LncRNA CBR3-AS1 Regulates of Breast Cancer Drug Sensitivity as a Competing Endogenous RNA Through the JNK1/MEK4-Mediated MAPK Signal Pathway

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Research

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Abstract

Background: Adriamycin (ADR) resistance is one of the main obstacles to improve the clinical prognosis of breast cancer (BRCA) patients. Long non-coding RNAs (lncRNAs) can regulate cell behavior, but the role of these RNAs in anti ADR of BRCA remains unclear. Here we aim to investigate the imbalance of a particular long noncoding RNA, lncRNA CBR3 antisense RNA 1 (CBR3-AS1), and its role in ADR resistance.

Methods: Microarray analysis of ADR-resistant BRCA cells was performed to identify CBR3-AS1. CCK8 assay and colony formation assay were used to detect the sensitivity of BRCA cells to ADR. Dual-luciferase reporter, RNA pull down, IHC and Western blot were used to verify the relationship between CBR3-AS1, miRNA and target genes. In vivo, the effect of CBR3-AS1 on BRCA resistance was observed by xenograft tumor model. The role of CBR3-AS1 in influencing ADR sensitivity was verified by clinical BRCA specimens, TCGA, CCLE, and GDSC databases.

Results: Here, we found that lncRNA CBR3-AS1 was significantly increased in BRCA tissues and was closely correlated with poor prognosis. CBR3-AS1 overexpression promoted ADR resistance in BRCA cells in vitro and in vivo. Mechanistically, we identified that CBR3-AS1 functioned as a competitive endogenous RNA by acting as a molecular sponge of miR-25-3p. MEK4 and JNK1 of MAPK pathway were the direct downstream proteins of CBR3-AS1 / miR-25-3p axis in BRCA cells.

Conclusions: In summary, our findings demonstrate that CBR3-AS1 plays a critical role in the chemotherapy resistance of BRCA by mediating the miR-25-3p, MEK4/JNK1 regulatory axis. The potential of CBR3-AS1 as an oncogene and therapeutic biomarker of BRCA was identified.

Background

Worldwide, BRCA (BRCA) is the most common malignant tumor in women [1–3]. Every year, an estimated 1 million BRCA new cases were diagnosed worldwide. Adriamycin (ADR) is considered to be one of the most effective drugs for BRCA, especially after tamoxifen treatment failed. However, its efficacy as a chemotherapeutic drug is greatly reduced due to intrinsic drug resistance and acquired drug resistance during treatment[4, 5]. The underlying mechanisms resulting in ADR resistance in BRCA remain poorly understood.

LncRNAs are a group of non-coding RNA molecules with a length of more than 200 nucleotides[6]. According to reports, lncRNAs are involved in many biological processes related to carcinogenesis and drug resistance[7–10]. For example, TINCR promotes epithelial-mesenchymal transition and trastuzumab resistance by targeting miR-125b [11]. HOTAIR mediates tamoxifen resistance by promoting estrogen receptor transcriptional activity [12]. DSCAM-AS1 mediates tumor progression and tamoxifen resistance by regulating estrogen receptor (ER)[13]. However, the role of lncRNAs in the process of ADR resistance of BRCA is still not fully explored.

LncRNA CBR3-AS1 is located in the antisense region of carbonyl reductase 3 (CBR3). It was initially discovered that CBR3-AS1 was abnormally expressed in prostate cancer cell lines and tissues[14–16]. Further studies showed that up regulation of CBR3-AS1 promotes the proliferation of prostate cancer cells and inhibit cells apoptosis. Subsequent studies found that overexpression of CBR3-AS1 was observed in gastric carcinoma, esophageal squamous cell carcinoma, osteosarcoma, colorectal cancer and retinoblastoma[17–21]. However, the biological function and clinical significance of CBR3-AS1 in BRCA drug resistance remain unclear.

In our study, we screened differentially expressed lncRNAs between ADR-resistant BRCA cells and its parent cells. We found that the expression of CBR3-AS1 was up-regulated in ADR-resistant BRCA cells, and its high expression was associated with poor prognosis of BRCA. The overexpression of CBR3-AS1 promoted, whereas knockdown of CBR3-AS1 significantly repressed the ADR resistance both in vivo and in vitro. Further researches showed that CBR3-AS1 functioned as a ceRNA (competing endogenous RNA) via binding miR-25-3p. JNK1 and MEK4 were downstream proteins of CBR3-AS1/miR-25-3p axis in BRCA cells. Our results confirm for the first time that CBR3- promoted ADR resistance of BRCA cells via regulation of CBR3-AS1/miR-25-3p/MEK4/JNK1 axis, indicates that CBR3-AS1 has potential as an oncogene and biomarker for BRCA treatment.

Methods

Access and analysis of public data

GSE20685 was downloaded from the GEO dataset (http://www.ncbi.nlm.nih.gov/geo/). The whole genome expression profiles and clinicopathological information of human cancer were downloaded from the Human Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/). The expression of lncRNAs in the cell lines was obtained from the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle). The IC_{50} values of cell lines to drug therapy were obtained from Genomics of Drug Sensitivity in Cancer (GDSC) database. All transcripts were normalized by $\log_2$ transformation. Diana lncbase (http://carolina.imis.athena-innovation.gr/diana_tools) was used to predict the binding of miRNAs and lncRNA. Bioinformatics tool analysis miRdb (http://www.miRdb.org/) was used to predict the binding of miRNA and mRNA 3'UTR. The RNAhybrid website (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) was used to predict the binding sites of miRNA and lncRNA. The bioinformatics website lncLocator (http://www.csbio.sjtu.edu.cn/cgi-bin/lncLocator.py) was used to predict the subcellular localization of lncRNA.

The correlation between genes was evaluated through pearson correlation test. The differences between BRCA tumor and normal samples was evaluated by unpaired t-test. Log-rank test was used to test the difference of survival rate among different groups of patients.

Patient specimens

BRCA specimens (n = 96) were obtained from hospitalized patients from November 2008 to June 2009. All patients were diagnosed with BRCA in the First Affiliated Hospital of China Medical University. Before surgery, the patient did not receive chemotherapy or radiotherapy. This study was approved by Ethics Committee of China Medical University (Approval number: AF-SOP- 07-1.1-01).

Cell culture

MCF-7, T47D, MDA-MB-231 and HEK-293T cell lines purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China), and MCF-7/ADR purchased from the Zhen Shanghai and Shanghai Industrial Co., Ltd (Shanghai, China). The cells were cultured in L-15 medium, DMEM medium or RPMI 1640medium (Hyclone, USA) and cultured in a humidified incubator at 37 ℃. Except the cells cultured in L-15 medium, the cells were cultured in the environment containing 5% carbon dioxide.

Microarray analysis

Microarray analysis was performed by Shanghai OE Biotech (China). The microarray was Agilent Human lncRNA 4*180K (Design ID: 076500). The raw microarray data were submitted to the GEO database (accession number: GSE155478). The LncRNAs were identified by gencode database (https://www.gencodegenes.org). The p-value and fold change value of t-test were used to screen differential lncRNAs. The screening criteria were up-regulated or down-regulated $\log_2 FC \geq 2.0$ and $FDR \leq 0.05$.

Gene set enrichment analysis

Data of CBR3-AS1 expression were obtained from BRCA patients with TCGA. Taking the median of cbr3-as1 expression as the cut-off point, BRCA cases were divided into two groups. The index of gene sequencing was set as "Pearson", the curve of top set was set to 150, and all other parameters were the default values.

Cell transfection

The cells were seeded on a 6-well plate and incubated for 24 hours. The fusion degree of cells reached 70–80%. Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA) transfection reagent (5μL) was dissolved in 125 μ l serum-free medium. 2 μ g DNA was diluted with 125 μl serum-free medium to prepare DNA (Hanheng, China) premix, and then 10μL p3000 reagent was added to mix well. The Lipofectamine 3000 was then mixed with DNA. After incubation for 5 minutes, the complex was added to the cells. After 4 hours, the fresh medium containing 10% serum was changed. The final transfection concentration of miRNA mimics (RiboBio, Guangzhou, China) was 50 μm and the final concentration of inhibitor was 100 μM. The final concentration of siRNA (RiboBio, China) transfection was 50 μM. p3000 was not used in the transfection process. The other steps were equivalent to DNA transfection.

In animal experiment, the plasmid was stably transfected. On the basis of the above steps, puromycin 1 mg/L was added to culture for one month, and stable transfected cell lines were selected for the

experiment.

Western blotting

Cells were lysed on ice in RIPA buffer (Beyotime, China). The lysate was centrifuged at 15000 g at 4 ℃ for 15 minutes. SDS polyacrylamide gel electrophoresis was used to isolate proteins and transfer them onto PVDF membrane. The membrane was blocked in 5% skimmed milk and incubated with the primary antibody in TBST at 4℃ overnight. Then incubated with a horseradish peroxidase-labeled secondary antibody at a concentration of 1:10000 for one hour. Finally, chemiluminescence was carried out using micro chemi 4.2 system (DNR Bio-imaging system, Israel). The details of all antibodies used were showed in Table S1

Luciferase assays

HEK-293T cells were transfected with MEK4-3'UTR-WT, MEK4-3'UTR-MU, JNK1-3'UTR-WT and JNK1-3'UTR-MU plasmids. At the same time, the four groups cells were transfected with mimic NC and mimic-miR-25-3p to form eight experimental groups. Finally, each group of cells were transfected with Renilla Luciferase plasmid. After 36 hours, the cells were harvested for luciferase analysis using a dual luciferase reporting and detection system (Promega, USA). The result were normalized against Renilla luciferase activity.

qRT-PCR

Trizol (Invitrogen, USA) was used to extract total RNA from cultured cells. For mRNA and lncRNAs, ReverTra Ace qPCR RT Kit (Toyobo, Japan) was used, and 200 ng of total RNA was used to synthesize cDNA at 10 μl reaction volume. For miRNAs, Bulge-Loop™ miRNA qRT-PCR Starter Kit (Ribobio, China) was used, and 1 μg of total RNA was used to synthesize cDNA at 10 μl reaction volume. The qRT-PCR of mRNA and lncRNAs used SYBR Green Realtime PCR Master Mix (Toyobo, Japan) with 12.5 μl reaction volume. The qRT-PCR of miRNAs used Bulge-LoopTM miRNA qRT-PCR Starter Kit (ribobio, China) with 20 μl reaction volume. The primers were listed in Table S2.

Colony formation assay

MCF-7/ADR and MCF-7 cells (1×10^3/well) in logarithmic phase were resuspended and inoculated in a 60 mm cell culture dish. When adding drugs, 100 nM ADR was added to MCF-7 cells, and 30 μM ADR was added to MCF-7/ADR cells to culture for 48 h. After, culture with normal medium. Two weeks later, the cells were washed with PBS twice and fixed with methanol, and stained with 0.1% crystal violet (Beyotime, China) for 30 minutes.

CCK8 assay

MCF-7/ADR and MCF-7 cells were transiently transfected into 96 well plate with 3×10^3 cells per well. After 24 hours, the cells were treated with ADR (Sigma Chemical Co, St. Louis, MO) for 48 hours. After incubation with 10 μl of CCK-8 reagent (dojindo, Japan) for 1 h, the spectrophotometer was measured at 450 nm.

ADR accumulation assay

According to the method of our previous articles[22], the ADR cumulative measurement was performed. Briefly, the cells were exposed to 5 μM ADR for 2 h, then wash the cells with PBS. Next, using the flow cytometer to measure ADR fluorescence to determine the intracellular ADR level.

Flow cytometric analysis of apoptosis

The MCF-7/ADR and MCF-7 cells apoptosis after ADR treated was evaluated using the Annexin-V APC/7AAD Iodide Detection Kit (BD Biosciences, USA) according to the manufacturer's instructions. Cells were then analyzed by MACSQuant (Miltenyi Biotec, Germany).

Animal assays

The GFP containing negative control plasmid and OE-CBR3-AS1 plasmid were transfected into MCF-7 cells, and then treated with puromycin to obtain stable expression cell lines. All BALB / c mice (4 weeks old) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). MCF-7 / control group and MCF-7 / CBR3-AS1 cells (1×10^7 cells / 100 μ L / nude mice) were subcutaneously injected into the axillary region of nude mice. When the subcutaneous tumor grew to 200-250mm^3 (16 days later), the treatment group nude mice were intraperitoneally injected with ADR (80 mg / kg), once every three days. All nude mice were treated for 6 times. The diameter of transplanted tumor was measured with digital caliper every week. The tumor volume was calculated as volume = $0.5 \times width^2 \times length$. All nude mice were humanly sacrificed at the end of the experiment, and the tumor tissues were collected for further study by qRT-PCR, IHC and Western blotting.

ISH (In situ hybridization) and IHC (Immunohistochemistry)

The assay was carried out according to the method mentioned in the previous study [23]. We collected BRCA tissues of patients from the First Affiliated Hospital of China Medical University for ISH assay. The ISH probes were ordered from BOSTER Biological Technology co.ltd (USA). We collected BRCA tissues from collected different groups of subcutaneous tissues for IHC assay. The details of all antibodies used were showed in Table S1.

Biotin pull-down

MCF-7 cell lysates were incubated with CBR3-AS1 or control probe (Sangon, China) conjugated to Streptavidin agarose resin beads (Thermo Fisher Scientific, USA) to generate probe bound dynabeads. After washing with buffer, add DNase I (20 U) and collect purified RNA. The purified RNA was analyzed by qRT-PCR. The whole experimental process and the buffer formulation were referred to Hsu et al[24]

Statistical analysis

Quantitative data were expressed as the mean ± SD of at least three independent experiments. All experimental values were evaluated using graphpad prism 8.3.0 (graphpad software). The unpaired t-test was used for statistical analysis between the two experimental groups. The relationship between CBR3-

AS1 and clinicopathological factors was tested by chi square test. In all cases, $P < 0.05$ was considered statistically significant.

Result

CBR3-AS1 is up-regulated in BRCA resistant cells, and is related to drug sensitivity and poor prognosis of BRCA

To identify whether the different expression of lncRNA can be induced in the course of BRCA drug resistance, we investigated the expression of lncRNA in MCF-7 cells and BRCA drug resistance MCF-7/ADR cells. Select lncRNAs with $\log_2 FC > 1$ and an FDR (false discovery rate) < 0.05 in microarray data. The results showed that, compared with MCF-7 cells, 949 lncRNAs were up-regulated in MCF-7 / ADR cells, while 915 lncRNAs were down-regulated (Fig. 1A-B). The top 20 lncRNAs with the most obvious up-regulation and down-regulation in MCF-7 / ADR are listed in Fig. 1C. Moreover, in the three groups of MCF-7/ADR cells and MCF-7 cells, differential expression of lncRNAs was used for principal component analysis (PCA). The results showed that differential expression of lncRNAs could effectively distinguish MCF-7/ADR and MCF-7 cells (Fig.S1 A). We further selected five lncRNAs (DSCR8, TP53TG1, CBR3-AS1, LINC01006, HAGLROS) that were not previously reported to be related to drug sensitivity of BRCA, and performed qRT-PCR to verify their expression levels (Fig. 1D). Among them, the expression of CBR3-AS1 was the highest in MCF-7 / ADR cells. In order to confirm the relationship between the expression of the five lncRNAs and the drug sensitivity of BRCA cells to ADR, we obtained the expression of lncRNAs in various BRCA cells from CCLE database and then obtained the IC_{50} of these BRCA cells to ADR from GDSC database. The results showed that the expression of CBR3-AS1 was positively correlated with the drug resistance of BRCA cells to ADR ($p < 0.05$, $r > 0.3$) (Fig. 1E, Fig. S1B). In order to verify this result, we performed CCK8 experiments on four BRCA cell lines, The IC_{50} of each cell lines to ADR was obtained (Fig. 1F). The results of qRT-PCR showed that CBR3-AS1 was highly expressed in BRCA cell lines with high IC_{50}, but low expressed in cell lines with low IC50 (Fig. 1G). In addition, we obtained the mRNA expression profile and clinical information of BRCA patients from the GSE20685 data set in GEO Datasets. and used the Kaplan-Meier plotter website to calculate whether the survival of patients with BRCA under different expression of the five lncRNAs was different. The results showed that in BRCA patients receiving chemotherapy, the prognosis of patients with high expression of CBR3-AS1 was worse (Fig. S1C). We also found that in TCGA database, CBR3-AS1 was up-regulated in BRCA tissues compared with adjacent normal tissues (Fig. 1H). Gene-set enrichment analysis (GSEA) showed that CBR3-AS1 was enriched in ABC transporter signal transduction pathway (Fig. 1I). Besides, the expression of CBR3-AS1 was significantly correlated with the expression of p-glycoprotein (P-gp, ABCB1) and BRCA Resistance Protein (BCRP, ABCG2) mRNA (Fig. 1J, K). The log-rank test of OS curve of BRCA patients in TCGA also found that compared with low expression of CBR3-AS1, high expression of CBR3-AS1 was significantly correlated with worse OS (Fig. 1L). We evaluated the expression of CBR3-AS1 in 96 BRCA samples from the First Affiliated Hospital of China Medical University by ISH. High and low expression were showed in Fig. 1M. There was a significant correlation between CBR3-AS1 level with tumor size and pathological

staging (Table 1). The log-rank test of the overall survival curve of these BRCA patients showed that the high expression of CBR3-AS1 group was significantly related to the worse prognosis of BRCA OS (Fig. 1N).

Table 1
The relationship between the expression of CBR3-AS1 and the clinicopathological characteristics of BRCA patients

Factors	Number (96)	CBR3-AS1 expression		χ2	[a]p-value
		Low (42)	High (54)		
Age (years)					
< 50	44	20	24	0.0959	0.7568
≥ 50	52	22	30		
Tumor size (cm)					
< 3	46	26	20	5.854	0.0155*
≥ 3	50	16	34		
ER					
positive	49	20	29	0.35	0.5541
negative	47	22	25		
PR					
positive	50	19	31	0.9567	0.328
negative	46	23	23		
Her2					
positive	51	22	29	1.92E-05	0.9965
negative	44	19	25		
Molecular typing					
Luminal A	33	15	18	0.5994	0.8966
Luminal B	32	13	19		
Her-2	18	9	9		
Basal-like	13	5	8		
staging					
I	22	15	7	7.127	0.0283*
II	44	17	27		

* $p < 0.05$

[a] Chi-square test

Factors	Number (96)	CBR3-AS1 expression		χ2	[a]p-value
		Low (42)	High (54)		
III	30	10	20		
* $p < 0.05$					
[a] Chi-square test					

Table 2
CCK8 assay on MCF-7 cells and MCF-7/ADR cells

medicine	IC_{50} (μM) ± SD				
	MCF-7	MCF-7/OE-CBR3-AS1	MCF-7/ADR	MCF-7/ADR-si-CBR3-AS1-1	MCF-7/ADR-si-CBR3-AS1-2
ADR	0.11 ± 0.02	0.28 ± 0.05	30.16 ± 3.13	8.54 ± 2.65	10.37 ± 1.94

Effect of CBR3-AS1 on drug sensitivity of BRCA cells

To identify the effect of CBR3-AS1 on the drug sensitivity of BRCA cells, we overexpressed CBR3-AS1 in MCF-7 cells with low expression of CBR3-AS1, and silenced CBR3-AS1 in MCF-7 / ADR cells with high expression of CBR3-AS1. The overexpression or silencing efficiency of CBR3-AS1 was evaluated by qRT-PCR. After transfection with CBR3-AS1, the expression of CBR3-AS1 was significantly up-regulated compared with the control group (Fig. S1A). By si-CBR3-AS1 siRNA transfection, CBR3-AS1 expression was significantly down regulated compared with si-control group (Fig. S1 B). The result showed that after overexpressed CBR3-AS1, the protein expression levels of P-gp increased significantly (Fig. 2A). After CBR3-AS1 was silenced, the protein expression levels of P-gp decreased significantly (Fig. 2B). Then we used flow cytometry to detect drug accumulation in cells. The overexpression of CBR3-AS1 significantly reduced the accumulation of ADR in cells (Fig. 2C), while the silencing of CBR3-AS1 significantly increased the accumulation of ADR in cells (Fig. 2D). Next, we used CCK8 cytotoxicity test to detect the change of drug sensitivity of cells. After overexpressed CBR3-AS1, the drug sensitivity of the cells was found to be decreased (Fig. 2E, Table 1). However, after CBR3-AS1 was silenced, drug sensitivity was significantly increased (Fig. 2F). Colony formation experiments showed that the ability of colony formation was significantly enhanced by over expression of CBR3-AS1 after adding ADR (Fig. 2G). However, compared with the control group, inhibition of colony formation was found in si-CBR3-AS1 cells (Fig. 2H). At the same time, the concentration of ADR with IC50 (48 h) was used to induce apoptosis, and flow cytometry showed that CBR3-AS1 overexpression significantly reduced apoptosis (Fig. 2I).

CBR3-AS1 regulates MEK4 / JNK1 expression through miR-25-3p

Bioinformatics tool analysis of Diana lncbase found that lncRNA CBR3-AS1 targeted miRNAs (Fig. 3A, Fig. S3A). The research has shown that have shown that activating miR-25-3p can resist the resistance of

chemotherapy[25]. qRT-PCR analysis showed that miR-25-3p was down-expressed in MCF-7/ADR cells (Fig. 3B). Bioinformatics tool analysis miRdb was used to discover the 3'UTR interaction of miR-25-3p with JNK1/MEK4 in MAPK pathway (Table S3). CBR3-AS1 has a significant positive correlation between JNK1/MEK4 mRNA expression in TCGA BRCA data (Fig. 3C). In order to determine whether miR-25-3p is a possible target for JNK1 and MEK4, luciferase reporter gene analysis was used. We constructed luciferase report vectors Luc-JNK1 and Luc-MEK4 containing 3'-UTR of JNK1 and MEK4 (Fig. 3D). Overexpression of miR-25-3p significantly reduced luc-jnk1 luciferase activity of MCF-7 cells (Fig. 3E), indicating that JNK1 and MEK4 are direct targets of miR-25-3p. The RNAhybrid website predicts that CBR3-AS1 has a binding site with miR-25-3p(Fig. 3F). RNA pulldown showed that CBR3-AS1 could directly bind to miR-25-3p in BRCA cells (Fig. 3G). Since the function of lncRNAs depend on their subcellular distribution, we examined the CBR3-AS1 subcellular localization. Bioinformatics website lncLocator predicted that CBR3-AS1 was expressed in both nucleus and cytoplasm (Fig. S3B). The results were verified by qRT-PCR (Fig. 3H). qRT-PCR analysis and Western blot showed that the overexpression of CBR3-AS1 increased the expression of mRNA and protein of JNK1 and MEK4 in MCF-7 cells after ADR was added (Fig. 3I, J), while the silencing of CBR3-AS1 may reduce the expression of mRNA and protein of JNK1 and MEK4 in MCF-7 / ADR cells (Fig. 3K, L). Meanwhile, overexpression of miR-25-3p decreased JNK1 and MEK4 expression in MCF-7 / ADR cells (Fig. S3C, D), while silenced miR-25-3p may increase JNK1 and mek4 expression in MCF-7 / ADR cells (Fig. S3E, F). In conclusion, CBR3-AS1 regulates the expression of MEK4 / JNK1 through miR-25-3p.

Overexpression of miR-25-3p reverses MAPK pathway activation and ADR resistance of BRCA cells induced by overexpression of CBR3-AS1

In order to determine whether miR-25-3p is involved in chemotherapy resistance of BRCA cells mediated by CBR3-AS1, CCK8 test was conducted with MCF-7 cells, and the results showed that miR-25-3p reversed the ADR sensitivity of decreased over expression of CBR3-AS1 (Fig. 4A). Colony forming experiments confirmed that miR-25-3p reversed the cell proliferation enhanced by overexpression of CBR3-AS1 after adding ADR (Fig. 4B). Apoptosis experiments confirmed that overexpression of cbr3-as1 led to a decrease in the number of apoptotic cells, which was reversed by miR-25-3p (Fig. 4C). qRT-PCR analysis and Western blot showed that the overexpression of miR-25-3p reversed the activation of the JNK1 and MEK4 caused by the overexpression of CBR3-AS1 (Fig. 4D-E).

In vivo experiments showed that overexpression of CBR3-AS1 promoted the drug resistance of BRCA cells to ADR

In order to determine whether the overexpression of CBR3-AS1 can still reduce the sensitivity of ADR to tumors in vivo, MCF-7 cells transfected with CBR3-AS1-cDNA or NC-cDNA were subcutaneously injected into nude mice for animal experiments. The experimental flowchart was showed in Fig. 5A. After 34 days, the expression level of CBR3-AS1 in subcutaneous tumor tissue was detected. The expression level of CBR3-AS1 in the transfected group was still significantly higher than that in the control group, indicating that the construction of the model was correct (Fig. 5B). In vivo imaging in mice showed that over

expression of CBR3-AS1 resisted the inhibitory effect of ADR on tumor cells (Fig. 5C). Compared with the control group, the tumor weight and volume increased significantly (Fig. 5D-F). Western blot showed that the overexpression of CBR3-AS1 increased the expression of JNK1 and MEK4 (Fig. 5G). JNK1 and MEK4 expression were also detected by IHC (immunohistochemistry) (Fig. 5H). These results showed that CBR3-AS1 promotes ADR resistance in BRCA through JNK1 / MEK4 in vivo.

Discussion

In the past few decades, BRCA was treated with standard chemotherapy after surgical removal, which greatly improved the overall survival of patients. However, cancer-related deaths are still on the rise due to BRCA metastasis and drug resistance. MAPK pathway plays a key role in the invasion, migration and metastasis of various malignant tumors[26–29]. Shen et al. found that the overexpressed LncRNA PCAT-1 can enhance cell division and inhibit cell apoptosis in myeloma cell through the p38 and JNK MAPK pathways[30]. Xu et al. found that ROR2 facilitates epithelial mesenchymal transition of BRCA by regulating MAPK / p38 signaling pathway [31]. Another study reported that circ-MAPK4 plays a key role in the survival and apoptosis of glioma cells by regulating miR-125a-3p and p38 / MAPK signaling pathway [32]. Sheng et al. found that MSI2 promotes EGF-induced Epithelial mesenchymal transition through ZEB1-ERK/MAPK signaling in pancreatic cancer[33].

JNK1 is one of the important genes in MAPK pathway. MAPK pathway genes act as integration points of various biochemical signals and participate in many biological processes, such as differentiation, proliferation, apoptosis and transcriptional regulation. [34, 35]. MEK4 can respectively phosphorylate the tyrosine residues of JNK stored in the cytoplasm, thereby activating JNK. The activated JNK quickly and significantly accumulates cells in the nucleus and causes downstream transcription factors c-Jun, ATF-22, and Elk21 Phosphorylation promotes expression of related genes[36].

Some studies have showed that CBR3-AS1 is involved in and regulates the occurrence and development of cancers. It is reported that CBR3-AS1 promotes osteosarcoma tumorigenesis and predicts its poor prognosis[17]. CBR3-AS1 promotes the development of colorectal cancer by regulating PI3K / Akt signaling pathway [18]. CBR3-AS1 plays an important role in the proliferation of esophageal squamous cells [19]. CBR3-AS1 is highly expressed in retinoblastoma and mediates the proliferation of retinoblastoma cells by regulating CBR3[21]. However, it is not clear how CBR3-AS1 affects the drug resistance of BRCA cells.

The ceRNA (competitive endogenous RNA) hypothesis believes that a large-scale transcriptional regulatory network may be formed between RNAs, which can affect biological processes [23]. Our study is based on the ceRNA hypothesis, that is, cbr3-as1 directly binds to miR-25-3p, thus targeting JNK1 / MEK4, activating MAPK pathway, and ultimately affecting the chemoresistance process of BRCA.

In this study, we determined that CBR3-AS1 is a new role in BRCA cell resistance. Overexpression of CBR3-AS1 confers chemotherapy resistance to BRCA cells in vitro and in vivo. It is worth noting that CBR3-AS1 is up-regulated in BRCA, while patients with high levels of CBR3-AS1 show poor overall survival. These

findings indicate that CBR3-AS1 can be used as a predictor of drug response in BRCA patients and may be a useful biomarker for clinically precise treatment.

Our study has some limitations. We did not detect the effect of CBR-AS1 on MCF-7/ADR apoptosis. This is because to make MCF-7/ADR cells apoptotic, a very large dose of ADR is needed. Because ADR itself has a strong fluorescence, in the case of large amounts of ADR, commonly used fluorescent dyes such as FITC, 7-AAD, PE and APC will be interfered, and it is difficult to get real results. Similarly, we did not use MCF-7/ADR in animal experiments. Due to the cardiotoxicity of ADR, it is difficult for mice to bear such a large dose. And MCF-7/ADR cells are also difficult to form tumors in mice.

Conclusions

CBR3-AS1 might be an important biomarker for evaluating the prognosis of BRCA patients. CBR3-AS1 increased the resistance of BRCA to ADR by competitively binding miR-25-3p with JNK1/MEK4 and enhancing the MAPK signaling pathway. The CBR3-AS1 / miR-25-3p / JNK1 and MEK4 axis will provide insights into BRCA drug resistance mechanisms and provide theoretical support for the search for new BRCA diagnostic markers and specific therapeutic targets.

Abbreviations

BRCA: breast cancer

TCGA: Cancer Genome Atlas

ADR: Adriamycin

CCK8: Cell Counting Kit-8

GEO: Gene Expression Omnibus

OS: overall survival

FC: fold change

ADR: Adriamycin

CCK8: Cell Counting Kit-8

MAPK: mitogen-activated protein kinase

JNK: c-Jun N-terminal kinase

MEK: mitogen-activated extracellular signal-regulated kinase

IHC: Immunohistochemistry

ISH: In situ hybridization

IC50: half maximal inhibitory concentration

CBR3-AS1: Carbonyl reductase 3 antisense RNA 1

Declarations

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of the first affiliated hospital of China Medical University (Approval number: AF-SOP-07-1.1-01). All animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University (Approval number: CMU2019265).

Consent for publication

All contributing authors agree to the publication of this article.

Availability of data and materials

The datasets generated and analysed during the current study are available in the Gene Expression Omnibus data sets (https://www.ncbi.nlm.nih.gov/gds) (GSE155487).

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Yan Wang conceived and designed the study. Ming Zhang performed experiments. Longyang Jang provides help for the specific ideas of the article. The data were analyzed by Xinyue Song and Ang Zheng. Hua Gao, Minjie Wei and Lin Zhao reviewed and edited the manuscript. All authors read and approved the manuscript.

Footnotes

[#]Ming Zhang and Yan Wang contributed equally to this work.

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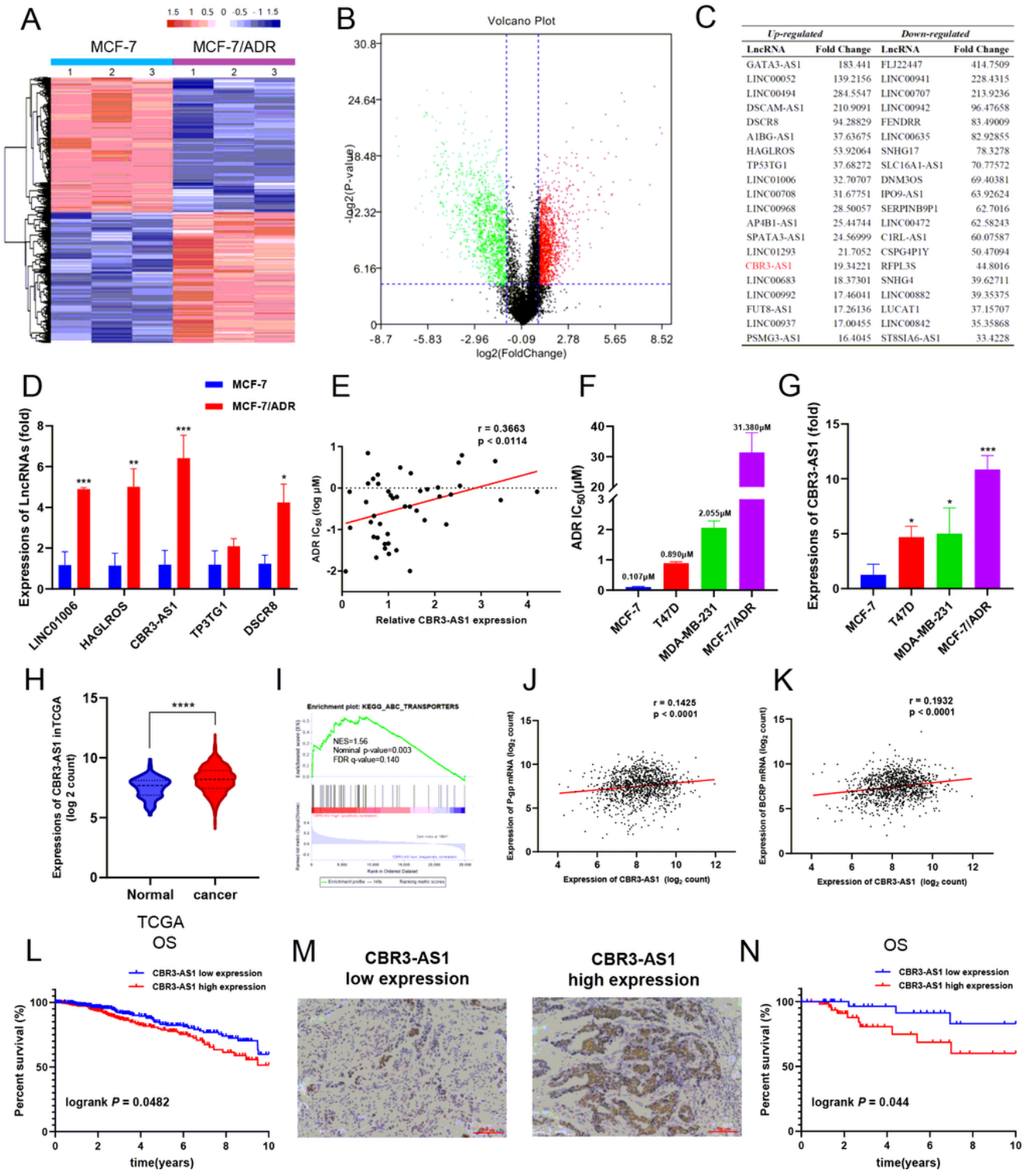
Figures



Up-regulated		*Down-regulated*	
LncRNA	**Fold Change**	**LncRNA**	**Fold Change**
GATA3-AS1	183.441	FLJ22447	414.7509
LINC00052	139.2156	LINC00941	228.4315
LINC00494	284.5547	LINC00707	213.9236
DSCAM-AS1	210.9091	LINC00942	96.47658
DSCR8	94.28829	FENDRR	83.49009
A1BG-AS1	37.63675	LINC00635	82.92855
HAGLROS	53.92064	SNHG17	78.3278
TP53TG1	37.68272	SLC16A1-AS1	70.77572
LINC01006	32.70707	DNM3OS	69.40381
LINC00708	31.67751	IPO9-AS1	63.92624
LINC00968	28.50057	SERPINB9P1	62.7016
AP4B1-AS1	25.44744	LINC00472	62.58243
SPATA3-AS1	24.56999	C1RL-AS1	60.07587
LINC01293	21.7052	CSPG4P1Y	50.47094
CBR3-AS1	19.34221	RFPL3S	44.8016
LINC00683	18.37301	SNHG4	39.62711
LINC00992	17.46041	LINC00882	39.35375
FUT8-AS1	17.26136	LUCAT1	37.15707
LINC00937	17.00455	LINC00842	35.35868
PSMG3-AS1	16.4045	ST8SIA6-AS1	33.4228

Figure 1

CBR3-AS1 is up-regulated in BRCA resistant cells, and is related to drug sensitivity and poor prognosis of BRCA. A, B Gene chip analysis of lncRNAs transcripts in MCF-7/ADR cells and MCF-7 cells. C The top 20 lncRNAs with the most obvious up-regulation and down-regulation in MCF-7 / ADR cells. D qRT-PCR analysis of five lncRNAs expression in MCF-7/ADR cells and MCF-7 cells. E The correlation between CBR3-AS1 expression and IC50 of ADR in 44 BRCA cell lines in the CCLE database and GDSC database. F

The IC50 value of ADR was detected for both sensitive and resistant cells by CCK8 assay. G CBR3-AS1 expression in four BRCA cell lines. H Expressions of CBR3-AS1 in normal tissues and BRCA tissues in TCGA. I GSEA analysis of CBR3-AS1 enrichment in ABC transporter signaling pathway. J, K Correlation analysis between CBR3-AS1 with P-gp mRNA and BCRP mRNA in TGCA database. L Kaplan Meier survival analysis based on CBR3-AS1 expression in BRCA patients ($p = 0.0482$). M CBR3-AS1 expression in BRCA patients by ISH. Original magnification, ×200. Scale bars, 100μm. N Kaplan Meier survival analysis of BRCA patients based on CBR3-AS1 expression in our cohort ($n = 96$, $p = 0.044$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

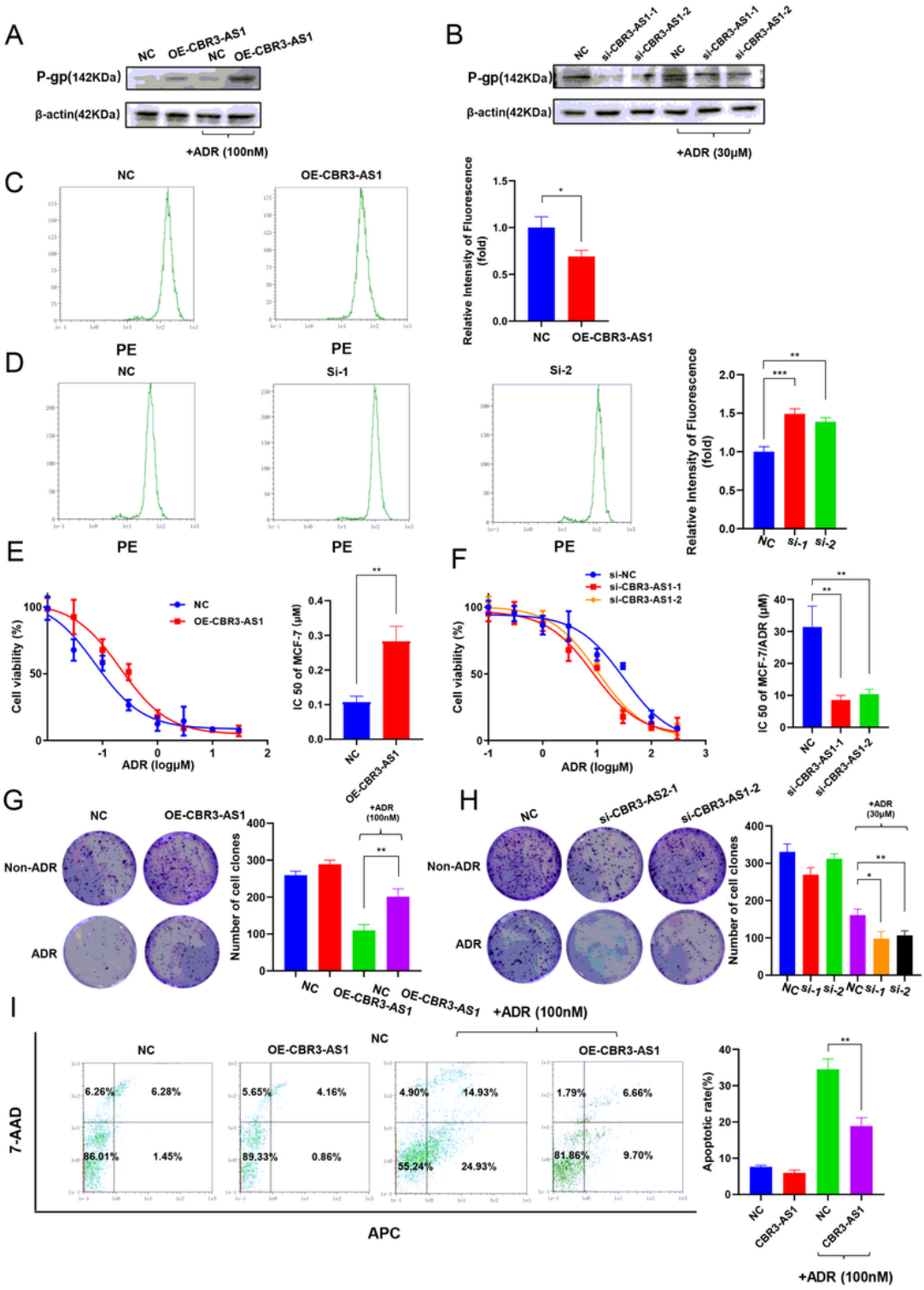


Figure 2

CBR3-AS1 affects BRCA cells drug resistance. A,B P-gp expression was measured by western blot after CBR3-AS1 overexpressed or was silenced. When adding drugs, 100nM ADR was added to MCF-7 cells, and 30μM ADR was added to MCF-7/ADR cells to culture for 48h. C, D Fluorescence intensity of ADR was detected by flow cytometry showing intracellular ADR accumulation. E,F Drug resistance was examined by CCK8 assays in cells transfected with CBR3-AS1 overexpression plasmid or siRNAs. G, H The cells

transfected with CBR3-AS1 overexpression plasmid or siRNAs were seeded onto plates. The number of colonies was counted on the 14th day after seeding. I Apoptosis rates of MCF-7 cells with ADR were detected at a concentration of 100nM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

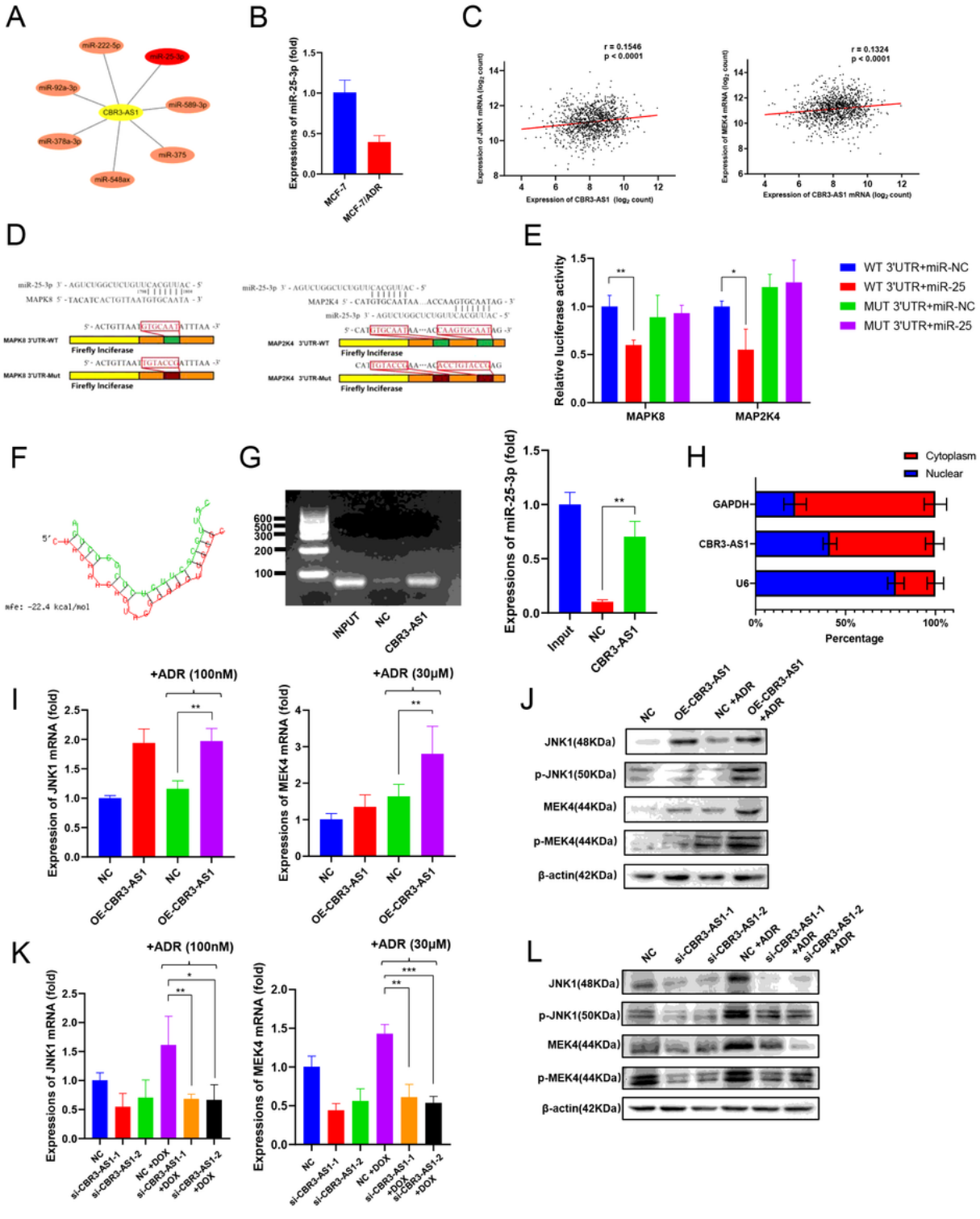


Figure 3

CBR3-AS1 functions as a sponge for miR-25-3p in MAPK pathway. A DIANA-LncBase website showed CBR3-AS1 targeted miRNAs. B The expressions of miR-25-3p in MCF-7 and MCF-7/ADR cells. C The

correlation of CBR3-AS1with JNK1 and MEK4 mRNA expression in TCGA BRCA data. D The binding sites of JNK1 and MEK4 3'UTR in miR-25-3p and the mutation sites of JNK1 and MEK4 3'UTR in the mutant plasmid. E Relative luciferase activity by luciferase reporter assays in MCF-7 cells co-transfected with wild-type (JNK1/MEK4-WT) or JNK1/MEK4-MUT and miR-25-3p or miR negative control (NC). F G RNA pulldown CBR3-AS1 directly bind with miR-25-3p. H RNA from nucleus and cytoplasm of MCF-7 cells was extracted separately, and the proportion of CBR3-AS1 contained in the cells was detected by qRT-PCR. I-J JNK1/MEK4 expression was measured by western blot and qRT-PCR after overexpressed CBR3-AS1 in MCF-7 cells. K-L JNK1/MEK4 expression was measured by western blot and qRT-PCR after CBR3-AS1 was silenced in MCF-7/ADR cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

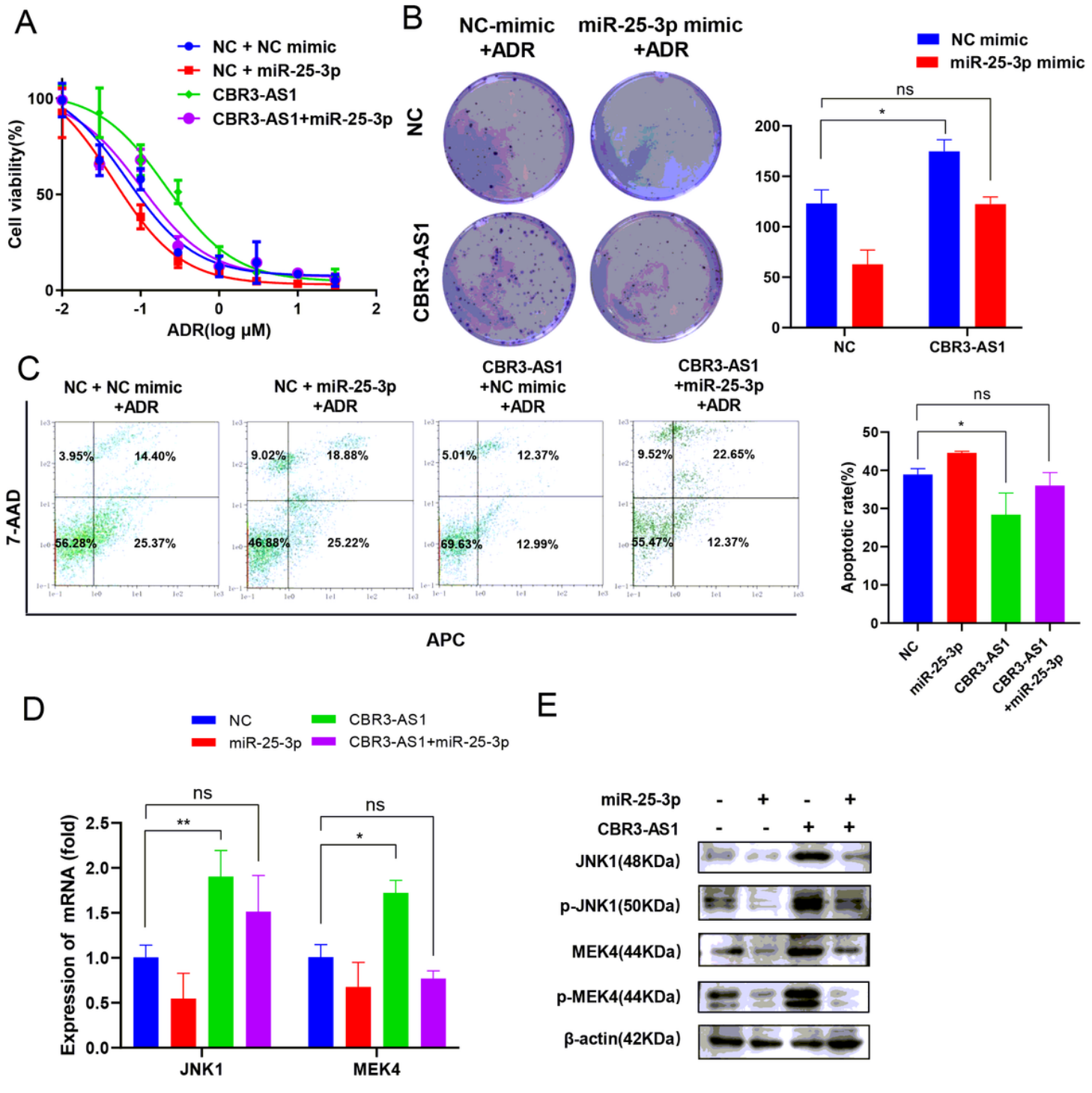


Figure 4

miR-25-3p overexpression reverses CBR3-AS1 overexpression induced activation of MAPK pathway and chemotherapy resistance in BRCA cells. A Drug resistance was examined by CCK8 assays in MCF-7 cells transfected with CBR3-AS1 overexpression plasmid and miR-25-3p mimic. B MCF-7 cells transfected with CBR3-AS1 overexpression plasmid and miR-25-3p mimic were seeded onto plates. The number of colonies was counted on the 14th day after seeding. C Apoptosis rates of MCF-7 cells with CBR3-AS1 or miR-25-3p overexpression were detected with ADR at a concentration of 200nM. D-E JNK1, MEK4 and P-gp expression was measured by western blot and qRT-PCR after overexpressed CBR3-AS1 and miR-25-3p. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

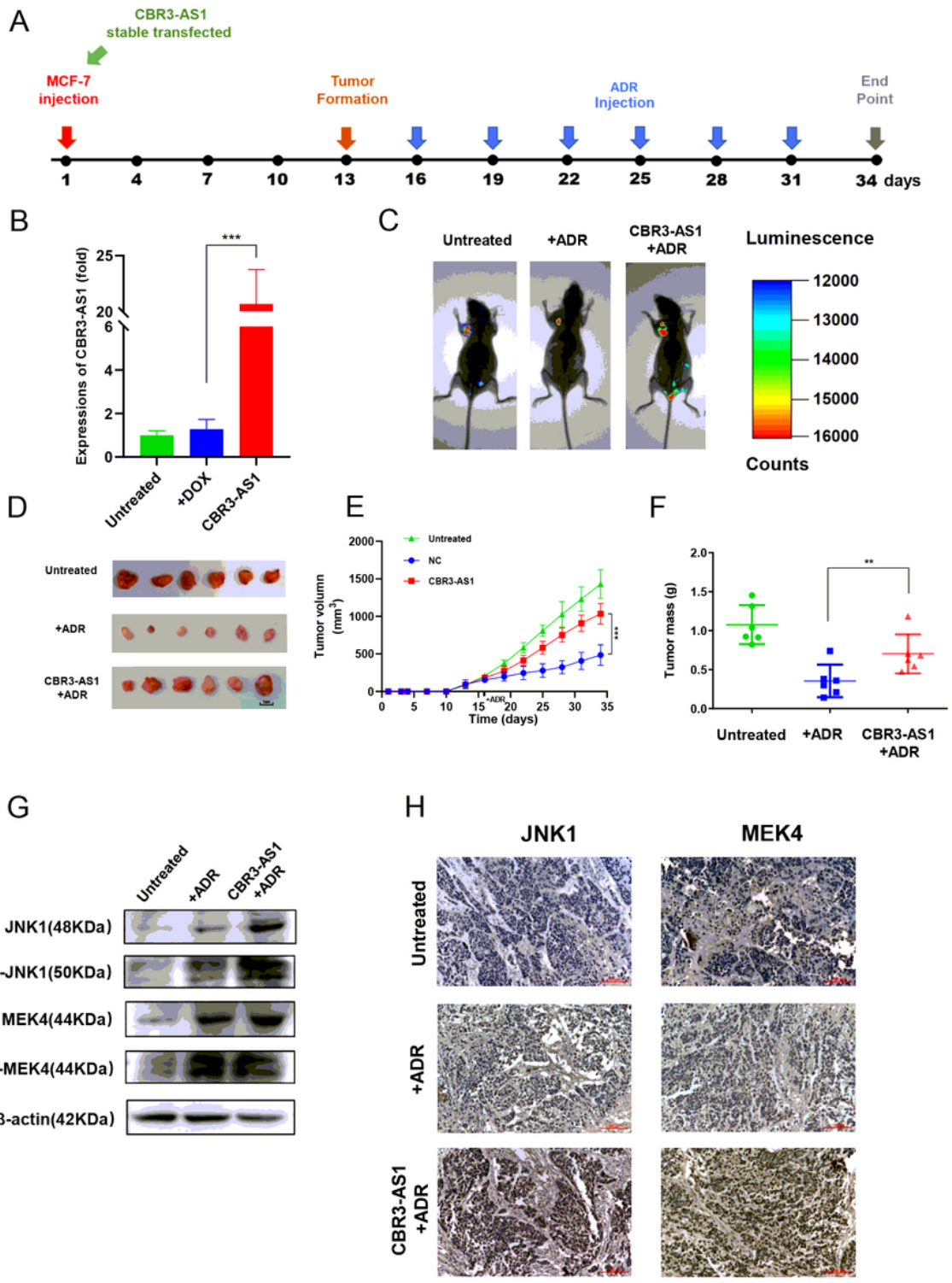


Figure 5

Overexpression of CBR3-AS1 promote ADR Resistance in vivo. A In vivo experiment schedule. B Live-imaging experiments Observed CBR3-AS1 overexpression CBR3-AS1 cells transplanted tumor. C Representative images of tumors at 5 weeks after subcutaneous transplantation when mice were euthanized. D Tumor growth curves of MCF-7 cells transfected with or without a CBR3-AS1 overexpression vector. E Tumor mass of MCF-7 cells transfected with or without a CBR3-AS1

overexpression vector. F IHC analysis of expression levels of JNK1 and MEK4 in respective groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

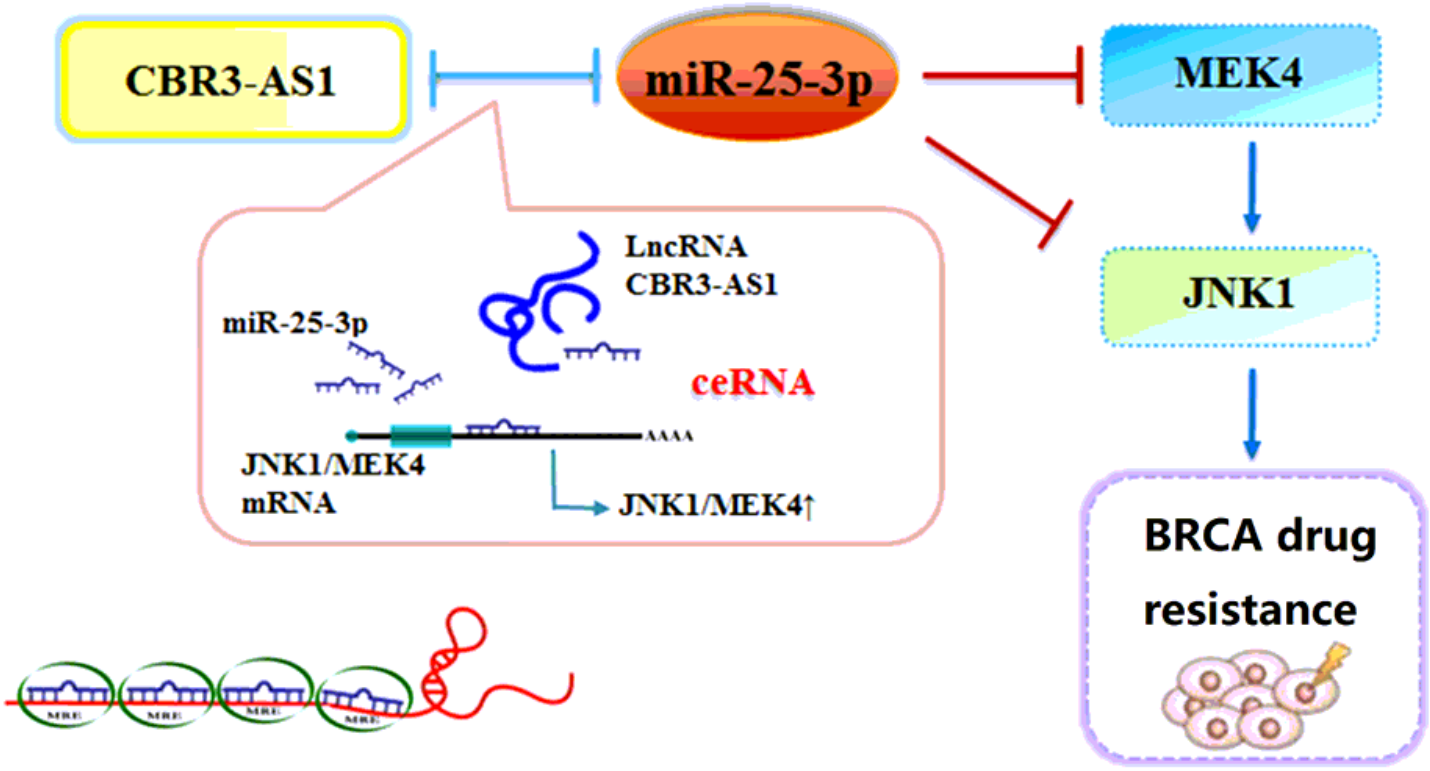


Figure 6

The schematic diagram indicating that CBR3-AS1 and JNK1 / MEK4 competitively bind to miR-25-3p, thus regulating MAPK signaling pathway to promote drug resistance of BRCA cells.

Supplementary Files

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