The Hypoxic Microenvironment of Breast Cancer Cells Promotes Resistance in Radiation Therapy

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The American Cancer Society has estimated an expected 279,100 new breast cancer cases, and an expected 42,690 breast cancer deaths in the U.S. for the year 2020. This includes an estimated 276,480 women who are expected to be diagnosed. Radiation therapy, also called ionizing radiation therapy, is one of the most frequently used methods in the treatment of breast cancer. While radiation therapy is used in the treatment of more than 50% of all cancer cases, tumor resistance to ionizing radiation presents a major challenge for effective cancer treatment. Most tumor cells are in a hypoxic microenvironment that promotes resistance to radiation therapy. In addition to radiation resistance, the hypoxic microenvironment also promotes cancer proliferation and metastasis. In this review, we will discuss the hypoxic microenvironment of breast cancer tumors, related signaling pathways, breast cancer stem-like cells, and the resistance to radiation therapy. Recent developments in our understanding of tumor hypoxia and hypoxic pathways may assist us in developing new strategies to increase cancer control in radiation therapy.

Keywords: breast cancer, radiation therapy, hypoxia, free radicals, superoxide ions, radiation resistance

INTRODUCTION

Breast cancer is the second leading cause of cancer death in women. The American Cancer Society has estimated an expected 279,100 new breast cancer cases and an expected 42,690 breast cancer deaths in the U.S. for the year 2020 (1). There have been many advancements in the diagnosis and treatment of breast cancer in the past few decades. However, more research is still needed to overcome cancer resistance to therapy and improve the prognosis of advanced-stage breast cancer.

One significant obstacle to improve prognosis is breast cancer recurrence that is often associated with metastasis (2, 3). Breast cancer recurrence is the return of breast cancer months to years after the completion of initial treatment. Some cancer cells survive initial treatment and become undetected. These cancer cells may multiply and repopulate in nearby or distant areas. As such, the three types of breast cancer recurrence are local, regional, and metastatic recurrence. Local recurrence is the return of cancer in the same area of the breast as initial cancer; Regional recurrence is the return of cancer in the lymph nodes near the original cancer location; Metastatic recurrence,

also called distant recurrence, is the return of breast cancer in areas distant from the original cancer site (1). Common metastatic sites include the bone, lungs, or brain, and these metastatic recurrences are the foremost cause of breast cancer death (4).

Radiation therapy is used as an adjunct therapy for many primary cancers and is one of the most frequently used methods in breast cancer therapy. Ionizing radiation targeted at breast cancer cells causes an interaction with water and O_2 molecules near and inside the cells. This interaction produces free radicals and superoxide ions, which in turn cause damage to the cancer cell's DNA and other macromolecules, and potentially induces cell death (5). The primary purposes of radiation therapy are to improve prognosis of primary treatments, to treat metastasized cancer cells, and to decrease the chance of recurrence. However, tumor resistance to ionizing radiation presents a major challenge for effective breast cancer treatment. Tumor resistance may be in part due to a hypoxic microenvironment that is common in tumors. Here, we outline the cellular response to ionizing radiation and signal transduction pathways induced by hypoxic conditions as targets to identify novel strategies to increase the efficacy of radiation therapy.
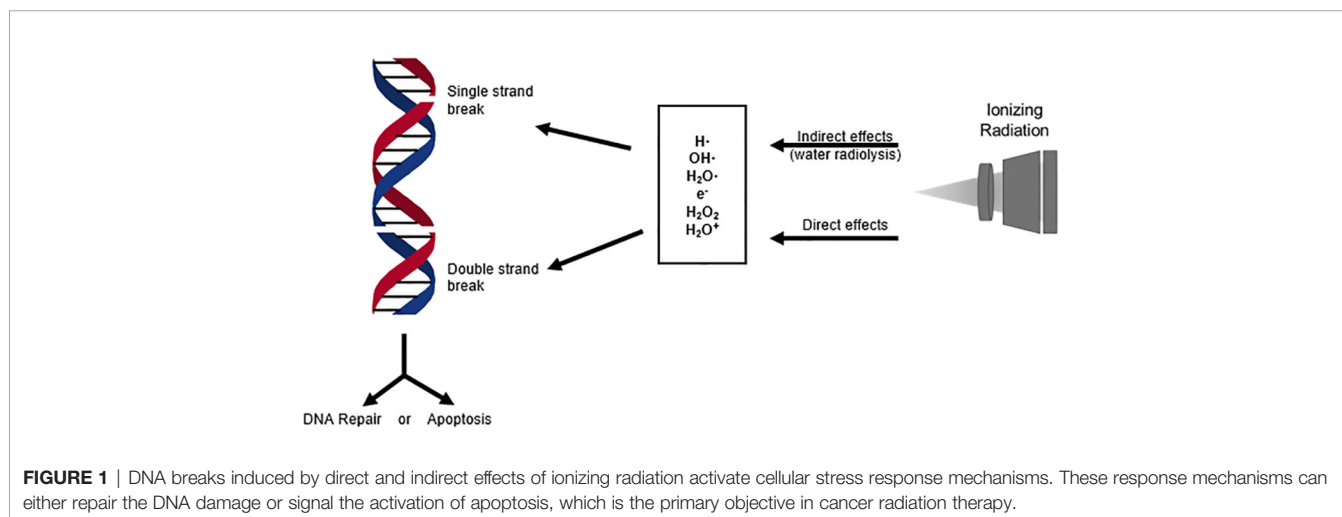
IONIZING RADIATION

Ionizing radiation in breast cancer therapy is greatly dependent on the damaging effects of low linear energy transfer (Low LET) radiation, such as X rays (5). There are direct and indirect effects of ionizing radiation. Direct effects of ionizing radiation are direct interactions between the particle and the targeted macromolecule, such as DNA (**Figure 1**), which eventually can lead to cell death (5, 6). Indirect effects of ionization consist of an intermediate step between radiation and the macromolecules, such as in water radiolysis. During water radiolysis in radiation therapy, water molecules are decompositioned by ionization radiation, and several types of free radicals are generated to damage macromolecules. These free radicals primarily include

the hydrated electron (e^-_{aq}), the hydrogen radical (H•), and the hydroxyl radical (OH•), which are highly reactive to the adjacent macromolecules (5). During radiaton therapy, the majority of deposited radiation will be absorbed by cellular water. This makes indirect ionization of water the primary cause of biological damage from radiation exposure (5).

THE HYPOXIC MICROENVIRONMENT

Tumor hypoxia, which is the lack of oxygen within a tumor, is one of the most common characteristics of the tumor microenvironment due to rapid cell growth and oxygen consumption (7). The hypoxic microenvironment in breast cancer requires the tumor to adapt in order to survive, and as such, tumor hypoxia has been closely associated with angiogenesis, metastasis, chemoresistance, and radiation resistance (8–10). Hypoxia has been recognized to activate many signaling transduction pathways, such as RAS/RAF/and mitogen-activated protein kinase (MAPK) (11). Hypoxia within the tumor microenvironment activates the heterodimer hypoxia-inducible factor 1 (HIF-1), a transcription factor consisting of two protein subunits, HIF-1α and HIF-1β. The expression and function of HIF-1α is regulated by oxygen concentration, while HIF-1β is constitutively expressed. Under normoxic conditions, HIF-1α is hydroxylated at proline residues 402 and 564, then ubiquitinated by prolyl-hydroxylase domain enzymes (PHD), which leads to proteasomal degradation (**Figure 2A**) (12–14). Under hypoxic conditions, HIF-1α is stabilized by dimerizing with HIF-1β (**Figure 2A**). Upon hypoxia, the HIF-1 heterodimer binds to the hypoxia response elements of multiple genes, which activates their transcription (**Figure 2A**) (8, 15). Many of these gene products participate in metabolism, such as, glycolytic enzymes, glucose transporters, antigenic growth factors, and carbonic anhydrases. The upregulation of these genes in breast cancer mediate a metabolic change from oxidative to glycolytic (11, 16, 17). Intratumoral hypoxia and alterations of the tumor microenvironmentare mechanisms that increase HIF-1α levels in breast cancer. In addition, the mutation and inactivation of tumor

FIGURE 1 | DNA breaks induced by direct and indirect effects of ionizing radiation activate cellular stress response mechanisms. These response mechanisms can either repair the DNA damage or signal the activation of apoptosis, which is the primary objective in cancer radiation therapy.
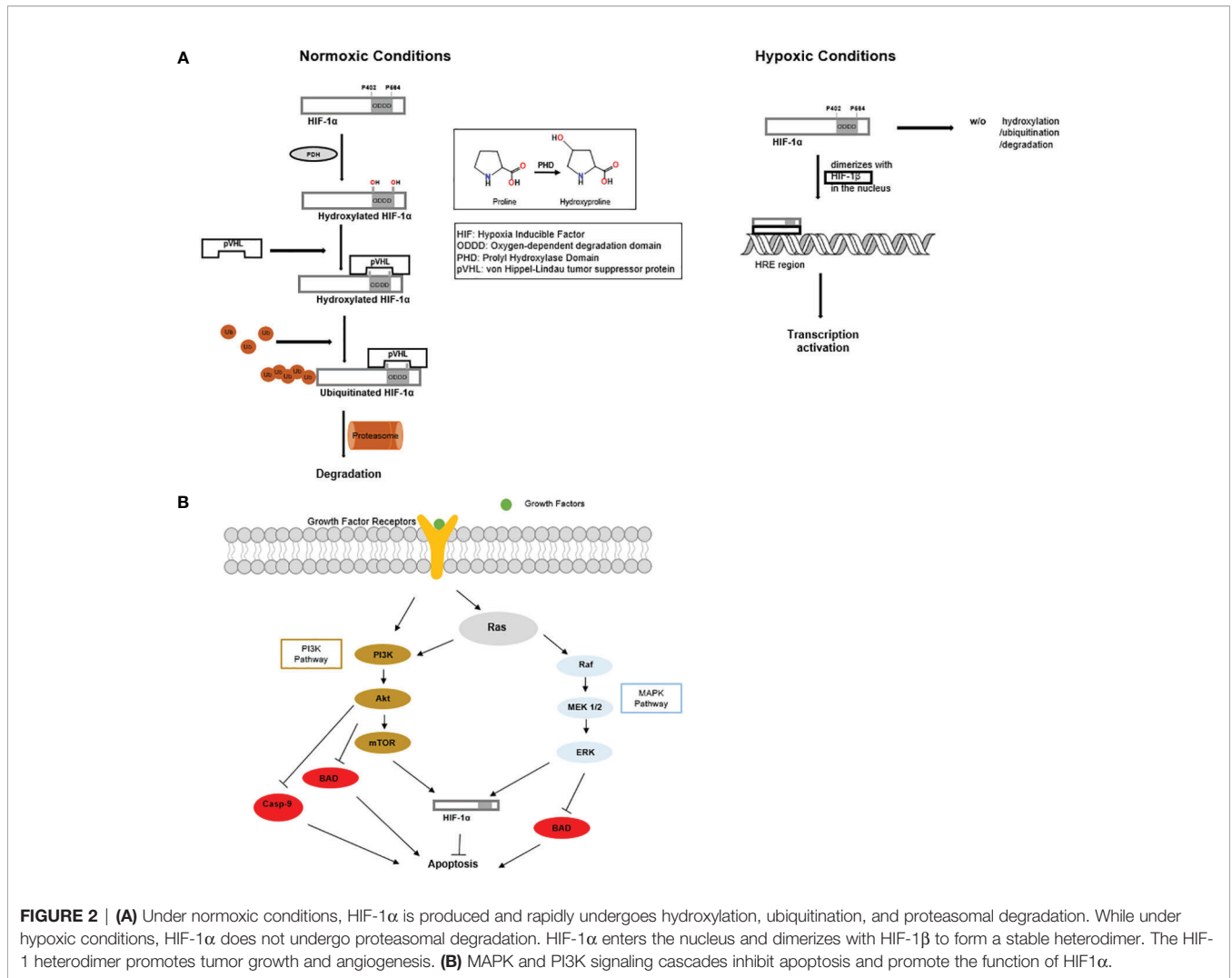
FIGURE 2 | **(A)** Under normoxic conditions, HIF-1α is produced and rapidly undergoes hydroxylation, ubiquitination, and proteasomal degradation. While under hypoxic conditions, HIF-1α does not undergo proteasomal degradation. HIF-1α enters the nucleus and dimerizes with HIF-1β to form a stable heterodimer. The HIF-1 heterodimer promotes tumor growth and angiogenesis. **(B)** MAPK and PI3K signaling cascades inhibit apoptosis and promote the function of HIF1α.

suppressor genes such as the von Hippel-Lindau tumor suppressor (pVHL), tumor protein p53 (p53)), and phosphatase and tensin homolog (PTEN) are associated with increased HIF-1α activity (18). It is important to note that although HIF-1α is widely recognized as the main regulator in tumor hypoxia, many additional factors, such as histone acetyltransferase (p300) and the CREB-binding protein (CBP) (19, 20), are essential to promote the comprehensive hypoxic response within the tumor microenvironment (11).

HYPOXIC AND ANTI-APOPTOTIC SIGNALING PATHWAYS

Hypoxic signaling within the tumor microenvironment is used by cancer cells to communicate with other cells and their extracellular environment. This communication within the hypoxic microenvironment is a highly explored area and is still not fully understood.

During hypoxia signaling, exosomes, the extracellular nanovesicles released by cells, play a vital role in communicating intercellular signals by way of paracrine signaling (7, 21). Tumor cells produce exosomes that contain several types of molecules from within the tumor cells, including miRNA, mRNA, DNA, proteins, and lipids that affect the activity of neighboring cells (21, 22). During hypoxia signaling, multiple pathways and even multiple cell types may crosstalk *via* exosomes. Boelens et al. showed that stromal cells released exosomes to communicate with breast cancer cells. This multi-signaling pathway uses both paracrine antiviral and juxtacrine neurogenic locus notch homolog protein 3 (NOTCH3) signaling to enhance breast cancer survival and therapy resistance. The communication is initiated by the stromal cells by increasing Ras-related protein Rab-27B (RAB27B) and transferring 5'-triphosphate RNA in exosomes. It results in the activation of retinoic acid-inducible gene 1 (RIG-l) antiviral signaling while simultaneously activates NOTCH3 receptors in breast cancer cells (23). The crosstalk between stromal and breast cancer cells signaling pathways converge as STAT1 promotes transcriptional responses to NOTCH3. This also promotes the

initiation of producing tumor cell subpopulations that are prone to therapeutic resistance. These findings also suggest that blocking the NOTCH pathway resensitizes tumor cells to radiation and may be a therapeutic target in the treatment of cancer (24). The role of exosomes in the breast cancer tumor microenvironment is still not fully understood, but there is an established correlation between the activation of hypoxic signaling and increased exosome production (7). Research is currently underway to determine whether tumor cell exosomes may be a novel target in cancer therapy.

Several signaling pathways participate in breast cancer resistance to radiation therapy, such as Ras/phosphoinositide 3-kinase (PI3K)/PTEN/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) (**Figure 2B**) (25), and Ras/Raf/ mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) (MAPK) (**Figure 2B**) (26, 27).

The activation of Akt, as part of PI3K pathway, could directly promote therapeutic resistance, including resistance to radiation (26). Akt is a kinase that is activated through the phosphorylation of its two residues, threonine 308 and serine 473 (28, 29). As a promoter of cell division and growth, Akt also plays a role in response to DNA damage (28). Akt can deactivate the BCL2 family member BAD by phosphorylation (30) and deactivate the cysteine protease Caspase-9. The deactivation of BAD and Caspase 9 is detrimental to the promotion of apoptosis, which is an essential factor of therapeutic resistance in breast cancer treatment. PI3-K/ Akt signaling pathway is dysregulated in breast cancer. Söderlund et al. showed that the stimulation of the epidermal growth factor erbB2 (HER2)/PI3-K/Akt with heregulin-B1 triggered the resistance to radiation-induced apoptosis in breast cancer (28). Furthermore, it was found that the inhibition of the PI3K signaling resulted in sensitizing breast cancer cell line BT-474, which overactivates PI3K pathway by overexpressing human epidermal growth factor receptor 2 (HER2). Independently, Steelman et al. showed that the PI3-k/PTEN/Akt/mTOR signaling cascade pathway was activated in breast cancer, therefore promoting its resistance to therapy. Consistently, the elevated levels of Akt-1 promoted resistance to doxorubicin, tamoxifen, and radiation. Interestingly, cells that were resistant to chemotherapy or radiation therapy harbored p53 mutations and expression of the downstream cyclin-dependent kinase inhibitor 1 (p21^{Cip1}). Also, ERK, an enzyme associated with cell development and proliferation, was induced by Doxorubicin therapy (26). A better understanding of the mechanisms of these signaling cascades, the activation and inhibition of Akt, may present promising therapeutic targets in the treatment of breast cancer.

The MAPK signaling pathway is known to promote cancer cell survival and limit the effectiveness of radiation therapy (31). Criswell et al. showed that ionizing radiation activated the insulin-like growth factor -1 receptor (IGF-1R), which in turn activated the MAPK signaling pathway, which upregulates secretory clusterin (sCLU) expression, a stress induced pro-survival protein. The study presented evidence that AG1024, an IGF-1R inhibitor blocked the induction of sCLU after radiation (31). More research is necessary to fully understand the role of the delayed EGF-1R/MAPK signaling pathway, but inhibition of IGF-1R may be a potential target in cancer therapy.

The mammalian target of rapamycin (mTOR) is another signaling pathway closely related to radiation resistance in breast cancer. mTOR and ribosomal protein S6 Kinase Beta-1 (p-S6K1) were found to be elevated in breast cancer cells (32, 33). CD44^{high}/CD24^{low} Michigan Cancer Foundation-7 (MCF-7) cells, a radioresistant breast cancer cell line, expresses higher levels of p-S6K1 than radiosensitive cells, suggesting a possible correlation between p-S6K1 and radiation resistance. Consistently, the inhibition of mTOR using everolimus increased radio-sensitivity in the CD44^{high}/CD24^{low} MCF-7 cells (32). In radio-sensitivity prognosis, p-S6K1expression levels may be a predictor of therapeutic response and may also be a potential target to increase radiation therapy sensitivity (32).

Micro-RNA-21 (miR-21) suppresses the functions of many tumor suppressor genes, such as tropomyosin 1 (TPM1) and PTEN, which are associated with proliferation, apoptosis and metastasis (34–36). In the research study by Anastov et al., T47D, a radioresistant breast cancer cell line, and MDA-MB-361, a radiosensitive breast cancer cell line, were studied in parallel, miR-21 was found to be significantly elevated in the T47D cells, suggesting miR-21 contributes to radioresistance of breast cancers. The study presented evidence that miR-21 knockdown improved radiation induced apoptosis and growth arrest in radiation resistant cells comparable to that of radiation sensitive cells (35). It is well accepted that the overexpression of the anti-apoptotic miR-21 can stimulate cell cycle progression in the G2/M checkpoint. However, it is not established that the correlation between miR-21 and G2/M checkpoint arrest promotes radiation resistance. Nevertheless, the inhibition of miR-21 may be a potential therapeutic target and its overexpression a possible prognosis indicator (35).

Long non-coding RNA (lncRNA) HOX Transcript Antisense RNA (HOTAIR) has been shown in several studies to participate in the promotion and metastasis of breast cancer (37, 38), and its single nucleotide polymorphism is a marker for breast cancer. HOTAIR is upregulated in breast cancer, links DNA damage and nuclear factor kappa B (NF-κB) signaling and takes part in p53 regulated DNA damage response. The link between HOTAIR and the p53 and NF-κB pathways correlate with the promotion of breast cancer and radiation resistance. HOTAIR binds with many miRNAs in various cancer types, causing an upregulation of the miRNA targets and deviations in signaling transduction. Braunstein et al. showed that binding lncRNA HOTAIR with miR-218 resulted in a phenotypical radiation-sensitive breast tumor. The research suggested that the inhibition of HOTAIR could be a novel target in breast cancer treatment (39).

CANCER STEM CELLS

In recent decades, a tumor cell population with cancer stem cell (CSC) phenotype has accumulated attention for its role in resistance to treatments. These cells have the ability to self-renew and initiate subpopulations of differentiated progeny (40). Cancer stem cells have been identified in a variety of tumors, including brain cancers, breast cancers, prostate, and melanoma (41). This population of cancer cells has presented evidence of

resistance to radiation therapy and chemotherapy (42–44). Research has presented evidence that the hypoxic tumor microenvironment is ideal for CSC survival (45). Further research is needed to better understand the role of the CSC phenotype subpopulation, but this subpopulation may be a new target to increase radiation sensitivity in cancer therapy.

Lin28 is a stem cell marker that is associated with radiation resistance in breast cancer (46). Apoptotic proteins poly(ADP-ribose) polymerase (PARP), caspase-3, and caspase-9 have significantly lower cleavage levels, thus less activation, in Lin28 overexpressing cells (46). As such, it was suggested that the overexpression of Lin28 mediated radioresistance by inhibiting radiation-induced apoptosis. Additionally, it has been shown that the Let-7 miRNA is downregulated in association with upregulation of Lin28 (47); and when the stabilized cells are transfected with Let-7 miRNA precursor, radiation sensitivity is resumed (46). Lin28 regulates Let-7 by directly interacting with the precesors of Let-7 family members (48). This suggests that Lin28 and Let-7 could be used as predictive biomarkers of response to radiation therapy.

The stem cell marker $CD44^+/CD24^-$ is recognized primarily in triple-negative breast cancer (TNBC) stem cells (49). $CD44^+/CD24^-$ has been associated with breast cancer resistance to ionizing radiation. In a 2011 study, Yin et al. showed that BRCA1 and Ataxia-Telangiectasia Mutated Kinase (ATM) activity are increased in $CD44^+/CD24^-$ cells (42). As an initiating factor for homologous recombination (HR), ATM is essential for the repair of radiation-induced double-strand DNA breaks (27, 50). ATM, a Ser/Thr kinase itself, is activated by autophosphorylation during double-strand DNA breaks. In $CD44^+/CD24^-$ cell lines and the primary culture of patient breast cancer cells, elevation in both expression and phosphorylation of ATM were found. Inhibition of ATM increased radiation sensitivity of the isolated $CD44^+/CD24^-$ cell, which suggests that ATM is a potential target to improve radiation sensitivity in breast cancer therapy (42).

Many studies have verified the breast cancer stem cell line with the $CD44^+/CD24^-/ALDH^+$ marker, and recently the high expression of aldehyde dehydrogenase ($ALDH^+$) was associated to therapeutic resistance. Croker et al. reported on the roles of $ALDH^+/CD44^+$ in breast cancer, where $ALDH^+/CD44^+$ was associated with chemoresistance, radiation resistance, poor prognosis, and played a role in metastasis (51). Considering that these stem cells are more resistant to therapy and promote proliferation in tumors (52), they may be more prone to distant metastasis. Additionally, it has been shown that this subset of cells expressed higher levels of therapy resistance proteins, p-glycoprotein, GSTpi, and/or CHK1 (51). Consistently, inhibition of $ALDH^+$ using diethylaminobenzaldehyde (DEAB) or all trans retinoic acid (ATRA) resulted in significantly improved radiation sensitivity, suggesting ALDH could be a potential target for improving therapeutic results (51).

CONCLUSION

As one of the most used methods to treat breast cancer, ionizing radiation in radiation therapy creates reactive oxygen species that cause cell damage and induce cell death. The hypoxic microenvironment of breast cancer cells promotes tumor cell proliferation, apoptosis resistance, metastasis, and resistance to radiation as well as other therapeutics. The overexpression and stabilization of the protein HIF-1α do not only result from low oxygen levels within the microenvironment, but also promote the advancement of hypoxia and facilitates tumor cell survival within the hypoxic microenvironment. The cancer cell's capacity to survive in a low oxygen environment presents a major challenge to effective radiation therapy. In addition, the adaptation to the hypoxic microenvironment also promotes additional alterations, including metabolic changes, mutations, signaling pathways, upregulation and downregulation of various cellular components. Many of these adaptations decrease radiation sensitivity.

Extensive studies have been performed to elucidate the hypoxic response mechanisms, anti-apoptotic pathways, and cascades that lead to resistance to radiation. This led to the discovery of promising therapeutic targets for drug development to sensitize tumors to radiations. Importantly, the presence of the radiosensitizing targets will be critical to predict the prognosis after radiaotherpy. To achieve these goals, a deeper understanding of the development of radiation resistance in breast cancers, especially for the subgroups, is needed to develop specified and personalized therapy.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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