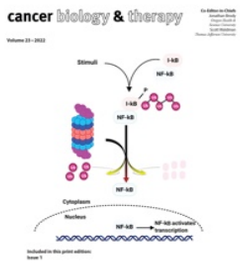

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Knockdown of NDUFAF6 inhibits breast cancer progression via promoting mitophagy and apoptosis

Shang Wu[a,b*], Xindi Ma[a,b*], Xiangmei Zhang[b,c], Kaiye Du[b,d], Chao Shi[a,b], Ahmed Ali Almaamari[e], Boye Han[e], Suwen Su[e], and Yunjiang Liu[a,b]

[a]Department of Breast Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, China; [b]Hebei Provincial Key Laboratory of Tumor Microenvironment and Drug Resistance, Hebei Medical University, Shijiazhuang, China; [c]Department of Research Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, China; [d]Radiotherapy Department, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, China; [e]The Key Laboratory of Neural and Vascular Biology, The Key Laboratory of New Drug Pharmacology and Toxicology, Department of Pharmacology, Ministry of Education, Hebei Medical University, Shijiazhuang, China

ABSTRACT

Background: While NDUFAF6 is implicated in breast cancer, its specific role remains unclear.
Methods: The expression levels and prognostic significance of NDUFAF6 in breast cancer were assessed using The Cancer Genome Atlas, Gene Expression Omnibus, Kaplan-Meier plotter and cBio-Portal databases. We knocked down NDUFAF6 in breast cancer cells using small interfering RNA and investigated its effects on cell proliferation and migration ability. We performed gene expression analysis and validated key findings using protein analysis. We also assessed mitochondrial activity and cellular metabolism.
Results: NDUFAF6 was highly expressed in breast cancer, which was associated with a poorer prognosis. Knockdown of NDUFAF6 reduced the proliferation and migration ability of breast cancer cells. Transcriptome analysis revealed 2,101 differentially expressed genes enriched in apoptosis and mitochondrial signaling pathways. Western blot results showed NDUFAF6 knockdown enhanced apoptosis. In addition, differential gene enrichment analysis was related to mitochondrial signaling pathways, and western blot results verified that mitophagy was enhanced in NDUFAF6 knockdown breast cancer cells. JC-1 assay also showed that mitochondrial dysfunction and reactive oxygen species content were increased after knocking down NDUFAF6. In addition, basal and maximal mitochondrial oxygen consumption decreased, and intracellular glycogen content increased.
Conclusions: Knockdown of NDUFAF6 resulted in apoptosis and mitophagy in breast cancer cells and NDUFAF6 may be a potential molecular target for breast cancer therapy.

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Breast cancer; NDUFAF6; prognosis; apoptosis; mitophagy

Introduction

The prevalence of breast cancer has seen a significant rise over the course of the last 40 years. During the most recent data years (2010–2019), there was a 0.5% rise in the rate,[1] and breast cancer has emerged as a prevalent form of cancer among women on a worldwide scale, with a significant annual mortality rate.[2] A subset of breast cancer patients still experience recurrence and distant metastasis despite advances in diagnosis and treatment.[3] Even though research on the pathogenesis of breast cancer has advanced significantly, the fundamental pathogenesis of breast cancer has to be explained.[4] A new age of personalized precision medicine based on many molecular subtypes of breast cancer has emerged in recent years as a result of the introduction of new medications and the ongoing advancement of precision medicine, replacing the earlier empirical model.[5,6] Therefore, it has become increasingly vital to identify more effective breast cancer treatment targets and to provide patients with individualized care.

The gene NDUFAF6, also known as NADH: ubiquinone oxidoreductase complex assembly factor 6, is a constituent of the mitochondrial respiratory chain complex 1.[7,8] Complex 1 of the respiratory chain of mitochondria is in charge of moving protons out of the inner mitochondrial membrane and transferring electrons from NADH to oxygen molecules, and recent studies have shown that NDUFAF6 can help NDUFS8 to enter complex 1 to fulfill its function, ultimately, this process generates the energy required by the cell.[9,10] Failure of the functionality of the gene that encodes the I component of the complex contributes significantly to aberrant respiratory chain function, resulting in tolerance to exercise that can vary from early-onset deadly conditions to adult-onset diseases such as Leigh's syndrome.[11,12] Meanwhile, mitochondrial metabolism is essential to supporting the existence of cells[13,14] and assisting tumors in adapting to hostile conditions.[15] On the other hand, tumors also increase

CONTACT Yunjiang Liu lyj818326@hebmu.edu.cn The Fourth Hospital of Hebei Medical University, 169 Tianshan Street, Shijiazhuang, Hebei, China; Suwen Su suswmk@hebmu.edu.cn The Key Laboratory of Neural and Vascular Biology, The Key Laboratory of New Drug Pharmacology and Toxicology, Department of Pharmacology, Ministry of Education, Hebei Medical University, Shijiazhuang, China
*Contributed equally.
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mitochondrial metabolism to promote their own growth, producing tricarboxylic acid cycle intermediates that are exported to the cytoplasm for lipid synthesis and nucleotide synthesis.[16,17] Thus, targeted modulation of mitochondrial metabolism appears to be a potential tumor therapy.[18,19] Meanwhile, immune factors play a major role in breast cancer development,[20] and NDUFAF6 can promote PD-L1 expression by inhibiting the NRF2 signaling pathway to respond to the immune response.[21]

In this research, we evaluated the levels of the mitochondria-related gene NDUFAF6 in breast cancer tissues and its association with prognosis. To investigate the impact of NDUFAF6 on the mitochondrial activity of tumor cells, we used small interfering RNA (siRNA) technology to suppress the expression of NDUFAF6 in two cell lines with breast cancer. Then we further searched for the alteration of tumor cell-related pathways by transcriptome sequencing, and found that when knocking down NDUFAF6, tumor cells could undergo mitochondrial dysfunction and thus lead to mitochondrial autophagy and apoptosis. These findings suggest that we can develop its corresponding small molecule inhibitors to play a therapeutic role in the treatment of breast cancer, which also provides a reference for our subsequent research on the important role of mitochondria in breast cancer.

Materials and methods

Source data

The initial bioinformatic analysis using The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases was conducted to identify NDUFAF6 as a candidate gene for further investigation, as it showed significant upregulation in breast cancer tissues and was associated with poor prognosis. From TCGA database, we retrieved the RNA sequencing transcriptome data from 1169 breast cancer patients and 111 normal tissues, and we used the form $\log_2$ (TPM +1) to represent their relative expression. GEO database was a functional genomics database with a broad scope. The expression profile of GSE42568[22] and GSE45827[23] from the GEO database was also analyzed, and the relative expression of different genes was shown in each of these two datasets. We used TCGA data to construct a Kaplan-Meier survival curve in order to assess the clinical importance of NDUFAF6, and we fund the optimal truncation value by calculating the minimum *P*-values. The study also investigated the associations between NDUFAF6 and the survival of breast cancer patients using data obtained from Kaplan-Meier plotters.[24] The cBioPortal[25] was a free internet resource that contains over 5,000 carcinoma specimens from 20 distinct cancer research projects and enabled the interactive examination of multivariate cancer genomics data.

Cell culture and cell transfection

RPMI1640 culture medium was used for MCF-7 and DMEM culture medium was used for MDA-MB-231, and 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin were added to the medium. A customized medium for normal breast epithelial cells (MCF-10A cells), sourced from Procell (China, item no. CM-0525), was used to culture the MCF-10A cells. GP-transfect-Mate (Shanghai GenePharma Co., Ltd.) was used to create NDUFAF6 knockdown cells by transfecting breast cancer cells. This was done in accordance with the instructions provided by the pharmaceutical manufacturer. GenePharma Co., Ltd (Shanghai, China) supplied the small interfering RNA (siRNA) targeting NDUFAF6 (Si-NDUFAF6) and siRNA targeting negative control (Si-NC). The Si-NDUFAF6 sequences consist of a sense strand known as 5′-GCCUGUGGCCAUUGAACUATT-3′ and an antisense strand referred to as 5′-UAGUUCAAUGGCCACAGGCTT-3′. The sequences corresponding to Si-NC were as follows: the sense strand 5′-UUCUCCGAACGUGUCACGUTT-3′ and the antisense strand 5′-ACGUGACACGUUCGGAGAATT-3′.

The extraction of RNA accompanied by quantitative real-time PCR (qRT-PCR)

Trizol (Takara, Japan) was used to extract RNA from whole cell lysates and the cDNA was generated using a reverse transcription kit (iGene, China) from the retrieved mRNA. All qRT-PCR was performed with the qPCR Mix (Promega, China). The primers were as follows: forward primer 5'-CACCCACTCCTCCACCTTTGAC-3' and reverse primer 5' -GTCCACCACCCTGTTGCTGTAG-3' for GAPDH; forward primer 5'-TGGCTCAGGCTGGTTAAAGACTC-3' and reverse primer 5'-ACAGGCTGATGTGGTGGATTGTC-3' for NDUFAF6.

Cell counting kit-8 assay (CCK8)

The present research evaluated the cellular proliferation capability using the CCK-8 test assay (Boster, China). The breast cancer cells were planted into 96-well plates at a cell density of 2000 cells per well for MDA-MB-231 and 5000 cells per well for MCF-7. The cells were subjected to incubation for 0, 24, 48, 72, and 96 h using a 10% CCK-8 solution. Following a period of incubation lasting one hour in the cell incubator, the optical density (OD) at 450 nm was determined with the use of a Varioskan LUX Multi-Purpose Enzyme Labeler (Thermo Fisher, US).

Colony formation and migration assays

The experiment included the inoculation of 2,000 transfected MCF-7 cells and 500 transfected MDA-MB-231 cells into 6-well plates with complete media for a duration of 2 weeks. Afterward, the specimens were put through a fixation treatment that lasted for 30 min by using 4% paraformaldehyde. This was followed by a staining procedure that lasted for 30 min and using crystal violet dye. The quantification of cell colonies was performed.

The assessment of cell migration capacity was conducted using Transwell chambers. The breast cancer cells that had been transfected were subjected to digestion using trypsin-EDTA (Solebo, China) and then reconstituted in a medium devoid of FBS. The top compartment was supplemented with a total of 40,000 counted cells, while the bottom chamber was supplemented with 600 μl of complete media containing 10%

FBS. At the conclusion of an incubation period of 24 h at a temperature of 37°C, cells that had not penetrated were removed using a cotton swab. Subsequently, the samples were fixed and stained using the same methods. Three arbitrary fields of view were captured on camera at a 100× magnification, and the number of cells was quantified using a microscope.

Transcriptome sequencing

The siRNA technique was applied to the MDA-MB-231 cells for a duration of 24 hours. Each group was sampled three times. Trizol (Takara, Japan) was used for RNA extraction, while mRNA-seq and data analysis were conducted by LC-Bio Technology CO. (Zhejiang, China). Specific test methods were described in the supplementary document.

Western Blot

RIPA buffer (Solarbio, China) combined with a protease inhibitor (Solarbio, China) was used for the purpose of cell lysis and protein separation. The BCA kit (boster, China), was used to perform the protein concentration test. The proteins in cell lysates were separated and transferred using 12.5% SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes underwent a 2-hour incubation period at ambient temperature using a 5% skimmed milk solution. Following this, the membranes were incubated overnight at 4°C with primary antibodies targeting Bax (proteintech, 1:8000), Bcl-2 (proteintech, 1:4000), Caspase-3 (boster, 1:1000), β-actin (proteintech, 1:10000), Parkin (boster, 1:1000), Pink1 (boster, 1:1000), and LC3B (boster, 1:1000). On the subsequent day, the membranes underwent a washing process using Tris Buffered Saline Tween. Subsequently, they were subjected to incubation with secondary antibodies for a duration of 1 hour at a temperature of 37°C. In the presence of an ECL luminous solution (Abbkine, China), the membranes were observed using a ChemiDocTMMP imaging system (Bio-Rad, US). All test results were repeated at least three times.

Mitochondrial membrane potential assay and reactive oxygen species (ROS) detection

The tumor cells underwent three washes with PBS 24 hours post-transfection with siRNA. The cells were subsequently placed in JC-1 working solution (Solebo, China) for a duration of 30 minutes at a temperature of 37°C in the absence of light. The fluorescence emitted by the cells was then examined using a fluorescence microscope (BX63F Olympus, Japan) and documented with pictures. Dichlorofluorescein diacetate (DCFH-DA, Beyotime, China) was used to quantify the total intracellular ROS. Cells were washed three times with PBS and then cultured in medium supplemented with 10 μM DCFH-DA. The samples that had undergone treatment were placed in a cell growth incubator for a duration of 30 minutes, with protection from light. The fluorescence emitted by the cells was then examined using a fluorescence microscope (BX63F Olympus, Japan) and documented with pictures. The fluorescence intensity was measured by image J software to evaluate the intracellular reactive oxygen species content and mitochondrial membrane potential changes.

Mitochondrial oxygen consumption assay

Oxygen consumption rate (OCR) was assessed using the oxygraph-2k (O2k, Oroboros Instruments, Austria). Breast cancer cells were resuspended using trypsin digestion 24 h after transfection, and the cells were resuspended using medium to achieve a cell density of 1 million/100 μl and immediately loaded into the O2k instrument for respiratory measurements. To the chamber this was added 5 nM oligomycin (Omy), 0.5uM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP)(titrated), 0.5uM rotenone (Rot), and 2.5uM antimycin A (Ama) sequentially, and the oxygen consumption rate was measured for the Si-NC group and Si-NDUFAF6 group for the corresponding time period.

PAS staining

Following a 24-hour transfection period, breast cancer cells were fixed for 30 min using a 10% paraformaldehyde solution. MCF-7 fixed cell samples were stained with PAS in accordance with the PAS kit's instructions (Solarbio, China). For fixed MDA-MB-231 cells, membrane-breaking solution (Servicebio, China) was added for 15 min and washed 3 times with ultrapure water. Then, PAS staining solution B (Servicebio, China) was stained for 20 min and washed 3–5 times with ultrapure water. Add PAS staining solution A (Servicebio, China) dropwise in the well plate to dip stain for 30–40 min, wash with ultrapure water for 3 times. Added PAS staining solution C dropwise (Servicebio, China) in the well plate to dye for 1 min, washed with water, baked dry and sealed the plate.

Transmission electron microscope

The MCF-7 cells of Si-NC and Si-NDUFAF6 were treated with 2.5% glutaraldehyde and thereafter exposed to 1% osmium acid for a duration of 2 hours. The specimens were subjected to dehydration using various concentrations of ethanol. Subsequently, they were embedded in epoxy resin and sliced into 80 nm sections. The cellular morphology was then examined using an 80 kV transmission electron microscope (HT7800, HITACHI, Japan).

Statistical analysis

The statistical analysis and data visualization were conducted using GraphPad Prism (version 9.5.0) and R statistical instruments (version 4.2.0). The data was analyzed using Student's t-test or analysis of variance. The *P*-values were consistently two-sided, with a *P*-values below 0.05 being deemed indicative of statistical significance.

Result

NDUFAF6 expression and prognostic potency of NDUFAF6 in breast cancer

The expression level of NDUFAF1–6 in breast cancer was first evaluated using RNA sequencing data obtained from the TCGA database. The findings indicated that there were substantial changes in the mRNA levels of the assembly factors of most mitochondrial complex I in comparison to normal tissues. Additionally, the expression of NDUFAF6 mRNA was dramatically increased in the breast cancer tumor group as compared to the normal group (Figure 1a). Additionally, we demonstrated using information from the GSE42568 database and the GSE45827 database that the expression of NDUFAF6 mRNA was considerably greater in breast cancer tissues compared to healthy tissues (Figure 1b,c). Concurrently, we quantified the mRNA expression levels of NDUFAF6 in MCF-10A cells, MCF-7 cells, and MDA-MB-231 breast cancer cells. The study revealed a significant increase in the expression of NDUFAF6 in breast cancer cells compared to normal breast epithelial cells. Furthermore, the level of expression in MCF-7 cells was found to be greater in comparison to that of MDA-MB-231 cells (Figure 1d). We utilized the TCGA database to generate Kaplan-Meier survival curves of overall survival (OS) and disease-specific survival (DFS) for breast cancer patients in order to determine the relationship between NDUFAF6 expression and breast cancer prognosis. Figures 1e,f revealed that a worse breast cancer prognosis was linked to increased NDUFAF6 expression (OS, hazard ratio (HR) = 1.98, 95% confidence interval (CI): 1.42–2.76, $p < .001$; DSS, HR = 1.80, 95%CI: 1.25–2.80, $p = .007$). Data from Kaplan-Meier Plotter also confirmed that higher NDUFAF6 expression was associated with a significantly poorer OS ($p < .05$, HR = 1.72, 95% CI: 1.24–2.38, Figure 1g) but not with relapse free survival (RFS) in breast cancer patients ($p = .23$, HR = 1.32, 95%CI: 0.83–2.09, Figure 1h). Through the use of the cBio-Portal database, the causes of the differential expression of NDUFAF6 in breast cancer cells and healthy tissues were

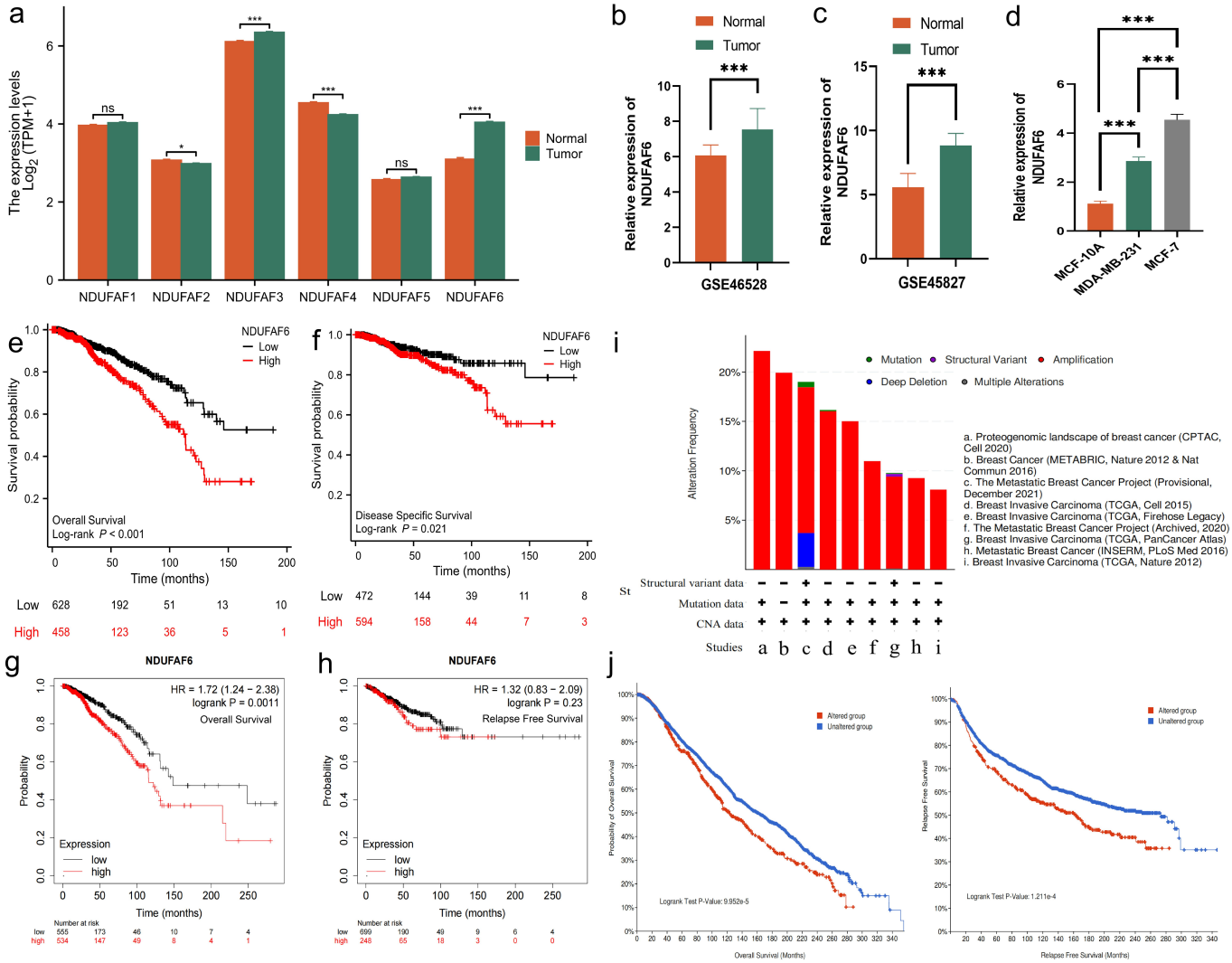

Figure 1. Expression of NDUFAF6 in breast cancer and its prognostic value. (a) NDUFAF1–6 expression levels in different tumor types from TCGA database. (b, c) expression of NDUFAF6 in breast cancer and in normal tissues in the GSE42568 and GSE45827 datasets of the GEO database. (d) NDUFAF6 expression in MCF-10A, MDA-MB-231 and MCF-7 cells. (e, f) overall survival and disease specific survival of breast cancer patients from TCGA database. (g, h) overall survival and relapse free survival curves of breast cancer patients from the Kaplan-Meier plotter database. (i) Genetic alterations of NDUFAF6 in breast cancer from neigh studies'data. (j) Overall survival and relapse free survival of breast cancer patients based on NDUFAF6 gene alterations. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

further investigated. After analyzing data from nine investigations, it was shown that amplification was the most common kind of genetic mutation in the NDUFAF6 gene. In Study "a", almost 20% of breast cancer patients exhibited NDUFAF6 gene amplification (Figures 1i). The Kaplan-Meier survival curve approach was then used to compare the prognosis of individuals who had NDUFAF6 gene changes versus those who did not. Poorer overall survival and recurrence-free survival were seen in patients with NDUFAF6 gene changes (Figures 1J).

Biological functions of NDUFAF6 in breast cancer cells

Through the use of siRNA methods, we further examined the importance of NDUFAF6 in tow breast cancer cells. We confirmed, using quantitative real-time PCR, that small interfering RNA dramatically reduced the expression of the NDUFAF6 gene (Figures 2a,e). The CCK-8 test findings revealed that the capacity of breast cancer cells to proliferate was dramatically decreased when the NDUFAF6 gene was knocked down (Figures 2b,f). The clone formation experiment provided further evidence supporting the notion that the proliferation capacity of breast cancer cells was notably decreased upon knockdown of the NDUFAF6 gene (Figures 2c,g). The Transwell test findings demonstrated that suppression of the NDUFAF6 gene dramatically decreased the capacity of breast cancer cells to migrate (Figures 2d,h).

Transcriptome analysis in NDUFAF6-knockdown breast cancer cells

To explore the potential regulatory mechanisms by which reduced NDUFAF6 expression induces diminished proliferation and migration in breast cancer cells. RNA sequencing technique was used 24 hours after the transfection of breast cancer cells with Si-NDUFAF6. Principal component analysis showed that the Si-NC and Si-NDUFAF6 groups could be significantly separated from each other, indicating reliable results (Figures 3a). A comprehensive set of 2101 differential genes were found, including 1203 genes that exhibited up-regulation and 898 genes that showed down-regulation (Figures 3b,c). Furthermore, the Gene Ontology (GO) analysis revealed that these differentially expressed genes (DEGs) were prominently associated with death, cell proliferation, migration, and cell signaling (Figure 3d). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the DEGs under investigation exhibited enrichment in many cancer-related signaling pathways, including PI3K-Akt, Rap1, Ras, MAPK, P53, and JAK-STAT (Figure 3e), and remained Gene Set Enrichment Analysis (GSEA) analysis further confirmed that knockdown of NDUFAF6 leads to apoptosis in breast cancer cells (Figure 3f). Furthermore, the western blot analysis revealed that the suppression of NDUFAF6 led to an elevation in the protein expression of

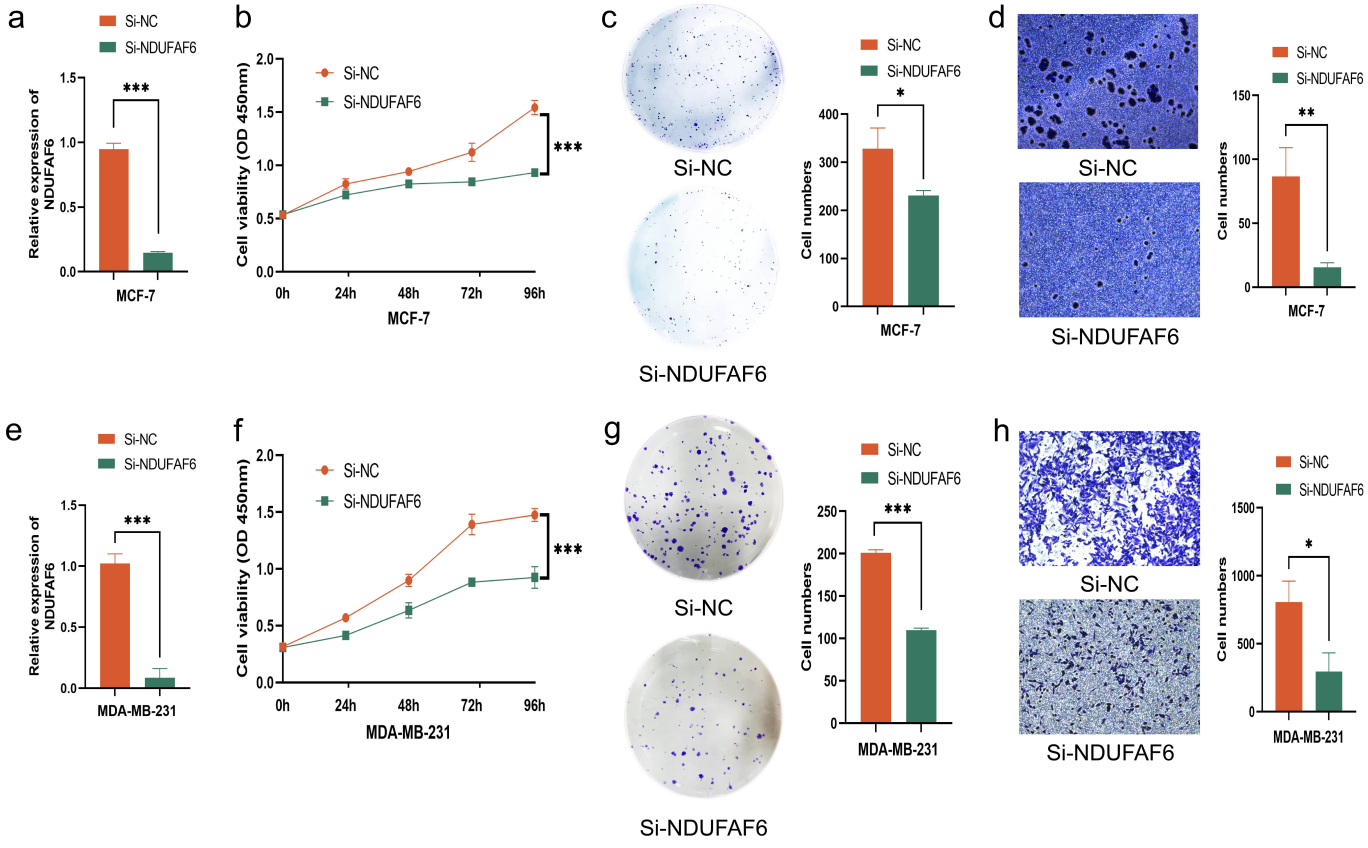

Figure 2. Effect of NDUFAF6 on breast cancer cell function. (a, e) NDUFAF6 mRNA expression decreased in MCF-7 and MDA-MB-231 transfected with NDUFAF6 siRNA by qRT-pcr. (b, f) CCK8 assay showed that knockdown of NDUFAF6 inhibited the proliferative activity of MCF-7 and MDA-MB-231 cells. (c, g) clone formation assays showed that knockdown of NDUFAF6 inhibited the proliferative activity of MCF-7 and MDA-MB-231 cells. (d, h) NDUFAF6 knockdown decreased the migration ability of MCF-7 and MDA-MB-231 cell (microscope, × 100). All experiments were repeated three times. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

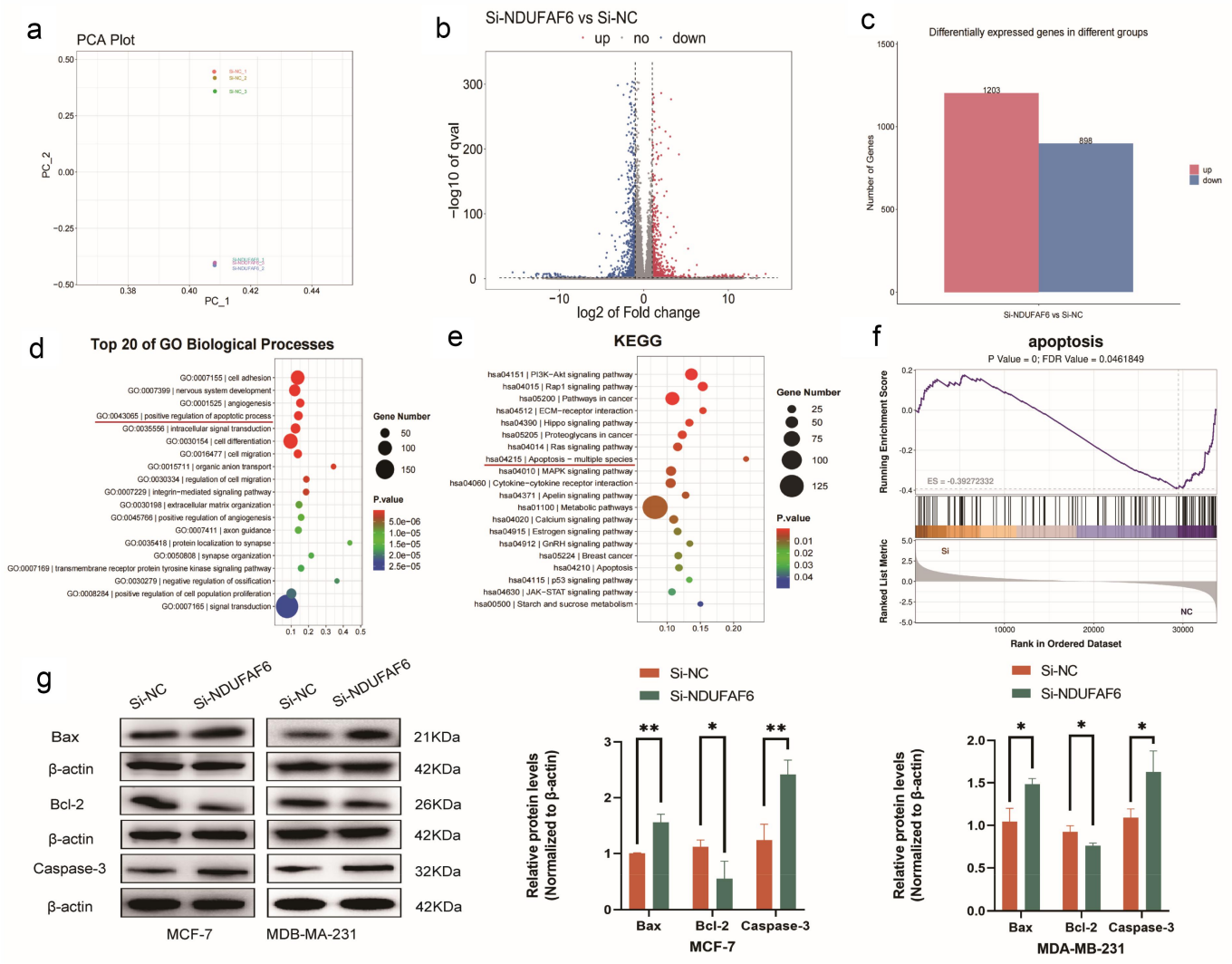

Figure 3. Transcriptome analysis of MDA-MB-231 cells with NDUFAF6 knockdown. (a-c) Principal component analysis (PAC) plot, differential gene volcano plot and number of up- and down-regulated differential genes after knockdown of NDUFAF6 gene in MDA-MB-231 cells. (d-f) GO, KEGG and GSEA analysis were used to analyze the differentially expressed genes in MDA-MB-231 cells after NDUFAF6 knockdown. (g) 48 h after knockdown of NDUFAF6, the apoptosis-related proteins Bcl-2, Bax and Caspase-3 were detected by Western blot. All experiments were repeated three times. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

Bax, while the protein expression of Bcl2 exhibited a reduction (Figure 3g). In addition, the downregulation of NDUFAF6 resulted in an upregulation of Caspase-3 expression in breast cancer cells. The findings of this study suggested that the activation of the apoptotic pathway occurred upon knockdown of the NDUFAF6 gene in breast cancer cells.

NDUFAF6 knockdown causes mitochondrial autophagy in breast cancer cells

We sought to explore whether apoptosis induced in breast cancer tumor cells after knockdown of NDUFAF6 was mitochondria-related. The results of RNA sequencing were analyzed by GO enrichment to find mitochondria-related pathways of interest and significance. GO enrichment analysis revealed pathways associated with mitochondria as shown in Figure 4a, and GSEA was enriched to the apoptotic mitochondrial changes signaling pathway (Figure 4b). The transmission electron microscopy findings revealed a notable augmentation in the quantity of autophagic lysosomes in the cells of the Si-NDUFAF6 group in comparison to the Si-NC group. Additionally, there was a reduction in the number of cristae in the mitochondria (Figure 4c). In addition, western blot results showed that knockdown of NDUFAF6 increased the expression of Parkin, Pink1 and LC3B-II proteins (Figure 4d). The aforementioned results demonstrated that knockdown of NDUFAF6 gene in breast cancer cells causes extensive mitochondrial autophagy.

Mitochondrial dysfunction and altered metabolism in breast cancer cells after NDUFAF6 knockdown

The cellular mitochondria's membrane potential was assessed using the JC-1 technique for both cell lines after NDUFAF6 knockdown. The findings indicated a considerable decrease in the red fluorescence intensity/green fluorescence intensity in the Si-NDUFAF6 group compared to the Si-NC group. This

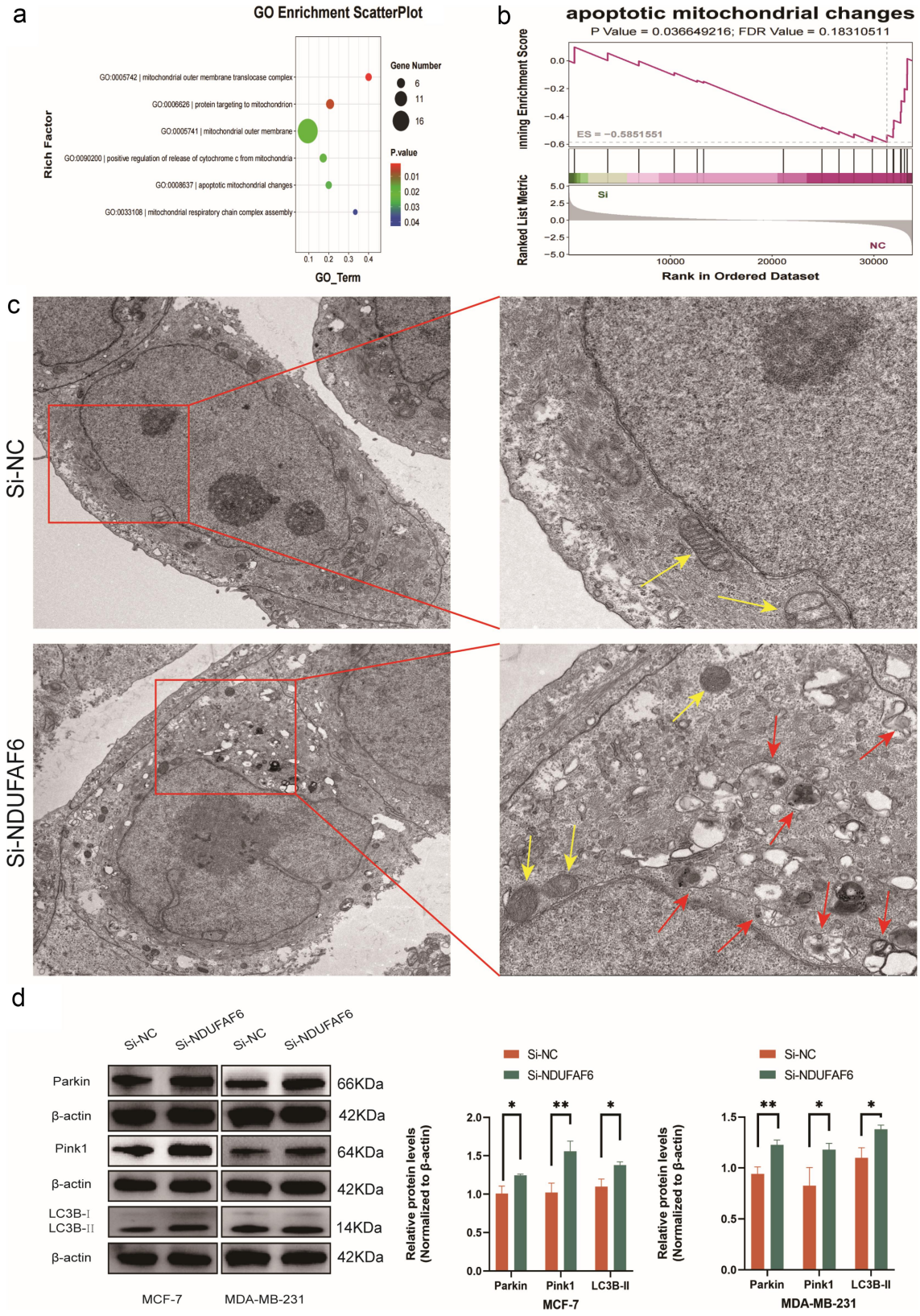

Figure 4. Knockdown of NDUFAF6 induced mitophagy in breast cancer cells. (a) GO enrichment analysis after NDUFAF6 knockdown revealed pathways related to mitochondria. (b) GSEA enrichment analysis after NDUFAF6 knockdown enriched "apoptotic mitochondrial changes". (c) Transmission electron microscopy of the hyperfine structure of Si-NC and Si-NDUFAF6 MCF-7 cells, with yellow arrows pointing to mitochondria and red arrows pointing to autophagic lysosomes. (d) 48 h after NDUFAF6 gene knockout, the expressions of mitophagy-related proteins Parkin, Pink1 and LC3B were detected by Western blot. All experiments were repeated three times. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

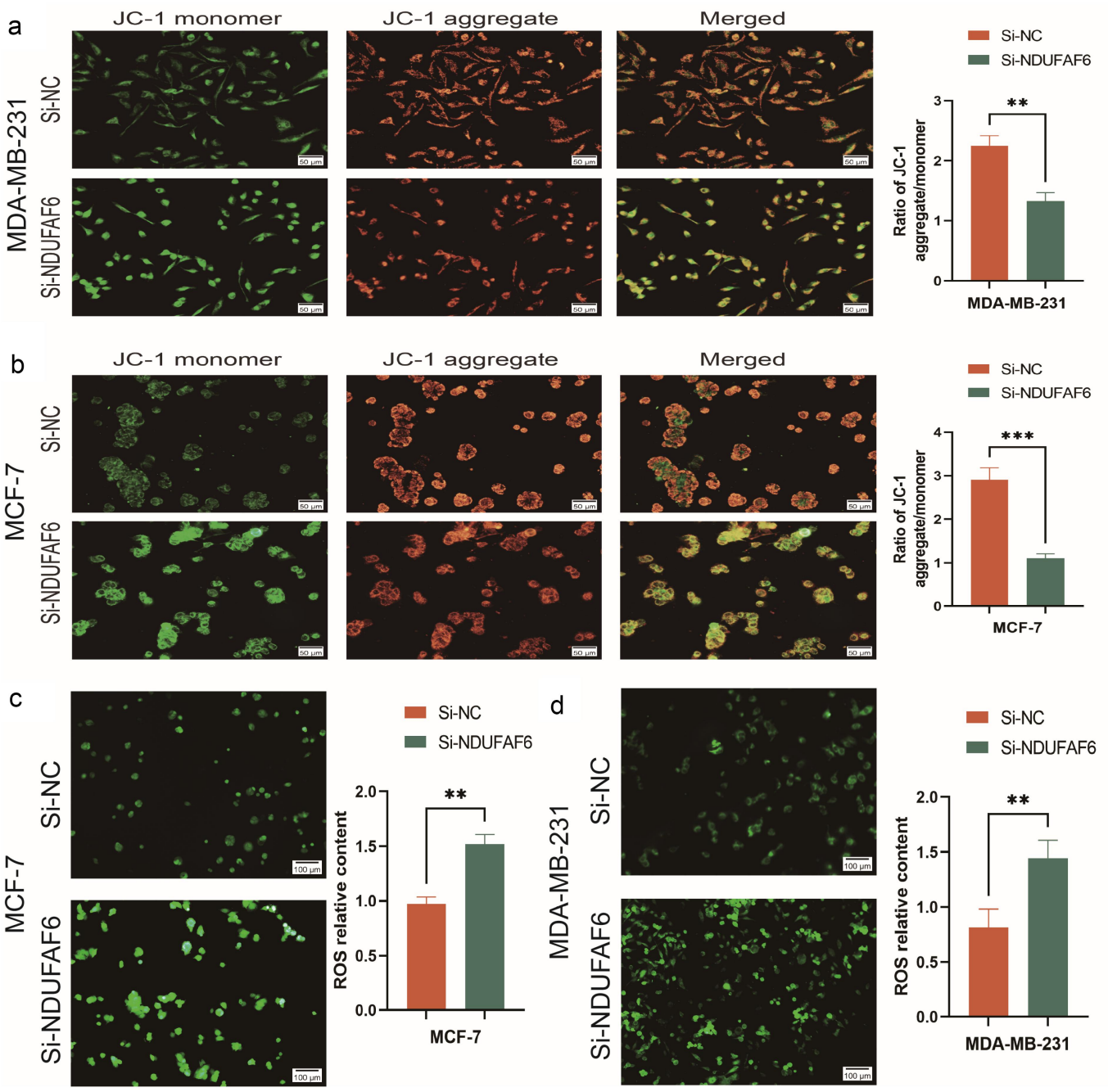

Figure 5. Changes in mitochondrial function and intracellular reactive oxygen species in breast cancer cells after knockdown of NDUFAF6. (a, b) JC-1 staining showed changes in mitochondrial membrane potential in breast cancer cells after knockdown of NDUFAF6 for 24 h. (c, d) DCFH-DA probe was used to detect the ROS level after 24 h of NDUFAF6 knockdown in breast cancer cells. All experiments were repeated three times. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

suggested a significant reduction in the mitochondrial membrane potential (Figures 5a,b). In this study, DCFH-DA staining was used to assess the intracellular ROS concentration. The fluorescence intensity exhibited a considerable rise during a 24-hour period of NDUFAF6 knockdown, in comparison to the control cells (Figures 5c,d), indicating that the content of intracellular ROS was significantly increased. In Figure 6a,b oxygen consumption rate reflects mitochondrial function. Basal mitochondrial respiration, maximal oxygen consumption (MOX) was decreased in both cell lines compared to the Si-NC group, whereas glycogen peroxynitrite-Schiff (PAS) staining showed higher glycogen content in the breast cancer cell lines of the NDUFAF6 knockdown group (Figures 6c,d), suggesting that knockdown of NDUFAF6 may affect cellular metabolism of glucose.

Discussion

Given that breast cancer remains one of the most prevalent malignant tumors in women and that its occurrence and development are the result of a multi-pathway and multi-factorial process, a thorough investigation into the disease's

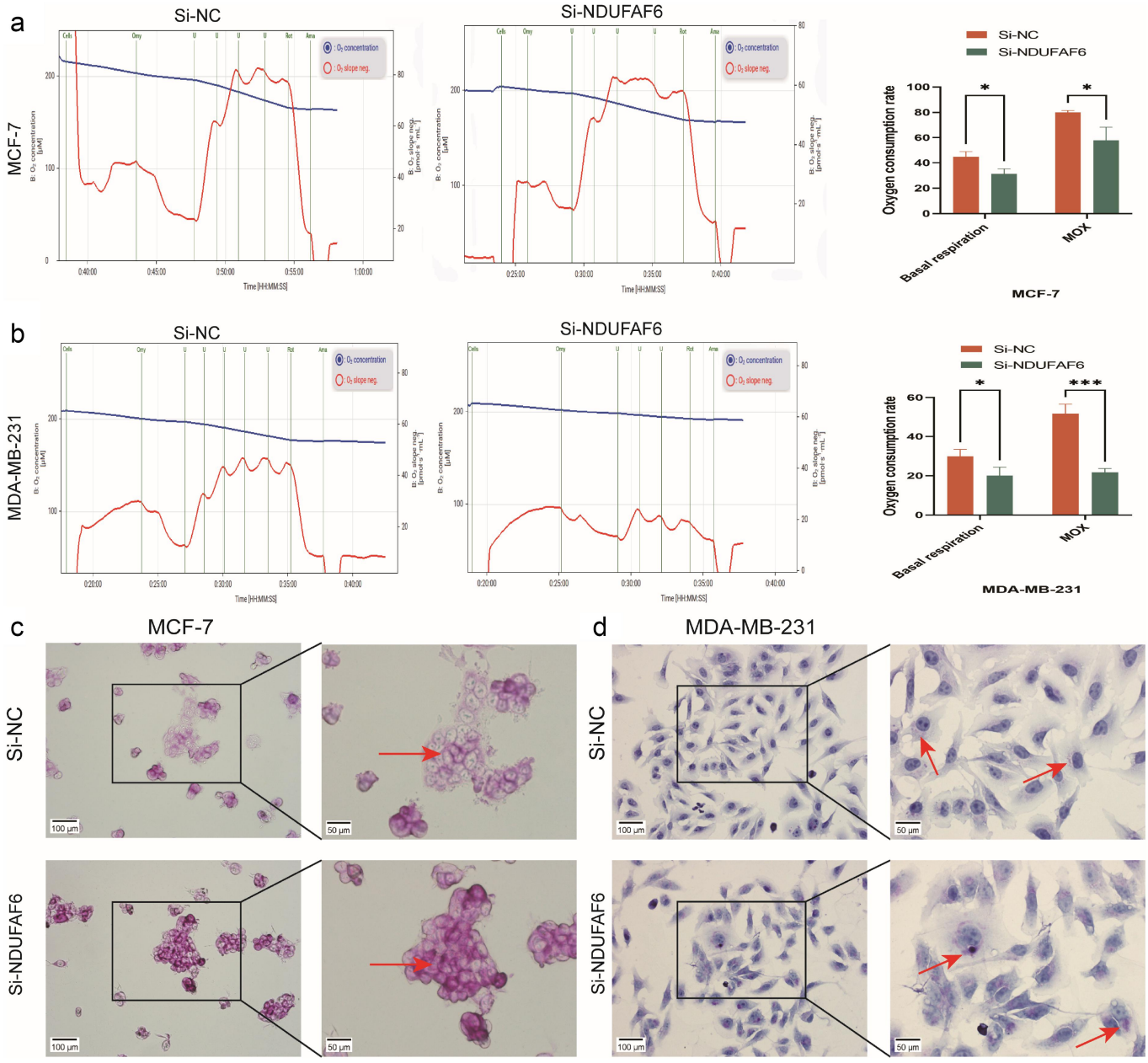

Figure 6. Knockdown NDUFAF6 can reduce the rate of oxygen depletion in breast cancer cells. (a, b) OCR of each group of breast cancer cells subsequent to the addition of various medications, and all experiments were repeated three times. (c, d) PAS staining was performed on MCF-7 cells and MDA-MB-231 cells, and the red arrow points to glycogen. *, $p < .05$. **, $p < .01$. ***, $p < .001$.

mechanism is crucial for both clinical diagnosis, treatment, and prevention.[26] The integration of bioinformatic tools, such as TCGA and GEO database analysis, is increasingly recognized as a critical step in cancer research for identifying and validating novel molecular targets with clinical relevance. Among several genes identified through database analysis, NDUFAF6 was prioritized due to its strong correlation with poor prognosis and its role in mitochondrial function, making it a promising candidate for therapeutic targeting.[21,27] Our results indicate that high expression of NDUFAF6 predicts poor prognosis in breast cancer patients. The observed phenomenon may be attributed to the mechanism by which NDUFAF6 enhances the expression of PD-L1 via the inhibition of the NRF2 signaling pathway.[21] A worse prognosis and immune evasion are associated with the presence of PD-L1, a critical immunological checkpoint, on neoplastic cells.[28] This finding provides an additional explanation for the worse prognosis seen in patients with elevated NDUFAF6 expression.

Previous research has shown that even when malignant cells are grown in an environment with plenty of oxygen, they still produce a lot of lactic acid instead of carbon dioxide and water, which is referred to as the Warburg effect as well.[29,30] Due of these, it is incorrectly believed that mitochondria have little bearing on the fast multiplication of malignant tumor cells.[31] Recent research has shown that mitochondrial metabolism is one of the essential requirements for tumor cell survival[13,32] and is intimately connected to carcinogenesis and proliferation.[33] Moreover, prior research has also shown that

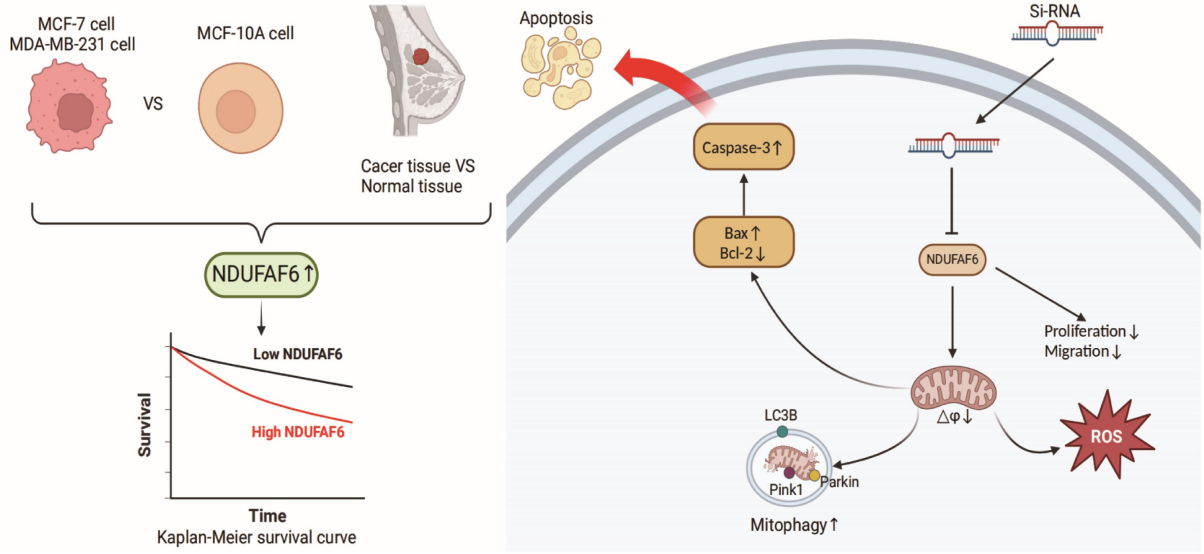

Figure 7. The role of NDUFAF6 in breast cancer.

the administration of pharmaceutical agents in breast cancer cells induces disturbances in mitochondrial energy metabolism, hence manifesting anti-tumor properties.[34] Our study showed that mitophagy could induce apoptosis in breast cancer cells and inhibit the proliferation and migration of breast cancer cells. Previous studies have shown that when mitochondrial function is severely impaired, intracellular autophagy activators are activated at the same time, causing the cell to initiate autophagy to degrade damaged mitochondria in order to prevent apoptosis.[35] However, if mitochondrial autophagy cannot be completely terminated, the formation of autophagic vesicles may promote the activation of caspases, further causing apoptosis.[36,37] This further indicated that knockdown of NDUFAF6 in breast cancer cells caused extensive mitochondrial dysfunction that could not be repaired in the short term. In addition, the focus of this study was on short-term cellular effects after NDUFAF6 knockdown and did not focus on long-term effects, and long-term effects may appear inconsistent with our expectations.

Complex 1 assembling component NDUFAF6, formerly known as c8orf38, was identified for the first time through phylogenetic investigation.[38] Individuals who possess mutations in the NDUFAF6 gene exhibit the manifestation of Leigh syndrome, a condition characterized by significantly diminished amounts of mitochondrial respiratory chain complex 1.[11] In a similar vein, the intentional suppression of the NDUFAF6 gene leads to a reduction in the quantity and levels of complex 1 in human fibroblasts.[38,39] These findings indicate that the knockdown of NDUFAF6 leads to a decline in the activity of the mitochondrial respiratory chain complex I, resulting in the impairment of its usual function. Respiratory chain complex 1 is essential for mitochondrial metabolism and is involved in a variety of biochemical processes, including the maintenance of the cellular redox state.[40,41] When there is a severe dysfunction in the mitochondrial respiratory chain complex I, the cell generates excessive ROS, which ultimately results in cellular demise.[42] Additionally, tumor cells have been found to produce a lot of mitochondrial ROS, which is a sign of mitochondrial dysfunction.[43] Tumor cells need elevated levels of ROS in order to consistently trigger pro-oncogenic pathways.[44] The same high antioxidant activity is also present in cancer cells to maintain redox homeostasis, and if cancer cells cannot control their ROS levels then they are vulnerable to oxidative stress-induced cell death.[45] Our results illustrate that after knockdown of NDUFAF6, cellular mitochondria were significantly dysfunctional, as reflected by a decrease in mitochondrial membrane potential, leading to a significant increase in intracellular ROS content, and the cells were unable to tolerate the higher ROS content and ultimately led to cellular dysfunction and apoptosis. Increasingly, research is focusing on cell death, for example by inducing ferroptosis in cells to increase their sensitivity to chemotherapeutic agents or to overcome cellular drug resistance,[46,47] and NDUFAF6 is an ideal target gene with potential to induce cell death. Meanwhile, the glycogen content in tumor cells was increased after knockdown of NDUFAF6, which may be related to the increased uptake of glucose by the cells. Prior research has shown that when the use of drugs causes mitochondrial dysfunction in breast cancer cells, cancer cells undergo a metabolic shift to obtain energy through enhanced glycolysis and have an increased uptake of glucose.[48]

Previous research has shown that the application of rotenone and its analogues to MCF-7 cells leads to increased levels of ROS, which in turn cause a reduction in Bcl-2 synthesis and an increase in Bax levels.[49,50] Our study also shows that by causing mitochondrial dysfunction in tumor cells also apoptosis is caused through the Bax/Bcl-2 pathway. Despite the potential efficacy of rotenone in targeting complex 1 and suppressing tumor cell proliferation, its considerable toxicity, particularly toward the central nervous system, remains a significant concern.[51] Hence, the use of rotenone as an anticancer medication in clinical settings is limited. If the NDUFAF6 gene under investigation exhibits similar effects to rotenone, it might potentially serve as a promising target for biotherapeutic

interventions. However, additional comprehensive investigations are required to provide a more thorough understanding of the precise function of the NDUFAF6 gene in breast cancer.

There are several limitations inherent in our investigation. There is no explicit mention of statistical power calculations, which could raise concerns about the robustness of the conclusions. Meanwhile, we did not perform deep bioinformatics analysis, such as GSEA enrichment analysis, because the amount of data in the public database is too large, and we will further improve it subsequently when the condition permits. The present study did not rule out a potential off-target effect of siRNA knockdown, and future studies may include the use of a second independent siRNA sequence targeting NDUFAF6 or CRISPR-Cas9 knockdown to confirm specificity. Insufficient animal models and clinical materials were used in our study to establish a definitive association between NDUFAF6 expression and prognosis, particularly in elucidating the relationship between NDUFAF6 and immunological invasion. Furthermore, further research is required to provide a more comprehensive understanding of the regulatory function of NDUFAF6 in breast cancer.

Conclusion

The findings of our investigation indicate a considerable increase in the expression of NDUFAF6 in breast cancer tissues. Elevated levels of NDUFAF6 expression were shown to be connected with worse prognosis in patients with breast cancer. Breast cancer cells with knockdown of NDUFAF6 develop mitochondrial dysfunction, leading to extensive mitochondrial autophagy and ultimately apoptosis (Figure 7). However, further in vitro studies and clinical trials are needed to validate the conclusions.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Yunjiang Liu http://orcid.org/0000-0001-7202-2004

Data availability statement

The raw data in this study may be obtained from the corresponding author upon reasonable request.

Authors contribution

Y. L, S. S, S. W and X. M conceived and designed the experiments. S. W, X. M and X. Z conducted the experimental study and wrote the manuscript. K. D, C. S, A. A and B. H assisted with the experiments and provided technical support. The final paper underwent a comprehensive review process and received approval from all contributing authors.

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