB1, circEPHA3.1 and circEPHA3.2. Four software tools, TargetScan v7.1, Miranda, Pita and RNAhybrid, were used to predict the potential microRNA targets on these 3 circRNAs.

miRNA sequencing and data analysis

The above extracted total RNA samples were separated by PAGE gel and 18-30 nt bands were selected for recovery. The MGIEasy Small RNA Library Prep Kit was used for the preparation of small RNA libraries. Please refer to the manual instructions for the procedure. The prepared libraries were sequenced on the MGI DNBSEQ sequencing platform to complete the sequencing of miRNAs. In the analysis of miRNA sequencing data, the filtered data were firstly compared with the mature miRNA sequences downloaded from the miRBase database and known miRNAs were found using BLAST, and then the expression levels of miRNAs were calculated and differentially expressed miRNAs between different groups were analyzed using DESeq2. Meanwhile, the miRNAs related to chemotherapy in the published literature were collected in PubMed database, and combined with the miRNA data related to doxorubicin and paclitaxel in Pharmaco-miR database for comprehensive analysis. Then miRNA target gene prediction and KEGG pathway enrichment etc. were performed.

Sample collection	Library preparation	Bioinformatics analysis	Result analysis
MDARES,MCF7-RES, MDA-MB-231, and MCF-7 cell lines were treated appropriately and RNA was extracted.	 MGIEasy RNA Library Prep set MGIEasy Small RNA Library Prep kit  DNBSEQ-G400 genetic sequencer	Software related to transcriptome, circRNA and miRNA analysis	Identification, prediction and functional analysis of Circ RNA and miRNA in docetaxel-resistant and sensitive cell lines

Result analysis

Identification and analysis of Circ RNA in both docetaxel-resistant (DRBC) and sensitive cell lines of breast cancer

a. Identification of CircRNA in the sample

The researchers aligned RNA sequencing reads to the human reference genome (hg19) and used the software CIRCexplorer for prediction. They detected a total of 8246 high-reliability exon circRNAs (containing at least two unique reverse spliced reads) (Figure 1A), and found that 58.9% of the circRNAs were shared between MCF and MDA cell lines (Figure 1B). The number of circRNAs and the genes that produce circRNAs in MDA cell lines was relatively fewer than those in MCF cell lines. However, there was no significant difference in the number of circRNAs and corresponding genes that are sensitive or resistant to docetaxel between the two cell lines (Figure 1C).

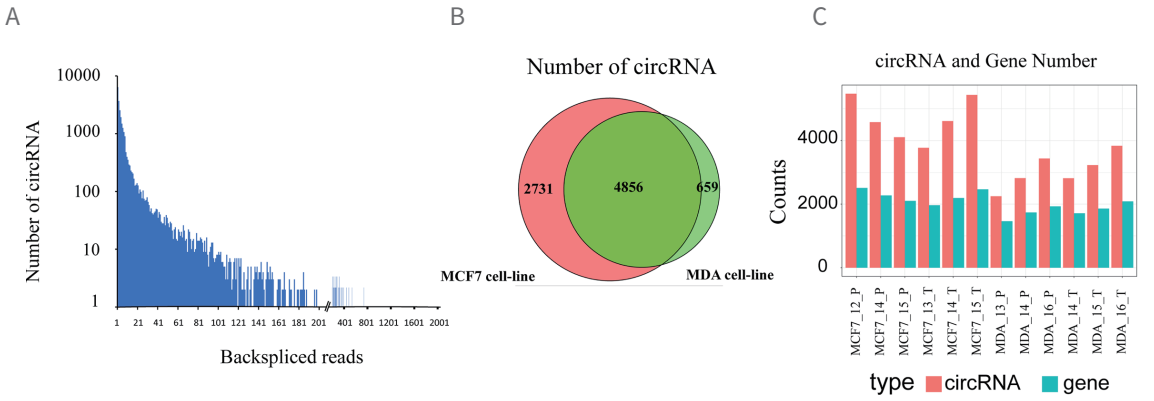

Figure 1. Identification of circRNAs in MCF and MDA cell lines. A. Number of reverse spliced sequences and circRNAs in the sample; B. Number of circRNAs shared between MDA and MCF cell lines; C. Number of circRNAs and genes generating circRNAs in each sample.

b. Identification and functional prediction of specific circRNAs in docetaxel-sensitive and resistant cell lines

The researchers analyzed the expression of circRNAs in docetaxel-sensitive and resistant cell lines and found that there were 380 circRNAs specifically expressed in docetaxel-resistant cell lines and 274 circRNAs specifically expressed in docetaxel-sensitive cell lines (Figure 2A). The abundance of specifically expressed circRNAs was evaluated by calculating the SRPBM value and the expression frequency of circRNAs in docetaxel-sensitive and resistant cell lines (Figure 2B). Figure 2C clearly shows the top ten most abundant circRNAs in both types of cell lines.

It is worth noting that among the 20 circRNAs with the highest abundance, researchers found that three circRNAs were produced by genes closely related to multidrug resistance in cancer cells: Circ.26318 was produced by the multidrug resistant gene *ABCB1*, and this circRNA was named "circABCB1"; circ.22881 and circ.5255 were produced by the multidrug resistant gene *EPHA3* and these two circRNAs were named "circEPHA3.1" and "circEPHA3.2", respectively. Further study found that the expression levels of the three newly discovered circRNAs were linearly positively correlated with their corresponding gene expression levels (Figure 2D), and the genomic structure of these three circRNAs and their expression levels in docetaxel-resistant and sensitive cells confirmed the existence of these three circRNAs (Figure 3).

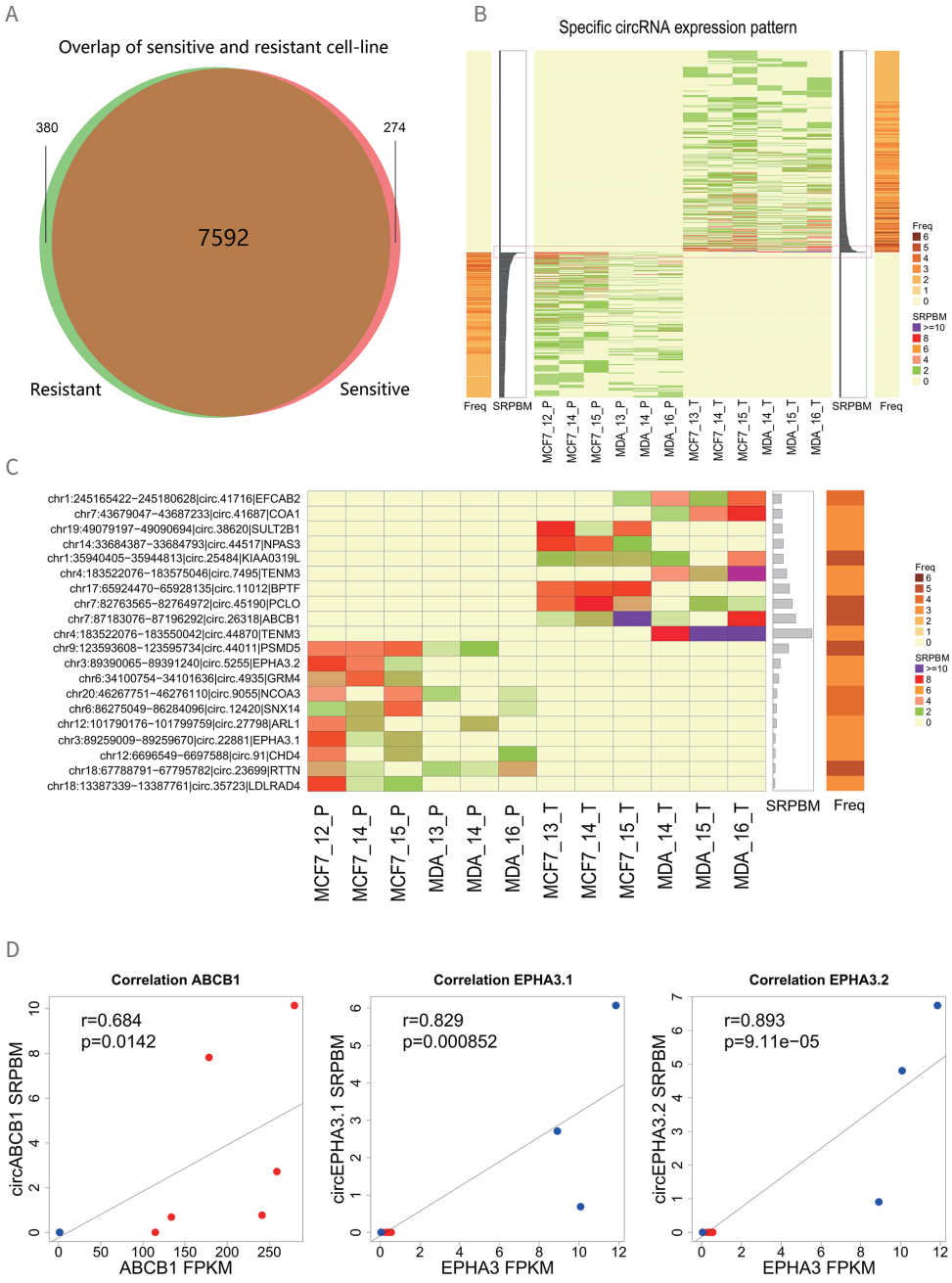

Figure 2. Identification and functional prediction of specific circRNAs in docetaxel-sensitive and resistant cell lines. A. The quantity of specific circRNA in docetaxel-resistant and sensitive cell lines; B. Evaluation of the abundance of specific circRNA; C. Top 10 abundant specific circRNA in docetaxel-resistant and sensitive cell lines; D. The linear relationship between circABCB1, circEPHA3.1, circEPHA3.2 and their corresponding gene expression levels.

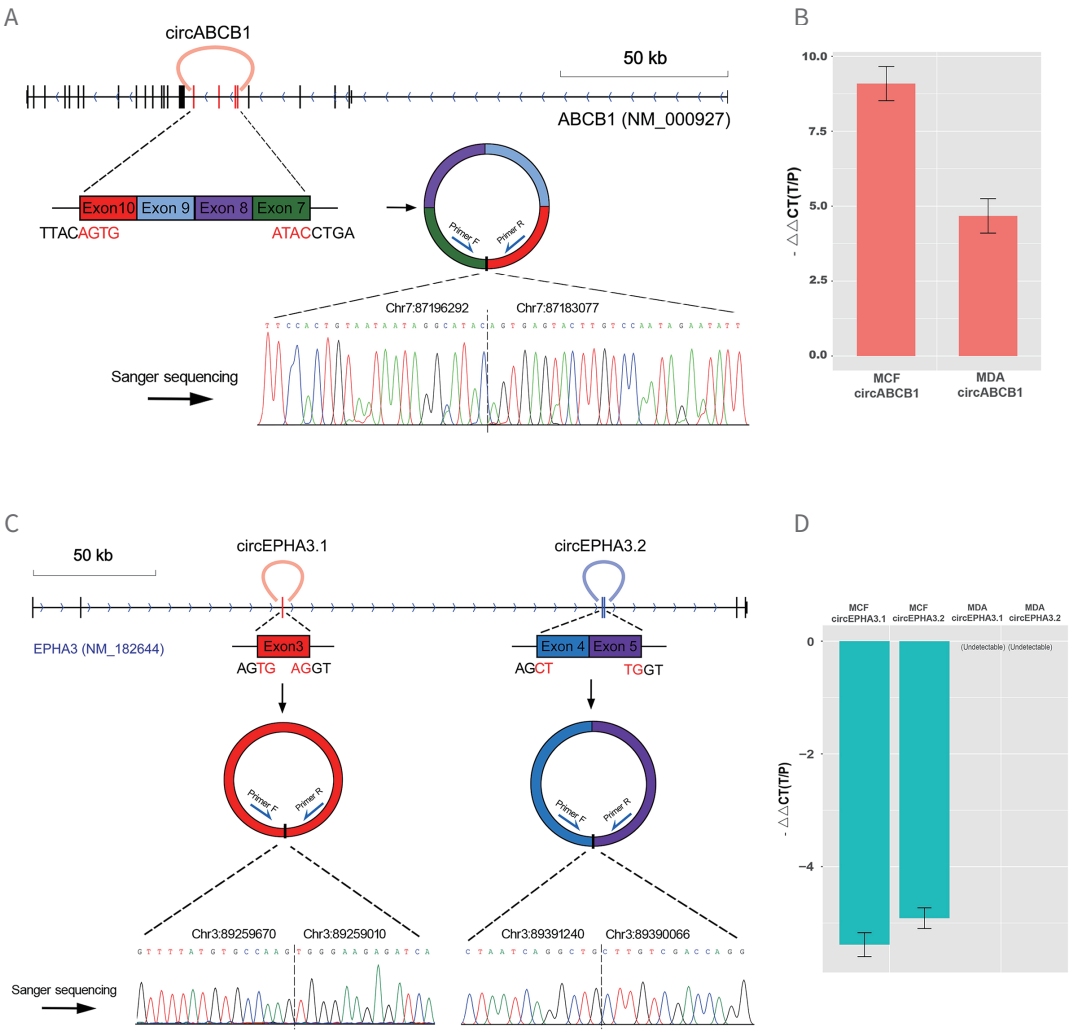

Figure 3. Gene structure and expression levels of circABCB1, circEPHA3.1, and circEPHA3.2. A. Gene structure of circABCB1; B. circABCB1 is significantly upregulated in docetaxel-resistant cells; C. Gene structure of circEPHA3.1 and circEPHA3.2; D. circEPHA3.1 and circEPHA3.2 are significantly downregulated in docetaxel-resistant cells.

Prediction of interaction between circABCB1, circEPHA3.1, and circEPHA3.2 and miRNA

This study used circRNA-miRNA binding response elements (MREs) to predict the interaction between the above three specific circRNAs and miRNAs, and to enhance the accuracy of the prediction, the author used four software programs, TargetScan v7.1, Miranda, Pita, and RNAhybrid, respectively, to build three interaction networks between circRNAs and miRNAs (Figure 4).

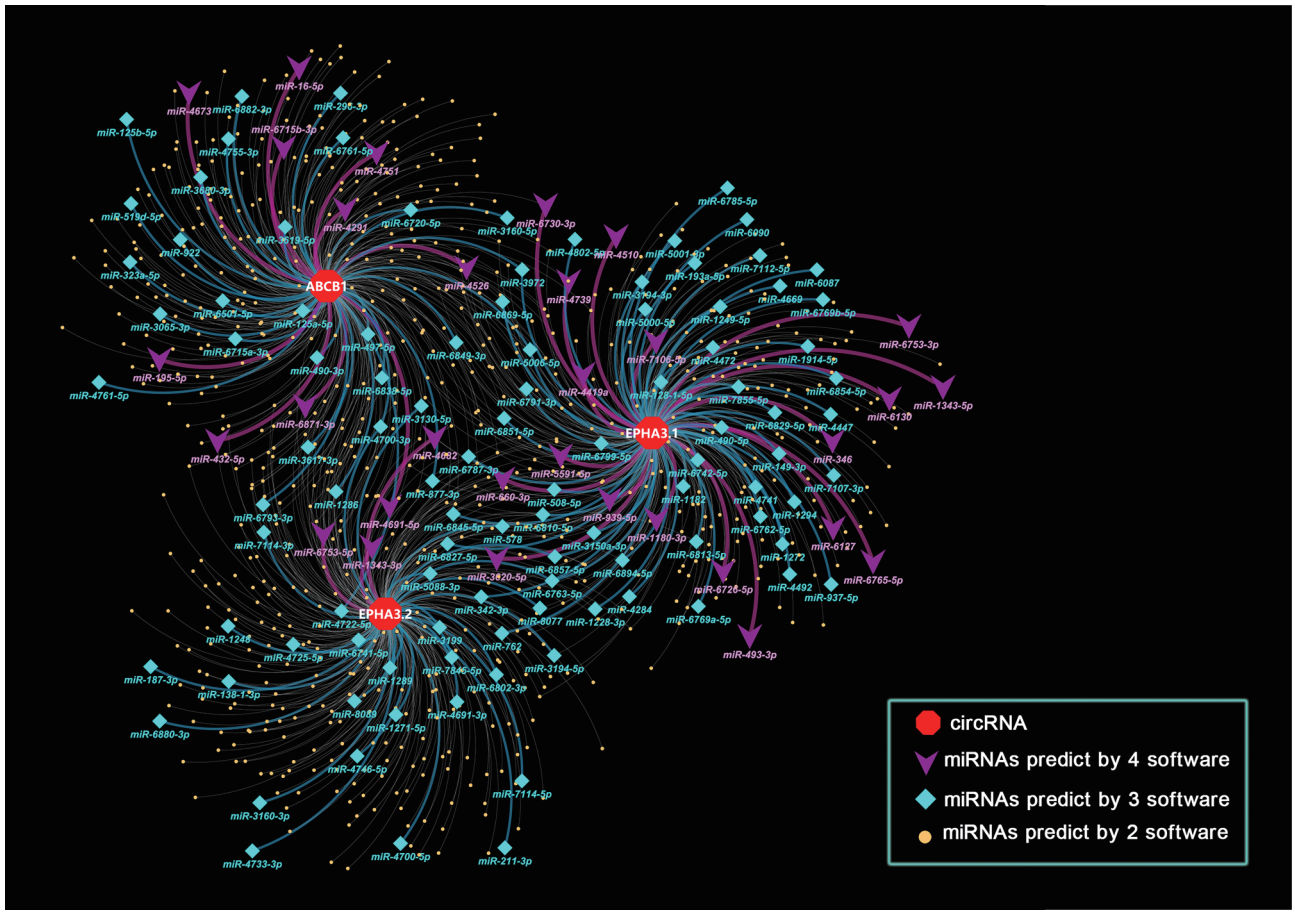

Figure 4. Interaction network between circABCB1, circEPHA3.1 and circEPHA3.2 and predicted miRNAs.

Identification, prediction and functional analysis of miRNA in docetaxel-resistant and sensitive cell lines

To further study the interaction between circular RNAs and miRNAs, the author constructed a library of small RNAs and sequenced them. In the sequenced sample, a total of 2104 miRNAs were identified, of which 88 miRNAs (44 downregulated and 38 upregulated) were significantly differentially expressed (SDE) in DRBC cells (Figure 5A). To further predict SDE miRNAs with potential functions, the author took the intersection of chemotherapy-related miRNAs reported in the literature, miRNAs predicted by software to interact with circABCB1, circEPHA3.1, and circEPHA3.2, and SDE miRNAs identified by sequencing (Figure 5B). It was found that among the SDE miRNAs related to chemotherapy response, 8 were predicted by at least two software programs to interact with circABCB1, circEPHA3.1, and circEPHA3.2.

The above results strongly indicate that three potential functions of circRNA - circABCB1, circEPHA3.1 and circEPHA3.2 - may promote chemotherapy resistance or sensitivity by regulating these miRNAs. The differential expression and chemotherapy-related information of the above 8 miRNAs are shown in Table 1. To reveal the function of these 8 miRNAs, the author con-

ducted enrichment analysis of their target genes in signaling pathways (Figure 5E), identifying a total of 13 significantly enriched pathways, including the PI3K-Ak and AGE-RAGE pathways which showed consistent enrichment results with the pathways of SDE miRNAs found in the same cell line. Based on the above results, the author believed that there was a regulatory pathway in the resistance mechanism of docetaxel, which was absorbed by at least 8 miRNAs through the PI3K-Akt signaling pathway, consisting of circABCB1, circEPHA3.1 and circEPHA3.2.

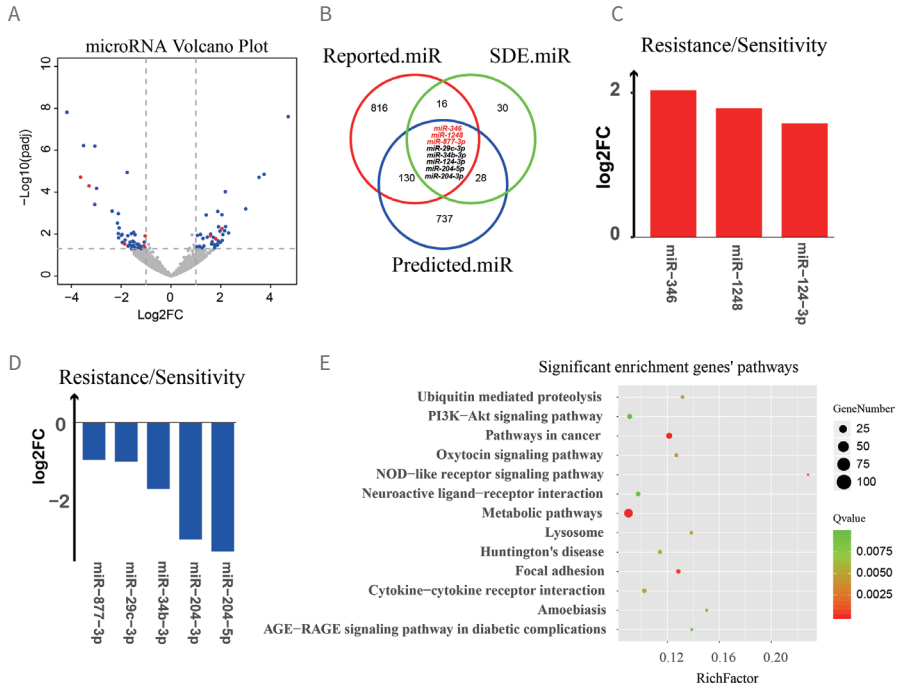

Figure 5. Identification, prediction and functional analysis of miRNA in docetaxel-resistant and sensitive cell lines. A. Screening conditions for miRNA volcano plot; B. Venn diagram of the intersection of miRNAs related to chemotherapy (red), miRNAs predicted by software to interact with circABCB1, circEPHA3.1, and circEPHA3.2 (blue), and SDE miRNAs identified by sequencing (green); C, D. Expression levels of 8 miRNAs in docetaxel cell lines; E. Signal pathway enrichment diagram of 8 miRNA target genes.

miRNA	log_2FC (Resistant/Sensitive)	P value	circRNAtarget	Publications associated with chemotherapy response
miR-346	2.04	$5.51x10^{-3}$	circEPHA3.1	Du L. et al. (40) Braun FK. et al. (41) Yang et al. (42)
miR-124-3p	1.58	$1.22x10^{-2}$	circEPHA3.2	Liu YX. et al. (43) He C. et al. (44) Khalil S. et al. (45)
miR-204-5p	-3.64	$1.95x10^{-5}$	circEPHA3.2	Bian Z. et al. (46) Yin Y. et al. (47)
miR-1248	1.78	$1.69x10^{-2}$	circEPHA3.2	Xu et al. (48)
miR-204-3p	-3.30	$5.01x10^{-5}$	circEPHA3.2	Chen PH. et al. (49)
miR-34b-3p	-1.87	$2.96x10^{-2}$	circABCB1	Zhou et al. (50) Hermeking et al. (51)
miR-29c-3p	-1.10	$3.61x10^{-2}$	circABCB1	Zhang et al. (52) Ma X. et al. (53)
miR-877-3p	-1.05	$1.25x10^{-2}$	circABCB1	Huang et al. (54) Li et al. (55)

Table 1. Differential expression and chemotherapy-related information of the 8 miRNAs.

Summary

This study conducted RNA and miRNA sequencing on two types of DRBC cell lines and their docetaxel-sensitive parent cell lines and discovered different potential biomarkers in docetaxel-resistant and sensitive breast cancer cells. It has been revealed for the first time that the circRNA produced by multidrug resistant genes in paclitaxel-resistant cancer cells. Moreover, this study also identified and investigated the potential functions of three circRNAs, CircABCB1, and circEPHA3.2. The potential interaction between circRNA and miRNA have been revealed and the findings suggested that CircABCB1, circEPHA3.1, and circEPHA3.2 might promote docetaxel resistance by absorbing eight chemotherapy-related SDE miRNAs through the PI3K-Akt and AGE-RAGE signaling pathways.

This study performed library preparation and sequencing for miRNA in docetaxel-resistant and sensitive cells with the MGIEasy Small RNA Library Prep Kit and DNBSEQ sequencing platform independently developed by MGI. The DNBSEQ sequencing platform developed by MGI is comprehensive, flexible, and capable of production scale. Additionally, it can effectively support sequencing applications and data analysis in fields such as scientific research, medical clinical study, judicial and agriculture.

DNBSEQ-G400 Genetic Sequencer

References

1. Huang PD, Li FY, Mo ZC, Geng CY, Stenvang JA, et al. Comprehensive RNA Study to Identify circRNA and miRNA Biomarkers for Docetaxel Resistance in Breast Cancer. *Frontiers in Oncology*, (2021) 11: 1713-1727.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* (2021) 0:1–41.
3. Yardley DA. Drug Resistance and the Role of Combination Chemotherapy in Improving Patient Outcomes. *Int J Breast Cancer* (2013) 2013:1–15.
4. Kastl L, Brown I, Schofield AC. Altered DNA Methylation is Associated With Docetaxel Resistance in Human Breast Cancer Cells. *Int J Oncol* (2010) 36:1235–41.
5. Hansen SN, Westergaard D, Thomsen MBH, Vistesen M, Do KN, Fogh L, et al. Acquisition of Docetaxel Resistance in Breast Cancer Cells Reveals Upregulation of ABCB1 Expression as a Key Mediator of Resistance Accompanied by Discrete Upregulation of Other Specific Genes and Pathways. *Tumor Biol* (2015) 36:4327–38.
6. Hansen SN, Ehlers NS, Zhu S, Thomsen MBH, Nielsen RL, Liu D, et al. The Stepwise Evolution of the Exome During Acquisition of Docetaxel Resistance in Breast Cancer Cells. *BMC Genomics* (2016) 17:1–15.
7. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, et al. Circrna Biogenesis Competes With Pre-mRNA Splicing. *Mol Cell* (2014) 56:55–66.
8. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, et al. Natural RNA Circles Function as Efficient microRNA Sponges. *Nature* (2013) 495:384–8.

Recommended Ordering Information

Category	Product	Cat. NO.
Instruments	Genetic Sequencer DNBSEQ-G400RS	900-000170-00
	MGISP-100RS Automated Sample Preparation System	900-000206-00
	MGISP-960RS Automated Sample Preparation System	900-000146-00
Software	MegaBOLT Bioinformatics analysis accelerator	900-000555-00
Library Prep	MGIEasy RNA Library Prep Set（16 RXN）	1000006383
	MGIEasy Small RNA Library Prep Kit（24 RXN）	940-000196-00
Sequencing Reagents	DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100)	1000016950
	DNBSEQ-G400RS High-throughput Sequencing Set (FCL SE50)	1000016941

MGI Tech Co.,Ltd

Building 11, Beishan Industrial Zone, Yantian District, Shenzhen, CHINA, 518083

+86-4000-688-114

en.mgi-tech.com

MGI-service@mgi-tech.com

Author：Lin Guo, Kaiyue Cen

Editor-in-Charge：Qiwei Wang

Reviewer：Yao Jiang

*1. For StandardMPS and CoolMPS: Unless otherwise informed, StandardMPS and CoolMPS sequencing reagents, and sequencers for use with such reagents are not available in Germany, Spain, UK, Sweden, Belgium, Italy, Finland, Czech Republic, Switzerland, Portugal, Austria and Romania. Unless otherwise informed, StandardMPS sequencing reagents, and sequencers for use with such reagents are not available in Hong Kong. No purchase orders for StandardMPS products will be accepted in the USA until after January 1, 2023.

2. For HotMPS sequencers: This sequencer is only available in selected countries, and its software has been specially configured to be used in conjunction with MGI's HotMPS sequencing reagents exclusively.

3. For HotMPS reagents: This sequencing reagent is only available in selected countries.