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Autophagy Inhibition to Increase Radiosensitization in Breast Cancer

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Abstract

Currently, many breast cancer patients with localized breast cancer undergo breast-conserving therapy, consisting of local excision followed by radiation therapy. Following radiation therapy, breast cancer cells are noted to undergo induction of autophagy, development of radioresistance, and enrichment of breast cancer stem cell subpopulations. It is hypothesized that inhibition of the cytoprotective autophagy that arises following radiation therapy increases radiosensitivity and confers longer relapse-free survival by eliminating tumor-initiating breast cancer stem cells. Therefore, we reviewed the controversial role of autophagy in breast cancer tumorigenesis and progression, autophagy induction by radiotherapy, and utilization of autophagy inhibitors to increase radiosensitivity of breast cancer and to target radioresistant breast cancer stem cells.

Keywords: Autophagy inhibition; Breast cancer; Radiosensitization; Breast cancer stem cells

Abbreviations:

ATG: Autophagy-Related Genes; BIF-1: BAX-Interacting Factor-1; UVRAG: UV Irradiation Resistance-Associated Gene; mTORC1: Mammalian Target of Rapamycin Complex 1; AMPK: AMP-Activated Protein Kinase; JNK1: c-jun NH2 Terminal Kinase; ER: Endoplasmic Reticulum; PERK: PKR-like eIF2α Kinase; ATF6: Activating Transcription Factor-6; PARP1: Poly(ADP-Ribose) Polymerase-1; HIF-1α: Hypoxia-Inducible Factor α; Akt: Serine-Threonine-Specific Protein Kinase B; BCSC: Breast Cancer Stem Cell; ROS: Reactive Oxygen Species

Introduction

Historically, mastectomy was the standard therapeutic option for all stages of breast cancer, the most common cancer occurring in women [1]. However, large randomized controlled trials have shown similar survival for early stage breast cancer patients when treated with breast-conserving therapy, which consists of local excision of breast tumor followed by radiation therapy, when compared to those treated with mastectomy [1-5]. Furthermore, in a study that examined mastectomy specimens, cancer cells were found more than two centimeters from the primary tumor mass in 41% of patients, indicating high risk of breast cancer recurrence after partial-mastectomy alone [6]. Therefore, post-operative radiation treatment, given to sterilize remaining cancer cells after surgery, is now an essential part of effective breast cancer therapy, as it significantly reduces the risk of recurrence of invasive breast cancers as well as ductal carcinoma in situ after breast conserving surgery [1,7]. Even after mastectomy, radiation treatment reduces local recurrence rates in patients with axillary nodal involvement and thus leads to an increase in overall survival rate [7].

Some tumor cells are seen to develop adaptive responses for survival despite excellent initial response to radiotherapy and become more treatment-resistant and invasive [8-10]. Therefore, it is imperative to find an adjunctive anti-cancer agent to increase radiosensitivity of breast cancer cells to improve the outcomes of many breast cancer patients, who are currently undergoing breast-conserving therapy. Although enhanced DNA repair mechanisms and overexpression of various survival signaling pathways may all contribute to radioresistance, many investigators are currently exploring autophagy as a cytoprotective factor leading to radioresistance [9-11]. Therefore, this review aims to outline the known roles of autophagy in cancer, mechanisms of autophagy induction by radiotherapy, and radiosensitization via incorporation of autophagy inhibitors in breast cancer therapy.

Autophagy Overview

Autophagy, a genetically regulated process controlled by autophagy-related genes (ATG), is described as a cell survival mechanism to maintain cellular homeostasis and to protect cells in the setting of cellular stresses, such as nutrient or growth factor deprivation, hypoxia and accumulation of reactive oxygen species, or DNA damage [12,13]. During periods of cellular stress, over 35 proteins come together as autophagy process undergoes stages of induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, degradation, and recycling [12,13]. Figure 1 summarizes the process of autophagy. Vesicle nucleation, initiated by the activation of class III PI3K/Beclin-1 complex, recruits proteins and lipids for formation of autophagosomal membrane [12]. This process is tightly regulated by multiple factors, including BAX-interacting factor-1 (BIF-1), ATG12L, and UV irradiation resistance-associated gene (UVRAG) [12-14]. Autophagosomes engulf cytoplasmic constituents and transport these components to lysosomes for degradation. Following the fusion of autophagosomes and lysosomes, lysosomal hydrolases degrade both the autophagosomal membrane and its cargo. Thus, autophagy plays

an essential role in preventing accumulation of deleterious materials as well as generating source of energy for the survival of cancer cells [15].

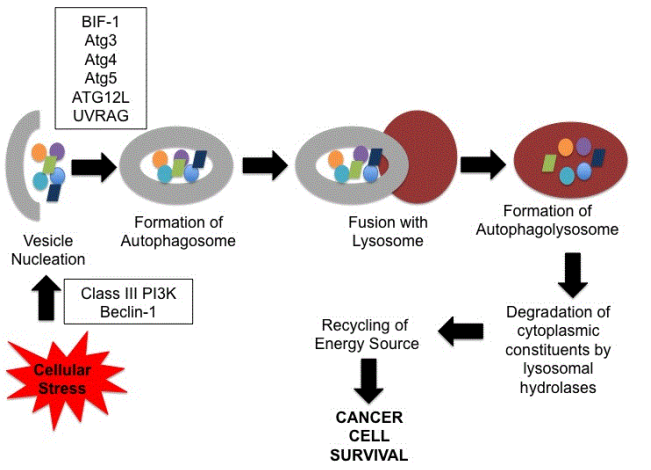

Figure 1: Diagram of the autophagy process. Under cellular stress, class III PI3K/Beclin-1 complex initiates vesicle nucleation and autophagosome formation. An autophagosome with cytoplasmic constituents fuses its membrane with a lysosome to form an autophagolysosome. Inside the autophagolysosome, cytoplasmic materials get degraded by lysosomal hydrolases, serving as a source of energy and allowing cancer cell survival during periods of hypoxia and nutrient deprivation, especially in the setting of therapeutic insult, such as radiation.

The response to cellular stress involves other pathways besides autophagy, such as cell cycle and growth control, as well as cellular survival and cell death pathways [13]. Therefore, there is a close integration between signals that regulate autophagy and those that regulate other stress signals [13]. The basal level of autophagy is maintained by mammalian target of rapamycin complex 1 (mTORC1), which functions as the sensor of the cellular nutrient state [12]. With starvation signal or withdrawal of growth factors, AMP-activated protein kinase (AMPK) becomes activated, leading to inhibition of mTORC1 and stimulation of autophagy [12,13,16]. Other kinases, such as c-jun NH2 terminal kinase (JNK1), which phosphorylates anti-apoptotic protein Bcl-2, resulting in a reduction in its affinity for Beclin 1, also play important roles in autophagy [13]. Furthermore, the unfolded protein response of the endoplasmic reticulum (ER) stress pathway is a potent stimulus of autophagy [17]. The ER membrane-associated proteins, PKR-like eIF2α kinase (PERK) and activating transcription factor-6 (ATF6) act as autophagy inducers [13,17]. Oxidative stress can activate PERK and directly inhibit mTOR or indirectly inhibits mTOR via activation of poly(ADP-ribose) polymerase-1 (PARP1) as a result of DNA damage [13,18,19]. During periods of cellular stress, the convergence of these pathways promotes autophagy induction.

Autophagy in Breast Cancer

Autophagy functions to remove damaged intracellular molecules and organelles, thus, suppressing genomic instability [12]. Removal of such damaging intracellular components prevents accumulation of deleterious alternations in the genome and limits initiation of cancer formation [12]. On the other hand, this process may also lead to autophagy-dependent cell death under stressful conditions [12,20]. Therefore, autophagy in the context of cancer is extremely complex and is presumed to differ in different stages of tumorigenesis and cancer progression.

Genetic studies have revealed that during the tumor initiation phase, tumor formation is inhibited by autophagy. Beclin1, a gene mapped to a tumor susceptibility locus and an important autophagy regulator, has been found to be deleted in a high percentage of human breast cancers [21,22], and its suppression has been associated with the spontaneous formation of various malignancies [23]. In addition, decreased Beclin1 expression was seen in human breast carcinomas, when compared to normal breast tissue [22]. Furthermore, autophagy maintains genome integrity, preventing the deleterious effects of reactive oxygen species that may lead to malignant transformations [12,21,22]. Beclin1 loss has been associated with accumulation other genomic abnormalities, including HER2 amplification and mutations in other tumor suppressors, such as p53 and PTEN [24]. Cells with intact autophagy are also able to protect themselves from oxidative stress by selectively eliminating the damaged mitochondria via mitophagy and by activating antioxidant-defense genes [25-27]. Apoptosis is the first line of defense against damaged cells. However, when apoptosis is reduced or inactivated, as commonly seen during tumorigenesis, cells rely significantly on autophagy [21]. All together, these studies support the imperative role of autophagy in suppression of genomic alterations and prevention of tumorigenesis.

Despite autophagy's role in inhibition of tumor formation, autophagy becomes a necessity for cancer cell survival in an established tumor [21,28,29]. Robust activation of autophagy is observed in cancer cells in response to various stressors, such as growth factor deprivation, hypoxia, and therapeutic stimuli [28]. This survival response by autophagy is especially dramatic when apoptosis is inhibited, as cancer cells remain dormant for a prolonged period of time before resuming growth once the environment returns to normal conditions [28]. Autophagy is induced in the setting of hypoxia within a tumor via hypoxia-inducible factor α (HIF-1α), and HIF-1α accumulation has been associated with decreased survival in breast cancer [30]. Regions of hypoxia are common in solid tumors, such as in breast cancers. As hypoxia selects for cancer cells that are resistant to apoptosis, it poses a major barrier to radiotherapy [21]. Additional study has shown that the induction of BNIP3, a downstream target of HIF-1α in hypoxic cells disrupts the Beclin1-Bcl-2 complex and releases Beclin1, thereby inducing autophagy as an adaptive survival mechanism during prolonged hypoxia [31]. Autophagy as a survival mechanism becomes increasingly crucial as tumor cells undergo anti-cancer therapeutic insults.

Autophagy and Radiotherapy

One of the approaches for enhancing the efficacy of radiotherapy has been utilization of radiation dose fractionation to determine optimal treatment schedule and maximize tumor cell death [32]. Autophagy in response to radiotherapy has resulted in varying responses in the survival of breast cancer cells. Some studies have shown that autophagy may play a role in cell death pathways that mediate radiation sensitivity. In the breast cancer cell line MCF-7 which lacks executioner caspase, radiation inhibits the rapamycin-sensitive mTOR pathway, resulting in changes in mitochondrial metabolism through autophagy induction, which ultimately leads to decreased cancer cell survival [33]. Another study has shown that the inhibition of apoptosis using small interfering RNAs against pro-

apoptoic proteins, as well as induction of autophagy via overexpression of ATG5 and Beclin-1, leads to increased radiation-mediated cell death of MDA-MB-231 breast cancer cells [34]. These studies have demonstrated autophagy-dependent cell death of breast cancer cells.

However, there is growing evidence that autophagy contributes to the resistance of breast cancer cells to radiation [35-37]. The UVRAG is now known to activate Beclin-1 and to confer anti-apoptotic activity during anti-cancer therapy via its effect on localization of Bax within tumor cells [37]. Furthermore, the serine-threonine-specific protein kinase B (Akt), a protein known to regulate survival in various cancers, is associated with downregulation of UVRAG and inhibition of autophagy through mTOR activation [38]. Previous study has shown that Akt1 overexpression sensitizes cancer cells to UV irradiation, resulting in the decreased viability of cancer cells [38]. A study comparing radioresistant MDA-MB-231 breast cancer cells with radiosensitive HBL-100 breast cancer cells has revealed significant induction of autophagy in radioresistant cells upon administration of radiation, which was not evident in radiosensitive cells [39].

Beclin 1 is a part of the class III PI3 kinase complex and thus plays an essential role in production of PI-3-phosphate and sorting of autophagosomal components as well as lysosomal enzyme transport [40]. As part of an ubiquitin-like protein conjugation system, Atg5 and Atg 12 proteins mediate large membrane-associated protein complexes that are required for the formation of complete autophagosomes [41-43]. Other autophagy-related proteins, such as Atg3 and Atg4 mediate protein-phospholipid complexes that are localized to autophagosomal membranes [44]. Hypoxia-induced autophagy leads to a marked accumulation of autophagosomes along with RNA induction of autophagy-related genes such as Beclin-1, Atg5, and Atg12, and ultimately, to radioresistance [45]. On the other hand, inhibition of autophagy-related genes with small interfering RNA results in retardation of DNA double-strand breaks repair, and thus, leads to radiosensitization [45].

Currently, there is sufficient evidence in preclinical studies that clinically relevant doses of radiation induce autophagy. Autophagy appears to be induced across a wide spectrum of tumor cell lines, regardless of whether tumor cells are considered to be radiosensitive or radioresistant [36]. While breast cancer cell lines such as MCF-7 and ZR-75 [35,36,46] have shown increased radiation sensitivity with autophagy inhibition, inhibition of autophagy neither sensitizes nor protects 4T1 and Hs578t breast cancer cells from radiation [36,47]. A more recent study has shown that enhanced radiation sensitivity via inhibition of autophagy requires functional p53 protein [10]. Whether autophagy can be utilized as a pharmacologic target for more effective breast cancer treatment in combination with radiation requires further investigation, as no similar study has been performed in a clinical setting.

Increase in Radiosensitivity with Pharmacologic Autophagy Inhibitors

With the accumulation of evidence demonstrating induction of autophagy by DNA-damaging agents, such as radiation therapy, autophagy inhibitors are being actively investigated as agents to enhance the efficacy of radiotherapy for solid tumors [35,39]. Pharmacologic autophagy inhibitors, 3-methyladenine and chloroquine, significantly increase the radiosensitivity of the radioresistant MDA-MB-231 cell line [39]. Autophagy may play a role in rescuing cells from radiation-induced cellular damage by delaying apoptotic death in response to DNA damage [48]. Therefore, autophagy inhibitors can lead to a reduction in the survival of tumor cells, especially during the recovery phase following radiation therapy. The enhanced radiation-induced apoptosis of breast cancer cells with autophagy inhibitors has been associated with the inhibition of TAK1, transforming growth factor-activated kinase 1 [49]. This study has demonstrated that TAK1 phosphorylation is induced by radiation and that MDA-MB-231 cells exhibit increased cytotoxicity upon inhibition of TAK1 [49].

In addition to apoptosis of cancer cells, radiation can result in senescence of cancer cells, a phenomenon of prolonged cell cycle arrest [50]. These senescent cells are metabolically active; therefore, they ultimately develop senescence-associated secretory phenotypes and secrete cytokines, chemokines, growth factors, and proteases, as well as insoluble extracellular matrix components that promote tumor progression via invasion and migration of neighboring non-irradiated cancer cells through JAK2-STAT3 pathway activation [50,51]. With autophagy inhibitors, 3-methyladenine and bafilomycin A1, irradiated cells undergo enhanced cell death by apoptosis, rather than senescence [50]. Therefore, autophagy inhibition may eliminate senescent cancer cells after radiation therapy and ultimately, inhibit tumor progression.

Breast Cancer Stem Cells and Radiation

The presence of breast cancer stem cells (BCSCs) has been associated with radioresistance and treatment failure after radiation therapy, resulting in the relapse of breast cancer [8]. The BCSC hypothesis proposes that a small subpopulation of tumor cells, with characteristics of self-renewal that lead to tumorigenesis is intrinsically more resistant to anti-cancer therapies [8,52,53]. In additional to self-renewal potential, enhanced DNA-repair capacity and reactive oxygen species (ROS) defenses have been associated with radioresistance in BCSCs compared to non-BCSCs [8]. Following radiation treatment, an increase in BCSC subpopulations has been observed *in vitro*, along with the activation of Notch-1 signaling pathway that promotes self-renewal in progenitor cells [54,55].

Moreover, inhibition of the Notch-1 pathway has shown to diminish the number of BCSCs [55]. Therefore, pharmacologic agents that inhibit Notch signaling are currently being investigated to overcome BCSC-mediated radioresistance [8]. In addition, lower levels of ROS were detected in BCSCs, indicating increased levels of radical scavengers [55]. BCSCs have also been observed *in vitro* and *in vivo* to accumulate less DNA breaks after radiation [56]. Therefore, it is hypothesized that the traditional cancer therapies reduce tumor bulk, but the inability to completely eradicate BCSCs results in the failure of complete remission [54,56,57].

Several contributing mechanisms for BCSC radioresistance have been reported in the literature. Radioresistance of BCSCs has been linked with genes involved in glutathione synthesis, such as glutamate cysteine ligase, glutathione synthetase, and FoxO1 [8]. Furthermore, depletion of glutathione has resulted in radiosensitization [8]. BCSCs in p53 null mutant mice have an increased capacity for DNA damage repair following radiation, and this effect is accompanied by down-regulation of PTEN and activation of Akt and Wnt/beta-catenin signaling pathways [58]. Inhibition of Akt has led to radiosensitization of BCSCs [58]. All together, these evidences support the need for future clinical investigations to target BCSCs to increase the efficacy of radiation therapy in breast cancer patients.

Autophagy in Breast Cancer Stem Cells

Although numerous factors contribute to the characteristics of BCSCs, autophagy has been linked to BCSCs survival and resistance to therapy [57]. Previously, breast tumors injected with serum-deprived mesenchymal stem cells have demonstrated higher cellularity and decreased apoptosis, with increased levels of autophagic activity in the surrounding areas [59]. Furthermore, BCSCs are seen to secrete various paracrine factors to support growth in the setting of serum deprivation, in parallel with upregulation of key autophagy regulators such as beclin-1, ATG10, and ATG12 [59]. Another study has demonstrated high expression of autophagy markers, Atg5, Atg12, and LC3-B in breast BCSCs, in associated with upregulation of JNK1 [60]. Inhibition of autophagy with 3-methyladenine can reverse the dormant phenyotpes of BCSCs [60]. Overexpression of beclin 1 and increased starvation-induced autophagy flux are also evident in mammospheres derived from breast cancer cells [61]. In addition, beclin1 is found to be essential in BCSC maintenance and tumorigenesis *in vivo* [61]. Recently, chloroquine has shown to inhibit autophagy and eliminate BCSCs in preclinical and clinical settings via inhibition of the JAK2-STAT3 pathway [62]. Therefore, targeting BCSCs via inhibition of autophagy may effectively increase radiosensitivity of breast cancers.

Clinical Trials

Chloroquine and hydroxychloroquine, two pharmacologic autophagy inhibitors are currently being investigated in several clinical trials (Table 1) for breast cancer treatment [63]. In the ongoing CAT clinical trial, 250 mg daily oral dose of chloroquine is being given in combination with taxane or taxane-like chemotherapy to advanced or metastastic breast cancer patients who have failed anthracycline based therapy. Overall response rates will be measured, and BCSCs will be quantified pre- and post- therapy. In the CuBiC clinical trial, patients waiting for surgery are receiving 500 mg oral chloroquine daily. Changes in proliferative and apoptotic responses, based on Ki67 and TUNEL assays, as well as autophagy markers of pre- and post-treatment biopsies, are being assessed. In the PINC trial, patients are receiving either standard dose chloroquine, 500 mg per week or low dose chloroquine 250 mg per week for 1 month prior to the excision of the breast tumor. The tumor response to treatment will be evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) criteria, using tumor measurements obtained from breast MRI, immediately preceding drug treatment and prior to surgery. There is yet another trial currently recruiting metastatic estrogen receptor positive breast cancer patients with progression of disease despite hormonal therapy, for hydroxychloroquine treatment in combination with hormonal therapy. The level of the autophagy marker LC3b will be evaluated in pre- and post-treatment biopsy samples, and the clinical benefit rate will be assessed. Another ongoing trial involves administration of daily hydroxychloroquine to breast cancer patients for 2-3 weeks between tumor biopsy and surgery, and hypoxia markers and autophagy markers will be quantified and compared between pretreatment biopsies and post-treatment surgical specimens.

Official Name of Clinical Trial	Clinical Trial Identifier	Intervention	Phase
Chloroquine With Taxane Chemotherapy for Advanced or Metastatic Breast Cancer Patients Who Have Failed an Anthracycline (CAT)	NCT01446016	Taxane or Taxane-like drugs) + Chloroquine	II
A Phase 2 Randomized, Double-blind Trial Evaluating the Effects of Chloroquine in Breast Cancer (CUBiC)	NCT02333890	Chloroquine	II
Study of the Efficacy of Chloroquine in the Treatment of (The PINC Trial)	NCT01023477	Chloroquine	I,II
Hydroxychloroquine in Metastatic Estrogen Receptor-Positive Breast Cancer Progressing on Hormonal Therapy	NCT02414776	Hydroxychloroquine in combination with the current hormonal therapy	I
Autophagy Inhibition Using Hydrochloroquine in Breast Cancer Patients	NCT01292408	Hydrochloroquine	II

Table 1: Autophagy inhibitors in clinical trial for breast cancer treatment.

Conclusion

Although the role of autophagy in tumorigenesis and tumor progression remains controversial, review of the literature supports the hypothesis that autophagy is an indispensable factor in the radioresistance of breast cancer, especially as a survival mechanism of BCSCs. To our knowledge, previous studies on this controversial topic have been limited to preclinical settings. Thus, future studies in clinical samples of breast cancer patients are necessary to validate preclinical findings. In addition to radiotherapy, the use of U.S. Food and Drug Administration-approved pharmacologic agents, such as chloroquine or hydroxychloroquine, as adjunctive therapy to suppress autophagy could be a potential breakthrough in breast cancer therapy. Furthermore, there are several other autophagy inhibitors such as N-acetylcysteine and 3-methyladenine that have shown promising results in other malignancies [64] that may benefit breast cancer patients. Therefore, targeting autophagy may be a novel therapeutic opportunity for more effective breast cancer treatment.

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