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# Pharmaceutically targeting hypoxia in the breast cancer microenvironment: Mechanistic and translational approaches

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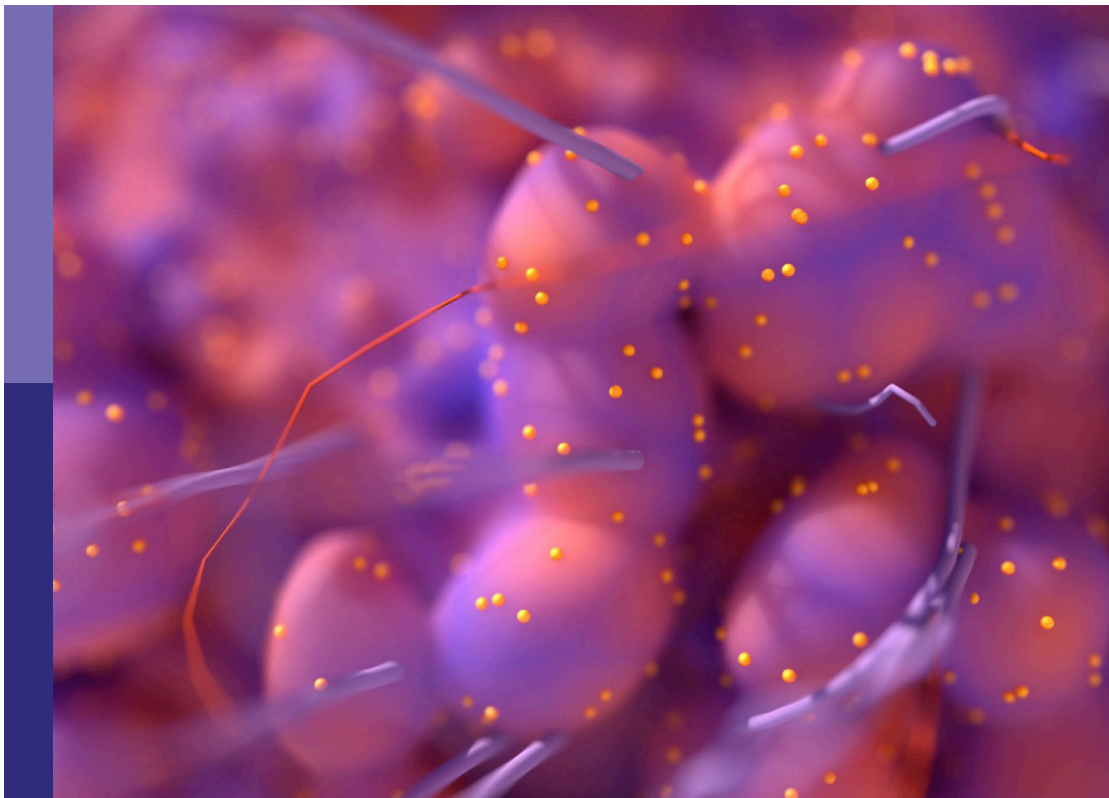

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# Pharmaceutically targeting hypoxia in the breast cancer microenvironment: Mechanistic and translational approaches

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# Editorial: Pharmaceutically targeting hypoxia in the breast cancer microenvironment: mechanistic and translational approaches

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KEYWORDS

**breast cancer, hypoxia, drug delivery, tumor microenvironment (TME), HIF-1α**

Editorial on the Research Topic

Pharmaceutically targeting hypoxia in the breast cancer microenvironment: mechanistic and translational approaches

Hypoxia and tumor microenvironment (TME) significantly influence breast cancer development, progression, immune response, angiogenesis, and metastasis. Advances in multi-omic techniques have significantly improved our understanding of the underlying mechanisms of hypoxia and the development of targeting strategies for treating breast cancer (1).

Mehraj et al. published the first research on this topic. Doxorubicin is effective against triple-negative breast cancer (TNBC), although the development of doxorubicin resistance is a significant challenge. They stated that some novel therapeutics can boost doxorubicin's efficacy while reducing its toxicity. Better TNBC therapy combinations may result from combining doxorubicin treatment with promising novel compounds or repurposed drugs. Additionally, the combined therapy will reduce the dosage and toxicity of doxorubicin. Adapalene, a third-generation retinoid, has shown promise in treating certain cancers. They examined the anti-cancer properties of adapalenein on TNBC cells, its combinatorial efficacy with doxorubicin, and the mechanism of action. Adapalene and doxorubicin synergistically reduce TNBC cell growth, colony formation, and migration. Adapalene and doxorubicin increased reactive oxygen species, causing Erk1/2 hyperphosphorylation and caspase-dependent cell death. Adapalene is a potential anticancer drug used alone or combined with current TNBC treatments (Mehraj et al.).

Continuing with drug remittance, Yong et al. discussed HIF-1α in the context of multidrug-resistant breast cancer. In their evaluation, *de novo* or acquired resistance remains a clinical challenge. Hypoxia is one mechanism of drug resistance. HIF-1α controls cell responsiveness to hypoxia. HIF-1α promotes tumor cell proliferation,

invasion, angiogenesis, anaerobic glycolysis, and multidrug resistance. Their study focused on drug-resistant breast cancer and HIF-1α-targeted treatment (Yong et al.).

Cheng et al. noted that fast proliferation and delayed angiogenesis cause intratumoral hypoxia in breast cancer. HIF, a transcription factor, mediates metabolic reprogramming, tumor angiogenesis, tumor cell proliferation and metastasis, gene instability, and other physiological and pathological processes in the hypoxic milieu. Hypoxia alters tumor cells' innate and acquired immunity to support tumor growth and suppresses immunological function. Thus, tumor microenvironment hypoxia offers a prospective target for breast cancer treatment resistance and poor efficacy. They also discuss the hypoxic mechanisms of breast cancer medication resistance and the latest HIF inhibitor-targeted medicines (Cheng et al.).

According to Singh et al., solid hypoxic tumor cells ferments glucose into lactate *via* aerobic glycolysis, which accumulates in the TME. Cancer cells fail to utilize lactate, so they release it into the TME, thereby increasing extracellular lactate and microenvironmental acidity. The cancer microenvironment also absorbs lactate under different pathophysiological circumstances. Lactate vanishes immediately in the cancer microenvironment, a mystery. Recent discoveries have illuminated the significance of lactic acidosis in cancer microenvironment. Lactate suppresses immunity and initiates angiogenesis and invasiveness in cancer cells *via* the *de novo* fatty acid synthesis pathway. In tumors that are normoxic, moderately hypoxic, and severely hypoxic, lactate reprograms the lipid biosynthesis pathway to create a metabolic symbiosis. In oxygen scarcity, highly hypoxic cancer cells cannot synthesize polyunsaturated fatty acids (PUFA) and release lactate into the TME. Lactate from the TME is taken up by the normoxic tumor cells and transformed back to PUFAs after a series of processes to be used by severely hypoxic cancer cells. Lactate plays a significant role in various biological processes, although its precise molecular mechanism remains elusive. This review examines the role of lactate in angiogenesis, invasiveness, immune suppression, and lipid synthesis reprogramming (Singh et al.).

Thomas et al.'s review takes a different method. Mutagenesis and cancer cell growth are known to occur in hypoxic microenvironments. The authors highlight the deliberate induction of localized hypoxia by the tumor cells to promote angiogenesis and production of growth factors that promote tumor growth and metastasis while promoting concurrent damage or mutagenesis of adjacent healthy tissue. Low oxygen levels reduce tumor-infiltrating lymphocyte (TIL) activation and recruitment, causing immunosuppression and immune surveillance. Hypoxic tumor endothelium suppresses the immune system in many ways, creating an immunosuppressive TME. Tumour endothelium anergy or non-responsiveness towards inflammatory signals precludes effector T cells from the TME. Tumour endothelium expresses endothelial-specific antigens and immunoinhibitory proteins such as Programmed death ligand 1, 2 (PDL-1, 2) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) to suppress T lymphocytes and promote regulatory T cells. The hypoxic microenvironment recruits immunosuppressive cells like the myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and Regulatory T cells (Tregs) in the TME. However, tumor blood vessels lack the organization of the normal tissue vasculature. Vascular normalization may improve tumor access in several tumor types and complement treatment. This paper briefly reviews immune-herbal therapy and immune-nutraceutical techniques that target tumor immunological evasion to boost immune response in the hypoxic TME. This study seeks to determine if these strategies can reduce breast cancer growth and prevent metastatic cell proliferation *via* new immunological switch points (Thomas et al.).

Luo et al. have demonstrated that breast cancer exhibits an upregulation and activation of HIFs due to persistent tumor hypoxia. Hypoxia-induced HIFs regulate glycolysis, angiogenesis, and metastasis, promoting breast cancer and poor prognosis by enhancing tumor invasion, metastasis, and drug resistance. Thus targeting the HIF pathway may enhance tumor targeting. This review analyzes the molecular mechanism associated with HIFs and the therapeutic strategies explicitly targeting HIFs in breast cancer. Drug delivery systems (DDSs) for targeting HIF are becoming more common due to the advances in nanotechnology and allied fields. They emphasized that HIF-targeted DDS may effectively target breast cancer, including DDS like liposomes, polymers, and metal- or carbon-based nanoparticles (Luo et al.).

In their study, Tang et al. provide an interesting take on the role of HIF1AN expression and breast cancer. HIF1AN reduces HIF-1α stability and transcription. Breast cancer patients with a decreased expression of HIF1AN exhibit reduced immunological infiltration and T-cell exhaustion and are correlated with an unfavorable prognosis. HIF1AN, clinical outcomes, and breast cancer immune involvement are not yet linked. Breast cancer cells expressed less HIF1AN than control specimens. HIF1AN expression serves as a predicted marker for breast cancer survival. In breast cancer, HIF1AN expression was linked to chemokines and immune cell infiltration, including neutrophils, macrophages, T helper cells, B cells, Tregs, monocytes, dendritic cells, and NK cells (Tang et al.).

Rastogi et al. evaluate NF-κB's complementing involvement in the tumor microenvironment. NF-B helps tumor formation and maintenance, while HIF-1α aids cell proliferation and angiogenic signaling. PHD-2 may be the oxygen-dependent regulator of HIF-1α and NF-κB. Without oxygen and 2-oxoglutarate, the proteasome degrades HIF-1α. This mechanism activates NF-κB, unlike PHD-2-mediated IKK hydroxylation, which deactivates it. In hypoxic cells, proteasomes protect HIF-1α, activating transcription factors related to metastasis and angiogenesis. Hypoxic cells accumulate lactate due to the Pasteur Effect. MCT-1 and MCT-4 cells transport lactate from the blood to non-hypoxic cancer cells in the lactate shuttle. Lactate, converted to pyruvate, fuels oxidative phosphorylation in non-hypoxic cancer cells. OXOPHOS cancer cells switch from glucose-to-lactate-facilitated oxidative phosphorylation. OXOPHOS cells had PHD-2. NF-κB activation is unexplained. Pyruvate, a 2-oxo-glutarate inhibitor, accumulates in non-hypoxic cancer cells. Pyruvate-mediated competitive reduction of 2-oxo-glutarate in non-hypoxic cancer cells inactivates PHD-2. NF-κB canonically activates. 2-oxoglutarate inhibits PHD-2 in non-hypoxic cancer cells. FIH blocks HIF-1α from transcription. Through pyruvate-mediated competitive inhibition of PHD-2, NF-B controls cancer cell growth and proliferation (Rastogi et al.).

Srivastava et al. reviewed the role of different hypoxia-targeting approaches in overcoming TNBC resistance. Based on biological and clinical data, TNBC-related mortality is high worldwide. Hypoxia modulates TNBC's glycolysis and angiogenesis pathways. Changes to these pathways promote cancer stem cell (CSC) enrichment and immune escape, which leads to tumor invasion, migration, and metastasis. Hypoxia also affects epigenetic plasticity and DNA damage response (DDR) to promote TNBC survival and progression. Hypoxia generates the low oxygen situation that alters HIF-1α signaling in the TME, allowing tumors to survive and resist treatment. Thus, suggesting the importance of target-based therapeutics to overcome TNBC's resistance. Chemotherapy, radiotherapy, immunotherapy, anti-angiogenic therapy, adjuvant therapy, photodynamic therapy, adoptive cell therapy, combination therapies, antibody-drug conjugates, and cancer vaccines may target HIF-1α. While improving therapy options, they also discussed the intrinsic mechanism and HIF-1α targeting concerns. The authors further discussed the future and major hypoxia-induced signaling-targeted TNBC resistance treatments (Srivastava et al.).

This compendium of papers will help readers understand how tumor hypoxia and the environment prevent breast cancer and offer therapeutic options.

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MS: Writing – original draft, Writing – review & editing. MP: Writing – original draft, Writing – review & editing. SR: Conceptualization, Investigation, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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# Adapalene and Doxorubicin Synergistically Promote Apoptosis of TNBC Cells by Hyperactivation of the ERK1/2 Pathway Through ROS Induction

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Doxorubicin is a commonly used chemotherapeutic agent to treat several malignancies, including aggressive tumors like triple-negative breast cancer. It has a limited therapeutic index owing to its extreme toxicity and the emergence of drug resistance. As a result, there is a pressing need to find innovative drugs that enhance the effectiveness of doxorubicin while minimizing its toxicity. The rationale of the present study is that combining emerging treatment agents or repurposed pharmaceuticals with doxorubicin might increase susceptibility to therapeutics and the subsequent establishment of improved pharmacological combinations for treating triple-negative breast cancer. Additionally, combined treatment will facilitate dosage reduction, reducing the toxicity associated with doxorubicin. Recently, the third-generation retinoid adapalene was reported as an effective anticancer agent in several malignancies. This study aimed to determine the anticancer activity of adapalene in TNBC cells and its effectiveness in combination with doxorubicin, and the mechanistic pathways in inhibiting tumorigenicity. Adapalene inhibits tumor cell growth and proliferation and acts synergistically with doxorubicin in inhibiting growth, colony formation, and migration of TNBC cells. Also, the combination of adapalene and doxorubicin enhanced the accumulation of reactive oxygen species triggering hyperphosphorylation of Erk1/2 and caspase-dependent apoptosis. Our results demonstrate that adapalene is a promising antitumor agent that may be used as a single agent or combined with present therapeutic regimens for TNBC treatment.

**Keywords: breast cancer, TNBC, doxorubicin, adapalene, Chou-Talalay, combination therapy, drug resistance**

## INTRODUCTION

Breast cancer (BC) is the most frequent cancer in women, with an estimated 2.2 million cases diagnosed in 2020 (1). Presently, BC is the main reason for global tumor-related deaths (2). Triple negative breast cancer (TNBC) is a highly invasive and aggressive BC subtype, accounts for 15% to 20% of all BCs, and lacks hormonal receptors and HER2 amplification (3). TNBC patients tend to show poor prognosis owing to its aggressive nature and limited therapies (4, 5). Additionally, TNBC has a high proclivity for rapid recurrence and the formation of therapy-resistant metastases, most often in the lungs, brain, lymph nodes, and bones, making treatment extremely challenging than other BC subtypes (3, 6). Current treatment strategies for patients with TNBC include tumor resection, radiation, and chemotherapy.

Anthracyclines and taxanes are the most utilized cytotoxic drugs, as are platinum-containing drugs. Unfortunately, several of these treatments have substantial side effects, and because tumor cells are innately adaptable, chemoresistance has developed as a problem (7–10). As a result, new effective treatments against TNBC are necessary. While doxorubicin (DOX) is an effective treatment for a range of tumors, its cumulative, dose-related adverse effects restrict its clinical use (11). Myelosuppression, cachexia, cardiotoxicity, and skeletal muscle damage are only a few adverse effects (12–14). As a result, patients who might benefit from ongoing therapy have to switch to a less effective medication. Moreover, due to the intrinsic genetic instability of malignant cells, which may quickly develop resistance, it is often ineffective to use single-drug therapy to treat cancer, particularly aggressive forms such as TNBC (15). Thus, modulation of DOX therapy is urgently needed, given the lack of available therapeutic regimens for TNBC.

Consequently, a combination of drugs with distinct modes of action is more efficient and may be able to effectively treat the disease (15). In addition, combination treatment demonstrates more significant or at least comparable effectiveness with concentrations lower of every single agent and reduces the chance of drug resistance by simultaneously targeting several signal transduction pathways essential to carcinogenesis (16). Therefore, combination therapy is viewed as a viable strategy that may impact the future development of more successful therapeutic regimens for TNBC.

Adapalene (ADA), a 3$^{rd}$ generation retinoid, is clinically used to treat acne vulgaris on a topical basis (17). In recent years, extensive research has analyzed the pharmacological properties of ADA and revealed its low toxicity and high stability in contrast to other retinoids (17). Studies report that ADA suppresses the growth of Hela, CC-531, and HepG2 cells and several malignancies both *in vitro* and *in vivo* (17–20). It has been reported that ADA treatment may increase ROS levels in cancer cells, which underlie the cancer cell killing activity of ADA (18). Based on the previous results, repurposing ADA for cancer treatment may be an effective therapeutic strategy.

In the present study, we investigated the anti-tumor potential of ADA in TNBC *in vitro* models and whether ADA can enhance the antitumor efficacy of doxorubicin in TNBC cells. The study's rationale was that combining emerging treatment agents or repurposed pharmaceuticals with doxorubicin might increase susceptibility and the subsequent establishment of improved pharmacological combinations for treating TNBC (15, 21). Additionally, combined treatment will facilitate dosage reduction, reducing the toxicity associated with doxorubicin (11). We found that ADA reduced tumor cell growth and proliferation and significantly enhanced doxorubicin-induced growth inhibition of these cells and that ERK1/2 activity is involved in their synergistic effect. Our results indicate that treating TNBC with a combination of ADA and DOX may be more successful than DOX alone.

## MATERIAL AND METHODS

### Cell Culture and Reagents

Cayman Chemical (Ann Arbor, Michigan 48108 USA) supplied doxorubicin (DOX) (Cat. No. 1160) and Adapalene (ADA) (Cat. No. 13655). Cell culture media DMEM (Dulbecco's Modified Eagle Medium), RPMI1640 (Roswell Park Memorial Institute Medium), & Fetal Bovine Serum (FBS) were procured from Gibco, Thermofisher Scientific USA. All the reagents used were of molecular grade or cell culture grade. TNBC cell lines (MDA-MB-231 and MDA-MB-468) and ER+ cell line MCF-7 were procured from the cell repository, National Centre for Cell Science (NCCS) Pune, India. Prof. Annapoorni Rangarajan (IISC, Bangalore, India) graciously provided the murine TNBC cell line 4T1. MDA-MB-231, MCF-7, and MDA-MB-468 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. The murine TNBC cell line, 4T1, was cultured in RPMI-1640 media with FBS (10%) and penicillin-streptomycin (1%). At 37°C, the BC cell lines were cultured in a humidified CO2 incubator.

### Single Drug Cytotoxicity Assay

A cell viability assay was performed to determine the anti-tumor effect of ADA and DOX and generate a dose-effect curve required for the Chou-Talalay model for designing binary drug combinations (22, 23). In 96-well plates, BC cells (MDA-MB-468, 4T1, MCF-7, and MDA-MB-231) were cultured at 3 x $10^3$ cells/well. Seven distinct concentrations of DOX, ADA, or drug vehicle (DMSO), each with four replicates, were given the next day. After 72 hrs of incubation, the drug solutions were replaced, and new media with 5mg/ml MTT reagent was added using the MTT assay kit (24) (Cat No V-13154, Thermofisher Scientific). The growth inhibition was evaluated using the equation below (eq 1):

$$\%\,\text{Inhibition} = \left[1 - \left(\frac{\text{OD treated Cells}}{\text{OD vehicle control Cells}}\right)\right] \times 100 \quad \text{Eq. 1}$$

Where "OD treated cells" defines the mean absorbance of cells incubated with therapeutics, "OD vehicle control" implies the mean absorbance of cells treated with a complete cell culture medium containing 0.1 percent DMSO.

### Constant-Ratio Cytotoxicity Test for Binary Drug Combinations

The single-drug cytotoxicity assay of DOX and ADA in BC models laid the groundwork for the combination study. Six

distinct equipotent DOX-ADA combinations were developed using the $IC_{50s}$ of two drugs and evaluated in four repetitions in different cell lines. As proposed by Chou and Talalay, the DOX-ADA combinations were designed using the equipotent constant-ratio design (or diagonal technique), as shown in **Table 1** (22, 25). Following a 72-hr treatment period, the cytotoxic effects of drugs as individual agents or in combination were evaluated. As indicated before in Eq.1, each treatment's percentage inhibition (effect) was calculated.

## Adoption of the Chou-Talalay Approach for Calculating the CI and DRI

The Combination Index (CI) value – a dimensionless variable used to identify and quantify the pharmacological interaction was computed by the CompuSyn software application, built on the combination Index Equation (Eq 2). When the CI value equals 1, an additive impact is obtained. Synergistic interaction is observed when the CI < 1 and antagonistic interaction when the CI > 1.

$$(CI)^2=\frac{(D)_1}{(Dy)_1}+\frac{(D)_2}{(Dy)_2}=\frac{(D)_1}{(Dm)_1\,[fa/(1-fa]^{1/m1}}+\frac{(D)_2}{(Dm)_2)\,[fa/(1-fa]^{1/m2}} \quad \text{Eq. 2}$$

Where (Dy)1 is the concentration of drug 1 that alone reduces cell viability by y percent, (Dy)2 is the drug 2 concentration that alone reduces cell viability by y percent, and (D)1 and (D)2 are the concentrations of drug 1 (D1) and drug 2 (D2) taken together that reduce cell viability by y percent. The values of (Dy)1 and (Dy)2 may simply be obtained by rearranging the Median-Effect Eq (2), as shown in Eq. 3

$$D = Dm\left[\frac{fa}{1-fa}\right]^{1/m} \quad \text{Eq. 3}$$

The dimensionless function, dose reduction index, or DRI, evaluates and indicates the magnitude by which the concentration of the individual agent in a drug combination may be lowered compared to the doses of each drug alone at a given fractional inhibition. It was generated automatically by the CompuSyn program for experimental drug combinations based on the DRI Equations (22), as shown in Eq. 4.

$$(DRI)_1=\frac{(Dy)_1}{D1},(DRI)_2=\frac{(Dy)_2}{D2},(DRI)_3=\frac{(Dy)_3}{D3}\ldots \quad \ldots .\text{etc}. \quad \text{Eq. 4}$$

DRI greater than 1 implies a desirable dosage decrease, DRI less than 1 suggests a detrimental dose reduction and DRI equal to 1 indicates zero dose reduction (22).

## Proliferation Assay

After assessing pharmacodynamic interactions, we examined the time-dependent effects of the synergistic drug combination DOX and ADA on cell proliferation. Cells were plated at 3 x $10^3$ cells/

**TABLE 1** | Experimental Design and data summary of the dose-effect curve and Chou-Talalay parameters of doxorubicin and adapalene drug combinations against breast cancer cell lines after 72 hrs treatment period.

| Cell Line | Doxorubicin(DOX) | Adapalene(ADA) | Fraction Affected(Fa) | Parameters | | | |
|---|---|---|---|---|---|---|---|
| | | | | m | Dm | r | CI |
| MDA-MB-231 | 0.1 * IC50 | 0.1 * IC50 | 0.16890 | 1.1 | 0.30 | 0.98 | 0.74914 |
| | 0.25 * IC50 | 0.25 * IC50 | 0.30804 | | | | 0.93978 |
| | 0.5 * IC50 | 0.5 * IC50 | 0.56324 | | | | 0.73811 |
| | 0.75 * IC50 | 0.75 * IC50 | 0.66716 | | | | 0.75161 |
| | **IC50 (0.28 µM)** | **IC50 (21.2µM)** | 0.77083 | | | | 0.63616 |
| | 1.25 * IC50 | 1.25 * IC50 | 0.79985 | | | | 0.68357 |
| MCF-7 | 0.1 * IC50 | 0.1 * IC50 | 0.27920 | 0.85 | 0.15 | 0.98 | 0.48507 |
| | 0.25 * IC50 | 0.25 * IC50 | 0.37246 | | | | 0.78334 |
| | 0.5 * IC50 | 0.5 * IC50 | 0.54634 | | | | 0.76621 |
| | 0.75 * IC50 | 0.75 * IC50 | 0.63769 | | | | 0.7869 |
| | **IC50 (0.14 µM)** | **IC50 (25.4 µM)** | 0.68579 | | | | 0.84762 |
| | 1.25 * IC50 | 1.25 * IC50 | 0.81200 | | | | 0.54222 |
| MDA-MB-468 | 0.1 * IC50 | 0.1 * IC50 | 0.22899 | 0.74 | 0.15 | 0.98 | 0.68975 |
| | 0.25 * IC50 | 0.25 * IC50 | 0.27907 | | | | 1.27003 |
| | 0.5 * IC50 | 0.5 * IC50 | 0.56781 | | | | 0.65398 |
| | 0.75 * IC50 | 0.75 * IC50 | 0.68200 | | | | 0.58359 |
| | **IC50 (0.13 µM)** | **IC50 (18.7 µM)** | 0.75925 | | | | 0.52201 |
| | 1.25 * IC50 | 1.25 * IC50 | 0.84601 | | | | 0.37243 |
| 4T1 | 0.1 * IC50 | 0.1 * IC50 | 0.25857 | 0.84 | 0.11 | 0.96 | 0.5536 |
| | 0.25 * IC50 | 0.25 * IC50 | 0.35729 | | | | 0.8538 |
| | 0.5 * IC50 | 0.5 * IC50 | 0.58695 | | | | 0.65922 |
| | 0.75 * IC50 | 0.75 * IC50 | 0.69310 | | | | 0.62439 |
| | **IC50 (0.11 µM)** | **IC50 (13.4µM)** | 0.70976 | | | | 0.76983 |
| | 1.25 * IC50 | 1.25 * IC50 | 0.82156 | | | | 0.51982 |

*m - Median; Dm - IC50; r- linear correlation coefficient CI - Combinational Index.*

well in a 96-well plate and treated with ADA or DOX alone or combined at a concentration below the $IC_{50}$. The proliferation of cells was determined after 24–72 hrs of incubation, using the Vybrant Proliferation Kit (Cat no. V-13154, Thermo Fisher Scientific USA).

## Colony Formation Assay

The effect of ADA, DOX, and their combined impact on the colony formation of cells was analyzed to assess the synergistic interactions further. Cells were plated at 1000–1500 cells per well in six-well plates (26, 27). After 48 hrs, fresh media was added and supplemented with therapeutics. The assay was performed for 14 to 18 days. The medium with therapeutics was replenished every three days, and colonies were observed in the wells using an inverted microscope. Once substantial colonies were formed, they were fixed with 3.7% paraformaldehyde (in PBS), and crystal violet (0.05%) was used for staining. Images of the plates were taken, and colonies were counted using the ImageJ application. The experiment was repeated three times for each cell type and treatment combination.

## Wound Healing Assay

Next, we investigated the individual and combined effect of DOX, and ADA, on the migration of the highly invasive TNBC cell lines MDA-MB-231 and 4T1 using the wound healing assay kit (Cat. no. CBA-120, Cell Biolabs, Inc., USA). The assay was performed in a 24-well plate, with cells seeded at 70% confluency and allowed to attach overnight with implanted scratch inserts. The scratch inserts were gently removed after 24 hrs, and the cells were washed with PBS. Fresh media with therapeutics was added, and cell migration was assessed after 48 hrs of treatment. Cells were fixed in 3.7% paraformaldehyde and stained with Giemsa stain. The cells were imaged, and the movement of cells into the wound site was examined and quantified using ImageJ software (28).

## Mammosphere Formation Assay

MDA-MB-231 cells as a single-cell suspension were seeded (1 x $10^4$ cells/well) onto ultralow attachment 6-well plates in 2ml DMEM/F12 (Gibco, 11320033) supplemented with 1 x B27 supplement (Invitrogen, 17504044) and SingleQuot™ (Lonza, CC-4136) (Gibco, 11320033) (Corning, 3471) (29). The next day, cells were treated with DOX, ADA alone, or in combination and cultured for five to ten days, with the medium being added every three days. The spheres were imaged using a phase-contrast inverted microscope (Nikon).

## Measurement of Reactive Oxygen Species

Next, we analyzed the accumulation of ROS upon treatment with therapeutics. MDA-MB-231 cells were seeded in 12-well plates and treated with ADA, DOX, or both for 24 hrs. Following staining with 10 μM DCFH-DA (Sigma) for 30 minutes in the dark, the cells were imaged using FLoid™ Cell Imaging Station (Thermo Fisher Scientific). Also, following staining, cells were collected and the fluorescence intensity was determined using an Agilent Fluorescence Spectrophotometer (30).

## Rhodamine-123 Staining Assay

Rhodamine 123 (Rh 123) staining was used to evaluate the mitochondrial membrane potential. As mitochondria transition from a polarized to a depolarized state during apoptosis, dye leakage occurs, leading to a decrease in the fluorescence intensity of Rh 123. MDA-MB-231 cells were seeded onto 24 well plates and treated with DOX, ADA, or combination of both for 24 hrs. Cells were stained for 15 minutes at 37 ℃ in the dark with 10 μM Rh 123 and washed thrice with 1x PBS and imaged using FLoid™ Cell Imaging Station. Also, following staining, cells were collected in PBS and fluorescence intensity was determined using Agilent Fluorescence Spectrophotometer (31). In some tests, cells were pretreated for 2 hrs with 5 mM N-acetyl cysteine (NAC) before exposure to the drugs.

## Western Blot Analysis

MDA-MB-231 cells were seeded in 6 cm dishes and treated with ADA, DOX, or both for 24 hrs. Following drug treatment, the cells were lysed with NP40 lysis buffer (Invitrogen, Thermo Fisher Scientific), supplemented with Halt™ Protease Inhibitor Cocktail using established protocols (32). Next, protein concentrations were measured using a BCA assay kit (PierceTM BCA Protein Assay Kit, Thermo Scientific Cat. No. 23227). Electrophoresis on SDS-polyacrylamide gels and electroblotting onto polyvinylidene difluoride membranes were used to separate the protein lysate. For 1.5 hrs at room temperature, 5% BSA was utilized for blocking. Specific primary antibodies against p-Erk1/2 (CST, Cat No. 4370, 1:2000), t-Erk1/2 (CST, Cat No. 4695 1:1000), PARP (CST, Cat No. 9542 1:1000), c-PARP (CST, Cat No. 5625, 1:1000), caspase-3 (CST, Cat No. 14220 1:1000), c-caspase-3 (CST, Cat No. 9664, 1:1000), caspase-9 (CST, Cat No. 9508, 1:1000), and c-caspase-9 (CST, Cat No. 52873, 1:1000) were used to probe protein bands. The binding of the primary antibody was detected using a secondary antibody coupled to horseradish peroxidase and visualized using an ECL kit (Bio-Rad, Hercules, CA). The immunoreactive protein bands were examined and normalized using GAPDH (CST, Cat No. 2118, 1:1000) as the loading control using ImageJ software.

## Annexin V Assay

Cells were grown in 12-well culture plates and treated with ADA, DOX, or both for 24 and 48 hrs. Next, floating and adherent cells were harvested and washed twice with ice-cold PBS. The washed cell samples were resuspended in 500 μl binding buffer containing 3 μl Annexin-V for 10 min and 2 μl 7-AAD for 15 min in the dark and, subsequently, evaluated for apoptosis (33, 34). Flow cytometry was performed at the Department of Biotechnology, National Institute of Technology, Rourkela Odisha, India, on a BD Accuri™ C6 Flow Cytometer. Apoptotic events were expressed as the percent of sub-G1 cells or the percent of apoptotic cells (combining early apoptotic Annexin V+/7-AAD − and late apoptotic Annexin V+/7-AAD+ cells).

## Cell Cycle Analysis

MDA-MB-231 cells were seeded at 50% confluency in 12-well plates and allowed to adhere overnight and serum-starved for cell cycle synchronization. Next, the cells were treated with DOX, ADA, or both for 24 and 48 hrs. Cells were trypsinized and fixed

in 75% ethanol following treatment. After washing the cells, PI (0.5 mg/ml) and RNase A (10 mg/ml) was used to stain and assess the effect on the cell cycle. Prior to flow cytometry, cells were filtered using a 70 μm cell strainer. Flow cytometry was performed at the Department of Biotechnology, National Institute of Technology, Rourkela Odisha, India, on a BD Accuri ™ C6 Flow Cytometer (35).

## Statistics

$IC_{50}$ values of compounds were calculated using non-linear regression analysis in GraphPad Prism. The statistical significance was analyzed using the one-way or two-way ANOVA in GraphPad Prism V 8.43, followed by Tukey multiple comparisons test. $P < 0.05$ was considered significant.

# RESULTS

## Single Drug Cytotoxicity Assay

MTT assay was carried out to evaluate the cytotoxicity of DOX and ADA alone against BC cell lines, and Graph-Pad prism v8 was used to produce dose-effect curves and obtain $IC_{50}$ values for DOX and ADA (**Figures 1A, B**). DOX and ADA were both cytotoxic to all breast cell lines dose-dependently. The $IC_{50}$ of DOX in MDA-MB-231, MCF-7, MDA-MB-468, and 4T1 was 0.28 μM, 0.14 μM, 0.13 μM and 0.11 μM respectively. DOX demonstrated high cytotoxicity in TNBC murine cell line 4T1. ADA showed an $IC_{50}$ of 21.18 μM, 25.36 μM, 18.75 μM, and 13.43 μM in MDA-MB-231, MCF-7, MDA-MB-468, and 4T1, respectively. Based on the $IC_{50}$ values, we designed an experimental setup for combination therapeutic evaluation.

## Cytotoxicity of Binary Drug Combination

The single-drug cytotoxicity assay fulfilled the Chou-Talalay method's criteria for commencing the *in vitro* pharmacodynamic drug interaction evaluation. We designed a constant-ratio combination approach or diagonal design. Cell viability was evaluated after 72 hrs of treatment (**Figures 1C–F**) The combination of DOX and ADA showed an enhanced reduction in cell viability of BC cells at very low doses, demonstrating positive drug-drug interactions of DOX and ADA. CompuSyn software was further utilized to calculate and quantify the CI, DRI values, and dose-inhibition curve parameters (**Table 1**). For MCF-7, MDA-MB-468, and 4T1, a flat sigmoidal ($m < 1$) curve was observed with an r-value (linear correlation coefficient) of approximately 0.97. MDA-MB-231 cells had a sigmoidal curve ($m > 1$) with approx. 0.99 for r. Also, the CompuSyn-calculated CI values for experimental points could achieve synergistic interactions as demonstrated with the CI less than 1 at precise combinations (**Table 1** and **Figure 2A**). The median-effect plots of drug combinations are shown in (**Figure 2C**).

## CompuSyn Software Simulation

A simulation algorithm was constructed using the median effect and combination index equations and the automation features of the CompuSyn software to simulate the estimated CI and DRI values at different fa levels. The simulated CI at different affected fraction levels was significantly synergistic, further validating *in vitro* results. The CompuSyn program also generated the Fa-Log CI plot, Fa-DRI plot, and isobolograms for each drug combination (**Supplementary Material**: CompuSyn reports). The simulated CI and DRI values at 50%, 75%, 90%, and 95% fraction affected are shown in (**Table 2**). Polygonograms at 50% fraction impacted levels were created to visually compare the kind and magnitude of drug interactions (**Figure 2B**). The solid line denotes synergistic interaction, the dashed line represents antagonistic interaction, and the thickness of the line indicates the degree of synergism or antagonism. Based on the simulated CI and DRI, it was further validated that all tested combinations exhibited synergistic interactions of varying magnitudes of inhibition, indicating that ADA acts in a synergetic manner with DOX.

## ADA Inhibits Proliferation and Enhances Sensitivity to DOX in TNBC Cells

We further evaluated the synergetic drug combination of DOX and ADA in a time-dependent manner. We proceeded with a single synergistic drug combination below individual $IC_{50}$ among several drug combinations designed earlier. The cell viability was analyzed at 24, 48, and 72 hrs using the Vybrant cell proliferation kit (Invitrogen, Thermofisher, USA) following the manufacturer's protocol. Combinatorial treatment significantly reduced cell proliferation compared to single-agent treatment (**Figures 3A–D**). The results demonstrate that DOX and ADA in combination enhance the anti-proliferative effect of each other synergistically. Moreover, the sensitivity of TNBC cells towards DOX significantly increased upon co-treatment with ADA. The trend was seen in all three time periods and all the four cell lines of BC.

## DOX and ADA Combination Disrupts Colony Formation and Migration of TNBC Cells

Experiments with colony formation in BC cell lines were utilized to validate further the anti-tumor activity and synergistic interactions of ADA with DOX. While treatment with DOX and ADA alone resulted in a decrease in colony formation, combined treatment with DOX and ADA resulted in a considerable reduction in colony formation compared to individual drug treatments. Further study found that the number of colonies in each treated cell line was equivalent when treated alone; however, the number of colonies was significantly reduced when treated in combination. The study also demonstrated that ADA as a single agent reduces tumor cell growth, inhibiting the colony formation of breast tumor cells (**Figures 4A–D**).

Cancer cells must infiltrate the ECM and undergo the multistep phenomenon of metastasis to colonize distant organs. As a result, blocking cell migration is a promising approach to prevent metastasis. This study aimed to determine

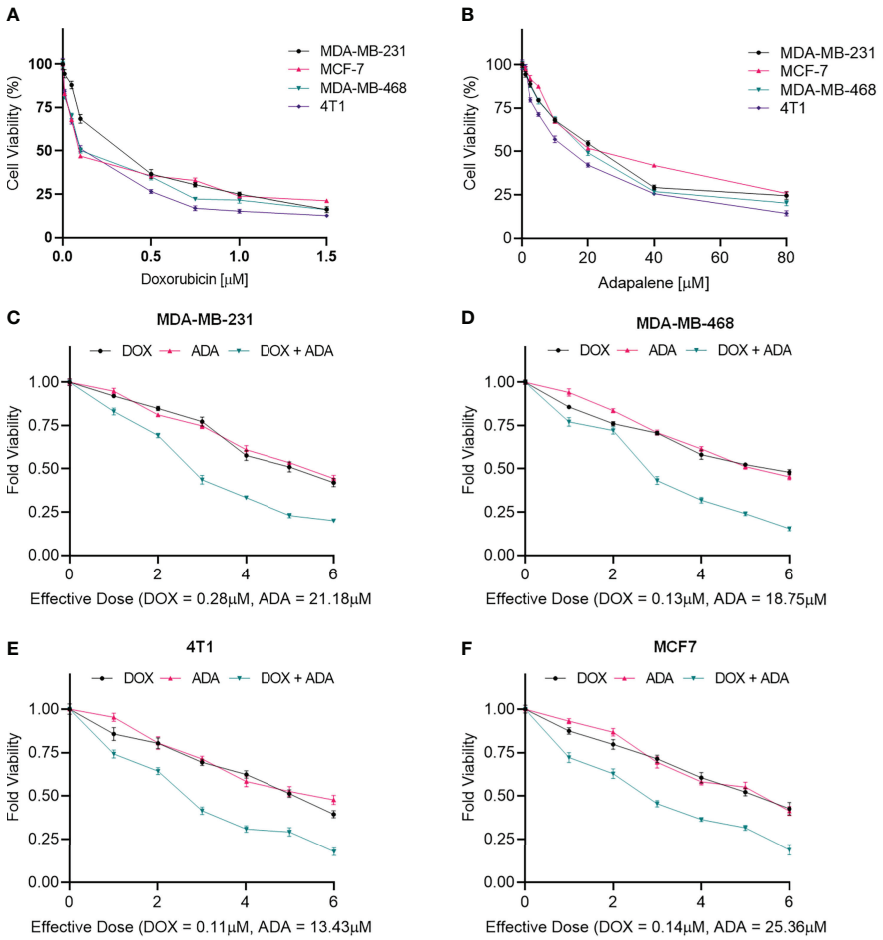


**FIGURE 1** | Doxorubicin and adapalene inhibited the growth of BC cells. Cell viability assay of BC cells treated with **(A)** Doxorubicin and **(B)** Adapalene. DOX and ADA both inhibited tumor cell growth in a dose-dependent manner. GraphPad prism was used for the calculations of the $IC_{50}$ values. Treatment with combination of DOX and ADA showed an enhanced reduction in cell viability of **(C)** MDA-MB-231, **(D)** MDA-MB-468, **(E)** 4T1 and, **(F)** MCF-7 cells. When ADA and DOX were used together, the cell viability was significantly reduced, demonstrating that the drugs had advantageous pharmacodynamic interactions with one another.

the effect of the combination of DOX and ADA on tumor cell motility. CytoSelect™ 24-Well Wound Healing Experiment Kit was used to perform the assay in 24 well plates. MDA-MB-231 and 4T1 cells were treated for 48 hrs with DOX or ADA alone or in combination, and migration of cells was assessed using ImageJ software. The combination of DOX and ADA significantly reduced migration compared to control cells or cells treated with DOX or ADA alone (**Figures 4E, F**).

Mammosphere assays are widely used *in vitro* to identify prospective cancer-initiating stem cells that can propagate clonally to form spheres in free-floating conditions (36). We evaluated the effect of DOX and ADA on spheroid formation. The combination of DOX and ADA significantly repressed the anchorage-independent growth of MDA-MB-231 cells and suppressed mammosphere formation, as shown in (**Figure 5A**). These results further support that combined treatment with DOX and ADA has significant tumor-reducing activity in TNBC.

## Combined Treatment With Adapalene and Doxorubicin Enhanced ROS Production and Impaired Mitochondrial Function

Next, we sought to elucidate the mechanisms driving the synergistic action of ADA and DOX in BC cells. Previously, it was suggested that most anticancer drugs act by modulating oxidative stress and regulating apoptosis (37). We determined the intracellular ROS levels using DCF-DA following treatment with DOX, ADA, or both. The results indicated that ADA elevated ROS levels in MDA-MB-231 cells, further intensified when DOX was added (**Figures 5B–D**). Additionally, we observed that DOX has a minimal influence at the concentration used in our study on ROS levels compared with ADA but dramatically augments ROS levels when combined with ADA.

ROS production is associated with disrupting the mitochondrial membrane potential (MMP), a critical step in initiating apoptosis, which can be detected using the Rh 123

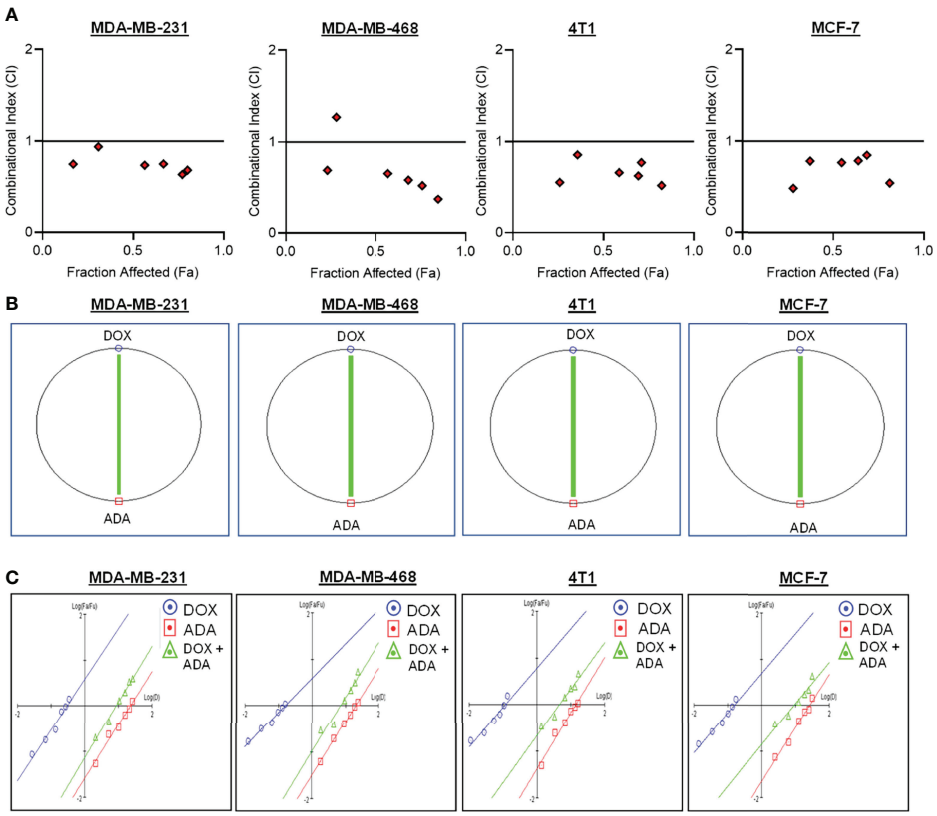


**FIGURE 2** | Doxorubicin and adapalene show synergistic pharmacodynamic interactions in BC models. **(A)** Combination Index (CI) plots of MDA-MB-231, MDA-MB-468, 4T1 and MCF-7 cells. The CI plots showed significant synergism between ADA and DOX in TNBC and ER+ MCF-7 cells. **(B)** Polygonograms of MDA-MB-231, MDA-MB-468, 4T1 and MCF-7 cells. **(C)** Median Plots of MDA-MB-231, MDA-MB-468, 4T1 and MCF-7 cells.

**TABLE 2** | Summary of CompuSyn simulated CI and DRI values for Doxorubicin and Adapalene combination in breast cancer cell lines at 50%, 75%, 90%, and 95% growth inhibition.

| Cell line | Drug CombinationDOX (D) + ADA (A) | CI Values at Inhibition of | | | | DRI Values at Inhibition of | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 50% | 75% | 90% | 95% | 50% | 75% | 90% | 95% |
| MDA-MB-231 | D + A | 0.75 | 0.70 | 0.65 | 0.62 | D = 2.64<br>A = 2.66 | D= 2.77<br>A= 2.80 | D= 3.22<br>A= 2.89 | D= 3.44<br>A= 3.03 |
| MDA-MB-468 | D + A | 0.65 | 0.52 | 0.44 | 0.40 | D= 3.26<br>A= 2.85 | D= 5.67<br>A= 2.90 | D= 9.87<br>A= 2.95 | D= 14.38<br>A= 2.98 |
| 4T1 | D + A | 0.64 | 0.65 | 0.67 | 0.71 | D=3.13<br>A=3.04 | D= 3.86<br>A=2.54 | D= 4.76<br>A= 2.13 | D= 5.49<br>A= 1.88 |
| MCF-7 | D + A | 0.66 | 0.75 | 0.8 | 1.0 | D= 3.13<br>A= 2.90 | D= 3.31<br>A= 2.19 | D= 3.5<br>A= 1.65 | D= 3.63<br>A= 1.37 |

staining. Compared to untreated controls, the cells treated with ADA, DOX, or both exhibited low fluorescence intensities for Rh 123. The MMP was lowest for the cells treated with the combination of DOX and ADA (**Figures 5E–G**). Additionally, membrane disruption was rescued by pre-treatment with NAC (5mM) for 2 hrs prior to DOX and ADA co-treatment (**Figures 5H, I**). These findings imply that oxidative damage, which disrupts the mitochondrial membrane potential, may contribute significantly to the increased lethality observed in the combination of ADA and DOX treatment of MDA-MB-231 cells.

## Hyperactivation of Erk1/2 Upon Treatment With DOX and ADA Triggers Intrinsic Apoptosis

Intracellular ROS production or oxidative stress generated by various anticancer therapies is well known to play a vital role in induced apoptosis *via* signalling cascade regulation (38). We

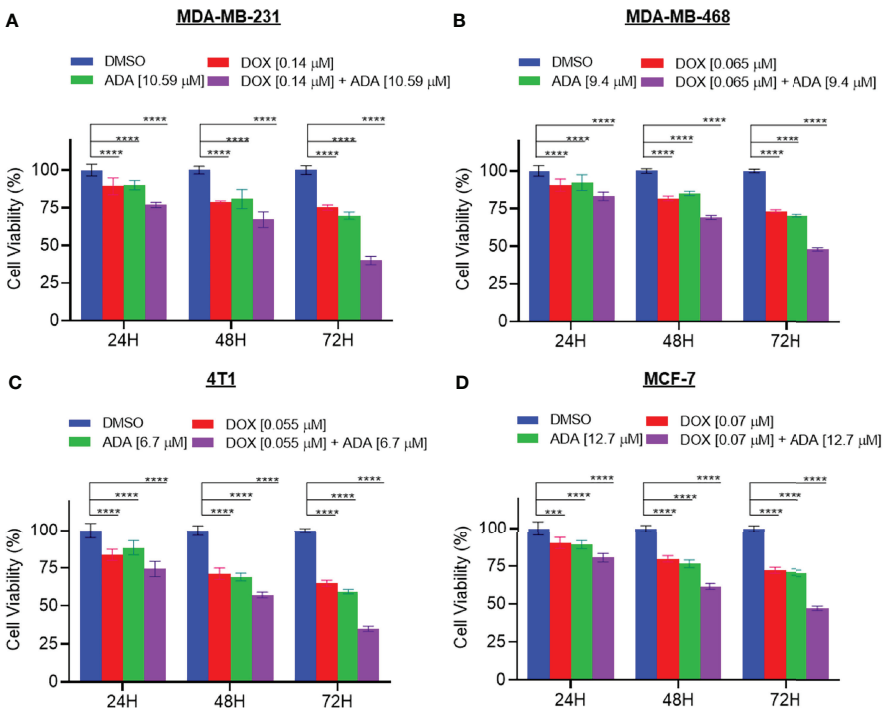


**FIGURE 3** | The combination of doxorubicin and adapalene synergistically reduces tumor cell proliferation. The combination of DOX and ADA inhibited proliferation of **(A)** MDA-MB-231, **(B)** MDA-MB-468, **(C)** 4T1 and, **(D)** MCF-7 in a synergistic manner. Data are mean ± SD. p-values were determined by two-way ANOVA followed by Tukey's multiple comparisons test (***$p < 0.001$; ****$p < 0.0001$). Significant reduction in cell viability was observed when treated in a time-dependent manner with combined treatment of DOX and ADA showing maximal effect. Data are representative of at least three independent experiments.

sought to investigate the effect of co-treatment of DOX and ADA on MAPK signalling. Combined treatment with ADA and DOX promoted hyperphosphorylation of Erk1/2 (**Figure 5J–K**). Notably, we showed that NAC (free radical scavenger) reduced ROS-driven phosphorylation of Erk1/2, supporting the involvement of stress induced by ADA in driving Erk1/2 phosphorylation (**Figure 5L–M**). These findings imply that ROS production is critical for the anti-tumor activity of ADA and its synergistic action with DOX.

Previously, it was shown that ERK1/2 phosphorylation is required for oxidative stress-induced apoptosis (38). Additionally, once apoptosis drivers are active due to mitochondrial membrane potential depletion, ERK1/2 may initiate caspase-mediated apoptosis. We sought to determine the expression of caspase 3, cleaved caspase 3, caspase 9, cleaved caspase 9, PARP, and cleaved PARP following treatment with DOX, ADA, or both. Combined treatment with ADA and DOX resulted in enhanced cleaved caspase 9, cleaved PARP, and cleaved caspase 3 (**Figures 6D–J**).

Additionally, we used Annexin-V and 7-AAD staining to determine the apoptosis-inducing capacity of ADA, DOX, or their combination. The flow cytometry study demonstrated that ADA promotes apoptosis in tumor cells and increases apoptosis upon combination therapy (**Figures 6A–C**). DOX also induced apoptosis, although the degree of apoptosis was much more significant in combination therapy than in single-agent treatment. Also, the necrotic cell population was high in cells treated with DOX for 48 hrs, while combination therapy reduced the necrotic cell population and enhanced apoptotic cells.

## Treatment With DOX and ADA Inhibits Cell Cycle Progression

Cells self-replicate *via* a process called the cell cycle. Since cell cycle arrest inhibits cancer cell proliferation, it may represent a critical method for cancer treatment. To evaluate whether cell cycle arrest contributes to the synergistic effects observed following combination treatment, the cell cycle profile of MDA-MB-231 cells was examined after 24 and 48 hrs of treatment with DOX, ADA alone, or in combination. Flow cytometry results demonstrated that ADA induced S-phase cell cycle arrest in MDA-MB-231 while DOX promoted the arrest of MDA-MB-231 cells in the G2/M phase **Figure 7**. In combination, DOX and ADA enhanced the arrest of cells in the S-phase of the cell cycle **Figure 8**.

# DISCUSSION

Combining targeted and conventional therapies are becoming a therapeutic standard for various haematological and solid cancers (15, 39). Due to toxicities and drug resistance, standard chemotherapeutic agents, such as doxorubicin, are restricted

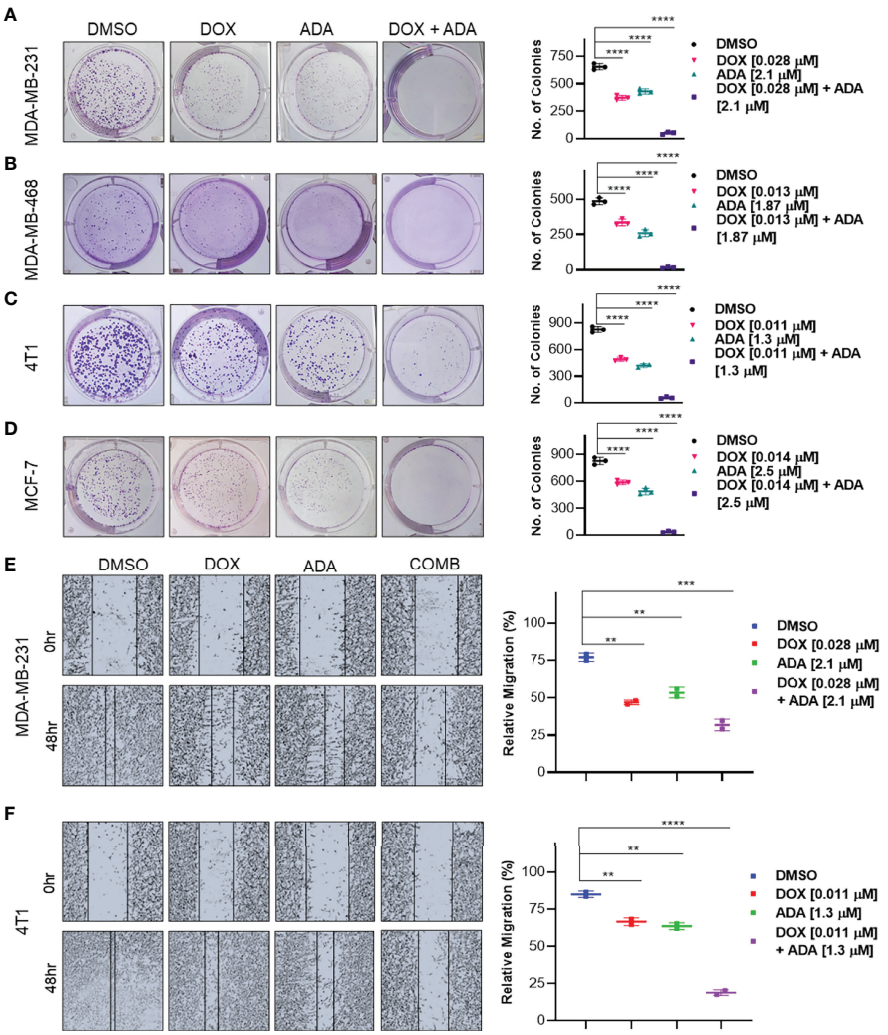


**FIGURE 4** | The combination of doxorubicin and adapalene inhibits colony formation and migration potential of TNBC cells. Representative images and quantification of colony formation assay data of **(A)** MDA-MB-231, **(B)** MDA-MB-468, **(C)** 4T1 and, **(D)** MCF-7 cells are treated with drug vehicle (DMSO), DOX, ADA, or a combination of DOX & ADA. The right panels show the quantification of colonies formed under each treatment condition described in the left panels. Data are mean ± SD. P values were determined by one-way ANOVA followed by Tukey's multiple comparisons test. Representative images and quantification of migration assay data of **(E)** MDA-MB-231, **(F)** 4T1. The right panels show the relative migration under control, single treatment and combination of DOX and ADA described in the left panels. Data are mean ± SD. P values were determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data are representative of at least three independent experiments. (**p < 0.001; ***p < 0.005, ****p < 0.0001).

(40). Additionally, resistance to doxorubicin is prominent in breast cancer and is typically associated with a multidrug resistance phenotype (41, 42). As a result, it is crucial to design combination treatment regimens comprising doxorubicin and chemosensitizer agents that improve rather than diminish its anticancer effectiveness while reducing its side effects. Recently, attention has been drawn to the combination treatment with conventional chemotherapeutic agents (15, 43). In this study, we demonstrated that the combined effect of ADA and DOX substantially increased apoptosis in TNBC cells. Increased intracellular ROS generation, Erk1/2 activation, and apoptosis were the primary mediators of this synergistic effect. Additionally, we observed a synergistic inhibitory effect of ADA and DOX on colony formation, cell migration, mammosphere formation, and cell proliferation.

Cancer cells produce and retain a larger proportion of reactive oxygen species (ROS) than normal cells. Increased reactive oxygen species (ROS) render tumor cells more sensitive to ROS-generating substances (37, 44). Studies have indicated that increasing ROS production in cancer cells inhibits tumor development and induces apoptosis. As a result, facilitating ROS is a promising therapeutic approach for cancer (21, 45, 46). Several chemotherapeutic agents have produced anticancer effects by activating the intrinsic apoptotic signalling. Mitochondria are both producers and targets of reactive oxygen species (ROS) (47). Excessive ROS generation may result in the loss of

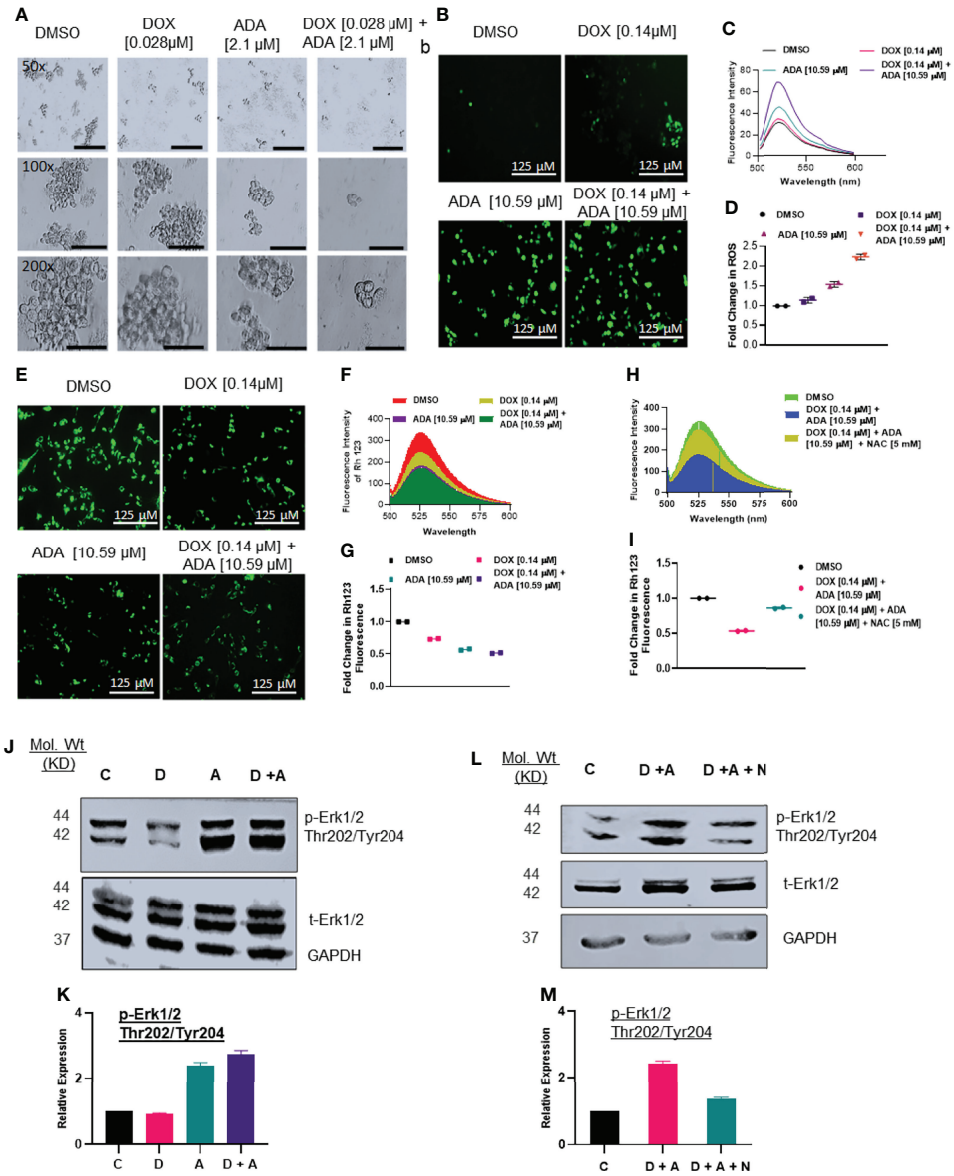


**FIGURE 5** | Doxorubicin and adapalene affect the anchorage-independent growth of MDA-MB-231 cells and enhance apoptosis. **(A)** Representative images of the spheroid assay. Treatment with the combination of DOX and ADA significantly reduced the growth of TNBC cells in ultra-low attachment plates and reduced mammosphere size (Bar: 500 μm (50X), 200 μm (100X and 200X). **(B)** DCF-DA staining, **(C)** fluorescence intensity & **(D)** fold change in ROS levels in MDA-MB-231 cells treated with ADA or DOX alone or in combination. **(E)** Rhodamine 123 staining, **(F)** fluorescence intensity & **(G)** fold change in Rh123 staining levels in MDA-MB-231 cells treated with ADA or DOX alone or in combination both showing decrease in mitochondrial membrane potential upon treatment. **(H)** Fluorescence intensity & **(I)** fold change in Rh123 upon treatment with DOX-ADA and DOX-ADA + NAC [5μM]. **(J, K)** Western blots of p-Erk1/2 (Thr202/Tyr204) on MDA-MB-231 with DOX/ADA or the combination of both. **(L, M)** Western blots of p-Erk1/2 (Thr202/Tyr204) on MDA-MB-231 with DOX-ADA or DOX-ADA + NAC [5μM]. Data are representative of at least two independent experiments. *C, control; D, doxorubicin; A, adapalene; A + D, adapalene + doxorubicin; A + D + N, adapalene + doxorubicin + NAC*.

mitochondrial membrane potential (MMP), allowing apoptotic effectors to escape (48). The mitochondria-mediated apoptosis pathway is dependent on cytochrome c release into the cytosol. It is required to form the apoptosome and activation of caspase 9, which leads to caspase 3 and caspase 7 activations. Caspase 3 and 7, the executors of the caspase family, break PARP, a characteristic of apoptosis. (49, 50). Our study demonstrates that ADA, either alone or combined with DOX, increases ROS generation, inducing MMP disruption. Pre-treatment with NAC reduced MMP disruption upon co-treatment of DOX and ADA. Additionally, we found that the inhibition of MMP by ADA and DOX activates the mitochondrial intrinsic apoptotic pathway *via* caspase 9 and caspase 3 activation and PARP cleavage, as shown in (**Figure 8**) diagrammatic summary. As a result, we infer that combination

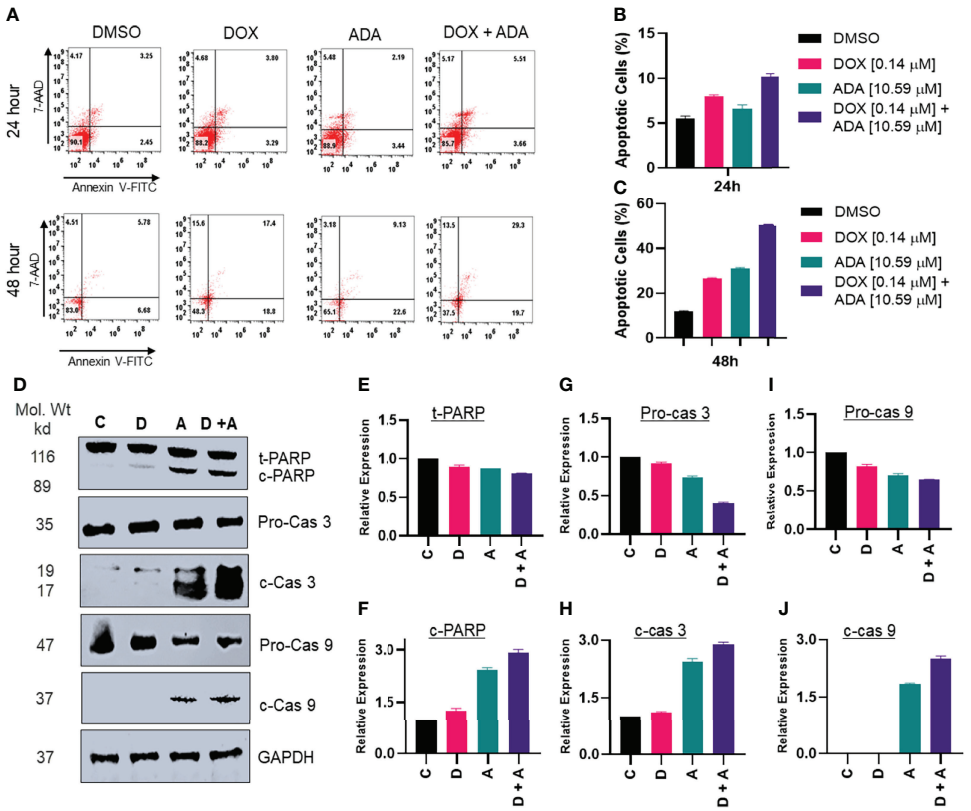


**FIGURE 6** | Adapalene and doxorubicin, in a synergistic manner, promote apoptosis. **(A–C)** Annexin V & &-AAD staining showed high apoptotic cells in plates treated with the combination of DOX or ADA or both after 24h or 48hr periods. **(D)** Western blots of expression levels of PARP, c-PARP, pro-caspase 3, c-caspase 3, pro-caspase 9 and c-caspase 9 on MDA-MB-231 with DOX/ADA or both for 24 hrs. Co-treatment showed enhanced PARP cleavage and activation of caspase 3 and caspase 9. C, *control (Treated with drug vehicle); D, doxorubicin; A, adapalene; A + D, adapalene + doxorubicin* **(E–J)** Relative expression plots of western blots.

therapy enhances ROS generation, mitochondrial malfunction, and eventually caspase-dependent death in TNBC cells.

The serine/threonine-protein kinase, ERK, is a member of the mitogen-activated protein kinases (MAPKs) family. Protein kinase mutations and dysregulation are involved in the pathogenesis of human illness and serve as a platform for developing therapeutic agonists and antagonists. Depending on the type of cell and stimulus, activation of ERK has been demonstrated to trigger apoptosis. Numerous anticancer drugs have been shown to activate ERK in various cancer cell types. Increased ROS accumulation linked with oxidative stress stimulates the Ras/Raf/ERK signalling pathway. The Ras/Raf/ERK pathway is activated in conjunction with the intrinsic apoptotic pathway, defined by the release of cytochrome c from the mitochondria and activation of the initiator caspase 9 (51, 52).

Herein, we found that ADA activates Erk1/2 in TNBC cells *via* increased ROS production, and this activation was rescued by pre-treatment with NAC. Additionally, we observed that combining ADA with DOX resulted in increased levels of (activated) p-ERK, unravelling the reason behind the synergistic effects of the DOX and ADA combination. Previously, it has been reported that drugs inducing ROS-mediated ERK activation sensitize anticancer therapies (53). For example, curcumin enhances the anticancer activity of cisplatin in bladder cancer cell lines *via* activating ERK1/2 through ROS-mediated signalling (54). Co-treatment with curcumin and cisplatin triggered activation of p53, apoptosis, and downregulation of survival proteins, which were reduced by NAC (a ROS scavenger) and U0126 (a MEK inhibitor). Also, curcumin and cisplatin caused apoptosis in bladder cancer cells *via* ROS-mediated activation of ERK1/2 (55).

Consequently, the growing concept that ERK1/2 may trigger cell death and the strategy/compound for enhancing pro-apoptotic ERK activity may provide a new therapeutic window for malignancies with oncogenic ERK signalling pathway activations. Chronic ERK activation, for instance, increases cell death in several cancer cell lines (55, 56). Additionally, altering ERK activity in a particular subcellular compartment may promote tumor cell death. (55). Notably, ACA-28 preferentially kills cancer cells with high ERK activity, while ACAGT-007 has shown better potency and selectivity against high-ERK melanoma cells *in vitro*. For ERK-induced apoptosis as an anti-cancer therapy, it is necessary to overcome the issue of selectively inducing apoptosis in cancer cells with abnormal ERK activity while preserving the survival of normal cells. In addition, the development of therapeutic molecules or the repurposing of existing drugs with the ability to enhance ROS-dependent ERK activation is a viable therapeutic approach for TNBC (55).

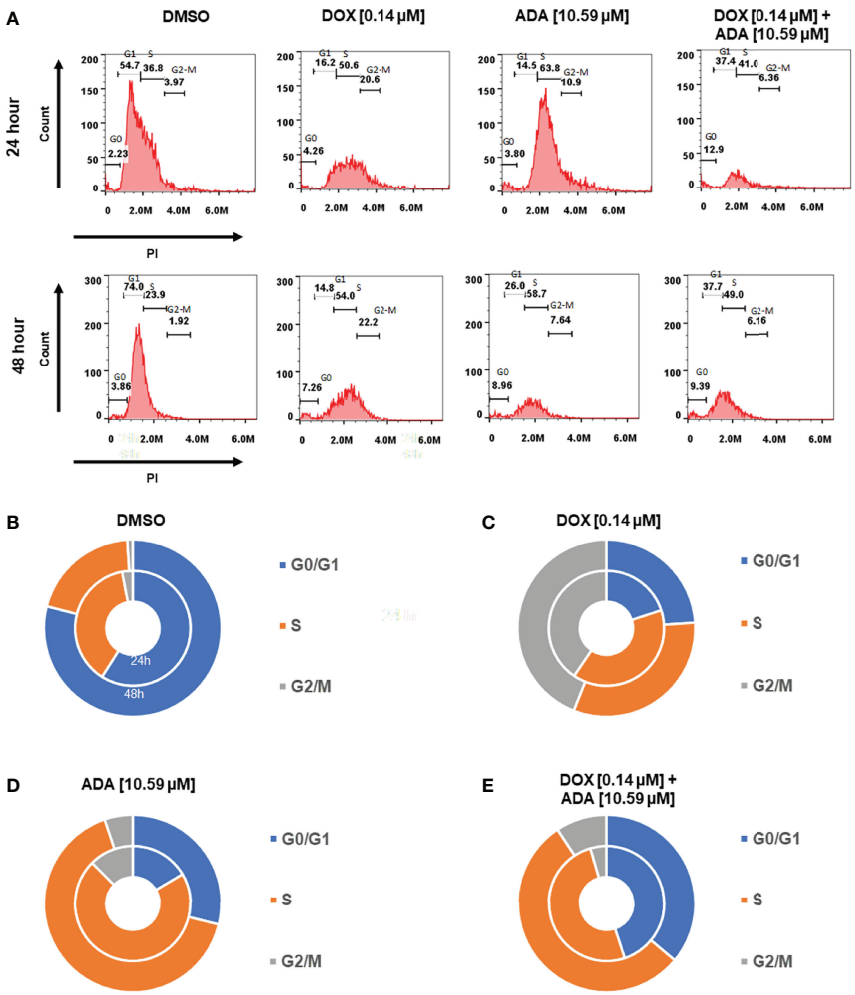


**FIGURE 7** | Doxorubicin and adapalene induce cell cycle arrest of MDA-MB-231 cells. **(A–E)** ADA upon treatment showed S-phase arrest of MDA-MB-231 cells, while DOX showed the arrest of MDA-MB-231 cells in the G2/M phase of the cell cycle. Upon Combination treatment with DOX and ADA, the arrest of cells enhanced in the s-phase.

Understanding the molecular mechanisms behind the anti-tumor activity of ADA and its pharmacodynamic interactions with DOX may help design ADA-DOX combination therapy. As previously documented, ADA triggers several pro-apoptotic responses that result in apoptosis in various tumor types (17). ADA promotes apoptosis in colorectal cancer cells *via* activating the caspase-3 and Bax/Bcl-2 pathways. Recent studies showed that ADA-mediated tumor growth inhibition occurs due to DNA damage. Melanoma cells treated with ADA produced increased levels of DNA damage marker, ϒ-H2AX (57). Apart from its increased ability to inhibit proliferation and promote apoptosis, it may have several physiological advantages over standard retinoic acid derivatives. ADA exhibits more anti-inflammatory effects *in vitro* and *in vivo* than other retinoids due to its suppression of lipoxygenase pathways (58). ADA is five times more stable to light than natural retinoids due to its chemical makeup.

Additionally, ADA has a safer profile than other retinoids with oral 5 g/kg $LD_{50}$ in rats and mice, significantly higher than 9-cis-retinoic acid + (58, 59). Also, high dosages of ADA administered orally have no adverse effects on the neurologic, hematologic, cardiovascular, or respiratory systems (17). The previous and present study findings demonstrate that ADA is a potent anti-tumor agent.

To summarise, we report that ADA is a potent anticancer agent and improved the anticancer activity of DOX *via* ROS-mediated hyperactivation of the Erk1/2 signalling pathway. These findings shed light on the molecular pathways through which ADA and DOX interact and imply that such combination therapy may become a more successful treatment for TNBC.

The validation of our results is restricted to the experimental methodology described, and unquestionably, additional screening studies are required to determine the optimal combination regimens. For example, the successive addition of drug

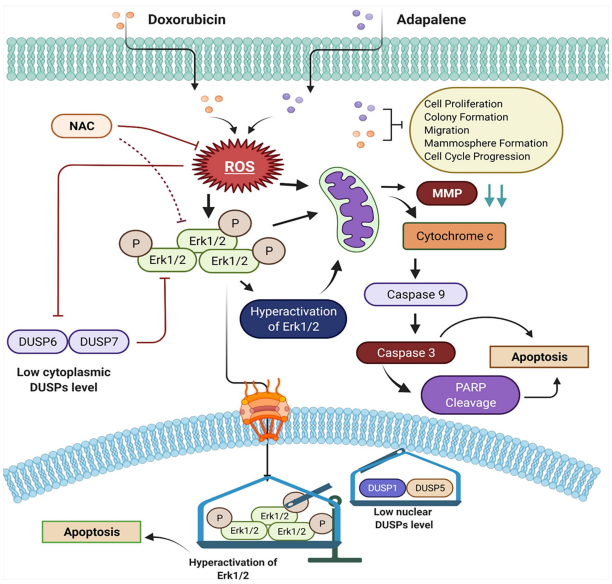


**FIGURE 8** | Schematic of the main findings of the study and possible mechanism of action of doxorubicin and adapalene in TNBC cells. The co-treatment with DOX and ADA resulted in enhanced ROS generation, leading to mitochondrial membrane potential disruption. Disruption of MMP triggers intrinsic apoptosis associated with PARP cleavage. Also enhanced ROS generation, triggers hyperactivation of Erk1/2 signalling, which further promotes apoptosis *via* mitochondrial death pathway.

combinations could drastically reverse the net effects. In addition, we suggest conducting additional *in vitro* pharmacodynamic interaction analyses based on a non-constant experimental design so that more potent combinations with a more favourable DRI can be achieved. In addition, further research might be done to determine the impact of the observed synergistic combinations on *in vivo* BC models and other cancer hallmarks. Our results indicate that ADA and DOX may have therapeutic potential for TNBC. The results of this study should be validated in relevant preclinical models of TNBC to determine their clinical importance.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

MM designed and supervised the study. UM performed the experiment, collected, analyzed the data, and wrote the manuscript. MM, NW, IM, MH, and MA performed the analysis and critically revised the manuscript. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.938052/full#supplementary-material

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# The role of hypoxia-inducible factor-1 alpha in multidrug-resistant breast cancer

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Breast cancer is the most common cancer in women worldwide with increasing incidence. Significant therapeutics advances in the field of breast cancer have resulted in a growing number of treatment options, whereas *de novo* or acquired resistance is still a persistent clinical challenge. Drug resistance involves a variety of mechanisms, and hypoxia is one of the many causes. Hypoxia-inducible Factor-1 Alpha (HIF-1α) is a key transcription factor which can regulate the response of cells to hypoxia. HIF-1α can trigger anaerobic glycolysis of tumor cells, induce angiogenesis, promote the proliferation, invasion, and migration of tumor cells, and lead to multidrug resistance. This review mainly discusses the role of HIF-1α in the drug-resistant breast cancer and highlighted the potential of HIF-1α -targeted therapy.

KEYWORDS

**breast cancer, drug, resistance, inhibition, hypoxia inducible factor-1 Alpha (HIF-1α), targeted drugs**

## Introduction

Breast cancer is the most common malignancy in women and the second leading cause of female cancer-related death after lung cancer (1). Its therapy methods mainly include surgery, endocrine therapy, chemotherapy, radiotherapy and targeted therapy based on the classification of tumors, among which drug therapy occupies an important part of the treatment of breast cancer. In the early 1990s, breast cancer mortality had declined due to its reduction in the risk, improvements in treatment and widespread use of early screening (2). However, the emergence of drug resistance during treatment in recent years has brought severe challenges for the survival of breast cancer patients (3). Resistance to anticancer drug therapy is caused by a variety of factors, which include tumor burden and growth kinetics; tumor heterogeneity; physical barriers; undruggable

cancer drivers; the many consequences of applying therapeutic pressures; the immune system and the microenvironment with hypoxia (4, 5). Hypoxia in the tumor microenvironment refers to a condition where the pressure of oxygen is lower than 5–10 mm Hg (6). Hypoxia is caused by an imbalance between oxygen consumption and oxygen supply due to rapid growth of tumor (7). As a hallmark of the tumor microenvironment, hypoxia occurs in a variety of tumors. It is well known that tumor hypoxia has a negative impact on treatment outcomes and prognosis. Hypoxia inhibits tumor cell proliferation, induces cell cycle arrest, and ultimately develops drug resistance because anticancer drugs preferentially target cells that are rapidly proliferating (8).

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that responds to hypoxia and is involved in several aspects of tumor progression, including metastasis, angiogenesis, drug resistance, and immune evasion (9). The constitutive HIF-1β/ARNT subunit and the highly oxygen-sensitive HIF-1α subunit constitute the HIF1 protein, while intracellular HIF-1α levels determine the activity of HIF-1 (10). After HIF-1α was initially discovered by identifying a hypoxia response element (HRE) in the 1990s, scholars have proven that it is a key regulator responsible for the induction of genes that facilitate adaptation and survival under low oxygen conditions (11). Overexpression of hypoxia-inducible factor-1 alpha (HIF-1α) is associated with drug resistance, poor prognosis, and a higher risk of metastasis in breast cancer patients (12). Currently, numerous small molecule inhibitors are under development, some of which are considered in clinical trials. For instance, in a phase II trial of echinomycin, a HIF-1α transcription inhibitor for metastatic non-small cell lung cancer, the response rate of patients treated with echinomycin was 5%, and the median survival was 24.3 weeks (13). Although the treatment did not satisfy the predefined expectations during the time, it revealed the feasibility of using HIF-1α as a potential target for cancer treatment. As research and development of drugs targeting HIF-1α are primarily based on its mechanism of action, exploring this aspect of breast cancer drug resistance is of great significance to the development of related drugs. It can provide a reference value for clinical combination therapy. Therefore, this review aims to investigate the role of HIF-1α in treating breast cancer drug resistance, emphasizing its potential as a therapeutic target, and forecast its inhibitors and clinical application prospects.

## Structure of HIF-1α

Hypoxia is involved in many pathological and physiological processes of the human body and acts as an important regulator. HIFs are an integral component of tumor adaptation in the hypoxic tumor microenvironment (14). So far, three types of HIFs have been identified in mammals. HIFs are heterodimeric proteins composed of an $O_2$-sensitive α subunit (HIF-1α, HIF-2α, and HIF-3α) and an $O_2$-insensitive β subunit (HIF-1β) and play a key role in the regulation of many genes transcribed in hypoxic conditions (Figure 1) (15). All three HIF-α genes are regulated by oxygen and bind to HIF-1β, but only HIF-1α and HIF-2α have been extensively studied (16). Although HIF-1α and HIF-2α share similar amino acid sequences and bind to the same HRE, they differ in several aspects (17).. First, HIF-1α is widely expressed, while HIF-2α is relatively tissue-specific (18).

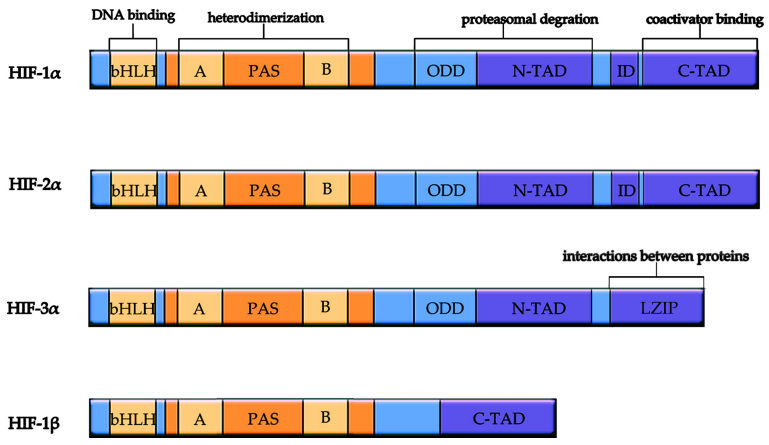


FIGURE 1
Schematic diagram of structure and function of HIF subunits. All HIF isoforms have a bHLH motif and two PAS domains responsible for heterodimerization. HIF-3α replaces C-terminal trans-activation domain with C-terminal Leucine zipper (LZIP) domain responsible for interactions between proteins. HIF-1β does not contain ODD domain for proteasomal degradation, N-TAD, and ID. bHLH, basic helix-loop-helix domain; PAS, Per/ARNT/Sim domain; ODD, oxygen-dependent degradation domain; ID, inhibitory domain. N-TAD, N-terminal transactivation domain; C-TAD, C-terminal transactivation domain.

Second, some studies showed that the oxygen dependence of HIF-1α and HIF-2α significantly differed as HIF-1α was more active and lasted for a shorter time under severe hypoxia, whereas HIF-2α was more active and lasted longer under moderate hypoxia (19–21).

HIF-1α and HIF-2α show non-overlapping antagonistic roles due to their unique regulators, different expression patterns, and gene targets (22). HIF-1α is ubiquitously expressed in hypoxic tissues, whereas HIF-2α is mainly expressed in a certain cell types, including vascular endothelial cells (ECs) and macrophages (23). The HIF transcription factors show disparate spatiotemporal regulation. For example, HIF-1α can be activated under acute and severe hypoxia (1-2% $O_2$), whereas HIF-2α is gradually accumulated under moderate hypoxia (5% $O_2$) (24). Moreover, genes which regulate cell death or anaerobic glycolysis appear to be predominantly controlled by HIF-1α, but genes which regulate erythropoietin synthesis (EPO) and tumor stemness or pluripotency are primarily regulated by HIF-2α (10). Furthermore, as to typical HRE mediated transcription, HIF subtypes also differentially regulate signaling pathways by interacting with proteins that do not contain PAS domains, such as β-catenin, p53, Notch intracellular domains, and c-myc proto-oncogene (23, 25, 26). Emerging data suggests that the HIF-α subtype is specific in multiple solid tumor types *e.g.*, glioblastoma, kidney carcinoma, and neuroblastoma, and HIF-α subtype may promote tumor progression (Figure 2) (23).

At present, there are few studies on HIF-3α, which may be related to the complex function due to a large number of different variants (27). The C-terminal leucine zipper (LZIP) domain responsible for the interaction between proteins was found to replace C-terminal trans-activated domain in a HIF-3α variant (28). It is generally believed that the HIF-3α gene is expressed as a selective splicing isomer, which can activate or inhibit HIF target gene (27).

HIF-1 contains HIF-1α subunit with 826 amino acids and the HIF-1β subunit with 782 amino acids. Both subunits belong to the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of transcription factors (29). N-TAD (N-terminal TAD) and C-TAD (C-terminal TAD), located in HIF-1α, are two transactivation domains (TADs) with rich acidic and hydrophobic amino acids (30). C-TAD is mainly responsible for regulating HIF-α transcription by interacting with the transcriptional co-activator protein CREB binding protein/P300 under hypoxia, whereas N-TAD is mainly a regulator for its stabilization (31, 32). The regions between the two TAD sequences are inhibitory domains (ID; Amino acids 576-785), which inhibit the transcriptional activation of TAD (33). HIF-1α contains the oxygen-dependent degradation (ODD) domain in upstream of the N-TAD region responsible for its degradation by the ubiquitin-proteasome pathway (34). HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator ARNT) is constitutively expressed in all cell types and is not regulated by oxygen levels (35). HIF-1β subunit lacks ODD and N-TAD domains and contains only C-TAD, and its structural differences are reflected in its function (30).

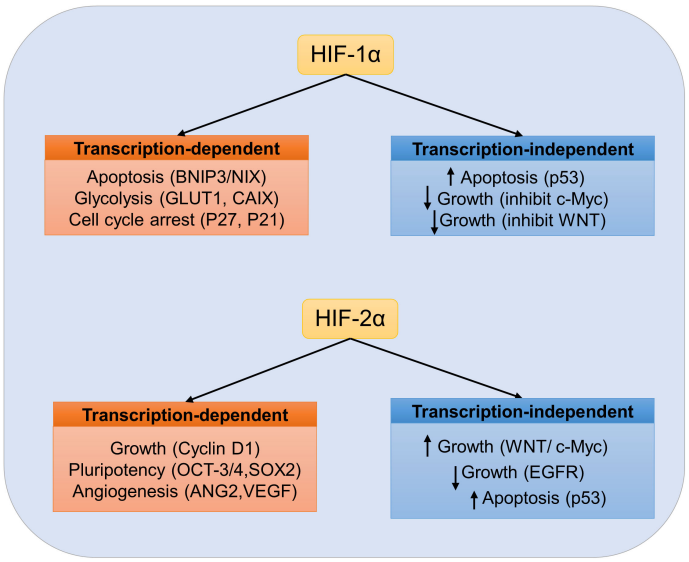


FIGURE 2
Specific role of HIF in tumor cells. Transcription-dependent and independent targets of HIF-1α or HIF-2α are listed. No overlapping HIF targets are listed. Depending on the cellular environment, activation of HIF transcription factors may have pro-tumor or anti-tumor effects.

## Induction of HIF-1α in breast cancer

HIF-1α stabilization was reported in tumors of varying origins, and functional analyses led to the perception of HIF-1α as an oncoprotein (36). HIF-1α is located in the cytoplasm and is easily degradable under normoxia conditions with a half-life of less than 5 min. However, many studies have found that HIF-1α enhances stability in the presence of hypoxia and maintains a set of mechanisms for stability and activation in the presence of normoxia. Hence, the mechanisms of HIF-1α stabilization and transcriptional activation under normal and hypoxic conditions are discussed based on: (1) classical oxygen-dependent pathways and (2) oxygen-independent pathways.

### Classical oxygen-dependent pathways

Under normal physiological conditions, HIF-1α is degraded in the body and cannot exert its biological effects. Hypoxia-inducer -α (HIF-α) protein inactivation is mainly regulated by FIH-1 and PHD through interactions with their specific N-TAD and C-TAD domains (37). FIH-1 is an oxygen-dependent enzyme that hydroxylates aspartic acid residues at position 803 (Asn803) in the transactivation domain of the HIF-1α C-terminal. The transcriptional activation function of HIF-1α was inhibited by blocking the binding of HIF-1α with CBP (CREB-binding protein)/P300 (38). Prolyl hydroxylase (PHDs) is also an oxygen-dependent enzyme that hydroxylates the key residue Pro564 and Pro402 of HIF-1α, located in the oxygen-dependent degradation domain (39). Subsequently, the E3 ubiquitin ligase Von Hippel Lindau protein (pVHL) binds to the ODD domain of HIF-1α subunit, recruiting a variety of ubiquitin proteins to form the ubiquitin ligase complex, leading to ubiquitination of the HIF-1α subunit (40). Finally, HIF-1α is degraded by the ubiquitin-linked protease complex pathway. The expression of PHDs varies from tissue to tissue, and the affinity for different HIF proteins varies, which may lead to the diversity of hypoxic responses. In addition to hydroxylation of Pro564, Pro402, and Asn803, lysine (Lys532) in the oxygen-dependent degradation domain is blocked by acetyltransferase arrest-defective 1 (ARD1) to promote tumor pVHL binding, leading to HIF-1α instability (41).

Under hypoxic conditions, FIH-1and PHDs activity is inhibited, resulting in decreased HIF-1α hydroxylation and repressed proteasomal degradation (42). HIF-1α stabilizes and dimerizes with HIF-1β present in the cytoplasm and nucleus of anoxic and normal cells to form HIF-1, which is then translocated to the nucleus (43). Heterodimer HIF-1 and co-activator CREB binding protein/P300 bind to hypoxia response element (HRE), which activates transcriptional activity of target genes such as VEGF, GLUT1, and MDR1 (Figure 3) (44). Therefore, HIF-1α does not degrade, leading to a rapid increase in intracellular protein levels (38).

### Oxygen-independent pathways

Most current studies focused on the relationship between cancer and HIF-1α in the context of hypoxia have limited insight because about 50% of advanced solid tumors lack hypoxic zones, and as a result, they remain able to activate HIF-1α (42).

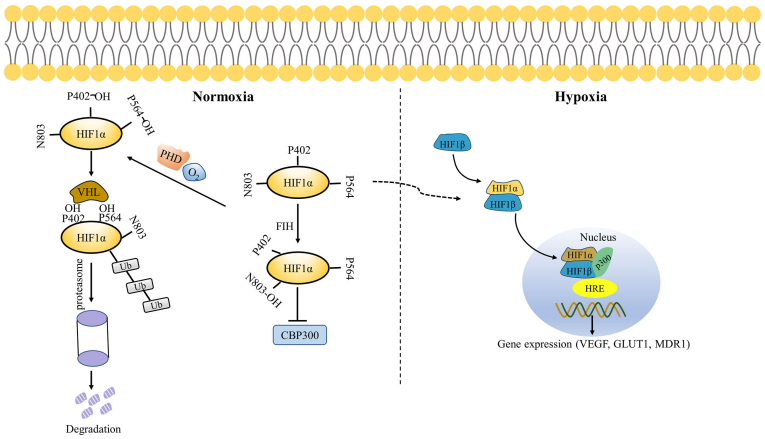


FIGURE 3
Oxygen-dependent regulation of HIF-1α. Under normoxic conditions, FIH hydroxylates HIF-1α Asn803 residues and blocks the binding of HIF-1α to CBP/P300, thereby inhibiting its transcriptional activation. PHD hydroxylates the key residues Pro564 and Pro402 of HIF-1α, resulting in pVHL binding to HIF-1α and ubiquitination of HIF-1α, which is ultimately degraded by the proteasome. Under hypoxia conditions, FIH and PHD are inactivated. HIF-1α and HIF-1β translocate to the nucleus, thus binding to p300 and hypoxia response elements (HRE) in the nucleus to activate gene transcription.

Therefore, exploring the stabilization and activation mechanism of HIF-1α in non-hypoxia conditions might help us to have a comprehensive understanding of its role in tumorigenesis, thus providing new targets for treatment.

Several pathways regulate HIF-1α stabilization and are thought to contribute to the intracellular accumulation of HIF-1α. For instance, it was reported that extracellular-signal-regulated kinase (ERK) was involved in the regulation of HIF-1α synthesis and transcriptional activation (45). In addition, ERK phosphorylates the co-activator CBP/P300 and increases the formation of HIF-1α/P300 complex, thereby stimulating its transcriptional activation (46). Some common genetic alterations in the oxygen-signaling pathway, such as loss of tumor suppressors p53, PTEN, and pVHL increase HIF-1α transcription, translation, or stability independently of O2 levels leading to tumor progression (47–49). A previous study reported that the loss of p53 enhances HIF-1α levels in human colon cancer, which may be explained by the role of p53 in promoting Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1α (50). Similarly, PTEN expression inhibited HIF-1α stabilization in glioblastoma-derived cell lines with evidence suggesting AKT regulation involvement, although AKT is indirectly associated with HIF-1α phosphorylation (51). As mentioned above, pVHL plays an important role in HIF-1α degradation. It was reported that HIF-1α stability was maintained, and HIF-1 was activated in VHL-deficient cells (52).Hsp90 inhibitors promoted effective ubiquitination and proteasome-mediated degradation of HIF-1α in RCC under normoxic and hypoxic conditions (53). Hsp90 directly binds to PAS domain of HIF-1α to induce conformational changes that enable HIF-1α to bind to HIF-1β, thereby initiating HIF-1α transactivation (54). Moreover, Hsp90 can stabilize HIF-1α by inhibiting its degradation.

## TME and HIF signaling and angiogenesis

Tumor microenvironment (TME) refers to the local biological environment of a solid tumor, consisting of both tumor cells, non-tumor cells, and extracellular matrix (ECM). In TME, there is a complex interaction and balance between tumor cells and non-tumor cells (55). Hypoxia, a hallmark of the TME, is caused by an imbalance between oxygen consumption and oxygen supply because of rapid tumor growth, which occurs in a variety of tumors including breast cancer (7). A key feature of the cellular response to hypoxia is the upregulation of multiple genes that promote angiogenesis/vascularization to increase oxygen delivery. This process is mediated by hypoxia-inducible factor (HIF-1α subunit) which can activate transcriptional responses under hypoxia (56, 57). Hypoxia-induced HIF-1α stabilization and accumulation can promote angiogenesis by increasing the expression of multiple pro-angiogenic genes. Vascular endothelial cell growth factor (VEGF) is one of its main target genes and is considered to be the main driver of angiogenesis. Particularly, VEGF can recruit endothelial cells to hypoxic and non-vascular areas and promote their proliferation (58). In addition to VEGF, HIF-1α regulates the expression of other angiogenic inducers (e.g., FGF, PDGF, and Ang-1/2) and angiogenic receptors (e.g., VEGFR, ANGPT receptor) (59–61). Meanwhile, HIF-2α plays an indispensable role in angiogenesis, which promotes vascular maturation (62).

HIF-1a not only mediates breast cancer angiogenesis but also leads to its metastasis, drug resistance, and poor prognosis. For example, HIF-1α signaling selectively supports breast cancer proliferation in the brain, which has been validated *in vivo* (63). In this study, nuclear HIF-1α staining was performed on breast cancer CTC-derived tumors growing in the brain and mammary gland, respectively. The results revealed that HIF-1α staining was approximately 11-fold increase in brain tumors in comparison with that in mammary tumors. Another study reported that mammary gland-specific deletion of Axl which is an HIF target can reduce HIF-1α levels in a HER2 + mouse model of breast cancer, thereby leading to a normalization of the blood vessels, a proinflammatory TME, and a reduction of lung metastases by inhibiting the hypoxia response of tumor cells (64). The *in vivo* data strongly suggests that HIF-1α plays a significant role in breast cancer metastasis.

In addition, some clinical randomized trials have demonstrated that HIF-1α can be used as a marker of poor prognosis and an independent predictor of drug resistance. For example, a clinical trial of 187 patients with T2-4 N0-1 breast cancer found that overall response to epirubicin and tamoxifen treatment decreased with increased tumor HIF-1α. The Kaplan-Meier curves showed that increased HIF-1α expression was associated with a significantly shorter disease-free survival (DFS) (65). Another clinical study enrolled 114 patients with T2-4 N0-1, estrogen receptor (ER) -positive breast cancer who were treated with letrozole. The response was assessed by measuring tumor size and detecting the presence of tumor cells in breast and axillary lymph nodes. The results found that 91 patients (81%) achieved disease response, 48 patients achieved complete clinical response (43%), and 22 patients did not achieve response (19%). Moreover, increased P44/42 MAPK and HIF-1α were found in patients without remission, suggesting that the increase in P44/42 MAPK and HIF-1α was a significant factor in treatment resistance in all leave-one-out iterations (63). A previous study also revealed that increased HIF-1α expression was associated with tamoxifen resistance. HIF-1α positivity was more common in contralateral breast cancer (CBC) during tamoxifen adjuvant therapy (N = 60) than in CBC without prior tamoxifen (N = 522) (32% (18/56) versus 17% (80/482) (64) These reports highlight the role of HIF-1α as a prognostic marker, but also demonstrate the positive association between HIF-1α overexpression and endocrine therapy resistance in breast cancer.

## HIF-1α contributes to drug resistance in breast cancer

Breast cancer is the most common malignancy in women and the second leading cause of female cancer-related death after lung cancer (1). Its therapy methods mainly include surgery, endocrine therapy, chemotherapy, radiotherapy, and targeted therapy based on the classification of tumors, among which drug therapy occupies an important part. However, drug resistance has become a major challenge in breast cancer treatment. Although the relationship between HIF-1α and drug resistance in breast cancer has been emphasized above, the mechanisms by which it induces resistance in chemotherapy, endocrine therapy, and targeted therapy remain to be clarified. This may be because HIF-1α is involved in various life activities in human cells. Studies suggested that HIF-1α may develop resistance to conventional therapies through a series of signaling pathways including drug effusion, tumor stem cell enrichment, autophagy and apoptosis (7, 66). Therefore, we will explore the mechanism of HIF-1α leading to breast cancer drug resistance from the above mentioned related signaling pathways.

### HIF-1α mediated overexpression of drug efflux proteins

A major cause of cancer MDR is the increased efflux of various ATP-dependent hydrophobic cytotoxic drugs, mediated by transmembrane transporters of ATP binding cassette (ABC) superfamily (67). ABC transporters are known as a complete family of membrane proteins, including many recognized drug transporters, such as the well-known multidrug resistance 1 protein (MDR1)/P-glycoprotein encoded by ABCB1 gene, MDR-related protein 1 (MRP1, encoded by ABCC1 gene) and G member 2 of ABC subfamily, also known as breast cancer resistance protein (BRCP), which is encoded by ABCG2 gene (68). The relationship between these three drug transporters and drug resistance in breast cancer has been extensively studied (69–71).

Using quantitative RNA microarray analysis, previous studies revealed an approximately 7-fold increase in MDR in epithelial cells exposed to hypoxia. Meanwhile, MDR1 gene detection identified the binding site between hypoxia-inducible factor-1 (HIF-1) and MDR1. Hypoxia-inducible MDR1expression was significantly inhibited, and the basic MDR1 expression was almost completely lost when HIF-1 expression was inhibited using antisense oligonucleotides (72). Over-expression of the multidrug resistance protein 1 (MDR1, also known as P-glycoprotein or P-gp) is associated with the resistance of taxane and anthracyclines, which are principle chemotherapeutic agents for breast cancer treatment (73). Both the gene encoding MRP1 (ABCC1) and ABCG2 gene encoding BCRP have a hypoxia response element upstream of the open reading frame, and the deletion of this locus prevents hypoxia-dependent activation (74, 75). In one study, western blotting analysis demonstrated increased mRNA and protein expression of MDR1 and MRP1 in SGC7901/HIF cells, whereas hypoxia-induced MDR1 and MRP1 were inhibited in SGC7901/si-HIF cells with HIF-1α knockdown, suggesting that HIF-1α expression can upregulate the expression of drug-resistant proteins MDR1 and MRP1 (76). Another study found that basic HIF-1α protein and BCRP mRNA and protein in AI (letrozole or exemestane)-resistant and HER2-transfected cells were higher than those in AI-sensitive HER2 parents under nonhypoxic conditions and BCRP mRNA in LTLTCa cells (AI-resistance breast cancer cells) treated with $CoCl_2$ (HIF-1α stabilizer) increased by about two times compared with the control group. Additionally, in the study, real-time PCR analysis of immunoprecipitated DNA after ChIP found that HIF-1α binds to the hypoxia response element (HRE) region of BCRP promoter in LTLTCa cells under non-hypoxia conditions and $CoCl_2$ significantly increased the binding of HIF-1α to BCRP promoter (77). However, the specific signaling pathway utilized by HIF-1α to regulate the expression of drug-resistant proteins remains unclear at present. The level of cell resistance to irinotecan and topotecan was correlated with the expression level of BCRP in cells, which was demonstrated in BCRP-overexpressed breast cancer cells (T47D) (78). These results suggest that HIF-1α expression and stabilization can increase mRNA and protein levels of MDR1, MRP1, and BRCP, which are involved in HIF-1α mediated drug resistance (Figure 4).

### HIF-1α mediated BCSC enrichment

Cancer stem cells (CSC) are a kind of cell subpopulation in solid tumors, which possess self-renewal, differentiation, and tumorigenic potential (79). HIF-1α has been reported as a prerequisite for chemotherapy resistance (paclitaxel and gemcitabine) of breast cancer stem cells by inducing ROS-dependent expression of HIF-1α and HIF-2α, leading to HIF-mediated expression of IL-6, IL-8, and MDR1, thereby promoting the survival of BCSCs (80). This study found that exposure of MDA-MB-231, SUM-149, and SUM-159 to paclitaxel increases the percentage of ALDH+ cells that exhibit stem cell properties *in vitro* and *in vivo* by 12-fold. All of the abovementioned effects can be eliminated by the HIF inhibitor digoxin or knockdown of HIF-1α. In addition, an assay of the ALDH activity of MDA-MB-231, SUM159, and MCF-7 cells, which were cultured at 21% $O_2$ (normoxia) or 1% $O_2$ (hypoxia), demonstrated that the percentage of ALDH+ cells per cell line increased by approximately two to three times and HIF-1α knockdown completely eliminated the hypoxia-induced ALDH+ population increase under hypoxic conditions (81). Furthermore, the authors speculated that

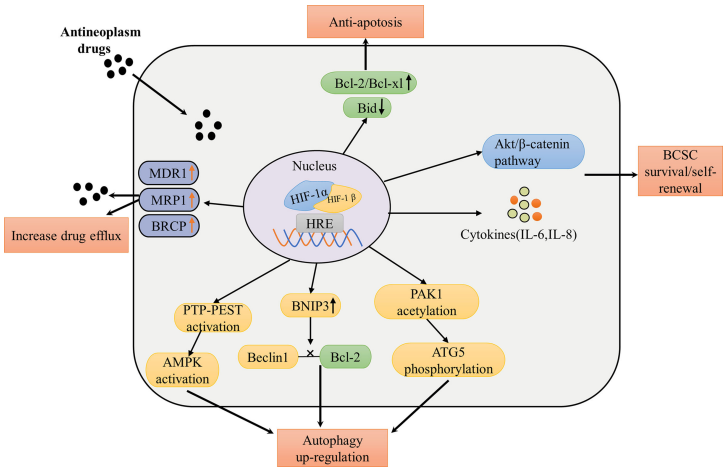


FIGURE 4
Summary of mechanisms and pathways of HIF-1α mediated drug therapy failure in breast cancer. The pathways of resistance to conventional treatment of HIF-1α include: increasing expression of drug efflux protein leads to drug efflux, increasing expression of anti-apoptotic protein and decreasing expression of pro-apoptotic protein enhance anti-apoptotic effect; phosphorylated Akt/β-catenin pathway and increased cytokine levels promote survival and self-renewal of breast cancer stem cells; promoting the expression of PTP-PEST to activate AMPK, increasing the expression of BNIP3 to interfere the interaction between Beclin1 and Bcl-2 and inducing the acetylation of PAK1to phosphorylate ATG5 promote the upregulation of autophagy. MDR1, multidrug resistance protein 1; MRP1, MDR-related protein 1; BCRP, breast cancer resistance protein; IL, interleukin; ATG5, autophagy-related 5; AMPK, AMP-activated protein kinase; Beclin1, a protein for regulating the formation of autophagosome membranes; Bcl-2 and Bcl-xl, antiapoptotic proteins; Bid, a pro-apoptotic protein; PAK1, p21 activated kinase 1; PTP-PEST, the protein tyrosine phosphatase (PTP)-PEST.

HIF-1α promoted stem cell enrichment, in part, through the Akt/β-catenin pathway, which was reported to be a key regulator of CSC self-renewal in breast cancer, because HIF-1α increased levels of both phospho-Akt and phospho-S552 -β-catenin in SUM159 cells, and β-catenin was inactivated (not phosphorylated) when HIF-1α was knocked down (81, 82). Thus, activation of HIF-1α can promote the proliferation and enrichment of tumor stem cells, leading to treatment resistance (Figure 4).

## HIF-1α mediated up-regulation of autophagy

Autophagy, also known as cellular self-digestion, is a cellular pathway that involves the degradation of proteins and organelles, with a complex relation to human disease and physiology (83). Autophagy in cancer is a double-edged sword, which can function as a tumor suppressor by preventing the accumulation of damaged proteins and organelles, and as a cell survival mechanism to promote the growth of established tumors under nutritionally deficient or hypoxic conditions (84).

HIF-1α mainly upregulates autophagy in cancer through the following pathways: promoting PTP-PEST expression to activate AMPK, increasing BNIP3 expression, and lastly, interfering with the interaction of Beclin1 with BCL-2, and inducing ELP3-mediated PAK1 acetylation, leading to subsequent PAK1-mediated ATG5 (autophagy-related 5) phosphorylation at T101 residue (Figure 4) (85–87). Some studies have revealed that hypoxia increases breast cancer cell resistance to doxorubincin (DOX) with activation of AMPK. Meanwhile, blocking the AMPK-ULK1 pathway can increase the sensitivity of breast cancer (BC) cells to doxorubicin (88, 89). Beclin1, a crucial regulatory protein for regulating autophagosome membrane formation, was upregulated in breast cancer, colorectal cancer, gastric cancer, liver cancer, and cervical cancer and has been associated with chemotherapy resistance (78, 84, 90). The present evidence demonstrated that Beclin1-knockdown breast cancer cells treated with paclitaxel increase cell death by inducing caspase-dependent apoptosis than the group without Beclin1 knockdown (91). In the study, the apoptosis rate of paclitaxel-treated breast cancer cells with Beclin1 knockdown was about 45%, while the apoptosis rate of the group without Beclin1 knockdown was about 33%, and western blot analysis showed that the expression of apoptotic protein caspase-3 increased in the former group. In addition, a study utilizing RT-PCR to measure ATG5 levels in 60 breast cancer tissues found that trastuzumab-resistant patients had higher ATG5 levels than trastuzumab effective patients (92). Similarly, elevated autophagy markers in drug-resistant breast cancer cells have been reported for tamoxifen and fulvestran (93, 94). In summary, it can be concluded that HIF-1α can lead to breast cancer resistance to endocrine drugs and cytotoxic drugs through upregulation of autophagy.

## HIF-1α-mediated inhibition of apoptosis

Apoptosis is a gene-regulated form of cell death that plays a role in biological processes, including embryogenesis, aging, and many diseases (95). Escape from apoptosis is one of the characteristics of cancer cells and is associated with chemotherapy resistance or tumor recurrence (96). At the molecular level, there are two main pathways of apoptosis: external signaling pathways dependent on the binding of death receptor–ligand and internal signaling pathways in response to various cellular stresses (97). Many proteins and cytokines are involved in apoptosis, including members of the B-cell lymphoma-2 (Bcl-2) family, inhibitors of apoptosis-associated proteins, cytochrome c, and the caspase family of proteases (97). Interactions between pro-apoptotic and antiapoptotic members of the Bcl-2 family may mediate the balance between cell survival and apoptosis.

Similar to Bcl-2 family, the role of HIF-1α in apoptosis is also double-sided: promoting apoptosis and inhibiting apoptosis (97). The pro-apoptotic alterations by HIF-1α include down-regulating the expressions of BNIP3, NIX, and NOXA, which belong to the members of the pro-apoptotic Bcl-2 family. In contrast, antiapoptotic effects include increased antiapoptotic proteins such as Bcl-2, Bcl-xL and Myeloid cell leukemia (Mcl-1) and decreased pro-apoptotic Bid, Bax, and Bak levels (98–100). The hypoxia-mediated downregulation of Bid in tumors is reported through HIF-1α dependent mechanisms and contributes to drug resistance (Figure 4) (101). HIF-1α was silenced by interfering RNA in HT29 and MEFs cells under hypoxia. At the same time, western blot analysis showed that the Bid protein expression was increased compared with the control group, indicating that the pro-apoptotic protein Bid expression was inhibited when HIF-1α expression was increased under hypoxia, partially explaining the antiapoptotic phenomenon induced by hypoxia. Simultaneously, hypoxia-induced reduction in Bid in HT29 and MEFs cells showed resistance to etoposide. In addition, inhibition of apoptosis induced by overexpression of antiapoptotic proteins is a core factor in acquiring multidrug resistance (MDR) in breast cancer (102). Increased expression of antiapoptotic proteins Bcl-2 and Bcl-xL in HCT116 cells under hypoxia and treatment with irradiation during severe hypoxia significantly improved cell survival scores, which could be ameliorated by Bcl-2 inhibitor ABT-263 (103). This result suggests that hypoxia can increase antiapoptotic proteins and thus resistance to treatment, but the specific mechanism of HIF-1α in this process remains to be explored.

# Targeting HIF-1α directly to overcome drug resistance

Hypoxia-induced overexpression of HIF-1α is an essential factor that induces drug resistance in breast cancer. Therefore, targeting HIF-1α is expected to overcome therapeutic resistance caused by HIF-1α in breast cancer and improve therapeutic efficacy. The drug mechanisms that directly target HIF-1α mainly include inhibiting transcription and translation of HIF-1α and promoting its degradation. Several potential approaches for targeting HIF-1α in breast cancer are described and summarized below (Table 1).

## Inhibitors of HIF-1α translation

KC7F2, a lead compound with a cysteamine center structure, has been reported to reduce HIF-1α protein levels in a dose-dependent manner (104). Western blotting analysis was performed on LN229 cells incubated with different concentrations of KC7F2 for 6 h under hypoxia conditions and demonstrated that HIF-1α protein levels specifically decreased with the increase of KC7F2 concentration, while β -actin level was basically unaffected. The same results were observed in U251MG, PC3, and MCF-7 cell lines.

Digoxin, a cardiac glycoside, which FDA has identified as a potential inhibitor of HIF-1 activity, has been reported to repress HIF-1α translation. In a study, Hep3B cells were exposed to vector (-) and 100 nM digoxin (+) under hypoxia, followed by a western blot analysis, displaying a significant decrease in HIF-1α protein levels in the digoxin exposed group (105). Another study using digoxin inhibiting HIF-1 from treating breast cancer modeling mice found that the group treated with digoxin had a 78% reduction in tumor growth and a 94% reduction in ipsilateral axillary LN metastasis compared to the control group (106). The mechanism by which cardiac glycosides inhibit HIF-1α may be ROS production

TABLE 1 Overview of drugs that inhibit HIF-1 activity reported in breast cancer.

| Mechanism | drug name | target | status | Ref |
|---|---|---|---|---|
| Inhibition of HIF-1α translation | KC7F2 | DNA binding | preclinical | (104) |
| | Digoxin | unknown | approved | (105, 106) |
| Inhibition of HIF-1α stabilization | AT-533 | Hsp90 | preclinical | (107) |
| | STA-9090 | | clinical trial | (108, 109) |
| Inhibition of the binding of HIF-1α to the HRE | Echinomycin (NC-13502) | HRE | suspendend | (59) |
| | liposomal-echinomycin | | preclinical | (60) |

leading to HIF-1α ubiquitination and degradation, which is still under investigation.

### Inhibitors of HIF-1α stabilization

HSP90 is a molecular chaperone, and its binding to HIF-1α stabilizes the activity of HIF-1α by blocking VHL-independent proteasome degradation and helping HIF-1α isodimer obtain appropriate conformation to recruit P300 (110). AT-533, a novel Hsp90 inhibitor, is considered a potential candidate for breast cancer treatment, as it inhibits breast cancer growth and angiogenesis by blocking HIF-1α/VEGF/VEGFR-2 signaling pathway (107). Ganetespib (formerly STA-9090) is also a unique Hsp90 inhibitor capable of rapidly inducing degradation of known Hsp90 client proteins (such as HIF-1α) (108, 109). In orthotopic MDA-MB-231 and MDA-MB-435 tumor models, Ganetespib treatment significantly impaired primary tumor growth and inhibited local tumor invasion and distant tumor metastasis to regional lymph nodes and lungs (111).

### Inhibition of the binding of HIF-1α to the HRE

HRE is the DNA binding site of HIF-1α, which promotes the expression of HIF-1α-related target genes. Echinomycin (NC-13502) has a strong hypoxic selective cytotoxicity by inhibiting the binding of HIF to VEGF promoter HRE but does not affect HIF to AP-1 or NF-κB promoter HRE (59). Chromatin immunoprecipitation studies have shown that echinomycin can also inhibit HIF-1 binding to DNA. It was reported that the liposomal -echinomycin can effectively inhibit HIF-1α transcriptional activity of primary and metastatic TNBC cells and inhibit tumor growth *in vivo* (60). In the study, liposome-echinomycin treatment in xenograft mice (MDA-MB-231 and SUM-159) significantly inhibited tumor volume and almost eradicated liver and lung metastases in both models compared with the control group.

## The limitations of clinical application of HIF-1α inhibitors

Although these compounds targeting HIF-1α have shown efficacy *in vitro*, HIF-1α inhibitors still have several limitations. First, differential expression of HIF-1α limits the efficacy of anti-HIF-1α therapy. Second, HIF-1α inhibitors monotherapy have limited efficacy (61). In a phase II clinical trial, 2ME2 NCD showed no efficacy in patients with renal cell carcinoma (61). Similarly, in another Phase II trial, 17-AAG (tanespimycin), a potential HSP90 inhibitor that increased HIF-1α degradation, did not achieve objective response rates in the treatment of metastatic RCC (62). Because no clinical trials investigated HIF-1α inhibitors monotherapy in breast cancer yet, the efficacy of HIF-1α inhibitors monotherapy in breast cancer may not satisfactory either. Finally, HIF-1α was measured by western blotting, real-time quantitative PCR (RT-PCR), and immunostaining. These detection methods are mainly used in cancer research. The clinical implementation requires invasive tissue biopsy which may not be feasible to patients with late-stage breast cancer. Therefore, non-invasive tests for HIF-1α are highly desired.

Given the limitations of HIF-1α inhibitors, the combination of HIF-1α inhibitors with chemotherapeutic agents or other agents may achieve an optimal efficacy. Because the relevant combination therapies in breast cancer still stay in the pre-clinical stage, we are unable to draw a solid conclusion yet. Nevertheless, a previous preclinical study reported that digoxin increased the sensitivity of triple negative breast cancer to paclitaxel and gemcitabine *in vivo* (80). Acriflavine, a HIF-1α dimerization inhibitor, has also been reported to enhance the antitumor activity of sunitinib in 4T1 breast cancer models (112). Additional data from *in vitro* and *in vivo* studies are urgently needed to determine whether the use of HIF-1α inhibitors in combination with current therapies may be beneficial for breast cancer patients. There is also an urgent need for combinations of HIF-1α inhibitors to be tested in clinical trials, especially in patients with drug resistance.

## Conclusion

Since the HIF family transcription factors were first discovered nearly 30 years ago, great progress has been made in understanding their regulation and role in physiology and pathophysiology. This achievement ultimately led to the awarding of the 2019 Nobel Prize in Physiology or Medicine for the discovery of HIF, the clinical approval of multiple therapies affecting upstream and downstream targets of the HIF subtype, and the clinical development of first-in-class selective inhibitors of HIF-2α. HIF subtypes may play complementary roles in driving tumor progression due to their nonoverlapping spatiotemporal regulation in tumor cells and tumor microenvironment (TME) cells. In brief, HIF-1α promotes metabolic reprogramming in tumor cells and TME cells, while HIF-2α induces an aggressive stem-like phenotype within tumor cells, and both contribute to angiogenesis and the production of tumor-licensed TME.

Tumor cell resistance to therapeutic drugs is a thorny problem, limiting the success in the clinical treatment of breast cancer. HIF-1α is upregulated in different breast cancer subtypes and is associated with poor prognosis and drug resistance in breast cancer. HIF-1α confers resistance to conventional therapies

through several signaling pathways involved in BCSC enrichment, drug outflow, apoptosis, and autophagy. At present, many compounds can directly or indirectly inhibit HIF-1α to alleviate drug resistance, but most of them are limited by poor efficacy or large toxic side effects *in vivo*, which may present challenges in the future. Due to the inherent limitations of cellular and animal models, a deep understanding of the role of HIF transcription factors in TME in clinical setting is critical to understanding the determinants of therapeutic resistance and to develop relevant compounds targeting them. Given these problems, we can consider the optimization method from the following three points. First, in addition to HIF-1α, HIF-2α has also been associated with histological grade, Ki67 expression, and multidrug resistance in breast cancer (113). Therefore, further exploration of HIF-2α may find another effective drug target. Second, HIF-1α varies between and within breast cancer subtypes and considering breast cancer patient selection may help screen for effective drugs. Finally, it can be considered that delivering drugs with nanoscale liposomes may reduce side effects and achieve both efficacy and safety. Most drugs remain in development, and some are in clinical trials, so combinations of drugs targeting HIF-1α and other drugs are unavailable. No single therapy can completely solve the problem of breast cancer drug resistance, so combination therapy is the best option in the future. Because inhibition of one HIF-α subtype tends to induce expression of the remaining subtypes, we propose that, in some cases, direct targeting of these two HIF subtypes may provide more benefits than targeting each subtype alone.

## Author contributions

FC and YW designed the conceptualization; LY and ST wrote the manuscript. HY, HZ, and YZ made manuscript review and critical comments. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Conducive target range of breast cancer: Hypoxic tumor microenvironment

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Breast cancer is a kind of malignant tumor disease that poses a serious threat to human health. Its biological characteristics of rapid proliferation and delayed angiogenesis, lead to intratumoral hypoxia as a common finding in breast cancer. HIF as a transcription factor, mediate a series of reactions in the hypoxic microenvironment, including metabolic reprogramming, tumor angiogenesis, tumor cell proliferation and metastasis and other important physiological and pathological processes, as well as gene instability under hypoxia. In addition, in the immune microenvironment of hypoxia, both innate and acquired immunity of tumor cells undergo subtle changes to support tumor and inhibit immune activity. Thus, the elucidation of tumor microenvironment hypoxia provides a promising target for the resistance and limited efficacy of current breast cancer therapies. We also summarize the hypoxic mechanisms of breast cancer treatment related drug resistance, as well as the current status and prospects of latest related drugs targeted HIF inhibitors.

KEYWORDS

**hypoxic microenvironment, hypoxia, breast cancer, target, drug resistance**

## Introduction

Breast cancer is the cancer type with the highest prevalence, and despite therapeutic advances, still has the second highest cancer-related mortality rate in women (1). One of the main reasons why tumors are difficult to treat is that tumor cells constantly adapt to the adverse environment in which they are exposed. Hypoxia is one of the typical adverse environment, which weakens the function of the tumor. However, malignant tumor cells are often able to compensate for the process of hypoxia and drive the occurrence of later more malignant disease behaviors (2).

Oxygen is essential for energy metabolism, which drives cellular bioenergetics (3). According to Data from a study describing the pretreatment oxygenation status, Oxygen tensions measured in normal breast tissue revealed a mean pO2 of 65 mmHg, whereas in

breast cancers of stages T1b-T4, the mean pO2 was 28 mmHg (4). The regions with low oxygen level is generally termed as hypoxic region, which is recognized as a typical microenvironment feature in nearly all solid tumors. Two mainly reasons leading to microenvironment hypoxia can be summarized as follows (1):As most tumor cells are in a state of rapid proliferation and high metabolism, oxygen consumption is far greater than supply, resulting in continuous decline of oxygen content in the microenvironment, and finally formation of hypoxia microenvironment (2, 5).Hypoxia tumor cells secrete vascular endothelial growth factor(VEGF)and other pro-vascular factors to accelerate the regeneration of tumor blood vessels. The density of tumor microvessels was increased, but these vessels were abnormal in structure, which made microvessels unable to regulate blood flow, resulting in hyperperfusion hypoxia (6).

The presence of hypoxic regions is one of the independent prognostic factors for breast and other cancers. Tumor cells, while adapting to hypoxia, lead to more aggressive and therapeutically resistant tumor phenotypes. Hypoxic tumor microenvironment can promote metastasis of tumor cells, inhibits the immune response to tumor cells and changes gene expression, ultimately limiting patient prognosis (7). For example, tumor-associated macrophages (TAMs) have been shown to be associated with poor prognosis of cancer and are predominately localized in the hypoxia regions of tumor. It was found that hypoxia-induced galectin-3 expression and secretion from TAMs promotes tumor growth and metastasis the in orthotopic syngeneic mammary adenocarcinoma model and metastasis model (8).

Considering such changes taken place in hypoxic tumor microenvironment, exploiting for selectively targeting hypoxic areas in breast cancer is an attractive strategy. Some mechanisms of hypoxia leading to drug resistance are being elucidated and drug delivery research has been moving to innovative strategies for breast cancer including engineered nanoparticle based drug/gene delivery systems (9–11). In this review, we briefly discussed microenvironmental changes caused by hypoxia, which are mainly metabolic, genetic and immune levels, and systematically summarized promising advances in targeted hypoxia therapy for breast cancer.

## Hypoxia-inducible factors and breast cancer

The response of cancer cells to hypoxia is principally ascribed to its transcriptional factors HIFs which includes three members, and they are heterodimers composed of an O2 sensitive α subunits (HIF-1α,or HIF-2α,or HIF-3α) and an O2 insensitive HIF-1β subunit (12, 13). HIF-1α is the most well-characterized isoform of the HIFs (14). In normoxiais, it is easily degraded by the ubiquitin-protease hydrolysis complex. Therefore, HIF-1α subunit is virtually undetectable in cells with normal oxygen saturation (15–17). Under hypoxia, degradation of HIF-1α subunit is inhibited and the 1α and 1β subunits form active and stable HIF-1, which is transferred into the nucleus to regulate transcription of multiple genes (18–20). HIF-2α and HIF-3α are two closely related homologues of HIF1α. HIF-1α and HIF-2α share very similar characteristics including their abilities to heterodimerize with HIF-1β, binding to hypoxia-inducible genes and transcriptional activation, but they show different specificity in different tissues and transcriptional targets (21–23). HIF-1α mediated mechanisms favor up-regulation and down-regulation of genes involved in tumor growth and malignant progression as well as epigenetic modification, while HIF-2α stimulates some, but not all, genes activated by HIF-1α. HIF-3α acts as a negative regulator of HIF-1α and HIF-2α mediated gene expression where it can dimerize with HIF-1β and indirectly inhibit HIF-1α and HIF-2α activity (24, 25).

Breast cancer shows extensive clinical and molecular heterogeneity. Prognostic factors are very important for outcome estimation in individual patients. HIF-1 is an important transcription factor in the adaptation of tumor cells to hypoxia, and directly or indirectly regulates cell proliferation and angiogenesis during the progression of tumor hypoxia microenvironment gene expression related to apoptosis and energy metabolism, whose transcriptional activity is a significant positive regulator of tumor progression and metastasis potential (25, 26). Many studies have shown that HIF-1α is overexpressed in breast cancer (27), and HIF-1α has been identified as an independent prognostic factor of breast cancer, and its high expression is significantly associated with poor DFS and OS in breast cancer patients (28–31). A meta-analysis of 5177 patients showed that high HIF-1α expression was associated with high Ki67 expression and strong VEGF expression in advanced breast cancer with lymph node metastasis positive lymph node status negative ER state ductal advanced histological grade (28).In another population-based case-control study evaluating breast cancer recurrence, HIF-1α expression may be associated with early recurrence in patients with ER-breast cancer (32).Additionally, patients with high expression of HIF may be resistant to chemotherapy and endocrine drugs, leading to treatment failure (31, 33).

## The growth and metastasis of breast tumor cells in hypoxic microenvironment

Hypoxia plays an important role in tumor growth and development, related processes include aerobic glycolysis,

angiogenesis, immune cells induced to aggregate, and epithelial-mesenchymal transition(EMT).

In the microenvironment of breast cancer, hypoxia activates metabolic changes, from oxidative phosphorylation to a more aerobic glycolytic metabolism (34). Maximum glucose uptake and efficient glucose utilization provide a foundation for glycolysis respiration, thus helping hypoxia cells adapt to the tumor microenvironment, and supporting biological activities such as tumor proliferation, invasion and migration (35, 36). Hypoxia activates transcription factors HIF-1α and FoxO1 and induces epigenetic reprogramming to up-regulate cytoplasmic phosphoenolpyruvate carboxylated kinase (PCK1), a key enzyme that initiates gluconeogenesis, triggering retrograde carbon flow from gluconeogenesis to glycogen decomposition and pentose phosphate pathways. The resulting NADPH promotes the production of reduced glutathione, leading to a moderate increase in reactive oxygen species (37). Tumor stem cells (CSCs) are strongly correlated with tumor progression, metastasis, recurrence and enhanced treatment resistance, and their maintenance of stemness benefits from glycolysis. Peng F et al. found that dehydrogenase kinase 1(PDK1), an important glycolysis enzymes, elevated through the H19/let-7/HIF-1α signal axis, and that downregulation of PDK1 significantly inhibits H19-mediated glycolysis and CSC maintenance. Interestingly, aspirin can significantly attenuate glycolysis and cancer stem-like features by inhibiting H19 and PDK1, providing a potential therapeutic strategy for breast cancer (38). IL-32, known as a pro-inflammatory cytokine, is overexpressed in many types of cancer and enhances tumor cell migration and invasion. Hypoxia-induced reactive oxygen species (ROS) enhances the expression of IL-32β, leading to the activation of IL-32β prolongation of Src, which is involved in the increase of glycolysis and the production of vascular endothelial growth factor (VEGF) under hypoxia (39). Therefore, inhibition of the above targets and pathways may be a therapeutic strategy for inhibiting glycolysis in breast cancer, thereby inhibiting the proliferation and metastasis of tumor cells (40).

Tumor cells grow out of control in tumor tissues, and their internal neovascularization network cannot be established in a timely and effective manner. Therefore, hypoxia controls tumor angiogenesis and malignant progression by regulating the expression of various carcinogenic molecules (41). HIF1α can directly induce the expression of VEGF at the transcriptional level and promote angiogenesis (42). Non-receptor protein tyrosine kinases Syk and Lck play an important role in signal transduction mechanisms of various cellular processes. And their cross-talk regulates hypoxia/reoxygenation (H/R) induces breast cancer progression and further regulates the expression of melanoma cell adhesion molecule (MelCAM) urokinase-type plasminogen activator (uPA) matrix metalloproteinase-9 (MMP-9) and VEGF (43). Immunohistochemistry of 45 patients of breast cancer showed that high levels of HIF1α were positively correlated with increased microvascular density (a measure of angiogenesis) (P=0.023) and with expression of angiogenic growth factors bFGF and PDGF-BB and receptor EGFR (44). Thus, drugs targeting HIF-1 may bind to different pathways that inhibit breast cancer growth, including angiogenesis and growth factors. Tumor-associated immune cells in the hypoxic microenvironment, also play a role in the expression of angiogenesis related signals. HIF-1α/VEGF-A axis is an important pathway for T cells to adapt to the hypoxia microenvironment Analysis of human breast cancer showed that VEGF-A expression was negatively correlated with CD8+ T cell infiltration, and there was a relationship between T cell infiltration and vascular formation (45). TAMs preferentially migrate to the hypoxic region and not only mediate the inhibition of T cells (46), but also directly upregulate angiogenic molecules(VEGF, FGF2, CXCL8, IL-8, type I receptor for VEGF, angiopoietin) or though upregulating of angiogenic modulators (COX2, iNOS, MMP7) to promote angiogenesis (47). In addition, under hypoxia, CAFs can activates VEGF promoters though a transduction pathway formed by HIF1α and its target gene, G-protein estrogen receptor (GPER) (48). Therefore, T cell, TAMs and CAFs play a role in hypoxia-dependent tumor angiogenesis.

Cell culture *in vitro* found that breast cancer cells under the condition of hypoxia training than the cells cultured under the condition of constant oxygen has significant motility (49). EMT is an important biological process for malignant tumor cells to acquire the ability of invasion and metastasis (50). Complete EMT made the epithelial cancer cells transforming into mesenchymal cells and occurring mesenchymal migration or amebic migration. Partial EMT retained the properties of both epithelial cells (cell adhesion) and mesenchymal cells (motility), leading to collective migration of cells, characterized by the presence of leader cells (mixed E/M state) and follower cells (epithelial state) at the front of the invasion, forming the body of the cell population (51). HIF-1α regulated many molecules involved in EMT, for example, HIF-1α regulated TGFβ1/SMAD3 signaling pathway, promoting breast cancer metastasis (52). E-cadherin promoted collective migration of mixed E/M phenotypes by inhibiting TGF-β, while activation of TGF-β leaded to single cell migration (53). Colony stimulating factor 1(CSF-1) played a key role in the control of EMT. Under hypoxia, HIF-1α induced a mixed E/M phenotype through its target gene CSF-1, promoting collective migration (54). Hypoxia leaded to the activation of EMT genes, including TWIST1, SLUG and SNAIL, by degrading PER2, which was considered to be a tumor suppressor, and disrupting the PER2 repression complex (55). X-C Motif chemokine Ligand 1 (XCL1) enhanced expression of HIF-1α and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, which induces EMT and imposes migration of breast cancer cells (56). Therefore, hypoxia-induced EMT is essential for invasion and metastasis of breast cancer cells. And EMT phenotypes are also associated with stem cell and drug resistance, so further exploration of the

molecular details of this process could help develop new therapeutic targets.

## Genomic instability of hypoxia

Intratumoral hypoxia promotes genomic instability, another hallmark of most cancers. It is estimated that up to 1.5% of the human genome is transcriptional responsive to hypoxia (57). In recent years, many genomic changes identified as responsive to hypoxia, may serve as prognostic or predictive markers or even as new therapeutic targets (58). Since increased activity of the HIF-1α pathway is associated with more severe intratumor hypoxia in basal-like breast tumors compared to other subtypes, the gene signature may guide the potential use of future antihypoxia drugs (59–61).

### Hypoxia related DNA

Tumor cells adapt to the hypoxia microenvironment by activating hypoxia-inducible factors to induce the expression of gene products, which are involved in angiogenesis, metabolic reprograming, tumor invasion and metastasis resistance, etc (62). In order to evaluate the changes of hypoxia-induced transcription profile of breast cancer cells, I Chae Ye exposed 31 breast cancer cell lines or normal human breast epithelial cells to either 20% or 1% oxygen. The result showed that in each cell line, more than 1000 genes are induced or inhibited in response to hypoxia, of which 42 genes have conserved responses to hypoxia (63). And all these gene responses under hypoxia were induced by HIF-1α or HIF-2α. Therefore, HIF, as the most important transcription factor in the hypoxic microenvironment, induces a series of changes at the gene level. These hypoxia gene features are meaningful prognostic markers for breast cancer patients and may provide a group of powerful hypoxia treatment targets for the clinic (59, 64, 65).

Studies showed that human breast cancer cells exposed to hypoxia are enough to induce the expression of ADAM12 in a HIF-dependent manner, leading to the shedding of HB-EGF outfield, enhancing EGFR signaling pathway propagation and downstream activation of focal adhesion kinase (FAK) to trigger the breast cancer cells of motility, invasion and metastasis (66). ZMYND8 is acetylated by HIF coactivator P300 in breast cancer cells. And then through the ZMYND8/P300/BRD4/HIF axis, increases angiogenesis, promotes breast tumor progression and metastasis (67). High mobility group box 1 (HMGB1), an important factor in cancer occurrence and development was up-regulated in breast cancer tissues. It regulated hypoxia-inducible factor 1 through the PI3K/AKT signaling pathway, resulting in angiogenesis and tumor migration of breast cancer cells (68). Analysis of previous clinical data shows that basal-like tumors which have the highest rates of metastasis and recurrence are among breast cancer tumors, are associated with higher JFK expression levels and poorer overall survival (69). HIF-1α protein can directly activate JFK transcription, which in turn leads to HIF-1α-induced glycolysis and make hypoxic breast cancer cells insensitive to chemo-radiotherapeutic treatment. In general, the HIF-1α-JFK axis enhances cell tolerance to hypoxia, promotes breast cancer cell survival (70). XBP1 drove TNBC tumorigenicity by regulating the expression of HIF-1α targets through RNA polymerase II recruitment (71). CLDN6 is a tumor suppressor gene for breast cancer. CLDN6, up-regulated by HIF-1α transcription, prevents HIF-1α desulfidation and ultimately leading to HIF-1α degradation through binding the transcription factor β-catenin in the cytoplasm (72).

### Hypoxia related non-coding RNA

MicroRNAs(miRNAs) are endogenous, small non-coding single-stranded RNAs that negatively regulate gene and protein expression primarily by binding to their selective messenger RNAs (mRNAs) (73, 74). Currently, several miRNAs expressed in the hypoxic microenvironment of breast cancer have been identified, which may indicate greater prognostic and therapeutic potential (75). MiR-210 is widely regarded as a powerful HIF target, which is a direct result of decreased oxygen tension in the microenvironment (75, 76). Its expression level in breast cancer samples can be used as an independent prognostic factor (77–79), playing a role in glycolysis, DNA repair, cell survival, immune prediction, chemotherapy resistance, etc. Du Y et al. found that miR-210-3p specifically participated in the Warburg effect (aerobic glycolysis) in TNBC through modulating the downstream glycolytic genes of HIF-1α and p53 (80). In addition, miR-210 inhibits the expression of e-cadherin by targeting the open reading frame region of E-cadherin mRNA and upregulation of e-cadherin transcriptional inhibitor Snail in hypoxic microenvironment, thereby promoting the metastasis, proliferation and self-renewal of breast cancer stem cells (81). Trastuzumab is part of the standard treatment for patients with HER-2 positive breast cancer, but not all patients respond to trastuzumab. An analysis of miRNA expression levels in plasma samples from breast cancer patients showed that circulating miR-210 levels were significantly higher in patients with residual disease than in patients with pathological complete response before neoadjuvant chemotherapy combined with trastuzumab ($P = .0359$). Therefore, circulating miR-210 level may be associated with trastuzumab sensitivity, tumor presence and lymph node metastasis (82). Chemotherapy resistance is also a serious clinical challenge in breast cancer. MiR-210 regulates JAK-STAT signal transduction pathway by targeting PIAS4, thus affecting the sensitivity of breast cancer to chemotherapy (83).

Most studies on miRNAs in hypoxic microenvironments focus on miR-210, but there are still other miRNAs that respond to hypoxia. Emma Gervin et al. showed that hypoxia can up-regulate miR-655 expression in human breast tumors, which is associated with poor prognosis. In MCF7-miR655 cell lines, the expression of PTEN(negative regulator of HIF-1α) and NFκB1 (positive regulator of COX-2 and EP4) were regulated by down-regulating transcription factors NR2C2, SALL4 and ZNF207, thereby enhancing oxidative stress induced EMT and vascular mimicry (84). In addition, hypoxia and tumor stem cells (CSCs) contribute to paclitaxel (PTX) resistance, the molecular mechanism may be related to miRNA. The experimental data of Liu JH et al. showed that miR-526b-3p attenuates breast cancer stem cell characteristics and chemotherapy resistance by targeting HIF-2α/Notch signaling pathway, which may be used to alleviate chemotherapy resistance in breast cancer (85). MiR-135b may act as a regulatory factor of hormone receptor α(ERα). MiR-135b regulates the protein levels of ERα and HIF1AN by interacting with the 3'UTR region of ERα and HIF1AN (86). Also, miR-153 finely regulated HIF-1α/VEGFA axis by binding to the 3 UTR of HIF1A mRNA, which directly inhibits HIF-1α expression. In this respect, miR-153 can be used for anti-angiogenesis therapy in breast cancer (87).

Long Noncoding RNAs (lncRNAs) are transcripts with more than 200 nucleotides in length but limited protein-coding capacity (88). In the hypoxic microenvironment of breast cancer, some lncRNAs affect the survival and growth of breast cancer cells by regulating HIFs related pathways, providing directions for the possibility of selectively targeted hypoxia therapy (89). TNBC is the most urgent pathological type to be explored, among which three lncrnas are related to hypoxia: IHAT, GHET1 and MIR210HG. LncIHAT promotes the survival of mouse TNBC cells and lung metastasis through the expression of proximal adjacent oncogenes PDK1 and ITGA6 in TNBC cells (90). LncRNA GHET1 leads to over activation of Hippo/YAP signaling pathway, promoting hypoxia-induced glycolysis proliferation and invasion of TNBC (91). MIR210HG directly binds to the 5'-UTR of HIF-1α mRNA, leading to an increase in HIF-1α protein level, thereby upregulating glycolytic enzyme expression (92). In addition to, Zheng F et al. demonstrated that HIF-1α antisense lncRNA HIFAL is essential for maintaining and enhancing HIF-1α mediated retrotranscriptional activation and glycolysis by introducing the PKM2/PHD3 complex into the nucleus. Clinically, targeting lncRNA HIFAL and HIF-1α significantly reduced their impact on tumor growth (93). LncRNA PCAT-1, elevated in breast cancer patients, directly interacts with the activated protein C kinase-1 (RACK1) protein to prevent RACK1 binding to HIF-1α, thereby protecting HIF-1α from RACK1-induced oxygen-dependent degradation of lncRNA (94). Rab11b-as1 enhances the expression of angiogenic factors including VEGFA and ANGPTL4 in hypoxia breast cancer cells by increasing the recruitment of RNA polymerase II, promoting tumor angiogenesis and distant metastasis of breast cancer *in vitro* (95). Hypoxia-induced lncRNA KB-1980E6.3 is abnormally up-regulated in clinical breast cancer tissues. The KB-1980E6.3/IGF2BP1/C-MYC axis maintained the stemness of BCSCs (96). LncRNA NEAT1 is a direct transcription target of HIF-2. It is induced by hypoxia to accelerate the proliferation of breast cancer cells, improve clone survival rate, and reduce apoptosis (97). One of the important mechanisms of lncRNA in hypoxia-related pathways is to antagonize the biological function of miRNA like a sponge (98). LncRNA MALAT1 in hypoxia response can be transcriptionally activated by HIF-1α and HIF-2α, acting as a molecular sponge for miR-3064-5p to promote tumor growth and migration of breast cancer cells (99). LncRNA Vcan-as1 compete with miR-106a-5p, promoting its progression by regulating the miR-106a-5P-mediated STAT3/HIF-1α pathway (100). Phosphoglycerate kinase 1 (PGK1) is an important part of the glycolysis pathway. Zhong Chu et al. found that hypoxia inhibits the expression of LINC00926 which activates the expression of PGK1 mainly through FOXO3A (101). Above, lncRNAs play an important regulatory role in the relevant pathways of breast cancer cells adapting to hypoxia, especially in triple negative breast cancer (102). Therefore, focus on hypoxia related lncRNAs of their potential impact on prognosis and treatment will help predicting new therapeutic agents and exploring mechanisms of drug intervention strategies.

Circular RNAs(CircRNAs) are single-stranded RNA transcripts without 5 caps or 3 polya-tails, but covalently closed ring structures formed by pre-mrna passage and delivery after delivery. CircRNAs mainly target miRNA, act as miRNA sponges, indirectly regulate functional proteins, and participate in cancer progression and hypoxia regulation (103). For example, circDENND4C, which is verified as a sponge for mir-200b and mir-200c, is up-regulated in hypoxia, boosting glycolysis, migration and invasion of breast cancer cells (104). CircRNF20 is highly expressed in BC under hypoxia, through circRNF20/miR-487a/HIF-1α/HK2 axis promoting Warburg effect (105). CircZFR acts as a sponge for miR-578 in BC tissues and cells, promotes the progression of BC malignancy by regulating miR-578/HIF-1α axis (106). Furthermore, Yanxia Zhan et al. screened circRNA differentially expressed between hypoxic and normoxic cancer-associated fibroblasts(CAFs) exosomes by array analysis. The expression of circHIF1A up-regulated in hypoxic CAFs. By which, miR-580-5p has been sponged to modulate dryness of breast cancer cells (107). In addition to competitively antagonizing miRNA, circRNA also has other mechanisms to play a role. CircWSB1 was up-regulated by HIF1α transcription and competitively binds to the deubiquitinase USP10, preventing p53 access to USP10 in BC cells, leading to the degradation of p53 and tumor progression of BC (108). Table 1

TABLE 1 Coding and non-coding transcriptome in hypoxic TME.

| DNA/RNA | Expression under hypoxia | Signaling pathways | Function | Reference |
|---|---|---|---|---|
| **DNA** | | | | |
| ADAM12 | Up-regulated | EGFR/FAK signaling pathway | Triggering motility, invasion and metastasis | (66) |
| ZMYND8 | Up-regulated | ZMYND8/P300/BRD4/HIF axis | Angiogenesis | (67) |
| HMGB1 | Up-regulated | PI3K/AKT signaling pathway | Angiogenesis | (68) |
| JFK | Up-regulated | HIF-1α-JFK axis | Enhancing cell tolerance to hypoxia | (69, 70) |
| XBP1 | Up-regulated | Recruitment RNA polymerase II | Driving TNBC tumorigenicity by regulating HIF-1α targets | (71) |
| CLDN6 | Up-regulated | Binding the transcription factor β-catenin | Leading to HIF-1α degradation | (72) |
| **miRNAs** | | | | |
| miR-210 | Up-regulated in TNBC | Downstream glycolytic genes of HIF-1α and p53 | Activating aerobic glycolysis | (80) |
| | Up-regulated in BCSC | E-cadherin mRNA | Up-regulating Snail, promoting the self-renewal of BCSC | (81) |
| | Up-regulated in patients with residual disease | – | Associated with trastuzumab sensitivity | (82) |
| | Up-regulated | JAK-STAT signaling pathway | Affecting the sensitivity to chemotherapy | (83) |
| miR-655 | Up-regulated | Regulating PTEN and NFκB1 by NR2C2, SALL4 and ZNF207 | Enhancing EMT and vascular mimicry | (84) |
| miR-526b-3p | Up-regulated | HIF-2α/Notch signaling pathway | Alleviate chemotherapy resistance | (85) |
| miR-135b | Up-regulated | 3'UTR region of ERα and HIF1AN | Regulating the protein levels of ERα and HIF1AN | (86) |
| miR-153 | Up-regulated | HIF-1α/VEGFA axis | Angiogenesis | (87) |
| **lncRNAs** | | | | |
| IHAT | Up-regulated in TNBC | PDK1 and ITGA6 | Promoting the survival of TNBC cells and lung metastasis | (90) |
| GHET1 | Up-regulated in TNBC | Hippo/YAP signaling pathway | Promoting hypoxia-induced glycolysis, proliferation and invasion | (91) |
| MIR210HG | Up-regulated in TNBC | 5'-UTR of HIF-1α mRNA | Upregulating glycolytic enzyme expression | (92) |
| HIFAL | Up-regulated | antisense RNA of HIF-1α | Enhancing HIF-1α mediated retrotranscriptional activation and glycolysis | (93) |
| PCAT-1 | Up-regulated | RACK1 | Protecting HIF-1α from RACK1-induced oxygen-dependent degradation of lncRNA | (94) |
| Rab11b-as1 | Up-regulated | RNA polymerase II | Enhancing the expression of angiogenic factors | (95) |
| KB-1980E6.3 | Up-regulated | KB-1980E6.3/IGF2BP1/C-MYC axis | Maintaining the stemness of BCSCs | (96) |
| NEAT1 | Up-regulated | a direct transcription target of HIF-2 | Accelerating proliferation, reducing apoptosis | (97) |
| MALAT1 | Up-regulated | miR-3064-5p | Promoting tumor growth and migration of breast cancer cells | (99) |
| Vcan-as1 | Up-regulated | miR-106a-5P-mediated STAT3/HIF-1α pathway | Activating the STAT3 pathway reversed miR-106a-5p-mediated antitumor effects | (100) |
| LINC00926 | Down-regulated | FOXO3A/PGK1 signaling pathway | Promoting hypoxia-induced glycolysis | (101) |
| **circRNAs** | | | | |
| circDENND4C | Up-regulated | mir-200b and mir-200c | Boosting glycolysis, migration and invasion | (104) |
| circRNF20 | Up-regulated | mir-487a/HIF-1α/HK2 axis | Promoting Warburg effect | (105) |
| circZFR | Up-regulated | mir-578/HIF-1α axis | Boosting malignant progression | (106) |
| circHIF1A | Up-regulated in CAF | mir-580-5p | Modulating dryness of BC cells | (107) |
| circWSB1 | Up-regulated | deubiquitinase USP10 | Leading to the degradation of p53 and tumor progression | (108) |

## Hypoxia-mediated immunosuppressive activity

Extreme hypoxia and aberrant HIF-1 activity in the tumor TME are obstacles to effective immunotherapy. In this setting, infiltration and activity of CD8+ T cells are reduced, whereas tumor associated macrophages(TAMs), regulatory T cells (Tregs) and bone marmo-derived suppressor cells (MDSCs) show higher activity. Hypoxic TME also impages cancer-associated fibroblasts (CAFs) and natural killer (NK) cell maturation and activity. Furthermore, hypoxic TME is positively correlated with immune checkpoint expression. These alterations suggest the need for hypoxic regulation as a

complementary targeting strategy for immune checkpoint inhibitor (ICI) therapy.

## Innate immunity

Hypoxia can negatively regulate innate antitumor cells in the microenvironment and some key mechanisms. TAMs adopt M1-like proinflammatory phenotypes in the early stages of tumorgenesis and mediate immune responses that inhibit tumor growth. Hypoxia induces the production of a large number of migration stimulators, such as VEGF, EGFR, CCL2, CCL5, CSF-1, oncostatin M, succinate, eotaxin and GM-CSF, produced in the stroma of tumor cells and hypoxic regions (109–112). These stimulators lead to the recruitment of TAM and transformation of M2-like (113), which further promotes its involvement in tumor support processes such as immunosuppressive angiogenesis. Hypoxic TAMs strongly upregulate the expression of REDD1. REDD1-mediated inhibition of mTOR can hinder glycolysis of TAMs and inhibit their excessive angiogenic response, thus forming abnormal blood vessels (114). HIF-1α is a positive regulator of macrophage-derived VEGF. Knockdown the HIF-1α in TAMs attenuates its pro-angiogenic response (115). In addition, it has been recently reported that HIF-1α can up-regulate the expression of PD-L1 in tumor-infiltrating macrophages, thereby promoting the immunosuppressive TME (116, 117).

NK cells are immune cells that kill both virus-infected and tumor cells without antigenic stimulation. The studies of Solocinski and Teng showed that hypoxic stress impaired NK cell cytotoxicity by reducing ERK and STAT3 phosphorylation (118, 119). Ni et al. found that the transcription factor HIF-1α can inhibit NF-KB signaling in tumor-infiltrating NK cells, which is drived by IL-18 to exert antitumor activity (120). However, Seon et al. presented evidence that NK cells stabilized and upregulated their target genes BNIP3, PDK1, VEGF, PKM2 and LDHA by HIF-1α under hypoxia, which activate the ERK/STAT3 pathway to reprogram preactivated NK cells. These reverse the impaired NK effector phenotype and generate necessary number of functional NK cells for adoptive cell therapy (121).

MDSCs have immunosuppressive activity, allowing cancer to escape immune surveillance and not respond to immune checkpoint blockade. HIF-1a enhances the expression of miR-210 in tumor-localized MDSC. MiR-210 regulates Arg1, Cxcl12 and IL16 at both mRNA and protein levels to enhance the immunosuppressant activity of MDSC *in vivo* (122). Deng et al. found that HIF-1α binding to a conserved hypoxic response element in the VISTA promoter, thereby upregulated VISTA in MDSCs. Antibody targeting or gene ablation of VISTA could alleviate MDSC-mediated T-cell inhibition and may mitigate the harmful effects of hypoxia on anti-tumor immunity (123).

Stromal fibrosis is a common event in hypoxic TME. CAFs are considered to be the main component of fibrous matrix and can be activated by tumor hypoxia (124). Hypoxia up-regulates the transcription target of HIF-1α, namely G protein estrogen receptor (GPER), that makes CAF-induced IL-1β to express IL1R1 in breast cancer cells (125, 126). HIF-1α/GPER signaling pathway is also involved in the regulation of VEGF expression in breast cancer cells and CAFs exposed to hypoxia (48). Knockdown GPER in CAFs inhibited the invasion of breast cancer cells induced by CAF conditioned medium (125). Figure 1

## Acquired immunity

Hypoxic TME inhibits the proliferation and differentiation of CD4+T cells and CD8+T cells mainly by inducing the recruitment and activation of regulatory T cells (T(reg)), initiating autophagy and depletion of T cells, jointly resulted in acquired immune suppression (127, 128). HIF-1 is a key metabolic sensor regulating the balance of T(reg) cells and T(H) 17 differentiation. HIF-1 enhances T(H)17 development through tertiary complex formation by recruiting IL-17 promoters with RORγt and P300. At the same time, HIF-1 weakens the development of T(reg) by binding Foxp3 for proteasomal degradation (129). In addition, tumor hypoxia induces the expression of CCL28, CXCL12 and CXCR4, selectively enhanced the recruitment of T(reg) cells, thereby inducing tumor tolerance and new angiogenesis (130–132).

Hypoxia impaired the ability of CD8+T cells in differentiation, proliferation, infiltration and lethality. VEGF-A is the main factor contributor to differential secretion from depleted CD8+T cells under hypoxia. It can promote the differentiation of PD-1$^{+}$TIM-3$^{+}$CXCR5$^{+}$ exhausted-like CD8+T cells and significantly affect the transport and killing ability of CD8+T cells (133). Reports have further shown that anti-VEGF treatment enhances CD8+T cell effector function and provides a mechanistic basis for combining anti-angiogenic and immunotherapeutic drugs in cancer treatment (134). Hypoxia reduces the O2 tension of CD8(+)T cells during activation, upregulates the expression of CD137(4-1BB) and CD25, secrets the immunosuppressive cytokine IL-10. These processes induces the phenotype of CD8+T cells conversing from effector cells to poor proliferation (135).

Hypoxia leads to T cell dysfunction, upon further antigenic stimulation, leads to a state similar to exhaustion. Hypoxia upregulates miR-24 in tumor cells and T cell, both endogenous and exogenous. Mir-24 inhibits the expression of MYC and FGF11 in T cells, thereby disrupting MFN1-mediated mitochondrial fusion. Loss of mitochondrial function generates intolerable levels of ROS, which promotes induction of T-cell exhaustion through phosphatase inhibition (136, 137). Adenosine and adenosine receptors(AR) are important components of hypoxia-related signaling pathways. Hypoxic

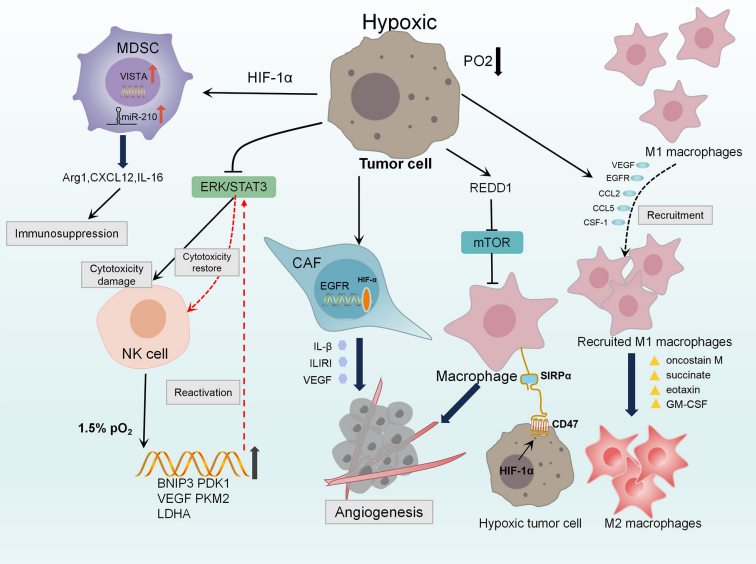


FIGURE 1
Diagram of the innate immunosuppression in hypoxic TME. Hypoxia induces the production of VEGF, EGFR, CCL2, CCL5, CSF-1 and other stimulators, leading to the recruitment and aggregation of TAMs. Oncostatin M, succinate, eotaxin and GM-CSF polarize M1 macrophages into M2 macrophages which demonstrate tumor-supporting and immunosuppressive functions. Hypoxia strongly up-regulates the expression of REDD1, it could inhibit mTOR to promote abnormal angiogenesis. HIF-1 directly up-regulates CD47, making breast cancer cells escape from macrophage-mediated phagocytosis through CD47-SIRPα axis. Hypoxia up-regulates GPER in CAFs, which is involved in the control of IL1R1, IL-β and VEGF, resulting angiogenesis and invasion of breast cancer cells. Hypoxia damages the cytotoxicity of NK cells by reducing the phosphorylation levels of ERK and STAT3. While Under 1.5% PO2, the ERK/STAT3 pathway reprograms preactivated NK cells through HIF-1α stabilization and higher expression of its target genes BNIP3, PDK1, VEGF, PKM2, LDHA to restore the cytotoxicity of NK cell. HIF-1a increases the expression of miR-210 in MDSC, regulating Arg1 Cxcl12 and IL16 to enhance immunosuppression of MDSC. Also, HIF-1a up-regulates VISTA in MDSCs mediating T cell inhibition.

TME up-regulates the expression of CD39 and CD73. The former is an exonucleoside triphosphate dihydrophosphate hydrolase (ENTPD1) that converts ATP/ADP to AMP. The latter is an exonucleoside 50 enzyme that converts AMP to adenosine (136, 138). Thus, hypoxic adenosine signaling negatively affects T cell activation and effects through adenosine A2A receptor (A2AR), inducing T cell apoptosis (139). At present, preclinical observations have shown that A2AR blockers and immune checkpoint inhibitors cooperate to induce tumor rejection with considerable results (140).

## Role of immune checkpoint blockade

Several important immune checkpoints have their own regulatory pathways. In hypoxic TME, almost all of them are directly transcriptional regulated by HIF. In the hypoxic adenosine pathway, CD73 encoded by NT5E gene is a key enzyme for adenosine production and has been considered as a potential immune checkpoint (141). Adenosine receptor has been found in DC, TAM, MDSC and NK cells, implying that adenosine produced by NT5E can inhibit cellular immune responses (142). Thus, NT5E has been identified as a target checkpoint molecule for novel tumor immunotherapy approaches (143). CD47 is an immunoglobulin overexpressed on the surface of cancer cells. CD47 forms a signaling complex with SIRPα expressed on phagocytes and other immune cells, which enables cancer cells to escape macrophage-mediated phagocytosis (144, 145). CD47 is directly regulated by HIF-1 in hypoxic breast cancer cells and plays an immune escape through the CD47-SIRPα axis (146). At present, 23 related drugs targeting CD47 have entered clinical trials and shown good effects (147). MiR-25 and miR-93 are two hypoxic response microRNAs. By targeting NCOA3, they down-regulate the expression of DNA sensor cGAS. This allows hypoxic tumor cells to escape the immune response elicited by the release of mitochondrial DNA, reveals direct link between hypoxic miRNAs and adaptive immune responses to hypoxic tumor microenvironment (148). Programmed death ligand 1(PDL1), which is expressed on the surface of cancer cells, binds to the receptor PD1 on the surface of CD8+T cells, thereby inactivating the antitumor response of CD8+T cells. Hypoxia significantly increases the expression of PD-L1 on MDSCs, TAMs and tumor cells. In addition, the upregulation of PD-L1 under hypoxia depends on the direct binding of HIF-1α to the transcriptional active HRE. Blocking PD-L1 under hypoxia enhances MDSC

mediated T cell activation. Therefore, blocking both PD-L1 and HIF-1α may be a promising approach for cancer immunotherapy (116). Figure 2

## Hypoxia-induced treatment resistance

Clinical studies have demonstrated that the components in the tumor hypoxic microenvironment are associated with poor prognosis in patients and can promote apoptosis and autophagy or inhibit DNA damage and mitochondrial activity through a number of signaling pathways associated with the failure of immunotherapy, chemotherapy, or radiation therapy (149, 150). This emphasizes that we need to decode the mechanism of hypoxia leading to drug resistance and take measures to promote sensitivity to treatment.

### Hypoxia and radiotherapy

There are good clinical evidences and systematic evaluations that hypoxia is a major negative factor influencing tumor radiation response (150). Preclinical studies in the early 1950s showed that cells can resist radiation damage when oxygen partial pressure is reduced below about 20 mmHg during irradiation (151). Radiation therapy kills cancer by producing ROS, which leads to DNA damage of recipient cells. However, in the case of hypoxia, free radicals produced by DNA under radiotherapy are reduced by molecules containing sulfhydryl group (SH), leading to DNA repair (152, 153). A great deal of efforts have been made to identify ways to overcome radiation resistance caused by hypoxia, including improving the availability of oxygen, increasing the sensitivity of radiotherapy or killing of hypoxia cells to improve the efficacy of radiotherapy.

Hypoxic activated prehaps (HAPs), also known as bioreduction prehaps, are chemically reduced to active compounds at low oxygen levels and target radiation-resistant hypoxic cells. Nevertheless, desirable results have not been achieved in HAPs coupled with radiation therapy (154), possibly due to the failure of the drugs to reach tumor hypoxic areas. Abbasi et al. designed a clinically applicable formulation of mixed manganese dioxide (MnO2) nanoparticles (MDNP) that uses biocompatible materials to react with endogenous H2O2 to regulate TME hypoxia. In a mouse model, approximately 40% of tumor-borne mice were tumor-free after a single treatment of MDNPs plus radiotherapy, 2.5 times lower than the dose required for treatment without MDNPs to achieve the same

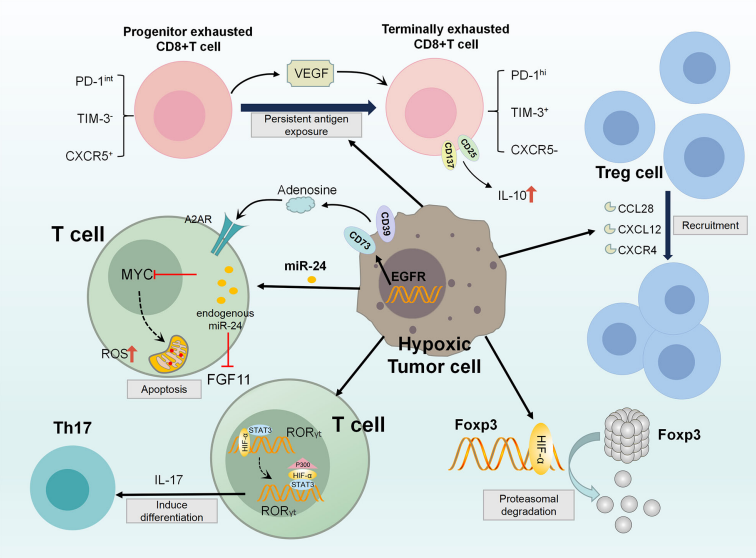


FIGURE 2
Diagram of the acquired immunosuppression in hypoxic TME. In the hypoxic TME, HIF-1 directly activates RORγt gene transcription in T cells, and then recruits P300 to the RORγt transcription complex to act as the promoter of the TH17 gene (IL-17). These activities promote TH17 differentiation. At the same time, HIF-1 attenuates T(reg) development by binding Foxp3 and targeting T(reg) for proteasomal degradation. Besides, tumor hypoxia induces CCL28, CXCL12, CXCR4 expression, enhancing T(reg) cell recruitment. VEGF-A is a major factor in differential secretion of depleted CD8+T cells under hypoxia, which can promote the differentiation of PD-1$^{+}$TIM-3$^{+}$CXCR5$^{+}$ terminally depleted CD8+T cells. In addition, hypoxia up-regulates the expression of CD137 and CD25, which secretes immunosuppressive cytokine IL-10, eventually inducing adverse T cell phenotype. MiR-24 upregulates in tumor cells and TIL, and inhibits MYC and FGF11 in CD8(+)T cell. Through the destruction of MFN1-mediated mitochondrial fusion, the generation of intolerable ROS levels, causing T cell exhaustion. Further, hypoxic TME up-regulates the expression of CD39 and CD73, which negatively affect T cell activation through adenosine signaling pathway.

efficacy (155). A newly prepared single-nanometer oxygen nanobubble water can overcome hypoxia-induced radiation resistance of cancer cells. Under hypoxia, MDA MB231 cells treated with oxygen nanobubble medium significantly inhibited hypoxia-induced HIF-1α and radiation resistance compared with normal medium (156). The upconversion nanoparticle coremesoporous silica shell structure (UCHMs) with the hypoxia activated pro-drug tirapazamine (TPZ) loaded within the cavity between the core and shell could act as excellent delivery vehicles of TPZ to the hypoxic centers of tumors, serve as highly effective radiosensitizer in the meantime, and subsequently kill hypoxic cells during culture. TPZ@UCHMs, this specially designed treatment can also effectively prevent potential hypoxia and reoxygenation, thus effectively inhibiting hypoxia and radiation-induced cell metastasis and tumor regeneration (157). In addition, hyperthermia (heat treatment at 39-45°C) can increase blood flow to improve tissue oxygenation, sensitize radiation through DNA repair inhibition, and can directly or indirectly kill cells by causing vascular damage. This combination therapy has potential clinical applications in the future, but the timing and sequence between radiation and hyperthermia and different action mechanisms caused by heating temperature and heating time need to be further explored (158).

## Hypoxia and chemotherapy

A large number of studies have found that HIF-1 was a necessary condition for chemotherapy resistance of breast cancer stem cells, and HIF-1α expression was correlated with pathological complete response (pCR) in chemotherapy patients (159, 159). Chemotherapy-induced HIF activity accumulated breast cancer stem cell populations through IL-6 and IL-8 signaling pathways and increased expression of multidrug resistance 1 (160). Samanta et al. demonstrated that the combination of HIF inhibitors can overcome breast cancer stem cell resistance to paclitaxel or gemcitabine *in vitro* and *in vivo*, leading to tumor eradication (160). Additionally, hypoxic TME can lead to drug resistance through down-regulation of chemotherapeutic drug targets by HIF-1, reducing the level of topoisomase IIalpha, an enzyme that generates DNA strand breaks when poisoned with etoposide, resulting in chemotherapy resistance of etoposide (161).

Treatment regimen based HIF-1 inhibition has been shown to rescue hypoxia-mediated chemotherapy resistance. Hypoxic-responsive polymeric drug nanoparticles(ICG@CPTNB) release camptothecin CPT by self-combustion in hypoxic regions, significantly improving the tumor growth inhibition efficiency of traditional chemotherapy (162). Based on the high reactivity of manganese dioxide (MnO2) to hydrogen peroxide (H2O2), a bioconjugated manganese dioxide nanoparticles (MAN-HA-MNO2) were targeted to the tumor hypoxia region. It could enhance chemotherapy response by stimulating TAMs to an M1-like phenotype and alleviating tumor hypoxia (163). A hypoxia-activated prodrug can be activated under hypoxia named YC-DOX. It's self-immolation releases doxoruin (Dox) and YC-1 cysteine, which respectively performs chemotherapy and down-regulates HIF-1α (164).

## Hypoxia and endocrine therapy

About 70% of breast cancer is caused by estrogen through estrogen receptor-α(ERα) (165). Therefore, aromatase inhibitor based endocrine therapy is an important treatment for breast cancer. HIF-1α gene has a typical ER binding element that responds to estrogen signaling, suggesting a direct regulatory link between ERα and HIF-1α pathway in breast cancer (166). Several studies have shown that HIF-1α makes tamoxifen (TAM) resistant to breast cancer cells of ERα+ (167–169). Baicalein helps overcome TAM resistance by promoting the interaction between HIF-1α and PHD2 and pVHL to reduce HIF-1α expression, thereby reducing aerobic glycolysis and reversing mitochondrial dysfunction (168). In addition, hypoxia further down-regulated ERalpha transcription through MAPK signaling and activation of ERK1/2. MEK1/2 inhibitors (U0126 or PD184352) could partially restore ERalpha expression through inhibition ERK1/2. Kronblad et al. demonstrated that U0126 combined with tamoxifen enhanced anti-estrogen effect in hypoxia (169). In a word, the direct and indirect regulatory pathways between ERα and HIF-1α may regulate hormonal responses in endocrine therapy, and it is significant to explore the targets in these pathways for overcoming endocrine resistance and enhancing of efficacy.

## Hypoxia and immunotherapy

Immunotherapy is a promising treatment for triple negative breast cancer (TNBC), but relapse and drug resistance are common (170). Baldominos et al. found that in primary breast cancer, tumor cells resistant to T cell attack are quiescent cancer cells (QCCs). Transcriptomic analysis revealed that QCCs block the function of T cells by regulating the local hypoxic immunosuppressive environment, thus forming a drug library of immunotherapy (171). As described above, adenosine signaling inhibits the activity of T cells and induces apoptosis of T cells through A2AR in hypoxic microenvironment. Inhibition of this pathway plays an important role in improving tumor immunotherapy which mainly through two mechanisms:(a)blocking immunosuppressive adenosine-A2AR mediated intracellular signaling *via* A2AR inhibitors; (b) attenuating HIF-1α mediated extracellular adenosine accumulation by oxygen mixture (142). A2AR blockers, adenosine inhibitors (e.g. CD39 and CD73), as well as hypoxia

targeting agents, are currently in clinical phase demonstrated that blocking the hypoxic adenosine-A2AR axis synergistically induces tumor rejection with immune checkpoint inhibitors, providing new hope for the majority of patients who do not respond to immunotherapy (172, 173). Wang Y et al. designed a hemoglobin-poly(Hb-PCL) conjugate self-assembled biomimetic nano red blood cell system(V(Hb)). The V(Hb)@DOX can bind to endogenous plasma haptoglobin (Hp) and specifically target the M2-type TAMs *via* the CD163 surface receptor. The O2 released by the Hb alleviates tumor hypoxia, which further augments the antitumor immune response by recruiting fewer M2-type macrophages (174). In addition, the PFC@lipo modified liposomes can effectively load and release oxygen, helping PD-1 antibody to break through the treatment bottleneck, significantly inhibiting the progression of breast cancer (175). Table 2

## HIF inhibitors

Targeting the HIF pathway is a direct and effective strategy for alleviating hypoxia in the tumor microenvironment (176). Especially triple negative breast cancer, which has high HIF transcriptional activity but poor response to existing therapies (177). There are two main classes of HIF inhibitors: Direct HIF inhibitors affect the expression or function of the HIF molecule, and indirect HIF inhibitors regulate other molecules in upstream or downstream pathways (such as AMPK, PHD, etc.), ultimately affecting HIF signaling (178). Compared with direct inhibitors, they affect many other pathways, so they are generally less selective for HIF-1α (149). Therefore, direct acting inhibitors of HIF-1α are receiving increasing attention as potential therapeutic agents that specifically target HIF-1 pathways in tumors. Direct HIF inhibitors act through a variety of mechanisms, including inhibiting mRNA expression and inhibiting HIF protein synthesis, affecting heterodimerization of HIF-1α and HIF-1β, inhibiting transcriptional activity of DNA, etc (179, 180). Several promising direct-acting small molecule inhibitors currently under study include: Acriflavone, which can affect HIF-1α dimeration and transcription activation (181), YC-1, Chetomin and Bortezomib, which can inhibit the interaction between HIF-1α and P300/CBP (182–184), and Echinom Ycin and NSC-50352 affect HIF-1α binding to DNA (185). In addition, FIH-1 regulation controls the transcriptional activity of HIF-1α through c-TAD (FIH-1-regulated domain), which is also a potential strategy to target hypoxia-induced

TABLE 2 Hypoxia-induced resistance related mechanisms and therapies.

| Hypoxia-induced resistance | Resistance mechanisms | Therapies | Function | Reference |
|---|---|---|---|---|
| Radiotherapy | 1.Free radicals reduced by molecules containing SH group, leading to DNA repair | HAPs | Chemical reduction to become active compounds that target radiation-resistant hypoxic cells | (154) |
| | | MDNP | Reacting with endogenous H2O2 to regulate TME hypoxia | (155) |
| | | Oxygen nanobubble | Inhibited hypoxia-induced HIF-1α and radiation resistance compared with normal medium | (156) |
| | | TPZ@UCHMs | UCHMs loaded with the hypoxic pre-activation drug TPZ is transported to the tumor hypoxic center, and at the same time serves as a highly effective radiosensitizer | (157) |
| | | Hyperthermia | Increasing blood flow to improve tissue oxygenation, sensitizing radiation through DNA repair inhibition | (158) |
| Chemotherapy | 1. Accumulating breast cancer stem cell populations through IL-6 and IL-8 signaling pathways<br>2. Increasing expression of multidrug resistance 1<br>3. Down-regulation of chemotherapeutic drug targets by HIF-1 | ICG@CPTNB | Releasing CPT by self-combustion in hypoxic regions | (162) |
| | | MAN-HA-MNO2 | Enhancing chemotherapy response by stimulating TAMs to an M1-like phenotype | (163) |
| | | YC-DOX | Releasing doxoruin and cysteine, respectively performing chemotherapy and down-regulating HIF-1α | (164) |
| Endocrine therapy | 1. HIF-1α gene has a typical ER binding element that responds to estrogen<br>2. Hypoxia down-regulates ERalpha transcription through MAPK signaling and activation of ERK1/2 | Baicalein | Overcoming TAM resistance by promoting the interaction between HIF-1α and PHD2 and pVHL to reduce HIF-1α expression | (168) |
| | | MEK1/2 inhibitors (U0126 or PD184352) | Restoring ERalpha expression, enhancing anti-estrogen effect through inhibition ERK1/2 | (169) |
| Immunotherapy | 1. Blocking the function of T cells by QCCs<br>2. Adenosine signaling induces apoptosis of T cells | A2AR blockers (CD39 and CD73) | Blocking adenosine-A2AR mediated intracellular signaling | (172, 173) |
| | | V(Hb)@DOX | Targeting the M2-type TAMs *via* the CD163, releasing O2 and recruiting fewer M2-type macrophages | (174) |
| | | PFC@lipo | Effectively loading and releasing oxygen | (175) |

malignancy (185). Although many direct inhibitors of HIF-1α have been proposed, none has entered clinical trials. The reasons for their lack of efficacy *in vivo* may be related to the heterogeneity of tumor cells, the complexity of hypoxic microenvironment, and the fact that only HIF-1α targets have been studied while few HIF-2α inhibitors (149). There is still a long way to go before HIF inhibitors can be used in the clinic.

## Conclusion

Hypoxia of the TME in breast cancer and other solid tumors are widespread phenomenon. In response to reduced oxygen tension, HIF1 and HIF-2 stabilize and mediate the hypoxic response, primarily by acting as transcription factors. HIF-1 influences important tumor characteristics, including: cell proliferation, apoptosis, angiogenesis, metabolism, genetic instability and immune response in TME. Therefore, hypoxia mediates resistance to radiotherapy, chemotherapy, endocrine therapy and immunotherapy, and is associated with poor prognosis in cancer patients. The elucidation of this important mechanism of hypoxia also brings new strategies for reversing resistance to current therapies and improving the efficiency of cancer treatment. At present, the main methods for targeting hypoxia are to improve the delivery efficiency by nanocarriers and directly or indirectly inhibit HIF, so as to alleviate tumor hypoxia and prevent HIF from causing tumor support and immunosuppressive effects through a series of signaling pathways. However, these specific targeted hypoxia drugs are still far from clinical practice. In the era of personalized precision medicine, more precise measurements are needed to distinguish between responders and nonresponders to hypoxia-targeted drugs, and more clinical trials are needed to determine whether hypoxia-targeted drugs alone or in combination with existing treatment regimens can increase survival in breast cancer patients.

## Author contributions

WC, XX, and YL designed the manuscript. WC wrote the manuscript. XX and YL drew the figures and tables. QC and CW revised the manuscript. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Targeting hypoxia-inducible factors for breast cancer therapy: A narrative review

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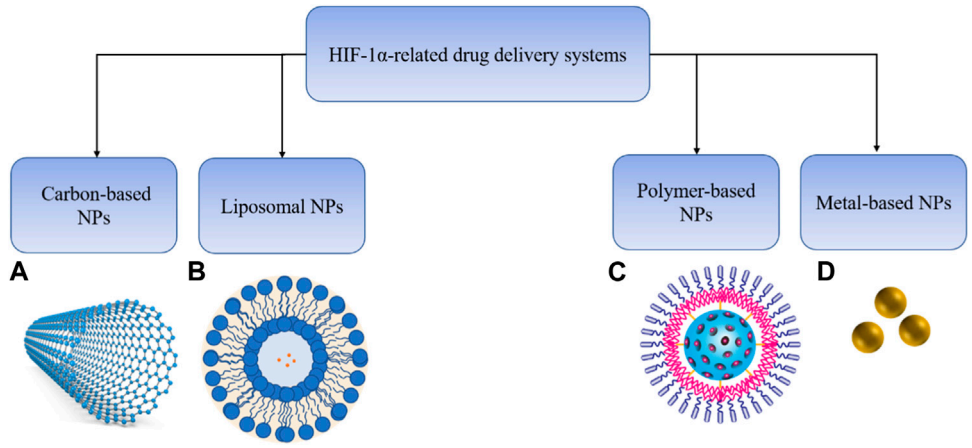
Hypoxia-inducible factors (HIFs), central regulators for cells to adapt to low cellular oxygen levels, are often overexpressed and activated in breast cancer. HIFs modulate the primary transcriptional response of downstream pathways and target genes in response to hypoxia, including glycolysis, angiogenesis and metastasis. They can promote the development of breast cancer and are associated with poor prognosis of breast cancer patients by regulating cancer processes closely related to tumor invasion, metastasis and drug resistance. Thus, specific targeting of HIFs may improve the efficiency of cancer therapy. In this review, we summarize the advances in HIF-related molecular mechanisms and clinical and preclinical studies of drugs targeting HIFs in breast cancer. Given the rapid progression in this field and nanotechnology, drug delivery systems (DDSs) for HIF targeting are increasingly being developed. Therefore, we highlight the HIF related DDS, including liposomes, polymers, metal-based or carbon-based nanoparticles.

KEYWORDS

**hypoxia-inducible factors, breast cancer, drug delivery systems, tumor microenvironment, angiogenesis, glycolysis**

**Abbreviations:** HIFs, hypoxia-inducible factors; DDS, newly discovered drug delivery systems; TNBC, triple-negative BC; BC, breast cancer; RBCm, anti-LDLR engineered red blood cell membranes; Sal, salidroside; ICG, indocyanine green; TME, the tumor microenvironment; PHD, prolyl hydroxylase domain protein; HREs, hypoxia response elements; BHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; ECM, extracellular matrix; CAF, cancer-associated fibroblasts; EMT, epithelial-mesenchymal transition; VEGF, vascular endothelial growth factor; VHL, VonHippel-Lindau; TAM, tumor-associated macrophages; TCA, enzymes of the tricarboxylic acid cycle; GLUT1, glucose transporter 1; HK2, Hexokinase 2; CSC, Cancer Stem Cell; BCSC, BC Stem Cell; Snail, zinc finger transcription factor; NP, nanoparticle; DOX, doxorubicin; ACF, acriflavine.




GRAPHICAL ABSTRACT

# 1 Introduction

Breast cancer (BC) is one of the major diseases affecting women's health and the leading cause of female death worldwide. According to the Global Cancer Statistics 2020 report, in terms of morbidity, the number of new cases of BC in 2020 reached 2.3 million, accounting for about 11.7% of the total cases (Sung et al., 2021). Triple-negative breast cancer (TNBC) is defined as BC lacking expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) and is classified as one of basal-like BC (BLBC) (Wolff et al., 2013). BC treatment is divided into systemic and localized treatment based on BC subtype and degree of metastasis. For non-metastatic BC, local therapy is mainly used to eradicate the tumor through surgical resection and radiotherapy, while for metastatic BC or more aggressive triple-negative BC, systemic therapy consisting of chemotherapy and immunotherapy are used to prevent tumor metastasis and recurrence (Waks and Winer, 2019). Although surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy have improved the survival and quality of life of BC patients in recent decades, the mortality rate of BC is high due to lack of therapeutic targets and chemotherapy resistance. Therefore, finding effective therapeutic targets and reducing drug resistance are indispensable in the treatment of BC (Veronesi et al., 2005; Barzaman et al., 2020).

The rapid proliferation of the tumor beyond its surrounding vasculature results in the normal oxygen level to drop to less than 2%, and the area with low oxygen is called the hypoxic area. Hypoxia promotes tumor plasticity and heterogeneity and a more aggressive and metastatic phenotype, which is seen in many solid tumors and is an important feature of the BC tumor microenvironment (Harris, 2002). Hypoxia-inducible factor (HIF) is a key marker of hypoxia and a core player involved in cell adaptation to hypoxia (Huang et al., 2017; Rani et al., 2022). Recently, nanoparticles (NP), as an effective drug delivery method have attracted special interest for cancer treatment. Various ongoing studies aim to optimize this method to ultimately reduce adverse reactions caused by traditional methods. So far, the NP used in drug delivery research for targeting HIF in BC includes liposomes NPs, polymers NPs, metal-based NPs or carbon-based NPs. Using NP for drug delivery has many advantages: 1) It improves the problems related to poor drug solubility and bioavailability; 2) It enhances the permeability of targeted drugs to cancer cells and slowly releases drugs; 3) NPs are very small (1–100 nm), non-toxic, biodegradable, and cancer drugs can be easily loaded onto these particles; 4) Delivery of multiple drugs with differing properties can be achieved (Farokhzad and Langer, 2009; Burgess et al., 2010). Compared with standard chemotherapy methods, nano carriers can significantly reduce the damage to healthy cells and tissues. Therefore, nano carriers may be used in clinical applications in the future in NP based drug delivery system (DDS) or in combination therapy. The main purpose of this review is to briefly summarize the mechanism of HIF-1 mediated angiogenesis, glycolysis, metastasis and drug resistance. Furthermore, we discuss the current therapeutic strategies targeting HIF-1, including HIF-1α inhibitors in preclinical and clinical studies, as well as small molecules targeting HIF-1α related signaling pathway. In addition, we emphasize the current progress in HIF related drug delivery systems.

# 2 Materials and methods

## 2.2 Data and processing

The expression profile and related clinical follow-up information of BRCA were download from The Cancer Genome Consortium (TCGA) database. A total of 1098 tumor samples and 113 normal samples were included.

## 2.2 Expression analysis

The expression of HIF1A in different subgroups was matched by clinical annotation information. All statistical analyses were implemented by R (v4.1.3) language. Statistical differences between each two subgroups were calculated by Wilcoxon test.

## 2.3 Literature search

We mainly used "NCBI-Pubmed" to conduct online literature search of all articles published in English over the past 10 years. The search words include "hypoxia and HIF", "HIF and breast cancer", "HIF and angiogenesis and breast cancer", "HIF inhibitor and breast cancer", sorted by "best match". Search results were selected by year ranging from the most recent to the earlier ones and also by impact factor of the article. Clinical studies were searched by key words "HIF and breast cancer" and article type set as clinical trials in Pubmed and also searched in ClinicalTrials.gov.

# 3 Hypoxia-inducible factors

Hypoxia-inducible factors (HIFs) are transcription factor responsible for activation of hypoxia genes. They are heterodimers belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factors, which are composed of an oxygen-regulated 120 kDa α subunit, and an oxygen-independent 91–94 kDa β subunit (Loboda et al., 2010). Three HIF-α subtypes (HIF-1α, HIF-2α and HIF-3α) have been reported, HIF-1α is the most classical and widely studied (Konisti et al., 2012). HIF-1α and HIF-2α share 48% amino acid sequence homology and similar domain arrangement, while there are different hypoxia-sensitivities for different prolyl hydroxylase sites (Pro564 and Pro402 in HIF-1α, Pro405 and Pro531 in HIF-2α) (Iyer et al., 1998; Jokilehto and Jaakkola, 2010). HIF-1α is thought to be a key coordinator of cancer cell responses to the hypoxic microenvironment by regulating metabolic reprogramming, angiogenesis, stem cell maintenance, matrix remodeling, metastasis and resistance to chemoradiotherapy (Schito and Semenza, 2016). Numerous studies have shown that the expression of HIF-1α was elevated in BC and high expression of HIF-1α predicts poor patient survival (Talks et al., 2000; Rajkovic-Molek et al., 2014; Cui and Jiang, 2019; Shamis et al., 2021). By analyzing TCGA data, we found that HIF-1α was highly expressed in TNBC. Moreover, its expression was higher in ER- and PR-compared with ER+ and PR + BC respectively (Figure 1). HIF-2α may play an important role in a variety of cells other than endothelial cells as well as in tumorigenesis Hu et al., 2003). HIF-3α mainly depends on other HIF complexes (Bristow and Hill, 2008). Under normal microenvironment, the HIFα-subunit is degraded with the aid of E3 ligase through hydroxylation by prolyl hydroxylase domain protein (PHD) and polyubiquitination of VonHippel-Lindau (VHL). Factor-inhibiting HIF-1α (FIH-1) is another transcriptional regulator of HIF-1α and HIF-2α, which interferes the binding of HIF to co-transcription factors (Rani et al., 2022). Under hypoxic conditions, PHD activity is reduced. So, HIF-α in cytoplasmic is accumulated and translocated to the nucleus, where α subunit dimerizes with β-subunit and induces transcription of target genes by binding to hypoxia response elements (HREs) in promoters (Figure 2) (Wang et al., 1995; Semenza, 2014; de Heer et al., 2020). Recently, it has been described that, HIF-1α and HIF-2α can be regulated in an oxygen-independent by regulators such as hypoxia-associated factor (HAF), small ubiquitin-related modifier (SUMO)-specific protease 1 (SENP1), and Int6/eukaryotic initiation factor (eIF) 3e (Hashimoto and Shibasaki, 2015). Enhanced expression of HIF-targeted genes is associated with many human diseases, including ischemic cardiovascular disease, stroke, chronic lung disease, and cancer (Semenza et al., 2000).

# 4 HIFs in the microenvironment of BC

TME is a complex network composed of different cell types, signaling molecules, and extracellular matrix components, which together coordinate tumor progression (Catalano et al., 2013). The cellular components of the TME include cancer cells, surrounding immune cells and endothelial cells, cancer associated fibroblasts (CAFs), etc. (Spill et al., 2016; Del Prete et al., 2017). Similar to most solid tumors, hypoxia is an inherent property of the BC TME. HIF-1, as the driver of hypoxia, plays a key role in the activation of CAFs and it promotes persistent chronic inflammation in the TME (Whitaker-Menezes et al., 2011; Martinez-Outschoorn et al., 2014; Mao et al., 2021). In addition, immune evasion is considered to be one of the main strategies for tumor survival in the TME. HIF-1 signaling suppresses the immune system in the hypoxic TME, allowing cancer cells to evade immune responses by triggering the expression of immunosuppressive molecules (Barsoum et al., 2014; Semenza, 2014; Schito and Semenza, 2016; Jiang et al., 2019; You et al., 2021). Activation of the HIF signaling pathway maintains oxygen homeostasis by mediating the expression of multiple genes involved in regulating many critical functions of cells, including growth, metastasis, drug resistance, and maintenance of stemness (Bao et al., 2012; Semenza, 2017; Chen et al., 2020).

## 4.1 The association of HIFs and angiogenesis in BC

When in the initial stage of tumor growth (tumor volume <0.5 mm), tumor obtains nutrients and oxygen by

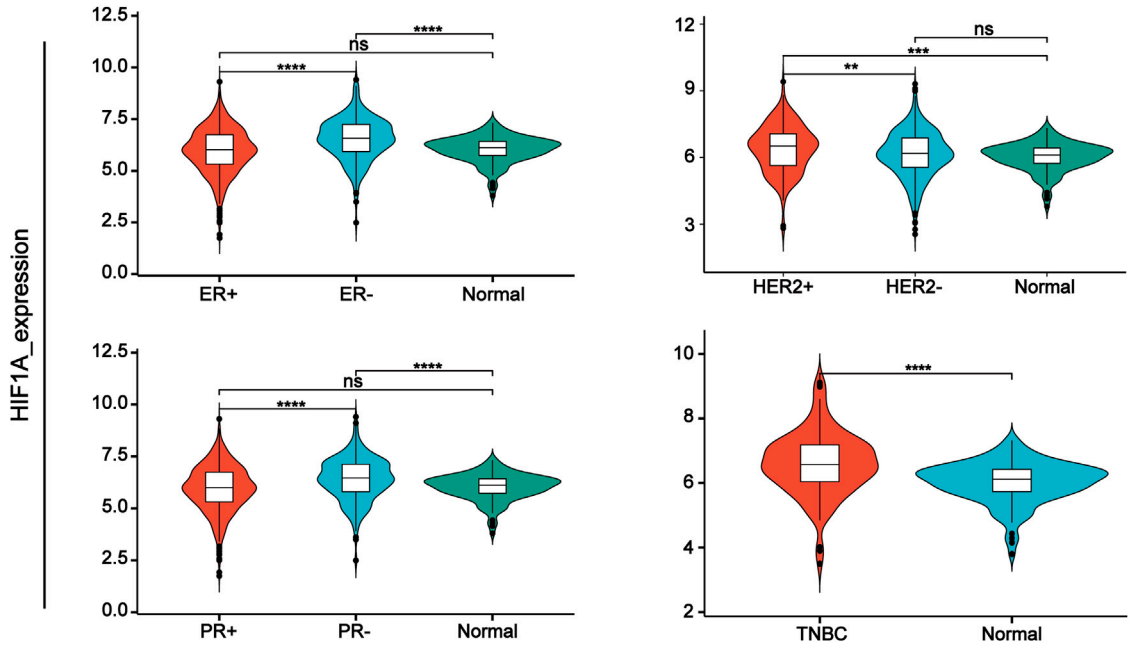



FIGURE 1
HIF-1α expression in different types of breast cancer tissue samples compared with normal tissue from TCGA database.

diffusion, when tumor masses grow larger than 0.5 mm, nutrients obtained by diffusion are insufficient to sustain tumor growth, and new vasculature is formed to maintain the growth state (Hanahan and Weinberg, 2000). Activation of this "angiogenic switch" will form a new vasculature, which is inevitable for the growth and metastasis of malignant tumors (Hanahan and Folkman, 1996). Compared to normal tissue, tumor vascular distribution results in abnormal vascular distribution (dilated, tortuous, disorganized) and dysfunction (hyper penetration, edema) (Rapisarda and Melillo, 2012; Viallard and Larrivee, 2017). And tumor angiogenesis perfusion is reduced, which in turn exacerbates the hypoxic environment and maintains HIF-1α stability (Rey et al., 2017). Angiogenesis is also known as basic condition for tumor progression, proliferation and metastatic spread. HIFs is an important hub for regulating angiogenesis (Hashimoto and Shibasaki, 2015; Olejarz et al., 2020). BC angiogenesis can be activated by HIFs-mediated downstream pathways, primarily vascular endothelial growth factor (VEGF) (Darbeheshti et al., 2021). VEGF as one of the key downstream targets of HIFs pathway belongs to the endothelial growth factor family and plays a central role in angiogenesis through its effects on endothelial cell migration, proliferation, permeability and survival (Semenza, 2000; Schoppmann et al., 2006; Kallergi et al., 2009; Ahluwalia and Tarnawski, 2012; Saponaro et al., 2013). A study showed that RAB11B-AS1, a long noncoding RNA, enhances the expression of VEGFA and ANGPTL4 in hypoxic BC cells in a HIF2α-dependent manner, leading to tumor angiogenesis and metastasis (Niu et al., 2020). Research evidence has also shown that hypoxia could induce the HIF-1α/G-protein estrogen receptor (GPER) in CAFs, which regulates VEGF and finally elicit hypoxia-dependent tumor angiogenesis (De Francesco et al., 2013). It has also been shown by Kallergi et al. (2009) that HIF-1α co-express with VEGF in patients with metastatic breast cancer. The direct link between HIF-1α and VEGF suggests that HIF-1α has a profound role in angiogenesis, Anti-angiogenic therapies such as VEGF inhibitor may cause drug resistance by increasing intratumoral hypoxia and upregulating HIF-1α. Clinical trials have been designed to test the efficacy of bevacizumab combined with HIF-1α to conquer drug resistance (Falchook et al., 2014; Jeong et al., 2014).

## 4.2 The association of HIFs and glycolysis in BC

Increasing research evidence suggest that cancer is not only a genetic disease but also a metabolic disease, in which glycolysis is an important player. It has long been recognized that although there are adequate oxygen levels in the TME, the metabolic demands of cancer cells are shifted from aerobic respiration to the uptake of glycolytic glucose, the reprogram known as the

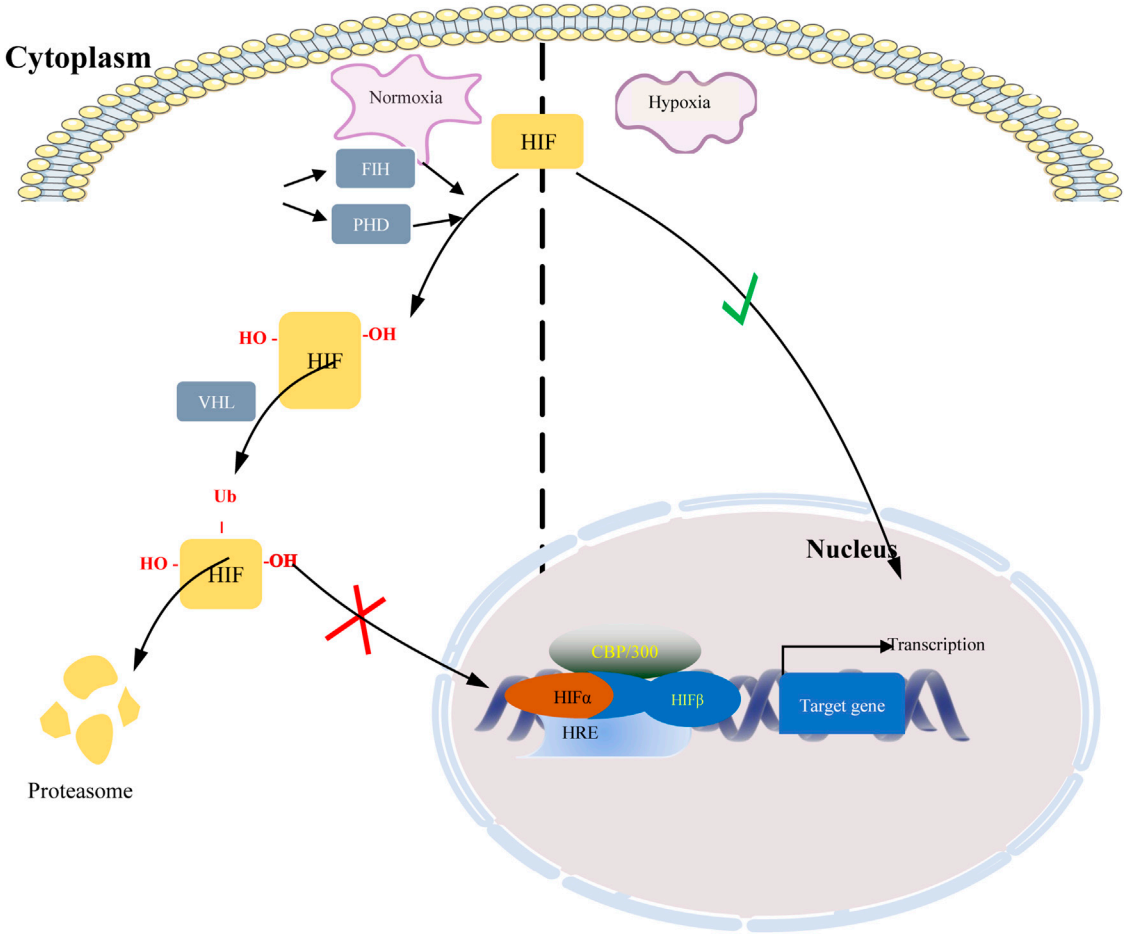



FIGURE 2
Regulation of HIF under normoxic and hypoxic conditions. When oxygen is abundant, HIF is hydroxylated by prolyl hydroxylase domain protein (PHD) enzymes at two specific proline residues, enabling it to bind VHL. VHL targets hydroxylated HIF subunits for ubiquitin-mediated proteasomal degradation. Under hypoxic conditions, inactivation of PHD and FIH-1 results in HIF stabilization and translocation into the nucleus where it stabilizes and dimerizes with HIF-1β, which together with the co-transcription factors p300 and CBP, drives hypoxia Transcription of target genes of response elements (HREs).

Warburg effect. Another study also showed that HIF-1α may drive glycolysis independent of hypoxia TME, the targets of HIF-1in the glycolytic pathway include hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and glucose transporter 1 (GLUT1, also known as solute carrier family A1, SLC2A1), and accelerate the process of glycolysis by downregulating the expression of enzymes of the tricarboxylic acid cycle, the factors that contribute to this situation may be pyruvate kinase isoform M2 (PKM2) physically interacts with HIF-1 and stimulates HIF-1 activity, but not pyruvate kinase isoform M1 (PKM1) (Christofk et al., 2008; Luo et al., 2011). When cancer cell metabolism is shifted to aerobic glycolysis, pyruvate is replaced by lactate and released into the TME, creating an immunosuppressive environment that promotes tumor cell growth, metastasis and invasion (Liberti and Locasale, 2016; El-Sahli and Wang, 2020).

Hexokinase 2(HK2) is an enzyme that catalyzes the phosphorylation of hexose, it is the first and the rate-limiting enzyme of the glycolytic pathway. CircRNF20 is a 499 bp circular RNA derived from RNF20 Gene that can promote tumor progression *via* miR-487a/HIF-1α/HK2 in BC (Cao et al., 2020). O-linked-N-acetylglucosaminylation (O-GlcNAcylation) is a type of glycosylation, which regulates glycolysis *via* HIF-1α/GLUT1 signal pathway in BC cells (Ferrer et al., 2014). Circular RNA circRBM33 inhibits the expression of downstream glycolysis-related proteins (HK2, GLUT1) through the miR-542-3p/HIF-1α axis, thereby preventing glycolysis and promoting BC cell apoptosis (Jiang et al., 2022). However, other studies have shown that Pyruvate dehydrogenase kinase 1 (PDK1) is a key switch of tricarboxylic acid (TCA) cycle in mitochondria. Signal-induced proliferation-associated 1 (SIPA1), a member of Rap1GAP family, promotes

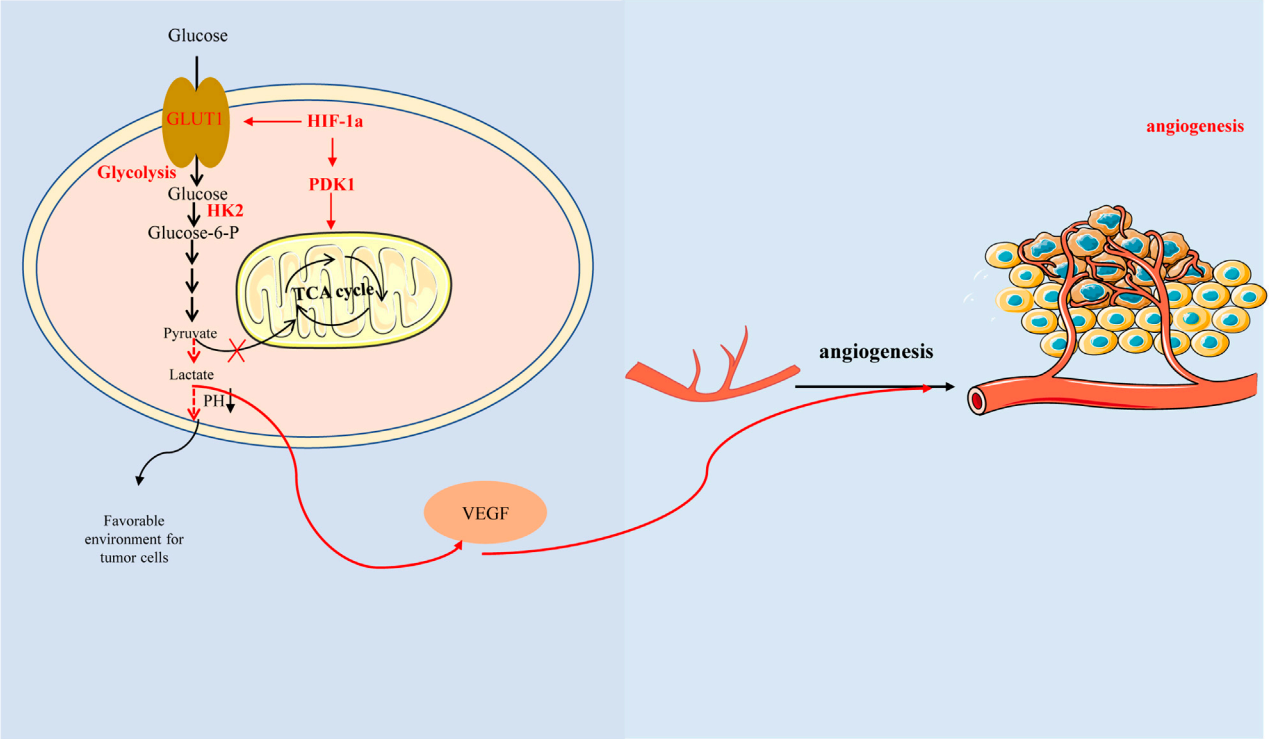



FIGURE 3
Hypoxia-inducible factor-1α promotes glycolysis by regulating key enzymes in the process of glycolysis, which produces lactic acid acidification microenvironment and affects glucose metabolism, thus promoting vascular endothelial growth factor expression and angiogenesis. In addition, HIF-1 α can also directly regulate tricarboxylic acid cycle and affect glucose metabolism with glycolysis-independent method.

aerobic glycolysis by regulating the SIPA1/HIF-2α/PDK1 axis, leading to tumor invasion and metastasis *in vivo* (Yao et al., 2021). Therefore, HIF-1 α plays an important role in glucose metabolism, and providing energy for cancer cells by controlling glucose metabolism may be another promising pathway.

Interestingly, here is a view that the 'glycolytic switch' occurs before the angiogenesis. Glycolysis could induce HIF-1α accumulation leading to high expression of VEGF (Figure 3) (Zare et al., 2021). Studies have reported that aerobic glycolysis can induce angiogenesis by producing lactate to acidify the extracellular environment and promote VEGF expression (Shi et al., 2001; Jung et al., 2011). Another study also suggested that lactate and pyruvate, the end products of glycolysis, regulate VEGF expression by increasing HIF-1α accumulation (Lu et al., 2002).

## 4.3 The association of HIFs with EMT and metastasis in BC

Tumor metastasis is a process by which cancer cells spread from the initial site of primary tumor growth to distant organs, where they survive, proliferate and form secondary tumors. EMT is an important part of tumor metastasis. It is the process of transformation from epithelial cells to cells with a mesenchymal phenotype through a specific program (Lee et al., 2006; Lee et al., 2011). Cancer cells that undergo EMT have strong invasive capacity and are resistance to apoptosis (Suarez-Carmona et al., 2017). Hypoxia is often an environmental feature of EMT, and activated HIF-1α induces cancer EMT through multiple molecules and pathways, including inflammatory cytokines, epigenetic regulators, and transcription factors (Bao et al., 2012). One study has shown that hypoxia induces HIF-1α expression, which induces the expression and activity of major transcription factors including TWIST, Snail, Slug, SIP1, STAT3, and ZEB, leading to the suppression of E-cadherin and induction of vimentin in BC cells. Inhibition of HIF-1α significantly enhanced the expression of E-cadherin (Zhou et al., 2016). Another report also revealed that hypoxia promoted the expression of Slug and Snail and decreased E-cadherin during HIF1-induced EMT through Notch pathway (Chen et al., 2010). He et al. (2020) demonstrated that hypoxia-induced HIF-1α regulated BC cells migration and EMT through the MiR-338-3p/ZEB2 axis. Moreover, research evidence showed that HIF-1α

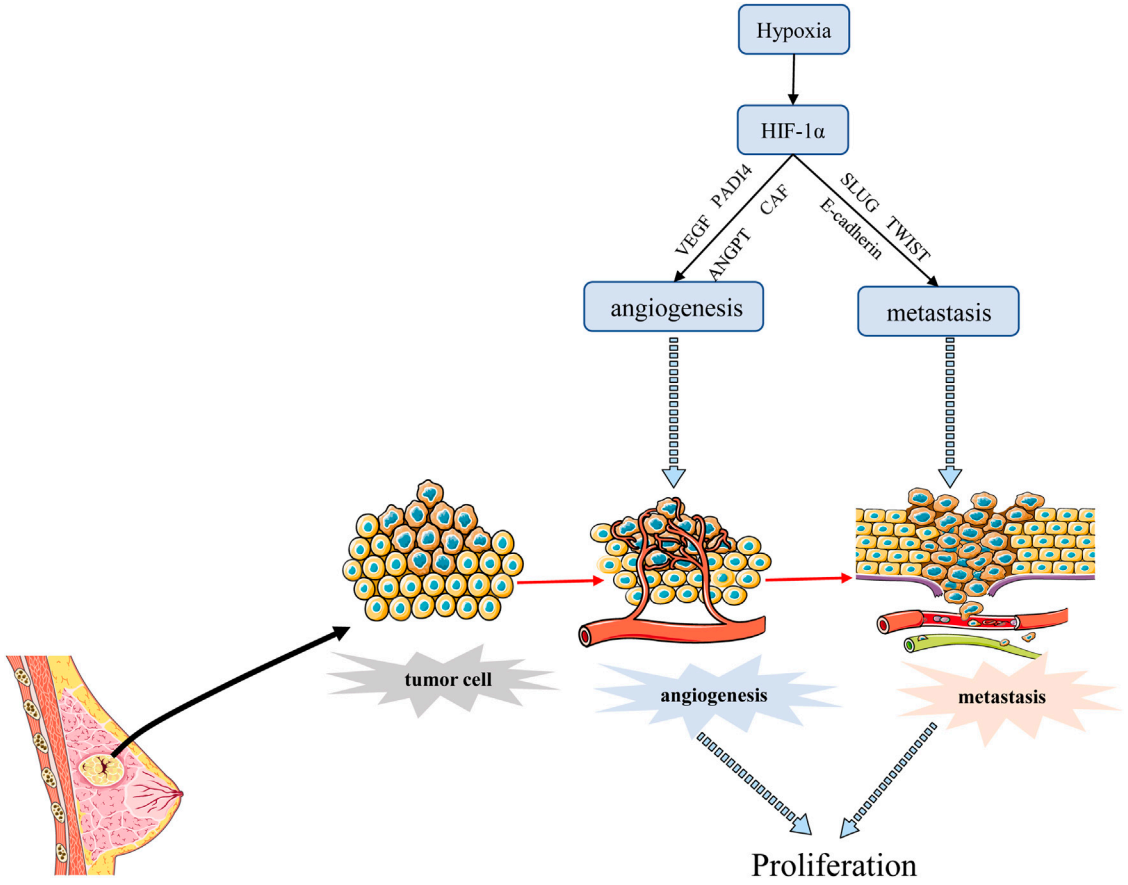



FIGURE 4
HIF-1α regulates the angiogenesis and metastasis of BC. HIF-1α promotes angiogenesis mainly by stimulating VEGF transcription, and promotes BC metastasis through EMT process. Angiogenesis can also promote transfer to normal tissues.

and integrin-linked kinase (ILK) formed a regulatory feedback loop which promoted EMT by modulating the expression of various EMT regulators/makers, including Snail, Zeb1, E-cadherin, and vimentin (Chou et al., 2015). Moon et al. (2021) found that MRPL52 acted as a transcriptional target of hypoxia-inducible factor (HIF-1α) and MRPL52 augmented epithelial-mesenchymal transition, migration and invasion of hypoxic BC cells by activating the ROS-Notch1-Snail signaling pathway. Angiopoietin-like protein ANGPTL4 is also a HIF-1α target that promotes lung metastasis when overexpressed in BC cells (Zhang et al., 2012). The above evidence suggested that HIF-1α acted as a crucial regulator of hypoxia induced EMT and metastasis through various mechanisms. In addition, angiogenesis is associated with metastasis because permeability and heterogeneous vascular systems contribute to the extravasation of tumor cells into normal tissues to escape the harsh hypoxia environment as shown in Figure 4. Metastasis is a major prognostic challenge for BC patients, and it may be a feasible way to inhibit BC metastasis by targeting HIF-1 α.

## 4.4 The association of HIFs and cancer stemness of BC

Cancer stem cells (CSCs) are a small subset of solid tumor cells with self-renewal and differentiation properties and tumorigenic potential and they spread to different parts of the body to form secondary tumors (Bai et al., 2018). Hypoxia may contribute to the formation of CSCs niches within tumors. Studies have confirmed that HIF targeting of cancer cell stemness-related genes may be a key inducer of stemness dynamics under pathological conditions. By increasing the expression of HIFs and enhancing the activity of HIFs, tumor cells acquire a stem phenotype and reach a higher degree of malignancy (Mohyeldin et al., 2010; Mathieu et al., 2011; Conley et al., 2012). Study has shown that the percentage of BC stem cells (BCSCs) is increased in a HIF-1 -dependent manner (Xiang et al., 2014). CD47 is a ubiquitously expressed cell surface glycoprotein belonging to the immunoglobulin superfamily, which is closely related to the self-renewal, tumorigenesis and chemotherapy resistance of BCSCs. When BC cells are in a

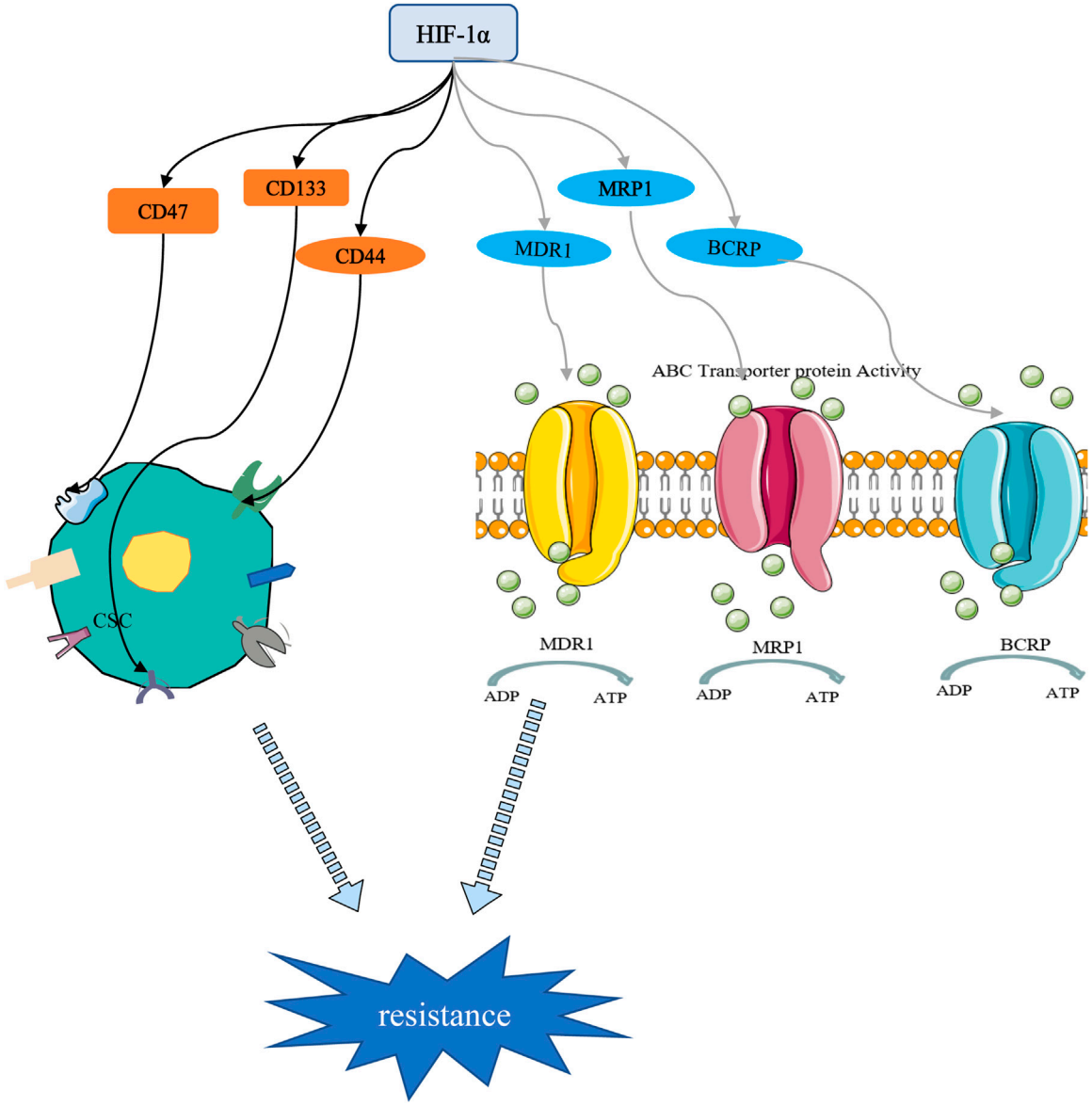



FIGURE 5
HIF-1α-mediated stemness and drug resistance. On the one hand, HIF-1α can induce drug resistance by regulating stem cell surface markers. On the other hand, HIF-1α promotes chemotherapy resistance through drug resistance-related proteins.

hypoxic environment, HIF induces the CD47 expression to promote breast CSC phenotype (Zhang et al., 2015). As a widely distributed transmembrane glycoprotein, CD44 is one of the important markers of CSCs. It has been reported that down-regulation of HIF-2α expression can reduce the stemness of BC cells through the CD44/PI3K/AKT/mTOR signaling pathway (Zhang et al., 2015). Moreover, HIF-1α can regulate BC cell stemness by regulating CD133+ stem cell population (Schwab et al., 2012). These studies highlight the important role of HIFs in the maintenance of BCSCs (Figure 5).

## 4.5 The association of HIFs and drug resistance in BC

Chemotherapy drugs are still the cornerstone of cancer treatment. Their killing effect on tumor cells is oxygen-dependent, and most of them kill cells by oxidizing free radicals and reactive oxygen species in the cells. Long-term or severe hypoxic conditions have been shown to promote resistance of tumor cells to chemotherapeutic drugs (Harris, 2002; Semenza, 2007). Increased drug resistance in hypoxic

TABLE 1 HIF-1α related clinical studies in BC.

| Drug | Status | Phase | Main Outcomes | Toxicity | NCT Number |
|---|---|---|---|---|---|
| Digoxin | Completed | Phase 2 | There was not enough data to analyze HIF-1alpha expression because of the limited tumor samples | No over grade 2 adverse event related to digoxin occurred | NCT01763931 |
| Vinorelbine | Completed | Phase 2 | The study was terminated early | Elevated liver enzymes (grade 3) 22.2%, Febrile infection (grade 5) 11.1% | NCT03007992 |
| Paclitaxel plus bevacizumab | Completed | | There was no significant difference between HIF-1alpha polymorphism and longer PFS in patients treated with paclitaxel and bevacizumab | | NCT01935102 |
| Bevacizumab, docetaxel | Completed | Phase 2 | | The rate of serious adverse events is about 18.06% and the rate of other adverse events is 98.61% in total | NCT00559754 |
| Propofol, Sevoflurane | Unknown | Not Applicable | | | NCT03005860 |

tumors has been reported both in cells and animal models. Resistance has been attributed to the upregulation of HIF-1, which was associated with poor overall survival (Campbell et al., 2019; de Heer et al., 2020). The transcription of numerous target genes can be activated by HIF-1, which promoted physiological changes associated with treatment resistance, including multidrug resistance 1 protein (MDR1), multidrug resistance-related protein 1 (MRP1), and BC resistance protein (BCRP) (Figure 5). Doublier et al. (2012) found that HIF-1 is activated and participates in the transcriptional activity of the MDR-1 gene, which promotes the resistance of MCF-7 cells to doxorubicin by regulating the MDR1/P-glycoprotein (P-gp, ABCB1) axis. MRP1 is an organic anion transporter. Study has shown that knockdown of HIF-1α attenuated cheomoresistance *via* affecting the expressions of apoptosis-related molecules such as Bax and Bcl-2 and drug transporters as P-gp and MRP1 (Wang et al., 2018). Besides, HIF-1-mediated chemoresistance is closely related to autophagy, apoptosis, stemness and glycolysis (Mimeault and Batra, 2013; Chen F et al., 2019; Li et al., 2020).

Collectively, these findings highlight the importance of HIFs in carcinogenesis and progression, which have prompted the scientific community to focus on the importance of HIF-1 and enable the discovery of new drugs that specifically inhibit HIF-1α or its target genes.

# 5 Therapeutic strategies targeting HIF-1α in BC

Here we summarize HIF-1α inhibitors that are in clinical trials, and various compounds that target HIF-1α or the HIF-1α pathway in basic research. Finally, we will introduce the latest drug delivery systems for HIF-1 α, which are designed to improve drug selectivity and ensure drug concentration.

## 5.1 HIF-1α inhibitors in clinical trials

We found some clinical studies on HIF-1α-related drug therapy for breast cancer as shown in Table 1. Unfortunately, they did not achieve some of the expected results. Three projects were completed in the second phase, but none of the projects successfully collected data on the response to HIF-1 α treatment, and two of them were terminated in advance. As for the reasons for early termination of the study, a small sample size and a small number of patients are the main causes, while serious adverse events caused by non-specific cytotoxicity are another possibility. As a result, more specific and safer preparations are needed for targeting HIF-1α in BC in clinic, which may take some time.

## 5.2 HIF-1α inhibitors under investigation

Since HIF-1α is closely related to the key processes in tumor progression and its expression is also associated with patient survival, it is not surprising that targeting HIF-1α has been extensively studied as possible therapeutic strategy against cancers. At present, there are no HIF-1α inhibitors approved by the FDA for BC treatment. The reported HIF-1α inhibitors for BC are still in basic research, so there is still an urgent need to discover novel HIF-1α inhibitors with sufficient potency, low toxicity, good druggability. HIF-1α inhibitors work by suppressing different processes in the HIF pathway: 1) HIF-1a protein accumulation 2) DNA binding 3) transcriptional activity 4) HIF-1a translation. Small molecule inhibitors targeting HIF-1α are shown in Table 2.

For example, based on Aryl Carboxamide Derivatives, 68 new aryl carboxamide compounds were synthesized and inhibitory effect was evaluated by dual luciferase-reporter assay. The results showed that compound 30 m was the most active inhibitor with the lowest cytotoxicity. It effectively attenuated hypoxia-induced HIF-1α protein accumulation in a

TABLE 2 HIF-1α inhibitors under investigation in BC.

| Compound | Dose | HIF-1 activity $IC_{50}$ | Cell growth inhibition $IC_{50}$ | Model | Duration of treatment | Routes of administration | Type of study | Mechanism | Results | References |
|---|---|---|---|---|---|---|---|---|---|---|
| KC7F2 | 40 uM | 15 uM | 20 uM | MCF-7 | 8–72 h | — | *in vitro* | decrease HIF-1a protein accumulation | Inhibit cancer cell growth in a dose-independent manner | Narita et al. (2009) |
| LXY6090 | 0.4 nM-100uM | 4.11 ± 0.4 nM | T47D: 245.7 ± 15.2 nM; MCF-7: 352.7 ± 14.2 nM; MX-1: 108.2 ± 2.1 nM | T47D, MCF-7, MX-1 | 16–96 h | — | *in vitro* | downregulate HIF-1a protein and mRNA level by promoting HIF-1a proteasome degradation | Inhibit breast cancer cells growth dose-dependently | Lai et al. (2016) |
| | 25 mg/kg/d to 100 mg/kg/day | — | — | Mouse model (MX-1) | 14 days | ip | *in vivo* | depress HIF-1a expression *in vivo* | Inhibit MX-1 cells subcutaneous xenograft tumors growth in a dose dependent manner | |
| Quercetin | 10–100 uM | — | — | SkBr3 | 1–8 h | — | *in vitro* | inhibiting HIF-1a protein accumulation | Did not affect cancer cell activity | Lee and Lee, (2008) |
| Aryl Carboxamide Derivatives (30 m) | 0.5–30 uM | 0.32 uM | — | MDA-MB-231 | 24 h | — | *In vitro* | inhibit HIF-1a protein accumulation and promote its degradation | Suppress cancer cells angiogenesis activity dose-dependently, and inhibit cancer cell invasion and migration | Liu et al. (2019) |
| | 15–30 uM/ 2 days | — | — | Mouse model (MDA-MB-231) | 3 weeks | ig | *in vivo* | inhibit HIF-1a protein accumulation | Inhibit lung colonization of tumor cells without obvious body weight loss in a dose-depended manner | |
| LXY6006 | 0.1–1 uM | 0.35 ± 0.11 nM | 1.3–249.7 nM | T47D, MD-MBA-231, MX-1 | 4–5 days | — | *in vitro* | inhibit HIF-1a nuclear accumulation | Arrest cell cycle, and hold back cancer cells growth | Lang et al. (2014) |
| LXY6006 | 60 or 120 mg/kg/ 6 days per week | — | — | Mouse model (MX-1 or MX-1/ Taxol) | 13 days | ig | *in vivo* | — | Arrest both normal and taxol-resistant breast cancer xenograft growth with slight body weight loss | |
| Aminoflavone | 0.06-1uM | — | — | MCF-7 | 16 h | — | *in vitro* | depress HIF-1a protein accumulation and decreases the rate of HIF-1α translation | Shows cytotoxic effect on breast cancer cells | Terzuoli et al. (2010) |
| Aminoflavone | 60 mg/kg/day | — | — | Mouse model (MCF-7) | 4 days | — | *in vivo* | AF inhibits HIF-1α expression | Inhibit cancer growth | |

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TABLE 2 (*Continued*) HIF-1α inhibitors under investigation in BC.

| Compound | Dose | HIF-1 activity $IC_{50}$ | Cell growth inhibition $IC_{50}$ | Model | Duration of treatment | Routes of administration | Type of study | Mechanism | Results | References |
|---|---|---|---|---|---|---|---|---|---|---|
| 7-Hydroxyneolamellarin A | 0.6–50 μmol/L | 23.0 ± 2.6 μmol/L | — | MCF-7,4T1 | 4–36 h | — | *in vitro* | inhibit HIF-1a protein accumulation | Suppress the cellular migration, invasion and proliferation dose-dependently | Li et al. (2021) |
| 7-Hydroxyneolamellarin A | 15 mg/kg/ 2 days | — | — | Mouse model (4T1) | 23 days | — | *in vivo* | inhibit HIF-1a protein accumulation | Inhibit HIF-1a and breast tumor growth with slightly body weight effect | Li et al. (2021) |
| DJ12 | 2.5–100 uM | 3.6 uM | 165–250 uM | MDA-468, ZR-75, MD435 | 16 h | — | *in vitro* | decrease HIF-1a transactivation and DNA binding | — | Jones and Harris, (2006) |
| Cardenolides | — | 21.8–64.9 nM | 30.5–68.8 nM | MCF-7 | 24 h | — | *in vitro* | inhibited HIF-1 transcriptional activity dose-dependently | cytotoxic effects on breast cancer cells | Parhira et al. (2016) |
| PX-478 | — | — | — | Mouse model (MCF-7) | — | — | *in vivo* | suppresses HIF-1a levels | antitumor activity | Welsh et al. (2004) |
| Methylalpinumisoflavone | 0.01–10 uM | 0.6 μM | — | T47D, MDA-MB-231 | 24–48 h | — | *in vitro* | inhibits HIF-1 activation by blocking the induction of nuclear HIF-1α protein | Inhibit tumor angiogenesis *in vitro*, cell migration, and chemotaxis | Liu et al. (2009) |





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dependent manner, which was demonstrated by its inhibitory potency on capillary-like tube formation (Liu et al., 2019). In another study, cardenolides were isolated and purified from latex and giant fir fruit of Calotropis gigantea, a medicinal plant. These cardenolides inhibited HIF-1α transcriptional activity and exhibited potent cytotoxicity with a dose-dependent manner in MCF-7 cells, but minimal inhibitory effect on normal human breast cells (Parhira et al., 2016).

## 5.3 Small molecule compounds targeting HIF-1α related signaling pathway in BC

Using extracts from medicinal plants and chemically synthesized derivatives have become the current trend in drug development. Table 3 summarizes the small molecule compounds targeting HIF-1-related signaling pathways in BC, which mainly act on key genes regulated by HIF-1 including those involved in glycolysis, angiogenesis and metastasis.

For instance, Honokiol (HNK), a natural compound, inhibited the glycolysis of BC cells and indirectly blocked tumor growth by targeting HIF-1α/GLUT1/PDK1/HK2 pathway (Yi et al., 2022). In another report, the researchers synthesized ionone alkaloid derivatives and identified the compound ION-31a with anti-metastatic activity of BC. Although ION-31a is a heat shock protein 90 (HSP90) inhibitor, it significantly inhibits BC metastasis and angiogenesis by HSP90/HIF-1α/VEGF/VEGFR2 signaling pathway (Ni et al., 2021).

## 5.4 Drug delivery system targeting HIF-1α in BC

Here we highlight the HIF related drug delivery systems, including liposomes NPs, polymers NPs, metal-based NPs or carbon-based NPs. A few experimental drug delivery systems targeting HIF-1α are contained in Table 4.

### 5.4.1 Liposomal NPs

Liposome NPs (LNPs), a kind of spherical vesicles with a size of several hundred nanometers, can encapsulate drug molecules with vesicles from phospholipid bilayer membranes. Liposomes have several additional advantages as nanocarriers for drug delivery applications. Liposomes protect the loaded drug from degradation, reduce the rate of drug release and the toxicity of drugs due to non-target distribution (Allen and Cleland, 1980; Senior and Gregoriadis, 1982; Bobo et al., 2016). LNPs are also potential delivery carriers for hydrophilic agents by encapsulating them in the inner core.

Acriflavine (ACF) is a kind of drug that inhibits Hypoxia-inducible factor (HIF) pathway and exerts cytotoxicity. One study demonstrated that compared with free drug, liposome encapsulated ACF showed similar cytotoxicity in 4T1 cells and decreased HIF activity *in vitro*. Compared with free ACF, liposome encapsulated ACF (ACF dose of 5 mg/kg) showed higher anti-tumor efficacy in an orthotopic model of murine breast cancer (4T1 cells) *in vivo* (Montigaud et al., 2018). R8 polypeptide, a small molecule cell-penetrating peptide, can carry macromolecular substances into cells and increase active targeting of drugs (Kang et al., 2017), R8GD modified daunorubicin liposomes plus R8GD modified emodin liposomes had small and uniform particle size and high drug encapsulation rate, which allowed the chemotherapeutic drug to selectively accumulate at tumor site. VM channels and metastasis are effectively inhibited compared with free drug in MDA-MB-435 cell, which may be related to down-regulation of metastasis related proteins, including HIF-1 α (Fu et al., 2020). In another report, researchers developed a new targeted liposome mitochondrial tropical material D-a-tocopheryl polyethylene glycol 1000 succinate-triphenylphosphine conjugate (TPGS1000-TPP) to encapsulate sunitinib and vinorelbine respectively. Targeted drug liposomes are accumulated in the mitochondria of invasive breast cancer cells or VM channel forming cancer cells. It can induce acute cytotoxic injury and apoptosis and down-regulated VM channel forming indicators (MMP-9, EphA2, VE cadherin, FAK and HIF-1 α) (Shi et al., 2015). Ying Li et al. found that a cationic liposome technology can rapidly release mesenchymal-epithelial transition to enhance the cytotoxicity of doxorubicin by reduce hypoxia stress *in vivo* and inhibit HIF-1α expression *in vitro* (Li Y et al., 2019). LNPs has been identified as an effective delivery model for peptide and siRNA-based BC gene therapy. Encapsulation of these peptides and siRNAs with LNPs prevents their degradation in the vasculature environment and allows targeted delivery by using target ligands. Emine Ş Alva et al. showed that chitosan coated liposome targeted HIF-1α siRNA and VEGF siRNA can improve the efficiency of gene silencing. The siRNA-based therapy of chitosan coated liposomes may have potential in cancer treatment (Hortobagyi et al., 2001; Salva et al., 2015). Ju et al. (2014) also reported liposomes modified with PTD (HIV-1) peptide, which contains epirubicin and celecoxib, to target vasculogenic mimicry channels in invasive breast cancer. In the study of Khan et al. (2019), phospholipids, as a component of liposomes, are also an easily synthesized, biocompatible and biodegradable carrier. They used phospholipids as shells to encapsulate doxorubicin and synthesize doxorubicin loaded oxygen nanobubbles (Dox/ONB), compared with free drugs. Dox/ONB significantly inhibited HIF-1α activity and increased ROS production to enhance the antitumor effect of doxorubicin under hypoxia in breast cancer cells.

Therefore, LNPs are very popular as nano-carriers of biodegradable drugs. These drugs can be encapsulated and protected until they reach the target cells, which is particularly important for peptides and siRNAs. In addition, in order to achieve better biocompatibility, LNP is usually coated with polymer, which increases the liposome size, and the drug

TABLE 3 HIF-1 related signaling pathway inhibitors in BC.

| Component | Dose | $IC_{50}$ | Model | Duration of treatment | Routes of treatment | Type of study | Mechanism | Results | References |
|---|---|---|---|---|---|---|---|---|---|
| Honokiol | 0–40 uM | — | MCF-7, MDA-MB-231 | 3–24 h | — | *in vitro* | downregulated HIF-1α protein expression | Inhibited cell proliferation and clonogenicity, as well as induced apoptosis of cancer cells | Yi et al. (2022) |
| | 25 mg/kg/day | — | Mouse model (MCF-7) | 4 weeks | ip | *in vivo* | decrease HIF-1α protein level | Suppressed tumor growth and HIF-1α-mediated glycolysis | |
| Sinomenine | 0.75 mM | — | stem-like side population (SP) cells gained from MDA-MB-231 | 24 h | — | *in vitro* | downregulating HIF-1α | Inhibit the migration and vasculogenic mimicry, and hold back epithelial-mesenchymal transition process | Song et al. (2022) |
| Polydatin (PD) combined with 2-deoxy-D-glucose (2-DG) | PD 100 μmol/L, 2-DG 5 mmol/L (4T1) or 10 mmol/L (MCF-7) | PD: 66.56uM(4T1)/ 103.1 uM (<CF-7), 2-DG: 5.53 mM(4T1)/ 8.67 mM (MCF-7). (24 h) | MCF-7 and 4T1 | 0–72 h | — | *in vitro* | inhibit HIF-1alpha/HK2 to suppress glycolytic metabolism | Induced cell apoptosis and inhibited cancer cells proliferation, migration and invasion | Zhang et al. (2019) |
| Polydatin (PD) combined with 2-deoxy-D-glucose (2-DG) | PD (100 mg/kg every other day), 2-DG (100 mg/kg ip every other day) | — | Mouse model (4T1) | 3 weeks | ip | *in vivo* | anti-proliferative and anti-angiogenic activity, promoted apoptosis | Inhibit cancer growth *in vivo* | Zhang et al. (2019) |
| bishonokiol A | 2.5–10 uM | — | MCF-7, MDA-MB-231 | 24–48 h | — | *in vitro* | hold back HIF-1a expression and its protein synthesis | Inhibit cancer cell invasion and migration | Li H. M et al. (2019) |
| | 100 mg/kg/ 3 days | — | Mouse model (MDA-MB-231) | 16 days | ip | *in vivo* | — | Antitumor activity and low toxicity | |
| Alkaloid derivative ION-31a | 0–75 uM | — | MDA-MB231, 4T1 | 24 h–48 h | — | *in vitro* | downregulate HIF-1α/VEGF signaling pathway | Inhibit cell migration, invasion, adhesion, and VEGF secretion | Ni et al. (2021) |
| Alkaloid derivative ION-31a | 25–100 mg/kg | — | Mouse model (4T1) | 26 days | ig | *in vivo* | — | Depress tumor growth and metastasis with slightly bodyweight change | |
| HS-1793 | 0–50 uM | MCF-7: 26.3 ± 3.2; MDA-MB-231: 48.2 ± 4.2 uM | MCF-7 and MDA-MB-231 | 24 h | — | *in vitro* | downregulate HIF-1a protein level and its target gene VEGF expression | inhibit cancer cells proliferation, and decrease the angiogenesis | Kim et al. (2017) |

(Continued on following page)

TABLE 3 (*Continued*) HIF-1 related signaling pathway inhibitors in BC.

| Component | Dose | $IC_{50}$ | Model | Duration of treatment | Routes of treatment | Type of study | Mechanism | Results | References |
|---|---|---|---|---|---|---|---|---|---|
| | 0–20 mg/kg/ twice a week | — | Mouse model (MDA-MB-231) | 4 weeks | ip | *in vivo* | downregulate HIF-1a protein level | Inhibit tumor growth, and suppress microvessel formation | |
| Salinomycin | 0–30 uM | — | MCF-7, T47D, MDA-MB-231, MDA-MB-468, 4T1 | 12–24 h | — | *in vitro* | decreased the HIF-1α transcription factor DNA binding activity | Inhibit cell proliferation, invasion, and migration | Dewangan et al. (2019) |
| | 5–10 mg/kg/ 3 days a week | — | Mouse model (4T1) | 3 weeks | ip | *in vivo* | inhibited hypoxia-induced HIF-1α/VEGF signaling axis | inhibits breast cancer growth and tumor angiogenesis | |
| HS-146 | — | — | MCF-7 | — | — | *in vitro* | depress hypoxia-induced HIF-1α/ VEGF signaling axis | Inhibit cancer cell proliferation, migration and invasion in a dose-dependent manner | Kim et al. (2020) |
| Baicalein | 0–25 uM | — | T-47D, BT-474 and ZR-75–1 | 24–72 h | — | *in vitro* | inhibit HIF-1α–mediated aerobic glycolysis and mitochondrial dysfunction | — | Chen et al. (2021) |
| | 30 mg/kg/ 3 days | — | Mouse model (MCF-7TR) | 30 days | | *in vivo* | inhibit HIF-1α–mediated aerobic glycolysis and mitochondrial dysfunction | Baicalein increases the inhibitory effects of TAM on the growth of MCF-7TR cells *in vivo* | |
| Chiral ionone alkaloid derivatives | 0–30 uM | 0.035 μM ± 0.004 | MDA-MB-231 | 0–24 h | — | *in vitro* | inhibit HIF-1α/ VEGF/VEGFR2/ Akt pathway | Depress cancer cell migration, adhesion, migration and invasion | Liu J. J et al. (2021) |
| Cardamonin | — | 24.458–52.885 uM | MDA-MB-231 | 24–72 h | — | *in vitro* | inhibit HIF-1a expression on mRNA and protein level | Inhibit cancer cell viability and promotes apoptosis | Jin et al. (2019) |
| | 3 mg/kg/day | — | Mouse model (MDA-MB-231) | 4 weeks | ip | *in vivo* | suppress HIF-1α/PDHK1 axis by inhibit the mTOR/p70S6K pathway | Inhibit tumor growth | |
| AT-533 | 0–75 uM | — | MDA-MB-231, MCF-7 | 12–72 h | — | *in vitro* | downregulate HIF-1α/VEGF signaling pathway | Inhibit breast cancer cells viability | Zhang et al. (2020) |
| | 10 mg/kg/ 2 days | — | Mouse model (MDA-MB-231) | 12 days | ip | *in vivo* | block the HIF-1α/VEGF/ VEGFR-2-mediated signaling pathway | Inhibit growth of breast cancer xenografts *in vivo* | |

(Continued on following page)

TABLE 3 (*Continued*) HIF-1 related signaling pathway inhibitors in BC.

| Component | Dose | $IC_{50}$ | Model | Duration of treatment | Routes of treatment | Type of study | Mechanism | Results | References |
|---|---|---|---|---|---|---|---|---|---|
| Rhaponticin | 0–100 uM | — | MDA-MB231 | 48 h | — | *in vitro* | decreased HIF-1α accumulation and HIF-1α nuclear expression | suppress cancer cells colony formation, migration, invasion and angiogenesis | Kim and Ma, (2018) |

TABLE 4 Drug delivery systems for targeting HIF-1α.

| Carrier and feature | Pharmaceutical ingredients | Cell line | References |
|---|---|---|---|
| HPDA | BEZ235 | 4T1 | Liu et al. (2022) |
| PLGA-NP | Curcumin | MDA-MB231 | Khan et al. (2018) |
| FA-BSA-MnO2 | DOX/siRNA | MCF-7 | Du et al. (2019) |
| PVCL-PVA-PEG | Betulinic acid | MDA-MB-231 | Qi et al. (2021) |
| SPION-TMC-ChT-TAT-H NPs | siRNA | 4T1 | Budi et al. (2021) |
| ONB | Dox | MDA-MB-231 | Khan et al. (2019) |
| Carbon nanoparticles | docetaxel | Walker256 | Liu W et al. (2021) |
| Liposomal | echinomycin | MCF-7/SUM-159/MDA-MB-231 | Bailey et al. (2020) |
| RBCm | Sal/ICG | 4T1 | Pan et al. (2022) |

release process may be affected by opening the phospholipid bilayer.

### 5.4.2 Metal-based NPs

Metal nanomaterials, also known as metal oxide nanomaterials, contain the core of magnetic and optical properties and the shell of the machine surface coating, which can make drugs gather in the local part of the body under the action of external magnetic field. Superparamagnetic iron oxide NP(SPION-NP) is a kind of magnetic nanomaterials. Researchers used SPION-NPs coated with thiolated chitosan (ChT) and trimethyl chitosan (TMC) and functionalized with hyaluronate (H) and TAT peptide for delivery of siRNA molecules against STAT3 and HIF-1α to cancer cells both *in vivo* and *in vitro*. The results indicated that tumor cell transfection with siRNA-encapsulated NPs robustly inhibited proliferation and migration and induced apoptosis in breast cancer cells (Budi et al., 2021). Similarly, researchers utilized superparamagnetic iron oxide-based NPs (SPIONs) combined with chitosan lactate (CL) and folic acid (FA) nanoparticles (NPs) loaded with TIGIT-siRNA and HIF-1α-siRNA for suppressing TIGIT and HIF-1α in tumor cells in another study. Results showed that cancer cells treated with TIGIT and HIF-1α siRNA-loaded SPIONs-CL-FA NPs strongly suppressed the TIGIT and HIF-1α expression and cancer angiogenesis (Fathi et al., 2021). At present, there are only a few studies on metal nanocarriers targeting HIF-1α in breast cancer and SPION has certain toxicity. More optimized metal nanocarriers may be developed in the future.

### 5.4.3 Polymer-based NPs

Polymer-based NPs (PNPs) have been extensively studied as drug delivery vehicles. PNPs are usually prepared by combining a copolymer with another polymer matrix. Polymer-based NPs can be synthesized from native polymers, such as hyaluronic acid, chitosan (Agnihotri et al., 2004; Choi et al., 2010), as well as synthetic polymers such as polyglycolic acid (PGA), poly (lactate-coethylene glycol) (PLGA). Polylactic acid (PLA), polyllactide-coethyl ester (PLGA) and chitosan are the most typical biodegradable and biocompatible polymers. Anticancer drugs can be incorporated into the surface of PNP by surface adsorption, chemical coupling or encapsulation. Curcumin is a NF-κβ inhibitor. A study reported that researchers fabricated biodegradable poly (lactic-co-glycolic acid) PLGA nanoparticles (NP) loaded with curcumin (cur-PLGA-NP). These nanoparticles effectively facilitated the targeting of curcumin by delivering to the tumor site in the form of nanoparticles in the hypoxic micro-environment. Compared with free curcumin, the nano-formulation group has increased solubility and anti-tumor activity, which can effectively improve the tumor hypoxic microenvironment and block the occurrence and development of tumors by suppressing HIF-1α (Khan et al., 2018). Botulinic acid (3β-

Hydroxy-20 (29)-lupaene-28-oic acid, BA) is a kind of pentacyclic triterpenoids with various biological activities such as antitumor, antiviral, anti-inflammatory and antioxidant. Due to poor solubility and low bioavailability, it cannot be used to effectively treat BC. In order to improve the antitumor activity of BA, researchers prepared polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol (PVCL-PVA-PEG) grafts Copolymer (Soluplus) encapsulated BA micelles, which inhibit the angiogenesis of BC cells by suppressing the HIF-1/VEGF/FAK signaling pathway (Qi et al., 2021). Similarly, Betulinic acid (3β-Hydroxy-20 (29)-lupaene-28-oic acid, BA) has excellent anti-cancer activity but low bioavailability for poor solubility. A polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol (PVCL–PVA–PEG) graft copolymer (Soluplus) encapsulated BA micelle (Soluplus-BA) was fabricated and results showed that Soluplus-BA micelles increased the inhibitory effect of BA on the angiogenesis by regulating the HIF-1/VEGF-FAK signaling pathway in breast cancer MDA-MB-231 cells (Qi et al., 2021). Recently a photodynamic therapy based on conjugated PNPs for BC has been reported (Liu et al., 2022). Ying Zhang et al. (2022) synthesized photochemical-responsive nanoparticle by incorporating DOX, curcumin (CUR), and perfluorooctyl bromide (PFOB) into poly (lactic-co-glycolic acid) (PLGA) *via* double emulsification (DOX-CUR-PFOB-PLGA). The synthesized composite nanoparticles with good ultrasound imaging induced MCF-7 cells apoptosis by downregulating AKT/HIF-1α signaling pathway. A drug delivery nanoplatform equipped with dual PI3K/mTOR inhibitor Dactolisib (NVP-BEZ235, BEZ235) and CAIX inhibitor 4-(2-aminoethyl) benzene sulfonamide (ABS) was designed to form HPDA-ABS/PEG-BEZ235/Ce6 (H-APBC) nanoparticles. The study showed that the H-APBC could produce ROS upon light irradiation and release of BEZ235 from H-APBC in acid microenvironment could mitigate PI3K/mTOR signal and resist HIF-1α-dependent tumor hypoxia adaptation (Liu et al., 2022). Photodynamic therapy (PDT) has become an emerging area of modern medicine. Studies have shown that the synergistic effect of PDT could enhance the effectiveness and reduce the limitations of the original treatment modality (Chen L et al., 2019; Xie et al., 2020).

### 5.4.4. Carbon-based NPs

Carbon nanotubes (CNTS) have a cylindrical shape with a long, hollow structure and a wall formed of graphene sheets. Carbon nanotubes have the advantages of thermal conductivity, optical and electrical properties. In addition, as nanocorbs. CNTS can act as excellent optical absorbers in near-infrared (NIR) light due to their tunable surfaces and unique thermal properties. Researchers designed a novel targeted multifunctional nanoplatform, which refers to docetaxel (DOC) and perfluorohexane (PFH) loaded onto carbon nanoparticles (CNs), and combined them with anti-HIF-1α antibody-modified PLGA nanoparticles (HPDC NPs) to achieve dual US/PA imaging-guided and laser-triggered *in situ* DOC release. HPDC NPs efficiently deliver CNs and DOC into lymph nodes to achieve their targeting behavior and the nanoparticles can be destroyed under NIR-I laser irradiation and subsequently release DOC molecules. This study not only provides targeted chemotherapy-hyperthermia synergistic therapy by laser-triggered, highly efficient *in situ* chemotherapeutic nano systems, but also represent a nano-delivery route that avoids additional damage from drug entry into the bloodstream (Liu W et al., 2021). Compared with metal-based NPs, carbon based NPs can be considered as a more promising DDS for cancer treatment and diagnosis. However, the preparation of carbon nanotubes is complex and there is a challenge in poor solubility and biodegradability of CNT (Mehra et al., 2008).

## 6 Conclusions and future perspectives

The recent in-depth refinement and diversify of treatments modalities for BC have led to significant control of tumors as well as improved patient prognosis. However, these treatments are considered as only temporary control of metastasis and primary tumors, and most patients often face recurrence and metastasis after treatment. HIF-1 may promote the development of BC through a series of downstream pathways, and its overexpression is related to tumor progression and BC mortality. For this reason, HIF-1 may be a potential therapeutic target in BC. However, HIF-1 inhibitors are very rare in clinic. Although more and more HIF inhibitors have been found, they are still inadequate as for drug selectivity and specificity. In addition, HIF has complex interactions among multiple pathways, which makes the clinical application of HIF inhibitors more challenging. Therefore, at this stage, we believe that it is a prerequisite to develop specific HIF-1 inhibitors and further clarify the regulatory pathway of HIF. In addition, the upstream governor of HIF-1 is also an attractive strategy, and a deeper understanding of the regulatory mechanism of the upstream regulator of HIF-1 will help us to start new therapeutic interventions. On the other hand, improving targeting specificity, overcoming solubility and reducing drug toxicity have attracted widespread attention on the drug delivery system based on nano-carriers, while only a few drugs based on nano-carriers are used in preclinical research stage. The toxicity of nano-carriers to the body and the metabolism of drugs loaded on nano-carriers is a complex topic. It may be necessary to find non-toxic nanoscale carriers and to test the metabolic changes of nanomaterials in *vivo* model. With the progress of nano-biotechnology and the development of cancer treatment, we believe that the difficulty of nano-carrier

in clinical treatment of BC will be broken through, and more drugs based on nano-materials will benefit BC patients. Overall, targeting hypoxia is a very promising way for cancer therapy but its real fulfillment requires time and great efforts.

## Author contributions

SL, YJ, and YZ contributed to write and edit the manuscript. AZ and SD performed bioinformatics analysis. XW, ML, FD, YC, and LG helped to collect documents. MC and WL designed the pictures and XL and YS helped to revise them. JS and ZX revised the manuscript and gave valuable suggestions. All authors have read and agreed to the published version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Hypoxia induced lactate acidosis modulates tumor microenvironment and lipid reprogramming to sustain the cancer cell survival

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It is well known that solid hypoxic tumour cells oxidise glucose through glycolysis, and the end product of this pathway is fermented into lactate which accumulates in the tumour microenvironment (TME). Initially, it was proclaimed that cancer cells cannot use lactate; therefore, they dump it into the TME and subsequently augment the acidity of the tumour milieu. Furthermore, the TME acts as a lactate sink with stope variable amount of lactate in different pathophysiological condition. Regardless of the amount of lactate pumped out within TME, it disappears immediately which still remains an unresolved puzzle. Recent findings have paved pathway in exploring the main role of lactate acidosis in TME. Cancer cells utilise lactate in the *de novo* fatty acid synthesis pathway to initiate angiogenesis and invasiveness, and lactate also plays a crucial role in the suppression of immunity. Furthermore, lactate re-programme the lipid biosynthetic pathway to develop a metabolic symbiosis in normoxic, moderately hypoxic and severely hypoxic cancer cells. For instance: severely hypoxic cancer cells enable to synthesizing poly unsaturated fatty acids (PUFA) in oxygen scarcity secretes excess of lactate in TME. Lactate from TME is taken up by the normoxic cancer cells whereas it is converted back to PUFAs after a sequence of reactions and then liberated in the TME to be utilized in the severely hypoxic cancer cells. Although much is known about the role of lactate in these biological processes, the exact molecular pathways that are involved remain unclear. This review attempts to understand the molecular pathways exploited by lactate to initiate angiogenesis, invasiveness, suppression of immunity and cause re-programming of lipid synthesis. This review will help the researchers to develop proper understanding of lactate associated bimodal regulations of TME.

KEYWORDS

**hypoxia, HIF-1α, lactate, angiogenesis, invasiveness, resistance, Immunity, Lipid reprogramming**

# 1 Introduction

Chemotherapy of solid malignant tumours has become a major challenge because of the development of resistance due to hypoxia-a condition characterised by lower amount of oxygen. In addition to imparting resistance to chemotherapy, it helps tumour cells acquire the most favourable environment, which supports their survival, even in oxygen- and nutrient-deficient environments (1). Various studies have reported that hypoxia-activated hypoxia-induced factor-1α (HIF-1α) functions at the gene level to enhance angiogenesis, metastasis, and invasiveness of cancer cells (2). HIF-1α also alters glucose, fatty acid, and amino acid metabolism to support cancer cell survival (3). Reprogramming of glycolysis has already been reported by Warburg group. They stated that cancer cells metabolise glucose only through glycolysis, even if there is sufficient oxygen supply which results in excessive accumulation of lactate (4). The continuous release of lactate in the tumour microenvironment (TME) makes it even more acidic (5). For many years, lactate has been recognised as a metabolic waste product which is toxic to cancer cells and is pumped out in the TME, but how TME pumped out the dump lactate is still not clear. Later, it was reported that malignant cells can utilise lactate secreted by nearby cancer cells. Recently, Brandon et al. reported that lactate fuels the tricarboxylic acid (TCA) cycle in normoxic cancer cells. *In-Vitro* and *In Vivo* studies on non-small cell lung carcinoma cells (NSC-LCs) demonstrated that, similar to glucose metabolism, lactate can also be utilised for energy production if the cancer cells have an adequate oxygen supply. Previous studies have already reported that tissue architecture and anatomical location also influence the use of lactate as a fuel. For instance, lung carcinoma cells can oxidise lactate in the TCA because lung tissue has a high level of perfusion and oxygenation (6). Later, Hui et al. in their study reported that glucose-derived lactate indirectly feeds into the TCA cycle. They carefully examined the fluxes of $C^{13}$-labelled lactate in mice *via* intravenous infusion. The results of this study showed the highest circulatory lactate turnover flux. Moreover, the circulatory turnover flux of lactate exceeded that of glucose by 2.5-fold in fasted mice and 1.1 fold in fed mice. To determine whether cancer cells can use lactate, they injected $C^{13}$-labelled lactate, glutamate, and alanine into genetically engineered lung and pancreatic tumour cells, and noted that the circulating lactate input in the citric acid cycle outpaced that of glucose. These studies clearly demonstrate that cancer cells can utilise circulating lactate (7). However, the mechanism through which cancer cells utilise lactate remains unclear.

Numerous studies have demonstrated an immunosuppressive role of lactate in cancer. It has been previously reported that a lactate-derived acidic TME in cancer has the potential to abolish cytotoxic T cells (CD8+ T cells) and natural killer (NK) cell anticancer immune responses. The acidic TME also interferes with the antigen presentation process of dendritic cells (DC) and halts maturation and differentiation (8). However, it remains a matter of discussion that how lactate protect cancer cells from the innate and adaptive immune responses and is there any way to trigger anticancer immune cells by regulating lactate acidosis?

Additionally, lactate is involved in angiogenesis and metastasis. Zhou et al. reported that lactate promotes neovascularization and neurogenesis *via* the Nuclear factor kappa-B (NF-kB) signalling lane. Although several studies have established the link between lactate and angiogenesis but induction of angiogenesis by lactate mediated signalling through activation of NF-kB pathway has not been well understood (9).

Lactate also help the cancer cell's invasion into the adjacent organs. Previous studies have delineated how lactate initiates invasiveness by inducing claudin-1 (Cln-1) expression through mitochondrial respiratory defects. However, the exact underlying mechanisms remain unknown (10). Later, An et al. also reported that elevated cytosolic enzymes, such as lactate dehydrogenase (LDHA), help tumour cells to become invasive. The results of this study confirmed that the overexpression of LDHA in pituitary oedema promotes cell invasion and proliferation (11).

Previously, in our lab, we have reported that hypoxia upregulates the fatty acid synthesis in mammary gland cancer cells. Results of the immunoblotting and metabolomics studies documented increased level of HIF-1α, sterol regulatory element binding proteins (SREBP) and fatty acid synthase (FASN) while level of prolyl hydroxylase-2 (PHD-2) was reduced significantly (12–14). Serum metabolomics profile also showed increased level of lactate and low density lipoproteins/very low density lipoproteins (LDL/LDL) and poly unsaturated fatty acids. We performed few more studies in the same direction and every time we noted increased level of lactate and fatty acids in carcinogen treated animals (15). Further we reported that hypoxia and lipid biosynthesis can be curtailed through activation of PHD-2. Interestingly we observed reduced level of HIF-1α, lactate, SREBP and fatty acids upon chemical activation of PHD-2 (16, 17). Based upon our observation we developed another hypothesis that lactate can be incorporated into lipid biosynthetic pathways because glucose alone is inefficient in meeting the increasing demand for fatty acids in malignant cells. But which pathway is exploited by cancer cells for incorporation of lactate into fatty acid synthesis-is still unknown.

From the above discussion, it is evident that lactate is the major metabolite that helps malignant cells suppress immunity, impart resistance to chemotherapy, and promote angiogenesis and metastasis. Although much has been discussed regarding the role of lactate in the abovementioned process, there are still several gaps in the literature that raise various questions. The current review aims to fill the gap in understanding the role of lactate in the aggressive transformation of malignant solid tumours and to answer the unrevealed questions. This review focuses on the synthesis of lactate-derived fatty acids in detail and its association as well as significant role in TME.

# 2 Development of hypoxia and activation of HIF-1α in solid tumours

Cancer cells immediately develop hypoxia as their distance continues to increase owing to an increase in tumour size. Owing to continuous pushing away from blood vessels, cancer cells face nutrient and oxygen deficiencies called hypoxia (18, 3). Normal cells undergo apoptosis in nutrient-deficient environments; however, cancer cells are immortal. They take help from HIF-1α (a cytoplasmic protein expressed ubiquitously) which is activated in

an oxygen-deficient environment, and is transported into the nucleus after dimerisation with their cytoplasmic subunits (19). In the nucleus, dimerised HIF-1α regulates the expression of several genes that play unique roles in cell cycle regulation, apoptosis, angiogenesis, metastasis, and invasiveness (20). HIFs-α also monitor the metabolism of glucose, fatty acid and amino acids specifically and wisely to sustain the survival of normoxic and hypoxic cancer cells in the TME. All of the aforementioned effects are discussed in detail in the preceding section of this review.

## 3 HIF-1α shifts the metabolism of glucose through glycolysis to enhance production of lactate

Firstly, Otto Warburg reported that cancer cells metabolise glucose under aerobic conditions. The incomplete oxidation of glucose to pyruvate leads to the accumulation of lactic acid which is pumped into the TME. Continuous pumping of lactate to the extracellular compartments increases the acidity of the TME, which indirectly functions under the instructions of HIF-1α to reprogram fatty acid and amino acid metabolism as a signalling molecule to induce metastasis, angiogenesis, invasiveness, and suppression of immunity (21). The exact molecular pathways exploited by the lactate to induce angiogenesis, invasiveness, immune suppression and fatty acid synthesis is discussed in much detail in the preceding section under individual headings.

## 4 Role of lactate in angiogenesis

An increase in the size of the tumour not only increases the suffering of patients but it also increases the proportion of hypoxic cancer cells. With an increase in tumour size, cells that were initially located near to blood vessels were slowly displaced away from the blood vessels. These cancer cells have a limited supply of oxygen and nutrients and become hypoxic (22). Usually, normal cells die under oxygen and nutrient deficiency conditions, but cancer cells uses alternative machinery; hence, new blood vessels are formed, and the process is known as angiogenesis (23). Several studies have demonstrated that lactate promotes angiogenesis but how does lactate initiates angiogenesis in cancer cells is yet unknown. Previous studies have reported that HIF-1α regulates the activation of various pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1, platelet-derived growth factor-B (PDGF-B), and overexpression of VEGF receptor genes, especially Fms-related receptor tyrosine kinases (FLT-1 and FLK-1), matrix metalloproteinases (MMP-2), TIE2(a receptor tyrosine kinase) receptor, and angiopoietins (ANG-1 and ANG-2). It has been shown that 47 of the total pro-angiogenic factors reported to date are regulated by HIF-1α (24, 25). Kishimoto et al. reported that neovascularization is indispensable for tumour growth and development. *In Vivo* and *In Vitro* studies on melanoma cells have shown that the angiogenic factor ANG is upregulated many-fold in hypoxic environments (26). Another study by Wang et al. reported that hypoxia effectively supports the initiation of angiogenesis in cancer cells. They cultured mouse breast carcinoma 4T1 cells under normoxic (21% oxygen) and hypoxic (1% oxygen) conditions. The results of the mRNA expression showed a higher level of angiogenesis-associated factors, such as VEGF, fibroblast growth factor-2 (FGF-2), PDGF-B, placental growth factor (PIGF), and ANG-2, in 4T1 cells cultivated in an oxygen-deficient environment. Hypoxic 4T1 cells treated with metformin showed reduced expression of these angiogenic factors and reduced the hypoxic prove-positive area. Hypoxia prove positive area is a tumor region highlighted by the antibodies. The results of this study are well supported by *In Vivo* experiments. Breast carcinoma was induced by injecting 4T1 cells into mammary gland tissue. Immunohistochemistry analysis demonstrated enhanced expression of proangiogenic factors which got significantly reduced after metformin treatment (27, 28). These studies clearly demonstrate the role of hypoxia in angiogenesis but how hypoxia regulates angiogenesis still not clear. More recently, it was reported that lactate in the tumour microenvironment signals neovascularization in hypoxic cancer cells. Consequently, decreased vascular perfusion in malignant tumours resulted in lower oxygen and glucose levels (29). To survive in this harsh environment, malignant tumour cells oxidise glucose anaerobically which results in excess production of lactate and a reduction in pH in the tumour microenvironment (30). The acidic tumour microenvironment stimulates cancer cells to secrete a wide variety of angiogenic factors to re-establish local blood supply. The study also reported that tumor associated macrophages (TAM) secrete similar pre-angiogenic factors under stressful conditions (31). Sonveaux et al. cultured bovine aortic endothelial cells (BAECs) to delineate the influence of lactate on angiogenesis in hypoxic cancer cells. NMR metabolomics revealed increased lactate levels in the hypoxic cells. Immunohistochemistry and western blotting revealed increased expression of monocarboxylate transporter-I (MCT-1 and 4). Hypoxic cancer cells oxidise glucose anaerobically, and the resultant lactate is pumped into the TME through MCT-4. Lactate is transported from hypoxic cancer cells to normoxic cancer cells, where it is absorbed by oxidative cancer cells (through the MCT-1 transporter) and utilised in the citric acid cycle for energy production. Furthermore, they performed *In Vivo* experiment to confirm whether lactate has any role in angiogenesis; they implanted Matrigel plugs subcutaneously in the flanks of mice. CD31 immunolabelling was performed and observed a 10 fold increase in endothelial colonisation (32). Kes et al. filled this gap and described the role of lactate in angiogenesis through a comprehensive review. They reported that lactate released by hypoxic malignant cells is taken up by tumour-associated macrophages (TAM) that secrete various cytokines to initiate neovascularization in cancer cells (33, 34). Although the above studies clearly demonstrate that lactate directly communicates with endothelial cells to initiate angiogenesis, the exact signalling pathways involved have not been properly resolved. Guo-Xiang and Kazlauskas cultured human umbilical endothelial cells (HUVECs) of veins to explore the signalling pathway using lactate to induce angiogenesis. The results of qRT-PCR and western blotting showed that lactate enhanced the expression of Axl, Tie2, and VEGFR-2 in endothelial cells, and these receptors were further stimulated by an autocrine mechanism. The expression of Gas6, Ang1, and VEGF increased several-fold in lactate-treated cells which further acted on Axl, Tie2, and VEGFR-2 receptors.

Activation of Axl, Tie2, and VEGFR-2 receptors further causes phosphorylation of PI3K and Akt, thus initiating angiogenesis (35). Axl, Tie2, and VEGFR-2 belong to a family of tyrosine kinase receptors and are involved in the regulation of diverse activities in endothelial cells. Tie1 and Tie2, endothelial cell-specific tyrosine kinase receptors, are indispensable for the maturation and remodelling of lymphatic and blood vessels (36). ANG-1 and ANG-2 are important ligands for the Tie2 receptors (37). Asahara et al. performed a corneal micropocket assay to explore the role of angiopoietin-1 and 2 in neovascularization. The results of the study reported that neither angiopoietin 1 nor 2 alone promoted angiogenesis, whereas the addition of VEGF alone initiated angiogenesis. It was also observed that the addition of angiopoietin-1 and 2 along with VEGF more aggressively initiated the process of angiogenesis (38). These studies not only revealed the role of ANGs but also demonstrated that VEGF is equally important for initiating angiogenesis (39).

Axl is another important tyrosine kinase receptor present in approximately all body tissues (40). Previous studies have reported that Axl plays a pivotal role in metastasis and angiogenesis in various cancers (41). It has also been reported that Gas6/Axl signalling pathways inhibit metastasis, invasion, angiogenesis, immune regulation, and stem cell maintenance (42). Axl overexpression has been reported in patients with NSCLC, along with poor invasion, metastasis, and drug resistance (43).

From these findings, it can be concluded that energy stress in hypoxic cancer cells initiates angiogenesis. Hypoxia-activated HIF-1α helps hypoxic cancer cells to overcome energy stress by inducing angiogenesis. HIF-1α can initiate neovascularisation in hypoxic cancer cells by enhancing the gene expression of VEGF by or by utilizing lactate as a signalling molecule (44, 19).

A.VEGF andDll4 mediated angiogenesis: In this mechanism the activation of various genes, such as VEGF which when expressed by hypoxic cancer cells, is secreted into the TME. VEGR from TME binds to VEGFR1/2) present on the endothelial tip cells and triggers the release of Delta-like ligand-4 (Dll4) present on the endothelial tip cells (Figure 1) (45). The Dll4 ligand binds to the Notch receptor present on stalk endothelial cells and triggers their proliferation and the subsequent sprouting of blood vessels (46). Although previous studies have reported that Dll4 acts a negative regulator of angiogenesis, exact role of Dll4 in angiogenesis remains undetermined.

B.Lactate mediated angiogenesis: Lactate can induce angiogenesis by both mechanism-directly and indirectly

B.1.Indirect mechanism: Lactate can indirectly induce angiogenesis by acting as a signalling molecule. Excess lactate produced by hypoxic cancer cells flows back into blood vessels and is taken up by the normoxic cancer cells. It is reused in oxidative phosphorylation for energy production, and some portion enters TAM and endothelial cells (EC) (47). Lactate can enter TAM directly through the MCT-1 transporter or act on the G-protein coupled receptor(GPCR) as a signalling molecule (48). Following the activation of GPCR and subsequent activation of adenyl cyclase, cAMP and Inducible cAMP Early Repressor (ICER) lead to the activation of various genes involved in the anti-inflammatory process initiated by TAM like Arginase-1(Arg-1), resistin-like molecule alpha1(Fizz-1),CD206, and VEGF molecules. Once inside the TAM, lactate acts through various mechanisms to an anti-inflammatory mechanism which moves towards the nearby blood vessel and binds to their defined receptors present on endothelial cells, subsequently triggers the sprouting of stalk/tip EC cells into new blood vessels (33).

B.2.Direct mechanism: Another mechanism that initiates angiogenesis involves the direct entry of lactate into endothelial cells through the MCT-1 transporter followed by the activation of various genes like Gas6,VEGF,Ang-1 and IL-8 (49). The gene products binds on their respective receptors (VEGFR, Tie2 and Axl) in autocrine mechanism through PI3/Akt pathways and regulate the expression of various genes involved in cell cycle of ECs like cycline-D1, P21 and Myc-1. Activation of cyclin-D1 triggers the DNA replication and thus mitosis in ECs. Continuous division in ECs eventually develop new blood vessels. This is how lactate initiate angiogenesis in hypoxic tumors. Inhibition of lactate circulation in TME can prevent angiogenesis in malignant tumors.

## 5 Role of lactate in induction of invasiveness

Invasiveness is another characteristic feature of cancer cells by which they invade nearby tissues after reaching a reasonable size. A continuous increase in tumour size eventually leads to a breach in the barriers between adjacent tissue cells (50). Invasiveness is also the first indication for the development of secondary tumours and distant metastases (51).

Basement remodelling and EMT are two well-known hallmarks of invasive behaviour in tumour cells. A considerable number of studies have documented the role of various factors in EMT; however, the role of lactate in tumour invasiveness is not well understood (52). Before discussing the role of lactate in invasiveness, we should take a look on the micro architecture of the basement membrane and epithelial layers.

The basement membrane is a layer of connective tissue just below the epithelial layers, comprising of collagen IV, collagen VII, and glycoproteins which provide mechanical support to tissue cells. The microscopic view of the epithelial layer shows that all cells that have epithelial tissue adhere to each other which is supported by various types of micronised junction proteins, such as adherent junctions, desmosomes, and junctions. Desmosomes remain connected to each other through intermediate filaments of cytokeratin, whereas cortical bundles allow the adherent junction to be in position. Integrin proteins linked to cytokeratin inside the cytoplasm help epithelial cells adhere to the basement membrane (53). During EMT, epithelial cells lose integrin proteins and detach themselves from the basement membrane.

However, the regulation and initiation of cancer cell invasiveness remain unclear. Recent studies have made remarkable progress in delineating the roles of various metabolic intermediates, among which lactate is the major metabolite involved in the induction of invasiveness in cancer cells. June-Hyungkim, while working on

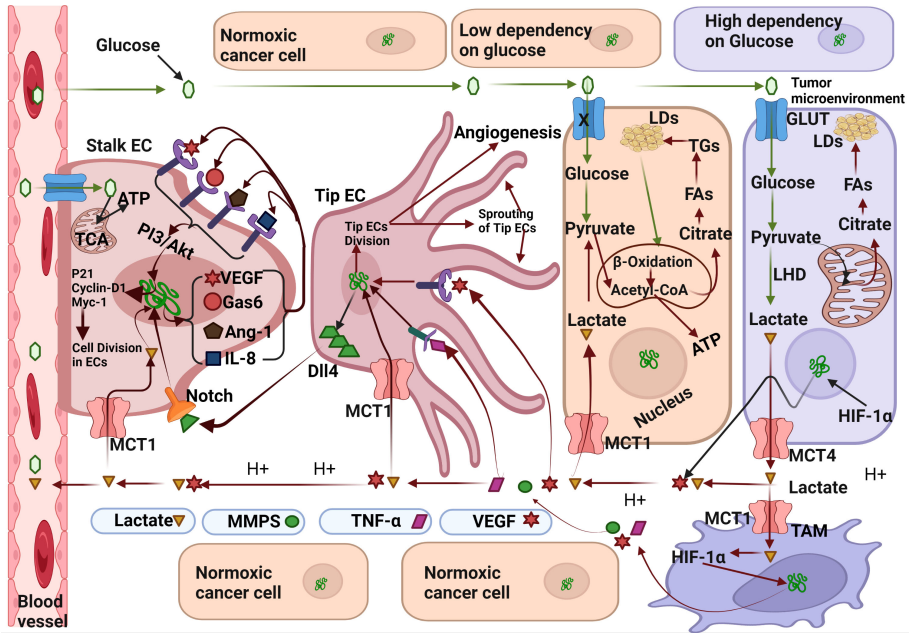


FIGURE 1
Molecular mechanism exploited by Lactate for angiogenesis induction. Hypoxic cancer cells can metabolise glucose only by glycolysis resulting in accumulation of lactate which is secreted in the tumor microenvironment (TME) through the monocarboxylate transporter-1(MCT-4). Lactate from TME flows towards nearby blood vessels and enters endothelial cells (EC) through MCT-1 transporter. After entering stalk epithelial cells, lactate upregulates the expression various pro-angiogenic genes like-Gas6, VEGF, Ang-1, and IL-8 which acts in the autocrine mechanism on their respective receptors. Gas-6 binds to the Tie2 receptor, VEGF binds to the VEFR2 receptor, Ang-1 binds to the Axl receptor, and IL-8 binds to the IL-8 receptor. Activation of VEGFR2, Tie2, IL-8 and Ang-1 receptors initiate cell division in stalk epithelial cells by activation of Cyclin-D/Myc-1/p21 genes and subsequently lead to the differentiation of stalk epithelial cells into new blood vessels. Fatty acids synthesised from lactate derived citrate in normoxic cancer cells transported towards the stalk ECs wherein used in β-Oxidation to full fill their energy needs. Lactate also enters tip epithelial cells and activates various genes which also regulate the cells division of tip ECs. A fraction of lactate also enters the tumor associated macrophages (TAM) through MCT-1 transporter and activates the inflammatory mediators like TNF-α, Vascular Endothelial Growth Factor and Metalloproteinases (MMPs). MMPs digest the stromal proteins, clears the road to sprouting blood vessels. Binding of VEGF and TNF-α also upregulates the expression of Delta-like-ligand 4(Dll4) which further regulate the differentiation of adjacent stalk ECs *via* NOTCH signalling pathway. Created by Biorender.

hepatoma cells, reported that lactate dehydrogenase B (LDHB) plays a compelling role in the process of invasiveness of hepatoma cells by activating the tight junction protein claudin-1 (Cln-1). This was the first study which documented the role of lactate in invasiveness (54).

Corbet et al., with their studies over cell line as well as on human patients suffering from metastatic cancer demonstrated that lactate acidosis activates the expression of transforming growth factor-β (TGF-β) which acts on the TGF-β receptor and subsequently contributes to the process of EMT of cancer *via* two pathways. First, TGF-β signals the activation of pSmaid2/3 which after being acetylated activates Snail to enhance activation of gene zinc finger E-box-binding homeobox 1 (ZEB1) activation. ZEB1 activation upregulates TGF-β expression. ZEB1 activates the Cadherin-2 (CDH2) and vimentin (VIM) genes, contributing to the development of anoikic resistance and invasiveness. Second, TGF-β also translocate CD36-a lipid transporter which transports long-chain fatty acids (LCFA) from extracellular sources. Accumulated LCFA perform several functions in the malignant cells. A portion of the LCFA is converted into Triacylglycerol's by the combination of diacylglycerols and Acetyl-CoA, which is stored as lipid droplets (LDs) in the cytosol. LDs also contribute to anoikic resistance. Some portions of LCFA is broken down into acetyl-CoA and transported to the mitochondria for use in β-oxidation for ATP production. Excess acetyl-CoA released from fatty acids (released from LDs) is further carry out the acetylation of Smad2/3 which activates ZEB1 and CDH2/VIM. Therefore, this study also gives a knowledge that why cancer cells require larger quantities of fatty acids than normal cells (55).

Kexin Sun et al, delineated the role of lactate and TGF-β in EMT process and cancer progression. This study was conducted on cancer-associated fibroblasts (CAF) and the human breast cancer cell line MDA-MB-231, as well as on nude mice, to unveil the role of oxidised ataxia-telangiectasia (ATM) in the regulation of glycolysis during hypoxia. Western blotting and immunohistochemistry revealed that CAF grown under hypoxic conditions showed higher expression of Glucose Transporter-1 (GLUT-1). Interestingly, higher expression of Oxidised ATM in a double-strand break (DSB)-independent manner was observed in CAFs under hypoxic conditions. Furthermore, to benefit cancer cells, oxidised ATM in CAF phosphorylates the Serine490 amino acid of GLUT-1 and thus promotes its translocation to the plasma membrane. This enhanced glucose uptake and utilisation in CAF results in the excess secretion of lactate in the tumour microenvironment. Co-culture of MDA-MB-231 and BT549 cells with CAF showed high levels of TGF-β, phosphorylated P38, MMP2, and MMP9. From this, the author inferred that excess glucose metabolism in CAF resulted in lactate

accumulation which is pumped in the TME, where it acts as a coupling metabolite and acts as a signalling molecule to enhance the expression of TGF-β1 and phosphorylated P38, MMP2, and MMP9 in order to accelerate the process of invasiveness (56).

Rattigan and colleague reported in their study that tumour cells and cancer associated fibroblasts and stromal cells cooperate each other in the TME leading to mutual existence. Glycolytic cells in the TME metabolise glucose through glycolysis and excess of lactate generated by these cells is readily taken up by the CAFs which is further converted to citrate (57). Konstantin et al. in their study reported that citrate, supplied by cancer-associated stromal cells, is indispensable for cancer cell metastasis. It has been reported that CAF express more citrate carriers on their surfaces which is purposely used for more citrate uptake to be incorporated for fatty acid synthesis or to fuel the citric acid cycle (58). Whitaker-Menezes et al. previously reported that tumour cells and associated fibroblasts can develop metabolic symbiosis to meet their energy requirements (59). Therefore, from the above studies, we can speculate that CAF might use lactate from hypoxic cancer cells and directly convert it into citrate which is further secreted in the TME and readily absorbed by nearby tumour cells for lipid synthesis (60).

In another study conducted by Young-Kyoung et al., on hepatoma cells (SNU354 and SNU423) reported that extracellular lactate can induced invasiveness. The study revealed that glycolytic tumour cells secrete excessive lactate, which enters nearby OXOPHOS cells, interferes with mitochondrial ribosomal proteins, and reduces the expression of mitochondrial ribosomal proteins L13 (MRPL13), leading to defective OXOPHOS and ROS generation. Excessive ROS activated nuclear Cln-1 gene expression and formation of Cln-1 protein which ultimately takes part in invasiveness (61). The study further validated the role of lactate in EMT process.

Lactate also causes the polarisation and activation of macrophages in solid tumours, such as pituitary adenomas, to initiate invasion and infiltration. Lactate from the TME activates macrophages through the mTORC2/ERK pathway and activated macrophages release CCL17 which initiates EMT through the CCL17/CCL4/mTORC1 pathway (62). A previous study conducted by Lin et al. also reported that lactate can induce EMT in cancer cells by activating TAM. TAM in TME secrete CCL5 which induces angiogenesis and EMT in cancer cells. A previous study also established a relationship between TGF-β and CCL5 and proved that TGF1β uses CCL5 to enhance glycolysis in cancer cells (63).

This can be concluded from the above discussion that lactate acidosis can initiate invasiveness through activation of TGF-β. Lactate acidosis in TME induces the upregulation of TGF-β in hypoxic cancer cells which is transported outside in the TME. TGF-β from TME acts on TGF-β receptors present on same cancer cells in autocrine mechanism. TGF-β receptor signalling leads phosphorylation of Smad2/3 and subsequently its acetylation. Acetylated Smad2/3 enters the nucleus where it regulates the expression of CDH2 and VIM genes. Protein product of these genes further participate in the EMT process. Acetyl-CoA required for acetylation of Smad2/3 is provided by the long chain fatty acids taken from dietary sources. Triglycerides released from the lipid droplets (LDs) can also confer the acetyl-CoA. High energy demand during EMT process is fulfilled by the β-oxidation of fatty acids released from the LDs (Figure 2).

## 6 Role of lactate in suppression of immunity

The immune system protects the body from damage caused by cancer or other pathogens by detecting and eliminating their respective cells (41). A special category of cells, including macrophages, natural killer cells (NK), and cytotoxic T lymphocytes, identifies and eliminates foreign cells (64). However, recent clinical studies have reported that immune cells fail to recognise tumour cells (65). Malignant cells in the TME remodel adaptive and innate immune responses for their own benefit. Various factors in the TME acts on the immune cells to remodel their metabolic, genetic, and epigenetic level and slowly transforms into resistant to immune cells (66). Owing to the high metabolic rate inside the TME, tumour cells secrete metabolic products such as lactate which favour cancer cells by suppressing immunity.

Studies have also reported that immunogenic cells in the TME assist the tumor cells in various ways to promote growth and development. In their comprehensive review, Wang described lactate as the main onco-metabolite that causes inactivation of immune cells and, thus, suppression of immunity. They reported that lactate acidosis in the TME suppressed the polarisation of M1 macrophages into M2 through an epigenetic mechanism. Lactate enters through the MCT-1 transporter and further binds with DNA, causing histone lysine lactylation (Kla) sites and consequently M2 polarisation. Macrophage polarization are very long debated program in context with host immune response in TME. Furthermore, they showed that a high lactate concentration in the TME disrupts the proton gradient in CD8+T cells, hindering the proliferation of effector T cells. Finally, they explained that lactic acid acts as a signalling molecule in both malignant and dendritic cells. Binding of lactate to GPR81 (G protein coupled receptor) present on dendritic cells causes decreased production of cAMP, IL6, and Il12, and suppression of the antigen presentation mechanism. While lactate binds to GPR81 present in cancer cells, it induces drug resistance and augments the expression of programmed death ligand(PD-L1) (67, 68).

Unlike macrophages, neutrophils function as a double-edged sword for cancer cells. On one hand, they induce apoptosis by employing hydrogen peroxide ($H_2$ $O_2$), reactive oxygen species (ROS), and tumour necrosis factor-α (TNF-α). On the other hand, these cells secrete inflammatory mediators, initiate angiogenesis, and thus promote tumour growth and development. Neutrophils can assist tumour cells in immune surveillance through extracellular trap formation (69). Hypoxia-induced lactate acidosis helps mobilise neutrophils towards the TME. Khati-Massalha et al., proposed a model for describing the mobilisation of neutrophils from the bone marrow in response to lipopolysaccharide (LPS) lactate in a mouse model of inflammation. They observed that neutrophil mobilisation significantly increased in the bone marrow in response to LPS injection. LPS acts on the toll-like receptors

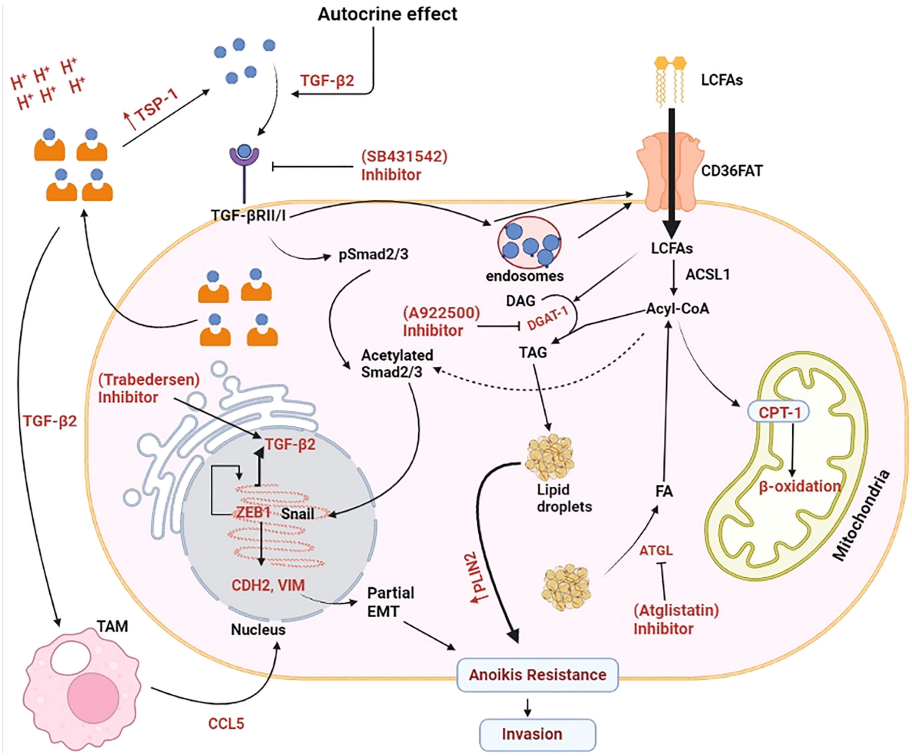


FIGURE 2
Molecular mechanism exploited by lactate for enhancing invasiveness in cancer cells. Lactate-induced acidosis augments epithelial-to-mesenchymal transition in solid tumours. Lactate in the tumor microenvironment (TME) enhances the synthesis and secretion of tumour growth factor-β2 (TGF-β2) which acts on tumour cells *via* an autocrine mechanism and phosphorylates pSmad2/3. After acetylation, pSmad2/3 activates Snail which enters the nucleus and enhances gene expression of N-Cadherin's (CDH2) and Vimentin (VIM). CDH2 protein enhances tumor cell motility and migration. VIM belongs to type-IIIrd intermediate filament proteins that maintains cell integrity and plays important role in cell migration, motility and adhesion and subsequently metastasis. TGF-β2 mediated overexpression of CAD and VIM proteins induces partial epithelial to mesenchymal transition (EMT) and Anoikis resistance in cancer cells and finally its transformation into invasive carcinoma cells. Tumour-associated macrophages (TAM) also assist in the EMT process by secreting CCL-5(chemokine cytokine) which further enhances the synthesis and secretion of TGF- β2. Long chain fatty acid (LCFA) taken from external sources provide Acyl-CoA responsible for the acetylation of pSmad2/3. Excess long chain fatty acids (LCFA) is stored as a lipid droplets (LDs) which ensures continuous supply of Acyl-CoA required for acetylation of Smad2/3 proteins. Increasing demand of energy in the form of ATP during EMT process is fulfilled by the β-oxidation of fatty acids released from the stored LDs. ACSL1, Acyl-CoA synthetase; ATGL, Adipose triglyceride lipase; CPT1, Carnitine palmitoyltransferase 1; DAG, Diacylglycerol; FA, Fatty acids; PLIN2, Perilipin 2; TAG:,Triacylglycerol; TSP-1, Thrombospondin-1. Created by Biorender.

present on neutrophils which stimulates glycolysis and enhances lactate production and secretion through the MCT-4 receptor. Secreted lactate binds to the GPR81 receptor on endothelial cells and decreases surface VE-cadherin levels, leading to higher bone marrow endothelial cell permeability in neutrophils (70). The same mechanism must be used by lactate in hypoxic tumours to enhance the permeability and infiltration of neutrophils in the TME.

Recently, Deng and colleagues, in their study on hepatocellular carcinoma cells, reported that neutrophils in the TME of solid tumours suppress T cell cytotoxicity. Lactate in the TME enters neutrophils *via* the MCT-1 transporter and enhances the expression of the death ligand PD-L1 *via* the activated NFkβ/COX-2 pathway. The study also reported that PD-L1 expression in neutrophils can be reduced by the selective cyclooxygenase-2(COX-2) inhibitor celecoxib. Furthermore, the combination of celecoxib and lenvatinib potentiates its anti-angiogenic action in hepatocellular carcinoma (71). However, it could be beneficial to minimise the level of PD-L1 expressing neutrophils in the TME. To evaluate the effect of PD-L1 inhibition, Guen et al. induced urothelial carcinoma in experimental animals by injecting MB49 murine urothelial cancer cell lines, and evaluated the tumour morphology with haematoxylin and eosin staining and immunohistochemistry. Animals were treated with PD-L1 antibody alone and in combination with 1-palitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG). The results of this study showed that the number of neutrophils infiltrating the tumour tissue decreased considerably in the PLAG- and PD-L1 antibody-treated groups and the population of cytotoxic T-cell cells increased substantially (72). This study clearly validated the role of PD-L1 in immune suppression and its association with lactate acidosis in solid hypoxic tumours. We can assume the same conditions in the hypoxic cancer of mammary gland as excessive accumulation of lactate is also reported in the TME of mammary gland tumors.

Gottfried et al. previously reported that lactic acid causes further differentiation of monocytes into tumour-associated dendritic cells (TADCs). They generated multicellular tumour spheroids (MCTS) using various tumour cell lines and cultured monocytes in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. They observed that monocytes penetrated MCTS and differentiated into TADCs. They concluded that external sources could modulate monocyte phenotype.

Furthermore, melanoma cells and prostate cancer MCTSs were co-cultured with lactic acid. They observed that the phenotype of DCs is similar to that of TADCs (73).

The adaptive immune system CD8+T cells (cytotoxic T cells) also become inactive in the acidic medium of hypoxic tumours. Fischer et al. collected serum samples from patients with malignant cancers such as breast cancer, gastric cancer, and lung cancer. Serum lactate levels were measured in individual samples and correlated with cytotoxic T-cell activity. The results of this analysis showed that T cell activity and the production of cytokines, such as interferon- γ (IF-γ)), decreased significantly in the acidic TME. T cells rely on glycolysis, and the resultant lactate is exported through the MCT-1 transporter; however, lactate acidosis imparted by hypoxic tumours causes inhibition of MCT-1 and thus accretion of lactic acid in T cells, and consequently their inactivation (74). Lactate also cause suppression of CD8+ T cell indirectly by inducing expression of TGF-β. Gu et al. reported that lactate in TME regulate the expression of TGF-β which helps the in suppression of immunity through T-regulatory cells (75). Further, Gunderson et al. illustrated the role of TGF-β in suppressing the activation of CD8+T cells through CXCR3. In their experiment on transgenic mice and tumour cell lines, they observed that TGB-β receptor-knockout mice exhibited higher CXCR3 and CD8+ T cell tumour infiltration than TGF-β receptor-positive cells. It was concluded that concluded that acidosis induced TGF expression which suppressed the CXCR3 expression and thus activation of CD8+ T cells in TME (76). These findings clearly showed that lactate can suppress the CD8+ T cells.

Natural Killer (NK) cells also destroy damaged, infected, and cancerous cells. This action is executed by NK receptors. All cells without major histocompatibility class-1 (MHC-1) on their surfaces were recognised and killed. NK cells release cytoplasmic granules containing granzyme and perforin which induce cell lysis in foreign cells (77, 78). Numerous studies have reported that cancer cells show decreased levels of MHC-1 which results in inactivation of NK cells (79). Lactate leads to the apoptosis of NK cells by decreasing intracellular pH which results in mitochondrial dysfunction and consequently programmed cell death. Lactate also blocks the synthesis and secretion of IFN-γ and interleukin-1 (IL-1) by NKT cells in the TME (41).

Husaain et al., studied the effects of lactate on MDSCs and NK cell function in a syngeneic Pan02 murine pancreatic cancer model. Considering that LDH-A is responsible for the conversion of glucose into lactate, LDH-A-deficient Pan02 (pancreatic cell lines) cancer cells were prepared by a knockdown process using Lentiviral vector –mediated hairpin RNA and injected into C57BL/6 mice. They observed smaller tumor in C57/6 mice compared than Pan02 treated mice. In addition, they noted a decrease in the number of MDSCs and NK cells in the spleens of LDH-A gene-deficient mice. *In vitro* exogenous supplementation with lactate increased the frequency of MDSC generation from mouse bone marrow, along with GM-CSF and interleukin (IL-6). *In Vitro* pre-treatment of NK cells with lactate impedes their cytotoxicity in both humans and mice. The results also showed a reduction in the expression of perforin, granzymes, and NKp46 proteins in the NK cells. Furthermore, they noted that mice developed fewer tumours when given access to only a ketogenic diet. This study clearly demonstrated the immunosuppressive role of lactate in MDSCs and NK cells (80). Serganova et al. first reported that LDH-A inhibition modulates the tumour immune response. They also noted reduced expression of HIF-1, Hexokinase 1and 2, and VEGF in LDH-A knockdown mice. Again, this study established a clear relationship between hypoxia, LDH-A, and tumour immunity (81).

## 7 Role of lactate in development of resistance against chemotherapy and radiotherapy

Lactate is currently recognised as the major oncometabolite which helps cancer cells to develop resistance to chemotherapy. Various studies have reported the acquired resistance caused by lactate. Lactate acidosis in TME can activate various receptors that contribute to the development of resistance to chemotherapy. Although c-MET is a receptor tyrosine kinase (RTK) that plays a vital role in the proper growth and development of normal cells, aberrant activation can sustain tumour growth and metabolism (82). Recently, Apicella et al. were the first to report that lactate in the TME assists cancer cells in acquiring and developing resistance to targeted therapies. According to the author, continuous and long-term treatment with Met or EGFR tyrosine kinase inhibitors caused excessive production of lactate which is taken up by CAF through the MCT4 transporter. Lactate in CAF activates NF-κB which enhances the transcription of hepatocyte growth factor (HGF). Previous studies have reported that HGF prevents apoptosis in both normal and cancer cells induced by various stimuli (83). When secreted into the TME, HGF binds to the MET receptor on the cancer cells and overcomes the inhibitory effect of receptor tyrosine kinase inhibitors (TKIs), such as sunitinib (84).

Another study conducted by Govon et al. demonstrated and noticed the resistance-imparting behaviour of lactate to cisplatin therapy in cancer cell lines. They selected a cell line with the potential to grow in lactate-containing culture medium and simultaneously treated it with cisplatin. It is well known that cisplatin is a platinum compound having the DNA damaging potential in cancer cells. The results of this study showed that cells grown in the lactate medium had a very low impact on cisplatin therapy. The efficacy of cisplatin was significantly reduced in the lactate-supplemented cells. Moreover, cells treated with lactate exhibited reduced DNA damage and increased levels of DNA repair genes. They reported that lactate can impart resistance to chemotherapeutic agents (85).

Park et al., studied the effects of lactate on breast cancer cell lines and reported that some cancer cells can stop using glucose and begin utilising lactate as an energy substrate, which confers resistance to PI3K/mTOR inhibitors. Furthermore, they reported that oestrogen-related receptor alpha (ERR-α) regulates the expression of various genes involved in lactate utilisation and uptake. Notably, the efficacy of inhibitors of the PI3K/mTOR pathway was substantially increased both *in vitro* and *in vivo* when ERRα antagonists were used (86).

In another study conducted by Qi Dong et al., etoposide administration exacerbated ROS production which indirectly reprogrammed glucose metabolism and enhanced lactate synthesis in NSC-LCs. The resultant lactate acidosis confers resistance to cancer cells by enhancing the upregulation of multiple resistance-associated protein 1(MRP-1), an AT-binding cassette (ABC) transporter protein. MRP-1 acts as a drug efflux pump in cancer cells and its expression increases in many types under the guidance of lactate acidosis (87).

Qu et al. reported in their study that lactate enhanced resistance to oxaliplatin in colorectal carcinoma (CRC) patients co-infected with *Candida tropicalis (C. tropicalis)*. They induced CRC carcinoma *via* xenografting of the colorectal cancer cell line SW480 in mice and validated their findings using various parameters such as apoptosis, immunohistochemistry, and western blotting. The results of this study showed a substantial increase in tumour burden in experimental animals treated with oxaliplatin and infected with *C. tropicalis*. Furthermore, they reported that lactate significantly altered the expression of mismatch repair proteins(MMR) as MSH1 and MSH2 through activation of the GPR81-cAMP-PKA-CREB axis (88). Leslie Amaral et al. worked in the same direction and also reported that lactate impart resistance to chemotherapeutic agents. They cultured *Saccharomyces cerevisiae* (S. cerevisiae) BY4741 cell lines in the presence and absence of glucose and lactate, and treated them with cisplatin. After 180 min of exposure to the respective treatments, they observed a reduced sensitivity to cisplatin in the presence of lactate. Furthermore, western blotting results showed higher phosphorylation of Rad4p in lactate medium-cultured cells, although no change in histone acetylation was observed (89). It is previously reported that Rad4p (a DNA repairing protein that belongs to xeroderma pigmentosum family) has important role in nucleotide excision repair (90). To understand the effect of lactate on gene expression in cancer cells, Govoni et al. selected a cancer cell line capable of growing under glucose-deprived conditions, and evaluated the effect of lactate acidosis on the DNA-damaging potential of cisplatin. They observed that the low efficacy of cisplatin in lactate-exposed cells was due to enhanced DNA recombination and the upregulation of DNA repair genes. They identified various genes in lactate-treated cell lines that participate in the mismatch repair and nucleotide excision pathways and restore cisplatin-induced DNA damage (85).

Xiaoping et al. collected samples from patients with NSCLC undergoing cisplatin treatment and analysed them using immunohistochemistry and RT-qPCR. The results showed that higher expression of osteopontin (OPN) protein which directly contributes to LDHA expression. Higher LDHA expression further enhances lactate acidosis, imparting resistance to cisplatin therapy. The same correlation between, lactate, and resistance to cisplatin was confirmed through *in vitro* studies conducted on SK-MES-1 and A549 cell lines (91). This study established a new relationship between OPN and lactate acidosis and resistance. OPN plays a crucial role in malignancy (92).

These studies clearly demonstrated that lactate plays a pivotal role in the emergence of resistance in cancer cells. It exploits various molecular pathways to induce resistance in cancer cells. When cancer cells are treated with chemotherapeutic agents such as cisplatin or tyrosine kinase inhibitors, they initially respond well, suggesting that they are drug-sensitive. However, continuous torture by chemotherapeutic agents forces cancer cells to develop a mechanism that counteracts drug response. Drug-sensitive cancer cells show increased cytoplasmic levels of OPN, which upregulates LDH expression. Overexpression of LDH enhances glucose oxidation to lactate which is pumped into the TME by MCT4. Lactate from the TME is absorbed by neighbouring cancer cells and CAF *via* MCT1. In CAF, lactate enhances NF-κB which acts on DNA, upregulates the synthesis and secretion of HGF cytokine which acts on the MET receptor and makes the tyrosine kinase receptor insensitive to tyrosine kinase inhibitors such as sunitinib and makes the cancer cells resistant to TKIs. Lactate from the TME enters directly through GPR81 and enhances the expression of mismatch repair genes such as MSH1 and MSH2 through the GPR81-cAMP-PKA-CREB axis. Lactate entering MCT1 acts on DNA and enhances the expression of multiple resistance transporter-I (MRT1) which acts as an efflux pump for cisplatin. Cisplatin exposure enhances ERRα expression, which allows cancer cells to oxidise lactate in the TCA cycle as an alternative to glucose. Lactate enhances the phosphorylation of Rad4P, which helps to repair DNA damage caused by cisplatin or ROS. Rad4P belongs to xeroderma pigmentosum group-C family-participate in nucleotide excision repair (Figure 3). Phosphorylated Rad4P carry out the repairing of damaged DNA of cancer cells treated with chemotherapeutic agents like cisplatin. This is how lactate acidosis renders the cancer cells insensitive to chemotherapeutic agents. Physician can enhance the efficacy of chemotherapeutic by co-administration of inhibitors of HGF and Rad4P.

# 8 Lipid reprogramming of cancer cells

## 8.1 Hypoxia induce lipid droplet accumulation in cancer cells

Recent work has pointed out that lactate is the principal oncometabolite which is essential for the continuous running of glycolysis and oxidative phosphorylation and as a precursor for biosynthetic processes in cancer cells (93). Lactate has also been reported to play an important role in gene regulation and expression, partitioning of energy substrates, and the regulation of cellular redox homeostasis. Although much has been reported regarding the role of lactate in the metabolic reprogramming of glycolysis, drug resistance, immune suppression, and invasiveness, very little attention has been paid to its role in lipid reprogramming. Recent studies have reported that lactate reprograms lipid metabolism in cancer cells (94). As lipids are indispensable for rapidly dividing cancer cells to form their plasma membrane along with other cell organelles (95). Fatty acids are abundantly required for nascent cancer cell membrane which cannot be accomplished alone by *de novo* fatty acid synthetic pathway (96). Cancer cells utilise lactate to produce more fatty acids. In addition, various studies also have reported that hypoxia in solid tumour induces the formation of lipid droplets which are can be used for energy production during oxygen availability (97). However, exact the mechanisms by which cancer cells use lacate in fatty acid synthesis remains unexplored.

However, recent studies have documented a role for lactate in lipid reprogramming. Corbet et al. reported that tumour acidosis in

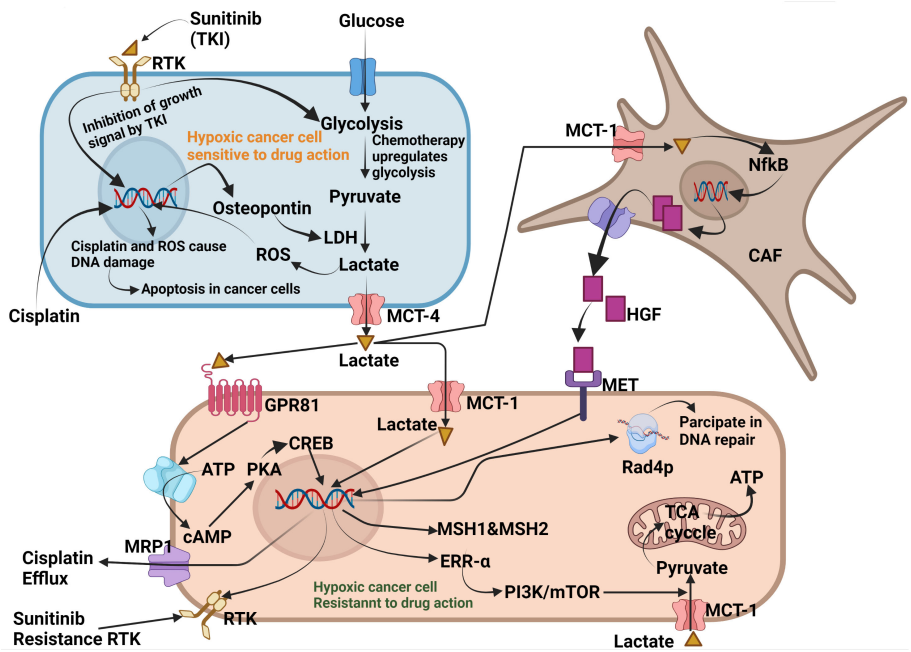


FIGURE 3
Molecular mechanism exploited by Lactate for developing resistance against chemotherapy. Exposure to cisplatin and tyrosine kinase inhibitors enhances cytoplasmic levels of osteopontin in cancer cells. Osteopontin enhances LDH expression which enhances glucose fermentation into lactate. Lactate from sensitive cancer cells is secreted into the TME *via* MCT4, where it is taken up by neighbouring cancer cells and cancer-associated fibroblasts (CAF). In lactate, NF-κB which enters the DNA, enhances the synthesis and secretion of the cytokine HGF. HGF from the TME binds to the MET receptor present on neighbouring cancer cells, making the receptor insensitive to tyrosine kinase inhibitors such as sunitinib. Lactate can also bind to GPR81 which enhances the expression of DNA mismatch repair proteins, such as MSH1 and MSH2, through the GPR81-cAMP-PKA-CREB axis. Cisplatin enhances cytoplasmic levels of oestrogen response receptor-α(ERRα), which helps cancer cells utilise lactate as an energy substrate. Lactate also phosphorylates Rad4p, which participates in the repair of cisplatin-induced DNA damage and ROS. Created by Biorender.

cancer cells favours lipid oxidation and synthesis under lactate acidosis. The results of the study also documented that acetyl-co-A derived through β-oxidation of fatty acids not only fuels the Krebs cycle but it also checks ROS production in mitochondria. In addition, acetyl-co-A resulting from β-oxidation lead non-enzymatic hyperacetylation of mitochondrial complex-I. Overall, it was postulated that fatty acid oxidation and synthesis takes place concomitantly in cancer cells in acidic environment which was enabled by the sirtuin-mediated deacetylation of histones and consecutively downregulation of acetyl-CoA carboxylase 2 (ACC2) (98). Pierre Sonveaux reported in their study that TME is heterogeneous with respect to oxygen and nutrient availability. The cells near the blood vessels have adequate nutrient and oxygen availability, whereas those located in the middle and periphery of the tumour are deficient in oxygen; hence, they are called hypoxic tumour cells. Hypoxic cancer cells metabolise glucose only through glycolysis, resulting in the accumulation of excess lactic acid, which is pumped into the extracellular environment, making it more acidic. Generally, normal cells undergo apoptosis in the presence of oxygen and nutrient deficiency. Cancer cells are immortal and do not initiate programmed cell death but develop a metabolic symbiosis with normoxic cancer cells (OXOPHOS cells). Lactate generated by hypoxic malignant cells is utilised by OXOPHOS cells and is further used in the TCA cycle. Simultaneously, OXOPHOS stops using glucose (Warburg effect) which is retained in hypoxic cancer cells (99). Another study reported the roles of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL), but not lactate, in breast cancer progression. When MCF7 and MDB-MB-231 cells were supplemented with LDL and VLDL, angiogenesis in the breast cancer cells was initiated. Although this study did not show any link between lactate and fatty acid synthesis, it proved that rapidly dividing cancer cells can take up circulating lipoproteins to accomplish their fatty acid needs, regardless of the raw material used in the synthesis of these fatty acids (100). Shen et al. reported a role for HIF-1α in LDL and VLDL receptor (VLDLR) regulation. In their study on MCF7, HepG2, and HeLa cells, LDL and VLDL levels were upregulated when cultured under hypoxia. The gene and mRNA expression of VLDLR increased significantly compared with that in cells cultured under normoxia. Furthermore, they confirmed the presence of an operational hypoxia response element (HRE) gene adjacent to the VLDLR gene over +405 exon 1 using dual luciferase and chromatin immunoprecipitation assays. The HRE of VLDLR responds to HIF-1α. In addition, knockdown of HIF-1αand VLDLR genes attenuated lipid accumulation in cultured cells, indicating a direct link between HIF-1α and LDL and VLDL (101). Sundelin et al. observed that VLDLR overexpression in human and mouse cardiomyocytes under hypoxic conditions results in detrimental lipid accumulation. After thoroughly mapping the 50-flanking region on the VLDLR promoter gene, they stated that the hypoxia-mediated increase in VLDLR protein expression depended on the HRE present between 162 to 158bp translation

site. This study rejected the previously described hypothesis that PPARc and SP1 binding sites on the VLDLR promoter region are not involved in the hypoxia-induced regulation of VLDLR expression (102).

Previously, Kodndo et al., found that both hypoxia and nutrient scarcity play critical roles in malignancy. However, the role of hypoxia-induced extracellular pH (pHe) in lipid biosynthesis has not been fully elucidated. His study on pancreatic cell lines (PANC-1 and ASPC-1) clearly documented the role of tumour acidosis in lipid biosynthesis. Reduced pHe in the external environment activates sterol regulatory element binding protein-2 (SREBP2) which is released from the endoplasmic reticulum and binds to the sterol response element in the nucleus. This leads to the activation of various genes involved in fatty acid synthesis (103).

In the above text, we have already discussed the study conducted by Corbet et al., who reported the effect of tumour acidosis on cancer progression by enhancing lipid droplet formation. They found that acidic pH encourages autocrine TGF-β signalling, which indirectly enhances the formation of lipid droplets (LD) and further helps in developing anoikic resistance, thus participating in EMT progression. In addition, TGF-β2 activation promotes epithelial-to-mesenchymal transition and lipid metabolism. Furthermore, it stimulated PKC-zeta-mediated translocation of CD36 which further enhanced fatty acid uptake, either stored in the form of triglycerides in the LD or utilised to generate ATP by oxidation. The study also described that distant metastasis can be prevented by inhibiting the mobilisation of fatty acids from the LD (55).

Bensad et al., reported that hypoxia in cancer cells activates various genes which directly and indirectly enhance lipid synthesis and storage. Further results of the study reported that HIF-1α induces the accretion of LDs in hypoxic cancer cells. These LD are rich in triglycerides (TGs) which are degraded to release fatty acids and used in ATP production by β-oxidation in the mitochondria, and can also be used to build the cell membrane of rapidly proliferating cancer cells. The link between LDs formation and hypoxia was established based on the overexpression of several surface proteins located on the LDs membranes. Hypoxia-inducible protein2(HIG2), Adipose differentiation-related protein(ADRP), and Perilipin-3(TIP47) constitute the surface of LDs. Of these, HIG2 and ADRRP are mainly induced by hypoxia. Role of FABP2/3 and ADRP in selective uptake of long-chain fatty acids was observed to be increased in hypoxic cancer cells (97).

Based on the above results, we hypothesised that hypoxia in cancer cells shifts glucose metabolism only through glycolysis. As a result, excess lactate accumulates in cancer cells and is secreted into the extracellular TME. An increase in the proton concentration in the TME stimulates the uptake of protons by nearby cancer cells, resulting in increased acidosis in these cells. Reduced acidosis further stimulates SREBP-1c present in the endoplasmic reticulum and translocates to the nucleus, where it activates genes that are indispensable for fatty acid synthesis. It has also been shown that excess glucose supplements in cancer cells can induce the accumulation of lipid droplets (LD) in cancer cells. Tirinato et al., while working on normal and colorectal cancer stem cells (CR-CRC), found that excess glucose induced LD and ROS production in cancer cells. Excess glucose is converted into palmitate which is further converted into triglycerides and cholesterol and finally encapsulated into LDs. TG and cholesterol from LD have been used for energy production (104).

Fabienne et al. obtained tumour specimens from patients with pancreatic cancer to understand the role of lactate and the expression of the low-density lipoprotein receptor (LDLR). They observed that LDLR expression increased many times in patients with pancreatic cancer. These results further supported that the higher expression of LDLR illustrates that malignant cells are metabolically active and eager to synthesise cholesterol. This further increased the risk of relapse. This study not only reported the role of cholesterol in cancer progression but also suggested that the synthesis and design of novel therapeutic agents against LDLR receptor-inactivating enzymes could be a new approach for the treatment and management of pancreatic carcinoma (105). Studies have also reported a role for cholesterol in cancer progression.

Later, many researchers worked on it to prove this hypothesis. Singh et al. induced breast carcinoma with MNU and DMBA (7-12 Benzanthracene), and a the serum metabolomic profile of experimental animals was analysed using Nuclear magnetic resonance (NMR). A large perturbation in serum metabolites was observed in toxicant treated animals. Overall, conclusion of the study was that hypoxic cancer cells reprogrammed their glycolytic pathway to enhance lactic acid production (106, 107). Interestingly, higher levels of lipids (polyunsaturated fatty acids) were also observed in the same animals with increased lactate level. Enhanced expression of HIF-1α, SREBP-1c, and fatty acid synthase (FASN) was noted with an increase in lactate acidosis (106). Recently, Minami et al. reported that glucose-deprived glioma stem cells (GSC) utilise lactate in the Krebs cycle to generate GTP. It has also been reported that choline, phospholipid, and fatty acid synthesis increases many-fold upon lactate supplementation in glucose-deprived cancer cells. In addition, cancer cells exhibit increased aggressiveness and metastasis following lactate consumption (108). This study clearly shows that cancer cells can reuse lactate for fatty acid synthesis. Luci et al. observed the same phenomenon in glial and neuronal cells. Under stress conditions, glial cells utilise glucose which is converted to lactate which is pumped out extracellularly and taken up by the neuron cells through the MCTs transporters and metabolised back to pyruvate-citrate so that it can be used in fatty acid synthesis. Excess fatty acids are not utilised by these cells, but are exported through apolipoproteins (ApoE/D) and stored in the form of LDs after being converted into TG and cholesterol. Under stressed conditions, glial cells and neuronal cells develop a metabolic symbiosis to minimise the ROS effect because excessive ROS can lead to neuronal degeneration in CNS disorders, such as Alzheimer's disease (109).

## 8.2 Role of lactate in causing lipid reprogramming in cancer cells

To understand lipid reprogramming in cancer cells, we must sit in the tumour microenvironment to closely observe metabolic changes at the molecular level in solid tumours. Furthermore, the TME is different in larger tumours than in smaller ones. First, we will understand the tumour microenvironment of smaller tumours. When a cell transforms from normal to malignant, it is located near the blood vessel, and all the tumour cells arising from these

cancer cells receive adequate amounts of nutrients and oxygen. Although all cancer cells in the tumour milieu are capable of metabolising glucose by glycolysis and OXPHOS, they prefer to metabolise glucose by glycolysis (Warburg effect). Consequently, excess lactate accumulates in tumour cells, which is dumped outside the TME through MCT-4), from where it is immediately washed away by the circulating fluid. This metabolic plasticity, which is maintained by cancer cells, is necessary for building the biomass. Rapid division of cancer cells requires replication, which requires ribose sugar as the major metabolite. Because ribose and other sugars are only formed by the pentose phosphate pathway (PPP), glycolysis is mandatory to run PPP continuously. The second most important requirement is for fatty acids. Fast running glycolysis yields extra pyruvate which in part is converted into acetyl-CoA and converted into citrate which is further utilised in fatty acid synthesis. Thus, palmitic acid is further converted into monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). These are further altered to form membrane phospholipids, such as phosphatidylcholine, phosphatidylinositol, and sphingolipids, which are used in the construction of plasma membranes of proliferating cancer cells. This is how glycolysis is reprogrammed to enhance lipid synthesis in cancer cells when the tumours are small.

Now, consider the TME of a solid hypoxic tumour. Unlike smaller tumours, the microenvironment in larger tumours is different in that some cancer cells are located near blood vessels to obtain sufficient amounts of nutrients and oxygen; hence, they are called OXPHOS cells. Some cells are located in a distant area at the periphery of the tumour and receive sufficient nutrients or oxygen; hence, they are called hypoxic cancer cells. In addition, there is a third category of cells located between OXPHOS and hypoxic cancer cells, which receive a partial amount of nutrients and oxygen, and are hence called moderately hypoxic cancer cells. Hypoxic cancer cells develop metabolic symbiosis to sustain nutrient- and oxygen-deficient environments. OXPHOS cells do not use glucose, unlike OXPHOS cells in small tumours, but they retard glycolysis. Spared glucose is then transported to severely hypoxic cancer cells. Severely hypoxic cancer cells can only metabolise glucose by glycolysis and thus produce excess lactic acid which is immediately exported to the TME, reducing its pH from 7.4 to 6.8. Continuously exported lactate flows towards OXPHOS cells and is taken up by these cells through the MCT-1 transporter. OXPHOS cells convert lactate back into pyruvate which is then utilised in the Krebs cycle. This phenomenon is known as the reverse Warburg effect (RWE). Lactate-derived pyruvate is further utilised for ATP production by the electron transport system. This also fuels the fatty acid synthesis pathway. The excess citrate produced is then converted into palmitate which is further modified into complex lipids such as triglycerides and cholesterol. Cholesterol is further utilised in the biosynthesis of signalling molecules, hormones, and cell membrane lipids such as phosphatidylinositol, phosphocholine, and sphingolipids. Fatty acids converted into LDL/VLDL are transported towards the hypoxic cancer cells and accumulated in the form of LDs (110).

From the above discussion we can conclude that cancer cells in TME develop a metabolic symbiosis for synthesis and utilization of fatty acids. Severely hypoxic cancer cells can extract energy only from glycolysis (high dependency on glucose), hence produce and secrete excess of lactate in the TME. Lactate from TME flows towards the normoxic cancer cells (low dependency on glucose) and is utilized in the TCA cycle after being converted back to pyruvate. Lactate derived citrate is also utilized in *de novo* fatty acid synthesis. In these cells, simple fatty acids are also converted into complex polyunsaturated fatty acids (PUFAs). PUFAs and Triglycerides are further converted into cholesterol and other structural lipids. Since severely hypoxic cancer cells cannot make complex lipids, these cells take up the complex lipids prepared by the normoxic cancer cells. Continuous accumulation if lipids in the hypoxic cancer cells are stored in the form of LDs. Hypoxic cancer cells can utilize FAs stored in the LDs during hypoxia re-oxygenation in β-oxidation to meet the high energy demand during rapid growth phase (Figure 4).

## 9 Conclusions

Cancer cells struggle with nutrients and oxygen as the tumour size increases to a reasonable size (3). Lactate secreted by hypoxic cancer cells initiates angiogenesis, and severely hypoxic cancer cells enhance the synthesis and secretion of VEGF which acts on epithelial cells present in nearby blood vessels. Binding of VEGF to ECs leads to their differentiation into stalk ECs and tip ECs (111). Lactate from the TME is also taken up by TAM, where it activates HIF-1 which enters the nucleus of TAM and activates various genes involved in angiogenesis. TAM secrete VEGF and TNF-α which bind to the tip of EC cells and enhance their proliferation. MMP secreted by TAM acts as a tissue-digesting enzyme, creating space for vessel formation (112). Lactate secreted from hypoxic cancer cells also enters stalk ECs, where it enhances the expression of Gas6, VEGF, Ang-1, and IL-8, which act on their respective receptors present on stalk cells in an autocrine mechanism, activate Myc, cyclin-D1 genes and subsequently induce cell division in stalk ECs. Tip ECs also secrete the Dll4 ligand which further enhances the proliferation of stalk ECs through the Notch signalling pathway. Lactate-mediated angiogenesis in solid tumours can be blocked by designing VEGF, Dll4, Gas6, IL-8, and Ang-1 inhibitors (113).

Tumour cells utilise lactate to initiate EMT. Lactate enhances TGF-β2 expression, which activates CAD and VIM gene expression. The inhibitors of TGF-β2, CAD, and VIM can prevent invasiveness. EMT can be further blocked by inhibiting LCFA uptake using transporter blockers (95, 114).

To protect themselves from chemotherapeutic agents, cancer cells develop resistance by using lactate as a mediator. Resistance to lactate can be abrogated by designing therapies against HGF cytokines which enhances HGF cytokines. MRT-1 inhibitors can prevent drug efflux from the target cells, rendering them sensitive to chemotherapy. Drugs that prevent Rad4p phosphorylation and antagonise MSH1 and MSH2 can overcome drug resistance by interfering with DNA repair mechanisms. ERR-α antagonists halt lactate utilisation in the mitochondria for energy production.

Cancer cells are self-sufficient to fulfil their fatty acid requirements. The metabolic symbiosis between OXPHOS, moderately hypoxic, and severely hypoxic cancer cells helps foster a harsh environment. Glucose is utilised by severely hypoxic cancer cells and rapidly fermented into lactate. Lactate is pumped into the

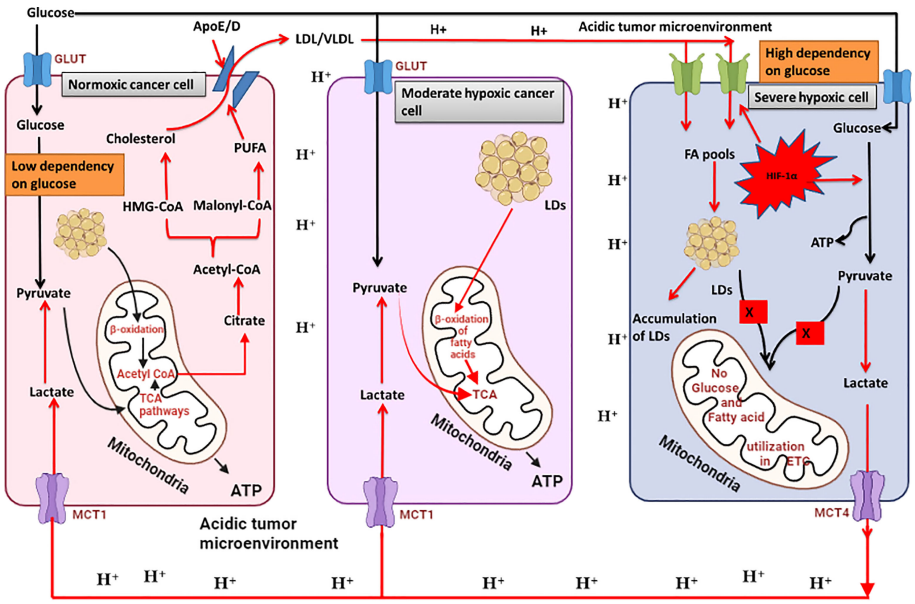


FIGURE 4
Metabolic symbiosis in cancer cells to utilize glucose and lactate for fatty acid synthesis and utilisation. Severely hypoxic cancer cells can oxidise glucose only through glycolysis which results in excessive production of lactate secreted in the tumour microenvironment. Enhanced glycolysis in these cells also results in lipid droplet accumulation. OXPHOS cancer cells can use lactate for oxidative phosphorylation by converting it back to pyruvate. Therefore, OXPHOS cells and severely hypoxic cancer cells develop a metabolic symbiosis for glucose and lactate utilisation. Excess lactate in OXPHOS cells is released as citrate which is utilised in fatty acid synthesis. Moderately hypoxic cancer cells can utilise fatty acids for ATP production through β-oxidation of fatty acids. Fatty acids accumulate in severely hypoxic cancer cells and are transported towards OXPHOS cells, where they are partly converted into fatty acids and utilised in β-oxidation for energy production. A large portion is converted into polyunsaturated fatty acids (PUFAs) which are further incorporated into plasma membrane synthesis. Created by Biorender.

tumour milieu and enters OXPHOS and moderately hypoxic cancer cells. Lactate is utilised by OXPHOS and moderately hypoxic cancer cells to fuel fatty acid synthesis and TCA cycle. Fatty acids formed by hypoxic and moderately hypoxic cancer cells are further modified for membrane lipid synthesis. Fatty acids can also be used in angiogenesis and invasion. Inhibitors of fatty acid synthesis are also beneficial in cancer therapy. Fatty acid synthesis inhibitors like HMG-CoA inhibitors (Statins), can be repurposed along with other anti-cancer drugs.

## Author contributions

LS and LN, performed the major writing work, and designed the graphical illustration mechanism. SM, SR, AD, and MA proofread the manuscript. MS and MC conceived the idea, performed final proofreading and prepared the final manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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# Glossary

| | |
|---|---|
| TME | Tumour microenvironment |
| HIF-1α | Hypoxia-induced factor-1α |
| TCA | Tricarboxylic acid |
| NSC-LC | Non-Small Cell Lung Carcinoma |
| CD8+ T cells | cytotoxic T cells |
| NK | Natural Killer cells |
| DC | Dendritic cells |
| NF-kB | Nuclear factor kappa-B |
| Cln-1 | Claudin-1 |
| LDHA | Lactate dehydrogenase |
| MNU | N-Methyl-N-Nitrosourea |
| NMR | Nuclear magnetic resonance |
| VEGF | Vascular endothelial growth factor |
| PGA-1 | Plasminogen activator inhibitor-1 |
| PDGF-B | Platelet-derived growth factor-B |
| FLT-1 and FLK-1 | Fms Related Receptor Tyrosine Kinases |
| MMP-2 | Matrix metalloproteinases |
| Tie2 | A receptor tyrosine kinase |
| ANG-1 and ANG-2) | Angiopoietins |
| EMT | Epithelial to mesenchymal transition |
| FGF-2 | Fibroblast growth factor-2 |
| PIGF | Placental growth factor |
| BAECs | Bovine aorta endothelial cells |
| MCT-1 and 4 | Monocarboxylate transporter-I &4 |
| TAM | tumour-associated macrophages |
| HUVECs | cultured human umbilical endothelial cells |
| Axl | A tyrosine kinase receptor |
| DLL4 | Delta-like Ligand-4 |
| EC | endothelial cells |
| GPCR | G-protein coupled receptor |
| Arg-1 | Arginase-1 |
| ICER | Inducible cAMP early repressor |
| Fizz-1 | Resistin-like molecule alpha1 |
| LDHB | Lactate dehydrogenase B |
| OSC | Oral Squamus Cell Carcinoma cells |
| TGF-β | transforming growth factor-β |
| ZEB1 | Zinc finger E-box-binding homeobox 1 |
| CDH2 | Cadherin-2 |
| VIM | Vimentin |
| LCFA | Long chain fatty acids |
| CAF | Cancer-associated fibroblasts |
| ATM | Ataxia-telangiectasia |
| GLUT-1 | Glucose Transporter-1 |
| DSB | double-strand-break |
| pmCIC | Plasma membrane citrate carrier |
| MRPL13 | Mitochondrial ribosomal protein L13 |
| ETC | Electron transport chain |
| OXOPHOS | Oxidative phosphorylation |
| ROS | Reactive oxygen species |
| ATP | Adenosine triphosphate |
| CCL4,5,6,10,17 | Motif chemokine 4,5,6,10,17 |
| PD-L1 | Programmed death ligand |
| H2H2 | Hydrogen peroxide |
| TNF-α | Tumor necrosis factor-alpha |
| LPS | Lipopolysaccharide |
| COX-2 | cyclooxygenase-2 |
| PLAG | 1-palitoyl-2-linoleoyl-3-acetyl-rac-glycerol |
| TADCs | Tumour-associated dendritic cells |
| MCTS | multicellular tumor spheroids |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| | |
| | |
| MHC-1 | major histocompatibility class-1 |
| c-MET | A receptor tyrosine kinase |
| HGF | Hepatocyte growth factor |
| TKIs | Tyrosine kinase inhibitors |
| ERR-α) | Estrogen-related receptor alpha |
| MRP-1 | Multiple resistance-associated protein 1 |
| ABC | AT-binding cassette |
| CRC | Colorectal carcinoma |
| MMR | Mismatch repair proteins |
| ACC2 | Acetyl-CoA carboxylase 2 |
| LDL | Low density lipoprotein |
| VLDL | Very low density lipoprotein |
| VLDLR | VLDL receptor |
| HRE | Hypoxia response element |
| pHe | Extracellular Ph |
| SREBP2 | Sterol regulatory element binding protein-2 |
| TGs | Triglycerides |
| LDs | Lipid droplets |

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| | |
|---|---|
| CR-CRC) | Colorectal cancer stem cells |
| LDLR | Low-density lipoprotein receptor |
| GSC | glioma stem cells |
| ApoE/D | Apolipoproteins |
| PPP | pentose phosphate pathway |
| PUFA | Polyunsaturated fatty acids. |

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# Low expression of HIF1AN accompanied by less immune infiltration is associated with poor prognosis in breast cancer

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**Background:** Hypoxia-inducible factor 1-alpha (HIF-1α) stability and transcriptional action are reduced by the hypoxia-inducible factor 1-alpha subunit suppressor (HIF1AN). Its inappropriate expression is associated with the development of cancer and immune control. It is yet unknown how HIF1AN, clinical outcomes, and immune involvement in breast cancer (BC) are related.

**Methods:** Using the GEPIA, UALCAN, TIMER, Kaplan-Meier plotter, and TISIDB datasets, a thorough analysis of HIF1AN differential expression, medical prognosis, and the relationship between HIF1AN and tumor-infiltrating immune cells in BC was conducted. Quantitative real-time PCR (qRT–PCR) analysis of BC cells were used for external validation.

**Results:** The findings revealed that, as compared to standard specimens, BC cells had significantly lower levels of HIF1AN expression. Good overall survival (OS) for BC was associated with higher HIF1AN expression. Additionally, in BC, the expression of HIF1AN was closely associated with the chemokines and immune cell infiltration, including neutrophils, macrophages, T helper cells, B cells, Tregs, monocytes, dendritic cells, and NK cells. A high correlation between HIF1AN expression and several immunological indicators of T-cell exhaustion was particularly revealed by the bioinformatic study.

**Conclusions:** HIF1AN is a predictive indicator for breast tumors, and it is useful for predicting survival rates.

KEYWORDS

**breast cancer, hypoxia-inducible factor 1-alpha subunit inhibitor (HIF1AN), prognosis, immune infiltrating cell, biomarker**

# Introduction

Breast cancer (BC) is a complicated disease with numerous classifications and exhibits both significant inter- and intra-tumor variations (1, 2). Globally, BC affects approximately 10% of women during the course of their lives (3, 4). Despite improvements in the diagnosis and treatment of BC, the management of the disease is still challenging and most patients have poor outcomes (5). ER (+) or ER (-) are the two hormone receptors used to classify BC, with the latter having fewer therapeutic decisions, especially for triple-negative breast cancers (TNBCs) (2, 6). Several studies have investigated the molecular pathways underlying the various hormonal states to reveal options for the treatment of BC.

In addition to surgery, chemo- and radiation therapy, inhibition of targeted pathways and combination immunotherapies are considered alternative treatment options (7). Given its high heterogeneity, not all BC patients benefit from immunotherapy. Researchers have demonstrated that the clinical efficacy of immunotherapy is partly influenced by the immunosuppressive tumor microenvironment (8). Therefore, specific immune-related biomarkers of BC should be explored to develop new immunotherapeutic targets and strategies to alleviate resistance.

The HIF-1A protein regulates the transcription of genes involved in response to hypoxia. The protein participates in processes the ensure the survival of cells under hypoxia (9, 10), and it has been shown that HIF1A may play a role in the development of tumor resistance to immunotherapy (11, 12). It is also involved in tumor development and metastasis (13). Hypoxia-inducible factor 1-alpha subunit suppressor (HIF1AN), also known as component suppressing HIF-1 (FIH-1), inhibits HIF-1 activity by hydroxylating the C-terminal trans-activation domain of the HIF-1α subunit, thus preventing HIF-1 from recruiting co-activators CPB/p300, which are important for the transcription of target genes (14, 15). Prior research suggests that HIF1A enhances cancer progression, spread, and metastasis by promoting angiogenesis and controlling cellular metabolism in hypoxic tumor conditions. HIF1A is upregulated in multiple malignancies and immune responses (16). Additionally, numerous studies have revealed that HIF1AN suppresses the growth of cancerous cells and might function as a potential tumor inhibitor in gastrointestinal and prostate cancer (17). The fundamental pathways *via* which HIF1AN prevents tumor growth and immunological interaction with BC are currently unknown.

To investigate the relevance of HIF1AN in BC, RNA sequencing and medical analysis process based on BC patients with identified HIF1AN collected from The Cancer Genome Atlas (TCGA) dataset. The link between HIF1AN and immunological infiltration was also investigated. This is the first in-depth investigation into the clinical, structural, and immunological features of HIF1AN gene expression.

# Materials and methods

## RNA-sequencing of patient data

Gene expression data of cases in which HIF1AN had been measured using HTSeq-FPKM or HTSeq-count, generated by the Breast invasive carcinoma (BRCA) projects, together with corresponding clinical information, were collected from the TCGA website. Normal BRCA samples and cases with an overall survival of <30 days were excluded. Level 3 HTSeq-FPKM data were transformed into transcripts per million reads (TPM) for subsequent analysis. Information from 1222 patients with BRCA was retained. Unknown or unavailable clinical data in the 1222 patients were considered to be missing values. All data used in the paper were acquired from TCGA, and hence ethics approval and informed consent were not required.

## Identification of DEGs and functional enrichment analysis

To obtain the differentially expressed genes (DEGs) for BRCA between the high and low HIF1AN expression groups, the expression profiles (HTSeq-counts) were analyzed using the DESeq2 R package (18). The threshold values used to identify DEGs were $|\log2FoldChange| > 1.5$ and $p.adj < 0.05$. Then the functional enrichment analyses, including Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway were performed using the clusterProfiler package in R. The thresholds were as follows: p.adj<0.05 and q value <0.2.

## Exploration of the expression of HIF1AN and its clinical relevance

To investigate the clinical relevance of HIF1AN, we explored the university of Alabama at Birmingham cancer data analysis portal (UALCAN). UALCAN (http://ualcan.path.uab.edu) a user-friendly portal, can facilitating the analysis in various tumor sub-groups based on individual cancer stages, tumor grade, race, body weight or other clinicopathologic features (19). In our study the associations between HIF1AN expression and significant clinical characteristics, including tissue type (healthy/tumor), breast cancer subtypes, stage of cancer (stages 1, 2, 3, and 4), lymph node stage (N0, 1, 2, and 3), and cancer cluster, are investigated.

## Survival analysis of HIF1AN in breast cancer

The Kaplan-Meier plotter Database (http://kmplot.com/analysis/) was used for survival analysis (20). We used the pattern of mRNA of gene chip in Breast cancer to explore the prognostic value of HIF1AN, including overall survival (OS), recurrence-free survival (RFS), and distant metastasis-free survival (DMFS). The hazard ratio (HR) and 95% confidence interval (CI) were calculated and the differences between the survival curves were examined using log-rank tests.

## Gene set enrichment analysis

To further investigate the functions of HIF1AN in breast cancer, Gene set enrichment analysis (GSEA) was conducted using the clusterProfiler package (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) in R (3.6.2). The low and high groups were determined according to the expression level of

HIF1AN, and gene set permutations were performed 1000 times for each analysis. The low and high groups were used as the phenotype label, and gene sets with adj.p-value <0.05 and FDR q-value <0.25 were considered to be enrichment significant.

## Analysis of immune cell infiltration and its correlation with HIF1AN

The immune infiltration analysis of BRCA was performed using single-sample GSEA (ssGSEA) with the GSVA package in R (3.6.2) (https://www.bioconductor.org/packages/release/bioc/html/GSVA.html) for 24 types of immune cells in the tumor samples. Spearman correlation was applied to explore the correlations between HIF1AN and the infiltration levels of T cell exhaustion and TAM related genes, WilCoxon rank sum tests were used to reveal the association of the infiltration of immune cells with the groups with different levels of expression of HIF1AN.

## Correlation analysis between HIF1AN expression and chemokines

To further clarify the role of HIF1AN in the interaction between breast cancer and immune system, we searched the Tumor-Immune System Interactions Database (TISIDB, http://cis.hku.hk/TISIDB/index.php) (21). The relationship between HIF1AN and chemokines (such as CCL2, CXCL8, CXCL16 and CCR2) were calculated by Spearman's correlation analysis in the database.

## Cell lines and culture

The normal breast epithelial cell line MCF10A, the human BC cell lines MCF-7, SKBR-3, and MDA-MB-453, and the Chinese Academy of Sciences' Cell Bank of Type Culture Collection. DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and a 1% (v/v) penicillin and streptomycin solution was employed to regularly cultivate SKBR-3, MCF-7, and MDA-MB-231 cells (Beyotime Institute of Biotechnology). A mammary epithelial cell environment (Procell Life Science & Technology Co., Ltd.) containing 10% horse serum, EGF, hydrocortisone, insulin, and 1% penicillin-streptomycin was employed to cultivate MCF10A cells. All cell lines were perfused employing conventional cell culture methods and grown in an incubator at 37°C with 5% CO2.

## Quantitative real-time PCR analysis

Using the RNAiso Plus Kit (cat. no. 9109; Takara Biotechnology Co., Ltd.), total RNA of the SKBR-3, MCF-7, MDA-MB-453, and MCF10A cells was extracted in accordance with the industrialist's recommendations. Following the industrialist 's instructions, 1,000 ng of total mRNA were retro transcribed into cDNA employing Takara Biotechnology Co., Ltd.'s PrimeScript™ RT Reagent Kit with the Genomic DNA Eraser (cat. no. RR047). To find out if each of the target genes was expressed, TB Green Premix Ex Taq (cat. no. RR420; Takara Biotechnology Co., Ltd.) was employed in a quantitative PCR (qPCR) assay using the LightCycler® 96 Instrument (Roche Diagnostics). The following primer pairs were used for qPCR: HIF1AN forward, 5'-GAGTGCCTCTACCCATACCCT-3' and reverse, 5'-TCGTAGTCGGGATTGTCAAAGT-3'; and GAPDH forward, 5'-CATTGACCTCAACTACATGGTTT-3' and reverse, 5'-GAAGATGGTGATGGGATTTCC-3'. qPCR was completed under the specified thermocycling situations: 95°C for 5 min, then 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The proportional mRNA expression levels were normalised to those of the housekeeping gene GAPDH using the conventional 2-Cq technique, and the comparative cycle limit of the housekeeping gene GAPDH was assessed as an endogenous control. The trial was carried out three times.

## Statistical analysis

R (software v.3.6.2) was used to perform the statistical analyses. WilCoxon signed-rank tests were used to analyze the expression of HIF1AN in non-paired and paired samples. The Kaplan–Meier method was applied to survival analysis, and the differences between the survival curves were examined using log-rank tests. Correlations between HIF1AN and other genes were identified using Spearman's correlation analysis. qRT-PCR results are presented as the mean ± standard deviation (SD) from the three independent experiments, t-test was carried out for statistical analysis with GraphPad Prism software version 7.0. A p-value <0.05 was considered as statistically significant.

# Results

## Expression of HIF1AN in various cancers and the differentially expressed genes in BC

The differential expression of HIF1AN between various cancers and nearby healthy tissue was assessed on the TCGA database. As shown in Figure 1A, the expression of HIF1AN was downregulated in most cancers such as breast cancer (BRCA), thyroid cancer (THCA), prostate adenocarcinoma (PRAD), and uterine corpus endometrial carcinoma (UCEC). It expression was in stomach adenocarcinoma (STAD), cholangiocarcinoma (CHO) and liver hepatocellular (LIHC).

To further verify the findings for BC, 1222 BC samples from the TCGA database were examined. HIF1AN expression levels were lower in BC (1109 samples) than in normal tissues (113 samples) (Figure 1B). Moreover, HIF1AN expression was lower than in matched adjacent normal tissue (Figure 1C). According to RT-qPCR analysis, the expression level of HIF1AN in all three types of BC cells (SKBR-3, MCF-7, MDA-MB-453) was significantly lower compared with that of MCF10A cells, which is consistent with the aforementioned results (Figure 1D). The 1222 BC patients were divided into two cohorts, elevated and low HIF1AN expression cohorts, depending on the median HIF1AN expression in BC tumors. The mRNA expression levels of the two cohorts were compared. In the elevated HIF1AN cohort, 949 mRNAs, comprising 31 elevated and 918 reduced genes, were identified as DEGs (absolute value of fold change >1.5, P < 0.05) (Figure 1E).

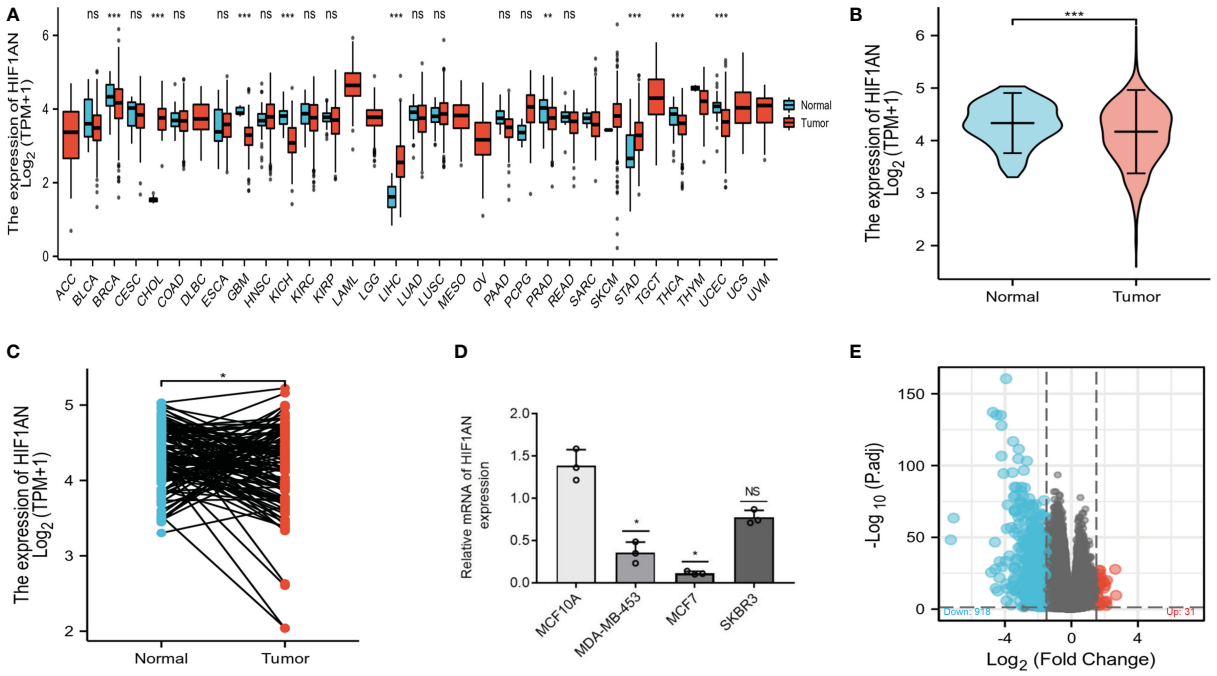


FIGURE 1
Stratified by HIF1AN levels, different mRNA expression patterns in BC patients. **(A)** The TCGA database-based expression of HIF1AN in various kinds of cancer. **(B)** According to the TCGA-BRCA data, HIF1AN expression was markedly reduced in BC cells as compared to healthy cells. **(C)** According to the TCGA-BRCA sets of data, HIF1AN expression was considerably reduced in associated BC tumour tissues as compared to neighbouring healthy tissue. ns, $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **(D)** Evaluation of HIF1AN mRNA expression levels in breast tissues epithelium and breast cancer cell lines using reverse transcription-quantitative PCR. Mann Whitney U analysis of the relationship between TCGA BRCA datasets' HIF1AN gene expression; **(E)** High- and low-HIF1AN expression cohorts' mRNA expression patterns are displayed. Volcano graphs are used to visualize information.

## Association of HIF1AN expression with clinicopathological features in BC individuals

The differential expression of HIF1AN in BC and healthy samples was explored by UALCAN as shown in Figure 2A. Compared to normal cells, the expression of HIF1AN was markedly significantly inhibited in BC cells (Figure 2A). For cancer patients with HIF1AN expression, the number of clinical and pathological factors, molecular subtypes, tumor phases (phase 1, 2, 3, and 4), and lymph node phase (N0, 1, 2 and 3) were examined. Figure 2B shows that compared to healthy tissues, the expression of HIF1AN was decreased in Luminal, HER2 positive, and triple negative BC. Furthermore, the expression of HIF1AN was decreased as the tumor level increased. Notably, middle-stage and late-stage BC had much lower expression of HIF1AN than early-stage BC (Figure 2C). HIF1AN expression was significantly decreased in BC than in all phases of lymph node phase specimens (Figure 2D). These findings suggest that the level of cancer is related to reduced HIF1AN.

## Decreased HIF1AN is linked to poor survival in breast cancer patients

The expression of HIF1AN in BC patients was lower than in healthy individuals. Therefore, there is a need to further investigate the relationship between HIF1AN and tumor rates. To assess the relationship between HIF1AN and prognostic outcomes in BC, KM survival curves were to explore the connection between HIF1AN and illness prognosis. In the group 226648-at, it is noteworthy that higher levels of HIF1AN expression were associated with better outcomes for BC (overall survival (OS): HR = 0.49, $p < 0.001$; recurrence-free survival (RFS): HR = 0.52, $p < 0.001$; distant metastasis-free survival (DMFS): HR = 0.55, $p < 0.001$) (Figure 3).

Next, using the PAM50 subtype approach, we evaluated the likelihood that HIF1AN expression would be present in various subtypes. The healthy breast-like subtype group showed better OS when HIF1AN was highly expressed (p=0.027). The luminalA, luminalB, and basal-like subtype groups, although, did not show any major changes, while the HER2 subtype cohort showed a tendency in the other direction (p=0.027). These findings imply that the outcome of various BC subtypes is correlated with the expression level of HIF1AN.

## Predicted biological function and pathways of HIF1AN in BC

Genes co-expressed with HIF1AN ($|logFC| > 1$, P.adj <0.05) were chosen to perform gene analyses once their bioactivity was determined. Epidermis progression, skin growth, epidermal cell differentiation, and keratinocyte differentiation were all considerably elevated in GO terms used to describe biological processes (BP) (Figure 4A). For the cellular component, extracellular matrix, vesicle lumen, and cytoplasmic vesicles that hold collagen were enriched (CC) (Figure 4B). Receptor-ligand action, enzyme suppressing activity, and channel activity were all

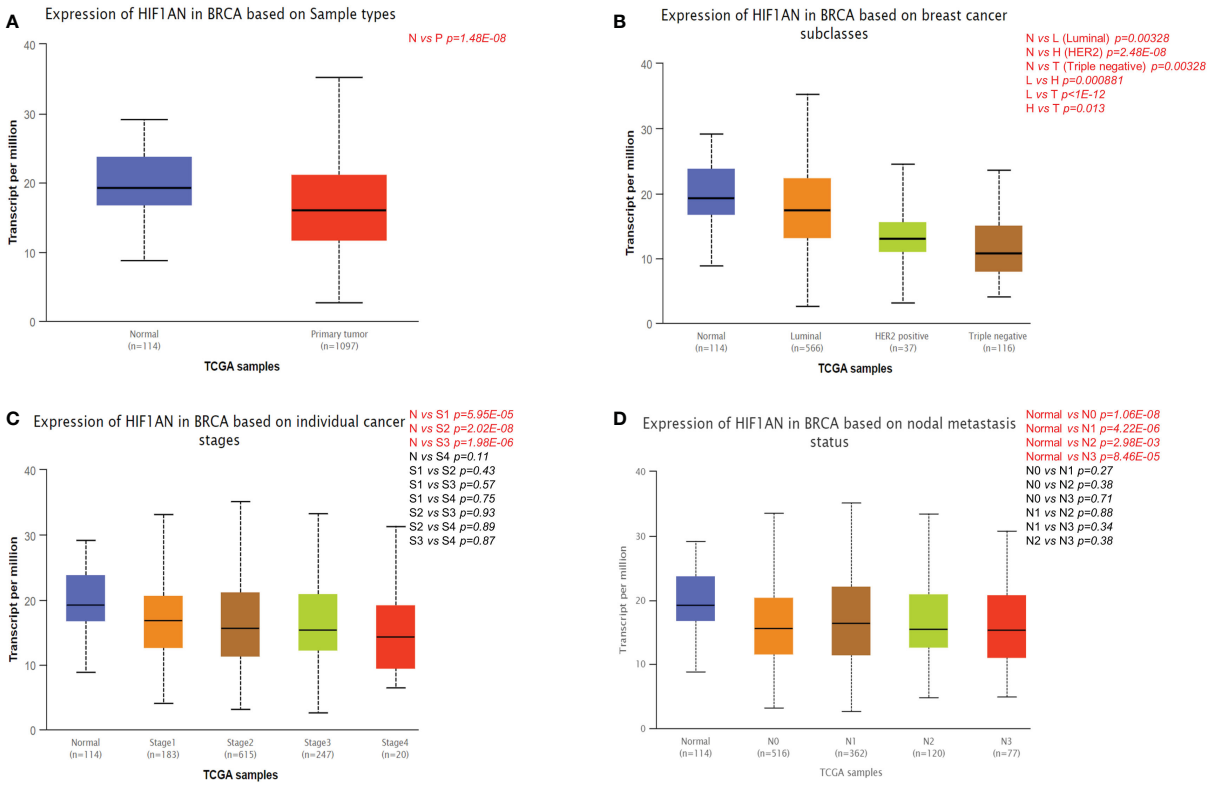


FIGURE 2
Correlation between HIF1AN expression level and clinicopathological variables of BC through the UALCAN datasets. **(A)** HIF1AN expression was remarkably downregulated in breast primary tumor than that in normal tissues. **(B)** HIF1AN expression was lower in luminal, HER2 positive and triple negative BC than in normal tissues. **(C)** HIF1AN expression in middle-stage and late-stage BC was substantially lower than in the early phase. **(D)** HIF1AN expression at all stages of lymph node stage specimens was substantially lower in BC than in healthy one; P, primary tumor; S1, stage 1; S2, stage 2; S3, stage 3; S4, stage 4.

highly enriched, according to the molecular function (MF) study (Figure 4C). Additionally, KEGG results suggests that the IL-17 signaling mechanism, neuroactive ligand-receptor activity, and cytokine-cytokine receptor interplay were dominant processes (Figure 4D). Generally, the findings suggested that HIF1AN and the genes its co-expresses may be involved in cell signaling, which may control BC's biological pathways.

Furthermore, GSEA was carried out using the normalized enrichment score (NES) and FDR (false discovery rate) q-value to clarify the potential biological mechanisms controlled by HIF1AN between elevated and reduced HIF1AN expression cohorts. As illustrated in Figure 5, a number of signal mechanisms, such as Notch signaling, cell-surface contacts, CD8 TCR downstream pathway, and chemokine signaling pathway, were substantially concentrated in the cohort with decreased HIF1AN expression.

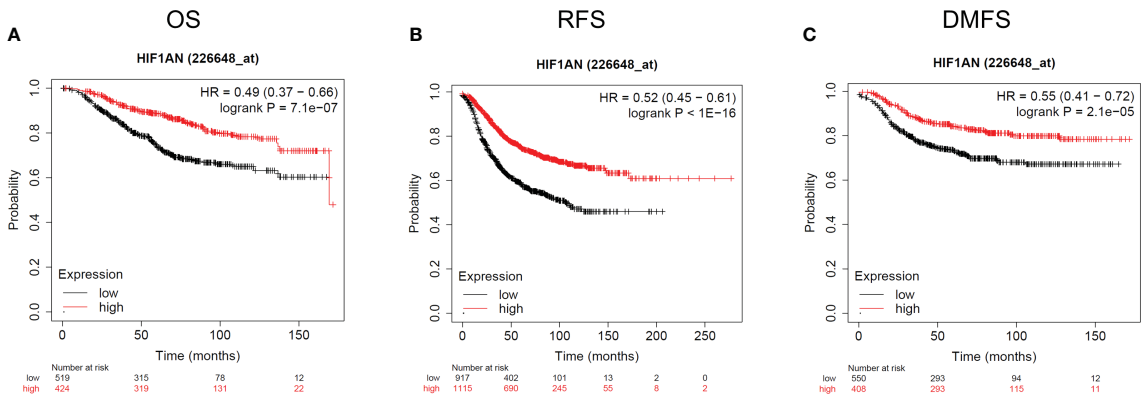


FIGURE 3
Kaplan-Meier survival curves comparing the elevated and reduced expression of HIF1AN in BC. Survival curves of OS **(A)**, RFS **(B)**, and DMFS **(C)**. OS, overall survival; RFS, recurrence-free survival, DMFS, distant metastasis-free survival.

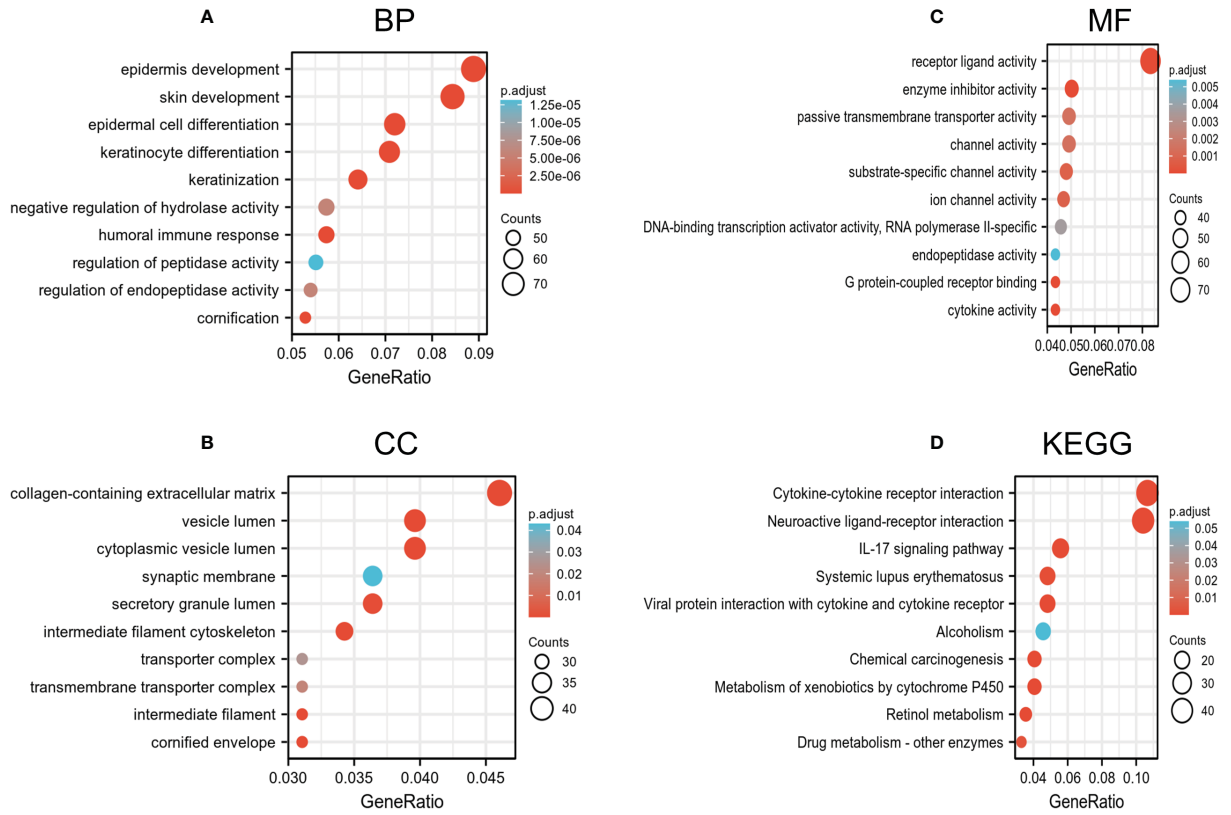


FIGURE 4
Go and KEGG enrichment test of genes associated with HIF1AN in BC cells in the TCGA-BRCA data. **(A–C)** Go enrichment evaluation revealed the BP (biological processes), CC (cellular components), and MF (molecular function) of co-expressed genes with HIF1AN. **(D)** substantially enriched KEGG terms derived from KEGG enrichment test of co-expressed genes with HIF1AN.

## Correlation of HIF1AN expression and immunity cells infiltration in BC

One of the main elements influencing tumor growth is immune infiltration. Notably, 24 different types of immune cells were found in breast tumor using ssGSEA. The relationship between immune cell infiltration and HIF1AN expression was then examined employing Spearman's analysis. Figure 6A reveals significant positive correlation between HIF1AN expression and Tcm cells (R = 0.320, P <0.001), eosinophils (R = 0.260, P <0.001), T helper cells (R = 0.330, P <0.001), and NK cells (R = 0.087, P = 0.004). However, there was a negative connection between HIF1AN and macrophages (R = -0.171, P <0.001), neutrophils (R = -0.146, <P 0.001), Th1 cells (R = -0.271, P <0.001), CD8 T cells (R = -0.169, P <0.001), and aDC cells (R = -0.251, P <0.001). Moreover, the rates of immune cell infiltration in various HIF1AN cohorts were assessed, including Tcm cells (Figure 6B), T helper cells (Figure 6C), Th17 cells (Figure 6D), eosinophils (Figure 6E), neutrophils (Figure 6F), and Treg cells (Figure 6G). The findings were in line with those shown in Figure 3A, demonstrating the significance of HIF1AN in immune infiltration of Breast malignancy.

The relationship between HIF1AN and several TIL indicators (neutrophils, T cells and associated variants, CD8+/CD4+ T cells, NK cells, B cells, monocytes, DCs, TAMs, M1 macrophages, and M2 macrophages) in BC was investigated using the GEPIA and TIMER datasets. It was found that most TIL identifiers were correlated with HIF1AN. Additionally, several functional T cells, particularly Tregs, Th1, Th2, Th17, Tfh cells, and fatigued T cells, were examined. Results showed that HIF1AN was particularly strongly correlated with TILs (Supplementary Table 1).

Interestingly, the findings suggested a correlation between HIF1AN in breast malignancy and PDCD1, LAG3, CTLA4, and GZMB of T cell exhaustion as well as chemokine ligand CCL2 of TAMs (Figures 7A, B). proving that HIF1AN may have a role in controlling T cell fatigue in breast tumors.

## Correlation between the HIF1AN and chemokines in BC patients

Chemokines regulate infiltration of immune cells (22). Here, we found that HIF1AN expression was correlated with chemokines. Particularly, HIF1AN expression was significantly ($p < 0.001$) linked to CCL2 (Cor = -0.338), CCL3 (Cor = -0.222), CCL4 (Cor = -0.303), CCL5 (Cor = -0.367), CCL8 (Cor = -0.264), CCL11 (Cor = -0.164), CCL13 (Cor = -0.297), CCL7 (Cor = -0.279), CCL17 (Cor = -0.264), CCL19 (Cor = -0.221), CX3CL1 (Cor = -0.351), CXCL9 (Cor = -0.234), CXCL10 (Cor = -0.282), CXCL13 (Cor = -0.21), and XCL2 (Cor =- 0.286). Furthermore, HIF1AN expression was also related with chemokine receptors ($p < 0.001$), including CCR1 (Cor = -0.203), CCR2 (Cor = -0.151), CCR5 (Cor = -0.179), CCR7 (Cor = -0.223), CCR10 (Cor = -0.275), CXCR3 (Cor = -0.285), CXCR4 (Cor = -0.273), CXCR5 (Cor = -0.211), CXCR6 (Cor = -0.26) and CX3CR1 (Cor = 0.187)

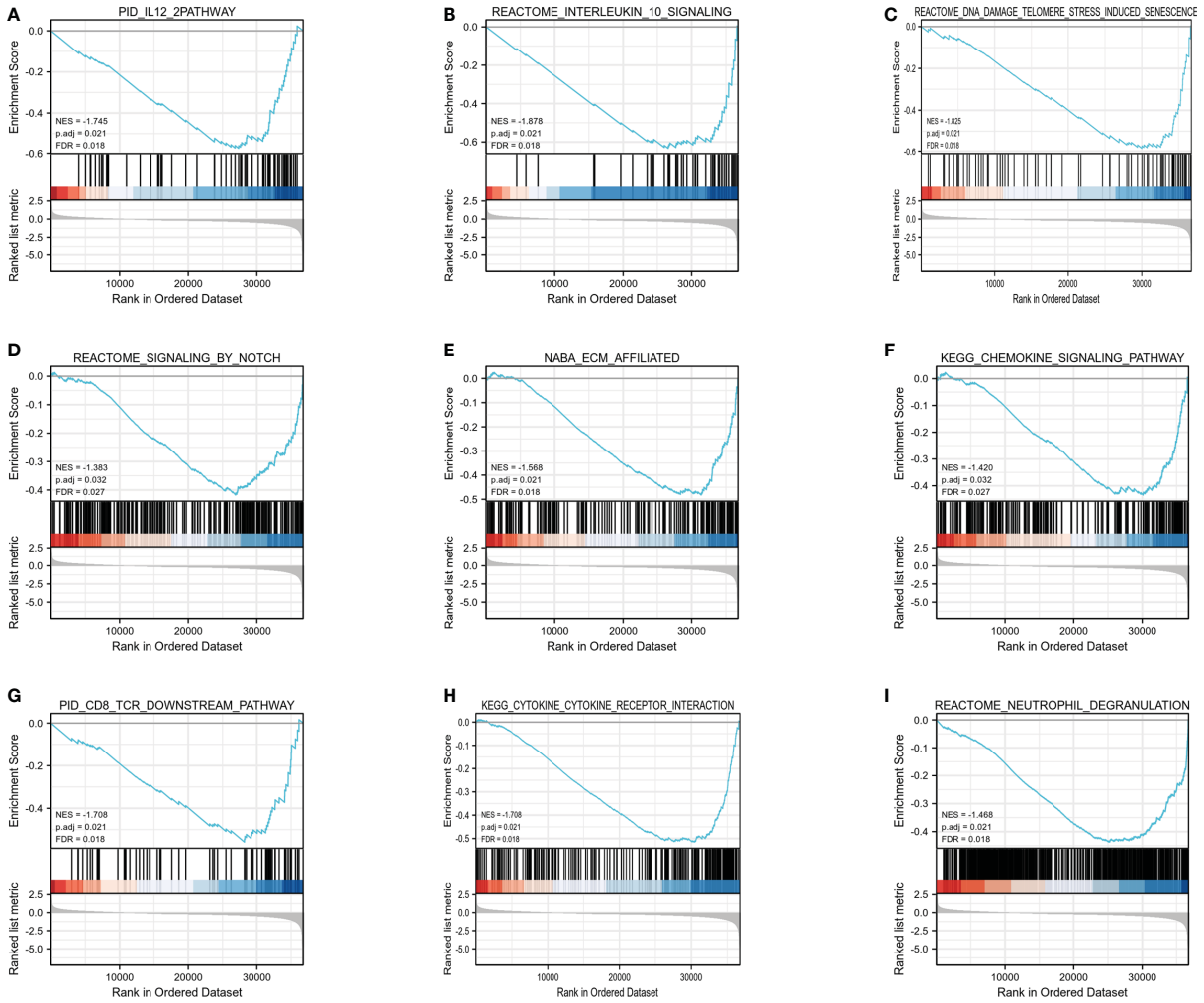


FIGURE 5
Enrichment plots from the gene set enrichment analysis (GSEA). **(A)** IL12 mechanism, **(B)** interleukin10 signaling, **(C)** DNA damage telomere stress induced senescence, **(D)** Notch signaling, **(E)** ECM affiliated, **(F)** chemokine signaling pathway, **(G)** CD8 TCR downstream pathway, **(H)** cytokine-cytokine receptor interaction, and **(I)** neutrophil degranulation were substantially enriched in HIF1AN-associated BC. NES, normalized enrichment scores; FDR, false discovery rate.

(Table 1). The above outcomes further proved that HIF1AN may modulate in breast malignancy.

## Discussion

Hypoxic stress is a characteristic of most solid tumors and is associated with adverse clinical outcomes (23). HIF1A is the main regulator of hypoxic response. Recent studies have demonstrated that the tumor immune escape is closely related to epithelial-mesenchymal transformation (EMT) of tumor cells and regulates the tumor microenvironment (24). In particular, HIF1A is stimulates the EMT in cancer cells, and modifies the immune function of tumor cells, and promotes immune escape (16). HIF1AN, an asparagine hydroxylase and an upstream regulation gene of HIF1A, acts as an inhibitor of HIF1A and is an important transcription factor that affects responses to hypoxia (17). Under normoxic conditions, HIF1AN inhibits the transcriptional activity of HIF1A, to prevent the transcription of the HIF1A-mediated gene. However, under hypoxic conditions, the inhibition is relieved, allowing HIF1A to recruit CBP/p300, resulting in expression of the target gene (25). Although HIF1AN has been well investigated in several kinds of malignant tumors, its clinical significance and possible regulatory role in immunity in breast malignancy is unknown.

In the current work, a bioinformatics analyses were done to examine the bioactivities of HIF1AN in BC. According to our studies, HIF1AN expression was downregulated in breast cancer females which correlated with poor prognosis (Figure 3). These findings further showed that decreased HIF1AN expression was directly linked to the level of immune cell, immunostimulator, immunological inhibitor, receptor, and chemokine infiltration in BC (Figures 6, 7, Table 1). Therefore, we concluded that HIF1AN may be a tumor suppressor with the potential to be a treatment target in women with breast cancer. It is also likely to be an indicator of immune infiltration in BC.

The expression of HIF1AN in BC was determined using a separate database. In normal cells and cancer cells, HIF1AN was considerably reduced in BC cells, and it was related to the tissue level

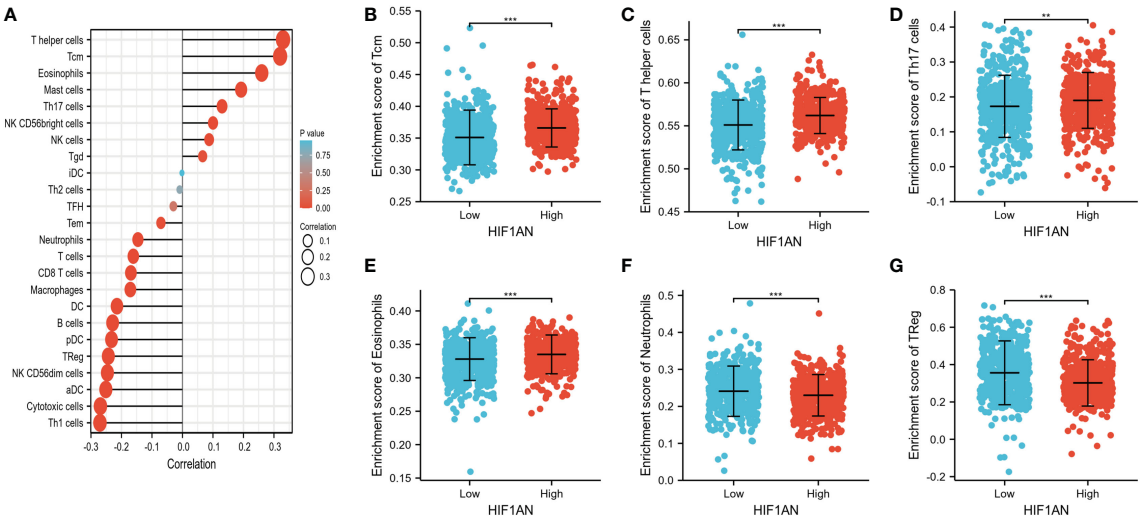


FIGURE 6
Correlation of immune cell infiltration and HIF1AN expression in BC females. **(A)** correlations among infiltration levels of 24 kinds of immune cell and HIF1AN expression profiles by Spearman's evaluation test. Illustrated is the comparison of infiltration levels of most correlated immune cells, containing Tcm **(B)**, T helper cells **(C)**, Th17 cells **(D)**, Eosinophils **(E)**, Neutrophils **(F)** and Treg **(G)** between high and low HIF1AN expression groups. DCs, dendritic cells; aDCs, activated DCs; iDCs, immature DCs; pDCs, plasmacytoid DCs; Th, T helper cells; Th1, type 1 Th cells; Th2, type 2 Th cells; Th17, type 17 Th cells; Treg, regulatory T cells; Tgd, T gamma delta; Tcm, T central memory; Tem, T effector memory; Tfh, T follicular helper; NK, natural killer; ns: P ≥ 0.05, *P < 0.05, ** P < 0.01, and *** P < 0.001.

(Figure 2). Furthermore, low expression of HIF1AN was correlated with negative clinical outcomes (Figure 3). These findings imply that HIF1AN may act as a tumor inhibitor in BC and slow the spread of breast cancer. Earlier studies also showed that HIF1AN deficiency increased VEGF expression in head and neck cancer, elevated the expression of HIF1AN to suppress the oncogenic progression of head and neck squamous cell carcinoma (26). Inhibition of HIF1Awas found to be a therapeutic strategy for the human colorectal cancer (17). Furthermore, a comparable investigation revealed that miR-135b-5p may promote the growth of ovarian cancer cells by suppressing HIF1AN the expression (27). These studies showed the cancer-inhibitory role of HIF1AN in other tumors, which was similar to the results of our analysis in BC.

Surprisingly, the PAM50 test showed that the effect of HIF1AN expression on survival rates varied across different breast cancer subtypes. Decreased HIF1AN expression, particularly in the

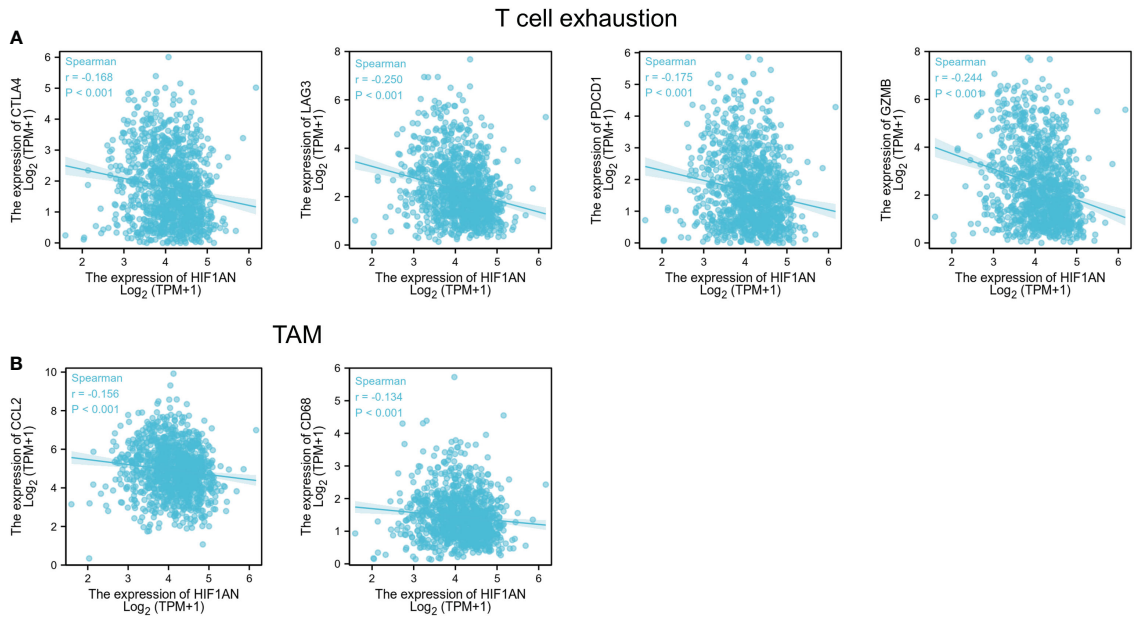


FIGURE 7
HIF1AN expression correlated with T cell exhaustion and markers include CCL2 and CD68 of TAMs in BC; Scatterplots of correlations between HIF1AN expression and gene indicators of **(A)** T cell exhaustion, **(B)** TAMs.

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TABLE 1 Correlation analysis between the expression of HIF1AN and Chemokines & Receptors in breast cancer at TISIDB datasets.

| Chemokines & Receptors | HIF1AN | |
|---|---|---|
| | Cor | *p* Value |
| CCL2 | -0.338 | <0.001 |
| CCL3 | -0.222 | <0.001 |
| CCL4 | -0.303 | <0.001 |
| CCL5 | -0.367 | <0.001 |
| CCL7 | -0.279 | <0.001 |
| CCL8 | -0.264 | <0.001 |
| CCL11 | -0.164 | <0.001 |
| CCL13 | -0.297 | <0.001 |
| CCL17 | -0.264 | <0.001 |
| CCL18 | -0.264 | <0.001 |
| CCL19 | -0.221 | <0.001 |
| CXCL8 | -0.19 | <0.001 |
| CXCL11 | -0.266 | <0.001 |
| CXCL13 | -0.21 | <0.001 |
| CXCL16 | -0.324 | <0.001 |
| XCL1 | -0.27 | <0.001 |
| XCL2 | -0.286 | <0.001 |
| CCR1 | -0.203 | <0.001 |
| CCR2 | -0.151 | <0.001 |
| CCR5 | -0.179 | <0.001 |
| CCR7 | -0.223 | <0.001 |
| CCR10 | -0.275 | <0.001 |
| CXCR3 | -0.285 | <0.001 |
| CXCR4 | -0.273 | <0.001 |
| CXCR5 | -0.211 | <0.001 |
| CXCR6 | -0.26 | <0.001 |
| CX3CR1 | 0.187 | <0.001 |

normal-like subtype, was associated with poor prognosis but opposite results were obtained in the HER2 subtype. These findings imply that HIF1AN has diverse functions in different BC types, but it is important to take into account sampling errors in the five BC subtypes.

TCGA datasets were subjected to GO and KEGG analyses of the HIF1AN-coexpressed genes and GSEA analyses of HIF1AN to better investigate the cellular roles and related concepts of HIF1AN in BC. Results of GO analysis revealed physiological systems associated with the formation of the epidermis, the extracellular structure that contains collagen, and receptor-ligand activity (Figure 4). The KEGG analyses revealed two major systems: cytokine-cytokine receptor interaction and neuroactive ligand-receptor relationship (Figure 5). A previous study showed that carcinogenesis and progression were thought to be influenced by neuroactive ligand-receptor interaction in several malignant forms, including glioma (25), renal cell carcinoma (28), colorectal cancer and hepatocellular carcinoma (29). HIF1AN might contribute to the neuroactive ligand-receptor interaction and cell signaling processes needed for BC to start and spread malignancy.

In the low HIF1AN expression phenotype, GSEA findings indicate that a number of routes were considerably dominated, including the chemokine signaling pathway, CD8 TCR downstream mechanism, interleukin 10 and ECM affiliated signaling (Figure 4). These mechanisms show a tight connection with cancer or the inflammatory reaction. Increased HIF1AN expression was associated with chronic colitis in a previous study (30).

To explore the immune infiltration status of BC and its association with HIF1AN, we assessed immune cell populations and their correlation with HIF1AN expression levels. Our results

illustrated that HIF1AN expression is negatively correlated with numerous immune cells. High HIF1AN levels are associated with decreased infiltration of several immunocellular markers including CD8 T cells, B cells, DC cells and neutrophils (Figure 6, 7 and supplementary materials). These results need to be further validated through *in vitro* and *in vivo* experiments. Nevertheless, they suggest that the role of HIF1AN in TILs attraction and cancer microenvironment through which immune cells regulate tumorigenesis, cancer development and metastasis, as well as affect the efficacy and/or resistance to chem- and immunotherapy (31). Immunosuppressive cells like Treg cells and neutrophils can inhibit antitumor response and high levels of these cell types in a hypoxic environment significantly modulate the immune microenvironment (32). In the present study, we observed a similar effect, with higher levels of Tregs and neutrophil cells found in BC females with low HIF1AN expression than in patients with elevated HIF1AN expression. This indicates that HIF1AN may be a favorable factor that modulate the immune microenvironment of BC patients.

Most studies have shown that immune checkpoints have cancer immunosuppressive effects and are the primary immunotherapeutic strategy (32). To date, multiple clinical studies have demonstrated the efficacy of immunosuppressants against PD1/PDL1 in various malignancies, including TNBC (33). However, resistance to immune therapy limits their clinical application (34). Therefore, improving malignant tissue response to immune checkpoint suppressors and cytokines has a significant impact in cancer treatment. It was demonstrated that low oxygen increased PD-L1 expression on macrophages in the tumor milieu (35). Additionally, it was shown that hypoxia significantly reduced the ability of CTLs to kill cancer cells (36). This is likely because HIF1A affects the sensitivity of malignant cells to CTL-mediated killing by increasing expression of NANOG and microRNA (miR)-210 (37, 38). Additionally, combining anti-PD-1 with reducing HIF1A concentrations by pharmacologically inhibiting Axl decreases the main tumour and metastatic loads in a preclinical model of HER2+ breast cancer, indicating a viable treatment strategy in BC (39). According to our findings, PD1, LAG3, CTLA4, and GzmB were all inversely correlated with T cell fatigue, as were elevated doses of HIF1AN expression. GzmB should be considered a sign of late T cell depletion. The exhausted T cells showed a diminished function in a hierarchical way (40). It is suggested that lower expression of HIF1AN is correlated with a higher level of T cell exhaustion markers, which indicates that the tumor may under a hypoxia state, the HIF1A is activated, and the T cells enter a cellular state called “Exhaustion”, they unable to clear the tumor cells. Therefore, decrease HIF1A activity by HIF1AN inhibition could provide an antitumorigenic microenvironment. Additionally, GzmB is an indicator for NK cell (Natural Killer)-mediated killing. One of the main methods by which NK cells destroy tumor cells is by producing cytotoxic granules containing perforin (PRF1) and GzmB. According to reports, lack of oxygen negatively affects NK-mediated killing in addition to impairing CTL-mediated death. Evidence indicates that ischemic cells preferentially activate phagocytosis to destroy the proapoptotic protein GzmB, which prevents the NK system from destroying cancer cells (41). Our findings, therefore, indicated that there is a bad correlation between GzmB expression and HIF1AN. This suggested that lack of oxygen cells may correspond with greater NK cellular function rather than a suppressive impact in reduced HIF1AN conditions.

Additionally, this study found that high expression level of HIF1AN were also negatively correlated with chemokines and receptors (Table 1). Most tumors produce two types of chemokines, CXC and CC. Studies have shown that CCL5 promotes tumor cell growth and inhibits paracrine and autocrine apoptosis of breast cancer (42). This indicate that decreased HIF1AN level may through chemokines regulate the tumor growth and apoptosis. Other studies have reported that HIF induced the release of proinflammatory and proangiogenic substances by breast cancer cells, adipocytes, infiltrating $CD8^+$ T cells, and other stromal cells, suggesting an intricate interplay between HIFs, proinflammatory factors derived from tumor and various TME cells, and angiogenesis that has yet to be fully elucidated (43, 44), and HIF1AN may also regulate angiogenesis and tumor microenvironment through chemokines and cytokines. Furthermore, chemokines and cytokines play an essential role in leukocyte recruitment. These results reflected that higher HIF1AN may correlated with lower level of immune response in the tumor microenvironment, and higher HIF1AN may also correlated with lower level of angiogenesis and proliferation, which indicate a better situation of the patient.

In conclusion, this study demonstrated that high HIF1AN expression may be associated with a favorable prognosis of BC patients. HIF1AN was also found to be involved in immune infiltration mechanisms, to modulate the tumor immune microenvironment. Further *in vitro* and *in vivo* investigation and validation experiments are necessary to confirm these observations. This study is the first systematic in-clinic investigation of core correlations of HIF1AN in BC, exploring its potential molecular mechanism and function in modulating the tumor microenvironment. Thus, HIF1AN is a potential prognostic factor and therapeutic target of BC.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Author contributions

FC and HW designed and managed the entire study; ST, DL, YF and LY collected and analyzed the data; ST and DL wrote the main manuscript text; LY, YZ, MG and XL performed the generated figures. HW assisted with the article revision. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1080910/full#supplementary-material

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# NF-κB mediated regulation of tumor cell proliferation in hypoxic microenvironment

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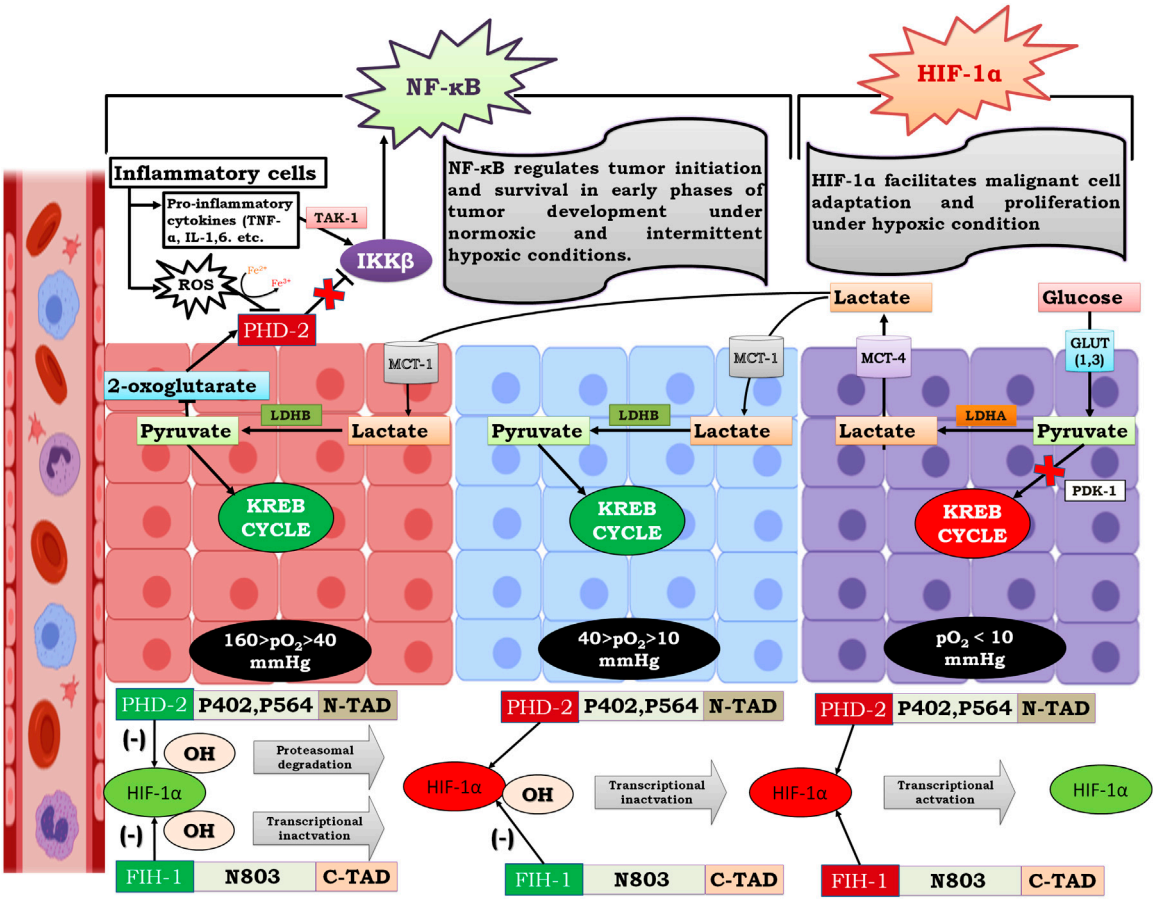
Hypoxia is caused by a cancer-promoting milieu characterized by persistent inflammation. NF-κB and HIF-1α are critical participants in this transition. Tumor development and maintenance are aided by NF-κB, while cellular proliferation and adaptability to angiogenic signals are aided by HIF-1α. Prolyl hydroxylase-2 (PHD-2) has been hypothesized to be the key oxygen-dependent regulator of HIF-1α and NF-transcriptional B's activity. Without low oxygen levels, HIF-1α is degraded by the proteasome in a process dependent on oxygen and 2-oxoglutarate. As opposed to the normal NF-κB activation route, where NF-κB is deactivated by PHD-2-mediated hydroxylation of IKK, this method actually activates NF-κB. HIF-1α is protected from degradation by proteasomes in hypoxic cells, where it then activates transcription factors involved in cellular metastasis and angiogenesis. The Pasteur phenomenon causes lactate to build up inside the hypoxic cells. As part of a process known as lactate shuttle, MCT-1 and MCT-4 cells help deliver lactate from the blood to neighboring, non-hypoxic tumour cells. Non-hypoxic tumour cells use lactate, which is converted to pyruvate, as fuel for oxidative phosphorylation. OXOPHOS cancer cells are characterized by a metabolic switch from glucose-facilitated oxidative phosphorylation to lactate-facilitated oxidative phosphorylation. Although PHD-2 was found in OXOPHOS cells. There is no clear explanation for the presence of NF-kappa B activity. The accumulation of the competitive inhibitor of 2-oxo-glutarate, pyruvate, in non-hypoxic tumour cells is well established. So, we conclude that PHD-2 is inactive in non-hypoxic tumour cells due to pyruvate-mediated competitive suppression of 2-oxo-glutarate. This results in canonical activation of NF-κB. In non-hypoxic tumour cells, 2-oxoglutarate serves as a limiting factor, rendering PHD-2 inactive. However, FIH prevents HIF-1α from engaging in its transcriptional actions. Using the existing scientific literature, we conclude in this study that NF-κB is the major regulator of tumour cell growth and proliferation *via* pyruvate-mediated competitive inhibition of PHD-2.

KEYWORDS

**lactate shuttle, cancer, HIF-1α, NF-κB, hypoxia, PHD2**




GRAPHICAL ABSTRACT

## 1 Introduction

Cancer is a genetic abnormality in which the body's old or flawed cells evade signals of programmed cell death and acquire uncontrolled replicative potential. Cancer can be categorized as solid tumors (carcinoma, sarcoma, melanoma, and lymphoma) and leukemia (no cell mass formation). However, all genetic aberrations that result in abnormal cell growth is not cancer. Mutation in cells results in the formation of "neoplasm".The neoplasm may either bud into an enormous cell mass that is not malignant as well as remains confined to a particular location is referred to as a "benign tumor" (like adenomas, fibroids, hemangiomas and lipomas) or may possess malignant characteristics and metastasize to nearby organs and tissues through blood and lymph referred to as a "malignant tumour" (like adenocarcinomas, basal cell carcinomas and squamous cell carcinomas) (Cooper and Hausman, 2007); Fares et al., 2020). Although there are several causes of cancer, inflammation remains a significant one. An essential contributor to the formation of malignant tumours is an exaggerated immune response to inflammation that results in a condition known as chronic inflammation (Karin et al., 2006; Schottenfeld and Beebe-Dimmer, 2006). TNF-, IL-1, IL-7, IL-8, IL-17, and other pro-tumorigenic cytokines and interleukins are secreted by infiltrating immune cells at the site of infection in a chronic inflammatory microenvironment (León et al.; Hung et al., 2012; Fu et al., 2015; Hospital, 2018; Seol et al., 2019).In combination with reactive oxygen species (ROS), these pro-tumorigenic cytokines lead to DNA damage by inducing genotypic changes in degraded tissue mucosa in favour of tumour initiation and development (Kryston et al., 2011; Kidane et al., 2014) (Figure 1). In the initial phase of tumor growth, oxygen supply is not a limiting factor for mutant cell survival because of easy access to pO2 from nearby vasculature. As the size of the tumor increases to more than 400μm, a hypoxic environment is created, especially at the center of cancer, because nutrients and oxygen can diffuse only up to a radius of 200 μm (Christensen et al., 2010; Grimes et al., 2014; Däster et al., 2017; Riffle and Hegde, 2017). In this regard, the tumor mass can be divided into three distinct zones: the non-hypoxic zone, intermittent hypoxic zone (OXOPHOS), and severe hypoxic zone. Bioenergetics in these three zones is highly interdependent and complex. Early transmuted (non-hypoxic) cells that make up the lining of blood vessels aerobically derive energy from glucose through glycolysis and oxidative phosphorylation. However, in fast-proliferating cells, $O_2$ is a limiting factor for oxidative phosphorylation.

Consequently, glycolysis is the only source of energy in rapidly proliferating tumor cells, which is evident from the increased intracellular accumulation of glycolysis's end product, pyruvate. Accumulated pyruvate is converted into lactate in hypoxic cells. The lactate generated is transferred into non-hypoxic and OXOPHOS cells through monocarboxylate transporter-1 (MCT-1). Hypoxic cells that form the core of the tumor mass are under the control of hypoxia-inducible factor -1α (HIF-1α). The stabilization and transcriptional activity of HIF-1α is controlled by two oxygen-dependent dioxygenases, namely, prolyl hydroxylase-2 (PHD-2) and factor inhibiting hypoxia-inducible factor-1 (FIH-1), through the N-terminal and C-terminal domains, respectively (Qutub and Popel, 2006; Brahimi-Horn and Pouysségur, 2007; Hu et al., 2007; Koh and Powis, 2012; Akanji et al., 2019).It would be appropriate to mention that previous studies have pointed out differential regulation of HIF-1α by NTAD and CTAD (Dayan et al., 2009); only CTAD is reported to directly bind with the co-activators CBP/P300 through the CH-1 region and regulate HIF-1α transcriptional activity. In contrast to CTAD, the mechanism that regulates the transcriptional function of NTAD is poorly understood. Moreover, it has also been reported that NTAD is responsible for gene specificity and regulates proteasomal degradation rather than transcriptional activation of HIF-1α (Dayan et al., 2006; Hu et al., 2007).

Studies have shown that as the oxygen gradient in solid tumors falls from 40 mmHg pO2 (5% $O_2$) in the non-hypoxic zone to 10 mmHg $pO_2$ (1% $O_2$) in the intermittent hypoxic (OXOPHOS) zone, PHD-2 loses its activity, whereas FIH-1 is inhibited only under severely hypoxic conditions <10 mmHg $pO_2$ (1% $O_2$) (Dayan et al., 2006; 2009). Thus, in the non-hypoxic zone where PHD-2 and FIH-1 are functional, HIF-1α is degraded and transcriptionally inactivated. In contrast, in the OXOPHOS zone, HIF-1α is stabilized due to the inactivation of PHDs but remains transcriptionally inactive due to the presence of FIH. In the hypoxic zone, HIF-1α is both stable and transcriptionally active due to the inactivation of both PHDs and FIH-1. As the $O_2$ concentration diminishes in the intermittent hypoxic and severely hypoxic zone during tumor development, HIF-1α becomes stabilized and progressively becomes transcriptionally active due to the inactivation of PHD first and FIH-1 second. In such cases, angiogenesis, glycolysis, fatty acid synthesis, migration, metastasis, malignant cell survival, and proliferation are regulated in non-hypoxic and intermittent hypoxic zones to support tumor progression. There is an intriguing possibility that during the early stage of tumor initiation and progression when oxygen tension is limiting for transcriptional activity HIF- 1α, other mechanisms could be responsible for regulating malignant cell survival and proliferation.

Research over the past decade has pointed out that NF-κB is a driver of inflammation that gives rise to cancer under a chronic inflammatory microenvironment (Pikarsky et al., 2004). Activation of NF-κB in both premalignant cells and cells of the microenvironment (phagocytes, T cells, and B cells) is crucial in the early stages of tumor development and progression (Wang et al., 2014). NF-κB is activated firstly in cells of tumor microenvironment in response to binding of pathogen-associated microbial patterns (PAMPs) and danger-associated molecular patterns (DAMPs) with toll-like receptors (TLRs). This association eventually activates the IKK complex, leading to transcriptional activation of NF-κB in cells of the microenvironment. In response to NF-κB actuation, cells in the tumor microenvironment secrete proinflammatory mediators, such as TNF-α and IL-1. These proinflammatory mediators act on their specific receptors present on premalignant cells and further lead to the induction of NF-κB in premalignant cells through the IKKβ-mediated canonical NF-κB signaling pathway (Martins et al., 2016). This canonical stimulation of NF-κB induces genes involved in cellular proliferation, survival, angiogenesis, and metastasis.

In this study, we propose to determine the possible role of lactate shuttle in the activation of NF-κB in non-hypoxic and intermittent hypoxic malignant cells. We have also directed our study to answer few specific questions.

a) How, NF-kappa B and HIF-1α play a crucial role in tumorigenesis and progression.
b) To what extent does factor inhibiting HIF-1α (FIH1) continue to function while Prolylhydroxylase-2 (PHD-2) activity is suppressed by the lactate shuttle in non-hypoxic and intermittently hypoxic cells?
c) Under normoxia, NF-κB is activated at the expense of HIF-1α due to the downregulation of PHD2 mediated by the lactate shuttle.
d) In the early stages of carcinogenesis, when oxygen tension is normal, HIF-1α is inactive. How can NF-κB promote tumour initiation and survival during this time?
e) PHD2 mediates NF-κB and HIF-1α double regulation, suggesting it may be a useful novel pharmacological target.

## 2 How inflammation contributes to the formation of neoplasm?

### 2.1 Inflammation

Inflammation is the first line of the body's defense mechanism that protects against infection and injury. Inflammation is defined by a sequence of responses involving vasodilation, migration of immune cells, and leakage of plasma proteins at the site of disease or injury. Phagocytic cells that arrive at the site of inflammation, especially neutrophils, macrophages, and dendritic cells, express pattern recognition receptors called "Toll-Like Receptors" (TLR's). The binding of inflammatory factors (such as cytokines, chemokines, PAMPs, and DAMPs) with TLRs triggers a signaling cascade that leads to the induction of NF-κB (Freedman et al., 2002; Tolle and Standiford, 2013; Chen et al., 2018). NF-κB is a primary transcriptional regulator of inflammation and regulates tissue repair and wound healing (Landén et al., 2016).

The inflammatory response to an infection or injury usually subsides after tissue repair; however, an exaggerated inflammatory response results in a condition known as chronic inflammation. During chronic inflammation, leaky vasculature and increased ATP requirement cause hypoxia. Under such a hypoxic inflammatory microenvironment, infiltrating immune cells at the site of infection or injury produce reactive oxygen species (ROS), which causes damage to the DNA by altering gene expression and genetic sequences. The accumulation of ROS and cytokines further elevates NF-κB activity to create a pro-tumorigenic

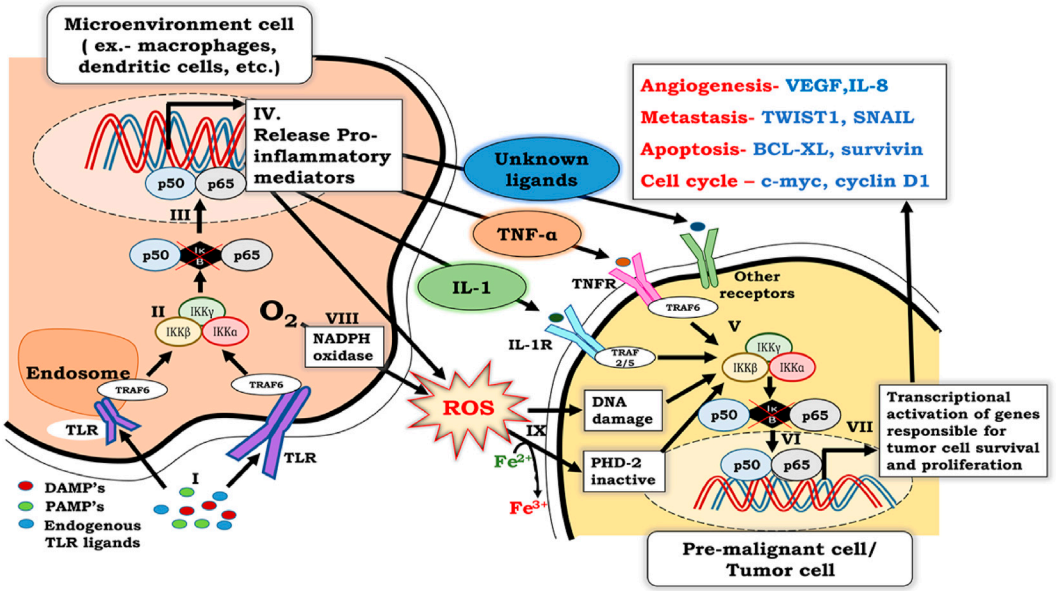



FIGURE 1
Crosstalk between NF-κB signaling in inflammatory cells and premalignant cells. I. NF-κB activation in inflammatory cells involves binding of PAMPs, DAMPs, and endogenous TLR ligands with TLR receptors, which are associated with TRAF-6. II. Binding of PAMPs, DAMPs, and endogenous TLR ligands with TLR's leads to activation of the IKKαβγ complex, which further results in phosphorylation, ubiquitination, and degradation of IκB. III. Degradation of IκB renders p50 and p65 heterodimers free to migrate to the nucleus and activate NF-κB. IV. Activation of NF-κB in inflammatory cells results in the secretion of pro-inflammatory cytokines, including TNF-α and IL- 1. V. Released pro-inflammatory cytokines bind to their specific receptors on pre-malignant cells and activate the IKKαβγ complex, which further results in phosphorylation, ubiquitination, and degradation of IκB. VI. Degradation of IκB renders p50 and p65 heterodimer free to migrate to the nucleus and activates the transcription of genes responsible for cell survival, proliferation, migration, metastasis, and angiogenesis. VII. ROS generated by phagocytic NADPH to counter infectious pathogens, along with the ROS generated by activation of NF-κB in inflammatory cells, enters the cytosol. VIII. Oxidative stress caused by large amounts of ROS not only damages DNA, but also ionizes $Fe^{2+}$ to $Fe^{3+}$. As a result, PHD-2, which requires $Fe^{2+}$ as a cofactor for its activity, is inactivated. ROS-induced DNA damage and PHD-2 inactivation further contribute to NF-κB activation in pre-malignant cells.

microenvironment that favors tumor initiation and development (Liou and Storz, 2010). Thus, the NF-κB pathway mediates the crosstalk between chronic inflammation and cancer, in which early transmuted malignant cells proliferate and form tiny tumors (Ben-Neriah and Karin, 2011).

## 2.2 NF-κB regulatory machinery

NF-κB has been reported to govern the expression of the immunoglobulin κ-light chain in B lymphocytes. Later, NF-κB was recorded as a group of transcription regulators consisting of NF-κBp65 (RelA), RelB, Rel-c, (p50/p105), and (p52/p100) subunits (Sen and Baltimore, 1986). These transcription factors comprise a preserved Rel homology domain (RHD) that facilitates them to undergo homo or hetero-dimerization (Perkins, 2012).The major heterodimer of NF-κB is p65/50, which remains segregated in the cytoplasm and tightly clubbed with an inhibitor of kappa B (IκB) family proteins (comprising IκBα, IκBβ, and IκBε) along with two precursors, that is, p105/IκBγ and p100/IκBδ) (Ghosh et al., 1995). The binding of NF-κB dimers to IκBs with the help of 7–8 ankyrin repeats (Malek et al., 2003) prevents nuclear translocation of NF-κB dimers and consequently inhibits their transcriptional activity (Marienfeld et al., 2003).

Pathways regulating NF-κB transcriptional activity include the canonical pathway (or classical pathway) and the alternative pathway (or the non-canonical pathway) (Sun, 2011; Sun et al., 2013; Dorrington and Fraser, 2019). The key regulator of these pathways is a cytoplasmic complex known as the "IKK complex." The IKK complex is composed of the catalytic subunits IKKα (or IKK1) and IKKβ (or IKK2) along with the modulatory subunit IKKγ (or NEMO). Both IKKα and IKKβ share 52% sequence chronology and 70% homology but play distinct yet pivotal roles in pan NF-κB regulation (Liu et al., 2012). NEMO/IKKγ has an N-terminal coiled-coil domain that interacts with IKKα and IKKβ and mainly functions as a regulatory subunit in the IKK complex. IKKα and IKKβ are essential regulators of IκBs, and their activation is necessary for all NF-κB signaling pathways, whether classical or alternative (Oeckinghaus et al., 2011). Release of NF-κBp65 dimer from the inhibitor of kappa B (IκB) requires phosphorylation of IKKα or IKKβ at specific serine residues in the activation loop, that is, serine-176(S-176) and serine-180(S-180) for IKKα and serine-177(S-177), and serine-181(S-181) for IKKβ. Once activated, IKKα or IKKβ, in turn, phosphorylates inhibitor of kappa B (IκBs), further leading to β-TrCP-dependent E3 ubiquitin ligase-mediated 26s-proteasomal degradation of inhibitor of kappa B (IκBs). Henceforth, the NF-κB dimer can migrate from the

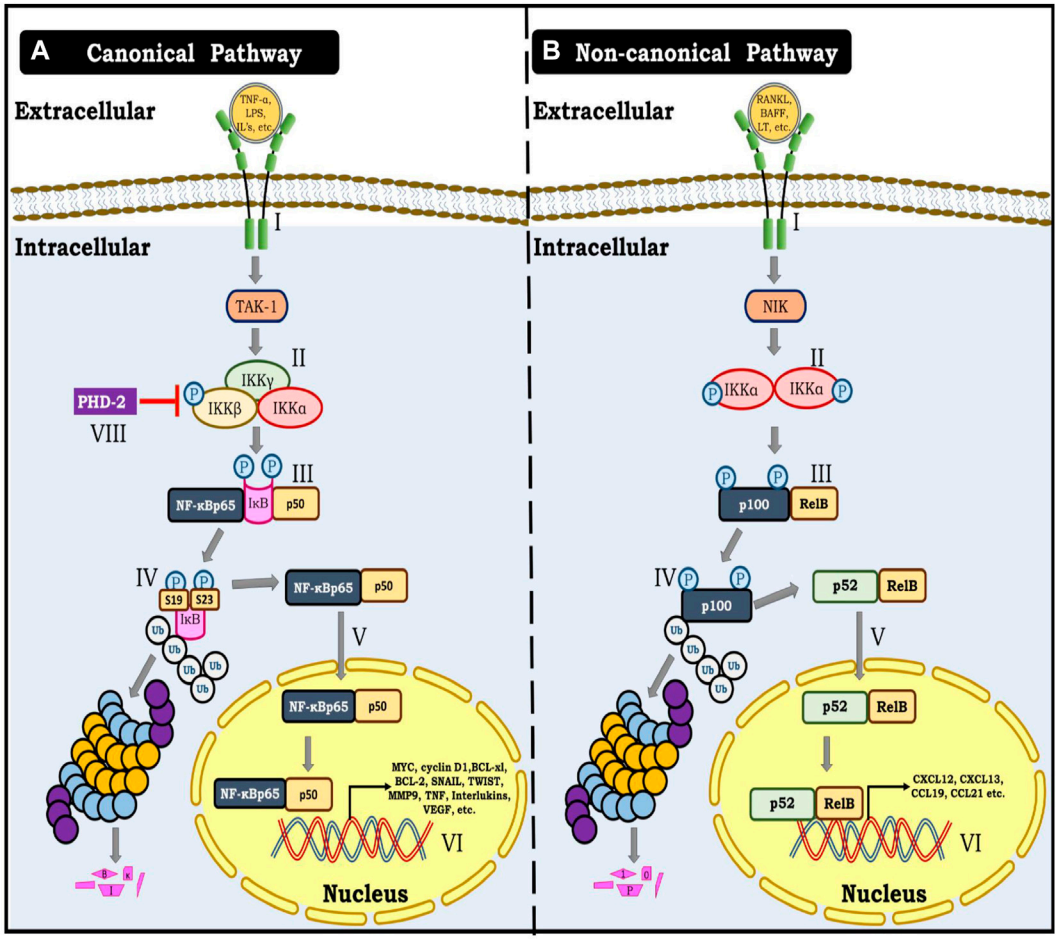



FIGURE 2
**(A)** Canonical NF-κB pathway. I- Represent binding of TNF-α, Interleukins, Lipopolysaccharides and other cytokines with their specific receptor resulting in activation of TAK kinase-1. II- Activation of TAK-1 result in phosphorylation of IKKB unit of the triad consisting of IKKα, IKKβ and IKKγ. III- Phosphorylation of IKKB further result in phosphorylation of IκB at Ser19 and Ser23. IV- This phosphorylation allows for ubiquitination and proteasomal degradation of IκB. V- This modification renders NF-κB dimer (p65 and p50) free to translocate to the nucleus. VI- NF-κB dimer (p65 and p50) further binds with specific response elements on DNA and triggers gene transcription of several genes involved in angiogenesis, metastasis, migration and cellular proliferation. **(B)** Non- canonical NF-κB pathway. I- denotes the binding of TNFR ligands to their respective receptors, which results in the activation of NF-κB inducing kinase-1 (NIK). II- NIK phosphorylates IKKα, causing it to be activated. III- Finally, activated IKKα phosphorylates p100. IV- IKKα mediated phosphorylation of p100 marks it for ubiquitination and proteasomal processing to yield p52. V-p52 forms an active dimer with Rel B and translocates to the nucleus. VI- p52 and Rel B dimer bind to the DNA and cause gene transcription.

cytoplasmic space to the nucleus, triggering gene expression (Gilmore, 2006; Dorrington and Fraser, 2019).

### 2.2.1 IκB kinase (IKK) function

The pivotal step in NF-κB induction is cytokine-inducible phosphorylation specific for amino-terminal regulatory serines at Ser32 and Ser 36 of IκBα or at Ser19 and Ser 23 in IκBβ. Even though various enzymes mediate phosphorylation of IκB, only IKK meets the acrostics of IκBα/β degradation, which includes fast signal induction and concurrent phosphorylation of the pair of serine residues, Ser32 together with Ser 36 of IκBα and Ser19 along with Ser 23 in IκBβ, respectively (Gilmore, 2006).

Another essential characteristic of IKK-mediated phosphorylation is the choice of serine as a target for phosphorylation over thionine, which matches IκBα/β degradation. As discussed previously, IKKα and IKKβ are catalytic subunits of the IKK complex, which are stimulated in cells in response to TNF-α and IL-1. Both subunits possess an activation loop in their kinase domains, similar to other protein kinases, along with a sequence homology between both kinases (Johnson et al., 1996; Mercurio et al., 1997). The activation loop contains specific sites on IKKα (S176, S180) and IKKβ (S177, S181), the phosphorylation of which results in a conformational change that is responsible for kinase activation (Ling et al., 1998). Moreover, replacing serine with alanine inhibits the activation of IKK, although a similar substitution with glutamic acid imitates activity equal to phosphoserine. It would be appropriate to mention that modification in S176 and S180 of IKKα to alanine, either on one or both of the serine residues, does not have any effect on TNF-α- and IL-1 mediated IKK activity (Mercurio et al., 1997; Delhase, 1999). This finding suggests that, in the case of proinflammatory stimuli, phosphorylation of IKKα is not necessary for stimulation of

the IKK complex. Research has also indicated that similar modifications of S177 and S181 in IKKβ to alanine abolished TNF-α-and IL-1 mediated IKK activity. Thus, activation of the IKK complex by proinflammatory stimuli depends entirely on the phosphorylation of IKKβ and not on IKKα.

### 2.2.2 The canonical pathway (classical pathway)

The canonical pathway is activated by the binding of TNF-α, interleukins, and chemokines to their specific receptors on cell membranes, which starts the IKK complex. The exact mechanism and the proteins involved in the activation of this complex are under investigation. However, stimulation by proinflammatory cytokines such as Ilβ1, TNF-α, and TLR ligands in the case of toll-like receptors (TLR) in macrophages and mouse embryonic fibroblasts (MEFs), triggers TGF-β-activated kinase 1 (TAK-1)-mediated phosphorylation of IKKβ at S177, which subsequently catalyzes autophosphorylation at S181, resulting in activation of the IKKβ complex. Furthermore, this activated cytoplasmic complex phosphorylates IκBα at serine residues S32 and S36, whereas for IκBβ at serine residues S19 and S23. This phosphorylation primes IκBs for ubiquitination by the (SCF)/(β-TCP) E3 ubiquitin ligase complex, followed by proteasomal degradation, which facilitates the release of NF-κB dimers. NF-κB dimers, released, are free to migrate to the nucleus and trigger transcription of genes involved in various cellular functions and bioenergetics, including cellular proliferation and metabolism (Gilmore, 2006; Oeckinghaus et al., 2011) (Figure 2).

### 2.2.3 The non-canonical or alternative pathway

Unlike the canonical NF-KB pathway, which is fast and activated by a wide range of inflammatory cytokines and other external factors, the non-canonical pathway is slow and only activated by a small subset of TNF-superfamily receptors, such as LTβR, BAFFR, CD40, CD30, CD27, RANK, TNFR2, FN14, and CD134. Henceforth, the biological functions of this pathway are more specialized and specifically associated with lymphoid organ and immune cell development, as well as immunological response and homeostasis (Sun, 2017).

Briefly, the non-canonical NF-κB pathway is activated by NF-κB inducing kinase (NIK). In the inactive state, NIK remains bound to TRAF3 which is complexed with TRAF2 and cIAP1/2. Further, cIAP1/2 ubiquitinates NIK, marking it for proteasomal degradation. However, binding of TNFR ligands (such as BAFF, RANKL, LTα1β2, TWEAK, etc.) with their specific receptors, TRAF3, TRAF2, and cIAP1/2 triad complex is recruited to the membrane binding site of the receptor-ligand complex, leaving NIK unbound. Moreover, cIAP1/2, instead of ubiquitinating NIK now ubiquitinates TRAF3 and marks it for proteasomal degradation which further facilitates the stabilization of NIK. As a result, NIK starts accumulating, and its level inside the cell increases. Consequently, NIK phosphorylates and induces IKKα homodimer. Activated IKKα in turn phosphorylates C-terminal serine residues (866 and 870) of the p100 subunit of inactive RelB and p100 heterodimer. The subsequent transduction mechanism involves p100 ubiquitination by the (SCF)/(β-TCP) E3 ubiquitin ligase complex followed by proteasomal processing to yield a p52 subunit. This event is followed by the association of p52 with RelB to form an active dimer which translocates to the nucleus and triggers gene transcription (Figure 2) (Sun, 2011).

Solid tumors primarily exhibit canonical signaling, whereas hematological cancers primarily exhibit non-canonical signaling (Kaltschmidt et al., 2018). Therefore, canonical signaling is the main subject of this review.

## 2.3 Biological effects of NF-κB in cancer cells

### 2.3.1 Effect of NF-κB on cell cycle

NF-κB is the critical modulator of the tumor microenvironment in the early stages of tumor development. NF-κB is integral to tumorigenesis, involving tumor initiation (formation of transformed cells), tumor promotion (early transformed cells proliferate rapidly and increase in both size and number), and tumor progression (transformed cells acquire malignant potential) (Pitot et al., 1981; Barcellos-Hoff et al., 2013). It takes approximately three to five oncogenic mutations for a normal cell to become malignant. This is because most oncogenic mutations are acquired *de novo*, and rarely, germline mutations result in cancer (Fearon and Vogelstein, 1990; Grivennikov et al., 2010).

Under non-hypoxic or intermittent hypoxic conditions, wherein HIF-1α is dormant, NF-κB initiates tumor growth by enhancing reactive nitrogen species and ROS generation that cause damage and impart oncogenic characteristics. Moreover, NF-κB activation results in cell cycle alterations and ensures that both DNA strands acquire mutations and transfer to progenitor cells (Ledoux and Perkins, 2014; Kiraly et al., 2015). NF-κB may also cause aneuploidy and epigenetic changes, resulting in tumorigenesis (Ren et al., 2011; Nakshatri et al., 2015). Further, by inhibiting p53 induced cell death, NF-κB activation favors the malignant transformation of DNA damaged cells (Gudkov et al., 2011). Henceforth, in solid tumors, NF-κB regulates the transformation of normal cells to premalignant cells and other premalignant cells to malignant cells by inhibiting p53 induced programmed cell death and enhancing the production of ROS and reactive nitrogen species.

### 2.3.2 Effect of NF-κB on programmed cell death

NF-κB suppresses apoptosis and enhances cellular proliferation in various cancer pathologies, including B-cell lymphoma, neuroblastoma, and breast carcinoma (Smith et al., 2014; Zhi et al., 2014; Knies et al., 2015). A common determinant among various tumors is the intrinsic NF-κB activity that confabs intransigence to cell death by up-regulating anti-apoptotic genes. Moreover, in the tumor microenvironment, NF-κB operates in a paracrine fashion to expedite tumor cell proliferation (Greten et al., 2004; Bassères and Baldwin, 2006). To repress apoptosis, NF-κB activates a group of targeted genotypes that impede distinct steps of the extrinsic and intrinsic apoptotic pathways. The targets of NF-κB-mediated suppression of apoptosis include inhibitors of apoptotic proteins such as XIAP, c-IAP1, and c-IAP2. It is relevant to mention that XIAP, c-IAP1, and c-IAP2 are involved in the inhibition of pro-caspase-9 and blockade of caspase-3 and caspase-7 activity. Among other targeted genes, BCL-2, BCL-XL, and NR13, which belong to the BCL-2 family (Liston et al., 2003; Fulda, 2014). Therefore,

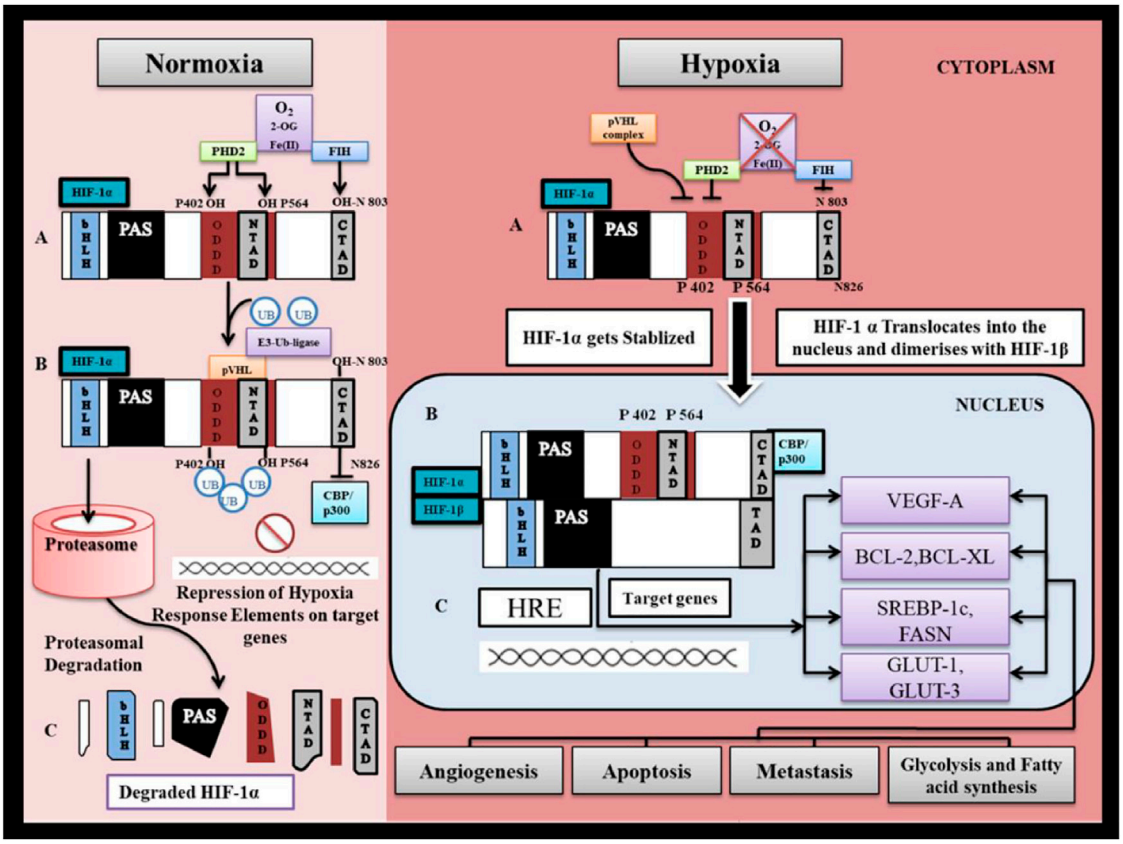



FIGURE 3
HIF-1α regulation under normoxia and hypoxia. I. Normoxia represents $pO_2$ > 40 mm Hg. (A) Under normal oxygen tension, PHD2 (prolylhydroxylase-2) hydroxylates HIF-1α at proline residues P402 and P564 and Factor Inhibiting HIF-1α hydroxylates HIF-1α at asparagine residue N803. in presence of O2 as substrate, Oxoglutarate as co-substrate and Fe as cofactor. (B) Prolyl Hydroxylation of HIF-1α allows for binding of pVHL to NTAD ( N-terminal transactivation domain) of HIF-1α. Binding of pVHL recruits ubiquitinin by activation of E3- ubiquitinin ligase which marks HIF-1α for proteasomal degradation. (C) HIF-1α is degraded by 26S proteasome thus preventing stabilization and accumulation of HIF-1α. Asparginyl hydroxylation of HIF-1α at CTAD (C-terminal transactivation domain) prevents binding of CBP/P300 to the CTAD (CH- 1 domain) and thereby inhibiting downstream mechanism involved in HIF-1α transactivation. II. Hypoxia represents $pO_2$ < 40 mmHg. (A) Under hypoxia HIF-1α escapes degradation and gets stabilized since PHD2 enzymes which are responsible for its proteasomal degradation are inhibited. (B) Stabilized HIF-1α gets accumulated in the cytosol till the oxygen tension reaches the level ($pO_2$> 10 mmHg) that it becomes limiting for FIH-1, such that CBP/P300 proteins are able to bind to CTAD. (C) HIF-1α translocate into the nucleus to bind with β-subunit that is constitutively expressed in the nucleus. HIF-1α further binds with hypoxia response element on targeted gene and trigger gene transcription.

NF-κB has a pivotal role in tumor cell survival by activating IAP's and BCL-2 family proteins. Therefore, by activation of inhibitors of apoptotic proteins and BCL-2 family proteins, NF-κB plays a pivotal role in malignant cell survival in solid tumors.

### 2.3.3 Effect of NF-κB on angiogenesis

Inflammation, as well as in an NF-κB dependent manner, stimulates angiogenesis. Research has shown that constitutive NF-κB activity regulates IL-8 and VEGF activity in cancer cells (Huang et al., 2000; Bonavia et al., 2012). Furthermore, NF-κB, in conjunction with iNOS, stimulates the production of proteases and NO, which has a pivotal function in inflammation-induced angiogenesis (Zhang et al., 2005; Costa et al., 2007). In addition, NF-κB-targeted genes such as fibroblast growth factor, IL-8, matrix metalloproteinase-9 (MMP-9), and others are involved in various steps in the regulation of angiogenesis (Zhang et al., 2005). It is pertinent to mention that MMP-2, 3, and 9 play an integral role in the breakdown of the basement membrane as well as in remodeling of the extracellular matrix, which not only facilitates cell migration but also favors either angiogenesis (endothelial cells) or metastasis (malignant cells) depending on the microenvironment (Huber et al., 2004). Furthermore, in the tumor microenvironment, cancer-associated fibroblasts (CAFs) facilitate the deposition of collagen and another extracellular matrix (ECM) components. CAFs are activated partially in an NF-κB-dependent manner and are vital in the actuation of proinflammatory genes that regulate TNF-α, interleukin (IL-1β and IL-6, VEGF, CXCL2, SDF-1, and many other chemokines, thereby enhancing angiogenesis (Disis, 2010; Erez et al., 2010; Kalluri, 2016). The above findings suggest that NF-κB, through stimulation of iNOS, cytokines, chemokines, and CAF in the tumor microenvironment, facilitates new vessel formation to support budding tumors.

### 2.3.4 NF-κB in invasion and metastasis

The key factor contributing to invasion in the case of solid tumors is acidosis. To survive under non-hypoxic and intermittent

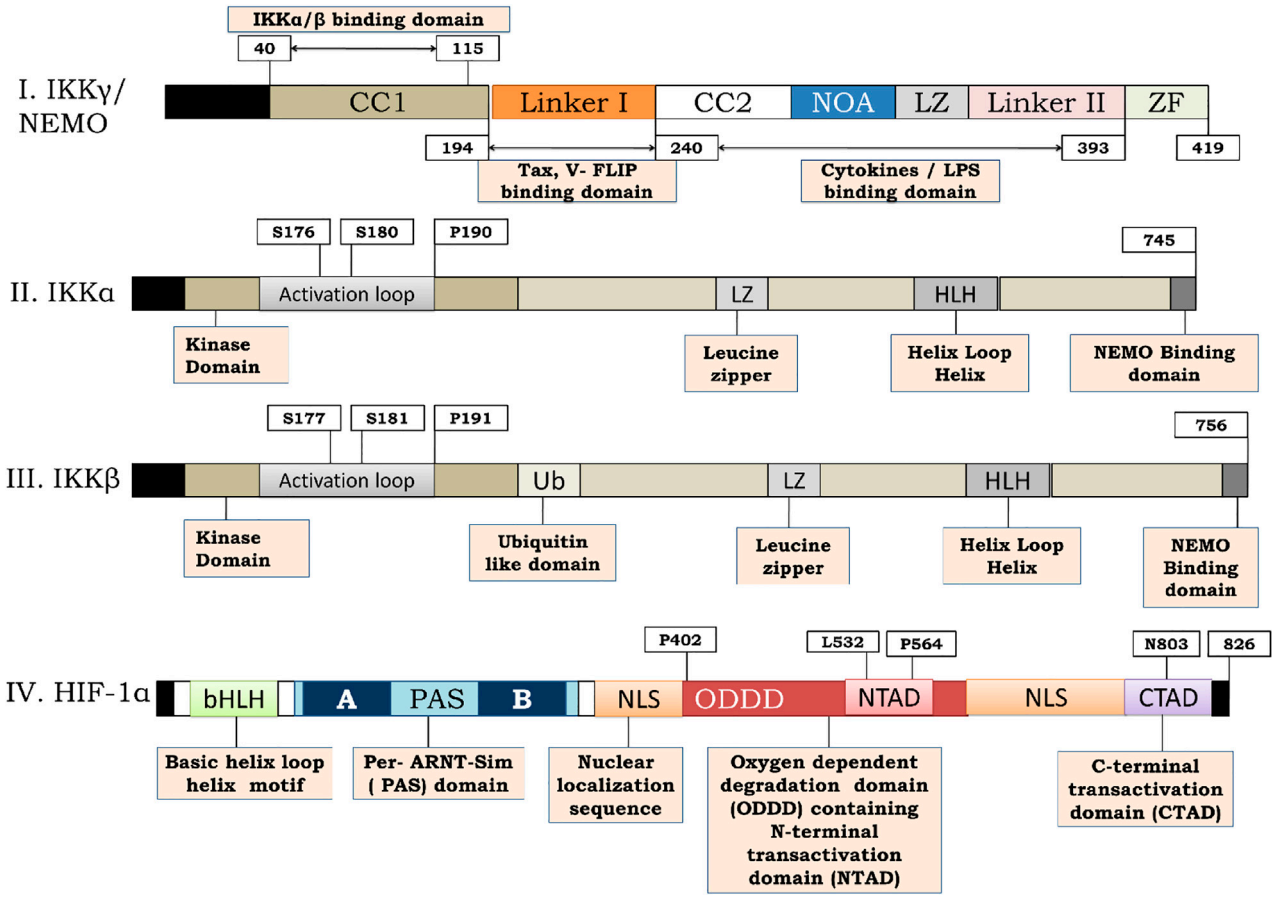



FIGURE 4
Structure of IKKγ, IKKα, IKKβ and HIF-1α. **(I)** Represents the structure of IKKγ or NEMO. IKKγ is a 419 amino acid dimeric fragment consisting of a series of parallel intermolecular coiled coil domains (represented as CC1 and CC2). The amino acid terminus (N-terminus) is vital for interaction with IKKα and IKKβ. Linker 1 serves for interaction with viral trans activators like HTLV-1 and Tax and V-Flip. C-terminus function for signal transmission while NOA and Zinc Finger (ZF) domains bind to polyubiquitin chains. **(II,III)** represents the structure of IKKα and IKKβ respectively. IKKα is a 745 amino acid fragment consisting of an activation loop from amino acid 176–180 whereas IKKβ is a 756 amino acid fragment consisting of activation loop from 177–181 on amino terminous. A ubiquitin like domain is present (from amino acid 307–384) on carboxy terminous of IKKβ but not on IKKα. The function of leucine zipper domain is to allow homo and heterodimerization of the kinases. The function of helix loop helix is less clear but it seems to be involved in the modulation of kinase activity. A 40 amino acid region at the extreme carboxy terminous of the kinases (AA-705–743) is required for their interaction with NEMO. **(IV)**- represents the structure of HIF-1α. HIF-1α is a 826 amino acid sequence consisting of basic helix loop helix motif and a PAS domain. The sequence possess a N-terminal trans activation domain (N-TAD) on Oxygen dependent degradation domain (ODDD) of HIF-1α along with C-terminal transactivation. The HIF-1α consists of prolyl hydroxylation site at Pro402 and P4 as w ro56ell as pVHL binding site at Leu532 respectively on that lie on ODDD. The CTAD of HIF-1α consists of Asparginyl hydroxylation site at N803.

hypoxic conditions malignant cells depend upon glycolysis despite the presence of O2, a phenomenon called the "Warburg Effect" (Warburg, 1956; Chandel, 2021). Whereas, under hypoxic conditions malignant cells utilize anaerobic glycolysis for energy production, a phenomenon called the "Pasteur Effect" (Kim et al., 2006; Milane et al., 2011). However, the end product of glycolysis, in both the cases, is lactic acid, resulting in the accumulation of lactic acid inside the cell (Gatenby et al., 2007). To survive, efflux of lactic acid from the cell by MCT-4 is necessary. The resultant increase in acidity and decrease in pH of the extracellular environment generates ROS and facilitates the activation of NF-κB (Gatenby and Gillies, 2004; Gupta et al., 2014).

NF-κB can directly trigger the expression of genes that stimulate epithelial to mesenchymal transition (EMT) and offer invasive characteristics to malignant cells, such as TWIST1, SLUG, and SNAIL (Wu and Zhou, 2009; Pires et al., 2017). Stimulation of these genes initiates neoangiogenesis and promotes EMT-induced cancer cell extravasation into the blood and lymphatic vessels. Therefore, preparing a protective pre-metastatic niche that allows for the survival and proliferation of metastatic initiating tumor cells (Psaila and Lyden, 2009).

Other mechanisms by which NF-κB promotes migration and metastasis is through upregulation of proto-oncogenes such as c-myc and cyclin D1 (You et al., 2002; Ledoux and Perkins, 2014), regulating selectins and integrins, which are essential players in invasion and colonization at distal sites (Collins et al., 1995; Nguyen et al., 2009), and increasing cell surface expression of the chemokine receptor CXCR4, thereby promoting metastasis and invasion (Helbig et al., 2003). From the above discussion, it is clear that lactic acidosis is a prominent regulator of NF-κB in solid

tumors, which provides cells with invasive potential and protects them with a pre-metastatic niche.

Till now, we have seen that hypoxia arising from an exaggerated immune response to inflammation activates NF-κB, which regulates downstream mechanisms of tumor initiation and development. Now, as the tumor size keeps on increasing, as it increases more than 400 μm, cells at the center of the tumor are under severely hypoxic conditions. To survive under such a hostile environment, these cells take the help of another transcription regulator that we will see in the following section of this manuscript.

# 3 How do tumor cells adapt under hypoxia?

## 3.1 Hypoxia

Perturbation in oxygen supply results in a condition termed hypoxia, characterized by reduced $pO_2$ in a particular tissue compared to $pO_2$ in well-vasculature tissues (Semenza, 2014). Tumor hypoxia results from either reduced oxygen delivery due to occlusion or leakage of blood vessels or increased oxygen consumption, which may be attributed to rapid cellular division (Vaupel et al., 2004).

## 3.2 Mechanistic regulation of HIF-1α

One of the significant factors contributing to the adaptation of malignant cells to the hypoxic tumor environment is the hypoxia-inducible factor (HIF) (Vaupel et al., 2004; Semenza, 2012). The master transcriptional regulator HIF belongs to a group of two bHLH domain-containing proteins from the PAS (PER-ARNT-SIM) family (Wang et al., 1995), with three isoforms: HIF-1, 2, and 3 (Wang and Semenza, 1995; Gu et al., 1998; Keith et al., 2012). Each isoform of HIF has two subunits, α, and β. The α-subunit is present in the cytoplasm and is regulated by hypoxia. The aryl hydrocarbon receptor nuclear translocator (ARNT), commonly referred to as the β-subunit, is integrally expressed in the nucleus and is induced by dimerization with the α-subunit (Jiang et al., 1996; Déry et al., 2005). HIF-1α acts as an oxygen sensor and is regulated by two sets of enzymes belonging to a class of 2- oxoglutarate, ascorbate, and iron-dependent dioxygenases called prolyl hydroxylases (PHDs) and factor inhibiting hypoxia-inducible factor-1 (FIH-1). Prolyl hydroxylase exists in three isoforms: PHD-1, 2, and 3. Among all PHDs, PHD-2 is the most prominent regulator of HIF-1α in solid tumors (Kaelin and Ratcliffe, 2008)].

Under non-hypoxic conditions ($pO_2$ > 40 mmHg) (Figures 3), HIF-1α undergoes proteasomal degradation by PHD-2 *via* a process that involves post-translational hydroxylation of HIF-1α at proline residues (P402 and P564) in the human sequence present in O2 dependent degradation (ODD) domain (Bruick, 2001; Schofield and Ratcliffe, 2005). Hydroxylation eventually allows the binding of von Hippel-Lindau tumor suppressor protein (pVHL) and recruitment of E3-ubiquitin ligase that combine to form the E3-ubiquitin ligase complex, which ultimately marks HIF-1α for degradation by the 26-S proteasome (Maxwell et al., 1999; Kaelin, 2003). On the other hand, FIH hydroxylates HIF-1α at an asparagine residue (N803) that abrogates the binding of histone acetyltransferases such as P300 and CBP (CREB binding protein (CBP) to the C-terminal transactivation domain (CTAD) on HIF-1α and blocks the transactivation of genes regulated by HIF-1α (Lando et al., 2002; McNeill et al., 2002).

Under hypoxic conditions such as ischemia and cancer, as the oxygen tension ($pO_2$) reaches below 40 mmHg, PHD2 is inactivated, followed by FIH (at $pO_2$ < 10 mmHg). In the $pO_2$ range of 40 and 10 mmHg, due to inactive PHD-2, HIF-1α escapes proteasomal degradation and subsequently stabilizes, and its cytoplasmic level increases. However, at this stage, the transcriptional function of HIF-1α is inhibited by FIH-1 activity (Koivunen et al., 2004; Stolze et al., 2004; Rani et al., 2022). As $pO_2$ deteriorates further and reaches below <10 mmHg, FIH loses its functional activity (Dayan et al., 2006). After that, cytoplasmic HIF-1α migrates to the nucleus, where it forms a dimer with HIF-1β. The HIF-1αβ dimer further binds with co-factor CBP/P300 to interact with HIF-1α responsive element "5′-RCGTG-3′" on target genes and triggers gene transcription (Semenza et al., 1996).

Apart from HIF-1α, hypoxia also activates NF-κB through phosphorylation of IKKβ (Koong et al., 1994). Moreover, evidence suggests that only canonical NF-κB signaling is oxygen-sensitive (Oliver et al., 2009; Taylor and Cummins, 2009). Interestingly, both HIF-1α and IKKβ were found to possess a highly conserved LxxLAP motif (Figure 4; Supplementary Table S1), which contains sites for prolyl hydroxylation. Research has shown that PHD-2 activity is necessary for the downregulation of NF-κB activity (Cummins et al., 2006; Takeda et al., 2011). Therefore, PHD-2 is a vital regulator of the oxygen sensing mechanism because it regulates the activity of both transcriptional factors important in regulating cellular responses to hypoxia. It is pertinent to mention that FIH-1 also hydroxylate various members of the NF-κB pathway, such as p105 and IκBα, but its significance on NF-κB remains elusive (Cockman et al., 2006).

## 3.3 Biological effects of HIF-1α in tumor cell

### 3.3.1 HIF-1α and glucose metabolism

HIF-1α was initially recognized as a transcriptional activator of erythropoietin gene (EPO), was found to upregulate the expression of genes encoding glycolytic enzymes and glycolytic flux (GLUT1 and GLUT3), indicating that HIF-1α is an essential factor contributing to the "Warburg effect" (Samanta and Semenza, 2018).

First, it seems that OXOPHOS inhibition results due to a lack of oxygen, but this is not the case. HIF-1α achieves maximum stability at 1% $O_2$ due to inactivation of FIH, but OXOPHOS can occur at even lower $O_2$ concentrations (0.1%–0.7%) (Kennedy and Jones, 1986; Wilson et al., 1998; Stolze et al., 2004). This provides sufficient evidence that HIF-1α is stable well before $O_2$ becomes limiting for OXPHOS. Therefore, HIF-1α has an instrumental role in the transfer of energy production from OXOPHOS to aerobic glycolysis.

It is well established that HIF-1α is the key to the production of nucleotides, amino acids, fatty acids, lipids, and glycogen for the synthesis of cellular components in hypoxic microenvironments. In such cases, glucose is required to maintain cell energetics and provide biosynthetic intermediates such as ribose-5-phosphate,

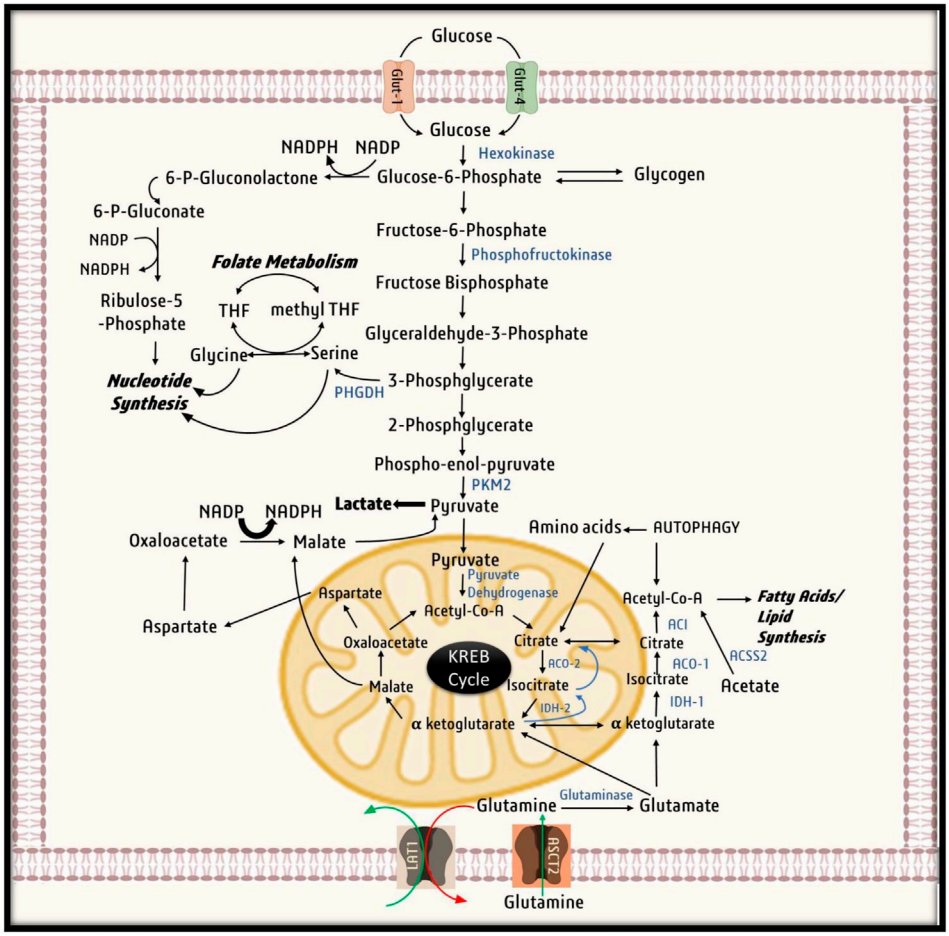



FIGURE 5
Utilization of Glucose by cancer cells. Cancer cell uptake Glucose inside the cell with the help of glucose transporters (GLUTs). Glucose then undergoes glycolysis, in first step of glycolysis, glucose is converted to Glucose-6-phosphate by Hexokinase, that is utilized by cancer cells for nucleotide synthesis and production of NADPH by PPP shunt. When in abundance glucose-6-phosphate is stored in form of glycogen. Glucose -6-phosphate then gets converted to 3-phosphoglycerate to provide for nucleotide synthesis through folate metabolism. Ultimately in the last step of glycolysis, phospho-enol-pyruvate is converted to pyruvate. Due to Warburg effect pyruvate is converted to lactate and excreted out of the cell. Under mild hypoxic conditions, pyruvate derived from malate aspartate shuttle and exogenous lactate as well as oxidative metabolism of glutamine runs the Krebs cycle to generate ATP. On the other hand, under severe hypoxia due to activation pyruvate kinase-1, pyruvate does not undergo conversion to citrate instead utilized for other vital process of cell survival. While citrate is aerobically generated from glutamine by conversion of glutamine to glutamate and ultimately to α-ketoglutarate. α-ketoglutarate is further converted by Isocitrate dehydrogenase-2 to isocitrate and then citrate by acoitase-2. Ultimately, citrate is converted to acetylcoA for fatty acid synthesis.

one carbon for nucleotide synthesis, and amino acids for protein synthesis resulting in an upsurge in demand for glucose. To counter such glucose crises, HIF-1α adapts to several mechanisms (Figure 5). Research has shown that malignant cells can compensate for the loss of glucose by utilizing intracellular glycogen for their survival and proliferation. Moreover, cancer cells can increase their glycogen accumulation by inducing enzyme glycogen synthase, which serves as a glucose reserve for the pentose phosphate pathway (Pescador et al., 2010). It has also been shown that HIF-1α upregulates the expression of glycogen phosphorylase, an enzyme required for glycogen metabolism. Research indicates that cancer cells target glycogen metabolism *via* the liver by glycogen phosphorylase to escape p53 dependent senescence *via* repression of ROS generation (Favaro et al., 2012).

Recent research has shown that some cancer cells, such as glioblastoma and breast cancer, utilize exogenous acetate to acetyl-CoA for lipid synthesis and biomass accumulation (Mashimo et al., 2014; Schug et al., 2015). The enzyme catalyzing this reaction is acetyl CoA synthase −2, an important target gene of HIF-1α and is integral to cancer cell survival and proliferation under hypoxic conditions (Kamphorst et al., 2014). These findings suggest that to meet the energy requirement of rapidly proliferating cells, HIF-1α shifts metabolism from oxidative phosphorylation to aerobic glycolysis. To manage glucose crises, HIF-1α facilitates glycogen and acetate by activating enzyme glycogen synthase and glycogen phosphorylase in hypoxic cancer cells.

### 3.3.2 HIF-1α regulator of cellular acidity and redox homeostasis

HIF-1α activates pyruvate dehydrogenase kinase-1(PDK1), which phosphorylates and renders mitochondrial pyruvate dehydrogenase ineffective. As a result, glycolytic pyruvate is not

converted to acetyl-CoA and NADH, decreasing the NADH flux to the electron transport chain (ETC) and production of ROS. The pyruvate thus accumulated undergoes conversion to lactate by the activity of lactate dehydrogenase A (LDHA) (Semenza et al., 1996; Papandreou et al., 2005). The lactate generated increases the cytosolic acidity and inhibits glycolysis. Therefore, lactate is effluxed out of the cell through a cell surface transporter known as MCT-4, which ultimately contributes to extracellular acidification and provides a favorable microenvironment for cellular proliferation. Henceforth, HIF-1α, through direct regulation of PDK1, LDHA, and MCT-4, effectively saves cancer cells from acidosis and harmful reactive oxygen species (Gatenby et al., 2007; Pinheiro et al., 2012; Doherty and Cleveland, 2013; Singh et al., 2023). Past research suggests that HIF-1α dependent expression of PDK-1 is required for metastatic colonization of breast cancer cells in the liver, contrary to lung or bone metastasis (Dupuy et al., 2015). Finally, to combat toxic ROS, HIF-1α indirectly induced the expression of glutathione. Since glutathione synthesis requires NADPH, HIF-1α triggers HMP shunt by initiating O-GlcNAcylation of glucose 6-phosphate dehydrogenase (Rao et al., 2015). Therefore, to protect cancer cells from harmful ROS, HIF-1α activates PDK-1, impairs the Kreb cycle, thereby reducing NADH flux to the electron transport chain and generating ROS. Moreover, HIF-1α also increases NADPH production from the HMP pathway to upregulate glutathione synthesis to maintain redox homeostasis.

### 3.3.3 Modulation of lipid and fatty acid synthesis

Fatty acid synthesis requires the activity of ATP citrate lyase for the conversion of Kreb-cycle-derived citrate into acetyl-CoA. Acetyl-CoA undergoes carboxylation in the presence of the enzyme acetyl-CoA carboxylase (ACC) to produce malonyl-CoA, which is subsequently grouped into long-chain fatty acids by the enzyme fatty acid synthase (FASN) (Pavlova and Thompson, 2016).

Many studies have reported that FASN activity is required for lipid biosynthesis and cellular proliferation in various cancer pathologies (Currie et al., 2013; Zaidi et al., 2013). Moreover, downregulation of FASN was found to promote breast cancer prognosis (Roy et al., 2020; Singh et al., 2021b; 2021a) (Singh et al., 2016; Roy et al., 2017; Singh M. et al., 2018; Devi et al., 2019a). It has been suggested that FASN activity is upregulated in response to an increase in the expression of SREBP-1c by HIF-1α (Furuta et al., 2008; Singh L. et al., 2018). Fatty acid, triacylglycerol, and cholesterol synthesis under hypoxia are supplemented by fatty acid uptake from the extracellular compartment by upregulating PPARγ and fatty acid-binding protein (FABP-3,7) whose transcription is regulated by HIF-1α (Krishnan et al., 2009; Bensaad et al., 2014). Furthermore, HIF-1α enhances the endocytosis of lipoproteins by increasing the number of cell surface receptors, LRP-1 (lipoprotein receptor −1) and VLDL receptor (VLDLR) (Perman et al., 2011).

In addition, fatty acids produced by overexpression of FASN are utilized for membrane synthesis by conversion into phospholipids or stored in the form of triacylglycerols in lipid droplets. Moreover, the esterification of free fatty acids to neutral TAGs and their storage in lipid droplets saves cells from lipotoxicity (Mukerjee et al., 2021). It prevents cancer cells from intratumoral hypoxia from harmful free radicals generated during the cycles of hypoxia and reoxygenation (Young et al., 2013; Bensaad et al., 2014; Yoo et al., 2014; Ackerman et al., 2018). During harsh times, cancer cells can utilize lipid droplets to produce signaling molecules, such as sphingosine 1, as well as for ATP production *via* β-oxidation in mitochondria (Ackerman and Simon, 2014). Previous findings suggest that hypoxia induces HIF-1α dependent lipid droplet accumulation in tumor cells (Mylonis et al., 2012; Kourti et al., 2015). Moreover, phosphorylation of HIF-1α can prevent lipid droplet accumulation and malignant cell proliferation under hypoxic (Mylonis et al., 2012; Karagiota et al., 2019). Henceforth, HIF-1α fulfills the fatty acid requirement of rapidly proliferating cells by inducing expression of FASN, PPARγ, and FABP-3.7 which increases the production of fatty acid and ensures their storage in the form of lipid droplets. These lipid droplets act as a reservoir of fatty acid during times of starvation and protect cells from lipotoxicity and harmful ROS.

### 3.3.4 HIF-1α mediated angiogenesis, metastasis and invasion

Under hypoxic conditions, HIF-1α regulates a vast set of genes and pro-angiogenic factors both in tumor mass and vascular endothelial cells, including vascular endothelial growth factor (VEGF) (Forsythe et al., 1996), angiopoietin-2 (ANGPT-2) (Simon et al., 2008), stromal-derived factor-1α (SDF-1α) (Ceradini et al., 2004), stem cell factor (SCF) (Bosch-Marce et al., 2007), and platelet-derived growth factor-β (PDGF-β) (Kelly et al., 2003). All of these are crucial players in the sequence of events involved in tumor angiogenesis. VEGF is pivotal not only for endothelial cell activation and proliferation through VEGF-R signaling but is also responsible for reduced vascular endothelial cell apoptosis by the upregulation of BCL-2 (Gerber et al., 1998). VEGF is also secreted in a paracrine fashion from pericytes in response to PDGF secreted from tumor cells (Reinmuth et al., 2001). HIF-1α also governs vascular tone by modulating NOS (Rey and Semenza, 2010). ANGPT-2 weakens the interactions between endothelial cells and smooth muscle cells, responsible for endothelial cell migration (Maisonpierre et al., 1997). *In vivo* investigations have reported that targeting HIF-1α remarkably inhibits tumor vascularization (Lee et al., 2009). HIF-1α induces metastasis, which is the primary cause of tumor-related deaths (Balamurugan, 2016). Limitations of tumor metastasis and invasiveness rests upon HIF-1α mediated transcriptional activation of matrix metalloproteinases (MMPs) and enzyme lysyl oxidase (Wong et al., 2011). Reports reveal that HIF-1α directly promotes epithelial to mesenchymal transition (EMT) by inducing the loss of E-cadherin (Krishnamachary et al., 2006; Zhang et al., 2014). It has been found that HIF-1α, through direct regulation of the TWIST gene, induces metastasis (Yang et al., 2008). CXCR-4 and urokinase-type plasminogen activator receptor (uPAR) are important receptor targets of HIF-1α in hypoxia-mediated metastasis (Staller et al., 2003; Büchler et al., 2009). The above studies suggest that the cells which are at the center of tumor mass require blood supply for transport of $O_2$, nutrients, and growth factors; therefore, HIF-1α activates angiogenic factors such as VEGF, ANGPT-2, SDF-1α, SCF, and PDGF-β to facilitate neoangiogenesis, endothelial cell proliferation, and endothelial cell migration. Moreover, HIF-1α also induces tumor dissemination to distal cites by directly activating TWIST, CXCR-4, and uPAR.

### 3.3.5 HIF-1α and apoptosis

Hypoxia plays a dual role in the regulation of apoptosis under graded oxygen conditions. A study reported that cells treated with staurosporine were less sensitive to apoptosis under severe hypoxia (0.1% oxygen) (Dong et al., 2003). HIF-1α has both pro-and anti-apoptotic effects. Pro-apoptotic activity occurs from continuous or extreme hypoxia, which arises from the interaction of HIF-1α with p53. The anti-apoptotic activity of-HIF-1α arises at the level of $O_2$ at which HIF-1α can dimerize with ARNT, thereby increasing the transcription of anti-apoptotic genes (Piret et al., 2002).

Another study reported that two different forms of HIF-1α should be held liable for various activities. The phosphorylated form of HIF-1α dimerizes with ARNT and triggers antiapoptotic genes. On the other hand, dephosphorylated HIF-1α interacts with p53, thereby stabilizing p53, which induces apoptosis *via* BAX overexpression (Suzuki et al., 2001). Apart from BAX, BNIP3 and NIX are also overexpressed at the transcriptional level during hypoxia. When oxygen is sparse, HIF-1α promotes mitophagy and inhibits mitochondrial biogenesis by upregulating BNIP3 expression and inhibiting c-Myc-dependent mitochondrial generation (Sowter et al., 2001; Zhang et al., 2007; Zhang et al., 2008). The above studies suggest that below 0.1% oxygen (wherein both PHD-2 and FIH are inactive), HIF-1α can dimerize with ARNT and activate transcription of genes that inhibit apoptosis. Whereas, pro-apoptotic effects of HIF-1α may be attributed to its cross-talk p53 which need further investigation.

## 4 Bioenergetics of normal cells *versus* cancer cells

The energy requirement of the cell is fulfilled by the catabolism of carbohydrates, proteins, and fat. The energy production starts with the catabolism of carbohydrates to glucose, which is further converted to pyruvate *via* a set of ten enzymatic reactions forming a pathway known as "Glycolysis" (DeBerardinis et al., 2008). In brief, glycolysis is a two phase process. First, is the "investment phase" in which ATP is utilized to produce high energy intermediates (such as conversion of glucose to glucose-6- phosphate by hexokinase and conversion of fructose -6-phosphate to fructose-1,6-bisphosphate by phosphofructokinase). Second is the "pay off phase" in which high energy intermediates are broken down to generate ATP molecules (such as conversion of 1,3-bis phosphoglycerate to 3-phosphoglycerate by phosphoglycerate kinase and conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase). Furthermore, glycolysis also generates NADH from conversion of glyceraldehyde-3-phosphate to 1,3-bis phosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chandel, 2015).

Overall, glycolysis contributes to the generation of four ATPs and two NADH molecules from one molecule of glucose. However, the utilization of two ATPs in the investment phase reduces the net gain to 2-ATPs and 2 NADH. Therefore, if we spare NADH, which requires oxygen for energy production, at least two ATPs are generated from substrate-level phosphorylation whenever a glucose molecule undergoes glycolysis (Bonora et al., 2012). The end product of glycolysis, pyruvate, yields different products under aerobic and aerobic conditions. In the absence of oxygen, pyruvate is transformed to lactic acid by lactate dehydrogenase A (LDHA) and is expelled out of the cell (Semenza et al., 1996).

In contrast, in the presence of oxygen, pyruvate is transformed to acetyl-CoA under the catalytic influence of the pyruvate dehydrogenase enzyme. This acetyl-CoA yielded from pyruvate (from glycolysis) combined with oxaloacetate to generate citrate. This marks the start of energy production, which is inside the mitochondria called the "Citric acid cycle." The citric acid cycle liberates essential metabolic intermediates, such as alpha-ketoglutarate, succinate, fumarate, malonate, and oxaloacetate. These intermediates are metabolic precursors of amino acids, nucleotides, fatty acids, hemes, and porphyrins, which are essential components for synthesizing cell membranes, DNA, proteins, and other macromolecules (DeBerardinis et al., 2008).

It is appropriate to mention that similar to glycolysis, the TCA cycle can generate two ATPs from substrate-level phosphorylation, which occurs when the succinic CoA synthetase enzyme catalyzes the reversible reaction of succinyl CoA to succinate (Galluzzi et al., 2010). The oxidation of acetyl-CoA to CO2 by the electron transport chain reduces NAD+ and FAD+ to form high-energy carriers NADH and FADH2 during the cycle. For this reason, the Kreb cycle, though it does not require oxygen for functioning, requires oxygen for the oxidation of NADH and FADH2 to yield NAD+ and FAD+ by an electron transport chain (or oxidative phosphorylation), which is coupled to the Kreb cycle (Martínez-Reyes and Chandel, 2020). In normal cells, ATP is generated mainly from OXOPHOS, accounting for nearly 89% of the total cellular energy, while substrate-level phosphorylation contributes 11% of the whole cellular energy. Approximately 32–38 total ATP molecules are generated from the complete oxidation of one molecule of glucose. Cancer cells switch from oxidative phosphorylation to glycolysis for most of their energy requirements. This prevents the generation of harmful reactive oxygen species (ROS) by ETC (ROS are the product of electron acceptation by $O_2$ in ETC) and facilitates rapid ATP supply to fast proliferating cells since glycolysis is a quicker process than oxidative phosphorylation. This dependency of cancer cells on glycolysis and excessive production of lactate from pyruvate, despite the presence of $O_2$, is termed the Warburg effect (Warburg, 1925; Epstein et al., 2014; Shestov et al., 2014; Warburg Berlin-Dahlem, 2016).

## 4 Energy generation and transcriptional regulation of tumor cell proliferation under graded oxygen tension: Role of lactate shunt

As previously discussed, as tumors grow away from blood vessels, areas that are distal to blood vessels or sometimes due to poor vasculature experience graded oxygen tension (Campillo et al., 2019). Various models have been proposed previously, which classify the tumor into two zones: the non-hypoxic zone, which has an adequate supply of oxygen to support tumor cell growth, and the second is the hypoxic zone, which lacks oxygen. In solid tumors, it is important to consider a third zone that is intermittent hypoxic simply because tumors have intermittently hypoxic patches. Therefore, to understand how cells of the same tumor respond differently to different $pO_2$, we bifurcated the tumor cells into three

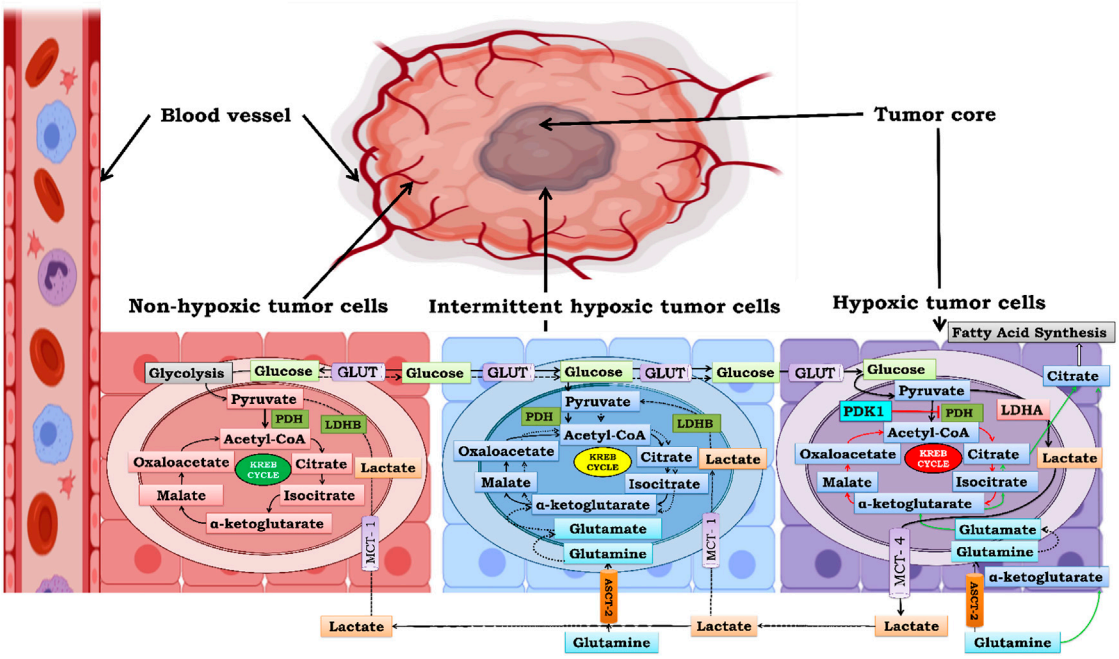



FIGURE 6
Metabolic Symbiosis under graded oxygen to produce ATP. Bioenergetics involved in solid tumor is highly complex. Cells which are closed to blood vessels have normal supply of oxygen and glucose therefore obtain energy through glycolysis and kreb cycle initially but as the size of the tumor increases the demand of glucose increases more and more to compensate for this ever increasing demand of glucose there is an upsurge in MCT-1 expression in non-hypoxic cells and they take up exogenous lactate from extracellular tumor microenvironment and convert it to pyruvate by the action of enzyme lactate dehydrogenase B (LDHB). and further this extemporaneously generated pyruvate is utilized to gain energy through Kreb cycle thus sparing glucose for utilization by hypoxic areas which are far from blood vessels. Second in line are intermittently hypoxic cells which utilize both exogenous lactate as well as oxidative glutamine metabolism for energy requirement. Ultimately the core of tumor is formed by severely hypoxic cells which lack access to both blood and oxygen therefore glycolysis is the only source for energy replenishment and therefore undergo rapid glycolysis and generate huge amount of lactate resulting in lactic acidosis. Therefore, in order to survive cancer cells, excrete this lactate out of the cell. This excreted lactate serves as pool of extracellular lactate which can be taken by non-hypoxic and intermittent hypoxic cells for energy production. Hence this cycle goes on and on leading to increase in tumor mass by rapid cellular proliferation and survival.

zones based on the oxygen gradient (Figure 6). The three zones are i) non-hypoxic zone ($40 < pO_2 < 160$ mmHg), ii) intermittent hypoxic zone ($10 < pO_2 < 40$ mmHg), and iii) severely hypoxic zone ($pO_2 < 10$ mmHg) (McKeown, 2014). To survive, cells in these zones cooperate with each other to fulfill their metabolic needs called "Metabolic Symbiosis." In recent times, lactate has evolved as the molecule which facilitates this "Metabolic Symbiosis" (Guppy et al., 1993). In the subsequent section, we will see how lactate facilitates cells to tackle energy crises in all three zones.

The non-hypoxic zone ($40 < pO_2 < 160$ mmHg) comprised oxygenated tumor cells. These cells have free access to $O_2$ because they lie close to the blood vessels. In the early phase of tumor development, these cells prefer glycolysis over oxidative phosphorylation because glycolysis can supply ATP faster to rapidly proliferating cells as compared to oxidative phosphorylation and also reduces the oxidative stress on tumor cells (Guppy et al., 1993; Pfeiffer et al., 2001). However, as the size of the tumor increases, a large amount of glucose is required not only to maintain cell energetics but also to provide biosynthetic intermediates such as ribose-5-phosphate, one carbon for nucleotide synthesis, as well as amino acids for protein synthesis, resulting in an increase in the demand for glucose. Therefore, oxygenated tumor cells reduce glucose uptake and increase lactate uptake from the extracellular environment during tumor development. Lactate is picked up from the extracellular environment through MCT-1 and is converted to pyruvate by LDHB. Pyruvate generated from lactate enters the Krebs cycle and provides energy through oxidative phosphorylation. This metabolic switch from glycolysis to oxidative phosphorylation provides a large number of ATPs and TCA cycle intermediates and makes glucose readily available for the survival of hypoxic cells that are distal to blood supply (Feron, 2009).

The intermittent hypoxic zone ($10 < pO_2 < 40$ mmHg) is the connecting link between the non-hypoxic zone and severely hypoxic zone, and therefore acts as a bridge that maintains a continuous supply of glucose from the non-hypoxic location the periphery to the severely hypoxic zone at the center. Similar to non-hypoxic cells, these cells rely mainly on the Kreb cycle and oxidative phosphorylation to generate ATP by utilizing pyruvate generated from lactate, thereby sparing glucose for utilization in severely hypoxic zones (Sonveaux et al., 2008; Nakajima and Van Houten, 2013). Therefore, these cells are also known as OXOPHOS cells. In addition to exogenous lactate uptake, OXOPHOS cells also take up glutamine for oxidative glutamine metabolism. In oxidative glutamine metabolism, glutamine is converted to glutamate by glutaminase, and glutamate dehydrogenase converts glutamate to

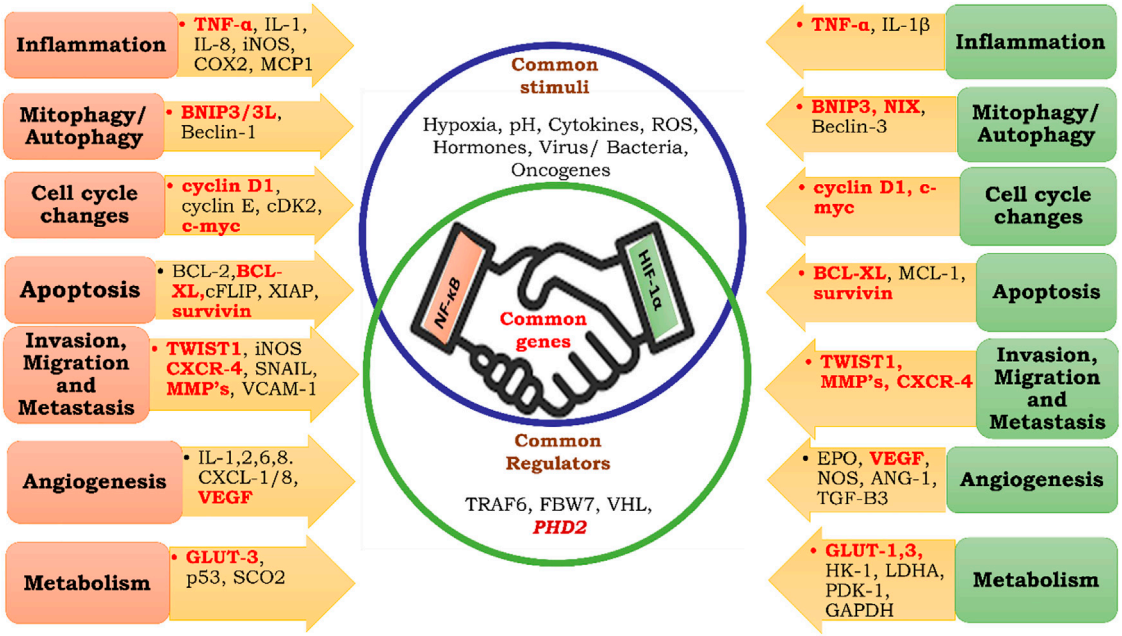



FIGURE 7
Common targets of HIF-1α and NF-κB. Inflammation, Mitophagy, Autophagy, Cell Cycle Changes, Apoptosis, Invasion, Migration, Metastasis, Angiogenesis, Metabolism.

the TCA cycle intermediate 2-oxoglutarate. Anaplerotically, 2-oxoglutarate undergoes oxidative glutamine metabolism, producing energy and rejuvenating the pool of Kreb cycle intermediate precursors of nucleotides, amino acids, and lipids (Moreadiths and Lehningert, 1984; DeBerardinis et al., 2007).

The severely hypoxic zone ($pO_2$ < 10 mmHg) consists of glycolytic cells that obtain energy from glycolysis only due to impairment of mitochondrial oxidative phosphorylation. Citrate deficit arising from the impaired mitochondrial function is partially fulfilled by "anaplerosis" from reductive glutamine metabolism in these cells (Moreadiths and Lehningert, 1984; DeBerardinis et al., 2007). Impairment of oxidative phosphorylation by mitochondrial defects is attributed to the activation of HIF-1α, which causes selective mitophagy to reduce oxygen consumption and ROS production under hypoxia (Zhang et al., 2008). Moreover, HIF-1α activates the enzyme pyruvate dehydrogenase kinase-1 (PDK1), which phosphorylates and thereby blocks the function of mitochondrial pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA, thereby uncoupling pyruvate from the Kreb cycle [114]. HIF-1α further induces the enzyme LDHA, which oxidizes pyruvate to lactate, and in the process, NADH is oxidized to NAD+ (Semenza et al., 1996). NAD + functions as an electron acceptor in the glycolytic pathway and therefore plays a vital role in the continuous running of glycolysis to support the energy requirements of rapidly proliferating cells (Bui and Thompson, 2006; Fan et al., 2011). Lactate, a by-product of glycolysis, is effluxed out of the cell by monocarboxylate transporter-4 (MCT-4), which is also a transcriptional target of HIF-1α (Pinheiro et al., 2012). Extracellular lactate accumulation favors migration and metastasis and acts as a reservoir of energy for non-hypoxic and OXOPHOS cells (Goetze et al., 2011; Dhup et al., 2012; Brooks, 2018).

From the above, it is clear that "metabolic symbiosis between the three zones is necessary for holistic growth and proliferation of cancer cells, and lactate shunt is the key to this metabolic adaptation." Compared to energy production, tumor growth and proliferation regulation under graded oxygen tension are much more complex. Studies over the past decade have emphasized HIF-1α as the central regulator of tumor cell survival, proliferation, angiogenesis, and metastasis under graded oxygen tension (Schofield and Ratcliffe, 2004). Here, we focus on the intricacies tangled in the modulation of HIF-1α in the non-hypoxic, intermittent hypoxic, and severely hypoxic zones.

In the non-hypoxic zone (40 < $pO_2$ < 160 mmHg), both PHD-2 and FIH-1 remain functional, which contribute to proteasomal degradation and transcriptional inactivation of HIF-1α, respectively (Maxwell et al., 1999; Bruick, 2001; Lando et al., 2002; Kaelin, 2003). In intermittent hypoxic zones (10 < pO2 < 40 mmHg), PHD-2 is inactivated while FIH-1 remains functional (Koivunen et al., 2004; Stolze et al., 2004). Therefore, how HIF-1α regulates the transcriptional factors responsible for cell growth and proliferation in these zones remains a question. Research postulates that Lactate uptake from the extracellular environment and its subsequent conversion to pyruvate by LDHB enzyme could be responsible for non-hypoxic activation of HIF-1α since pyruvate acts as a competitive inhibitor of oxoglutarate (α-ketoglutarate), which is a co-substrate for enzymes PHD-2 and FIH-1. Therefore, reduced levels of α-ketoglutarate might lead to the inactivation of PHD-2 and FIH-1, thereby facilitating the stabilization and transcriptional activity of HIF-1α (De Saedeleer et al., 2012). Notably, it has been found the Michaelis constant (Km) of FIH for the substrate α-ketoglutarate is 55–60 μM, which is just double that

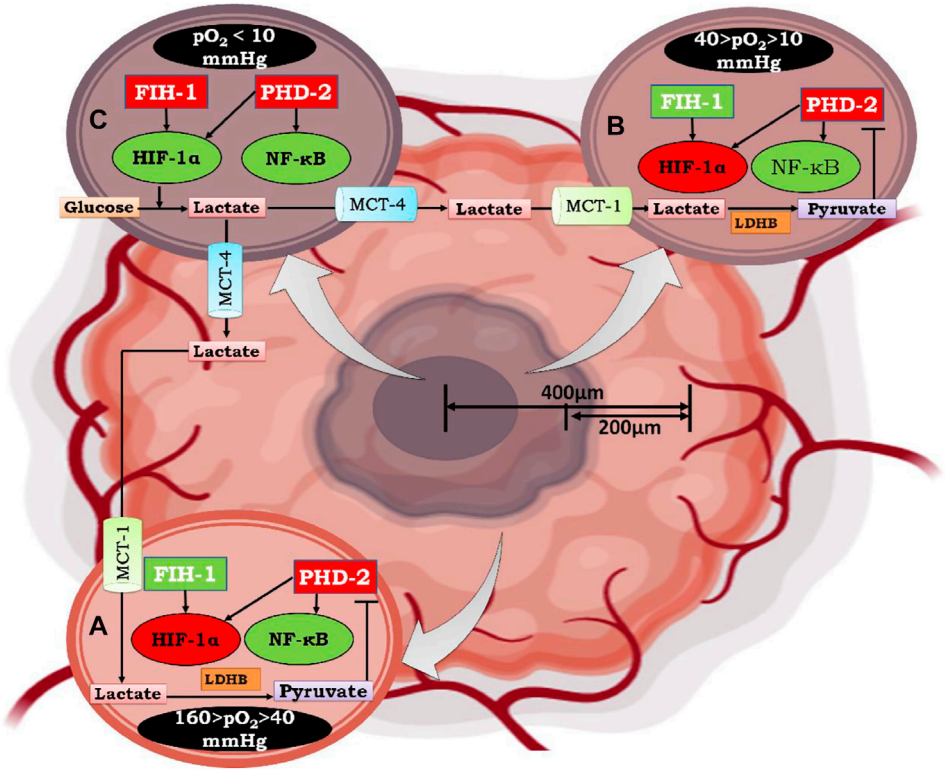



FIGURE 8
Mechanism of Lactate shuttle mediated activation of NF-κB under graded oxygen tension. As the tumor size increases to more than 200 μm, the diffusion of oxygen decreases, which marks the beginning of the condition known as "intratumoral" hypoxia. Moving from the periphery to the core (400 μm), cells are under graded oxygen tension, which ranges from 160 mmHg to less than 10 mmHg. Based on this, cells can be divided into three zones. In Figure A. represents a cell that lies in the non-hypoxic zone (160 > $pO_2$ > 40 mmHg), B. represents a cell that lies in the intermittent hypoxic zone (40 > $pO_2$ > 10 mmHg), and C. represents a cell that lies in a severely hypoxic zone ($pO_2$ < 10 mmHg). In a non-hypoxic cancer cell (A) and intermittent hypoxic cancer cell (B), effluxed lactate enters through MCT-1 and is converted to pyruvate by the enzyme lactate dehydrogenase B. The conversion of lactate to pyruvate causes competitive inhibition of 2-oxoglutarate by pyruvate, which is the substrate for enzyme Prolylhydroxylase-2(PHD-2). Henceforth, PHD-2 fails to degrade IKKβ, resulting in the activation of NF-κB, which further regulates the downstream mechanism of cancer cell survival, growth, and proliferation. In contrast, HIF-1α, which assumes stability due to inactivation of PHD-2, does not undergo transcriptional activation because of hydroxylation at N803 of its C-TAD by factor inhibiting HIF-1α (FIH-1). Although FIH-1, similar to PHD-2, belongs to the group of 2-oxoglutarate dependent dioxygenases, has a Km value of 55-60μM for 2-oxoglutarate, which is double that of PHD-2, whose Km value for 2-oxoglutarate is 25 μM. Moreover, FIH-1 remained active up to an oxygen tension of 10 mm Hg. Therefore, FIH-1 effectively inhibits HIF-1α transcriptional activity in non-hypoxic and intermittent hypoxic zones. Henceforth, it can be postulated that in these zones cell survival and proliferation is under influence of NF-κB. In hypoxic cancer cells, lactate accumulates due to excessive glycolysis, which results in an increase in intracellular acidity. In order to cope with intracellular acidity, hypoxic cancer cells efflux lactate into the extracellular environment through monocarboxylate transporter-4 (MCT-4). Moreover, in hypoxic cancer cells, both PHD-2 and FIH-1 were inactive due to very low oxygen tension ($pO_2$ < 10 mmHg). Thus, HIF-1α is both stable and transcriptionally active along with NF-κB. Therefore, cell survival and proliferation are influenced by both HIF-1α and the inflammatory partner NF-κB.

of PHD-2, whose Km value for α-ketoglutarate is 25 μM (Koivunen et al., 2004).

Moreover, it has been found that FIH remains functional up to $pO_2$ = 10 mmHg, while PHD-2 is inactivated as soon as $pO_2$ reaches <40 mmHg. Therefore, even if PHD-2 is inactivated by competitive inhibition of α-ketoglutarate by pyruvate, FIH-1 remains active and prevents HIF-1α transcriptional activity (Rani et al., 2022). Thus, pyruvate-induced competitive inhibition of α-ketoglutarate will only contribute to the stability of HIF-1α but not to the functional activity of HIF-1 α in non-hypoxic cells. Now, a question still remains: how does tumor cell survival, proliferation, angiogenesis, and metastasis occur in the truancy of HIF-1α. This raises an intriguing possibility that other transcriptional regulators are involved that facilitate cellular adaptation when oxygen tension is limiting for HIF- 1α.

In severely hypoxic zones ($pO_2$ < 10 mmHg), both PHD-2 and FIH-1 are inactivated in response to oxygen. As a result, HIF-1α becomes stable and activates genes responsible for tumor cell growth survival.

## 5 Different hypotheses for NF-κB activation and their future endorsement: Role of PHDs

### 5.1 PHDs as a dual regulator of HIF-1α and NF-κB

While HIF-1α is an extensively studied transcriptional regulator under hypoxia, other transcriptional regulators exist (Nakayama

and Kataoka, 2019). NF-κB is also a transcriptional regulator. NF-κB is a crucial modulator of angiogenesis, metastasis, migration, cell survival, and proliferation (Gilmore, 2006), but its role in conjunction with HIF-1α has not been studied extensively. A few studies have established a link between HIF-1α and NF-κB; however, the results have been contradictory. Although it appears that both HIF-1α and NF-κB work hand in hand, establishing a well-defined mechanism has proven elusive (BelAiba et al., 2007; Nam et al., 2011; Azoitei et al., 2016).

Interestingly, PHDs that regulate HIF-1α activity were also found to regulate NF-κB signaling through IKKβ. An *in vitro* study showed that inhibition of PHDs moderately activated IKKβ in cells cultured under hypoxic conditions (Walmsley et al., 2005). It would be appropriate to mention that NF-κB activation is regulated by IKKβ induced phosphorylation and degradation of IκB inhibitors (IκBα and IκBβ). It has been reported that ischemia leads to hypoxia, which can suppress PHD1 function, thereby enhancing IKKB expression for NF-κB activation (Cummins et al., 2006; Xie et al., 2014).

It was also found that PHD-2 downregulation increased NF-κB-mediated expression of IL-8 and angiogenin in certain tumors. The same study also reported that stimulation of NF-κB activity by TNF-α treatment was impaired in the presence of PHD-2, which further confirms that PHD-2 is a negative modulator of NF-κB (Chan et al., 2009). Moreover, a functional link between both ROS and PHD2 pathways was provided by showing that the conversion of NAD + to NADH and H+ by LDHB was converted from lactate to pyruvate.

Competitively inhibits PHD. but also stimulates NAD(P) H oxidase (Végran et al., 2011). NAD(P) H oxidase generates ROS from a pool of NADH. ROS generated may contribute to the inhibition of PHD-2, leading to an increase in basal NF-κB activity (Gerald et al., 2004; Pan et al., 2007). From the above findings, there appears to be a negative regulatory loop between PHDs and NF-κB.

## 5.2 Various mechanisms of NF-κB activation

Endothelial cells have an increased expression of MCT-1, which allows them to take up a large amount of lactate from the extracellular environment. Although MCT-1 operates bi-directionally depending on the concentration gradient, the unidirectional movement of lactate is maintained by its higher affinity for lactate (Km = 3–6 mM) and an ever-increasing extracellular pool of lactate effluxed from glycolytic cells through MCT-4 (Km = 25–30 mM) (Halestrap, 2013; Benjamin et al., 2018).

This effluxed lactate is converted to pyruvate in the presence of the enzyme LDHB to run the Krebs cycle for energy production, thereby sparing glucose for distal hypoxic areas [171] (Figure 7). However, LDHB converts NAD+ to NADH and H+, thus generating NAD(P) H oxidases. NAD(P) H oxidases produce large amounts of ROS. Moreover, pyruvate, which is a competitive inhibitor of 2-oxoglutarate, inhibits PHD2. Both excessive ROS production and inhibition of PHD2 contribute to NF-κB activation (Gatenby and Gillies, 2004). It is pertinent to mention that MCT-4 plays a pivotal role in NF-κB activation indirectly by effluxing lactate out of the cell, which is further taken up by MCT-1 from the extracellular environment.

Specific blocking of MCT-1 could be a possible mechanism to inhibit NF-κB activity by preventing competitive inhibition of PHD-2 and generation of ROS. On the other hand, specific blockage of MCT-4 leads to decreased extracellular lactate levels, creating a negative feedback loop, thereby inhibiting lactate uptake by MCT-1. Blocking both MCT-1 and 4 could be a more dynamic approach because it would disrupt the metabolic symbiosis of lactate both intracellularly and extracellularly, rendering NF-κB ineffective.

Activation of NF-κB by this mechanism is dependent on the uptake of lactate; however, there is a need to investigate the level at which cells would prefer to take lactate instead of glucose when both are available in abundance. Secondly, cells could hire other mechanisms to overcome the effect of blockage of MCTs and increase intra-or extracellular acidity.

Modern research has focused on inhibiting the enzyme LDHB, a crucial enzyme involved in converting lactate to pyruvate. Inhibition of the activity of this enzyme has resulted in decreased disease prognosis in breast, colon, and ovarian tumors. This enzyme not only replenishes NADPH for fatty acid synthesis but also maintains redox homeostasis. This enzyme indirectly activates HIF-1α because of the competitive inhibition of PHD2 by pyruvate. However, whether competitive inhibition of pyruvate leads to activation of NF-κB remains a matter of research (Brisson et al., 2016; Mishra and Banerjee, 2019). Few researchers have suggested that competitive inhibition of PHD2 does not activate NF-κB in oxidative tumor cells but activates HIF-1α (De Saedeleer et al., 2012; Van Hée et al., 2015). It has been shown that PHD2 activation results in decreased activation of HIF-1α and NF-κB; thus, how the inhibition of the same regulating factor results in reduced expression of HIF-1α and not of NF-κB requires further investigation.

# 6 Unresolved riddle and proposed hypothesis.

It has been known for quite some time that inflammation, when left unchecked, might eventually result in cancer. The precise mechanism driving this event, however, remains unclear. Many studies (Höckel and Vaupel, 2004) have pointed to hypoxia as a prognostic factor that changes normal cells into cancerous ones in a chronic inflammatory milieu. Previous studies have implicated NF-B and HIF-1α as two significant transcriptional regulators in this transition. But how they interact is still a mystery. The two transcription factors control separate aspects of cell growth and division. While HIF-1α promotes malignant cell adaptability and proliferation, nuclear factor-kappa B (NF-kappa B) is essential for tumour initiation and survival (Dolcet et al., 2005; Masoud and Li, 2015). Many studies have looked into the method by which these two proteins cooperate, however the results have been inconsistent. While some research suggests that HIF-1α activates following NF-κB suppression, others show that the opposite is true. Still other research suggests that NF-κB regulates the HIF-1-mediated response. Overall, little is known about HIF-1's involvement with its inflammatory companion NF-κB. Basal NF-κB activity is essential for HIF-1α activation, and this has been stressed by a small but vocal group of researchers in recent years, although there is not yet enough evidence to back up these claims. Moreover, it is pretty understandable that sufficient oxygen is available in the early

phase of tumorigenesis because the affected area is close to the blood vessels. Therefore, HIF- 1α undergoes proteasomal degradation due to the presence of PHD2. Although non-hypoxic stabilization of HIF-1α and NF-κB is very high on the cards by inhibition of PHD2 by ROS and TCA cycle intermediates (Lee et al., 2016), the presence of FIH is likely to seize the transcriptional activity of HIF-1α, while NF-κB undergoes transcriptional activation. Therefore, in the initial phase of tumors, it could be hypothesized that cellular proliferation is HIF-1α independent and NF-κB-dependent. It is pertinent to mention that PHD2 inhibition is necessary for the transcriptional activity of both HIF-1α and NF-κB.

Past research suggests that NF-κB and HIF-1α share commonly targeted genes (Rocha, 2007; Park and Hong, 2016) (Figure 7); therefore, they both may be regulated by similar mechanisms. Despite the presence of FIH-1, angiogenesis, metastasis, migration, and other phenomena necessary for cellular proliferation take place. Therefore, it could be hypothesized that until oxygen becomes limiting for FIH, NF-κB regulates tumor cell growth and expansion through pyruvate-mediated competitive inhibition of PHD-2 (Figure 8).

## 7 Conclusion

To regulate HIF-1α and NF-κB, the PHD-2 is the typical checkpoint. Because of this, activating PHD-2 either directly or indirectly may represent a novel approach to treating solid tumours. PHD-2 may be directly activated by small-molecule chemical activators (Roy et al., 2018; Devi et al., 2019a; Roy et al., 2019). (Devi et al., 2019b). It is worth noting that there are just a few of compounds thought to be PHD-2 activators, and none of them have made it to market as of yet. In addition, the response in tandem with NF-κB downregulation is still not clear.

Activating PHD-2 in a roundabout way may be accomplished by focusing on MCT-1 and LDHB. Recent studies have indicated that inhibiting MCT-1 has a significant impact on cancer treatment, although their effect in tandem with PHD-2 activity has yet to be studied. As with Akt inhibition, LDHB inhibition was found to be useful in cancer therapy, but selective LDHB inhibitors have not been discovered as of yet. Consequently, it is important to study the impact of novel selective inhibitors of LDHB on carcinogenesis. The current study elucidates the function of NF-κB in the first stages of tumour development and proliferation, when HIF-1α is inactive due to elevated pO2. This review also sheds light on how, under normal oxygen tension, downregulation of PHD-2 by the lactate shuttle activates NF-κB but not HIF-1α. The review highlights PHD-2 as a dual down regulator of HIF-1α and IKK, and proposes that, like HIF-1α, PHD-2 causes hydroxylation and proteasomal degradation of IKK.

## Author contributions

SR collected the literature and wrote the manuscript; SA, MA, and AS contributed to creating the scientific illustration, mechanism, and expanded outline of the manuscript; and MS edited and proof read the manuscript. GK conceived the manuscript and approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1108915/full#supplementary-material

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## Glossary

**TNF-α** tumor necrosis factor-α
**IL** interleukin
**ROS** reactive oxygen species
**PAMPs** pathogen associated molecular patterns
**DAMPs** damage/danger associated molecular patterns
**TLRs** toll like receptors
**NF-κB** nuclear factor kappa light chain enhancer of activated B- cells
**OXOPHOS** oxidative phosphorylation
**MCT-1** monocarboxylate transporter-1
**MCT-4** monocarboxylate transporter-4
**LDHA** lactate dehydrogenase-A
**LDHB** lactate dehydrogenase-B
**GLUT-1** glucose transporter-1
**GLUT-3** glucose transporter-4
**HIF-1α** hypoxia inducible factor-1α
**HIF-1β** hypoxia inducible factor-1β
**PHD-2** prolylhydroxylase-2
**ODDD** oxygen dependent degradation domain
**pVHL** Von-hippel-Lindau tumor repressor protein
**FIH-1** factor inhibiting hypoxia inducible factor 1α
**CBP** CREB binding protein
**CTAD** C- terminal transactivation domain
**HRE** hypoxia response element
**2-OG** 2- oxoglutarate
**TCA** tricarboxylic Acid cycle
**IκB** inhibitor of nuclear factor kappa B
**IKK** inhibitor of nuclear factor kappa-B kinase
**NEMO** NF-κB essential modulator
**MEFs** mouse embryonic fibroblasts
**TAK-1** transforming growth factor -β- activated kinase-1
**SCF** SKP1-cullin-1-F- box protein
**βTrCP** beta transducing repeats containing protein
**VEGF** vascular endothelial growth factor
**MMP's** matrix metalloproteinases
**CAF's** cancer associated fibroblasts
**ECM** extracellular matrix
**CXCl-2** C-X-C motif chemokine ligand 2
**SDF-1** stromal cell-derived factor-1
**c-myc** cellular myelocytomatosis oncogene
**CXCR4** C-X-C chemokine receptor type 4
**bHLH** basic helix loop helix
**PAS** PER-ARNT-SIM
**ARNT** aryl hydrocarbon receptor nuclear translocator
**ATP** adenosine triphosphate
**ETC** electron transport chain
**PDK-1** pyruvate dehydrogenase kinase-1
**IDH-1** isocitrate dehydrogenase-1
**IDH-2** isocitrate dehydrogenase-2
**ACO-1/2** aconitase-1/2
**ACl** ATP- citrate lyase
**ACC** acetyl CoA carboxylase
**FASN** fatty acid synthase
**SREBP-1** sterol regulatory element binding protein-1
**$PPAR_{\gamma}$** peroxisome proliferator receptor-γ
**FABP** fatty acid binding protein
**LRP-1** lipoprotein receptor related protein
**VLDLR** very low density lipoprotein receptor
**TAGs** Triacylglycerols
**AGPAT2** acylglycerol-3-phosphate acyl transferase-2
**DAG** diacylglycerol
**NHE1** sodium hydrogen exchanger-1
**Ca9** carbonic anhydrase-9
**BAFFR** B-cell activating factor receptor
**CD** cluster of differentiation
**RANK** receptor activator of nuclear factor κ B
**TNFR2** tumor necrosis factor receptor 2
**FN14** fibroblast growth factor-inducible 14
**TWEAK** tumour necrosis factor (TNF)-like weak inducer of apoptosis

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# Enhancement of immune surveillance in breast cancer by targeting hypoxic tumor endothelium: Can it be an immunological switch point?

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Breast cancer ranks second among the causes of cancer-related deaths in women. In spite of the recent advances achieved in the diagnosis and treatment of breast cancer, further study is required to overcome the risk of cancer resistance to treatment and thereby improve the prognosis of individuals with advanced-stage breast cancer. The existence of a hypoxic microenvironment is a well-known event in the development of mutagenesis and rapid proliferation of cancer cells. Tumor cells, purposefully cause local hypoxia in order to induce angiogenesis and growth factors that promote tumor growth and metastatic characteristics, while healthy tissue surrounding the tumor suffers damage or mutate. It has been found that these settings with low oxygen levels cause immunosuppression and a lack of immune surveillance by reducing the activation and recruitment of tumor infiltrating leukocytes (TILs). The immune system is further suppressed by hypoxic tumor endothelium through a variety of ways, which creates an immunosuppressive milieu in the tumor microenvironment. Non responsiveness of tumor endothelium to inflammatory signals or endothelial anergy exclude effector T cells from the tumor milieu. Expression of endothelial specific antigens and immunoinhibitory molecules like Programmed death ligand 1,2 (PDL–1, 2) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) by tumor endothelium adds fuel to the fire by inhibiting T lymphocytes while promoting regulatory T cells. The hypoxic microenvironment in turn recruits Myeloid Derived Suppressor Cells (MDSCs), Tumor Associated Macrophages (TAMs) and T regulatory cells (Treg). The structure and function of newly generated blood vessels within tumors, on the other hand, are aberrant, lacking the specific organization of normal tissue vasculature. Vascular normalisation may work for a variety of tumour types and show to be an advantageous complement to immunotherapy for improving tumour access. By enhancing immune response in the hypoxic tumor microenvironment, *via* immune-herbal therapeutic and immune-nutraceuticals based approaches that leverage immunological evasion of tumor, will be briefly reviewed in this article. Whether

these tactics may be the game changer for emerging immunological switch point to attenuate the breast cancer growth and prevent metastatic cell division, is the key concern of the current study.

KEYWORDS

tumor endothelial cells (TECs), hypoxia inducible factors (HIFs), myeloid derived suppressor cells (MDSCs), T regulatory cells (Treg cells), angiogenesis, hypoxic tumor microenvironment, immunological switch point

# 1 Introduction

The most prevalent types of cancer diagnosed and the main reason for deaths due to cancer among women is breast cancer (BC) (1). The detection and treatment of BC have undergone a number of advancements in recent years. Since the early 1990s, there has been a 39% decrease in breast cancer mortality thanks to a combination of better screening, earlier detection/diagnosis, and anti-cancer medicines that have made substantial advances. The prognosis for patients with advanced-stage breast cancer can be improved, though further investigations are required to overcome the threat of cancer resistance to treatment.

Aggressive breast cancers with hypoxic cores account for 40% of cases; these tumours are highly metastatic, and resistant to the majority of treatments. Breast cancer stem cells (BCSCs) multiply in a hypoxic tumor microenvironment, which results in a variety of epigenetic changes that retain cancer stem cells in an undifferentiated state and aid in its development and recurrence (2).Unchecked tumor cell growth outgrows the surrounding vascular system, which leads to a reduction in oxygen delivery relative to demand. Chronic hypoxia refers to this restriction on oxygen diffusion, whereas acute hypoxia occurs when blood arteries abnormally close, leading to reduced perfusion (3–5). Chronic hypoxia causes DNA breakage, malfunctions in the mending systems and mutagenesis (6). On the other hand, short-term hypoxia boosts the generation of Reactive Oxygen Species (ROS), tumor survival, and spontaneous metastasis (7, 8). Cancer cells have demonstrated radio-resistance *in vitro* and *in vivo* under both chronic and acute hypoxia (9). Hypoxia-inducible factors (HIF), which is a transcription factor, serves as the catalyst for tumor growth (10, 11). HIFs are heterodimers made up of the oxygen-sensitive HIF–α and a subsequently expressed HIF–β (12). There are three isoforms; HIF1 and HIF2 are well known, while HIF 3 is also present but has not been well researched in relation to cancer (13). HIF-1 and HIF-2 under normoxic circumstances undergo hydroxylation at particular proline residues. This causes the tumor suppressor protein Von-Hippel Lindau (VHL) protein to bind to HIF 1 and 2, which then makes it easier for them to be degraded by the ubiquitin-proteaosome system (11, 14, 15). A variety of genes encoding proteins are used for anaerobic energy production, vascularization, extracellular matrix (ECM) remodelling, suppression of apoptosis, and metastasis progression. They are transcribed when hypoxia occurs due to which HIF–1 and HIF–2 get dimerized with the subunit. They (HIF–1 and HIF–2) are then translocated to the nucleus and where they activate hypoxia responsive elements (HRE) (11, 16). Patients with breast cancer who had higher levels of HIF expression in their primary tumor biopsies are more likely to develop metastases to the bone, lungs, liver, brain, and local lymph and causes major portion of breast cancer related mortalities (17).

Through a series of sequential multistep processes the original tumour transforms into a secondary tumour at a distant site (18). The EMT transcription factors (EMT-TFs), mainly belonging to the SNAIL, TWIST, and ZEB families, which play significant roles in all processes of tumour metastasis, cause the epithelial to mesenchymal transition (EMT), which is critical for cancer spread (19).

EMT type-1, type-2, and type-3 subtypes have been extensively investigated in a variety of physiological and pathological processes. Type -1 EMT is the exchange of Epithelial to Mesenchymal cells and associated with events in embryonic phase such as implantation, embryogenesis and organ development, while Type -2 is the transformation of epithelial to mesenchymal cells that occurs during wound healing and fibrosis driven by inflammation. Type-3 EMT is the transition of epithelial cells to mesenchymal cells and is active in different types of cancers including breast cancer. Therefore, Type-3 EMT is also known as "oncogenic epithelial-mesenchymal transition" (20). Different subtypes of breast cancer show distinct metastatic organ tropisms governed by different molecular mechanisms. Along with distant lymph nodes, common target organs for breast cancer metastasis include the bone, lung, liver, and brain (21). Though all breast cancer subtypes show bone metastasis, Luminal A and B have bone as their major metastatic site. Luminal B subtype is more probable than luminal A subtype to have bone as the first site of relapse when compared to other subtypes. Incidence of bone metastasis is much higher in luminal subtype tumors than in HER-2 positive and basal like subtypes. HER -2 positive subtype is more often positively tropic to liver and luminal B and basal-like subtypes present higher levels of lung -specific metastasis (22). Triple negative breast cancer (TNBC) is often associated with visceral metastases including lung, liver and brain (23). Additionally, it has been reported that hypoxia triggers EMT in different type of cancers including breast cancer, prostate cancer and oral cancer (24). Studies by Peng, Jianheng, et al. (2018). clearly show that TGF-1 and Suppressor of Mothers Against Decapentaplegic (SMAD3) expression levels were both dramatically raised by HIF-1 in breast cancer cells, however SMAD3 overexpression had no effect on either of these proteins' expression levels (25). Moreover, HIF-1α upregulated the expression of EMT transcription factors, SWIFT and SNAIL. In case of SWIFT in breast cancer cell lines, HIF-1α could

directly bind to proximal promoter of SWIFT and enhance transcription (26). Hypoxia triggers a significant up-regulation of angiogenic growth factors and their receptors, which causes endothelial cell migration with enhanced vascular permeability and promotes tumor angiogenesis. Due to the leaky vessels and haphazard arrangement, tumor and stromal cells have limited access to nutrients and oxygen during transformation and proliferation (27). However, tumors make up for this by producing metabolic intermediates that act as precursors for biosynthetic pathways, which allows cancer cells to adapt to these circumstances in the tumor microenvironment (TME) and continue to grow and multiply. Glucose metabolism is switched from the tricarboxylic acid pathway to the oxygen independent glycolysis through the activation of glycolytic pathway regulators such as glucose transport proteins (GLUTs), hexokinase 1 and 2 (HK1, 2), and pyruvate dehydrogenase kinase 1 (PDK1) (28–30). The role of HIF–1 in reshaping the topography of the ECM under hypoxia by collagen deposition and promoting the increased expression of remodelling enzymes such prolyl-4-hydroxylases and lysyl oxidases, which leads to ECM stiffness and metastasis, has been determined by studies. An increasing body of research indicates that hypoxia promotes the chemo-invasive and metastatic potential of breast cancer by activating metalloprotease 2 and 9 (MMP-2 and MMP-9), which also degrade ECM (31–33). According to multiple studies, hypoxic stress is expected to stimulate VEGF (Vascular Endothelial Growth Factor), a crucial regulator of angiogenesis (34). Immunosurveillance in breast cancer, as in many other tumor forms, is functionally represented by the presence of tumor infiltrating lymphocytes (TILs) into the neoplastic cellular mass (35). According to Ono et al., 2012, when compared to the HER–2-/HR+ subtype, TILs were significantly greater in TNBC (triple negative breast cancer) and HER–2+/HR- breast cancer subtypes. Furthermore, compared to TNBC with low TILs levels, the pathological complete response (pCR) rate was considerably higher in TNBC with high TILs scores (36). It has been found that low oxygen levels in the TME cause TILs to become less activated, which results in immunological suppression and less immune detection. Since hypoxia-associated transcription factors have long been suggested as a viable target for immunostimulatory therapy and immunological detection, numerous researchers have found therapeutic strategies for inhibiting these factors (37). In the TME, it has been demonstrated that overexpression of HIF-1 specifically affects many aspects of the immune cell's capacity to fight tumors (38). The foregoing discussion has briefly outlined the role of hypoxia in the aggressiveness of breast cancer, specifically in the transformation of normal endothelium into tumorigenic endothelium, as well as the various therapeutic approaches by which immune modulators can specifically target breast cancer and how hypoxic tumor endothelial cells can be targeted within the TME.

## 2 Hypoxia- induced immunosuppression in tumor microenvironment

Growing body of data indicates that, a hypoxic microenvironment may protect tumors from immunological treatments as well as naturally occurring anti-tumor immune responses by limiting anti-tumor immune effector cells and encouraging immune escape. It is clear that hypoxia affects immune cells either directly or indirectly, supporting the TME in a direction that is immunosuppressive (39–41). Understanding the disease biology of Breast Cancer (BC) requires an understanding of the immune surveillance in the tumor microenvironment since it has the potential to either facilitate the eradication of disease or encourage tumor growth. This dual role is sustained by the dynamic interactions between diverse immune effector cells, tumour cells, stromal cells, and soluble substances in the tumour microenvironment (42). Selection favours tumor variants with the genetic trait of escaping immunological detection, while variants lacking it are wiped off by immune surveillance. In addition to genetic variation, deficiencies in antigen presentation methods, T-cell receptor (TCR) signalling, interferon (IFN) signalling pathways, and expression of the MHC class I protein has been lost or mutated, along with tumour antigens (43, 44), which will impair the immune surveillance mechanism in the tumor microenvironment. Inhibition of tumor antigen-specific T cells by intratumoral myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs), and the switch from an anti-tumorigenic T-helper type 1 (TH1) to a pro-tumorigenic T-helper type 2 (TH2) immune response are additional factors that significantly support tumor growth. Furthermore, a variety of soluble factors like TGF-β, IL–10 etc are released by tumor/stromal cells inhibit T-cell activation and dendritic function while promoting stromal remodelling and angiogenesis (45, 46). The hypoxic core of the tumors attracts more pro-tumorigenic leukocytes, including MDSCs, tumor-associated macrophages (TAMs), and regulatory T cells. This double down the body's natural immune defensive measure to evacuate the aggressive tumor (47) (Figure 1). An in-depth study using Balb/c mice found that intra-tumoral hypoxia increases HIF-1 activation, which then sequentially activates the PDL-1, CD73, and CD47 genes to reduce the recruitment and activity of CD8+ T cells, NK cells, and macrophages, ultimately causing activity to evade both innate and adaptive immunity (48). Chemokines and their related receptors are another important hypoxic hotspot (49), which in turn affect tumor endothelial cells and increase the over expression of VEGF, CXCL12, and its receptor CXCR4, making all of them function on endothelial cells in an autocrine way. The expression of the pro-inflammatory chemokines CCL5 and CCL2 increased in HIF–1-deficient tumors, which in turn increased the infiltration of cytotoxic lymphocytes into the tumor (50). Additionally, the CXCR4-CXCR12 axis promotes metastasis to distant organs (51). Thus, hypoxic stress in TME along with activation of HIF transcription factors, is a principal cause for breast cancer angiogenesis, metastasis, immune suppression and overall poor survival rate. In the subsequent sections of this review, a detailed mechanism of hypoxia induced vascular abnormalities that activate endothelial cells (ECs) associated with tumor cells and immune suppression is furnished.

## 3 Hypoxia, a prerequisite to vascular alterations in the tumor microenvironment

Tumor vasculature is different from healthy blood vessels in a number of aspects, including irregular structural dynamics, high

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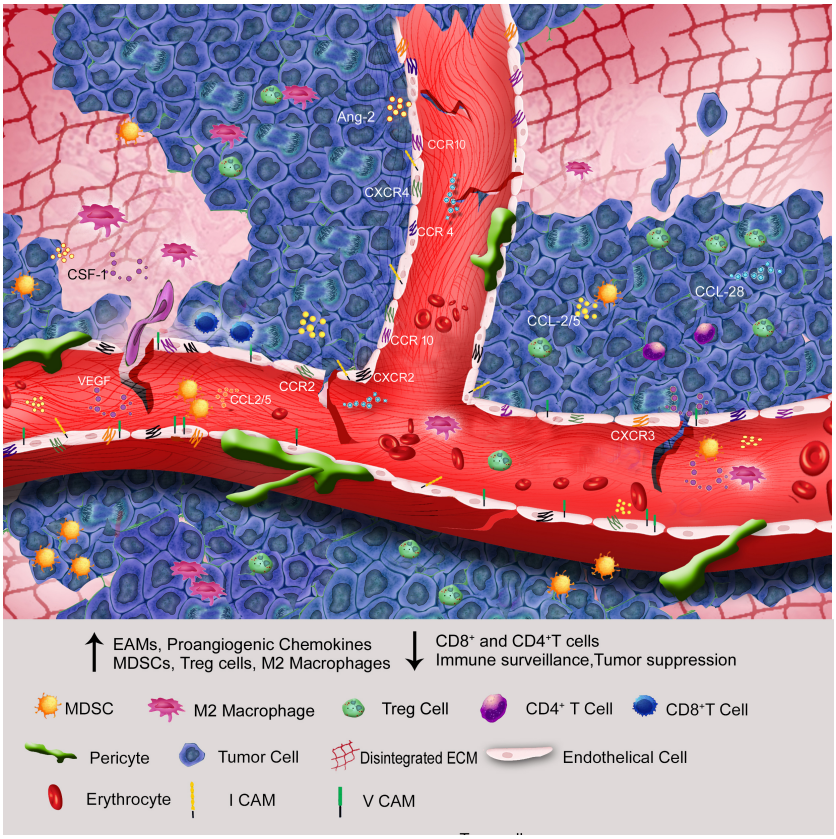


FIGURE 1
Through endothelial anergy, elevation of proangiogenic chemokines, cell adhesion molecules, and ECM disintegration, the hypoxic core of solid tumors attracts more immunosuppressive cells than immunostimulatory cells to the tumor microenvironment.

permeability, and convoluted arteries, whereas healthy blood vessels are well-organized and provide for the best perfusion of nutrients and oxygen (52). Specialized mural cells called pericytes are seen in the normal vasculature, whereendothelium covers them uniformly (53). Patchy hypoperfusion and blood vessel leakage are caused by the loosening, unstable, and tumor-related pericyte phenotype (54).

Endothelial cells interact with tumor cells in numerous ways to promote angiogenesis (55) (Figure 2). They serve as the connecting link between cancer cells and immune cells (56). Proangiogenic substances like Vascular Endothelial Growth Factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PGF), and angiopoietin are released by tumor cells to initiate angiogenesis. According to studies, the hypoxic tumor microenvironment might boost VEGF synthesis, which in turn promotes the growth of new blood vessels (57). When the microenvironment experiences a lack of oxygen, endothelial cells and pericytes are stimulated to create VEGF, which functions in an autocrine and paracrine manner to increase the recruitment and activation of endothelial cells in the tumor site (58). Angiopoietin-2 (Ang–2) plays a critical function in destabilising vasculature for normal or pathological angiogenesis and is also up-regulated by hypoxia. It is only expressed at sites of vascular remodelling. Numerous studies have documented the essential function of Ang-2, a ligand for the endothelial cell-specific tyrosine kinase Tie2, in the vascular permeability and blood vessel instability that leads to tumor growth (59, 60). Thus, newly formed blood vessels have an uneven thickness of the basement membrane, a loose interaction between pericytes and endothelial cells, an increase in interstitial pressure, and ultimate vascular leakage (47). One of the main processes behind angiogenesis is the angiopoietin/Tie (tyrosine kinase) signalling pathway, which is composed of growth factors called angiopoietins. These include Ang-1, a strong angiogenic growth factor that communicates with Tie2, and Ang-2, a vascular disruptor with a negative effect that also uses Tie2 as a conduit (61). To cause pericyte separation from the basement membrane and migration, Ang-2 and Tie2 bind in the hypoxic tumor microenvironment. Mice lacking in pericytes had higher Ang–2 levels, suggesting that pericytes may control Ang–2 levels and limit vascular permeability. This finding highlights the importance of Ang–2 in reducing vessel leakiness (62, 63). In a different study, it was reported that reduced pericyte coverage increased IL-6 expression in the hypoxic tumor microenvironment and MDSC transmigration and circulating malignant cell phenotype (64). Rgs5 overexpression in pericytes and endothelium seen in the hypoxic tumor microenvironment causes the vasculature to become unstable. Better pericyte coverage, less vascular leakage, and adequate oxygen perfusion was all observed in the Rgs5-deficient mice (65). In-depth research found that the development of a receptor complex made up of PDGF-R and VEGF-R2 during PDGF-induced angiogenesis is what causes VEGF to activate VEGF-R2 and suppress PDGF-R signalling in vascular smooth muscle cells (66), which then

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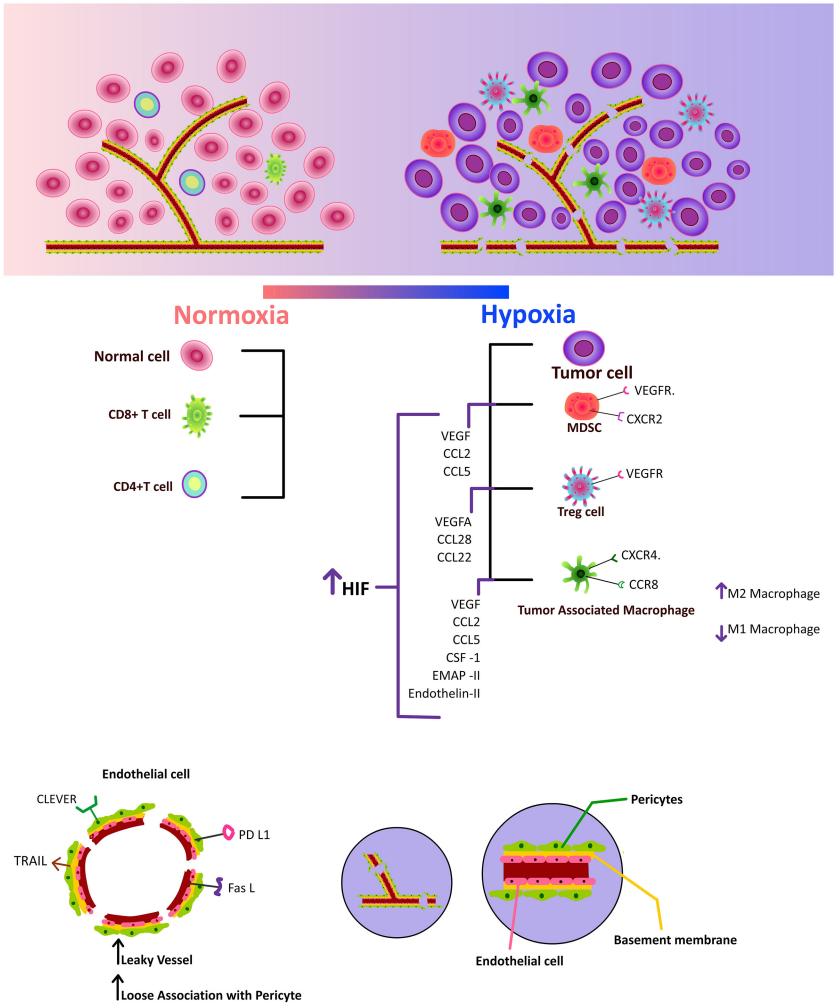


FIGURE 2
Increased hypoxia inducible factor, which upregulates pro-angiogenic factors and chemokines, draws immunosuppressive cells to the tumor microenvironment. Additionally, increased CLEVER, TRAIL, PD-L1, and FAS-L expression on tumor endothelium impairs effector T cells.

decouples neo-vasculature from pericyte covering. Researchers have shown that inhibiting VEGF and Ang–2 jointly results in tumor necrosis, vascular regression, intra-tumoral phagocyte antigen presentation, and a reduction in breast cancer-brain metastases (67, 68).

## 4 Endothelial cells in tumors are the "switch point", aren't they?

Endothelial Cells (ECs) ECs have specific functions based on their locations and exhibit distinct heterogeneity across vascular beds. They have a critical role in the regulation of immune responses, inflammation, angiogenesis and actively control the degree of vascular relaxation and constriction (69). ECs that line tumor blood vessels are initially derived from the surrounding tissue, and in due course of the tumor progression, they reprogram to a tumoral phenotype (70). Normal physiologic conditions do not often need the activation of ECs, but in tumors, hypoxia and other inflammatory signals cause ECs in the tumor microenvironment to become active, causing aberrant angiogenesis and impeding normal immune surveillance (71). Numerous inflammatory reactions in the tumor microenvironment, such as inflammatory cytokines, chemokines, reactive oxygen species, etc., directly or indirectly stimulate the tumor endothelium. Tumor endothelium has a very different genetic profile than healthy endothelium (Table 1), with the main variations influencing a number of cell adhesion molecules (such as ICAM1, VCAM1, E-selectin), antigen presentation, and chemokines (such as CCL2, CCL18, CXCL10, and CXCL11) and cytokines involved (such as TNFα, IFNγ, and IL-1) in immune cell recruitment. All of these elements have a deleterious impact on the immune surveillance of tumor cells in the tumor microenvironment. This unique character of TEC that enables them to avoid immune cell extravasation and, unresponsiveness of TECs to pro-inflammatory stimulation is Endothelial cell anergy, also called vascular immune checkpoint (72). ECs lining tumour blood arteries have a very different metabolic profile from ECs in normal tissue. A stronger dependence of tumour ECs on glucose metabolism is supported by the transcriptional elevation of the glycolytic pathway gene, PFKFB3, in comparison to other normal EC.

According to a report, tumor ECs and tumor associated macrophages (TAMs) engage in fierce competition for glucose, and the tumor ECs' intake of the metabolite boosts the angiogenic response in the tumor microenvironment (73). TECs produce energy through aerobic glycolysis and fatty acid oxidation, rather than oxidative phosphorylation. This metabolic reprogramming enables the tumor ECsto check the production of reactive oxygen species (ROS), and also allows the production of ATP more rapidly than through oxidative metabolism (74). Alam et al. (2014) has identified suprabasin as a new marker for TECs. Suprabasin, an upstream component of the AKT pathway, was substantially expressed in TECs compared to normal ECs and linked favourably with TECs' capacity for migration and tube formation (75). On the other hand, microarray and immunohistochemical studies revealed that biglycan is a specific marker of TEC and an autocrine angiogenic factor of TECs (76). Because of the aggressive behaviour of tumor endothelial cells and their particular molecular, cytogenetic, and metabolic characteristics, the tumor microenvironment can therefore selectively draw in immune suppressive cells. The role of TECs in increasing immunological suppression is discussed in the following sections of this review.

## 5 Tumor endothelium driven immunesupression and tumor progression

The success of immunotherapy depends on adequate immune cell infiltration, and low immune cell infiltration reduces the effectiveness of immunotherapy. Despite the fact that angiogenesis triggered by tumors is crucial for the growth of solid tumors, mounting evidences indicate that it also aids in immune evasion by fostering a highly immunosuppressive TME by increasing the proportion of T reg cells and MDSCs. Furthermore, by preventing dendritic cell (DC) maturation and T cell growth, VEGF impacts immunological responses in TME (77). Leukocytes are extravasated to tissues from blood arteries during typical inflammation. These leukocytes in circulation were halted and firmly adhered to the endothelium cells by a multi-step procedure. This mechanism calls for the endothelial adhesion molecules (EAM), E-selectin (rolling), ICAM1 and VCAM1 (firm rest), VE-cadherin and CD31 (trans-endothelial migration). In addition to EAMs, surface antigens (such as HLA molecules) must be upregulated, pro-thrombotic endothelial cell changes (such as the loss of the surface anticoagulant molecules thrombomodulin and heparan sulphate), cytokines (such as IL–6, IL–8, and MCP–1) production, and changes in vascular tone (such as the loss of vascular integrity and expression of vasodilators) are associated with inflammation (78). When inflammatory cytokines like TNF, IFN, and IL–1 are released, normal ECs are activated. This causes an up-regulation of adhesion molecules, which in turn triggers the extravasation of leukocytes. However, the continual secretion of pro-angiogenic molecules VEGF and bFGF in hypoxic microenvironments adversely affects this process. Even when TNF is present, they prevent ICAM1, VCAM1, and E-selectin from being overstimulated (79–81). The hypoxia inducing factors favour the heterogeneity as well as inflammation of tumor endothelial cell (82). *In vitro* and *in vivo* studies by Tellier et al. (2015) showed that ECs exposed to hypoxia expressed tumor-promoting pro-inflammatory cytokines and chemokine such as IL-6, IL-8, and CXCL1 (83). In addition, hypoxic tumor niche affects protein glycosylation and favours recruitment of immune suppressive cells to TME, thereby impacting tumor progression (84). Research shows that the tumor endothelium downregulates the expression of endothelial adhesion molecules (EAM) such as ICAM1/2, VCAM1, E-selectin, and CD34. This lessens immune cell infiltration or leucocyte extravasation in the TME, enabling the TME to adopt an immune evasion strategy and accelerate tumor growth (72, 79, 85). Hypoxic tumor microenvironment alters the tumor endothelium leading to its diversity and also induces its "stemness". This induction of stemness is favourable for cancer stem cells, which can drive tumor initiation and progression (86). Moreover, breast cancer stem cells enhance its tumorigenic phenotype (87). Cancer stem cells and their role in tumor progression is a vast horizon and beyond the scope of this review. Stemness genes such stem cell antigen-1 (Sca-1), MDR-1, and aldehyde dehydrogenase (ALDH) have been found to have elevated expression in TECs. This tumour stromal stem-like cell population affects the TEC population in the TME (57). With higher VEGF expression, ovarian, oesophageal, and colorectal tumors showed worse

TABLE 1 Factors converting normal endothelium to tumor endothelium.

| Factors converting Normal endothelium to Tumor endothelium | |
|---|---|
| Antigen presentation | ·TECs act as Antigen Presenting Cells (APCs) |
| Recruitment of immune suppressive cells | ·Chemokines - CCL2, CCL18, CXCL10, and CXCL11<br>·Cytokines - TNFα, IFNγ, and IL-1 |
| Endothelial anergy | ·Unresponsiveness to inflammatory stimulation<br>·Downregulation of adhesion molecules- ICAM-1/-2, VCAM-1, E-selectin, and CD34 |
| Higher dependence on glucose metabolism | ·Elevation of glycolytic pathway gene, PFKFB3.<br>·Aerobic glycolysis and fatty acid oxidation |
| Expression of specific markers | ·Suprabasin<br>·Biglycan |
| Overexpression of Immune checkpoint molecules | ·PDL-1 - Overexpression |
| Overexpression of molecules to prevent effector T cells | ·Overexpression of TRAIL and CLEVER |

survival rates and a higher chance of relapse. VEGF synthesis in tumors affects tumor behaviour by reducing T cell numbers in addition to promoting angiogenesis. In pre-clinical tumor models, it was found that using anti-VEGF antibodies increased the recruitment of T lymphocytes into the tumor microenvironment (88, 89). Increased nitric oxide (NO) in the TME has been found to upregulate VEGF in solid tumors and further regulate the expression of several adhesion molecules involved in the interaction between EC and leukocytes. FoxP3+ T regulatory (Treg) cells predominated and there was little CD8+ infiltration in solid tumors of both humans and mice that express specific Fas ligands in the tumor vasculature. Similarly, blockade of Fas-Fas L signalling increased intra-tumoral CD8+ T cells and subsequent reduction in tumor size (90). It is now understood that the tumor endothelium plays a crucial dual role in the development of the tumor by favouring a niche where inflammation is increased by upregulating EAMs and by negatively regulating the influx of leucocytes through endothelial anergy (Figure 3). The role of hypoxia in attracting immune suppressive cells to the TME and its relationship with cancer endothelium are discussed in the forthcoming sections of this review.

## 5.1 Myeloid-derived suppressor cells

MDSCs are one of the main immunosuppressive elements in TME. Their activation and expansion coincide with metastasis and progression in many types of cancer (91). Those patients with higher levels of MDSCs in the tumor microenvironment have exhibited higher metastatic burden and poor survival (92).Various inflammatory cytokines such as IL-13, IL-4, and transforming growth factor (TGF–β)- are linked to MDSC proliferation, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF), Prostaglandin 2 (PGE2), IL-6, stem cell factor, and vascular endothelial growth factor (VEGF) are linked to MDSC activation (93).The expression of VEGF receptor on MDSCs, explains the correlation between the up-regulation of VEGF in hypoxia and the accumulation of MDSCs in the TME (94). The presence of hypoxia in the tumor microenvironment boosts the production of CCL 5, which in turn stimulates the HIF 1α and VEGF signalling pathways. Hypoxia in the tumor microenvironment increases the production of CCL 5, and which in turn activates VEGF signalling mechanism. The expression of PD L-1 in MDSCs is increased by VEGF production in the tumor microenvironment, which limits the recruitment of cytotoxic lymphocytes (85). In line with this, a related study showed that numerous MDSC chemoattractants were upregulated in the VEGF overexpression group, suggesting that the immunosuppressive effects of VEGF are partially mediated by MDSC recruitment into the tumor microenvironment (95). Similarly, lymphatic endothelial cells (LECs) have shown to recruit MDSCs to the tumor microenvironment and increase tumor progression in TNBC cells *via* pro-angiogenic receptor CXCR2 on MDSCs. According to the study, tumor-derived vascular endothelial growth factor-C (VEGF-C) stimulated LECs to produce chemokines, which in turn helped MDSCs find their way to lymph nodes. Additionally, LEC-released chemokines increased lymphatic invasion by upregulating VE-cadherin phosphorylation

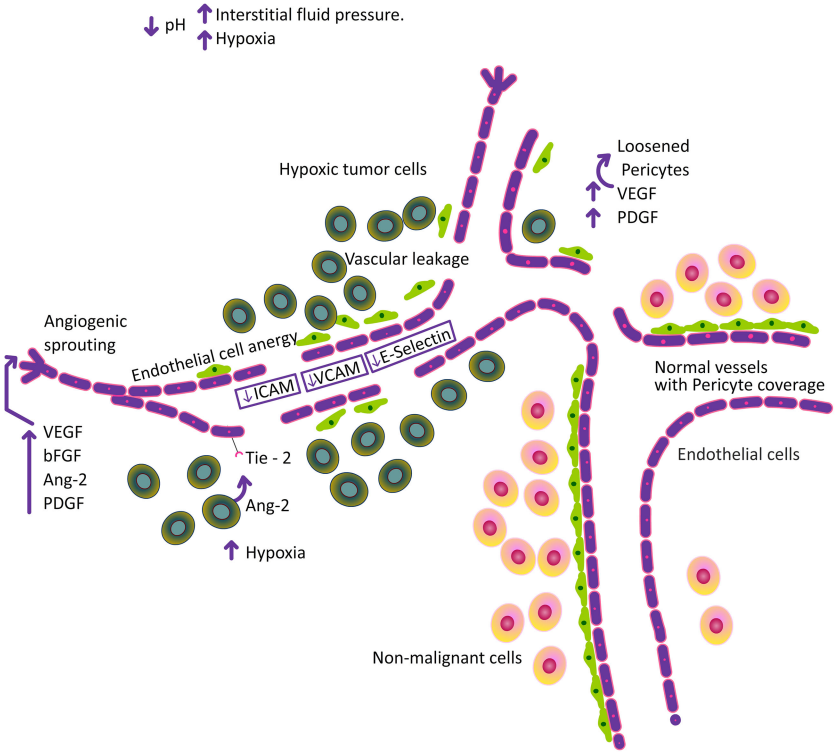


FIGURE 3
Increase in hypoxia, alter the pH and increase in interstitial fluid pressure followed by structural abnormalities in the tumor vasculature.

and junction disruption, which in turn increased serum amyloid A1 (SAA1) expression in breast cancer cells (96). In a recent study by Roberts et al. (2022) reported that co-culturing of murine TNBC-4T1 cells with MDSC and murine LECs (iLECs) in culture inserts showed an increase in MDSC invasion in the presence of iLEC (97). Monocyte/M-MDSC recruitment to malignancies is mostly facilitated by CCL2 and CCL5 (C-C motif ligand 2/5) chemokines. There is evidence that CCL2 is crucial for recruiting PMN-MDSC as well (98). Apart from endothelial cells, the absence or presence of immature pericytes, a component of vasculature also acts as a signal for MDSC recruitment in breast cancer patients (64). Taken together, MDSC play a critical role in tumor immune suppression where its activity is tightly regulated by hypoxia *via* endothelial receptors and growth factors, which overall aids in tumor progression. The trafficking of MDSCs by TECs has not been elicited clearly this far. Thus, establishing this link may offer novel targets of anti-tumor therapies.

## 5.2 Macrophages

Macrophages make up a significant portion of the leukocyte infiltrate, which is found in all cancers to variable degrees (99). Macrophages, which are derived from the blood compartment, are renowned for their flexible and variable genomes (100). Overall, macrophage matrix metalloproteinase-12 dampens inflammation and neutrophil influx in arthritis (101). Depending upon their nature of activation and the TME, macrophages can either increase or inhibit the immune responses that fight cancer. According to reports, VEGF stimulates the growth of endothelial cells in breast cancer and attracts macrophages through the VEGF receptor. The synthesis of several pro-angiogenic substances by recruited macrophages, such as VEGF, tumor necrosis factor (TNF), and thymidine phosphorylase (TP), is able to accelerate angiogenesis in the TME. This is a form of mutual activation/dependence between TAM and tumor endothelial cells in the TME (102). Tumor cells and stroma, which make up the hypoxic core of the tumor microenvironment, promote the production of VEGF, CCL2, CCL5, CSF-1, EMAP-II, endothelin-2, SEMA3A, oncostatin M, and eotaxin. The overproduction of migratory molecules promotes macrophage infiltration of the tumor (103).Macrophages can be classified as pro inflammatory-immune stimulatory (M1) or alternatively activated anti-inflammatory-immunosuppressive (M2) macrophages depending on the environment in which they are recruited.It has been shown that the recruitment of TAM into the TME is a poor prognostic indicator for overall survival and treatment effectiveness (104). In addition, non-responsive tumor EC-derived IL-6 is a cytokine that encourages macrophage M2-like polarisation in the tumor microenvironment. The EC biomarker ESM1, which is linked to a poor prognosis in human gastrointestinal and hepatocellular carcinomas, is also highly expressed in tumor ECs in a number of mouse tumor models. Furthermore, studies have shown that ESM1 induces ECs to express ICAM1, which draws and polarises M2 macrophages toward the tumor microenvironment (105–107). As tumors grows, increased levels of hypoxia cause M1-polarized macrophages to secrete less pro-inflammatory mediators including IL-1, TNFα, and CCL17 and accelerate macrophage differentiation toward the M2-like phenotype. Despite the fact that hypoxia does not affect the proportion of different macrophage subsets, it does cause the M2-like macrophage subset to activate the transcription of pro-tumor genes, including growth factors like FGF2, PDGF, and VEGF (108, 109). Therefore, the dichotomy in macrophage differentiation into M1 and M2 type can have varied outcome on tumor progression *via* hypoxia and angiogenesis machineries (110). The polarisation of macrophages from the anti-tumor M1 phenotype to the pro-tumor M2 phenotype is clearly influenced by hypoxic stress. Focusing on TAMs that are produced by hypoxia and their trafficking across tumor endothelium may be beneficial given that it has been suggested that TAMs and cancer endothelium interact. The elucidation of this mechanism might produce brand-new indicators and therapeutic outcomes that are promising.

## 5.3 T cells

Hypoxic TME can regulate T cell response in two major ways depending upon the T cell subset. Effector T cells, in the form of $CD4^+$ and $CD8^+$ T cells, play a critical role in resolving tumor growth by releasing inflammatory cytokines or by direct lysis of tumor cells. However, these effector T cell responses undergo immune suppression in the tumor microenvironment which is typically hypoxic (111). Multiple factors such as tumor growth factor (TGF-β), IL-10, VEGF, indoleamine 2, 3-dioxygenase (IDO) and arginase contribute to their immune suppression (112). Another mechanism by which hypoxia mediated immune suppression in effector T cells is through the loss of expression of co-stimulatory molecules (CD80, CD86 and CD40) on dendritic cells that interact with T cells and promote immune tolerance in them (113). Cytotoxic $CD8^+$ T cells are capable of infiltrating tumors making them critical for tumor clearance. However, under hypoxic conditions, these $CD8^+$ T cells also undergo immune suppression *via* defective antigen presentation of tumor cells through low MHC expression, down regulation of transporter associated with antigen processing protein (TAP) and tumor antigen (114–116). This tumor hypoxia specifically promotes the immunosuppressive function of T regulatory cells including its migration and activation at the tumor site (117). Treg cells are characterized by the expression of specific cell surface molecules (such as CD25, GITR, CTLA4) and nuclear transcription factor (FOXP3) (118), mediate immune suppression by downregulating activated T cell function through increased production of immunosuppressive cytokines such as IL-10 and TGF-β or *via* interaction between CTLA-4 on Tregs and CD80/86 on antigen presenting cells or by sequestering IL-2 from naïve T-cells by its IL-2 receptor (CD25) on Tregs (119). Hypoxia induced HIF-1α results in increased expression of FOXP3 in Treg cells (120) and at the same time, also promotes CCL28 in the tumor microenvironment. This CCL28 binds to its cognate receptor CCR10 on Treg cells and thereby promotes the migration of Treg cells (117). HIF-2α is also involved in Treg stability as HIF-2α-deficient Tregs are functionally defective at suppressing effector T-cell function (121). Tumor infiltration by T lymphocytes has shown to increase overall

survival in different types of cancer such as colorectal, ovarian, breast and melanoma. Tumor endothelial cells act as a major barrier for the extravasation of effector T lymphocytes into the tumor niche through the downregulation of ICAM1 and VCAM1. Furthermore, TECs can increase the expression of molecules such as common lymphatic and vasculature endothelial receptor 1 (CLEVER1) on their surface to recruit immunosuppressive Treg cells (80). (Figure 2) Interestingly, the expression of TRAIL and FasL on TECs selectively kill effector T cells while not hampering Treg cells. The pre-clinical studies by actively immunizing tumor endothelial expressing antigens *via* DNA vaccines and protein pulsed DCs successfully inhibited tumor growth and increased the infiltration of $CD8^+$ T cells in the TME. Also, DNA vaccines targeting tumor endothelial marker 1 (TEM1), specific TEC expressing antigen could escalate intratumoral infiltration of endogenous CD3+ T cells (122). Thus, tumor induced hypoxia and tumor associated endothelial cells mediate immune suppression by directly or indirectly regulating T cell functions, which promotes tumor growth and invasiveness.

## 5.4 Tregs

Immunosuppressive T cells known as regulatory T (T reg) cells control homeostasis and self-tolerance by limiting erroneous immune responses (123). One of the essential immune cells favouring tumor growth and regulating the immunesurveillance is the CD4+CD25+Foxp3+ Treg cell (124). FoxP3, a sign of Treg activity, has been proposed as a marker of tumor progression and metastasis in breast carcinoma. In order to determine the progression and prognosis of BC, measuring Tregs recruitment and activity has been proven to be a useful approach (125). Hypoxia has been proven to associate with the infiltration of regulatory T cells in breast tumor microenvironment by the upregulation of CXCR4 receptor on Tregs. Hypoxic stress induced expression of CXCR4 by activating HIF pathway has been reported in different stromal cells including endothelial cells in TME. By increasing the expression of FOXP3, a lineage transcriptional regulator of Tregs, HIF-1 may also indirectly stimulate CXCR4 expression.

By increasing the expression of FOXP3, a lineage transcriptional regulator of Tregs, HIF-1 may also indirectly stimulate CXCR4 expression. Thus, there are opportunities for clinically targeting Tregs by blocking CXCR4 to stratify patients for anti-HIF therapies (126). Mounting evidences suggests the prime role of hypoxia in stimulating the secretion of cytokines and chemo- attractants from cancer cells and tumor associated macrophages, including CCL28, CCL22 and IL-10, that recruit Treg cells from the circulation. Hypoxia induces the expression of CD 73 on various cell types including T regs, and actively involves in the generation of immunosuppressive metabolite adenosine which negatively affects T cell function (127, 128). Treg infiltration in tumor locations can be correlated with increased microvessel density and upregulation of angiogenesis indicators like VEGF in breast and endometrial malignancies, illustrating the relation between Tregs and tumor angiogenesis (127). According to Andrea Facciabene et al., hypoxic intraperitoneal tumors recruit Treg cells, which impair effector T cell activity and promote tumor angiogenesis *via* VEGF-A. Furthermore, CCL28 secreted by hypoxic tumor cells attract Treg cells in to the tumor niche. Treg cells can directly contribute to the overproduction of VEGF-A and can promote the proliferation and recruitment of endothelial cells (129). In fact, Tregs contribute to angiogenesis indirectly by inhibiting Th1 effector T cells and secreting interferon-induced chemokines like CXCL9, 10 and 11 as well as angiostatic cytokines like TNF and IFN. Another work by Andrea Facciabene et al. shown that depletion of Treg cells reduce VEGF upregulation and angiogenesis (52). Increased expression of CCR8 was noticed in tumor infiltrating Tregs compared to circulating T regs. A promising immunotherapeutic strategy for the treatment of breast cancer would involve targeting CCR8 to prevent the migration of tumor-resident Tregs (130). Targeting T reg cells in tumors using selective immunotoxin against CD 25 (Treg marker) increased CD8+ T cell-dependent antitumor immune response in experimental tumor models (131). T reg cell elimination and subsequent anti-VEGF therapy, restored IFN-production in CD8+ T cells and enhanced the antitumor response from anti-VEGF therapy in tumors (132). Although there are lot of studies depicting hypoxia mediated T reg infiltration, there are no confirmatory studies on the role of hypoxic cancer endothelium, especially breast cancer endothelium in recruiting regulatory T cells to the TME.

## 5.5 Immune checkpoint inhibitors

Recently, immune checkpoint blockade therapy has gained attention since it allows patients' natural immune systems to combat cancer. Immune checkpoint molecules like CTLA4, PD1, PDL–1, LAG3, and TIM–3 inhibit the immune response in different tumor types at different phases of tumor development (133). A significant development in the field of cancer immunotherapy is the discovery ofproteins like programmed cell death protein 1 (PD1), programmed cell death ligand 1 (PDL–1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA4). These molecules block the signals that result from the activation of the T cell receptor (TCR), which eliminates cytotoxic T cells (CTLs) and blocks anti-tumor immunity.

The FDA recently approved the use of two mouse antibodies (immune checkpoint inhibitors) known as anti-CTLA-4 and anti-PD-1 for the treatment of humans (134). Immune checkpoint (IC) molecules are found in TME cells including cancer cells, immune cells, and stromal cells like TEC. It has been discovered that TECs express PD-L1 and PD-L2 along with other well-known immunoinhibitory molecules such TIM-3, which raises the possibility that they may be able to directly suppress T cell activation when the vasculature is present. It has been shown that pro-inflammatory cytokines like IFN and TNF encourage PDL–1 up-regulation on ECs. Although the most often used biomarker in immune-oncology to determine treatment options and patient stratification is now PDL–1 expression by cancer cells, its therapeutic value has not yet been determined (82). Studies conducted *in vitro* and *in vivo* by Barsoum I et al. gave the information for the upregulation of PDL-1 in an HIF dependent

way. By utilising glyceryl trinitrate (GTN), an agonist of nitric oxide (NO) for signalling, they were also able to prevent the HIF-1 accumulation and the hypoxia-dependent PDL-1 production and Cytotoxic T Lymphocyte resistance (135). The expression of PD-L1/2 may begin to make T lymphocytes lethargic and exhausted before they even enter the cancer microenvironment. In the pancreatic neuroendocrine tumour model and the polyoma middle T oncoprotein (PyMT) breast cancer model (RT2-PNET), the concomitant administration of anti-VEGFR2 and PDL–1 antibodies promoted the development of specialised vessels known as High Endothelial Venules (HEVs), which support lymphocyte trafficking and enhance T-cell infiltration. Even before T cells penetrate the tumour microenvironment, the expression of PDL–1/2 may start to cause them to become anergic and worn out. In the pancreatic neuroendocrine tumour model and the polyoma middle T oncoprotein (PyMT) breast cancer model (RT2-PNET), the combination of both anti-VEGFR2 antibodies and PDL–1 antibodies stimulated the formation of specialised vessels called High Endothelial Venules (HEVs), which contribute to lymphocyte trafficking and improved T-cell infiltration (129).

It has proven that Immune checkpoint inhibitors or drugs could increase immune surveillance. Thus, combination therapies targeting Immune checkpoint and metabolism of cancer endothelium could be a promising strategy to reduce tumor progression.

## 6 Trans-endothelial migration

Due to metastasis and disease recurrence, breast cancer is the cancer that claims the lives of more women than any other (136). Journey of tumor cells across the endothelial membrane is a key step in the process of tumor invasion and metastasis. Tumor cells cross the vascular membrane stimulated by several cytokines and growth factors such as Transforming Growth Factor-Beta (TGF-β) superfamily of proteins,Bone Morphogenetic Proteins (BMPs). This is called Endothelial-mesenchymal transition (EndMT). EndMT induced by TGF-β shows a decrease in the expression of endothelial markers such as VE-cadherin, claudin and zona-occludens 1 (ZO-1) and an elevation in EMT transcription factors such as Snail, Slug and ZEB-1 which marks metastasis. TGF-β promotes ECs to change into CAF-like cells, which results in the loss of endothelial adhesion molecules and remodelling of the endothelium cytoskeleton *via* the Rho and Rac-1 signalling pathway, which is the primary characteristic of EndMT (76, 137, 138). It has been found that hypoxia associated with inflammation or tissue damage can also cause EndEMT (139). Rokana, et al. (2021) have recently studied the role of ICAM in trans-endothelial migration in breast cancer and also assessed the therapeutic efficacy of anti-ICAM1 neutralizing antibody on breast tumor cell aggregation and trans-endothelial migration. This anti-ICAM treatment inhibited the cluster formation of TNBC cells in suspension. Clinical data also showed high levels of ICAM1 mRNA expression in breast tumors which might mediate distant metastasis. This makes ICAM as a potential therapeutic target in TNBC metastasis (140). S100P and Ezrin, two members of the S100 family of short calcium binding proteins, also encourage the trans-endothelial migration of triple negative breast cancer cells. Furthermore, S100P activity has been linked to a variety of malignancies, a poor prognosis, metastasis and recurrence, and a low rate of survival in TNBC patients (141). An investigation on the SDF-1/CXCR4 axis in a breast carcinoma model revealed their role of hypoxia. It was confirmed that in a hypoxic tumor niche activation of HIF leads to the transcription of an array of HIF target genes including SDF-1 and CXCR4 which contributes to tumor cell migration and adhesion to endothelial cells in breast cancer cells. Moreover, the study also illustrated that SDF-1 binding to CXCR4 stimulated tube formation in endothelial cells, which point towards its role in angiogenesis and trans-endothelial migration (142). Since metastasis and related poor survival is a hallmark of breast cancer, pathways targeting trans-endothelial migration could have a significant impact in clinical trials.

## 7 Recent strategies to overcome hypoxic endothelium driven immunosuppression

The hypoxic microenvironment and impaired immune response to cancer cells by innate immune cells continue to be major obstacles for immunotherapy. Recent advancements in nano-immunotherapy, however, might lessen immunosuppression brought on by hypoxia and enhance systemic antitumor immune responses to eradicate metastatic breast cancer cells. Similar to this, *in situ* $O_2$ generation, $O_2$ delivery, normalisation of tumor vasculature, and mitochondrial-respiration inhibition could be the alluring therapy to overcome hypoxia-driven immune suppression for preventing the growth and progression of metastatic breast cancer cell lines. Monoclonal antibodies, immune modulators, and biodegradable bio-nanomaterials could address the problems in a very satisfactory way when used in conjunction with such methodologies. Nowadays, there is a great deal of interest in using nanomaterials as treatments to target the downstream pathways that lead to hypoxia-driven immune suppression in breast cancer (143). In this sense, PD1 receptor antagonist delivered in nanoform has the potential to effectively address the problem of hypoxic endothelium-driven immunosuppression during the treatment of breast cancer. The specific ligand antagonist of the upstream hyper-expressive biomarkers, which is responsible for the immunosuppressive state caused by hypoxic endothelium around the breast cancer microenvironment, can be used to develop new strategies. This may be a new strategy to improve immune monitoring in the treatment of breast cancer, especially for managing disease progression. Additionally, by preventing hypoxia in TME, such an approach could activate the immune cells, strengthen immune surveillance, and destroy breast cancer cells. By disrupting the hypoxia-mediated cancer signaling pathway, they may specifically target the hypoxic TME (144). Through the generation of oxygen-derived free radicals, radiation destroys tumor cells by causing DNA fragmentation. Due to the absence of oxygen and a reduction in DNA repair processes, hypoxia confers resistance to radiation therapy and reduces the

effectiveness of radiation. The main cause of chemotherapy resistance in hypoxic tumors is the fact that many of the commonly used medications need oxygen to release the deadly free radicals that kills the tumor cells (145). In addition, another study that looked at the connection between HIF-1α/CAIX and the response to epirubicin found a strong correlation between high HIF-1α expression and a subpar response to treatment (146). This gives a clear insight that hypoxic pathway is involved in significant events that have a direct impact on the efficacy of numerous treatment methods. In the earlier sections of the review, we addressed the role of pro-angiogenic factor stimulation that causes tumor-associated endothelial cells to become anergic, losing the capacity to react to inflammatory signals and rendering them unable to activate EAMs (79, 147). It was discovered that this anti-infiltration barrier helped tumors avoid being destroyed by the immune system. Consequently, it is thought that using angiogenesis inhibitors to encourage leukocyte infiltration into the tumor is a useful way to increase the effectiveness of ICIs (148, 149). Bevacizumab, a VEGFA neutralizing antibody, has been shown to increase the number and activation of DC (149–151) as well as the number of cytotoxic T cells (152, 153) and to reverse VEGF-induced T cell exhaustion (154). Whereas, Sutinib, a TKI of VEGFR and other kinases, was shown to decrease the number of MDSCs and T reg cell (155, 156). Xiaodong et al. (2020). identified a tumor endothelial specific marker CLEC14A, which specifically recruits T reg recruitment and subsequently enhance immune suppression in the TME. CLEC 14 A specific CART cells, exhibited substantial decrease in tumor growth through IFN-γ indicating their antitumor potential (157).

## 8 Future perspectives

TNBCs exhibit a markedly increased HIF transcriptional activity and a subpar response to the existing therapeutic strategies (158). Therefore, it makes sense to speculate that a novel therapeutic approach to treat TNBCs might involve targeting hypoxia in the TME. Preclinical investigations indicate that the combination of cytotoxic chemotherapy with molecules that block HIFs is particularly promising. Therapeutic studies with Digoxin and acriflavine, two HIF-1α inhibitors, demonstrated reduced initial tumor development, vascularization, invasion, and metastasis in breast cancer animal models. Furthermore, digoxin prevents HIF–1α dependent transcriptional responses that encourage cancer stem cell (CSC) resistance to chemotherapy, which causes tumor regression in TNBC when combined with paclitaxel or gemcitabine (159). Numerous synthetic and natural substances have been shown to block the regulation of HIF-1 on downstream target genes by lowering HIF-1 mRNA levels, accelerating the protein's breakdown, and preventing HIF-1 and HIF-1 dimerization (160, 161) (Table 2). An effective anticancer therapy may include normalizing the tumor vasculature rather than destroying it. Anti-angiogenic drugs must be dosed carefully during vascular normalization in order to reverse the aberrant phenotype of the tumor vasculature and increase blood flow and oxygenation. Through vessel maturation and the alleviation of immunosuppression brought on by hypoxia and/or VEGF, it has been demonstrated that vascular normalization enhances immunological responses (16, 162). It indicates that restoring the structural and functional integrity of the tumour vasculature is a viable method for polarising TAMs to an anti-tumor phenotype (163). The hypoxic tumor endothelium is hyperglycolytic, thus, targeting tumor endothelial metabolism might offer a novel therapeutic strategy. Glycolysis is the energy source for endothelial sprouting in angiogenesis rather than oxidative phosphorylation for ATP production. Thus, blocking PFKFB3, a key molecule involved in endothelial glycolysis pathway, reduced vessel sprouting and angiogenesis (164). VEGF and PFKFB3, which are both implicated in the TEC's glycolytic pathway, were downregulated when tumor-cell-specific cyclooxygenase (COX-2) was pharmacologically inhibited. The restoration of glucose metabolism in TECs and the reduction of tip cells, filopodia, and branching are affected by COX-2 inhibitor therapy-induced inhibition of PFKFB3-mediated endothelial cell motility (165). The role of anti-angiogenic drugs in regulating the tumor vasculature and reducing hypoxia-induced immune suppression has been shown in a number of preclinical investigations. These anti-angiogenic therapies have also been successful in overcoming endothelial anergy, which results in normalized production of endothelial adhesion

TABLE 2 Hypoxia and vascular targeting compounds.

| HIF Targeting Novel Compounds | Action | Vascular targeting Compounds | Action |
|---|---|---|---|
| Digoxin<br>Acriflavine | Prevents HIF -1 α dependent transcriptional responses -Tumor regression<br>Prevents dimerization of HIF-1α – Prevents tumor vascularization | Bevacizumab - VEGFA neutralizing antibody | Increase the number of DCs, Tc cells |
| Sanguinarine,<br>Elemene (C15H24),<br>Isoliquiritigenin (ISL),<br>Cardamonin,<br>Anhydroicaritin (AHI),<br>Melittin (MEL),<br>Fucoidan,<br>Curcumin,<br>Arsenic sulfide (As4S4),<br>Acriflavine, Ganetespib, and Echinomycin | Prevents the dimerization of HIF-1α and HIF-1β | Sutinib - TKI of VEGFR | Decrease the number of MDSCs and Treg cells |
| | | TEC specific marker - CLEC 14 A specific CART cells | Reduction in tumor growth via IFN-γ |
| | | Tumor-cell-specific cyclooxygenase (COX)-2 Inhibitor | Downregulation of PFKFB3, involved in hyper-glycolysis of TEC<br>Downregulation of VEGF |
| | | PFKFB3 blocker - 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (**3PO**) | Reducing hyper-glycolysis in TECs<br>Normalisation of tumor vessels |

molecules, which are necessary for leukocyte trans-endothelial migration into the TMC. Treatment that targets the VEGF pathway may result in increased immune infiltration and ICAM1 overexpression in renal cell carcinoma (166). Since TECs have higher glycolytic rates than typical proliferating ECs, reducing glycolysis by blocking PFKFB3 with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) may be able to suppress tumor growth. According to studies, 3PO therapy increased the effectiveness of chemotherapy, aided in the normalisation of tumor vessels, tightened the EC barrier to prevent cancer metastasis, and encouraged tumor vessel normalisation. In mouse tumor models, proliferating ECs were treated with the weak mitochondrial uncoupler Embelin, which resulted in reduced mitochondrial oxidative phosphorylation, inhibited tumor growth, and reduced microvessel density (164). The endothelium of tumors significantly differs from endothelium of healthy tissues in a number of aspects, including metabolic reprogramming, alternative morphology, cytogenetics, and molecular genetics (82). So, a viable strategy to restore normal tumor vasculature involves attacking tumor endothelium. The research carried out by our group has also shown a rise in the infiltration of effector T cells, which in mouse TNBC inhibit the pro-angiogenic CXCR2 receptor. Targeting CXCR2 or other pro-angiogenic expressed on tumor endothelium may produce breakthrough outcomes in accordance with the promising results. (Unpublished data). Similar results were seen *in vitro* when a novel synthetic quinoline derivative was administered to block the pro-angiogenic chemokine (Unpublished data). Targeting the molecules involved in the regulation of tumors would open up the potential of a wide range of therapeutic options, normalising vascular function, and improving immune surveillance. Future therapeutic implications may benefit from knowledge of the variations in chemokines or pro-angiogenic substances produced by NECs and TECs in a hypoxic environment, as well as the expression of these receptors in normal and malignant cells.

## 9 Conclusion

In the light of the foregoing discussion, it will be wise to more effectively employ therapeutic alternatives in BCs with treatment resistance. Understanding the control of selective immune cell trafficking *via* hypoxic tumour endothelium may also be necessary. Clinical studies have found that the early preventative methods for the development of new blood vessels have only limited effects. The establishment of resistant mechanisms and enhanced tumor hypoxia are the causes of this insufficient efficacy. The development of effective treatment combinations can be facilitated by an understanding of how tumors use hypoxic endothelium cells to evade the immune system. Hypoxia regulating molecules and Immune Checkpoint Inhibitors (ICIs) should be combined to improve the prognosis of patients with hypoxic breast cancer in the light of the role played by the hypoxic TME in immune evasion. Therefore, molecules linked to selective trafficking may offer brand-new prognostic criteria to justify the use of particular immunotherapy regimens in conjunction with vascular targeting therapeutics. Though it will take time to investigate and comprehend such issues, promising research on anti-angiogenic adjuvant immunotherapy techniques offers hope for the improvement of treatment ways to improve the outcomes of breast cancer patients. A concerted effort from all investigators is imperative to usher in an era free from the woes of life-threatening diseases such as the BCs.

## Author contributions

SB and PP conceived the concept and contributed writing and editing of the review. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Glossary

| | |
|---|---|
| TME | Tumor Microenvironment |
| BC | Breast Cancer |
| HIF | Hypoxia Inducible Factor |
| VHL | Von-Hippel Lindau |
| EMT | Epithelial to mesenchymal transition |
| EMT-TFs | EMT transcription factors EMT transcription factors |
| TNBC | Triple negative breast cancer |
| TGF-β1 | Transforming growth factor-β1 |
| SMAD3 | Suppressor of Mothers against Decapentaplegic |
| GLUTs | Glucose Transport Proteins |
| HK1, 2 | Hexokinase 1 and 2 |
| PDK1 | Pyruvate Dehydrogenase Kinase 1 |
| ECM | Extra Cellular Matrix |
| ROS | Reactive Oxygen Species |
| BCSCs | Breast cancer stem cells |
| MMP-2 and MMP-9 | Metalloprotease 2 and 9 |
| VEGF | Vascular Endothelial Growth Factor |
| TILs | Tumor Infiltrating Lymphocytes |
| TCR | T-cell receptor |
| IFN | Interferon |
| MHC | Major Histocompatibility Complex |
| MDSCs | Myeloid-Derived Suppressor Cells |
| Tregs | T regulatory cells |
| TH1 | T-helper type 1 |
| TH2 | T-helper type 2 |
| TAMs | Tumor-Associated Macrophages |
| NK cells | Natural Killer Cells |
| PDL-1 | Programmed Death Ligand-1 |
| CXCL12 | C-X-C Motif Chemokine Ligand 12 |
| CXCR4 | C-X-C Motif Chemokine Receptor 4 |
| CXCR12 | C-X-C Motif Chemokine Receptor 12 |
| CCL5 | CC-chemokine Ligand 5 |
| CCL2 | CC-chemokine Ligand 2 |
| bFGF | basic Fibroblast Growth Factor |
| PGF | Placental Growth Factor |
| Ang-2 | Angiopoietin-2 |
| ECs | Endothelial Cells |
| ICAM1 | Intercellular Adhesion Molecule 1 |
| VCAM1 | Vascular Cell Adhesion Molecule 1 |
| TNFα | Tumor Necrosis Factor α |
| IFNγ | Interferon γ |
| IL-1 | Interleukin 1 |
| PFKFB | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 |
| Sca-1 | stem cell antigen-1 |
| MDR-1 | Multi-Drug Resistance Mutation 1 |
| ALDH | Aldehyde Dehydrogenase |
| NO | Nitric Oxide |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |
| PGE2 | Prostaglandin 2 |
| LECs | Lymphatic Endothelial Cells |
| SAA1 | Serum Amyloid A1 |
| TEM1 | Tumor Endothelial Marker 1 |
| CTLA4 | Cytotoxic T Lymphocyte-associated Antigen 4 |
| TCR | T cell receptor |
| HEVs | High Endothelial Venules |
| EndMT | Endothelial-mesenchymal transition |
| ICIs | Immune Checkpoint Inhibitors |

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# Hypoxia: syndicating triple negative breast cancer against various therapeutic regimens

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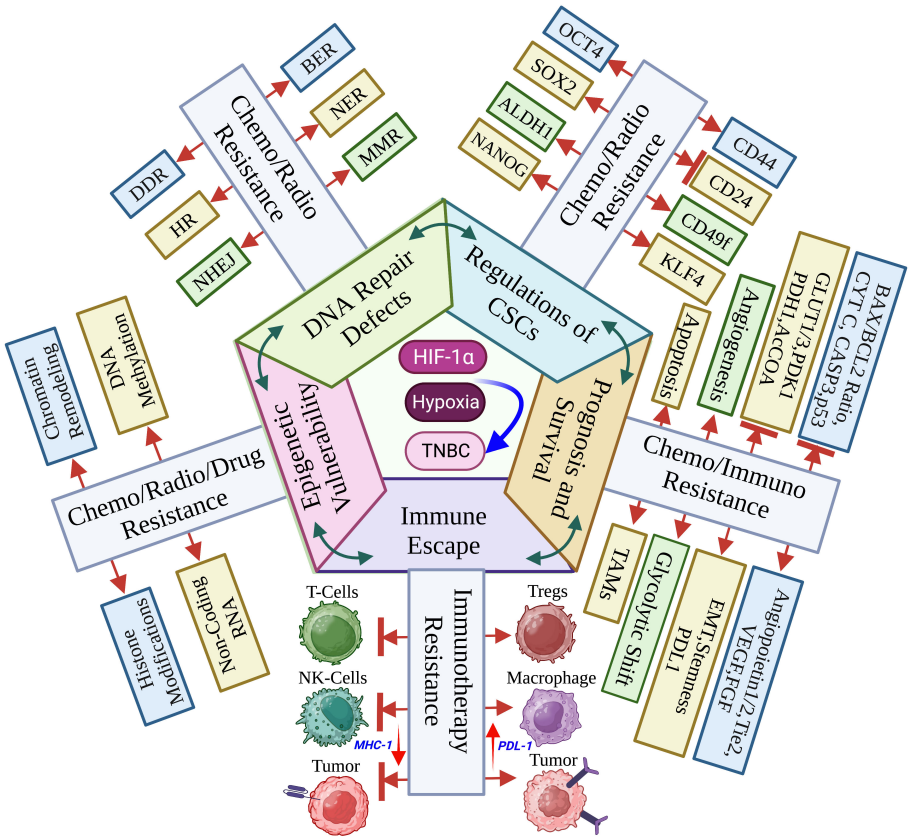
Triple-negative breast cancer (TNBC) is one of the deadliest subtypes of breast cancer (BC) for its high aggressiveness, heterogeneity, and hypoxic nature. Based on biological and clinical observations the TNBC related mortality is very high worldwide. Emerging studies have clearly demonstrated that hypoxia regulates the critical metabolic, developmental, and survival pathways in TNBC, which include glycolysis and angiogenesis. Alterations to these pathways accelerate the cancer stem cells (CSCs) enrichment and immune escape, which further lead to tumor invasion, migration, and metastasis. Beside this, hypoxia also manipulates the epigenetic plasticity and DNA damage response (DDR) to syndicate TNBC survival and its progression. Hypoxia fundamentally creates the low oxygen condition responsible for the alteration in Hypoxia-Inducible Factor-1alpha (HIF-1α) signaling within the tumor microenvironment, allowing tumors to survive and making them resistant to various therapies. Therefore, there is an urgent need for society to establish target-based therapies that overcome the resistance and limitations of the current treatment plan for TNBC. In this review article, we have thoroughly discussed the plausible significance of HIF-1α as a target in various therapeutic regimens such as chemotherapy, radiotherapy, immunotherapy, anti-angiogenic therapy, adjuvant therapy photodynamic therapy, adoptive cell therapy, combination therapies, antibody drug conjugates and cancer vaccines. Further, we also reviewed here the intrinsic mechanism and existing issues in targeting HIF-1α while improvising the current therapeutic strategies. This review highlights and discusses the future perspectives and the major alternatives to overcome TNBC resistance by targeting hypoxia-induced signaling.

KEYWORDS

**Hypoxia, HIF-1, TNBC, immune escape, DNA damage response, chemotherapy, immunotherapy, cancer vaccines**




GRAPHICAL ABSTRACT
Hypoxia within the TNBC tumor microenvironment stabilizes the HIF-1α to decide the cancer cell fate, genetics, metabolism, immune response and clinicopathology. (Created via BioRender.com).

## 1 Introduction

Breast cancer (BC) is the 2nd most common leading cause of cancer-related deaths, mostly diagnosed in young women. It accounts for over 43,000 estimated deaths annually among women in the US alone (1, 2). The stratification of breast carcinoma involves histological features, including the expression of markers such as estrogen receptor (ER) (3), progesterone receptor (PR) (4), and human epidermal growth factor receptor 2 (hEGFR2) (5). Furthermore, six intrinsic subtypes of TNBC, such as basal like, HER2 enriched, luminal A, luminal B, normal like and claudin low have been identified by high-throughput transcriptomic and genomic sequencing (6). These subtypes display distinct features in molecular portraits as well as clinical outcomes (5, 7, 8). TNBC is the small claudin-low subset of BC. It has a high histological grade, high epithelial-mesenchymal transition (EMT) marker enrichment, and high metastasis rates, including aggressive cancer stem cell-like features. In addition, they also have low luminal differentiation power and low expression of cell-cell adhesion molecules but are highly hypoxic in nature, making TNBC the most aggressive and deadliest subtype of BC (9–12). The term "negative" in TNBC refers to a very uncommon BC subtype that does not express ER, PR, and hEGFR2 (11, 13–16).

Epidemiological data analysis reveals that premenopausal women under the age of 40 are the primary suspects of TNBC occurrence, and approximately 20% of all BC patients are under the young age (13, 14). The TNBC patient's survival time is comparably shorter than that of patients with another subtype of BC. The mortality rate of TNBC patients is also significantly high, and around 40% of deaths occur in TNBC patients within five years after the first diagnosis (13, 15). TNBC is a highly heterogenous subtype, and because of its aggressiveness and invasiveness, approximately 46% of TNBC patients have distant metastasis. Patients diagnosed with TNBC are more likely to develop distant metastasis within three years of diagnosis. Besides, the overall survival rate of metastatic patients is low, and based on the available data, the average median survival is only 13.3 months. The studies also demonstrate that the chances of tumor recurrence after surgery are as high as 25%. Brain and visceral organ metastasis also have been reported in metastatic TNBC patients. Most distant metastasis happen in the third year following diagnosis (16–18). TNBC patients have a shorter average time to relapse (19–40 months) than non-TNBC patients (35–67 months). According to published statistics, the death rate of TNBC patients after tumor recurrence is as high as 75% compared to non-TNBCs (16, 19).

Since heterogeneity, aggressiveness, and hypoxia create a favourable microenvironment for TNBC to grow and spread faster than other types of invasive BC, therefore, planning an effective treatment strategy for TNBC patients' remains a herculean task. Although, in recent years, much research has been focused on identifying the specific targets of TNBC, the need for well-defined molecular targets in TNBC has resulted in limited therapeutic options. Currently, TNBC patients treatment mainly relies on standard therapies for TNBC, such as surgery, chemotherapy (CT), radiotherapy (RT), and photodynamic therapy (PDT) (20, 21). Therefore, identifying new therapeutic targets for TNBC is an urgent need and a high priority for society. Studies have investigated and identified several therapeutic molecules targeting oncogenic signaling pathways, including the PI3K/AKT/mTOR pathway and Src/Wnt signaling, to check their effectiveness in treating TNBC (22, 23). In addition, the alterations of BC genes 1 and 2 (BRCA1/2) and DNA damage-responsive (DDR) genes, including dysfunction of epigenetic and immune regulators, have also been used as an inhibitory index to predict treatment response in TNBCs (13, 24). Moreover, several studies also demonstrated a promising result by following a combination drug therapy strategy where they use targeted cancer drugs combined with chemotherapy or radiotherapy, and a few are in clinical trials (25, 26). Although the current treatment strategies are significantly effective, unfortunately, the overall treatment outcomes are highly variable, and it could be because of the highly heterogeneous nature of TNBC. Therefore, there is an urgent demand for alternative and accurate therapeutic strategies with improved efficiency, either alone or in combination with other therapies. HIF-1 is a key heterodimeric transcription factor of hypoxia. It consists of an oxygen-sensitive α subunit and a constitutively expressed β subunit. It is the master regulator to induce oncogenes and inactivate tumor suppressor genes functionality. It is widely regulated by inflammatory mediators released by tumor stromal cells TNBC that allow cellular adaptation against hypoxia (27, 28). Several studies established and proved that an intra-tumoral hypoxic environment creates a negative impact on the survival of BC patients and is associated with tumor aggressiveness and heterogenic phenotypes, which further induce a high risk of metastasis and provide a shielding barrier against various therapies such as chemotherapy, radiotherapy, and immunotherapy which suggest that hypoxia makes TNBC resistant to different treatments (28–31). Available evidence also supports the hypothesis that the elevation of HIF-1α expression in TNBC may provide a suitable environment for TNBC to grow in hypoxic conditions (32). Therefore, targeting hypoxic cancer cells seems to be a plausible idea for treating TNBC. Studies have also revealed that in TNBC, HIF-1α regulates the various complex biological processes and activates the transcription of several target genes involved in regulating angiogenesis, cellular metabolism, stem cell differentiation, and immune cell migration. The activation of these pathways further induces the expression of downstream gene products associated with stemness and EMT that have been further proven to be hyperactivated in TNBC by various research groups (33–35). Therefore, targeting HIF-1α could be a significant potential therapeutic option.

The pattern of HIF-1 expression in TNBC as well as the mechanism by which HIF-1 accelerates the disease are reviewed. This review also examines how breast cancer stem cell (BCSC) enrichment and immune evasion are affected by HIF-1 in the regulation of angiogenesis, invasion, and metastasis. It has also been investigated how HIF-1 affects TNBC through chemotherapy, immunotherapy, anti-angiogenic therapy, adjuvant therapy, PDT, adoptive cell therapy, antibody drug conjugates, cancer vaccines and also in combination therapies. The internal mechanisms as well as prospective therapeutic medicines that target HIF-1 are also reviewed.

## 2 The linkage between TNBC and hypoxia

The hypoxia-related mechanism is one of the distinguishing features of the cancer signaling system (34, 36). Each stage of the metastatic process is constrained by the hypoxic tumor microenvironment, which also regulates different cancer phenotypes (31, 37). Intratumorally, hypoxia is the major critical microenvironmental factor that is associated with TNBC and its invasiveness, metastasis and mortality (38). Additionally, disorganization of the tumor vasculature during tumor growth, is also associated with fluctuation of oxygen and glucose levels, leading to a heterogenous state of hypoxia, aerobic and anaerobic glycolysis (39, 40). Therefore, it is fundamental to correctly define the term 'hypoxia', presumed as an unusual system accompanying advanced malignancy by absolutely different mechanisms like chronic permanent inflammation or cell death pathways. Yet, the local hypoxia is deliberately produced by tumor cells to induce angiogenesis, hence directing the growth factors (30, 41). In chronic hypoxia, cells remain in a state above the diffusion limit of oxygen due to increased distance caused by tumor expansion (42, 43). This oxygen fluctuation within the tumor stipulates cancer cells for both aerobic and anaerobic glycolysis (44). Amplified glycolysis with or without oxygen is an important indicator for cancer and serves as a connecting link between TNBC and hypoxia (45, 46). Hypoxic microenvironment response in TNBC is tightly regulated by HIFs, which contain either HIF-1α or HIF-2α with a constituent expression of the HIF-1β subunit. Their elevation is associated with an increased risk of metastasis and mortality (34, 47). HIF-1α, HIF-2α, or both cause the activation of hypoxia-inducible genes, and their translational product is involved in several steps of the TNBC invasion and metastasis (48, 49). Under normoxic conditions, HIF-1α subunits are finally degraded by the proteosome, whereas hypoxia inhibits prolyl hydroxylases (PHD) and factor-inhibiting HIF-1 (FIH-1), key components required in the steps involved in the proteasomal degradation of HIF-1α, leading to HIF-1α stabilization and translocation to the nucleus, where they dimerize with HIF-1β and finally bind with hypoxia response elements within the promoters of target genes (50–52). In TNBCs, HIF target genes are highly expressed, whereas the expression of progesterone, estrogen and human epidermal growth factor receptors are deficient. Thus, TNBCs respond poorly to several current therapeutic regimens (53, 54).

Giatromanolaki et al. claimed that the overexpression of HIF-1α is closely related to the immune response and adverse prognosis of BC and also inhibits the proliferation and survival of cytotoxic T cells and the expression of IL-2 and IFN-γ cytokines (55).

## 2.1 TNBC hypoxia in relation with prognosis and survival

In TNBC, hypoxia plays a critical role in the prognosis and survival of cancer cells *via* the mediation of angiogenesis, glycolytic shift, apoptosis, and the recruitment of tumor-associated macrophages (TAMs). Hypoxic conditions upregulate the angiogenic growth factors and their receptors, leading to increased vascular permeability due to endothelial cell migration, and thus mediating TNBC angiogenesis. This phenomenon of angiogenesis is achieved *via* HIF pathways by regulating several pro-angiogenic genes such as angiopoietin-1 and 2, tunica intima endothelial kinase 2 (Tie2), vascular endothelial growth factor (VEGF) and their receptor, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), etc. (56). Besides, hypoxia causes the accumulation of TAMs, exhibiting the cancerous phenotype. TAMs also secrete angiogenic growth factors, leading to angiogenesis and prognosis (57, 58).

Hypoxia induces apoptosis by regulating several pro- and anti-apoptotic pathways either by HIF-dependent or independent mechanisms. Hypoxia reduces the bax/bcl-2 ratio as well as cytochrome c release and caspase-3 activity, thus inhibiting the pro-apoptosis pathways. In addition, hypoxic conditions favor the selection of p53 mutant cells having elevated levels of bcl-2, which is a well-known inhibitor of apoptosis, thus causing the decline in the p53 and bcl-2 ratio (p53/bcl-2), which further increases the mutation rates in clone populations (59, 60). This endless cycle promotes the prognosis of TNBCs (61).

Hypoxia modulates the expression of glucose transporters like GLUT-1 and GLUT-3 as well as glycolytic enzymes such as hexokinase 1, hexokinase 2, and phosphoglycerate kinase 1 (PDK1), causing a glycolytic shift from oxidative phosphorylation to glycolysis. HIF-1α plays a fundamental role in this metabolic adaptation (62). HIF-1α induces the expression of PDK1, which after phosphorylation inhibits pyruvate dehydrogenase (PDH), a key enzyme converting pyruvate to acetyl-CoA. In anaerobic glycolysis, this pyruvate is forced to be metabolized into lactate. Thus, hypoxic conditions reduce the amount of acetyl-CoA available to enter the Krebs cycle or TCA cycle, leading to a reduced amount of substrate availability for mitochondrial respiration as well as oxygen consumption (63–65). It is a well-known fact that cancer cells immediately use glycolysis, even when sufficient oxygen is available. This dependency on inefficient aerobic glycolysis is known as the Warburg effect, which promotes tumor prognosis and survival (Figure 1).

## 2.2 Hypoxia in regulation of cancer stem cells in TNBC

CSCs are a small heterogenous subset with self-renewal characteristics. These cells have a tremendous power to differentiate into all other specific cell types within the tumor tissues and can survive after therapy (66, 67). Specific biomarkers such as $CD44^{high}/CD24^{low}$, CD49f, and aldehyde dehydrogenase 1 (ALDH1) define the BCSCs in TNBC and are predominantly associated with a poor survival rate in TNBC patients (68, 69). Several analytical reports on human breast carcinomas have proved that $CD44^{high}CD24^{low}ALDH1^{high}$ CSCs predominantly associated with TNBCs and are significantly associated with tumor recurrence (69, 70). The hypoxia environment in TNBCs induces several stress-responsive genes which modulate the CSCs to activate their self-renewal and anti-apoptotic phenotype properties. These properties play a crucial role in tumor growth, immune evasion, metabolic reprogramming, drug resistance, and constraining clinical outcomes by modulating the transcription of several target genes (71). Hypoxia-activated pathways in the tumor microenvironment, such as HIF, CD133, CD24, CD47, DLK1, and mixed lineage leukemia 1 (MLL1), are the most essential contributing vital factors to CSC generation and maintenance (72–74). Increasing published evidences has supported and proved that HIF-1α is the central regulator of induction and maintenance of self-renewal and anti-apoptotic phenotypic properties of various CSCs such as octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), NANOG (encodes an NK2-family homebox transcripton factor), and Krüppel-like factor 4 (KLF4) (35, 75, 76). Published reports strongly suggests that HIF-1α directly binds to the promoter region of CD24 and induces CD24 overexpression which further accelerates tumor formation and metastasis (77, 78). Additionally, the direct binding of HIF-1α to CD47 and CD133 activate several gene transcription factors that inhibit the phagocytic activity of macrophages and promote the production of CD133+, respectively, which maintain the OCT4 and SOX2-mediated CSC pool of TNBC (79). However, there is controversial evidence in gastrointestinal cancer cells where hypoxia-induced HIF-1α expression decreases CD133 expression. Still, during normoxic states, inhibition of mTOR signaling in gastrointestinal cancer cells reduces the HIF-1α expression that overexpresses CD133 (79).

Moreover, increasing evidence also suggests that HIF-1 transactivates the RNA demethylase ALKBH5 to encode N6-methyladenosine demethylase and increases the stability of NANOG mRNA in BC (80, 81). Additionally, HIF-1 also induced A2BR and activates protein kinase C to transcribe IL-6, IL8, and NANOG, which further promotes stemness, as Lan et al. reported (82). HIF-1α also regulates the 4-trimethylaminobutyraldehyde dehydrogenase, which is associated with cancer cells metastasis, self-renewal, and resistance in BC (80, 83). In turn, HIF-1α expression is also regulated by aldehyde dehydrogenase 1A1 (ALDH1A1) *via* retinoic acid signaling in TNBC (83). Studies also suggested that HIF-1α induces the JAK/STAT3 signaling pathway, which can upregulate IL-6 and NANOG while promoting the production of VEGF, responsible for the self-renewal ability and maintenance of the CSC phenotype (81, 84). Another study reported by Lee et al., showed that the production of reactive oxygen species (ROS) in TNBC *via* amplification of MYC and MCL1 overexpress the HIF-1α expression and promotes stemness and chemoresistance in TNBC (85). Crowder et al. discussed the correlation between the

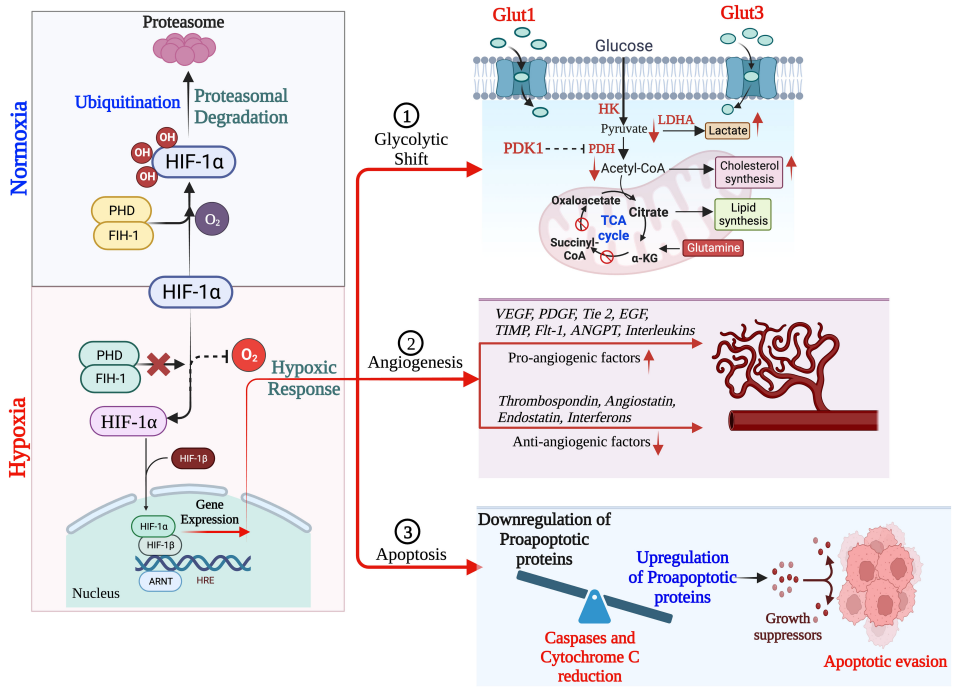



FIGURE 1
Schematic overview of HIF-1α regulation leading to hypoxia, resulting into adaptation of metabolic pathways, inducing angiogenesis and apoptosis. (Created via BioRender.com).

antioxidative enzyme superoxide dismutase 2 (SOD2) expression and the expansion of BCSCs in hypoxic conditions. They suggested that TNBCSCs might resist radiation *via* a SOD2-mediated mechanism (86). The tumor microenvironment pH is also associated with CSC's survival in various cancer types, including TNBC. Interestingly, the pH of TME is tightly regulated by the hypoxia-inducible protein carbonic anhydrase IX (CAIX) by improving the acids transport within the tumor, further increasing the BCSCs survival, expansion and tumor invasiveness (87, 88). Published reports suggest that hypoxia upregulates the CAIX, further enhancing their downstream mTORC1 signaling pathway responsible for regulating triple negative breast cancer stem cells (TNBCSCs) stemness and EMT genes such as Snail and NOTCH (89).

HIF-1α also regulates the expression of ERs, which is a critical indicator of the hypoxic response of BCSCs. Harrison et al. have shown that higher expression of estrogen receptors (ER) is also regulated by HIF-1α and activates the hypoxic responsive factor for the maintenance and proliferation of BCSCs and stimulates the upregulation of Notch genes (90). An interesting study was conducted by Xing et al. group to analyze the expression pattern of Notch ligands in BC patients. They revealed that the expression of Notch and Jagged2 is significantly upregulated in the hypoxic breast tumors, which suggests that they might also regulate the TNBCs maintenance and proliferation and provide critical evidence that Notch and Jagged2 should act as a potential prognostic marker for future clinical applications (91).

Recent advances in BC research have also shown that hypoxia induces the involvement of microRNAs (miRNAs) in regulating the response of BCSCs (35, 92, 93). Hwang-Verslues et al. reported for the first time that miRNA-495 increases the colony-formation ability, invasive capacity and tumor formation capacity, which further regulates the tumor aggressiveness and hypoxic response of BCSCs (94). Several studies have shown that HIF-1α upregulation in TNBC controls cancer metastasis, CSC self-renewal, and invasion. As a result, the data imply that HIF-1α acts as a direct or indirect upstream regulator of BCSCs in TNBC under low oxygen conditions. This suggests that HIF-1α could be a new target for removing CSCs, which would enhance therapeutic approaches (Figure 2).

## 2.3 Hypoxia induces immune escape in TNBC

Tumor immune escape allows tumor cells to survive and grow after evading the host immune system by several mechanisms. It has been reported that hypoxia may induce immunogenic cell death (ICD) within a tumor (95). Earlier investigations have shown that immune escape caused by hypoxia has poor prognostic results and have highlighted the hypoxia pathways as prospective therapeutic targets. Hypoxia signaling has intricate and contradictory functions in triggering immunological escape, encouraging tumor growth and the potential for metastasis, as well as boosting some immunogenic aspects of the tumor microenvironment. Under hypoxic conditions, tumor immune escape involves HIF-1α overexpression (96). HIF-1α inhibits immune cells tumor killing function, which is mediated by regulatory cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-

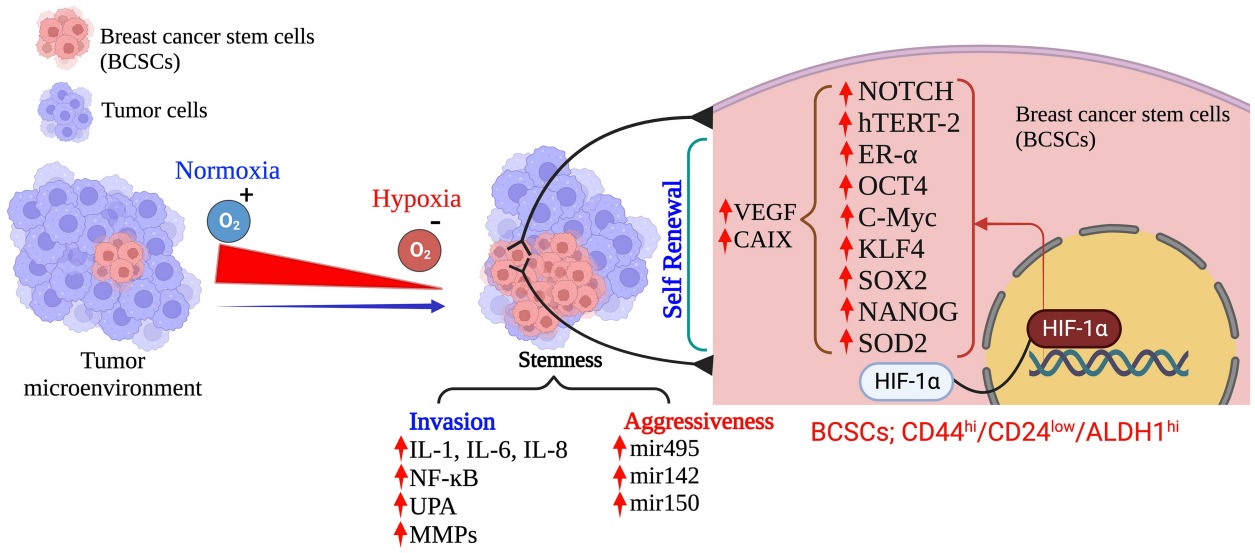



FIGURE 2
Hypoxia: Determinant of the fate of BCSCs in TNBC. (Created via BioRender.com).

1), transforming growth factor-β (TGF-β), CC-chemokine ligand 5 (CCL5), and VEGF in TNBC. In addition, the transcription factor Foxp3 and immune checkpoint molecules like PD-1 also participate in the tumor immune escape mechanism through activation and infiltration of immunosuppressive cells (97).

Myeloid-derived suppressor cells (MDSCs) in TNBC can control tumor-killing cells and immunosuppressive cells by secreting cytokines and suppressing anti-tumor immunity. HIF-1 drives immune evasion and encourages MDSC recruitment. There isn't much research in TNBC on HIF-1's modulation of myeloid suppressor cells. HIF-1 controls the communication between TNBC cells and MSCs, leading to the control of MDSCs recruitment. MSCs create CCL5 and bind to CCR5 on TNBC cells in a mouse model (98). The CSF1 receptor on MSCs is simultaneously bound by the cytokine CSF1 produced by basal cells (49). To encourage the recruitment of MDSCs, HIF-1 increases CSF1 and CCL5 signaling.

TNBC has higher levels of TAMs, which are strongly linked to a poor prognosis. By producing immunosuppressive molecules, including IL-10 and TGF-β, M2 macrophages have an immunosuppressive effect (99). HIF-1 drives the development of an immunosuppressive milieu and stimulates the polarization of TAMs towards the M2 phenotype (100). Granulocyte-monocyte stimulating factor plays a variety of activities in TAMs, according to prior research. A high concentration of GM-CSF has an immunosuppressive impact by enriching M2 macrophages, whereas a low concentration has an anti-tumor effect by stimulating dendritic cells (DCs) (101). High levels of GM-CSF are generated in TNBC cells under the control of HIF-1 and NF-κB, attracting additional macrophages, and polarizing them into M2-type macrophages (102). Macrophage CSF1, which is secreted by MDA-MB-231 TNBC cells and binds to its receptor on mesenchymal stem cells (MSCs), aids in the attraction of TAMs and MDSCs. Through preserving CCL5/chemokine receptor type 5 (CCR5) communication between MSCs and MDA-MB-231 TNBC cells, HIF-1 controls the expression of CSF-1 (52). As a result, HIF-1 can promote the polarisation of TAMs to the M2 type *via* regulating GM-CSF and CSF1 in TNBC. The T-regulatory (Tregs) cell transcription factor Foxp3 is essential. HIF-1 can regulate the aggregation of immunosuppressive Tregs in TNBC *via* regulating forkhead box P3 (FoxP3) and the C-X-C motif chemokine receptor 4 (CXCR4). *Via* co-regulatory proteins like co-stimulators, transcription factors, co-repressors, and chromatin remodelers, Foxp3 controls the restrictive activity of Tregs (103). CXCR4 is widely expressed on the Treg cell surface and regulates the recruitment of Tregs (104). In TNBC, HIF-1 directs downstream Foxp3 expression by binding to HREs while indirectly enhancing CXCR4 expression by acting on regulatory regions upstream of the CXCR4 transcription start site (105). In patients with TNBC, enrichment of CD8+T is directly linked with improved clinical prognosis and a higher immunological response because CD8+T cells are essential anti-tumor immune cells (106). The tumor-killing ability of HIF-1 in CD8+ T cells is controversial because HIF-1 overexpression in CD8+ T cells increases the level of infiltration and tumor-killing ability of CD8+ T cells (101). The dysfunction of CD8+ T cells was caused by HIF-1's suppression of immunological effector gene expression under hypoxic settings through histone deacetylase (HDAC-1) and polycomb repressive complex 2 (PCR2)-mediated histone alterations (102). Moreover, under the control of HIF-1, tumor cells produce more adenosine in a hypoxic microenvironment. Adenosine inhibits T cell proliferation and toxicity, promotes T cell death, and inhibits anti-tumor immunological function *via* interacting with adenosine A2A receptors (103). In summary, HIF-1 inhibits CD8+ T cell activation and immune infiltration in TNBC while largely promoting immunological escape. By generating the overexpression of VEGF and PD-1 and encouraging the release of adenosine from tumor cells, HIF-1 regulates the epigenetic mechanism of immune effector genes. This reduces infiltration

and impairs CD8+ T lymphocytes capacity to attack tumors (Figure 3).

## 2.4 Hypoxia in relation with epigenetic vulnerability in TNBC

Accumulating evidence has also demonstrated that hypoxia-mediated increased expression of HIF is closely linked with manipulating epigenetic plasticity in the tumor cellular system and subsequently induces immune dysfunction (104, 105). Recently, transcriptional and epigenomic analyses by Cong et al. group have shown that Bromodomain-containing protein 4 (BRD4) is the epigenetic regulator. Its dysfunction is critical in mediating several transcription factors by HIF-1α in hypoxic conditions. The study also proved a close association of BRD4 dysfunction with the malignant progression of various tumors; thus, it could be a promising target for different cancer treatments (107). They have also shown that selective degradation of BRD4 subsequently down-regulates the expression of CAIX, a crucial hypoxia-mediated pH gene regulator. Further, it has been proved that overexpression of CAIX induces the acidic environment in various tumors, including TNBC, to adapt the hypoxic environment. Hypoxia-mediated CAIX overexpression also causes a marked reduction in VEGF levels, a master regulator of angiogenesis (107). Another study by Ma et al. demonstrated that hypoxia induces chromatin remodeling in TNBC by the interaction between HIF-1α with HDAC1 and the concurrent PRC2. This interaction epigenetically suppresses the effector gene's function, which subsequently impacts immune homeostasis and disturbs immune tolerance (102). Chromatin immunoprecipitation (ChIP) assay data demonstrated that hypoxia induces the enrichment of HDAC1 and PRC2. However, it does not change in HDAC 2 and HDAC3 at the effector gene IFN-γ and TNF promoters in the T cells and NK cells (102). Some data suggest that enrichment of HDAC1 and PRC2 by HIF1α-mediated pathways suppresses the functionality of various effector immune cells. Therefore, targeting these pathways through either genetic depletion or therapeutic intervention may be a revolutionary strategy to overcome alteration in immune cell homeostasis. In addition, it might inhibit the disturbance of immune tolerance in TNBC and enhance checkpoint immunotherapy responses. Further, several studies have shown that bromodomain and extra-terminal domain (BET) protein reads histone-acetylation and recruits various transcription factors in TNBC to adapt to hypoxic conditions (108, 109). Ongoing investigations have also shown that hypoxia increases H4ac and H3K27ac, which are associated with transcription and BET protein binding in the HIF targets such as CA9, VEGFA, and LOX promoters (107, 110, 111). Luo et al. discussed and demonstrated selective and specific interactions of HIF-1α with histone demethylase jumonji domain-containing protein 2C but not with HIF-2α (112). By this selective interaction, HIF-1α recruits JMJD2C to the hypoxia response elements of HIF-1 target genes, further reducing the histone H3 trimethylation at lysine 9, and enhancing HIF-1 binding to hypoxia response elements. The enhancement of HIF-1 binding to hypoxia response elements activates the transcription of PDK1, L1CAM, GLUT1, LOX, LOXL2, and LDHA mRNA in human BC biopsies (112, 113). Lambert et al. suggested that lysine demethylases 4C (KDM4C) encoded by JMJD2C interact with HIF1α and are involved in metabolic remodeling and metastasis (114). Overall, based on emerging research, epigenetic plasticity plays a critical role in stimulating HIF-1, which further mediates the transactivation of genes that code for proteins implicated in immunological and metabolic tolerance reprogramming in TNBC.

## 2.5 Hypoxia and DNA repair defects in TNBC

Hypoxic tumor microenvironment down-regulates or deregulates the DNA repair pathways by inducing modifications in several transcriptional, translational, post-translational and epigenetic mechanisms (115). This down-regulation or deregulation causes defects in DNA repair pathways linked to extensive genomic

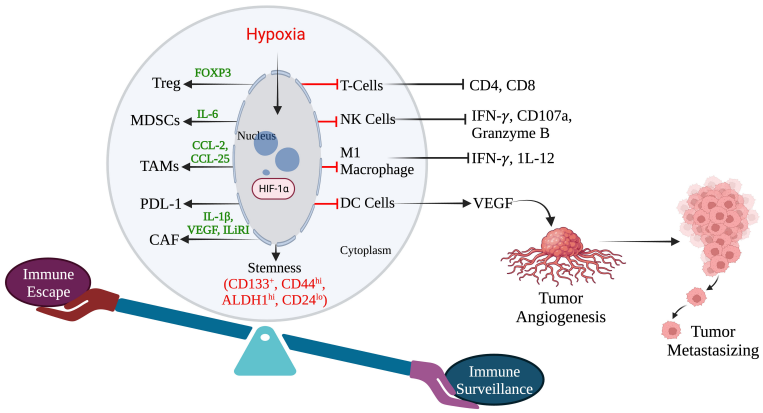



FIGURE 3
Hypoxia induces immune escape in TNBC. (Created via BioRender.com).

rearrangements with a high rate of mutation burden, DNA hyper-replication stress, fragile site induction, and microsatellite instability (MSI). These DNA repair anomalies further lead to the tumor becoming more aggressive and are associated with a significantly worse prognosis/survival in TNBC (116). Therefore to protect the cell against hypoxia-induced replication stress and DNA damage, three primary DNA damage response (DDR) kinases, which include DNA-dependent protein kinase, ataxia telangiectasia and Rad3-related (ATR) protein, and ataxia-telangiectasia-mutated (ATM) kinases are responsible and becomes activated through post-translational modifications (117). Studies have demonstrated that genetic or chemical depletion of ATM or Chk2 in tumor cells has reduced clonogenic survival and increased apoptosis after exposure to hypoxia (118–120). Additionally, hypoxia also activates the ATR/Chk1 response, which subsequently causes pan-nuclear induction of phosphorylation of H2AX (γH2AX) and p53 (121, 122). Further, an emerging study has also demonstrated that depletion of ATR/Chk1 followed by exposure to hypoxia or re-oxygenation significantly reduces cell survival by increasing apoptosis. The study has also unraveled that DNA-PK is activated in hypoxic cells and phosphorylates at Ser2056 of the catalytic subunit that regulates HIF-1 expression (123). The study has demonstrated that ER, PR, and HER2 deficiency in TNBC leads to ATM response hyperactivates (124). Hyperactivation of ATM is predominantly associated with high invasiveness and metastasis of TNBCs by inducing the expression of EMT markers such as Snail and vimentin and reducing the expression of E-cadherin and cytokeratin, which exclusively characterized the epithelial cells (124). Another interesting study demonstrated that hypoxia induces the activation of oxidized ATM, which is independent of DNA damage-mediated ATM activation in TNBCs. Hypoxia-dependent activation of oxidized ATM accumulates the citrate in the cytoplasm. This extracellular accumulation of citrate stimulates the signaling pathway to activate the AKT/ERK/MMP2/9 crucial signaling axis for cell growth, survival, motility and metabolism in TNBC. These findings unravel that oxidized ATM is significantly responsible for TNBC hyperproliferation, invasion and metastasis (125). In addition, available reports have also shown a significant overexpression of ATR and CHK1 in TNBC tissues and promoted tumor progression (126). Meyer et al. demonstrated that an ATR/CHK1 mediated-DDR response prevents the replication stress and induces the resistance of homologous recombination-deficient (HRD) TNBC to mitomycin C (127). This study also suggests that ATR/Chk1 DDR might be a primary mechanism that induces chemoresistance in HR-deficient TNBC (127). Emerging studies have also observed aberrant genetic alteration in other DDR pathways, such as a high prevalence of p53 functional insufficiency and BRCA1/2 mutations (126, 128, 129).

Moreover, HRD is a crucial clinicopathological feature of the BRCA1/2 mutated TNBC, and several studies have proven that specific ATR inhibitors are highly efficient in sensitizing HR-proficient as well as HR-deficient TNBC cells against radiotherapy and inhibit the TNBC proliferation (130, 131). Rad51 is an essential protein for HR that triggers the initiation of the HR process at the sites of DNA damage to repair the damaged DNA. Studies have demonstrated that the knockdown of Rad51 or treatment with a Rad51 inhibitor can enhance the sensitivity to proton therapy and induce proton-mediated clonogenic cell death in TNBC cells (132). Several investigations also revealed that complement 1q binding protein (C1QBP) is highly expressed in hypoxic TNBC and promotes the progression of TNBC. In addition, hypoxia mediated high expression of C1QBP, stabilizes the MRE11 protein (DDR protein) in MRN complex (MRE11/RAD50/NBS1) and inhibiting MRE11 exonuclease activity which makes TNBC resistance to chemotherapy (133). Study has also demonstrated that blocking RAD50 within the MRN complex sensitizes CSCs and chemo-resistant BT-549 and MDA-MB-231 TNBC cell lines to chemotherapeutic drugs (3, 134). Recent studies have also shown that BRCA1 is critical in resolving DNA double-strand breaks (DSB) by HR repair, particularly DSB associated with cross-links at the end of DNA replication forks. Moreover, available reports suggest that mutation or deficiency of BRCA1 genes altered the HR-related gene, which further leads to HR deficiency in TNBC, makes TNBC more sensitive to the specific therapies that generate cross-links or DSBs fragments, including platinum drugs, alkylating agents, anthracyclines and PARP inhibitors (134, 135).

Non-homologous end joining (NHEJ) repair pathway is another crucial pathway that recognizes DSBs *via* Ku70/80 heterodimer in association with DNA-PKcs to repair damaged DNA in various cancer cell normal cells (136). Although it's still debated, the exact effects of hypoxia on NHEJ still need to be explored. It is most likely that several hypoxia-mediated molecular mechanisms altered the crucial NHEJ protein expression and deregulated or downregulated functionality of NHEJ-associated proteins. Studies have demonstrated that long noncoding RNA (lncRNA) overexpressed in TNBC and hyperactivated the NHEJ pathway by supporting Ku80 and DNA-PKcs to repair of DSBs and promote tumorigenesis (137). Interestingly, the hypoxia-mediated amplification of EGFR activity and P53 mutation in TNBC is also responsible for the high expression of lncRNA in non-homologous end-joining pathway 1 (LINP1) in TNBC (137). Zhang et al. demonstrated by RNA-immunoprecipitation assays (RNA-IP) that the association between LINP1 and Ku80 or DNA-PKcs induced by ionizing radiation (IR) exposure to tumor cells. However, blocking LINP1 radio sensitizes the TNBC tumor for radiotherapy (137).

Hypoxia also alters the mismatch repair (MMR) pathway by mutating the major MMR proteins MLH1 and MSH2, including MSH6 (138, 139). Severe hypoxic conditions downregulate MLH1 and MSH2 in a HIF-independent manner. However, in moderate hypoxic conditions, MSH2 and MSH6 downregulate in HIF and P53-dependent manner (138, 140). Although it's unclear, available reports suggest that hypoxia-mediated MMR downregulation also requires HDAC activity (141). These mutations or deregulation further led to microsatellite instability and are characterized by decreased or increased repeated nucleotide sequences (138–140).

Moreover, a high incidence of MSI observes in various tumor models, such as colorectal, ovarian, stomach, urothelial, central nervous system, and adrenal gland. The high incidence of MSI is further responsible for developing several malignant mutations and tumor evasion. However, based in published reports so far, only limited data are available on disease prevalence, and the prognostic significance of MMR-d/MSI-H in BC. Additionally, in TNBC, a low incidence rate of

MMR-D/MSI-H is observed. Recent clinicopathological studies conducted in 440 patients with TNBC demonstrated no correlation between MMR-d/MSI-H and clinicopathological parameters such as PD1/PDL-l immune checkpoint expression and survival. Another study also revealed that low expression MMR-D/MSI-H characteristics in TNBC may not be a practical predictive marker for immunotherapy by using immune checkpoint inhibitors of PD1/PDL-l (139).

Emerging studies also demonstrated the hypoxia mediated reduction of several base excision repair (BER) factors expression such as OGG1, MYH, POLB, APE1, RPA, PCNA and ASCIZ/ATMIN in TNBC which leads the TNBC BER-deficient (BER-d). Moreover, induction of oxidative or alkylating glycosylation *via* low protein production in TNBC is significantly associated with oxidative or alkylating glycosylases through low protein production (142, 143). The available report suggests that a combination of PARP inhibitors that directly impact BER signaling and other relevant therapies that generate ROS and induce defective glycosylation would be more precise and targeted therapies for TNBC treatment (142, 144).

The effects of hypoxia on nucleotide excision repair (NER) remain debatable. Several NER gene expressions, such as XPA, XPB, XPD and XPG, do not change after hypoxia exposure (115). However, some evidence indicates that severe hypoxia suppresses the NER capacity and demonstrates hypermutability to UV irradiation (145). Another study conducted in HCC1806 and MDA-468 TNBC cells showed the inactivation of major NER proteins such as ERCC1, XPA, and XPF (146). These proteins play an essential role in recognizing and excision bulky base lesions. The inactivation of scaffold protein, XPA, induces severe sensitivity to UV radiation and a high risk of carcinogenesis (147). These studies suggest that the induction of deregulation of NER in TNBC could offer revolutionary new targets for the treatment of TNBC either alone or in combination with other therapies. In summary, understanding of DNA repair defects in TNBC can potentially be used to overcome resistance to treatment.

# 3 TNBC and treatment strategies

TNBC harbors a highly heterogenous and aggressive behavior with a distinct metastatic pattern. It also contains a high mutational burden and activates various tumor initiation signaling pathways. TNBC is the highest malignant BC subtype with a poor clinical outcome. Therefore, the current treatment options are limited to surgery, chemotherapy, and radiotherapy. The limited clinical ramifications of TNBC have lagged other types of BC. Due to its hyper-progression and aggressiveness, it remains challenging BC to treat and minimal options are available for treating this form of BC. Many efficacious treatments for most BC are limited to inhibiting the growth-stimulating effects of PR, ER, and HER2. Finding novel and potent therapies for TNBC remains a crucial clinical need because it lacks these growth-stimulating receptors. To develop specific efficacious drugs, new experimental approaches need to be adequately investigated in pre-clinical and followed by clinical trials platform on patients diagnosed with TNBC. Figure 4 summarizes the tumor hypoxia mechanism and current treatment approaches for TNBC.

## 3.1 Radiotherapy

In 1920, Hall et al. showed that the tissue inadequately supplied with oxygen was highly resistant to ionizing radiation (IR) (148, 149). In 1953, Gray's seminal paper demonstrated that the preclinical study reported that decreasing hypoxia enhanced oxygen distribution and increased radio sensitivity. However, these findings facilitate the way for several studies summarizing the mechanism of radio resistance induced by hypoxia. On the whole, HIF-1 and radiotherapy have a complicated, two-sided relationship.

Following radiotherapy, which induces hypoxia-dependent HIF-1 expression in the tumor, causes vasculature damage and a consequent oxygen level deficit. Earlier, Moller et al. demonstrated that following IR, the murine tumor model showed increased HIF-1 expression. As a result, the irradiated mouse tumors gave the impression of radiation-induced reoxygenation and displayed relatively high oxygenation levels in the tissue (150). Authors found two unexpected results were found in the same study: (a) ROS activation led to HIF-1 stabilization, and (b) the increased translation of HIF-1 transcripts owing to reoxygenation caused the breakdown of monomers referred to as "stress granules". Previous studies endorsed ROS-directed HIF-1 stabilization, most likely by decreasing PHD enzyme activity (151, 152). However, another hypothesis emerged as P13/AKT/mTOR pathways, which result in increased HIF-1α expression, and Ras/Raf/ERK/MEK pathways, which always attribute ROS-mediated regulation of HIF-1 expression.

Additionally, the stabilization of the HIF-1 protein during IR is linked to heat shock protein 90 (Hsp90) (153, 154). In response to IR, endothelial cells underwent a stress reaction that resulted in either recovery or function loss and cell death. The critical destiny is determined by a number of variables, such as intrinsic TME characteristics, fractionation schedule, and total dose (155). VEGF is at the centre of this interaction as an HIF-1 target gene and a crucial regulator of tumor vascularization (156). Radiation-induced fibrosis, or RIF, is a dose-limiting postradiotherapy consequence. In reaction to radiation exposure, the body undergoes an aberrant wound-healing process (RIF), which leads to a self-replicating fibroproliferative condition (157). IR-induced DNA damage triggers an initial inflammatory response, which is followed by endothelial cell failure and hypoxia. This causes aberrant collagen and other extracellular matrix protein buildup as well as an atypical activation of fibroblasts (sometimes referred to as the activated state of myofibroblasts). TGF- is an important participant in this process, and one of the critical RIF-initiating events is ROS-mediated post-translational activation of TGF- β (158).

One of the most notable instances of how HIF-1-mediated metabolic reprogramming might directly counteract radio-resistance in relation to radiation is glycolysis-induced activation of the pentose phosphate pathway (PPP). PPP activation decreases oxidized glutathione, restores nicotinamide adenine dinucleotide phosphate (NADPH), and shields cancer cells from ROS (159). In a recent

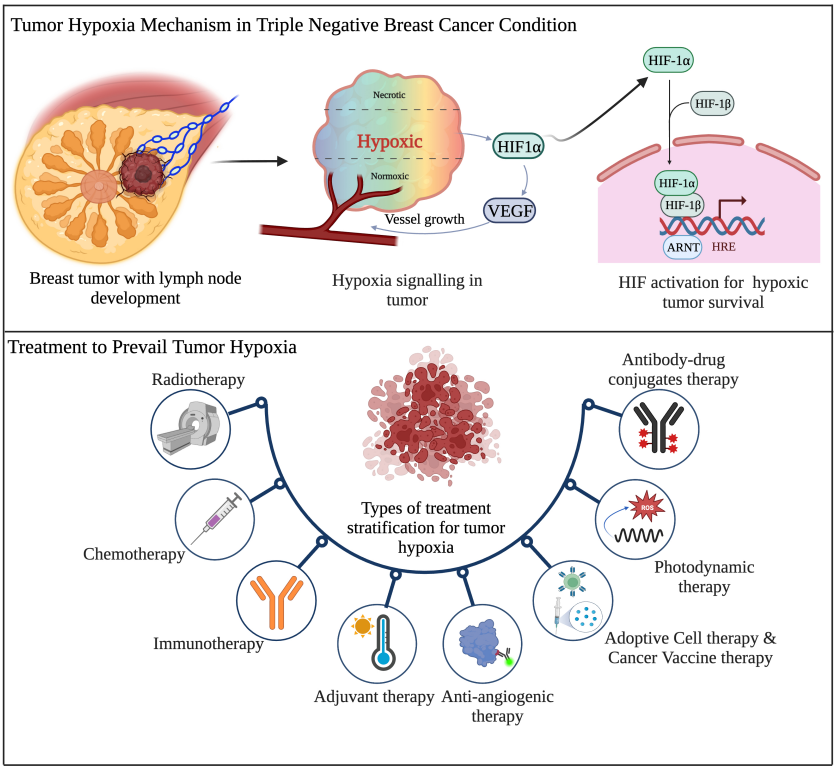



FIGURE 4
Schematic overview of tumor hypoxia mechanism and existing therapeutic regimens in TNBC. (Created via BioRender.com).

clinical trial looking at first-time tumor hypoxia in SBRT, Song et al. first showed a clinical study investigating tumor hypoxia in SBRT with high doses of single fraction radiation delivered to patients with lung cancer (160). Overall, there is still much to learn about the importance of tumor hypoxia in the era of hypofractionation therapy. Stereotactic body radiation (SBRT) replaced traditional fractionated radiotherapy techniques, and this change sparked a new quest for hypoxia-modifying radiotherapy drugs. Image-guided radiation, intensity-modulated radiotherapy, volumetric modulated arc treatment, targeted combinatorial drug therapies, and immunotherapy are examples of contemporary innovations in radiotherapy delivery and imaging approaches that have improved radiotherapy's therapeutic index (161, 162).

The conventional radiotherapy involves the usage of either external beam, like that of a regular x-ray or more feasible internal radiation known as brachytherapy, in which a sealed radiation source is inserted to target the cancerous area. However, Smith et al. demonstrated that a high increased risk of subsequent mastectomy after brachytherapy treatment compared with external beam therapy (163). Proton beam therapy (PBT) has a lower administrative dose than conventional radiotherapy and allows the majority of the radiation dose to be explicitly focused on the tumor, is also more frequently used. This can reduce the needless irradiation of nearby normal tissues, which will lessen the likelihood of side effects. Emerging studies demonstrated a dosimetric comparison between brachytherapy and PBT and intensity modulated proton radiotherapy technique (IMPT) and suggested a comparability similar dose effect in BC patients and also suggested to use of PBT for significant outcome (164) (Table 1). Modern developments in clinical radiotherapy technology are aimed at enhancing the capabilities of the radiotherapy machines and altering the local mode of radiotherapy to maximize the accuracy of irradiating tumor tissue while minimizing damage to healthy tissue.

Ultra-high dose rate (UHDR) radiotherapy called FLASH radiotherapy (FLASH-RT) has been expected as a new method in recent years (Table 1). In multiple trials, radiation toxicity to the surrounding healthy, normal tissues was markedly decreased, and tumor growth was suppressed, with tumor control on par with conventional dose rate irradiation. It is generally acknowledged that FLASH irradiation has great future potential and is perhaps the most significant discovery in the history of radiation treatment, despite some researchers' skepticism over FLASH-effectiveness RT's in treating cancer patients (182).

## 3.2 Chemotherapy

Chemotherapy aims to weaken the cancer cell defenses against apoptosis, mitotic catastrophe, autophagy, and necrosis in order to promote cancer cell death. Apoptosis and autophagy, to start, are genetically programmed. Second, passive reactions to extreme cellular mistreatment include necrosis and mitotic catastrophe (218). The majority of chemotherapy medicines cause DNA damage. Apoptosis is the main method of cell death in reaction to DNA damage, even if medications that cause DNA damage can

TABLE 1 Clinical trials evaluating different therapeutic approaches in patients with TNBC.

| S. No. | Type | Treatment | TNBC patient population | Mechanism of Action | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|---|
| 1. | CHEMOTHERAPY | Paclitaxel | 52 | p53/p21 pathway or Raf-1 kinase activation pathway | II | Completed | (165) |
| 2. | | Nab-paclitaxel | 903 | Inhibit of tumor growth | II/III | Completed | (165, 166) |
| 3. | | Docetaxel | 127 | Attenuate the effect of BCL-2 and BCL-XL gene | II/III | Completed | (167) |
| 4. | | Tesetaxel | 674 | PDL-1 Inhibitor | III | Completed | (168) |
| 5. | | Doxorubicin | 52 | Blocking the topoisomerase 2 | 1/1b | Completed | (169, 170) |
| 6. | | Epirubicin | 53 | Inhibit totpisomerase II activity | II | Completed | (171) |
| 7. | | Pegylated liposomal doxorubicin | 39/113 | Blocking the topoisomerase 2 | 1/III | Completed | (172, 173) |
| 8. | | Cyclophosphamide | 40 | Inhibiting humoral 1 and 2 | II | Completed | (174) |
| 9. | | Cisplatin | 47 | Inhibit the DNA synthesis | II | Completed | (174, 175) |
| 10. | | Carboplatin | 647 | Inhibit the DNA synthesis | II | Completed | (176) |
| 11. | | Eribulin mesylate | 762 | Reversing epithelial-mesenchymal transition to mesenchyal-epethilial transition. | III | Completed | (177) |
| 12. | | Capecitabine | 434 | Inhibit thymidine monophopshate (ThMP) synthesis | II/III | Recruiting | (178) |
| 13. | | Gemcitabine | 50 | Activates p38 MAP kinase pathway | II | Completed | NCT02435680; Novartis (Novartis Pharmaceuticals), 2021 |
| 14. | | Fluorouracil | 647 | Inhibit thymidylate synthesis (TS) | III | Completed | NCT01216111; Zhimin Shao, Fudan University, 2020 |
| 15. | | Ixabepilone | 91 | Microtubule inhibitor, blocks cell growth by stopping cell division. | – | Recruiting | (179) |
| 16. | | Taxanes | - | Microtubule inhibitor and inactivate HIF-1α pathway | - | - | (180) |
| 1. | RADIOTHERAPY | External beam radiation therapy (EBRT) | - | Radiation to destroy cancer cells | I | Unknown | (163, 164, 181) |
| 2. | | Brachytherapy (149) | - | Limits radiation treatment to the tissue surrounding the lumpectomy | I | Unknown | (163, 164, 181) |
| 3 | | FLASH-RT | - | Limits the radiation toxicity to the surrounding healthy, normal tissues. | - | - | (182) |
| 4 | | IMPT | - | Radiotherapy technique to treat tumors in layers of spots at varying depths by altering the number localized proton dose deposition, energy penetration, and magnetic deflection. | - | - | (164) |
| 5 | | PBT | - | Reduce the needless irradiation of nearby normal tissues and side effects | - | - | (164) |

*(Continued)*

TABLE 1 Continued

| S. No. | Type | | Treatment | TNBC patient population | Mechanism of Action | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|---|---|
| 1. | ANTIANGIOGENIC THERAPY | | Bevacizumab | 54 | Angiogenesis agent by Inhibits the VEGF binding to it cell surface receptor | II | Completed | (183) |
| 2. | | | Lenvatinib | 31 | Multiple receptor inhibition VEGFR-1, VEGFR-2, VEGFR-3, FGFR1, FGFR-2, FGFR-3, FGFR-4, PDGFRa, RET and c-KIT. | II | Recruiting | (184) |
| 3. | | | Apatinib | 32 | Inhibits VEGFR-2 receptor reduce tumor vasculature | I | Completed | (185) |
| 4. | | | Cabozantinib | 35 | Inhibits VEGFR-1/2 and -3, TRNKB, FLT-3,KIT,TIE-2, MET,AXL and RET | II | Completed | (186) |
| 5. | | | Anlotinib | 30 | Dual signaling blockade VEGFR2 and MET pathways | I | Unknown | (187) |
| 1. | ADJUVANT THERAPY | | Accelerated radiotherapy with carbogen and nicotinamide | MDA-MB-231 TNBC xenograft tumors | Radiation with carbogen to destroy cancer cells | 0 | Preclinical | (188) |
| 2. | | | Hypothermia | 2 | Heat tissue high as 113 $^{0}$ F to kill cancer cells | 0 | Unknown | (189) |
| 1. | PHOTODYNAMIC THERAPY (PDT) | | Protoporphyrin IX | TNBC cell lines (HCC1395, BT-20, MDA-MB-231, and Hs578T) | Inhibits Ras/MEK pathway. | Preclinical | *In-vitro* | (190) |
| 1. | IMMUNOTHERAPY | Immune-checkpoint inhibitors (ICI) | Pembrolizumab | 32/84/170 | Inhibit PD-1 pathway. | Ib/II/III | Active | (191–193) |
| 2. | | | Atezolizumab | 115/41 | Blocking its interaction with PD-1 and B7-1. | I/II | Active | (194) |
| 3. | | | Avelumab | 58 | Blocking its interaction with PD-1 and B7-1. | I | Active | (195) |
| 4. | | | JS001 | 20 | PD-1 Inhibitor | I | Active | (196) |
| 5. | | | Nivolumab | 51 | Blocking interaction with PDL1 and PDL2. | I/II | Active, Not Recruiting | (197) |
| 6. | | | Durvalumab | 45 | Blocking interaction with PDL1 with PD-1 and CD80. | II | Completed | (198) |
| 1. | | T-Cell Targeted Modulators | Anti-CTLA4 | 35/129 | Inhibit CTLA4 and suppress natural killer cell maturation. | I/II | completed | (199) |
| 2. | | | Anti-LAG3 | 363 | Inhibit LAG3 | II | Completed | (200, 201) |
| 3. | | | Anti-TIGIT | - | Inhibit TIGIT | 0 | Not yet | (202) |
| 4. | | | Anti-CD137 agonistic antibody | - | Suppress CD137 receptors. | 0 | Not yet | (203) |
| 5. | | | Anti-OX40 agonistic antibody | - | Suppress OX40 receptors | 0 | Not yet | (203) |
| 6. | | | Anti-CD40agonistic antibody | - | Suppress CD40 receptors | 0 | Not yet | (203) |
| 1. | | | | - | | - | Not yet | (204) |

*(Continued)*

TABLE 1 Continued

| S. No. | Type | Treatment | TNBC patient population | Mechanism of Action | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|---|
| | Immunomodulators | Acetylsalicylic acid | | Disrupts NFkappaB-IL6 signaling axis and inhibits cyclooxygenase (COX) enzyme. | | | |
| 2. | | COX2 inhibitors (indomethacin) | - | Disrupts cancer-cell fibroblast signaling. | - | Not yet | (205) |
| 3. | | Recombinant IFN-alpha-2b activating. TLR3 receptors | - | Stimulates JAK-STAT pathway. | - | Not yet | (206) |
| 4. | | A2AR antagonists | - | Decreases the immunosuppressive mechanisms such as Tregs, CTLA-4, TGF-beta and COX2, eicosanoid mediators. | - | Not yet | (207) |
| 5. | | CSF-1R inhibitors | - | Target M2 macrophages/TAM | - | Not yet | (208) |
| 6. | | Anti-TGF-beta antibodies | - | Promotes T cell infiltration. | - | Not yet | (209) |
| 7. | | L-NMMA (pan-NOS inhibitor) | 15/24 | Increases circulating IL-6 and IL-10 cytokines, in contrast, CD15+ neutrophils and decrease in arginase. | I/II | Recruiting | (210) |
| 8. | | Oncolytic reoviruses | - | Selectively replicate in cancer cells and then kill them without damaging the healthy cells by enhancing the recruitment of innate immune function and inducing tumor cell apoptosis. | - | Not yet | (211) |
| 9. | | Anti-IL1beta antibodies | - | Decreases IL-6 production through a transglutaminase 2/NF-κB pathway. | - | Not yet | (212) |
| 10. | | Poly-ICLC | - | Increase cytokines and immune response. | - | Not yet | (213) |
| 11. | | Anti-IL-6R antibodies | - | Decreases the breast cancer cell aggressiveness. | - | Not yet | (214) |
| 1. | ADOPTIVE CELL THERAPY (ACT) | EGFR/CD276 | 30 | Induces T cell activation | I | Recruiting | (215) |
| 2. | | ROR1-targeted CAR T cell (LYL 797) | 54 | Harbors synthetic Notch receptors specific for EpCAM or B7-H3 (expressed by ROR1-expressing tumor cells) and reported that these CAR-Ts safely mediated efficient tumoricidal activity without toxicity | I | Recruiting | (215) |
| 3. | | NKG2DL-targeting CAR-grafted gamma delta (gd) T cells | 10 | Secretes cytokines and chemokines and exhibiting cytotoxicity | I | Recruiting | (215) |
| 4. | | c-met-RNA CART T cells | 6 | Reduces the proliferation and migration capacity of TNBC | 0 | Recruiting | (215) |
| 5. | | CART-TnMUC1 cells | 16 | Target antigen-dependent cytotoxicity and released cytokines, chemokines, and granzyme B | I | Recruiting | (215) |
| 6. | | Anti-meso-CAR vector transduced T cells | 20 | | I | Recruiting | (215)) |
| 7. | | Mesothelin-specific chimeric antigen receptor positive T-cells | 186 | Activate T-cells. | I | Recruiting | (215) |
| 8. | | PD-1+ TILS | 20 | Inhibition of T cell function and depletion of T cells | i/ii | Recruiting | (102, 215) |
| 9. | | TC-510 | 115 | Elicits T cell response through mesothelin. | i/ii | Recruiting | (215) |

*(Continued)*

TABLE 1 Continued

| S. No. | Type | Treatment | TNBC patient population | Mechanism of Action | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|---|
| 1. | CANCER VACCINE (CV) | Dendritic cell vaccine | 23 | Induces the IFN-γ-production by CD4+ T cells. | II | Active/not recruiting | (211) |
| 2. | | AE37 peptide therapeutic vaccine | 29 | Activate CD4+ immune response and stimulate T-helper cells against HER2/Neu expressing cancer cells. | II | Active/not recruiting | (211) |
| 3. | | Neoantigen personalized DNA vaccine | 18 | Induces the number of neoantigen-specific cytotoxic T cells. | I | Recruiting | (211) |
| 4. | | PVX-410 | 20 | Induces cytotoxic T lymphocytes (CTLs) to target specific tumor associated antigens such as highly over-expressed tumor antigens XBP1, CD138 and CS1. | II | Recruiting | (211) |
| 5. | | GP2 | 456 | Activate CD8+ response against the HER2 antigen | II | Completed | (216) |
| 6. | | Nelipepimut-S | 275 | Stimulate cytotoxic T lymphocytes to lyse of HER2-expressing cancer cells. | II | Completed | (216) |
| 7. | | Tecemotide | 400 | Stimulate an antigen-specific cellular immune response against MUC1+ cancer cells. | II | Completed | (216) |
| 8. | | AS/OBI-821 | 349 | Reduces the Tregs, therefore increases the humoral response. | II | Completed | (216) |
| 9. | | H/K-HELP | 12 | Increases the IFN-γ-production by CD4+ T cells and induces Th1 dependent induces cellular and humoral immune responses. | I | Completed | (216) |
| 10 | | P10s-PADRE | 24 | Induces the expression of CD16, NKp46 and CD94 expression on NK cells and a serum content of IFN-γ produced by CD4+ T cells. | 1/II | Recruiting | (211) |
| 11. | | Galinpepimut-S | 90 | Stimulate CD8+ and CD4+ T-cell responses. | II | Recruiting | (211) |
| 12. | | KRM-19 | 14 | Stimulate cytotoxic T lymphocytes and induces the IFN-γ-production by CD4+ T cells. | II | Completed | (216) |
| 13. | | Tumor lysate-pulsed DC 23 vaccine | 29/21 | Induces the IFN-γ-production by CD4+ T cells. | II | Completed | (216) |
| 14. | | RO7198457 (iNEST) | 272 | Enhances anti-tumor activity of atezolizumab (anti–PD-L1) by increasing the number of neoantigen-specific cytotoxic T cells. | II | Active/not recruiting | (211, 217) |
| 15. | | NANT cancer vaccine (NCV) | 79 | Enhances immunogenic cell death by activating the T cell and NK therapy and also reduces the Tregs | I | Active/not recruiting | (216) |
| 16. | | Elenagen | 27 | Reduces in the population of suppressive cells in the TME, including regulatory T-cells (Tregs) or myeloid-derived suppressor cells (MDSCs). | I/II | Completed | (216) |
| 17. | | p53MVA | 11 | Induces the frequencies and persistence of p53-reactive CD8+ T cells. | I | Active/not recruiting | (216) |

also cause necrosis as an alkylating agent or autophagy as an etoposide (219). It is well known that hypoxia lowers the effectiveness of chemotherapy treatments since these drugs need oxygen to act as an electron acceptor in order to kill cells.

Hypoxic tumor cells are discussed in this review as a way to avoid chemotherapy (220). Hypoxia drastically altered the transcription of cells, principally by activating HIF-1. HIF-1 is made up of two subunits: HIF-1/ARNT, which is constitutively stable and HIF-1, which is oxygen-sensitive. Low oxygen levels enable the development of active HIF-1 by preventing post-translational changes of the HIF-1 subunit. Through controlling expression, angiogenesis, autocrine growth factor signaling, invasion, and treatment failure often due to the presence of ABC transporters, HIF-1 contributes to metabolic reprogramming (221, 222). In addition, the HIF-1 subunit helps control p53. Its main function is to control the expression of numerous genes that code for proteins, which helps to control apoptosis. The preservation of genomic integrity depends heavily on the transcription factor p53. An et al. were the first to discover that HIF-1 is essential to the p53 pathway. The stability of the p53 protein under extreme hypoxia conditions was determined by the authors in previous study (223). In addition, they noted that the phosphorylation of HIF-1 has a dual

role function in controlling apoptosis. According to Suzuki et al., the dephosphorylated form of HIF-1, which predominates in severe hypoxia and has a greater affinity for ARNT than the phosphorylated version of HIF-1, is necessary for p53 stabilization through HIF-1. On the other hand, research by Pan et al. and others have demonstrated that even intense hypoxia is insufficient to stabilize the p53 gene without the secondary holding provided by rigorous hypoxia, such as food shortage and pH collapse (224, 225). For many years, the only way to completely eradicate tumor cells and prevent their development and proliferation by chemical agents used in cancer therapy was through chemotherapy. Chemotherapy's main strength and most compelling flaw is its inability to distinguish between cancer cells and healthy cells, which results in severe toxicity and side effects. Cancer treatment has changed significantly during the past 20 years from broad-spectrum cytotoxic medications to tailored treatments (226). Targeted medications now have a higher potency and lower toxicity as compared to traditional chemotherapeutic drugs since they can directly target cancer cells while protecting healthy cells. Targeted medications can be broadly categorised into two groups: (a) small compounds like imatinib, which the US Food and Drug Administration (FDA) licenced for clinical use in 2001 (227); and (b) macromolecules such monoclonal antibodies, polypeptides, nucleic acids, and antibody-drug conjugates (228, 229). It is a well-known medication that will be easily developed and has entered a golden stage of development, which has been supported by the approval of targeted drugs.

Over the last 20 years, there has been a significant increase in targeted FDA-approved medicines for cancer treatment. Small-molecule targeted drugs, on the other hand, have several advantages over macromolecule targeted drugs in terms of cost, pharmacokinetic properties (PK), patient compliance, drug storage, and market availability. In the United States and China, 89 small anticancer molecules have been approved. Small molecules for anticancer drugs face numerous challenges, including drug resistance and a low response rate. Many strategies for administering chemotherapeutic medications can now be used to extend life. Chemotherapeutic drugs are commonly administered in a combinational approach. For various types of cancer, various combinations are available. In this review, we will focus on a few drugs that are commonly used to treat various cancer types, such as doxorubicin, cyclophosphamide, cisplatin, 5-fluorouracil, and others (Table 1). In general, chemotherapeutic agents are administered to patients who may be able to withstand the treatment. Because less cell death is observed in tumor masses, current clinical setup chemotherapeutic regimens are used to treat cyclic tumors. As a result, dose reputation is required to reduce tumor size. There are a few drawbacks to the duration and frequency of chemotherapies, which are limited by patient toxicity (230).

## 3.3 Immunotherapy

Hypoxia refers to solid tumors and attributes the selection of intrusive and destructive malignant clones displaying resistance to RT, traditional chemotherapy, or small molecule targeted therapy. The recent clinically applicable immunotherapy-based checkpoint inhibitors (ICPIs) and chimeric antigen receptor (CAR) T- cells, has evidently altered the prognosis for certain tumors (231). Notably, hypoxia triggers the angiogenesis and causes immunosuppression, which is termed another dilemma of hypoxia-induced immune resistance. While these treatment strategies reveal both a promise and a despair in terms of efficacy and safety in phases of clinical trials, they correspond to the future solution to appreciate the efficacy of immunotherapy in contrast to hypoxic and therapy-resistant solid tumors.

However, based on the prediction, tumor hypoxia has shown poor outcomes across all types of cancer. Despite the success of T-cell immune checkpoint blockade in treating melanoma, abrasive adenocarcinomas of the prostate and pancreas are mostly resistant to CTLA-4 and PD-1 antibody treatment in the mice and humans. Previously, Midan et al. reported that hypoxic zones of the tumors endure infiltration by T cells, even in the context of vigorous infiltration of T cells in normoxic regions of the same tumor (4). Beyond the dearth of admissibility to tumor-specific T-cells, hypoxia energizes the foundation of an extremely interdependent network of immunosuppressive stromal cells. Based on the Midan et al. finding it was noted that the critical population of myeloid-derived suppressor cells (MDSCs) and myofibroblasts which act together to suppress T-cell responses and intervene in immunotherapy resistance (4).

Tumor hypoxia primarily affects antitumoral immune activity by inhibiting the native immune system and immune killing mechanisms. Many studies have concentrated on immunosuppressive elements in the tumor microenvironment, including MDSCs, Treg cells, and TAMs in the hypoxic zone of solid tumors (232). Inside the hypoxic TME, HIF-1, a key hypoxia transcriptional factor, controls MDSC activity and differentiation. According to research by Norman et al. on this subject, enhanced HIF-1-dependent arginase activity and nitric oxide production in tumor-dependent MDSCs make them more immunosuppressive than splenic-derived MDSCs (233). Another study discovered evidence that HIF-1 controls PD-L1 expression by directly attaching to components that have hypoxia-responsive properties in the proximal promoter of PD-L1 (234). Atezolizumab is the first FDA approved ICI monoclonal antibody for the treatment of mTNBC which targets PDL-1 and later pembrolizumab is also approved for mTNBC treatment in combination with chemotherapy based on the positive clinical trials result with atezolizumab and pembrolizumab monotherapy in TNBC (215). There are several clinical trial studies are registered on clinicaltrial.gov which implies that using ICI either alone or in combination with other therapy could be a promising strategy in TNBC treatment which is summarizes in Table 1. In addition to ICI several other combinations with immunotherapies such as immunomodulators (acetylsalicylic acid, indomethacin, IFN-α2b etc.), T-cell targeted modulators (CART-TnMUC1, TC-510 etc.) are still under investigation and these may contribute to the development of precision immunotherapy for TNBC (215) (Table 1).

## 3.4 Adjuvant therapy

The main challenge in overcoming tumor hypoxia in a clinical setting is to increase oxygen delivery. Horsman et al. previously

showed that hyperbaric oxygen (HBO) treatment involves breathing 100% oxygen 2-4 times daily at normal atmospheric pressure. The results showed increased saturation of hemoglobin and oxygen levels in the circulation (235–237). In general, HBO treatment is administered during or shortly before radiation therapy. Previously, in the 1970s, Chaplin et al. reported that, when compared to normal air, patients with head and neck squamous cell carcinoma responded better to HBO treatment in terms of local control in a multi-center randomized trial (238).

On the other hand, carbogen breathing produced disparate results, which might be explained by variations in the number of patients who underwent carbogen breathing (239). Although the treatment for high-risk brain stem glioma in pediatric patients was well tolerated, there was no evidence of any benefit when radiation therapy was added. Siemann et al. previously reported that the combination of nicotinamide, a vitamin B3-derived molecule, and radiation appears to target both acute and chronic hypoxia (240). Additional research suggests that nicotinamide reduces ACT hypoxia by sporadically preventing vascular shut-down. Van Laarhoven et al. conducted ARCON trials, and other groups demonstrated improved patient survival, particularly in bladder and laryngeal cancer (241–243). However, the ARCON trials have demonstrated the efficacy of carbogen breathing as an adjuvant therapeutic regimen (244).

Interestingly, one of the adjuvant treatments called hyperthermia (HT) involves heating tissue over physiological temperatures (40–450°C). Although HT can be given directly to tumor masses, it is typically utilized as an adjuvant therapy alongside chemotherapy or radiation therapy due to technical challenges in obtaining cytotoxic temperatures (153, 154). However, in patients, ultrasonography is used to administer HT superficially or intravenously. Microwaves, radio frequencies, and electromagnetic radiation are all examples of electromagnetic radiation. In the 1970s and 80s, HT's positive impacts were seen in primary and secondary cells in the culture system, as well as in the preclinical evaluation of animal models and patients. Previously, five randomized trials combining radiation and HT exhibited benefits in recurrent melanoma, cervical and BC patients (155–157). HT has also been shown in studies to be beneficial in adolescent and pediatrics patients with various types of tumors, including soft tissue sarcoma, malignant germ cell tumors, and chondrosarcomas.

The impact of HT in DDR is another alternative to improve the current radiotherapy strategy, and it can significantly radiosensitize the tumor cells. According to earlier studies, HT stimulates the ATM and γ-H2AX pathways and increases the expression of p53 (158, 159). As a result, HT induction is an important pathway in DDR and plays an early role in DDR responses. Another intriguing study found that HT has a direct beneficial effect in combination with DDR-targeted therapy by inhibiting the homologous recombinant repair pathway. In addition, it also deactivates the NHEJ pathway by suppressing the interaction between Ku80 and BRCA2 at DSB damage sites (160). A clinical trial is underway with HT and Olaparib combination therapy for BC patients (NCT03955640). The hypothesis is that HT could modify the immune system *via* systemic treatments, promoting the expansion of the MHC class I. Published reports demonstrated that HT induces the significant infiltration of cytotoxic T, B, and NK cells (161, 162, 245). The preceding studies demonstrated that combining HT with immuno- and radiation therapy may improve treatment efficacy. Mild HT is one of the potential adjuvant treatments to overcome tumor hypoxia. To gain a more precise understanding, we must investigate the molecular-level relationship between HT and tumor oxygenation in depth. Furthermore, to identify therapeutic targets and understand the underlying mechanisms (HIF pathways, ROS, heat shock proteins, and EMT) of heat resistance pathways that could be used as therapeutic targets in cancer patients (218–220).

## 3.5 Anti-angiogenic therapy

As already discussed in Section 2.1, the role of angiogenesis in cancer survival and progression, we can estimate that targeting the angiogenesis could be a possible approach to combat TNBC. While reviewing the literature, we found that several angiogenesis inhibitors are clinically available against different types of advanced solid cancers. These inhibitors are generally either monoclonal antibodies or small molecule-based tyrosine kinase inhibitor, which target the VEGF and receptors. Angiogenesis inhibitors act by blocking the activity and expression of pro-angiogenic factors, secreted by tumor cells by targeting their receptors. Consequently, these inhibitors reduce the amount of nutrients available for tumor growth, and promote tumor vasculature normalization, and increase the delivery of cytotoxic chemotherapy (246–248). Unfortunately, these angiogenesis inhibitors failed to respond against BC when comparing the patient's survival outcome to that of other solid tumors. Although research is ongoing, several clinical trials are underway to explore the angiogenesis inhibitors clinical outcomes in BC and TNBC patients.

In clinical trials, in the subgroup analysis, TNBC patients had shown a significant improvement in overall response rate in the E2100 and Avado trials; however, no statistical differences were observed in the Ribbon 1 trial, in which bevacizumab, humanized anti-VEGF monoclonal antibody, was given in combination with either second-line treatment by using chemotherapy or bevacizumab plus paclitaxel for first-line treatment or bevacizumab is added to neoadjuvant chemotherapy (249–251). In these three trials, 684 patients with TNBC were enrolled, and a meta-analysis was performed. In these three trials, 684 patients with TNBC were enrolled, and a meta-analysis was performed. This exciting study has clearly shown a marginal increase in progression-free survival. The overall objective response rate was also statistically increased, and there was a trend towards improved overall survival (226). Similarly, several other monoclonal antibodies, such as ramucirumab, have also been studied in several clinical trials, but no improvement in the overall survival of patients has been observed (227).

Besides, few small molecule-based tyrosine kinase inhibitors were studied in several clinical trials. There are several inhibitors such as bevacizumab, lenvatinib, apatinib, cabozantinib have been

shown a positive clinical response in BC patients including TNBCs which are summarized in Table 1. In addition, a few other examples include, sorafenib, vandetanib, sunitinib, axitinib, pazopanib and cediranib, which are approved in several other cancers, like advanced renal cell carcinoma, hepatocellular carcinoma, soft-tissue sarcoma, gastrointestinal stromal tumors, advanced pancreatic neuroendocrine tumors, medullary thyroid carcinoma etc. Till now, these inhibitors were studied either as alone or in combination of first- and second-line treatment in various studies, but no significant improvement in overall survival in BC patients has been observed. All these inhibitors generally target the classical angiogenic pathway by targeting VEGF and VEGFR, and gave suboptimal results (228, 229). Thus, in our view, there is a need to explore novel anti-angiogenic approaches, such as targeting pericytes for vascular normalization, miRNA utilization and usage of immunotherapeutic drugs.

## 3.6 Photodynamic therapy

Another emerging and constantly developing method to treat cancer is photodynamic therapy (PDT), which involves using low to medium-energy monochromatic light to photo-excite subsequently applied photosensitizers (PS) interacting with the oxygen and producing ROS. The interaction between light and tissue is *via* absorption, scattering, reflection and refraction. Tissue's optical properties determine the distribution of treatment light, as most of the light is transmitted at near-infrared wavelengths. PDT uses light with a wavelength of 600-800 nm, and it is a well-known fact that light with longer wavelengths has been absorbed to a greater extent; therefore, one of the limitations of PDT is its therapeutic depth, which is less than a centimeter (252–254).

At its early stage, PDT is well established and accepted in dermatology such as non-melanoma skin cancers, pre-malignant conditions like actinic keratosis and Bowen's disease (255). Besides, it is also accepted in non-dermatologic condition like head and neck cancer (256), low grade prostate cancer (257) and pancreatic cancer (258, 259). But now, there have been reports describing PDT as suitable options for treating cutaneous metastases from BC as well as primary BC (239, 260). PDT combined with traditional antitumor therapies show much promising effect in improving patient outcome and reducing the unwanted side effects. The combination of light with rhodamine 123 and its platinum complex, indocyanine green (261), meso-tetra hydroxyphenyl chlorine and zinc phthalocyanine has been proven very effective in *in -vitro* studies (262–265). Recently, Chou et al. study the effect of combination of PDT and bio reductive therapy in targeting TNBC with an aptamer functionalized nano formulation (23). This new therapeutic strategy, which utilized the combination of protoporphyrin IX and tirapazamine, performed well in both hypoxia and normoxia, and hence could be a promising medical procedure for effective treatment of TNBC (Table 1). In summary, the synergistic effect of PDT and traditional therapies could enhance the therapeutic effect and even can prove to be a better way to tackle TNBCs.

## 3.7 Adoptive cell therapy and cancer vaccines

Recently, adoptive cell therapy (ACT) and cancer vaccines have been proposed as future therapy approaches, which can cure various cancer stages, including TNBC. Adoptive cell therapy in TNBC mainly covers three types of ACT which include three types of therapy: tumor-infiltrating lymphocyte (TILs), engineered T cell receptor, and chimeric antigen receptor therapy (CAR-T) which is strongly correlated with the infiltration of T-cells in TNBC (266). These all ACT based on similar principles where patients' natural t-cells have been modified genetically in ex-vivo condition and injected back into the patient's body to make them tumor antigen-specific and accelerate their ability to kill cancer cells by triggering the cytotoxic immune response (266, 267). CART-T cells improve the effective tumor transport of engineered activated T-cells and overcome antigenic heterogeneity and the broad repertoire of immune escape mechanisms occurring in advanced TNBC. However, certain issues need to be addressed, such as identifying tumor-specific antigens (TSAs) rather than tumor-associated antigens (TAs) and optimizing the adverse effects of cell lysis for immune hyper-activation (215). Currently, CAR-T cell therapies have been FDA-approved for the treatment of various cancer-type patients, including TNBC, and a considerable number of clinical trials are testing CAR constructs against multiple tumor antigens in TNBC, which are summarized in Table 1.

Cancer vaccines also target TAs to accelerate tumor-specific immune responses through active immunization by generating cytotoxic CD8+ T-cell (CTLs) and other effector immune responses such as NK and dendritic cell responses (266). These vaccines consist of either peptides, carbohydrates, recombinant DNA or RNA, whole cells, or dendritic cells (DC), which summarizes in Table 1. In addition, neoantigen vaccines use peptides that are specific to mutations in the tumor and not present in normal cells, therefore have been shown to elicit robust immunogenic responses because of high tumor mutational burden (TMB) and further activates tumor antigen specific CD8+ and CD4 + T cells (266). Emerging evidence suggests that these cancer vaccines, in combination with ICI and chemotherapeutic agents, may boost the anti-tumor immune response. The current clinical trials using cancer vaccines in combination with ICI and chemotherapeutic agents are summarized in Table 1.

## 3.8 Antibody drug conjugates

ADC are immunoconjugative drugs which are specifically engineered by using three pre-defined immune components a) cytotoxic drugs, b) a chemical linker moiety and c) a humanized monoclonal antibody specifically recognizing neoplastic epitopes on tumor cells and overexpressed definite antigens {trophoblast cell surface antigen 2 (trop-2), receptor tyrosine kinase-like orphan receptor (180), human epidermal growth factor receptor (HER) etc.} (215, 268). These ADC drugs are degraded once it recognizes and conjugates with specific antigens in the highly acidic metabolic

TME (268). ADC's high target specificity and potency feature defines its novelty in personalized therapeutic approaches. Emerging evidence also suggests that a high therapeutic index compared to traditional chemotherapies and their specificity against selective tumor populations make the ADCs a promising partner for targeted agents in combination therapies (268, 269). However, several preclinical and clinical data have shown and suggested high pharmacological properties and improved survival benefits, several limitations still need to be improved, such as recognition of specific binding antigens, optimization of the drug-to-antibody ratio (DAR) and release of the chemical linker in tumor cells and their toxicities etc. Song Hua et al., 2010 have shown that novel anti-HIF-1α ADC nano micelles filled with paclitaxel precisely target and selectively kill the stomach cancer cells having high expression of HIF-1α and suggesting that HIF-1 ADC could be great potential in various clinical settings (270). Several clinical studies have so far been ongoing based on preclinical antitumor activity in both neoadjuvant and metastatic settings in the TNBC cohort, and trop-2 targeted sacituzumab govitecan is the first FDA-approved ADC for the mTNBC treatment (215, 269). Table 2. summarizes the ongoing clinical trials of ADCs and their analogues in locally advanced or metastatic TNBC.

## 3.9 Combination therapies

TNBC lacks expression of some generalized targeted receptors such as estrogen, progesterone, and HER2 receptors, making it difficult to target with conventional therapies. However, combination therapy involves the simultaneous use of multiple treatment modalities, such as chemotherapy, targeted radiotherapy, and immunotherapies, to enhance efficacy and overcome resistance mechanisms by targeting multiple signaling pathways and tumor vulnerabilities. The combination treatment approaches mostly involved tailored strategies based on individual patient characteristics and the tumor's molecular profile, leading to precise therapy to improve patient outcomes. Paclitaxel and nab-paclitaxel are among the frequently used chemotherapy options, but their resistance is one of the major reasons for the failure and relapse of TNBC (271). Therefore, currently, several clinical trials are undergoing where the combination of paclitaxel or nab-paclitaxel with immune checkpoint inhibitors such as atezolizumab, cobimetinib, or PARP, AKT, PI3K, or VEGF inhibitors has been administered, leading to a significant increase in mean objective survival and response rate. Besides, the combination of chemotherapy and immune checkpoint inhibitors followed by adjuvant therapy are also under clinical trials, exhibiting significant positive responses. Similarly, the combination of anti-angiogenic therapy like lenvatinib, apatinib etc., with several inhibitors also exhibits positive responses in undergoing clinical trials. Ongoing research and clinical trials continue to explore innovative combination regimens, offering hope for improved survival rates and a brighter future for TNBC patients, and we have summarized such clinical trials revolving around combination therapy in Table 3.

# 4 Hypoxia: a main culprit to nullify the various cancer treatment strategies

Normal tissues generally require a steady supply of oxygen and nutrients to stay alive and remove waste through metabolism. The solid tumor, unlike normal tissue, has dysfunctional vasculature (285, 286). The rate of tumor progression, stroma composition, and pathological vasculature all contribute to a hypoxic environment in the tumor microenvironment, which impairs immune cell function. Furthermore, hypoxia creates selection pressure by promoting cell growth alongside genetic machinery having malignant potential (287). As a result, hypoxia causes EMT, which leads to cell mobility and metastasis (288, 289). Moreover, metabolism of tumor cell reforms after hypoxia, leading to cell quiescence (30, 64). This condition alters transport or distribution and is resistant to radiotherapy, chemotherapy, immunotherapy, and adjuvant therapy (31, 290). Chemotherapy and radiotherapy affect proliferating tumor cells, especially in normoxic states, but hypoxic cells survive these antineoplastic therapies. Thomlinson and Gray et al. previously proposed that hypoxia is a "diffusion-limited chronic hypoxia" (291).

The top preclinical evaluation studies demonstrated increasing tumor oxygenation by modifying oxygen delivery by allowing tumor-bearing rodent models to inhale either (95% $O_2$ + 5% $CO_2$) carbogen or 100% oxygen. According to the data, the tumor grew significantly after radiation (292). Many preclinical studies previously reported that cancer cells were more malignant in hypoxic conditions. Earlier, Young et al. *in-vitro* studies demonstrated that cells were kept for 18–24h in hypoxic conditions and injected into the mice (293). In such cases, injected cells reach the lungs and form lung nodules; additionally, they reported that the level of hypoxia in the primary tumor directly increases the number of metastases in tumor-injected mice, regardless of whether the hypoxia was natural or induced (294, 295). According to the previous report, two separate clinical trial studies on how hypoxia influences the malignant progression of cancer cells were conducted. The study's findings revealed that the oxygenation status of the patients was assessed using the Eppendorf electrode before the regimen. Previously, only one study on a cervix cancer patient who underwent surgery was reported (296). In the other group, most soft-tissue sarcoma patients underwent surgery (297). In such cases, injected cells reach the lungs and develop lung nodules. In addition, they also reported that the level of hypoxia in the primary tumor directly aggravated the number of metastases in tumor-injected mice regardless of whether that hypoxia was natural or induced (294, 295). Based on the previous report, two separate clinical trial studies were conducted on how hypoxia influences cancer cells' malignant progression. The study outcome revealed that the oxygenation status of the patient's assessed by applying the Eppendorf electrode before the regimen. Earlier, only one study was reported on a cervix cancer patient who underwent surgery (296). In the other group, most patients with soft-tissue sarcoma underwent surgery (297). Both studies found that patients who had previously received oxygenation treatment had an overall

TABLE 2 Development of antibody-drug conjugates (ADC) and ongoing clinical trials for TNBC treatment.

| S.No. | Treatment | Target | Cleavable linker | TNBC cases | Cohort | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|---|---|
| 1. | Sacituzumab govitecan | Trop-2 | SN-38 | 108 | mTNBC | II | ORR: 33.3%; 5.5 mo. | (211, 215, 269) |
| 2. | Datopotamab deruxtecan | Trop-2 | Deruxtecan | 44 | mTNBC | I | Recruiting | (215) |
| 3. | SKB264 | Trop-2 | Moderate cytotoxic belotecan-derivative | 48 | mTNBC | I-II | ORR: 35.3% | (211, 215) |
| 4. | Mirvetuximab soravtansine | Folate receptor $\alpha$ | Tubulin-disrupting maytansinoid DM4 | 44 | TNBC | I | Active | (215) |
| 5. | Ladiratuzumab vedotin (SGN-LIV1a) | Zinc transporter LIV-1 | Monomethyl auristatin E (MMAE) | 310 | mTNBC | 1b/II | Recruiting | (211, 215, 269) |
| 6. | NBE-002 | ROR1 | Anthracycline-derivative PNU-159682 | 100 | TNBC | I/II | Recruiting | (215) |
| 7. | VLS-101 | ROR1 | Monomethyl auristatin E (MMAE) | 210 | TNBC | II | Recruiting | (215) |
| 8. | CAB-ROR2-ADC (BA3021) | ROR2 | Conditionally active biologic (CAB) | 120 | TNBC | I/II | Recruiting | (215, 269) |
| 9. | Anti-CA6-DM4 immunoconjugate (SAR566658) | CA6 | DS6 | 23 | mTNBC | II | completed | (269) |
| 10. | Camidanlumab tesirine | CD25 | Pyrrolobenzodiazepine | 44 | mTNBC | I | Recruiting | (215) |
| 11. | Praluzatamabravtansine | Cd166 | Tubulin-disrupting maytansinoid DM4 | 125 | mTNBC | II | Recruiting | (215) |
| 12. | Vobramitamab duocarmazine (MGC018) | CD276 (B7-H3) | Duocarmycin | 143 | mTNBC | I/II | Recruiting | (215) |
| 13. | Anti-EGFR-immunoliposomes-DOX | EGFR | Doxorubicin | 48 | TNBC | I | ORR: 33%; PFS: 12mo. | (211, 215) |
| 14. | AVID 100 | EGFR | Cleavable linker with DM1 | 90 | TNBC | Ia/Iib | Terminated | (215, 269) |
| 15. | Trastuzumab dreuxtecan | HER2 | Topoisomerase I inhibitor | 278 | mTNBC | II | Recruiting | (215, 269) |
| 16. | Patritumab dreuxtecan | HER3 | Topoisomerase 1 inhibitor payload, an exatecan derivative (DXd) | 120 | mTNBC | I | Recruiting | (215) |
| 17. | Anetumab Ravtansine | Mesothelin (MSLN) | Maytansinoid tubulin inhibitor DM4 | 173 | TNBC | Ib | Active/not recruiting | (215) |
| 18. | Cofetuzumab peledotin | Protein tyrosine kinase 7 | Auristatin | 18 | mTNBC | I | ORR: 16.7%; mPFS: 2mo. | (215) |
| 19. | Enfortumab vedotin | Nectin-4 | Monomethyl auristatin E (MMAE) | 288 | mTNBC | II | Recruiting | (211, 215) |
| 20. | BT8009 | Nectin-4 | Monomethyl auristatin E (MMAE) | 329 | TNBC | I/II | Recruiting | (215) |
| 21. | TH1902 peptide | Sortilin | Docetaxel-peptide conjugate | 70 | mTNBC | I | Recruiting | (215) |
| 22. | Rovalpituzumab Tesirine | Delta like protein 3 (DLL-3) | Cytotoxic pyrrolobenzodiazepine (PBD) | 182 | TNBC | I | Active/not recruiting | (211) |

mTNBC, metastatic TNBC; pCR, Pathological complete response; PFS, Progression free survival; mPFS, mean progression free survival; ORR, Objective response rate; mOS, Mean objective survival, ITT, Intention to treats; mo., month.

higher survival rate. Patients with higher levels of hypoxia had significantly worse survival outcomes based on their pretreatment oxygenation status (296, 297). Later clinical studies revealed that using an eppendorf electrode causes significant hypoxia in leiomyomas, myometrium, and leiomyosarcomas, all originating in premenopausal women (298). Solid tumors directly contribute to cancer's malignant properties and are a hallmark of hypoxia (299, 300). It is well understood that HIFs instantly activate tumors through HIF transcription factors, which promote changes in the expression of VEGF and CAIX levels, both of which are required for unstable and anaerobic energy production (301). In general, HIFs promote the expression of multiple genes involved in metabolic

TABLE 3 Current combination treatments in TNBCs.

| S. No. | Treatment | TNBC cases | Cohort | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|
| A. | Current clinical trials of ICIs involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Nab-paclitaxel+atezolizumab | 33 | mTNBC | Ib/III | ORR: 39.4%, mPFs: 5.5mo, mOS 14.7mo. | (203, 272) |
| 2. | Ipatasertib and atezolizumab plus either nab-paclitaxel | 26 | mTNBC | Ib | ORR: 73% | (203) |
| 3. | Ladiratuzumab vedotin + pembrolizumab | 26 | mTNBC | Ib/II | ORR: 54% | (203) |
| 4. | Durvalumab + trastuzumab deruxtecan | 21 | mTNBC | Ib/II | ORR: 66.7% | (203, 273) |
| 5. | Eribulin + pembrolizumab | 167 | mTNBC | Ib/II | ORR: 23.4, mPFS: 4.1mo, mOS: 16.1mo. | (203, 274) |
| 6. | Atezolizumab+taxanes+MEKi | 902 | Locally advanced/ mTNBC | II | Active/ORR: 29%-34% | (275) |
| 7. | Pembrolizumab +MEKi | 12 | Locally advanced/ mTNBC | I/II | Recruiting | (275) |
| 8. | Cobimetinib and atezolizumab + either nab-paclitaxel/paclitaxel | 63 | mTNBC | II | ORR: 31.7% | (203, 276) |
| 9. | Entinostat + atezolizumab | 40 | mTNBC | II | ORR: 10%; mPFS: 1.68mo; mOS: 9.4mo. | (203) |
| 10. | Lenvatinib + pembrolizumab | 31 | mTNBC | II | ORR: 29% | (203) |
| 11. | Paclitaxel + atezolizumab/placebo | 651 | mTNBC | III | ORR in ITT: 53.6 *vs.* 47.5% | (203, 277) |
| 12. | GX-17 + pembrolizumab | 30 | mTNBC | Ib/II | ORR: 13.3% | (203) |
| 13. | Nab-paclitaxel+atezolizumab/placebo | 902 | mTNBC | III | ORR: 45.9% | (277) |
| 14. | Pembrolizumab + nab-paclitaxel/paclitaxel/gemcitabine/carboplatin | 882 | mTNBC | III | PFS: 9mo. | (19, 275) |
| 15. | Pembrolizumab + gemcitabine/carboplatin | 87 | mTNBC | II | Pending | (275) |
| 16. | Pembrolizumab + eribulin mesylate | 167 | mTNBC | Ib/II | ORR: 25%; PFS: 4.1mo. | (275) |
| 17. | Nivolumab after Cyclophosphamide/cisplatin/doxorubicin | 66 | mTNBC | II | ORR: 35% (Doxorubicin); | (275) |
| 18. | Atezolizumab +nab-paclitaxel | 900 | Locally advanced/ mTNBC | III | ORR: 53%; OS: 25mo | (166, 275) |
| 19. | Atezolizumab + paclitaxel | 600 | Locally advanced/ mTNBC | III | Pending | (275) |
| 20. | Atezolizumab + gemcitabine/carboplatin or capecitabine | 540 | Locally advanced/ mTNBC | I | Recruiting | (275) |
| 21. | Atezolizumab + paclitaxel followed by atezolizumab +AC or EC | 2,300 | Locally advanced | III | Recruiting | (275) |
| 22. | Neoadjuvant pembrolizumab + paclitaxel and AC | 114 | Locally advanced | II | Recruiting | (275, 278) |
| 23. | Neoadjuvant pembrolizumab + chemotherapy combination (Nab-paclitaxel, paclitaxel, doxorubicin, Cyclophosphamide, carboplatin | 60 | Locally advanced | I | Completed, pCR: 60% | (275) |
| 24. | Neoadjuvant pembrolizumab + paclitaxel-carboplatin followed by adjuvant pembrolizumab | 1,174 | Locally advanced | III | pCR: 64.8% | (279) |

*(Continued)*

TABLE 3 Continued

| S. No. | Treatment | TNBC cases | Cohort | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|
| 25. | Paclitaxel ± Pembrolizumab followed by adjuvant thaerpy | 114 | Early Stage | II | pCR: 60% *vs.* 22% | (203) |
| 26. | Nab-paclitaxel+durvalumab/placebo followed by endocrine therapy +durvalumab/placebo | 174 | Early Stage | II | pCR in ITT: 53.4% *vs.* 44.2% | (203) |
| 27. | Pembrolizumab+anthracycline+taxane-based chemotherapy ± carboplatin followed by adjuvant chemotherapy | 60 | Early Stage | ib | pCR overall: 60% | (203) |
| 28. | Nab-paclitaxel+atezolizumab/placebo followed by adjuvant chemotherapy+atezolizumab/placebo | 313 | Early Stage | III | pCR in ITT: 58% *vs.* 41% | (203, 280) |
| 29. | Anthracycline, taxane and carboplatin+Pembrolizumab/placebo followed by adjuvant chemotherapy/endocrine therapy | 1,174 | Early Stage | III | pCR: 63% *vs.* 55% | (203, 281) |
| 30. | Nab-paclitaxel+acarboplatin ± atezolizumab | 280 | Early Stage | III | pCR in ITT: 43.5% *vs.* 40.8% | (203) |
| 31. | Neoadjuvant atezolizumab+paclitaxel+carboplatin followed by atezolizumab +AC or EC | 1520 | Early Stage | III | Recruiting | (203) |
| 32. | Atezolizumab + carboplatin + nab-paclitaxel | 278 | Early/Locally advanced/ mTNBC | III | Active/not recruiting | (215) |
| 33. | Atezolizumab + neoadjuvant chemotherapy | 1550 | TNBC | III | Active/not recruiting | (215) |
| 34. | Atezolizumab + nabpaclitaxel | 184 | Locally advanced/ mTNBC | III | Active/not recruiting | (215) |
| 35. | Atezolizumab + chemotherapy | 572 | Locally advanced/ mTNBC | III | Recruiting | (215) |
| 36. | Atezolizumab + adjuvant anthracycline/taxane based therapy | 2300 | Locally advanced/ mTNBC | III | Recruiting | (215) |
| 37. | Atezolizumab + ipataseritib and paclitaxel | 242 | mTNBC | III | Active/not recruiting | (215) |
| 38. | Avelumab as adjuvant or post-neoadjuvant | 474 | Locally advanced/ mTNBC | III | Active/not recruiting | (215) |
| 39. | Camrelizumab + Chemotherapy | 581 | Locally advanced/ mTNBC | III | Recruiting | (215) |
| 40. | Serplulimab + chemotherapy | 522 | Locally advanced/ mTNBC | III | Not recruiting | (215) |
| 41. | Toripalimab + nab-paclitaxel | 531 | Locally advanced/ mTNBC | III | Recruiting | (215) |
| 42. | Carelizumab + nab-paclitaxel + apatinib *vs.* Carelizumab+nab-paclitaxel *vs.* nab-paclitaxel | 80 | Locally advanced/ mTNBC | III | Recruiting | (215) |
| 43. | TQB2450 + anlotinib hydrochloride/paclitaxel | 332 | TNBC | III | Not recruiting | (215) |
| 44. | Anti-Globo-H-Vaccine adagloxad simolenin (OBI-822)/OBI-821 | 668 | Early Globo-H+ TNBC | III | Recruiting | (215) |
| B. | Current trials of combination chemotherapeutic agents involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Ixabepilone+capecitabine *vs.* capecitabine | 443 | mTNBC | III | PFS: 4.2 *vs.* 1.7mo.; OS: 9.0 *vs.* 10.4 mo. | (215) |
| 2. | Pacitaxel+carboplatin *vs.* cyclophosphamide+epirubicin+fluorouracil +docetaxel | 647 | TNBC | III | DFS: 86.5 *vs.* 80.3% | (215) |

*(Continued)*

TABLE 3 Continued

| S. No. | Treatment | TNBC cases | Cohort | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|
| 3. | Docetaxel+epirubicin ± lobaplatin | 125 | TNBC | II | pCR: 93% *vs.*73% | (215) |
| 4. | Cisplatin+gemcitabine *vs.* paclitaxel+gemcitabine | 236 | mTNBC | III | PFS: 7.7 *vs.* 6.47mo. | (215) |
| C. | Current trials of PARP inhibitor involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Veliparib/Paclitaxel/carboplatin *vs.* Paclitaxel/carboplatin *vs.* Paclitaxel | 634 | Early Stage | III | pCR: 53% *vs.*58% | (215) |
| 2. | Veliparib+carboplatin | 72 | Locally advanced/ mTNBC | II | pCR51%*vs.*26% | (215) |
| 3. | Paclitaxel/carboplatin ± olaparib | 527 | Early stage TNBC | II/III | pCR15 to 20% | (275) |
| 4. | Olaparib + Pembrolizumab *vs.* carboplatin/gemcitabine | 932 | Locally advanced/ mTNBC | II/III | Recruiting | (282) |
| 5. | Olaparib + durvalumab | 17 | mTNBC | I/II | ORR: 58.8%; mPFS: 4.9mo; mOS: 20.5mo. | (203, 283) |
| 6. | Niraparib + pembrolizumab | 45 | mTNBC | I/II | ORR: 29.0%; mPFS 2.3mo. | (203, 284) |
| 7. | Atezolizumab + olaparib | 81 | Locally advanced/ mTNBC | II | Active/Not-recruiting | (275) |
| 8. | Iniparib+ gemcitabine/carboplatin | 80 | Early Stage TNBC | II | pCR: 36% | (215) |
| D. | Current trials of AKT inhibitor involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Paclitaxel ± ipatasertib | 450 | Locally advanced/ mTNBC | III | PFS: 9.3mo; ORR: 47% | (282) |
| 2. | Paclitaxel+ipatasertib or placebo | 124 | Locally advanced/ mTNBC | II | PFS: 6.2 *vs.* 4.9 mo. | (215) |
| 3. | Ipatasertib+paclitaxel or placebo | 151 | Early stage TNBC | II | pCR: 17% *vs.* 13% | (215) |
| 4. | Paclitaxel ± capivasertib | 800 | Locally advanced/ mTNBC | III | Recruiting | (282) |
| 5. | Paclitaxel + capivasertib or placebo | 140 | mTNBC | II | PFS: 5.9 *vs.* 4.2 mo. | (215) |
| 6. | Paclitaxel/ipatasertib/Atezolizumab *vs.* Paclitaxel/ipatasertib *vs.* Paclitaxel | 450 | Locally advanced/ mTNBC | III | Recruiting | (282) |
| E. | Current trials of PI3K inhibitor involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Nab-paclitaxel ± alpelisib | 566 | Locally advanced | III | Pending | (282) |
| 2. | Paclitaxel+buparlisib or placebo | 416 | mTNBC | II/III | PFS: 8.0 *vs.* 9.2 mo. | (215) |
| 3. | Camrelizumab+apatinib or intermittent apatinib | 40 | mTNBC | | PFS: 3.7 *vs.* 1.9 mo. | (215) |
| F. | Current trials of VEGF/VEGFR inhibitor involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Paclitaxel+carboplatin *vs.* cyclophosphamide+carboplatin+/or bevacizumab | 443 | mTNBC | III | pCR: 93.5% *vs.* 73.0% | (215) |
| 2. | Anthracycline+taxane± bevacizumab | 493 | TNBC | III | pCR: 50% | (215) |
| 3. | Paclitaxel+doxorubicin+ bevacizumab±;carboplatin | 315 | TNBC | II | pCR: 53% *vs.* 36.9% | (215) |

*(Continued)*

TABLE 3 Continued

| S. No. | Treatment | TNBC cases | Cohort | Clinical Phase | Status | References |
|---|---|---|---|---|---|---|
| G. | Current trials in combination with cancer vaccine involving patients with metastatic/early stage TNBC | | | | | |
| 1. | Pembrolizumab+PX-410 | 20 | TNBC | I | Active/ Not-recruiting | (215) |
| 2. | Pembrolizumab+p53-MVA | 11 | TNBC | I | Active/ Not-recruiting | (215) |
| 3. | Durvalumab+PX-410 | 22 | TNBC | I | Active/ Not-recruiting | (215) |
| 4. | Durvalumab+neo-antigen DNA vaccine | 18 | TNBC | I | Active/ Not-recruiting | (215) |
| 5. | Durvalumab+nab-paclitaxel+neo-antigen DNA vaccine | 70 | TNBC | II | unknown | (215) |
| 6. | Atezolizumab+neo-antigen DNA vaccine | 272 | TNBC | I | Active/ Not-recruiting | (215) |

ICIs, Immune checkpoint inhibitors; mTNBC, metastatic TNBC; pCR, Pathological complete response; PFS, Progression free survival; mPFS, mean progression free survival; ORR, Objective response rate; mOS, Mean objective survival, ITT, Intention to treats; mo., month.

management, pH balance, angiogenesis, and cell apoptosis, all of which contribute to tumor survival.

As a result, Figure 5 clearly explains the mechanism, as mentioned earlier. HIFs play a role in tumor blood recovery *via* vascular protection and promote nutrient supply to solid tumors, which become one of the most difficult to treat, leading to resistance to chemotherapy, radiotherapy, immunotherapy, and adjuvant therapy.

## 4.1 Targeting hypoxia: a new tactic to improve current TNBC therapy

A key goal of TNBC therapy has been to target hypoxia, which inhibits several tumor characteristics, including metastasis, radioresistance, and chemoresistance (302, 303). A study that has been published suggests that many hypoxia-related genes (HRGs) and their mediators, HIFs, may be used as therapeutic targets and prognostic indicators in BC (34, 303).

HIF1α is a well-established key target regulating the TNBC, and its expression is regulated by various signaling pathways like NF-κB, PI3K/Akt/mTOR, RAS-RAF-ME-ERK and JAK-STAT. Studies have found that under hypoxic conditions, HIF1α induced STAT3 *via* JAK or adenylate receptor 2B pathway, which upregulates the IL-6 and NANOG to maintain the CSC phenotype and also enhances the production of VEGF, required for the self-renewal ability of CSCs (79). Studies have also demonstrated that in hypoxic conditions, HIF-1α activates the Sonic Hedgehog signaling pathway to induce the production of CSC markers in cholangiocarcinoma cells which can be blocked by HIF-1α inhibition (304). Emerging studies also demonstrated that HIF1α suppress the ERK activity and induces the P38 activity, which further upregulates NANOG and KLF4 to promote the development of breast CSCs. Several studies have been conducted to identify targetable molecules from these signaling pathways that characterize various inhibitors or drug molecules (304, 305). The astonishing fact is that some of these signaling pathways can be targeted by already approved therapeutics or inhibitors under clinical trials alpelisib is an approved inhibitor while buparlisib is under clinical trial, and both inhibit class I PI3K. Similarly, several inhibitors target VEGF, EGFR, PARP, and cell cycle and have shown significant outcomes in TNBC patients. Table 4 summarizes potential inhibitors and drug molecules against molecular targets and

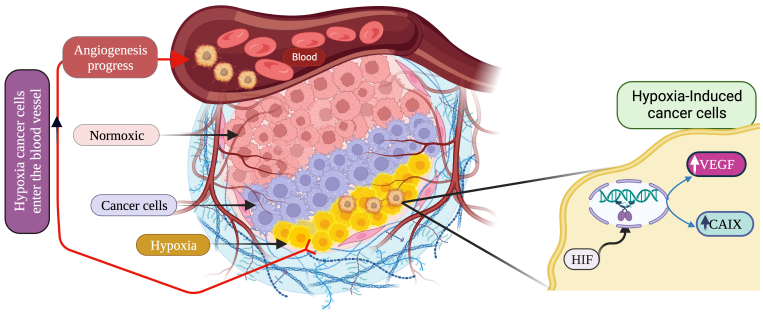



FIGURE 5
Schematic description of hypoxic cancer microenvironment and angiogenesis progression. (Created *via* BioRender.com).

signaling pathways involved in the progression of hypoxia-induced TNBC.

TNBC patients exhibit higher mortality rates, and it has already been studied that overexpression of HIF-1α is associated with poor prognosis in various cancer (302). Here, we have explored a publicly available gene expression dataset (GSE103091, subseries GSE58812) to study the effect of hypoxia-related gene expression on mortality (303, 304). This dataset contains gene expression molecular subtyping of TNBC samples from 107 patients (78 alive and 29 dead). We have explored the expression of HIF and VEGFs and genes for glucose transporters. As shown in Figure 6, the overall presentation of HIF-1α is significantly higher in the patients who died due to TNBC than in the alive patients. However, there is no significant relationship between HIF-3α with the mortality.

Similarly, the expression of VEGF-A and GLUT-1 significantly ($p = 0.001$ and 0.02, respectively) differs in both cohorts. This study suggests a possible association between hypoxia-related gene expression and mortality. However, other factors like age, cancer grade, metastatic etc., haven't been considered and may impact the conclusion. But hypothetically, there is a strong correlation between hypoxia-related gene expression and mortality, and it needs to be validated in larger cohorts.

## 4.2 The potential significance of targeting HIF-1α in different therapies

The association between HIF-1α and TNBC strongly suggests the possibility of novel targeted therapy in combination with chemotherapy, anti-angiogenic therapy, and immunotherapy for TNBC treatment (97, 102). HIF-1α reflects its potential to improve the current therapeutic outcome because of its extensive biological activities, particularly its function in angiogenesis, activation, and enhancement of tumor stem cells among other processes. The enrichment of BCSCs in tumors generated by various chemotherapeutic treatments is highly correlated with the increase of HIF-1, which is the major hurdle against chemotherapy. Clinical evidences support that some molecules like selenium, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) in combination with low-dose chemotherapeutic agents significantly induced the degradation of HIF-1α and limits the BCSCs enrichment which may increase TNBC chemotherapy resistance (97, 310).

It is well documented that HIF-1 is stabilized in hypoxic conditions, and transcriptionally controls the lactate dehydrogenase A (LDHA) gene, which is associated to glycolysis

TABLE 4 Potential inhibitors and drugs for hypoxia induced TNBC.

| S. No. | Inhibitors/Drugs | Mechanism of Action | References |
|---|---|---|---|
| A. | Tropomycin receptor kinase (TRK) Inhibitors | | (203, 215) |
| 1. | Larotrectinib | Binds to Trk and prevent neurotrophin-Trk interaction and Trk activation | |
| 2. | Selitrectinib | Inhibitor of Trk receptors | |
| 3. | Repotrectinib | Inhibitor of Trk receptors | |
| B. | Human epidermal growth factor receptor (HER) Inhibitors | | (203, 215) |
| 1. | Netatinib | Inhibit Growth factor receptors | |
| C. | Phosphoinositide 3-kinase (PI3K) Inhibitors | | (203, 215) |
| 1. | Alpelisib | Inhibit class I PI3K p110α | |
| 2. | Taselisib | PI3K Inhibitor targeting PI3Kα/δ/γ | |
| 3. | Buparlisib | Inhibits class I PIK3 in ATP-competitive manner | |
| 4. | Sapanisertib | Inhibitor of raptor-mTOR (TOR complex 1 or TORC1) and rictor-mTOR (TOR complex 2 or TORC2) | |
| 5. | Ipatasertib | Inhibit PI3K pathway | |
| 6. | Uprosertib | Binds to and inhibits the activity of Akt, which may result in inhibition of the PI3K/Akt signaling pathway | |
| 7. | Samotolisib | Inhibitor of certain class I phosphoinositide 3-kinase (PI3K) isoforms and mammalian target of rapamycin kinase (mTOR) in the PI3K/mTOR signaling pathway | |
| 8. | Copanlisib | Inhibit PI3K-α and PI3K-δ isoforms | |
| 9. | Eganelisib | Inhibits gamma isoform of phosphoinositide-3 kinase | |
| 10. | Gedatolisib | Inhibits both PI3K and mTOR kinases | |
| 11. | GDC-0941 | Inhibit PI3K pathway | |
| 12. | NVP-BKM120 (BKM-120) | Inhibit PI3K pathway | |
| 13. | BEZ235 (NVP-BEZ235) | Inhibit PI3K/mTOR pathway | |

*(Continued)*

TABLE 4 Continued

| S. No. | Inhibitors/Drugs | Mechanism of Action | References |
|---|---|---|---|
| 14. | GDC-0980 | Inhibit PI3K/mTOR pathway | |
| D. | Protein kinase B (PKB/AKT) Inhibitors | | (203, 215) |
| 1. | Ipatasertib | Inhibits AKT pathway | |
| 2. | Capivasertib | Inhibits AKT pathway | |
| E. | Mammalian target of rapamycin (mTOR) Inhibitors | | (203, 215) |
| 1. | Everolimus | Inhibits mTOR | |
| 2. | Vistusertib | Inhibits mTOR 1/mTOR2 | |
| 3. | Gedatolisib | PI3K/mTOR inhibitor | |
| F. | Mitogen-activated protein kinase (MEK) Inhibitors | | (203, 215) |
| 1. | Trametinib | Inhibits MEK pathway | |
| 2. | Binimetinib | Inhibits MEK pathway | |
| 3. | Selumetinib | MEK 1/2 inhibitor | |
| G. | Cyclin-dependent kinase 4 and 6 (CDK4/6) Inhibitors | | (22, 203, 215) |
| 1. | Palbociclib | Inhibits CDK4/6 | |
| 2. | Abemaciclib | Inhibits CDK4/6; G0/G1 arrestor, induce chromatin condensation | |
| 3. | Ribociclib | Inhibits CDK4/6; G0/G1 arrestor, induce apoptosis | |
| H. | Checkpoint kinase 1 (CHK1) Inhibitors | | (22, 203, 215) |
| 1. | LY2880070 | Inhibits CHK 1 | |
| 2. | Prexasertib | Inhibits CHK 1 and induced Homologous recombination deficiency | |
| I. | WEE1 Inhibitors | | (22, 203, 215) |
| 1. | AZD1175 | Inhibits WEE1 | |
| 2. | ZN-c3 | Inhibits WEE2 | |
| 3. | MK1775 | Inhibits WEE 1 kinase, G2/M arrestor; sensitize cells to cisplatin | |
| J. | Checkpoint kinase 2 (CHK2) Inhibitors | | (203, 215) |
| 1. | LY2606368 | Inhibits CHK2 | |
| K. | Androgen Receptors (AR) Inhibitors | | (22, 306, 307) |
| 1. | Bicalutamide | Inhibits AR | |
| 2. | Enzalutamide | Inhibits AR | |
| 3. | Abiraterone | Inhibits AR | |
| 4. | Enobosarm | Inhibits AR | |
| 5. | Darolutamide | Inhibits AR | |
| 6. | 17-DMAG | HSP-90 inhibitor, regulate the stability of AR | |
| 7. | VT464 | Involved in synthesis of AR | |
| L. | Atxia telangiectasia and Rad3-related (ATR) Inhibitors | | (203, 215) |
| 1. | Ceralasertib | Inhibits ATR | |
| M. | RAD51 Inhibitor | | (203, 215) |
| 1. | CYT-0851 | Inhibits RAD51 | |
| N. | Poly (ADP ribose) polymerase (127) Inhibitors | | (22, 203, 215) |
| 1. | Olaparib | Inhibit PARP | |

*(Continued)*

TABLE 4 Continued

| S. No. | Inhibitors/Drugs | Mechanism of Action | References |
|---|---|---|---|
| 2. | Talazoparib | Inhibit PARP | |
| 3. | Veliparib | Inhibit PARP | |
| 4. | Rucaparib | Inhibit PARP | |
| 5. | Niraparib | Inhibit PARP | |
| 6. | Pamiparib | Inhibit PARP | |
| 7. | Fluzoparib | Inhibit PARP | |
| 8. | Iniparib | Inhibit PARP1 | |
| O. | Carbonic anhydrase IX (CAIX) Inhibitors | | (308) |
| 1. | SLC-0111 | Inhibitor of carbonic anhydrases IX/XII; resulted in CSCs and EMT inhibition in TNBC cell lines | |
| 2. | DTP348 | CAIX inhibitor/radiosensitizer, inhibits HIF-1α in TNBC by targeting Hsp 90 | |
| P. | Cell cycle Inhibitors | | (203, 215) |
| 1. | Trilaciclib | Inhibits CDK4/6; G)/G1 arrestor | |
| 2. | Etoposide | Inhibits CDK4/6; G)/G1 arrestor | |
| 3. | PF-06873600 | Inhibitor of CDK4/6 | |
| 4. | Abemaciclib (Verginio) | Inhibitor of CDK4/7 | |
| 5. | Prexasertib | Inhibits CHK 1 and induced Homologous recombination deficiency | |
| Q. | Vascular endothelial growth factor/receptor (VEGF/VEGFR) Inhibitors | | (203, 215) |
| 1. | Anlotinib | Inhibitors of VEGFR 1/2 and FGFR 1/4 | |
| 2. | Apatinib | Inhibitors of VEGFR 2 | |
| 3. | Afatinib | Inhibitors of ErbB family of receptors (EGFR/ErbB1, HER2/ErbB2, ErbB3, and ErbB4) | |
| 4. | Lenvatinib | Inhibitors of FGFR 3 and decreases the phosphorylation of downstream molecules of the FGF signaling pathway (such as FRS2, Erk, and p38 MAPK), and induced PARP cleavage | |
| 5. | Erlotinib | Reduces VEGF promoter activity | |
| 6. | Famitinib | inhibitor of targeting VEGFR2, PDGFR and c-kit | |
| 7. | Pyrotinib | Irreversible pan-ErbB receptor tyrosine kinase inhibitor that targets hEGFR (HER) 1, HER2, and HER4 | |
| 8. | Bevacizumab | Inhibitors of VEGFR 2 | |
| R. | Epidermal growth factor receptor (EGFR) Inhibitors | | (22, 203, 215) |
| 1. | Dasatinib | Block BCSCs enrichment and Src activation | |
| 2. | Gefitinib | Inhibits AKT and MEK pathway | |
| 3. | Sorafenib | Modulates the SHP-1/STAT3 axi | |
| 4. | Nimotuzumab | Inhibitor of EGFR pathway and has low immunogenicity | |
| 5. | Panitumumab | Inhibitor of EGFR pathway | |
| 6. | SCT200 | Inhibitor of EGFR pathway | |
| S. | g-Secretase Inhibitors | | (215) |
| 1. | AL101 | Inhibitor of NOTCH 1, 2, 3, and 4 | |
| 2. | PF-03084014 | Inhibitor of g-secretase inhibitor | |
| T. | AXL Kinase Inhibitors | | (215) |
| 1. | Bemcentinib | AXL kinase inhibitor; inhibits Axl phosphorylation | |
| U | Hedgehog pathway Inhibitors | | (215) |

*(Continued)*

TABLE 4 Continued

| S. No. | Inhibitors/Drugs | Mechanism of Action | References |
|---|---|---|---|
| 1. | Vismodegib | Hedgehog (Hh) pathway inhibitor | |
| V. | CXCL8 and CXCR1/2 Inhibitors | | (203, 215) |
| 1. | Reparixin | Allosteric inhibitor of CXCR1, reduced the CSC content of human BC | |
| W. | Hypoxia-activated prodrugs (HAP) of DNA-damaging cytotoxins | | |
| a. | DNA breakers | | (309) |
| 1. | Tirapazamine | Produces hydroxyl or and benzotriazinyl radicals as the DNA damaging reactive species in hypoxic cells | |
| 2. | SN30000 | Selective activation to a DNA-reactive radical species under hypoxia | |
| b. | DNA alkylators | | (309) |
| 1. | TH-302 | Cellular reductases that generate a radical anion through 1-electron reduction | |
| 2. | PR-104 | Exploit hypoxia and HR defects in tumors, with translational implications for TNBC and other HR-deficient malignancies | |
| 3. | SN30548 | Exploit hypoxia and HR defects in tumors, with translational implications for TNBC and other HR-deficient malignancies | |

and supports an acidic milieu. In patients with TNBC, this acidic milieu changes increases the CD8+ T cell counts and the generation of IFN, which is linked to a better clinical result and a stronger immunological response (311). Therefore, to improve the acidic microenvironment through HIF-1/LDHA targeting may restore the cytotoxic effect of CD8 cells to enhance the impact of immunotherapy in TNBC (97, 312). HIF-1α interaction with HDAC1 and concurrent PRC2 dependency epigenetically suppress the effector genes and induces the immune dysfunction in TNBC which results resistance to immunotherapy. A recent study in syngeneic and humanized TNBC mouse model has shown the efficacy of PD-1 blockade combined with HIF-1α and HDAC1 inhibition by PX478 and ENT respectively to reverse the anti-PD-1-resistant TNBC and significantly reduces tumor metabolic activity and metastasis (102, 313). Some clinical studies have showed that anti-angiogenic therapy alone is not recommended as the first-line treatment for metastatic TNBC since it increases the likelihood of TNBC invasion and metastasis. Consequently, inhibiting HIF-1 can enhance clinical efficacy by preventing invasion and metastasis-induced anti-angiogenic treatment. When used with the anti-angiogenic drug avastin, Guo et al. discovered that selenium with omega-3 polyunsaturated fatty acids decrease angiogenesis and metastasis *via* preventing COX-2 overexpression induced by HIF-1 (168). Furthermore, the anti-angiogenic drug bevacizumab hyperactivates the Wnt/β-catenin signaling in response to HIF-1α's high expression in TNBC because of aberrant expression of frizzled 7 (Fzd7), a key receptor for Wnt/β-catenin signaling's key receptor that induces cell invasiveness and metastasis (314, 315). Consequently, using an anti-Fzd7 antibody (SHH002-hu1) to target hypoxia adaptation-related proteins VEGFA and Glut1 expression as well as HIF-1 transcriptional activity will decrease TNBC cells' acclimation to hypoxia and counteract the negative effects of anti-angiogenic medicines (314).

Under hypoxic conditions, irradiation can increase HIF-1 expression, which would lead to radio resistance (316). There are some confirmed reports suggesting that after radiation therapy, the availability of oxygen and glucose is increased in solid tumors, which activate HIF-1 and promote EMT that is HIF-1 dependent. Because of the translocating cells' proximity to the blood arteries, which allows them to absorb nutrition and oxygen, tumor recurrence may be made easier (317, 318). The HIF-1 dependent translocation and migration of the surviving cells towards radio-protected blood vessels may indicate a specific role for HIF-1 in both local tumor recurrence and distant tumor metastasis after radiation therapy. Recent findings also suggest that HIF-1 inhibition utilizing HIF-1 inhibitors, may enhance radiosensitivity, chemosensitivity, immunosenstivity that could potentially provide advantages to methods of therapeutic treatment for hypoxic malignancies (316, 319). Table 5 summarizes several potential synthetic compounds and natural products that have clinically proven to inhibit the HIF-1α activity at the transcriptional and translational level in TNBC, such as inhibiting the mRNA level of HIF-1α and their dimerization with HIF-1β as well as accelerating the degradation of the HIF-1α protein.

## 5 Conclusion and future prospective

A preponderance of evidence supports the notion that different histological and molecular subtypes of TNBC signify its heterogeneity and aggressiveness. Several genetic and transcriptomic alterations define each subtype of TNBC, and they can be potentially targeted for a unique therapy. Recent advancements in targeting hypoxic-tumor microenvironments by suppressing HIF-1α transcription and oxidative phosphorylation have yielded promising results. Besides, anti-angiogenesis inhibitors and hypoxia-activated pro-drugs gained a lot of attention. Moreover, recent studies confirmed that TNBC also causes hypoxia-dependent genetic changes in DDR pathways, which

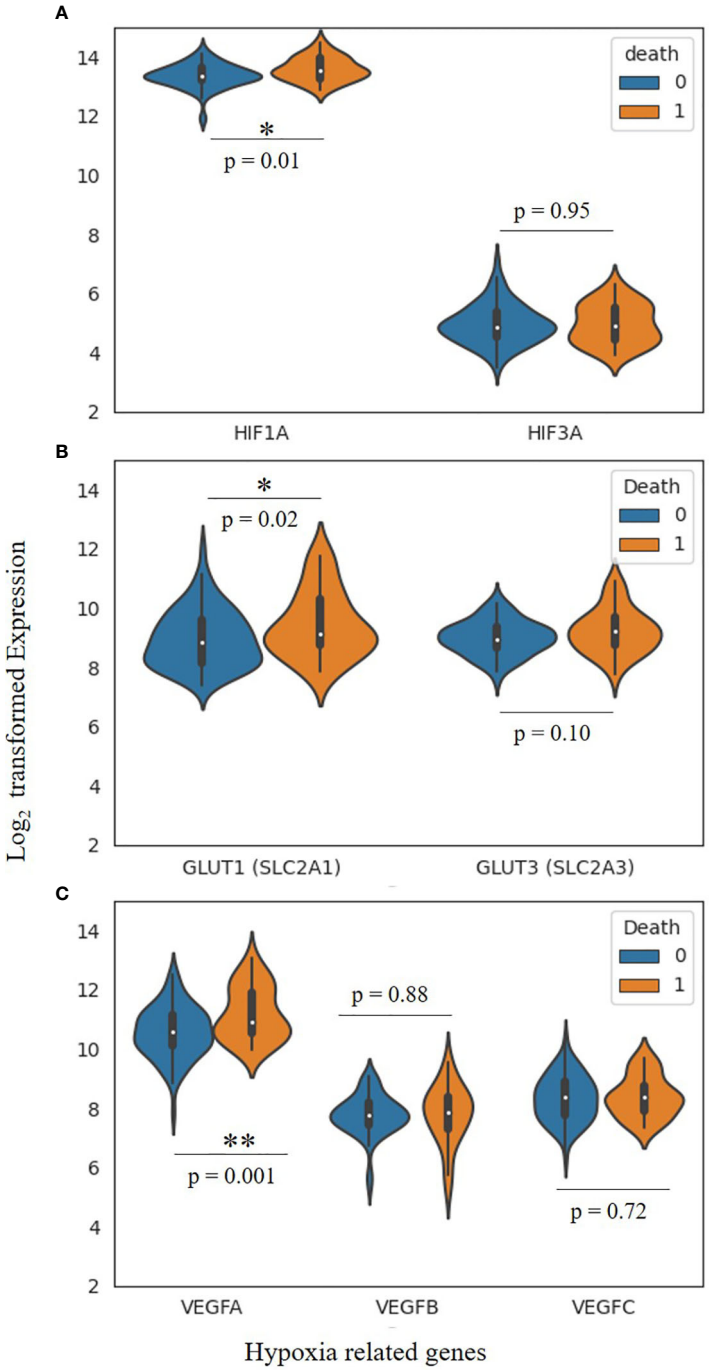



FIGURE 6
Hypoxia-related gene expression and morality in TNBC patients. **(A)** Violin plot exhibiting the expression of HIF-1α and HIF-3 α, **(B)** GLUT-1 and GLUT-3 Glucose transporters (genes SLC2A1 and SLC2A3) and **(C)** Vascular endothelial growth factors, VEGFA, VEGFB as well as VEGFC. Two-tailed T-test has been aplied for the significance (*, *P < 0.05*, **, *P < 0.005*). Cohort contains n=107 TNBC pateint samples (alive 79, dead 28).

suggests the possibility of predictive biomarkers. Combining DDR inhibitors with other therapy, including radiotherapy, chemotherapy, immunotherapy, PDT, and adjuvant therapies, can optimize their efficacy in TNBC treatment. TNBC also characterize by complex immunological landscape vulnerability through defects in the DDR pathway, which induces high TMB, anti-tumor immune suppressive features, as well as adaptive immune resistance *via* the expression of corresponding inhibitory ligands against immune checkpoints such as the PD1-PDL1 interaction. Therefore, DDR deficiencies offer potential therapeutic leverage for TNBC treatment by combining DNA/DDR-targeted therapies with cytotoxic anti-tumor immune cells, leading to favorable immune effects. Combining immune-checkpoint inhibitors, chemotherapy, and radiotherapy with HIF-1α inhibitors or its downstream target inhibitors like Trk, PI3K, PARP, CAIX etc., maybe a significant potential to match the high standard of clinical benefit in TNBC. In

TABLE 5 Summary of related drugs/inhibitors targeting HIF-1α.

| S.No. | Agents | Mechanism of action | Target gene/ Signaling | Therapeutic strategies | Pre-clinical/ Clinical trial status | References |
|---|---|---|---|---|---|---|
| 1. | Acriflavine | Inhibits premetastatic niche of TNBC by blocking HIF-1α | HIF-1α/LOX | Monotherapy | Preclinical | (97, 320) |
| 2. | As4S4 | Inhibits the TNBC metastasis by scavenging ROS and reduce thr transcription level of HIF-1α | ROS/HIF-1α | Monotherapy | Preclinical | (97, 321) |
| 3. | Cardamonin | Inhibits the transcription of HIF-1α | mTOR/P70s6k/HIF-1α | Monotherapy | Preclinical | (97, 322) |
| 4. | Digoxin (DIG) | Blocks the accumulation of HIF-1α and HIF-2α in hypoxic cells and block chemotherapy-induced expression of IL-6, IL-8, and MDR-1, and blocked BCSC enrichment | HIF-1α/VEGF | Monotherapy | Preclinical | (318) |
| 5. | Diallyl Trisulfides (323) | Inhibits the translation level of HIF-1α and inhibits the TNBC metastasis | HIF-1α | Monotherapy | Preclinical | (97, 324) |
| 6. | Elemene (C15H24) | Reduce the stability of HIF-1α | ROS/HIF-1α | Monotherapy | Preclinical | (97, 325) |
| 7. | Ganetespib | Induces the HIF-1α protein degradation and controls the angiogenesis, metabolism, invasion, and metastasis in TNBC | Hsp90/HIF-1α/SDF1/ VEGF/GLUT1, HK2/ PDK1/ALD1A1, ALD1A3/MMP9 /P4HA1/P4HA2/ ANGPTL4/ LICAM/LOX | Monotherapy | Preclinical/ II | (97, 326) |
| 8. | Isoliquiritigenin (ILTG) | Inhibits the expression of HIF-1α and VEGF and inhibits the TNBC metastasis | PI3K/Akt/HIF-1α/ VEGF//NF-κB | Monotherapy | Preclinical | (327) |
| 9. | Nanoliposomal echinomycin | Blocks the activity of HIF-1α | HIF-1α/VEGF | Monotherapy | Preclinical | (97, 328) |
| 10. | Melittin | Inhibits the transcription of HIF-1α by inhibiting NF-κB expression | HIF-1α/VEGFA/ NF-κB/LDHA | Monotherapy | Preclinical | (328) |
| 11. | Sanguinarine | Induces the proteasomal degradation of HIF-1α | HIF-1α/STAT3 blocker | Monotherapy | Preclinical | (329) |
| 12. | Amphotericin B | Suppress the binding of HIF-1α/p300 complex to HRE | HIF-1α/p300/FIH1 and PI3K/mTOR | Monotherapy | Approved for clinical use | (320, 330) |
| 13. | Apigenin | Inhibits expression of HIF-1α and VEGF | PI3K/AKT/p70S6K1 and HDM2/p53 | Monotherapy | II | (331) |
| 14. | YC-1 | Inhibits HIF-1α synthesis and blocked angiogenesis and an inhibition of tumor growth | HIF-1α | Monotherapy | Preclinical | (332) |
| 15. | Pleurotin (PX-12) | Inhibits the proto-oncogene (Trx-1) which further blocks the activity of HIF-1α | Trx-1 and thioredoxin 1 | Monotherapy | II | (333) (334) |
| 16. | Polyamides | Modulates the HIF-1alpha activity at transcriptional level | HIF-1α | Monotherapy | N/A | (335) |
| 17. | 2-phenethyl isothiocyanate (PEITC) | Down-regulates theHIF-1α with reduction of ROS and by induction of Nrf2 signaling | HIF-1α/Nrf2/MMPs 2 & 9/VEGF | Monotherapy | II | (320, 336) |
| 19. | PX-478 | Inhibits the expression of HIF-1α and HIF-1 transcription factor activity | HIF-1α/VEGF/GLUT-1 | Monotherapy | I | (337) |
| 20. | Silibinin | Inhibits the HIF-1α synthesis and induces the metabolic crisis in triple-negative breast cancer cells by modulating EGFR-MYC-TXNIP axis | mTOR/p70S6K/4E-BP1 | Monotherapy | Approved | (338) |
| 21. | Wondonin | Induces the proteasomal degradation of HIF-1α by increasing the interaction of HIF-1α and pVHL | HIF-1alpha/pVHL/ ERK1/2//Akt | Monotherapy | Preclinical | (339) |

*(Continued)*

TABLE 5 Continued

| S.No. | Agents | Mechanism of action | Target gene/ Signaling | Therapeutic strategies | Pre-clinical/ Clinical trial status | References |
|---|---|---|---|---|---|---|
| 22. | Sulphoraphane | Decrease the HIF-1α and VEGF expression by inhibition of STAT3/HIF-1α/VEGF signaling | HIF-1α | Monotherapy | II | (340) |
| 23. | Cardenolides | Inhibits the expression of HIF-1α and HIF-1 transcription factor activity | HIF-1α | Monotherapy | Approved for clinical use | (341) |
| 24. | DIM (3,3'-Diinolylmethane) | Downregulates the mRNA expression of HIF-1α | HIF-1α/TRAF2/p38 MAPK | Monotherapy | III | (342) |
| 25. | Pseudolaric acid (186) | Inhibits angiogenesis and reduces HIF-1α by promoting proteasome-mediated degradation | JNK/SAPK and p53 and HIF-1α/VEGF/KDR | Monotherapy | Preclinical | (343) |
| 26. | Andrographolide | Suppress COX-2 expression and angiogenesis *via* inactivation of HIF-1α/p300 signaling and VEGF pathway | HIF-1α/p300/VEGF | Monotherapy | III | (344) |
| 27. | Curcumin | Inhibits the expression of HIF-1α and HIF-1 transcription factor activity by degrading ARNT in cancer stem-like cells and reduces the proliferation and metastasis of TNBC cells | Hedgehog/Gli1/HIF-1α | Monotherapy | II | (345) |
| 28. | Echinomycin | Inhibits HIF-1α transcriptional activity of primary and metastatic TNBC cells | HIF-1α | Monotherapy | Rejected after Phase II trial | (328) |
| 29. | Flavopiridol (alvocidib) | Inhibiting HIF-1α gene transcription | HIF-1α | Monotherapy | III | (346) |
| 30. | GA and analogs | Induces the proteasomal degradation of HIF-1α | HIF-1α/Hsp90 | Monotherapy | II | (347) |

addition, combining these inhibitors with emerging antibody-drug conjugates, cancer vaccines, or adoptive cell therapy followed with existing treatments may be a significant step towards precision therapy and extend overall clinical benefits. Therefore, to determine the solid TNBC combination therapy regimens, it is pertinent to access the immune-molecular expression of HIF-1α and its associated mutational analysis in hypoxic TNBC. Although, HIFs have already been largely explored, but their downstream effector signaling, as well as other pathways like MYC, TP53, and KRAS, should be further explored in the surge of potential therapeutic targets. A more in-depth understanding of the TNBC hypoxic microenvironment, its molecular nature and its effect on tumor prognosis and survival will surely help in early detection and accurate treatment. 10,000 human genome projects will definitely aid in designing precise medicine based on the individual genome as well as tumor specificity.

## Author contributions

Conceptualization: NS and SU; writing and original draft preparation: NS, SU, RSS, and PKP; writing—review and editing: NS, SU, RSS, and RS; visualization: NS, SU, RSS, and RS; supervision and critically reviewed: NS, SU, RSS, and RS. All authors contributed to the article and approved the submitted version.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

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