NEOPLASIA
www.neoplasia.com
Volume 32 Number xxx Month 2022 pp. 100821

Original Research

Hypoxia induces docetaxel resistance in triple-negative breast cancer via the HIF-1α/miR-494/Survivin signaling pathway ☆

Hongchang Li [a]; Xianhao Su [a]; Jindong Li [a]; Weiyan Liu [a]; Gaofeng Pan [a]; Anwei Mao [a]; Jiazhe Liu [a]; Qing Zhang [a]; Longhua Rao [a,*]; Xiaofeng Xie [b,*]; Xia Sheng [c,*]

[a] Department of General Surgery, Institute of Fudan Minhang Academic Health System, Minhang Hospital, Fudan University. 170 Xinsong Rd, Shanghai, China
[b] Department of General Surgery, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 528 Zhangheng Rd, Shanghai, China
[c] Department of Pathology, Institute of Fudan-Minhang Academic Health System, Minhang Hospital, Fudan University. 170 Xinsong Rd, Shanghai, China

Abstract

Cytotoxic chemotherapy is the major strategy to prevent and reduce triple-negative breast cancer (TNBC) progression and metastasis. Hypoxia increases chemoresistance and is associated with a poor prognosis for patients with cancer. Based on accumulating evidence, microRNAs (miRNAs) play an important role in acquired drug resistance. However, the role of miRNAs in hypoxia-induced TNBC drug resistance remains to be clarified. Here, we found that hypoxia induced TNBC docetaxel resistance by decreasing the miR-494 level. Modulating miR-494 expression altered the sensitivity of TNBC cells to DTX under hypoxic conditions. Furthermore, we identified Survivin as a direct miR-494 target. Hypoxia upregulated survivin expression. In a clinical study, the HIF-1α/miR-494/Survivin signaling pathway was also active in primary human TNBC, and miR-494 expression negatively correlated with HIF-1α and survivin expression. Finally, in a xenograft model, both miR-494 overexpression and the HIF-1α inhibitor PX-478 increased the sensitivity of TNBC to DTX by suppressing the HIF-1α/miR-494/Survivin signaling pathway *in vivo*. In conclusion, treatments targeting the HIF-1α/miR-494/Survivin signaling pathway potentially reverse hypoxia-induced drug resistance in TNBC.

Neoplasia (2022) 32, 100821

Keywords: miR-494, HIF-1α, Survivin, TNBC, Drug-resistance

Introduction

Breast cancer (BC) is the most common cancer and the second leading cause of cancer-related deaths in women worldwide. According to cancer statistics, BC accounted for 30% of cancers in females and an estimated 276,480 new cases and more than 42,000 deaths occurred in 2020 [1]. Triple-negative BC (TNBC) (ER-, PR- and HER2-) accounts for 15–20% of BC cases. Compared with other BC molecular subtypes, TNBC is more aggressive, has a worse prognosis, and often occurs in young women [2]. The lack of hormone receptors and HER2 overexpression render TNBC unsuitable for endocrine and HER2-targeted therapy. Consequently, cytotoxic chemotherapy is the major strategy used to prevent and reduce TNBC progression and metastasis [3–5]. Recent studies have identified various molecular mechanisms and signaling pathways related to TNBC metabolism, proliferation, and survival, which lead to chemotherapy resistance [6,7]. Therefore, studies elucidating the molecular factors related to acquired resistance to chemotherapies are important.

The acquisition of resistance to chemotherapy is a complex process involving changes in the tumor microenvironment, and its underlying mechanism has not yet been fully elucidated. Previous studies have shown that tumor hypoxia is a condition of low oxygen levels in solid tumors that increases the resistance of cancer cells to chemotherapy and radiotherapy

Abbreviations: miRNA, microRNA; BC, Breast cancer; TNBC, Triple-negative Breast cancer; DTX, docetaxel; PI, propidium iodide; FITC, fluorescein isothiocyanate; shRNA, small hairpin RNA; qRT-PCR, Quantitative real-time PCR; OE, overexpression; KD, knock down.

* Corresponding authors.
E-mail addresses: raolonghua@fudan.edu.cn (L. Rao), xiexf@shutcm.edu.cn (X. Xie), shengxia_021@fudan.edu.cn (X. Sheng).

☆ Hongchang Li, Xianhao Sun, Jindong Li contributed to the study equally.

Received 26 February 2022; received in revised form 30 June 2022; accepted 6 July 2022

https://doi.org/10.1016/j.neo.2022.100821

[8,9]. Approximately 50–60% of advanced solid tumors, including breast cancer, contain hypoxic areas that are usually associated with shorter survival [10]. Hypoxia affects the growth dynamics, angiogenesis, migration, endoplasmic reticulum (ER) stress, and aggressive characteristics of breast cancer cells [11,12]. In addition, many reports indicate that hypoxia increases the chemoresistance of cancer cells by activating the HIF-1α pathway [13,14]. These findings indicate that HIF-1α plays a key role in regulating tumor chemosensitivity.

Current studies have revealed that miRNAs also play a role in the process of drug resistance in tumors, although the molecular mechanisms underlying this phenomenon are numerous and remain unclear [15,16]. miR-494 is a carcinogenic miRNA that drives tumor progression and drug resistance in certain cancer types, including colorectal cancer [17] and hepatocellular carcinoma [18]. Interestingly, we performed miRNA microarrays and found that miR-494 was significantly downregulated in TNBC under hypoxic conditions (Fig. 1), indicating that it may play a role in drug resistance under hypoxic conditions. Survivin is the smallest member of the inhibitor-of-apoptosis protein (IAP) family and plays key roles in cell division and cell survival. According to previous studies, high Survivin expression levels are associated with drug resistance [19]. Yun et al. found that Survivin was downregulated by miRNA-494 in gastrointestinal stromal tumors [20].

In the present study, we investigated the role of miRNA-494 in TNBC resistance under hypoxic conditions. The HIF-1α/miR-494/Survivin signaling pathway was responsible for hypoxia-mediated DTX resistance in TNBC. Treatments targeting the HIF-1α/miR-494/Survivin signaling pathway may affect the sensitivity of TNBC to DTX.

Materials and methods

Cell lines and reagents

The human breast cancer cell lines MB-231 and MB-468 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a 5% CO_2 atmosphere at 37°C with Leibovitz's L-15 (Thermo Fisher) medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). Hypoxic conditions were achieved by placing the cells in a 1% O_2, 94% N_2, and 5% CO_2 multistage incubator (Sanyo, Osaka, Japan).

The miR-494 mimics, negative control mimics, miR-494 inhibitors, and negative control inhibitors were all purchased from Exiqon Inc. (Woburn, MA). The cDNA encoding the miR-494 precursor was cloned into the pMCS-CMV lentiviral vector purchased from GeneChem, Shanghai, P.R. China. The HIF-1α knockdown shRNA plasmid (HIF-1α KD) and Survivin knockdown shRNA plasmid (Survivin KD) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The HIF-1α overexpression plasmid (HIF-1α OE) and Survivin overexpression plasmid (Survivin OE) were purchased from Addgene (Cambridge, MA). 293T packaging cells were cotransfected with pPackH1 packaging plasmid mix (SBI, Mountain View, CA) and lentiviral vectors using Fugene HD (Promega, Madison, WI). Viruses were harvested 48 h later and used to infect target cells.

Patient samples

Human BC, TNBC and corresponding nontumor (NC) colon tissue samples were collected at the time of surgical resection at Minhang Hospital, Fudan University, China, from January 2010 to December 2011. None of the patients included in the study had received neoadjuvant therapy before surgery. Samples were immediately snap frozen in liquid nitrogen and stored at −80°C. Written informed consent was obtained from the patients, in accordance with the institutional guidelines, before sample collection, and the study was approved by the Committees for the Ethical Review of Research at the Minhang Hospital, Fudan University, China. The methods were performed in accordance with the approved guidelines.

Cell viability and apoptosis assays

Breast cancer cells were seeded into 96-well plates and treated with different agents. After 48 h, a CCK-8 assay was used to evaluate cell viability (Dojindo Molecular Technologies, Inc., MD, USA). The absorbance of each well was measured at 490 nm and 630 nm using a spectrophotometer (Bio-Rad, Hercules, CA). Apoptosis was detected using a DNA fragmentation ELISA kit (Roche, Indianapolis, IN).

miRNA microarray analysis

Total RNA was extracted from MB-231 and MB-468 cells cultured under normoxic or hypoxic conditions with TRIzol reagent (Invitrogen, Carlsbad, CA), and the miRNA fraction was further purified with the mirVana^{TM} miRNA isolation kit (Ambion, Austin, TX). A miRCURY^{TM} Array Labeling kit (Exiqon, Vedbaek, Denmark) was used to label the isolated miRNA with Hy3, and samples were hybridized on a miRCURY^{TM} LNA microRNA Array (v 8.0, Exiqon). A Genepix 4000B scanner (Axon Instruments, Union City, CA) was used to acquire microarray images, and Genepix Pro 6.0 software (Axon Instruments) and Excel were used for data processing and analysis.

Quantitative RT–PCR

Each sample was cut into 5-mm^3 sections, frozen tissues were finely ground into particles, and then total RNA was extracted from the tissue particles with TRIzol (Invitrogen). Total RNA was extracted from cultured MB-231 and MB-468 cells with TRIzol (Invitrogen). The concentration of total RNA was quantified by measuring the absorbance at 260 nm. The expression of mature miRNAs was assayed using stem-loop RT followed by real-time PCR analysis. All reagents for stem-loop RT were obtained from Applied Biosystems (Foster City, CA). The relative amount of each miRNA was normalized to the U6 snRNA. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The results are presented as fold changes for each miRNA relative to its control. The miR-494 expression level in tissues was calculated using the $2^{-\Delta Ct}$ method. The primers for miR-494 and the U6 snRNA used for stem-loop RT–PCR were purchased from QIAGEN Inc. (Valencia, CA).

Luciferase activity assay

The wild-type Survivin 3'UTR and sequence containing mutations in the putative miR-494 putative target sites were cloned into the pGL3-promoter vector. A total of 3×10^4 cells were cotransfected with 500 ng of pGL3-Survivin-WT or pGL3-Survivin-Mut constructs along with miR-494 mimics. Each sample was cotransfected with 50 ng of pRL-SV40 plasmid expressing Renilla luciferase to monitor the transfection efficiency. A dual luciferase reporter assay system was used to detect luciferase activity 48 h after transfection. The relative luciferase activity was normalized to the Renilla luciferase activity.

Western blot analysis

Cells were lysed with cell lysis buffer (Cell Signaling Technology, US). Proteins were resolved on SDS-PAGE gels and analyzed using western blotting. All antibodies were used at a working concentration of 1 mg/ml after dilution in PBS containing 5% skim milk. The membrane was further probed with a goat anti-rabbit or anti-mouse IgG-HRP secondary antibody (Abcam, 1:1000), and the protein bands were visualized using enhanced chemiluminescence (Millipore, USA). Protein bands were quantified using ImageJ software.

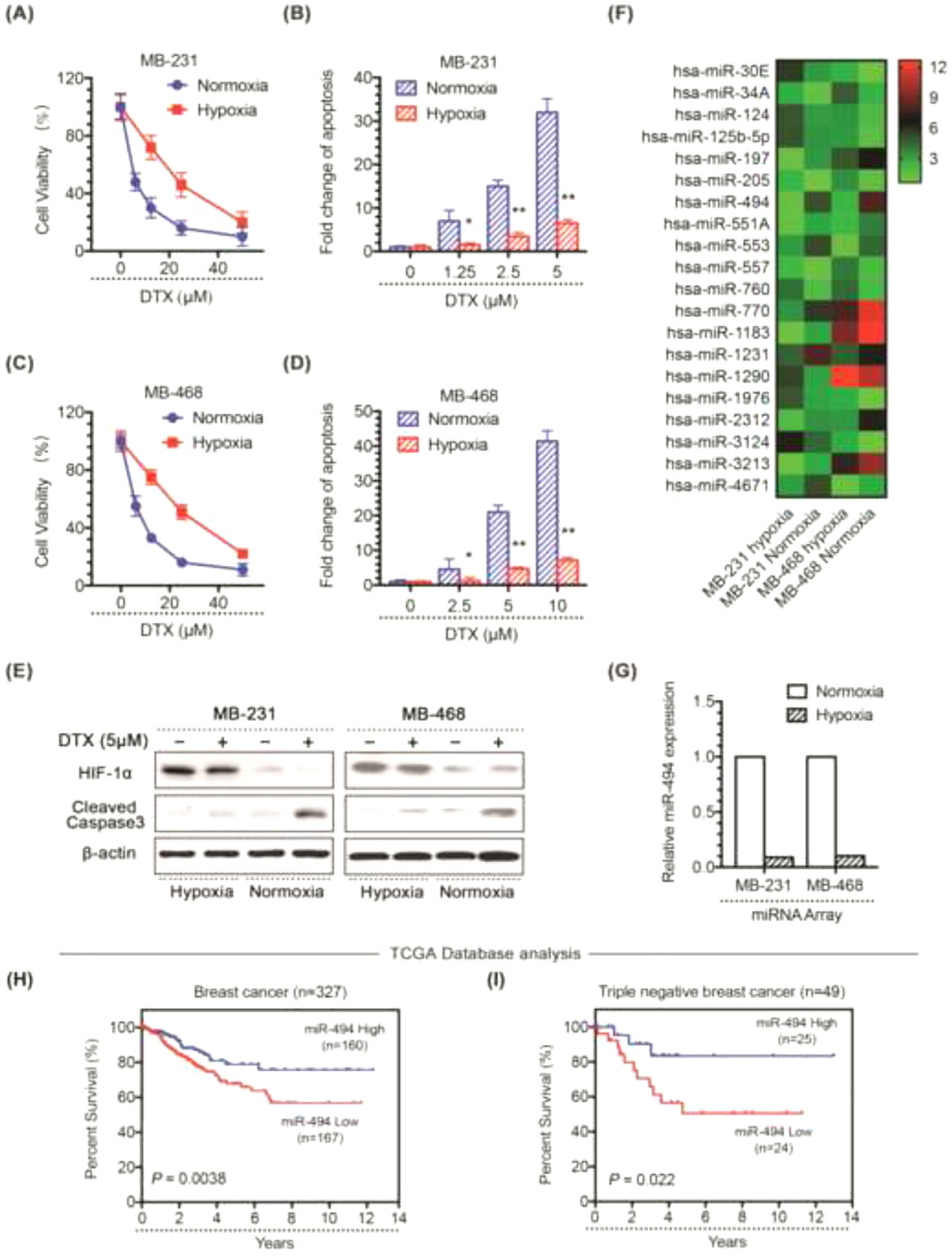

Fig. 1. Hypoxia induces Triple-Negative Breast Cancer docetaxel resistance via miR-494. (A) Hypoxia significantly decreased the growth-inhibitory effect of docetaxel (DTX) by CCK-8 assay, (B) reduced apoptosis level of DTX by DNA fragmentation assays in Triple-Negative Breast Cancer (TNBC) cells MB-231; (C) Hypoxia significantly decreased the growth-inhibitory effect of DTX (D) reduced apoptosis level of DTX in TNBC cells MB-468; (E) Protein levels of HIF-1α and cleaved Caspase 3 pathway were determined by Western blot. (F) The heatmap showed 20 differentially expressed miRNAs in hypoxic vs. normoxic TNBC cells by miRNA microarray ($p < 0.05$). (G) miRNA microarray results showed miR-494 expression levels significantly decreased in hypoxic TNBC cells. Kaplan-Meier analysis of overall survival in patients with variable miR-494 expression according to the data from the TCGA database: (H) Breast Cancer ($n = 327$), $P = 0.0038$; (I) TNBC ($n = 49$), $P = 0.022$. $^{*}P < 0.05$, $^{**}P < 0.01$. Each bar represents the mean ± SD of three independent experiments.

In vivo *xenograft model*

MB-231 cells (2×10^6) containing the miR-494 or control vector were injected into the flanks of male athymic nude mice (4-5 weeks old). Two weeks after the injection, DTX (10 mg/kg) or vehicle was injected intraperitoneally every 5 days for 4 weeks. DTX (10 mg/kg) or PX-478 (20 mg/kg) was administered to other groups. Tumor volumes were measured at the beginning of the treatment and every 4 days until the mice were euthanized. The estimated tumor volumes (V) were calculated using the formula $V = W^2 \times L \times 0.5$, where W represents the largest tumor diameter in centimeters and L represents the next largest tumor diameter. The tumors were dissected, weighed, and then immediately fixed with formalin for immunohistochemistry.

All procedures were approved and supervised by the Institutional Animal Care and Use Committee of Minhang Hospital, Fudan University, P.R. China. All animal studies were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Immunohistochemistry

The tissues were fixed with 10% formalin, embedded in paraffin, and sliced into 5-mm thick sections. TUNEL staining and immunohistochemical staining for Ki67, Survivin and HIF-1α were conducted as described below. Sections were deparaffinized and incubated with 3% H_2O_2 in water for 10 min to quench endogenous peroxidase activity. Antigens were detected using the heat-induced antigen retrieval method. Slices were incubated with 5% normal goat serum in TBS (0.05 M Tris-HCl and 0.5 M NaCl, pH 7.4) for 1 h at room temperature and incubated with primary antibodies in TBS overnight at 4°C. The appropriate secondary antibodies were incubated with sections for 1 h at room temperature. The EnVision (K4007, Dako) signal enhancement system was used to develop the bound antibodies. The sections were counterstained with Harris' hematoxylin, dehydrated and mounted. Images were captured with a microscope (Leica, Wetzlar, Germany).

Statistical analysis

Error bar was expressed as the mean ± standard deviation (SD) of three independent exprement. Statistical analysis was performed by T-test to assess the significance of differences between groups, values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were accepted. The Spearman rank statistical test and the Mann-Whitney test were used for statistical analysis of tissue samples to assess the significance of differences between groups.

Results

Hypoxia induces docetaxel resistance in triple-negative breast cancer through miR-494

Hypoxia is a common feature of the tumor microenvironment that activates the HIF signaling pathway in cancer cells. According to previous reports, hypoxia regulates the expression of many genes involved in angiogenesis, tumor growth, metastasis, metabolic reprogramming, and treatment resistance [21,22]. We confirmed whether hypoxia induces drug resistance in triple-negative breast cancer (TNBC) cells by treating the human TNBC cell lines MB-231 and MB-468 with chemotherapeutics commonly used in the clinic and culturing the cells under hypoxic or normoxic conditions. Compared to normoxia, hypoxia impaired the cytotoxic effect of docetaxel (DTX) on both MB-231 (Fig. 1A) and MB-468 cells (Fig. 1C). In addition, hypoxia significantly reduced the DTX-induced apoptosis of TNBC cells (Fig. 1B and D). Furthermore, the western blotting (WB) results showed that hypoxia decreased the levels of cleaved caspase 3 in both TNBC cell lines (Fig. 1E). miRNAs play key roles in cancer drug resistance [23,24]. We compared the miRNA expression profiles of two TNBC cell lines, MB-231 and MB-468, cultured under hypoxic or normoxic conditions using a 2578 miRNA microarray of human mature miRNA probes to study the role of miRNAs in the development of hypoxia-induced drug resistance in TNBC cells. The heatmap and Table 1 show differences in the expression levels of 20 miRNAs in hypoxic cells compared with normoxic cells, and the trends were the same in the two TNBC cell lines (Fig. 1F).

The expression of miR-494 was reduced very significantly in both cell lines cultured under hypoxic conditions, suggesting that miR-494 played a certain role in the development of acquired resistance to DTX in TNBC cells (Fig. 1G). The Kaplan–Meier survival curve analysis showed that the overall survival time of patients with high miR-494 expression was significantly longer than that of patients with low miR-494 expression who were diagnosed with breast cancer (BC, $n = 327$) and TNBC ($n = 49$) in The Cancer Genome Atlas (TCGA) database (Fig. 1H and I). Taken together, hypoxia induces docetaxel resistance in TNBC cells and miR-494 may play certain roles in this process.

Hypoxia decreases miR-494 expression, conferring resistance to DTX in TNBC cells

Consistent with the microarray data, RT–qPCR analyses confirmed reduced miR-494 expression in hypoxic cells compared with normoxic cells (Fig. 2A).

We transfected miR-494 mimics into hypoxic TNBC cells (Fig. 2B) or added inhibitors to normoxic TNBC cells (Fig. 2C) to determine whether hypoxia reduced miR-494 expression and subsequently altered the sensitivity of TNBC cells to DTX. The miR-494 mimics reversed DTX resistance induced by hypoxia (Fig. 2D), increased apoptosis levels (Fig. 2F), and increased the cleavage of Caspase 3 (Fig. 2H). In contrast, in TNBC cells cultured under normoxic conditions, inhibition of miR-494 prevented DTX-induced apoptosis, as evidenced by the right shift of the growth inhibition curve (Fig. 2E), significantly reduced apoptosis (Fig. 2G), and decreased the levels of cleaved Caspase 3 (Fig. 2I). These data showed that the regulation of miR-494 expression altered the sensitivity of hypoxic and normoxic TNBC cells to DTX.

Survivin is a target of posttranscriptional repression by miR-494 in TNBC cells

Survivin is a member of the inhibitor-of-apoptosis protein (IAP) family that plays keys role in cell division and cell survival. High Survivin expression is reported to be associated with drug resistance in TNBC. Based on our results, hypoxia induced Survivin expression at both the mRNA and protein levels (Fig. 3A and B). These results indicate that Survivin is potentially key point in the pathway by which hypoxia mediates TNBC drug resistance.

We used miRDB (http://mirdb.org), miRWalk (http://mirwalk.umm.uni-heidelberg.de) and TargetScan (http://www.targetscan.org) to predict the target genes of miR-494. Using the miR-494 recognition sites in the 3' UTRs, all three algorithms revealed that Survivin was one of the common candidate target genes (Fig. 3C). We assessed whether miR-494 directly targets Survivin by cloning the Survivin 3′UTR fragment containing the miR-494 binding site into the luciferase reporter system. A plasmid lacking the miR-494 binding site was used as a negative control (Fig. 3D). The results showed that miR-494 inhibited the luciferase activity of the Survivin 3′UTR (Fig. 3E).

In addition, WB results showed that miR-494 mimics reduced the level of the Survivin protein in hypoxic TNBC cells and increased its levels in miR-494 inhibitor-transfected normoxic TNBC cells (Fig. 3F). Immunofluorescence (IF) staining revealed a similar effect of miR-494 (Fig. 3G). Taken together, miR-494 targeting of Survivin is required for hypoxia-mediated TNBC DTX resistance.

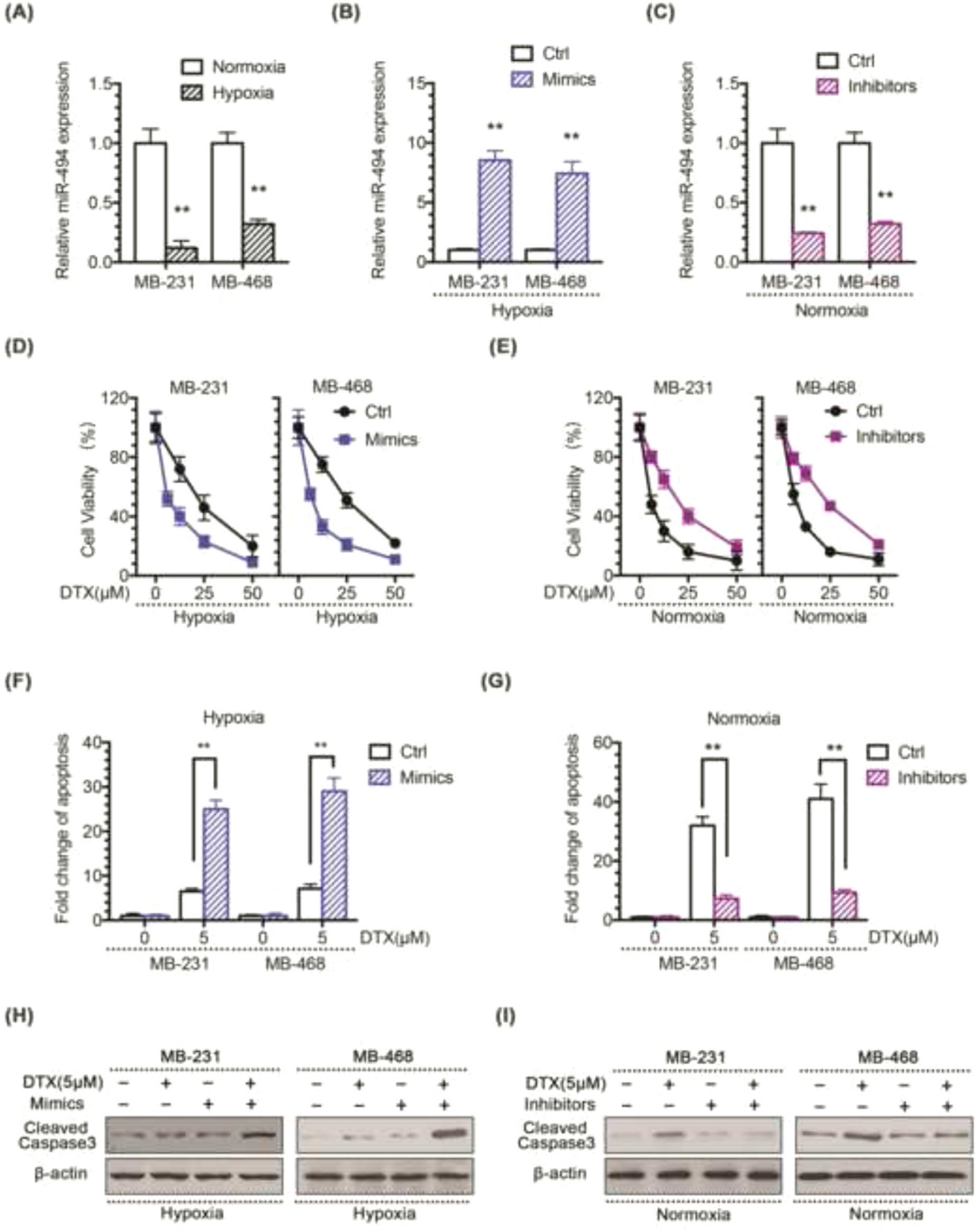

Fig. 2. Hypoxia decreases miR-494 expression confer the resistance of TNBC cells to DTX. (A) qPCR validation showing miR-494 expression level was significantly decreased in hypoxic TNBC cells. (B) MiR-494 expression level was significantly increased after transient transfection of miR-494 mimic in hypoxic TNBC cells as measured by qPCR. (C) MiR-494 expression level was significantly decreased after transient transfection of miR-494 inhibitor in normoxic TNBC cells as measured by qPCR. (D) Overexpression of miR-494 induced by mimics transfection significantly enhanced the growth-inhibitory effect of DTX in hypoxic TNBC cells by CCK assay; (F) increased hypoxic TNBC cells apoptosis level to DTX by DNA fragmentation assays; (H) and increased cleaved Caspase 3 level by western blot analysis. (E) Down-regulation of miR-494 induced by inhibitors transfection significantly decreased normoxic TNBC cells growth under DTX treatment by CCK assay; (G) decreased normoxic TNBC cells apoptosis level to DTX by DNA fragmentation assays; (I) and decreased cleaved Caspase 3 level by western blot analysis. $^{*}P < 0.05$, $^{**}P < 0.01$. Each bar represents the mean ± SD of three independent experiments.

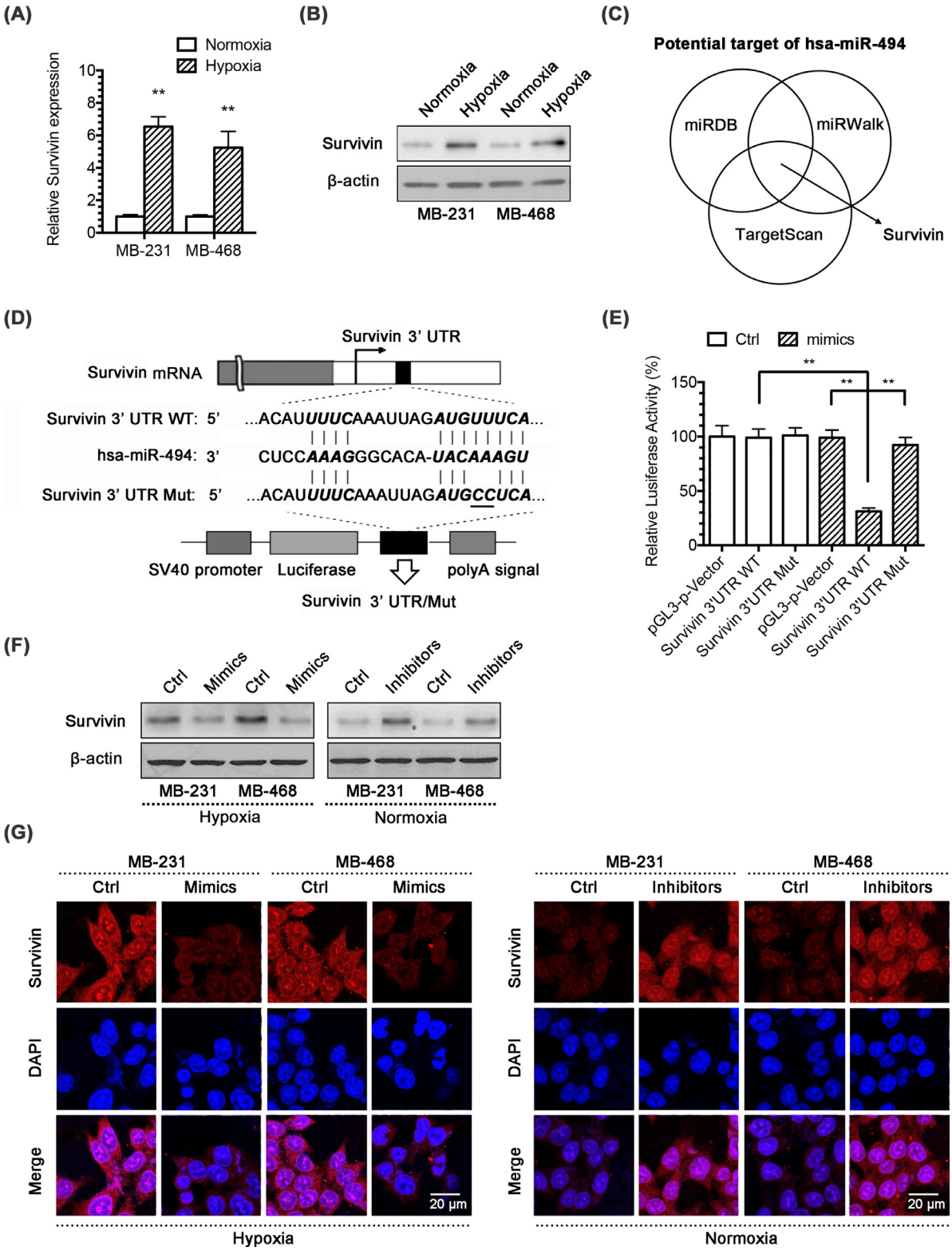

Fig. 3. Survivin is a target of post-transcription repression by miR-494 in TNBC cells. (A) Survivin mRNA expression level was increased in hypoxic TNBC cells by qPCR; and (B) Survivin protein expression level was increased in hypoxic TNBC cells by WB; (C) Hsa-miR-494 was the intersection miRNA of miRDB, miRWalk and TargetScan databases targeted Survivin. (D) The wild-type and mutant variant of the putative miR-494 target sequences of the Survivin gene. (E) Two copies of the wild-type and mutant miR-494 target sequences were fused with a luciferase reporter and transfected into control oligonucleotide and miR-494 mimics infected HEK293T cells. MiR-494 significantly suppressed the luciferase activity of the wild-type Survivin 3'UTR. Survivin protein expression level was found regulated directly by miR-494, as reflected by the decreased Survivin expression in hypoxic TNBC cells after transient transfection of miR-494 mimic, and increased Survivin expression in normoxic TNBC cells after miR-494 inhibitor transfection via (F) WB, or (G) Immunofluorescence. $^{*}P < 0.05$, $^{**}P < 0.01$. Each bar represents the mean ± SD of three independent experiments.

Table 1

miRNAs differentially expressed in hypoxic/normoxic TNBC cells.

miRNA	MB-231 Hypoxia/Normoxia	MB-468 Hypoxia/Normoxia
hsa-miR-30E	1.68	4.16
hsa-miR-34A	2.20	1.82
hsa-miR-124	1.54	1.65
hsa-miR-125b-5p	1.45	2.05
hsa-miR-197	0.28	0.78
hsa-miR-205	3.13	2.35
hsa-miR-494	***0.23***	***0.31***
hsa-miR-551A	0.43	0.61
hsa-miR-553	0.62	0.26
hsa-miR-557	2.41	1.86
hsa-miR-760	2.18	2.02
hsa-miR-770	0.62	0.70
hsa-miR-1183	0.29	0.74
hsa-miR-1231	0.58	0.75
hsa-miR-1290	1.70	1.20
hsa-miR-1976	1.61	3.84
hsa-miR-2312	0.59	0.49
hsa-miR-3124	1.40	3.58
hsa-miR-3213	0.33	0.77
hsa-miR-4671	0.62	0.33

The HIF-1α/miR-494/Survivin signaling pathway is responsible for hypoxia-mediated DTX resistance in TNBC cells

In the studies described above, we found that hypoxia reduced miR-494 expression to decrease the sensitivity of TNBC cells to DTX. Hypoxia-inducible transcription factor 1α (HIF-1α) is important for regulating the coordinated adaptive response to hypoxic conditions, and thus we must determine whether HIF-1α regulates miR-494 expression. In subsequent experiments, decreases in HIF-1α expression induced by the HIF-1α inhibitor PX-478 or HIF-1α KD plasmid increased miR-494 expression in hypoxic cells (Fig. 4A). In contrast, HIF-1α overexpression reduced miR-494 expression in cells cultured under normoxic conditions. (Fig. 4B). Overexpression of HIF-1α in TNBC cells treated with PX-478 significantly reduced miR-494 expression (Fig. 4C).

Therefore, miR-494 inhibition in hypoxic TNBC cells rescued the diminished Survivin expression observed after reducing HIF-1α expression (Fig. 4D). In contrast, HIF-1α reversed the decrease in Survivin expression induced by miR-494 mimics. We studied the function of survivin in hypoxia-mediated DTX resistance in TNBC cells by transfecting cells with Survivin overexpression (OE) or Survivin knockdown (KD) plasmids. An increase in miR-494 expression rendered hypoxic TNBC cells sensitive to DTX treatment again, and overexpression of survivin reversed this effect (Fig. 4F) and reduced apoptosis (Fig. 4H). In contrast, KD-Survivin resensitized normoxic TNBC cells with miR-494 inhibitor-induced DTX resistance in (Fig. 4G and I). Based on these results, the HIF-1α/miR-494/Survivin signaling pathway mediates hypoxia-induced DTX resistance in TNBC cells.

The HIF-1α/miR-494/Survivin signaling pathway in human TNBC specimens

We studied the HIF-1α/miR-494/Survivin signaling pathway in human TNBC by detecting the expression levels of HIF-1α, miR-494 and Survivin in 43 pairs of human TNBC specimens (adjacent normal tissues and tumor tissues) using qPCR and investigated the miR-494 expression levels in 125 pairs of human BC specimens. The Kaplan–Meier curve analysis showed that the overall survival of the patients with low miR-494 expression was obviously longer than that of patients with high miR-494 expression who were diagnosed with both BC and TNBC (Fig. 5A and B). First, we detected a negative correlation between the expression of miR-494 and Survivin (Fig. 5C) or HIF-1α (Fig. 5D) and a positive correlation between the expression of Survivin and HIF-1α (Fig. 5E) in TNBC specimens. Additionally, Survivin expression negatively correlated with miR-494 expression (Fig. 5F); likewise, miR-494 expression negatively correlated with HIF-1α expression (Fig. 5G). However, HIF-1α expression was positively correlated with Survivin expression (Fig. 5H). Finally, miR-494 expression was reduced in tumor tissues compared with their adjacent tissues (Fig. 5I), and both Survivin and HIF-1α expression were increased in the tumor tissues (Fig. 5J and K). In summary, the HIF-1α/miR-494/Survivin signaling pathway is also active in primary human TNBC, and miR-494 expression is negatively correlated with HIF-1α and Survivin expression.

Modulating the HIF-1α/miR-494/Survivin signaling pathway alters the sensitivity of TNBC in vivo

We established a TNBC xenograft model by subcutaneously implanting isogenic MB-231 cells to analyze the effect of the HIF-1α/miR-494/Survivin signaling pathway *in vivo*. Tumor-bearing mice were divided into 4 groups (i.e., vehicle, DTX, miR-494 OE+DTX and PX-478+DTX groups). The tumor-bearing mice were monitored for 4 weeks until they were euthanized. Tumor growth curves showed that both miR-494 and PX-478 significantly increased the response of TNBC cells to DTX (Fig. 6A), which led to a reduction in tumor size and weight (Fig. 6C).

Furthermore, none of the four groups lost weight or died (Fig. 6B). In addition, qPCR results showed that PX-478 upregulated miR-494 levels (Fig. 6D), and survivin mRNA levels were downregulated in the miR-494-overexpressing TNBC groups and PX-478 group (Fig. 6E). Immunohistochemical staining showed that both miR-494 overexpression and HIF-1α inhibition significantly increased the inhibitory effect of DTX on TNBC cell growth, which was verified by reduced Ki67 levels, increased apoptosis detected using TUNEL staining, and decreased survivin expression in tumors (Fig. 6F). In conclusion, we confirmed that regulating

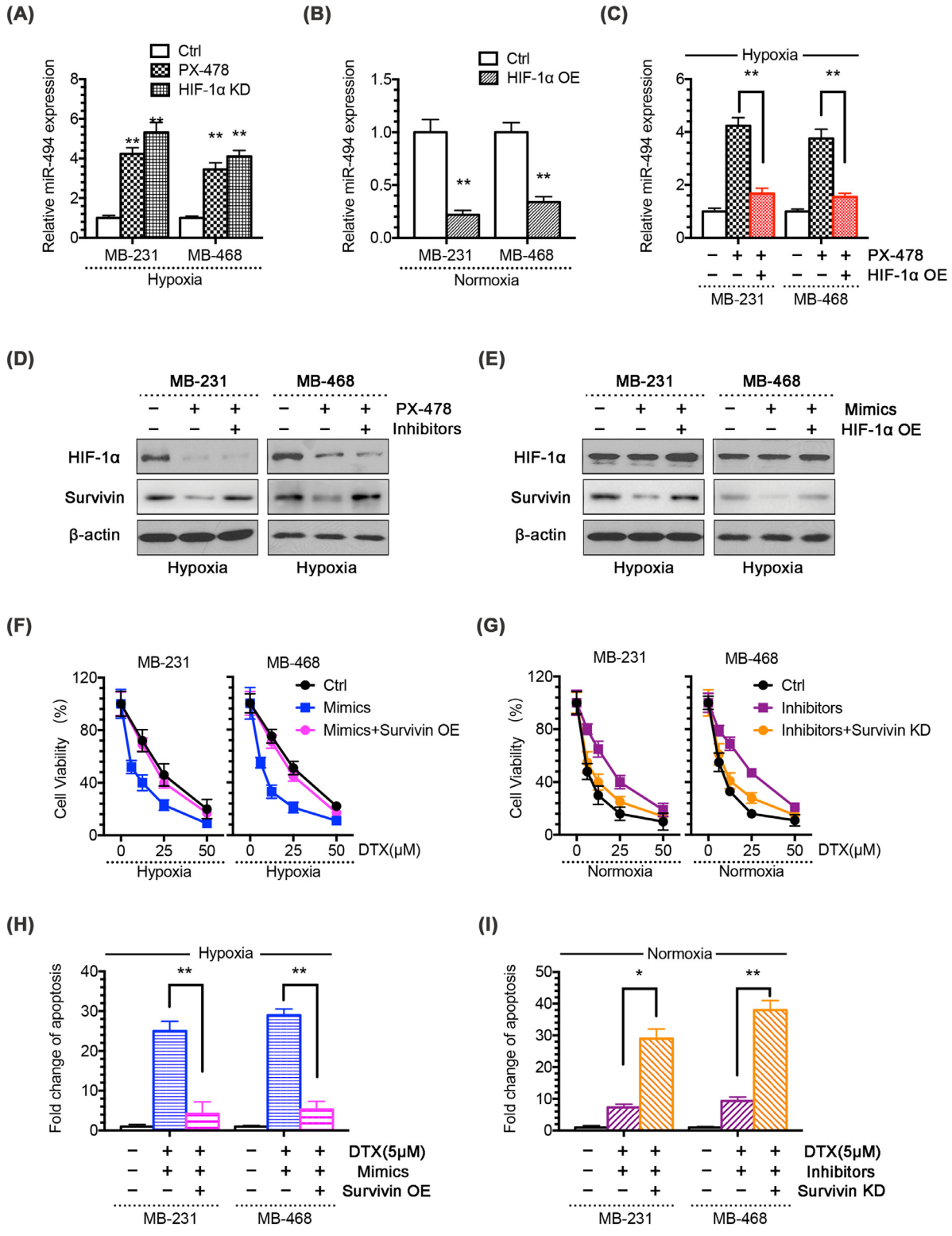

Fig. 4. HIF-1α/miR-494/Survivin signaling pathway may be responsible for hypoxia-mediated TNBC DTX-resistance. qPCR analysis of miR-494 expression (A) in hypoxic TNBC cells treated with HIF-1α inhibitor PX-478 or HIF-1α knockdown (KD) plasmid; (B) in normoxic TNBC cells treated with HIF-1α overexpression (OE) plasmid; (C) in hypoxic TNBC cells treated by PX-478 with or without HIF-1α OE plasmid. (D) Western blot analysis of the Survivin protein level from hypoxic TNBC cells treated with PX-478, with or without miR-494 inhibitor. (E) Western blot analysis of the Survivin protein level from hypoxic TNBC cells treated with miR-494 mimics, with or without HIF-1α OE plasmid. Overexpression of Survivn significantly (F) decreased the growth-inhibitory effect of DTX combined with miR-494 in hypoxic TNBC cells by CCK-8 assay; (H) and decreased hypoxic TNBC cells apoptosis level by DNA fragmentation assays; Inhibition of Survivin significantly (G) increased the growth-inhibitory effect of miR-494 inhibitor in normoxic TNBC cells to DTX by CCK-8 assay; (I) increased apoptosis level by DNA fragmentation assays. $^{*}P < 0.05$, $^{**}P < 0.01$. Each bar represents the mean ± SD of three independent experiments.

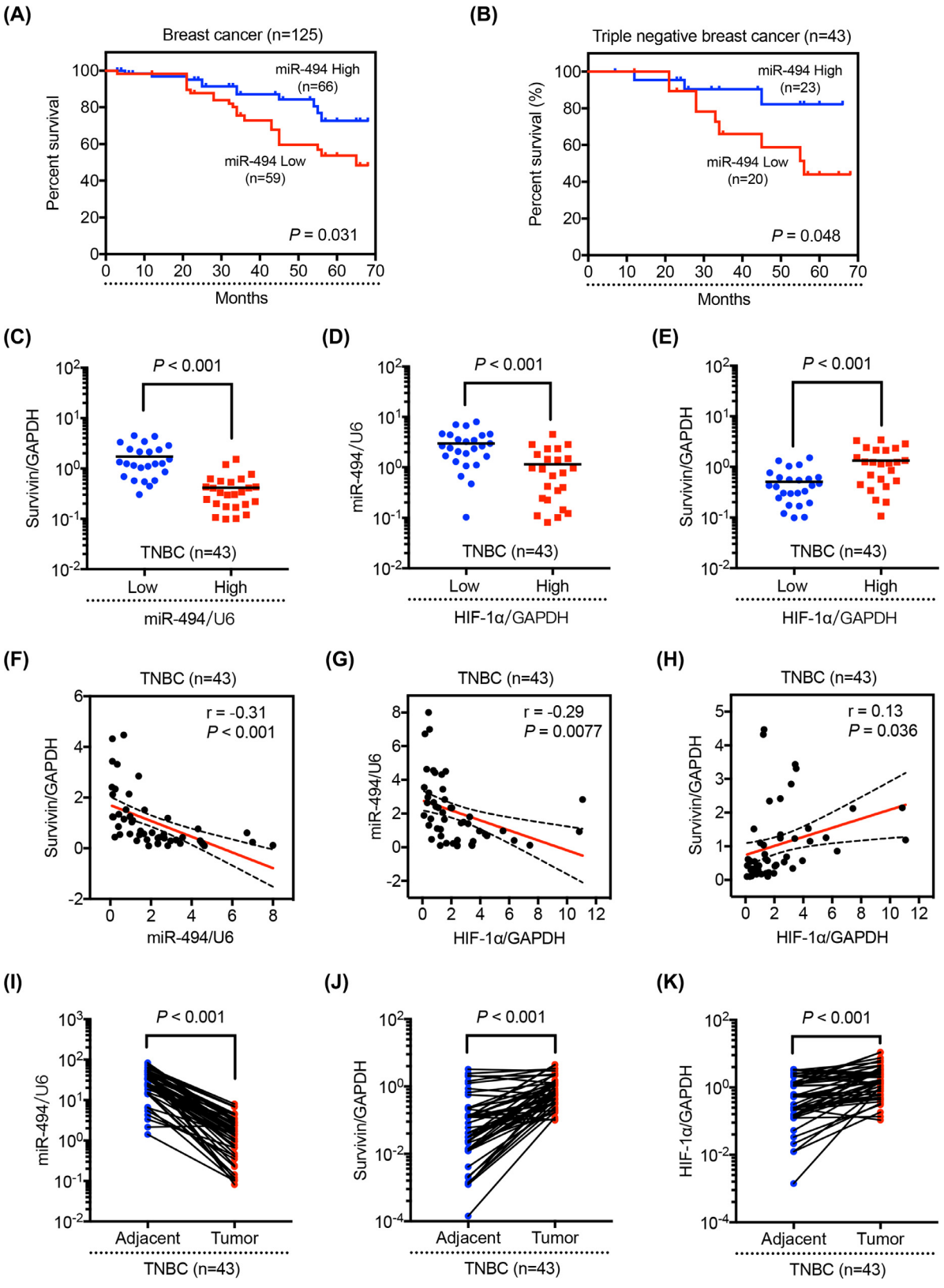

Fig. 5. Expression of HIF-1α/miR-494/Survivin signaling pathway in human TNBC specimens. Survival analysis based on the expression of miR-494 of selected BC specimens ($n = 125$, P=0.031), (B) or TNBC specimens ($n = 43$, $P = 0.048$). MiR-494 expression levels were inversely correlated with (C) Survivin and (D) HIF-1α in TNBC specimens, and (E) Survivin expression levels were positively correlated with HIF-1α in TNBC specimens. Expression levels of miR-494 and (F) Survivin and (G) HIF-1α were inversely correlated among all TNBC specimens ($n = 43$) as indicated by two-tailed Pearson's correlation analysis, respectively, and (H) expression levels of HIF-1α and Survivin were positively correlated among all TNBC specimens ($n = 43$). Relative expression levels of (I) miR-494, (J) Survivin and (K) HIF-1α were detected in adjacent and tumor tissues ($n = 43$) via qPCR. Results are representative of three experiments.

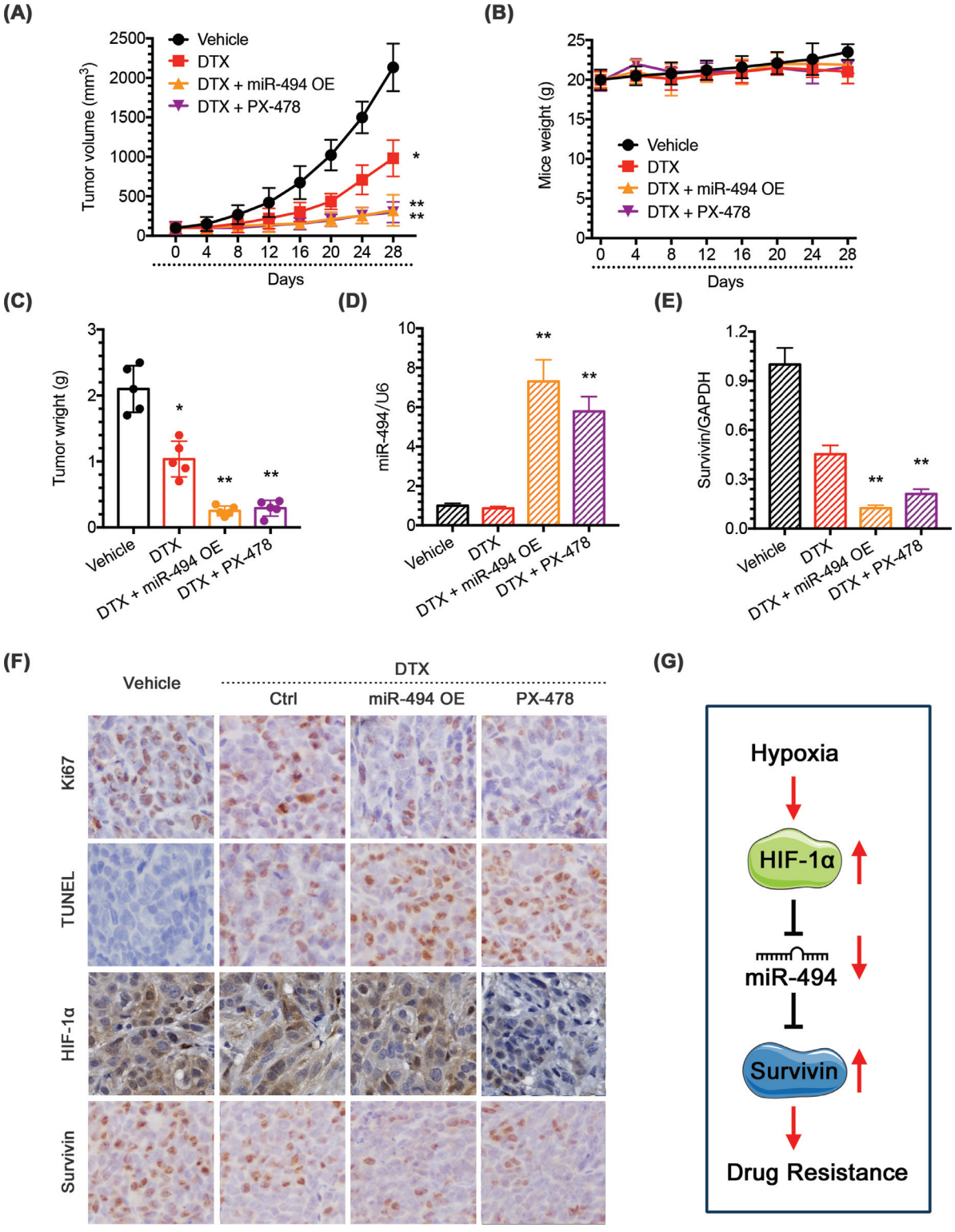

Fig. 6. Modulation of HIF-1α/miR-494/Survivin signaling pathway expression alters the sensitivity of TNBC cells to DTX in Xenograft mice model. Overexpression of miR-494 or HIF-1α inhibitor (PX-478) increased the effectiveness of DTX in the inhibition of tumor growth *in vivo*. (A) Xenograft tumor growth curves, (B) mice weight and (C) tumor weights. qPCR analysis of (D) miR-494 expression and (E) Survivin expression *in vivo*. Overexpression of miR-494 or HIF-1α inhibitor (PX-478) reduced (F) Immunohistochemical analysis of Ki67, TUNEL, HIF-1α and Survivin in tumors. (G) Schematic representation of HIF-1α/miR-494/Survivin signaling pathway expression alters the sensitivity of TNBC cells to DTX. $^{*}P < 0.05$, $^{**}P < 0.01$. Each bar represents the mean ± SD of three independent experiments.

the expression of the HIF-1α/miR-494/Survivin signaling pathway indeed altered the sensitivity of TNBC cells to DTX *in vivo*.

Discussion

Cytotoxic chemotherapeutics such as taxanes and anthracyclines are effective in patients with TNBC and significantly reduce the tumor burden [25]. Unfortunately, most TNBC cases relapse after standard chemotherapy, and metastatic lesions show resistance, limiting the effectiveness of treatment and the survival of patients with advanced TNBC [26]. The response of cancer to chemotherapy is regulated not only by the inherent sensitivity of cancer cells but also by the tumor microenvironment [27]. Here, we identified a hypoxia-triggered signaling pathway that involves HIF-1α-mediated miR-494 repression, which contributes to the drug resistance of TNBC. We believe this study is the first to illustrate the chemosensitization function of miR-494 in TNBC *in vivo*.

Hypoxia increases HIF-1α expression, which in turn modulates the expression of many genes that affect tumor growth, metastasis, and drug resistance to ensure that cancer cells survive under hypoxic conditions [27–29]. Therefore, HIF-1α represents a compelling treatment target for cancer [30]. Under hypoxic conditions, TNBC cells exhibited resistance to DTX. Recently, the HIF-1α-specific inhibitor PX-478 was identified to selectively inhibit hypoxia-induced HIF-1α mRNA expression and translation [31]. Previous studies have shown that hypoxia causes CRC to develop resistance to OXA, and the combination therapy of OXA and PX-478 reverses this resistance [32]. In HR−/HER2+ breast cancer, HIF1A was upregulated and selected as a treatment target. The combination of PX-478 with neratinib significantly inhibits tumor growth [33]. Here, PX-478 increased miR-494 expression. This result was confirmed in a xenograft model. We found that PX-478 effectively increased DTX sensitivity in TNBC cells by inhibiting HIF-1α/miR-494/Survivin signaling *in vivo*. Therefore, our research provides a basis for the use of the combination of PX-478 and DTX to treat TNBC.

MiRNAs improve the clinical management of patients with breast cancer and are easy-to-detect biomarkers that predict response to treatment. In fact, analyzing tissue levels or circulating miRNA levels in patients' serum or plasma may help identify patients who may respond and change treatment strategies for those who are already resistant [34]. To date, several miRNAs related to breast cancer resistance have been identified, such as miR-128 [35], miR145 [36] and miR-181 [37]. Furthermore, it is reported that miR-494 expression is inhibited in cardiomyocytes under hypoxia conditions [38]. In the present study, miR-494 expression was significantly reduced in hypoxic cell lines and TNBC tumor tissues. Moreover, reducing HIF-1α expression using the HIF-1α inhibitor PX-478 or HIF-1α KD plasmid increased miR-494 expression in cells cultured under hypoxic conditions. The expression of miR-494 altered the sensitivity of both hypoxic and normoxic TNBC cells to DTX. In a clinical study, miR-494 was expressed at low levels in TNBC. The total survival time of patients with high miR-494 expression was significantly longer. The expression of miR-494 negatively correlated with HIF-1α expression levels. Taken together, miR-494 plays a certain role in hypoxia-induced docetaxel resistance in TNBC. Tian et al. [39] found that miR-494 is a potential therapeutic target that suppresses drug resistance in acute myeloid leukemia cells. Combined with our study, docetaxel combined with miR-494 might be a strategy for treating TNBC, and miR-494 might represent a detectable biomarker to predict docetaxel resistance in TNBC.

Survivin, an apoptosis inhibitor protein, inhibits cell apoptosis and promotes cell division [40]. Survivin expression is related to development in normal tissues [41], and almost no expression is observed in most terminally differentiated tissues. The abnormally high expression of survivin in cancer cells makes it an ideal target for anticancer therapy. Survivin was the target of miR-494 in gastric cancer cells [42] and sporadic gastrointestinal stromal tumors [43]. We found that survivin expression was upregulated under hypoxic conditions. miR-494 targeted Survivin in hypoxia-mediated DTX resistance in TNBC cells. Similarly, we detected increased Survivin levels in the tumor tissues. We observed a negative correlation between the expression of Survivin and miR-494 and a positive correlation between the expression of Survivin and HIF-1α in TNBC specimens. In addition, survivin overexpression has been correlated with tumor prognosis and is considered a biomarker that is negatively correlated with the clinical outcome and drug resistance in patients with many cancers [44]. Our results show that the HIF-1α/miR-494/Survivin signaling pathway is active in primary human TNBC and that survivin potentially represents a promising therapeutic target in TNBC DTX resistance.

Conclusions

Taken together, our study reveals a novel role for miR-494 in DTX-induced TNBC chemoresistance under hypoxic conditions, providing a convincing preclinical theoretical basis for the design of innovative therapeutic strategies that will selectively target the HIF-1α/miR-494/Survivin axis to weaken the resistance and progression of TNBC.

Data Availability

The datasets used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study do not require ethical approval.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

This work was supported by grants from the High level key Specialist Doctors Under the Medical Teaching Research Collaborative Health Service System in Minhang District (No. 2020MZYS02); Project of Shanghai Municipal Health Commission (No. 20204Y0107); Fundamental Medical Project of Minhang Hospital of Fudan University Project Foundation (No. 2021MHJC05 and No. 2022MHBJ05); Minhang District medical specialty project (No. 2020MWFC03); Minhang District Science and Technology Committee project (No. 2021MHZ065).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100821.

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020;**70**:7–30.

[2] Yao H, He G, Yan S, Chen C, Song L, Rosol TJ, et al. Triple-negative breast cancer: is there a treatment on the horizon? *Oncotarget* 2017;**8**:1913–24.

[3] Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol* 2016;**13**:674–90.

[4] Marra A, Viale G, Curigliano G. Recent advances in triple negative breast cancer: the immunotherapy era. *BMC Med* 2019;**17**:90.

[5] Pandy JGP, Balolong-Garcia JC, Cruz-Ordinario MVB, Que FVF. Triple negative breast cancer and platinum-based systemic treatment: a meta-analysis and systematic review. *BMC Cancer* 2019;**19**:1065.

[6] Montor WR, Salas A, Melo FHM. Receptor tyrosine kinases and downstream pathways as druggable targets for cancer treatment: the current arsenal of inhibitors. *Mol Cancer* 2018;**17**:55.

[7] Qi F, Qin WX, Zang YS. Molecular mechanism of triple-negative breast cancer-associated BRCA1 and the identification of signaling pathways. *Oncol Lett* 2019;**17**:2905–14.

[8] Piret JP, Cosse JP, Ninane N, Raes M, Michiels C. Hypoxia protects HepG2 cells against etoposide-induced apoptosis via a HIF-1-independent pathway. *Exp Cell Res* 2006;**312**:2908–20.

[9] Xiong H, Nie X, Zou Y, Gong C, Li Y, Wu H, et al. Twist1 enhances hypoxia induced radioresistance in cervical cancer cells by promoting nuclear EGFR localization. *J Cancer* 2017;**8**:345–53.

[10] Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metast Rev* 2007;**26**:225–39.

[11] Liverani C, De Vita A, Minardi S, Kang Y, Mercatali L, Amadori D, et al. A biomimetic 3D model of hypoxia-driven cancer progression. *Sci Rep* 2019;**9**:12263.

[12] Chen X, Iliopoulos D, Zhang Q, Tang Q, Greenblatt MB, Hatziapostolou M, et al. XBP1 promotes triple-negative breast cancer by controlling the HIF1alpha pathway. *Nature* 2014;**508**:103–7.

[13] Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res* 2002;**62**:3387–94.

[14] Comerford KM, Cummins EP, Taylor CT. c-Jun NH_2-terminal kinase activation contributes to hypoxia-inducible factor 1alpha-dependent P-glycoprotein expression in hypoxia. *Cancer Res* 2004;**64**:9057–61.

[15] Zheng T, Wang J, Chen X, Liu L. Role of microRNA in anticancer drug resistance. *Int J Cancer* 2010;**126**:2–10.

[16] Gomes BC, Rueff J, Rodrigues AS. MicroRNAs and cancer drug resistance. *Methods Mol Biol* 2016;**1395**:137–62.

[17] Zhang Y, Guo L, Li Y, Feng GH, Teng F, Li W, et al. MicroRNA-494 promotes cancer progression and targets adenomatous polyposis coli in colorectal cancer. *Mol Cancer* 2018;**17**:1.

[18] Pollutri D, Patrizi C, Marinelli S, Giovannini C, Trombetta E, Giannone FA, et al. The epigenetically regulated miR-494 associates with stem-cell phenotype and induces sorafenib resistance in hepatocellular carcinoma. *Cell Death Dis* 2018;**9**:4.

[19] Li F, Aljahdali IAM, Zhang R, Nastiuk KL, Krolewski JJ, Ling X. Kidney cancer biomarkers and targets for therapeutics: survivin (BIRC5), XIAP, MCL-1, HIF1alpha, HIF2alpha, NRF2, MDM2, MDM4, p53, KRAS and AKT in renal cell carcinoma. *J Exp Clin Cancer Res* 2021;**40**:254.

[20] Yun S, Kim WK, Kwon Y, Jang M, Bauer S, Kim H. Survivin is a novel transcription regulator of KIT and is downregulated by miRNA-494 in gastrointestinal stromal tumors. *Int J Cancer* 2018;**142**:2080–93.

[21] Sun Y, Zhou Z, Yang S, Yang H. Modulating hypoxia inducible factor-1 by nanomaterials for effective cancer therapy. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2021;**14**:e1766.

[22] Barthel L, Hadamitzky M, Dammann P, Schedlowski M, Sure U, Thakur BK, et al. Glioma: molecular signature and crossroads with tumor microenvironment. *Cancer Metastasis Rev* 2021;**41**:53–75.

[23] Santos P, Almeida F. Role of exosomal miRNAs and the tumor microenvironment in drug resistance. *Cells* 2020;**9**(6):1450–67.

[24] Wang S, Li MY, Liu Y, Vlantis AC, Chan JY, Xue L, et al. The role of microRNA in cisplatin resistance or sensitivity. *Expert Opin Ther Targets* 2020;**24**: 885–897.

[25] Zelnak A. Overcoming taxane and anthracycline resistance. *Breast J* 2010;**16**:309–12.

[26] Andreopoulou E, Sparano JA. Chemotherapy in patients with anthracycline- and taxane-pretreated metastatic breast cancer: an overview. *Curr Breast Cancer Rep* 2013;**5**:42–50.

[27] Piret JP, Cosse JP, Ninane N, Raes M, Michiels C. Hypoxia protects HepG2 cells against etoposide-induced apoptosis via a HIF-1-independent pathway. *Exp Cell Res* 2006;**312**:2908–20.

[28] Liu Z, Wang Y, Dou C, Xu M, Sun L, Wang L, et al. Hypoxia-induced up-regulation of VASP promotes invasiveness and metastasis of hepatocellular carcinoma. *Theranostics* 2018;**8**:4649–63.

[29] Pawlus MR, Hu CJ. Enhanceosomes as integrators of hypoxia inducible factor (HIF) and other transcription factors in the hypoxic transcriptional response. *Cell Signal* 2013;**25**:1895–903.

[30] Adams JM, Difazio LT, Rolandelli RH, Lujan JJ, Hasko G, Csoka B, et al. HIF-1: a key mediator in hypoxia. *Acta Physiol Hung* 2009;**96**:19–28.

[31] Palayoor ST, Mitchell JB, Cerna D, Degraff W, John-Aryankalayil M, Coleman CN. PX-478, an inhibitor of hypoxia-inducible factor-1alpha, enhances radiosensitivity of prostate carcinoma cells. *Int J Cancer* 2008;**123**:2430–7.

[32] Xu K, Zhan Y, Yuan Z, Qiu Y, Wang H, Fan G, et al. Hypoxia induces drug resistance in colorectal cancer through the HIF-1α/miR-338-5p/IL-6 feedback loop. *Mol Ther J Am Soc Gene Ther* 2019;**27**:1810–24.

[33] Ryu JS, Sim SH, Park IH, Lee EG, Lee ES, Kim YH, et al. Integrative *in vivo* drug testing using gene expression signature and patient-derived xenografts from treatment-refractory HER2 positive and triple-negative subtypes of breast cancer. *Cancers* 2019;**11**:574.

[34] Di Cosimo S, Appierto V, Pizzamiglio S, Silvestri M, Baselga J, Piccart M, et al. Early modulation of circulating MicroRNAs levels in HER2-positive breast cancer patients treated with trastuzumab-based neoadjuvant therapy. *Int J Mol Sci* 2020;**21**.

[35] Zhu Y, Yu F, Jiao Y, Feng J, Tang W, Yao H, et al.. Reduced miR-128 in breast tumor-initiating cells induces chemotherapeutic resistance via Bmi-1 and ABCC5. Clinical cancer research: an official journal of the, 17. American Association for Cancer Research; 2011. p. 7105–15.

[36] Gao M, Miao L, Liu M, Li C, Yu C, Yan H, et al. miR-145 sensitizes breast cancer to doxorubicin by targeting multidrug resistance-associated protein-1. *Oncotarget* 2016;**7**:59714–26.

[37] Zhu Y, Wu J, Li S, Ma R, Cao H, Ji M, et al. The function role of miR-181a in chemosensitivity to adriamycin by targeting Bcl-2 in low-invasive breast cancer cells. *Cell Phys Biochem Int J Exp Cell Phys Biochem Pharm* 2013;**32**:1225–37.

[38] Ning S, Li Z, Ji Z, Fan D, Wang K, Wang Q, Hua L, Zhang J, et al. MicroRNA494 suppresses hypoxia/reoxygenation induced cardiomyocyte apoptosis and autophagy via the PI3K/AKT/mTOR signaling pathway by targeting SIRT1. *Mol Med Rep* 2020;**22**(6):5231–42.

[39] Tian C, Zheng G, Zhuang H, Li X, Hu D, Zhu L, et al. MicroRNA-494 activation suppresses bone marrow stromal cell-mediated drug resistance in acute myeloid leukemia cells. *J Cell Phys* 2017;**232**:1387–95.

[40] Altieri DC. Survivin - the inconvenient IAP. *Semin Cell Dev Biol* 2015;**39**:91–6.

[41] Garg H, Suri P, Gupta JC, Talwar GP, Dubey S. Survivin: a unique target for tumor therapy. *Cancer Cell Int* 2016;**16**:49.

[42] Xu S, Li D, Li T, Qiao L, Li K, Guo L, Liu Y. miR-494 sensitizes gastric cancer cells to TRAIL treatment through downregulation of survivin. *Cell Physiol Biochem* 2018;**51**(5):2212–23.

[43] Yun S, Kim WK, Kwon Y, Jang M, Bauer S, Kim H. Survivin is a novel transcription regulator of KIT and is downregulated by miRNA-494 in gastrointestinal stromal tumors. *Int J Cancer* 2018;**142**(10):2080–93 May 15.

[44] Rödel F, Sprenger T, Kaina B, Liersch T, Rödel C, Fulda S, et al. Survivin as a prognostic/predictive marker and molecular target in cancer therapy. *Curr Med Chem* 2012;**19**:3679–88.