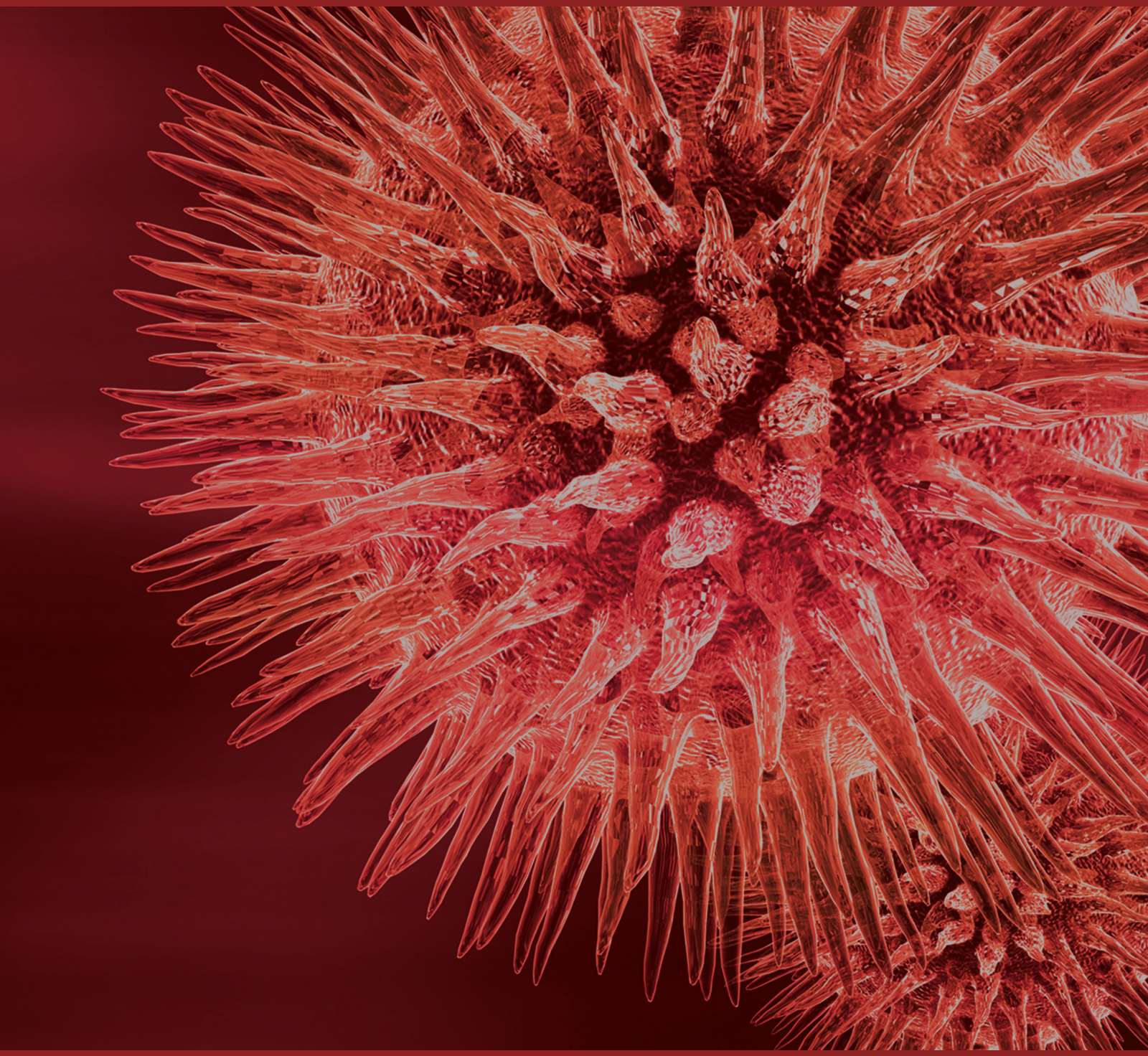
BioMed Research International

Control of Autophagy in Cancer

Guest Editors: Arkadiusz Orzechowski, Saverio Bettuzzi, Patrycja Pawlikowska, and Beata Pająk



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Editorial

Control of Autophagy in Cancer

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Autophagy (macroautophagy) is a self-degradative physiological mechanism observed in insufficiently nourished or injured cells and often activated to meet energy requirements or for cleaning up damaged organelles. Cancer cells, which are known to require more energy and nutrients than benign counterparts, may eventually take advantage of autophagy for survival. This special issue of this journal provides an updated view on the role of autophagy in the pathogenesis of cancer. The expert authors who have been invited to help with this task will describe the molecular mechanisms of autophagy with a special focus on tumor cells biology, dealing with those natural compounds which have potential anticancer activity because of being known to induce or inhibit autophagy.

The review entitled "Tumor Suppression and Promotion by Autophagy" addresses the issue of how autophagy affects tumorigenesis. It is apparent from several studies that tumor suppressor genes that negatively regulate mTOR, such as PTEN, AMPK, LKB1, and TSC1/2, stimulate autophagy. Conversely, oncogenes that activate mTOR, such as class I PI3K, Ras, Rheb, and AKT, inhibit autophagy. All together this suggests that autophagy is a tumor suppressor mechanism. Nevertheless, autophagy also functions as a cytoprotective mechanism under stress conditions, including hypoxia and nutrient starvation. These phenomena may promote tumor growth and induce resistance to chemotherapy in established tumors.

Another review entitled "The Importance of Autophagy Regulation in Breast Cancer Development and Treatment" summarizes the current knowledge on autophagy regulation in breast cancer, describing up-to-date anticancer strategies correlated with autophagy. During breast cancer development autophagy exerts different effects at cancer initiation and progression due to superimposition of signaling pathways of autophagy and carcinogenesis. Inhibition of autophagy may enhance the effectiveness of currently used anticancer drugs and other therapies (like radiotherapy). However, the promotion of autophagy can also induce death and, hence, elimination of cancer cells and reduction of tumor size. Thus, in the development of cancer, autophagy is regarded as a double-edged sword.

The review paper entitled "Calcium Homeostasis and ER Stress in Control of Autophagy in Cancer Cells" points to calcium ion homeostasis and starvation as the major factors influencing autophagy in tumors. Several Ca^{2+} channels like voltage-gated T- and L-type channels, IP3 receptors, or CRAC are involved in autophagy regulation as well as glucose transporters, mainly from GLUT family, which are often upregulated in cancer. Signals from both Ca^{2+} perturbations and glucose transport blockage might be integrated at UPR and ER stress activation. Thus modulation of autophagy might be a promising anticancer therapy. However, whether inhibition or activation of autophagy leads to tumor cell death or not appears to be a context-dependent matter.

The review entitled "Roles of Autophagy Induced by Natural Compounds in Prostate Cancer" focuses on prostate cancer (PCa), one of the most common cancers in aged men. Natural compounds showing low toxicity to benign tissue associated with specific anticancer effects at physiological levels *in vivo* are receiving increasing attention for prevention and/or

treatment of PCa. Current evidence shows that some natural compounds may exert their action by modulating autophagy in PCa cells. Since mTOR activity can be directly or indirectly modulated by a number of upstream signaling pathways, it is mandatory to uncover the mechanisms through which these natural compounds inhibit the Akt/mTOR pathway and regulate the cell fate.

The review "Elaborating the Role of Natural Products-Induced Autophagy in Cancer Treatment: Achievements and Artifacts in the State of the Art" illustrates how the tumor suppressive action of natural products-induced autophagy may lead to cell senescence and apoptosis-independent cell death, also inducing complement apoptotic cell death by robust target-specific mechanism. Technicalities at detecting autophagy may affect the quality of the data; therefore it is suggested that rational criteria should be set up for monitoring natural products-induced autophagy in cancer cells. The action of autophagy-inducing natural products should be highlighted in future studies because it could become clinically relevant.

The paper entitled "Nucleofection of Rat Pheochromocytoma PC-12 Cells with Human Mutated Beta-Amyloid Precursor Protein Gene (*APP-sw*) Leads to Reduced Viability, Autophagy-Like Process, and Increased Expression and Secretion of Beta Amyloid" describes observations obtained from tumor pheochromocytoma PC-12 cells. These cells (immune to apoptosis) became sensitive to cell death following human *GFP* vector + *APP-sw* gene expression. Reduced cell viability was accompanied by higher expression of Aβ 1–16 and elevated secretion of Aβ 1–40. At the ultrastructural level autophagy-like process was demonstrated to occur in *APP-sw*-nucleofected cells with numerous autophagosomes and multivesicular bodies but without autolysosomes. Summing up, human *APP-sw* gene is harmful to PC-12 cells and cells are additionally driven to incomplete autophagy-like process.

In the following paper entitled "Combined Epidermal Growth Factor Receptor and Beclin1 Autophagic Protein Expression Analysis Identifies Different Clinical Presentations, Responses to Chemo- and Radiotherapy, and Prognosis in Glioblastoma" the authors investigated the expression of EGFR and Beclin1 in 117 glioblastoma undergoing postoperative chemo- or radiotherapy. Clinical cases are classified according to the level of expression of EGFR and Beclin1 and compared with clinical data. It is suggested that low expression of EGFR associated with high expression of Beclin1 could be a useful biomarker for the identification of a patient subgroup with relatively favorable clinical presentations and prognosis. This information supports the rationale for possible combined EGFR/Beclin1-targeted therapies.

Finally, the article "Gene Network Exploration of Crosstalk between Apoptosis and Autophagy in Chronic Myelogenous Leukemia" renders a graphical illustration of the most relevant gene networks for the exploration of functional links and potential coordinated regulations of gene expression related to apoptosis and autophagy in CML. In the CML-specific network, the link between E2F3 and AKT3 demonstrated a possible cell response to oncogenic stress which is active during the proliferation of hematopoietic cells. It is important to note that E2F3 and AKT3 were both the predicted targets of miR-15, whose deletion was proved to be associated with cancer promotion. The central role of E2F2 was further confirmed by the normal-specific transcription factor regulatory signature network. In the normal-specific miRNA regulatory signature network, the apoptotic balance was strengthened by the coregulation of BAK1 and BCL2 by miRNAs. As a normal-specific composite regulatory signature, the E2F2-BAK1-PIK3R5 motif may constitute the core mechanism controlling cell cycle progression, apoptosis, and autophagy. This hypothesis is worth further investigations in the future.

Overall, the current issue of this journal highlights the contribution of autophagy in tumorigenesis and describes which natural compounds are more promising in the future for chemoprevention and anticancer therapy on the basis of their ability to modulate autophagy.

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Review Article

Roles of Autophagy Induced by Natural Compounds in Prostate Cancer

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Autophagy is a homeostatic mechanism through which intracellular organelles and proteins are degraded and recycled in response to increased metabolic demand or stress. Autophagy dysfunction is often associated with many diseases, including cancer. Because of its role in tumorigenesis, autophagy can represent a new therapeutic target for cancer treatment. Prostate cancer (PCa) is one of the most common cancers in aged men. The evidence on alterations of autophagy related genes and/or protein levels in PCa cells suggests a potential implication of autophagy in PCa onset and progression. The use of natural compounds, characterized by low toxicity to normal tissue associated with specific anticancer effects at physiological levels *in vivo*, is receiving increasing attention for prevention and/or treatment of PCa. Understanding the mechanism of action of these compounds could be crucial for the development of new therapeutic or chemopreventive options. In this review we focus on the current evidence showing the capacity of natural compounds to exert their action through autophagy modulation in PCa cells.

1. Introduction

In Europe, prostate cancer (PCa) is the first most frequent diagnosed malignancy and the third-leading cause of cancer death in men [1]. Although patients with an early androgen-dependent and localized tumor have a good prognosis, the survival rate decreases notably when the tumor eventually becomes androgen-independent and progresses to a hormone-refractory disease leading to metastasis formation. At present, patients with hormone-sensitive PCa at early-stage can be treated with surgery, radiotherapy, and/or hormonal therapy (i.e., surgical or medical castration). Nevertheless, the disease can progress into castration-resistant and metastatic PCa, for which the only treatment option is chemotherapy with docetaxel. Therefore, further investigations are required to elucidate the mechanisms underlying onset and progression of PCa and to develop new strategies for therapy and prevention. Increasing evidence supports a key role of autophagy in cancer development, drawing researchers' attention because of its potential implication as a drug target in anticancer treatments.

2. Autophagy and PCa

2.1. The Autophagic Machinery and Its Regulation. In eukaryotic cells, proteins are degraded through two major proteolysis systems: the proteasome degradation and autophagy. The ubiquitin-proteasome system is the major catabolic pathway for short-lived proteins, while autophagy is a process through which long-lived proteins, damaged organelles, and other waste intracellular material are delivered to lysosomes for degradation. Autophagy is constitutively active at low levels in order to preserve cellular homeostasis but strongly induced by stressful conditions, such as nutrient deprivation, growth factor depletion, oxidative stress, hypoxia, irradiation, and anticancer drug treatments. Under these stressful conditions, autophagy is believed to act primarily as a first protective response. Nevertheless, autophagy may also participate in cell death, constituting an alternative caspase-independent cell death mechanism called type II (or macroautophagy-related) programmed cell death [2, 3]. The importance of autophagy in physiology and pathophysiology is underlined by the finding of an association of autophagic dysfunctions

with the development of important diseases including neurodegenerative disorders, myophaties, and cancer [4].

Autophagy is an evolutionarily conserved dynamic cellular catabolic process. Many autophagy-related (Atg) proteins take part in the various steps of the autophagic pathway. So far more than 36 Atg genes have been characterized in yeast and the majority of them have orthologues in mammals. Many mammal-specific proteins with multiple functions in autophagy have also been identified [5]. When autophagy is induced, Atg proteins associate following a hierarchical order (Figure 1); in mammals the first step is the formation of a preautophagosomal structure which seems to localize on endoplasmic reticulum (ER), where the uncoordinated-51-like kinase (ULK) and the class III phosphatidylinositol 3 kinase (PI3KCIII) complexes are early recruited to start the double-membrane structure nucleation [6]. The ULK complex is composed of ULK1/2 (homologs of yeast protein kinase Atg1), Atg13L, Atg101, and FIP200. Once activated, it recruits other Atg proteins and interacts with Atg9L1 and the PI3KCIII complex. Atg9L1 is a trans-membrane protein that cycles between the trans-Golgi network and the endosomes, and during starvation it localizes on autophagosomes, regulating the autophagosome size [7]. The PI3KCIII complex consists of Beclin 1, vacuolar protein sorting 15 (Vps15) and class III PI3K (Vps34). The PI3KCIII complex, through the activation of the Vps34 enzymatic activity, enriches the double-layer structure of membranes with phosphatidylinositol 3-phosphate (PI3P), which is essential for vesicle nucleation and recruiting of PI3P-interacting Atg proteins, such as Double-FYVE-containing protein 1 (DFCP1) and WD-repeat protein interacting with phophoinositides (WIPIs) (both homologues of yeast Atg18). Afterwards, during the later steps of autophagy, two ubiquitin-like protein conjugation systems participate in the elongation and maturation of autophagosome: the Atg12-Atg5-Atg16L complex and the microtubule-associated protein 1 light chain 3 (LC3)-phophatidylethanolamine (PE) complex. Atg7 and Atg10 catalyze the conjugation between Atg12 and Atg5. Then Atg12-Atg5 complex interacts noncovalently with Atg16L forming a tetrameric structure through the homooligomerization of Atg16L [5]. This final multimeric complex localizes on the outer membrane of the autophagosome and is released from the membrane just before or after the completion of autophagosomes. The second ubiquitin-like molecule is LC3 (the mammalian Atg8 homologue), which is first hydrolyzed by Atg4 to LC3-I (cytosolic form). After that, Atg7 and Atg3 mediate the conjugation of LC3-I with PE producing the lipidated form LC3-II. The Atg12-Atg5-Atg16L complex cooperates facilitating the conjugation of LC3-I with PE. LC3-II displays an apparently symmetrical distribution on both sides of the phagophore membrane [5]. The LC3-II molecules residing on the cytoplasmic side of the autophagosome are delipidated by Atg4 in order to be recycled, while the LC3-II located inside the autophagosome is degraded after fusion with the lysosome. The autophagosome maturation continues with the fusion of endosomes to form amphisomes; at last, the fusion with lysosomes generates autolysosomes, which will degrade the entrapped content.

Several extracellular (e.g., nutrient status, hormonal and therapeutic treatment) and intracellular (e.g., metabolic stressors and accumulation of misfolded proteins) stimuli are able to activate autophagy and many signaling pathways are involved in the regulation of the autophagic process (Figure 2). The mammalian target of rapamycin (mTOR) pathway is the most studied pathway regulating autophagy. The mTOR pathway involves two functional complexes: the mTOR complex 1 (mTORC1) that is an important controller of cell growth and proliferation and plays a major role in controlling autophagy, and the mTOR complex 2 (mTORC2) that is not directly implicated in autophagy modulation. The mTORC1 pathway is a key sensor of nutrient and energy status and is regulated by signals such as growth factors, amino acids and stressors. Mainly under nutrient-rich conditions, mTORC1 directly interacts with and phosphorylates ULK1 negatively affecting the ULK complex formation. Conversely, starvation inhibits mTORC1 leading to dephosphorylation-dependent activation of the ULK complex, which then translocates from the cytosol to the phagophore [8]. In addition to mTORC1, AMP-activated protein kinase (AMPK), another cell key energy sensor, can play a major role in transmitting autophagic signaling. AMPK is activated by the increase in cellular AMP/ATP ratio occurring during nutrient deprivation or hypoxia, and positively regulates the ULK complex both by direct phosphorylation of ULK1 and inhibition of mTORC1 via a pathway involving tuberous sclerosis complex 1 and 2 (TSC1/2) [8]. The PI3KCIII complex is another major point of regulation of autophagy induction. The association of Beclin 1 to the other subunits of the PI3KCIII complex is a key event for the induction of PI3P synthesis by Vps34. Beclin 1-Vps34 connection is regulated by the interaction with Bcl-2, Bcl-XL, Mcl-1 and Rubicon, which act as inhibitors, and with Atg14, UV radiation resistance associated gene (UVRAG), Bax-interacting factor-1 (Bif-1), vacuole membrane protein 1 (VMP1) and Ambra-1, which behave as activators [9, 10]. The dynamic interaction between Beclin 1 and its binding proteins is further regulated by post-translational modifications. For instance, the phosphorylation of Beclin 1 by the death associated protein kinase (DAPK) triggers the dissociation of Beclin 1-Bcl-XL/Bcl-2 complex, allowing Beclin 1 to interact with Vps34 [11], while Beclin 1 phophorylation by Akt inhibits autophagy [12]. Moreover, the phosphorylation of Bcl-2 by c-Jun N-terminal kinase 1 (JNK1) or extracellular signal-regulated kinase (ERK) reduces Beclin 1-Bcl-2 interaction leading to autophagy activation [13]. Downstream the ULK and the PI3KCIII complexes, LC3 can be down-regulated via phosphorylation by protein kinase A (PKA) or protein kinase C (PKC) [14, 15].

2.2. The Role of Autophagy in PCa. The role of autophagy in cancer is controversial and still not completely clarified: it has been described as a double-edged sword because of its involvement in both cell survival and tumor suppression, depending on cell type, genetic context, stage of tumor development and nature of the stressor [16, 17]. As mentioned above autophagy is an evolutionarily conserved process that allows cells to respond to changed environmental conditions preserving cellular homeostasis. This function is

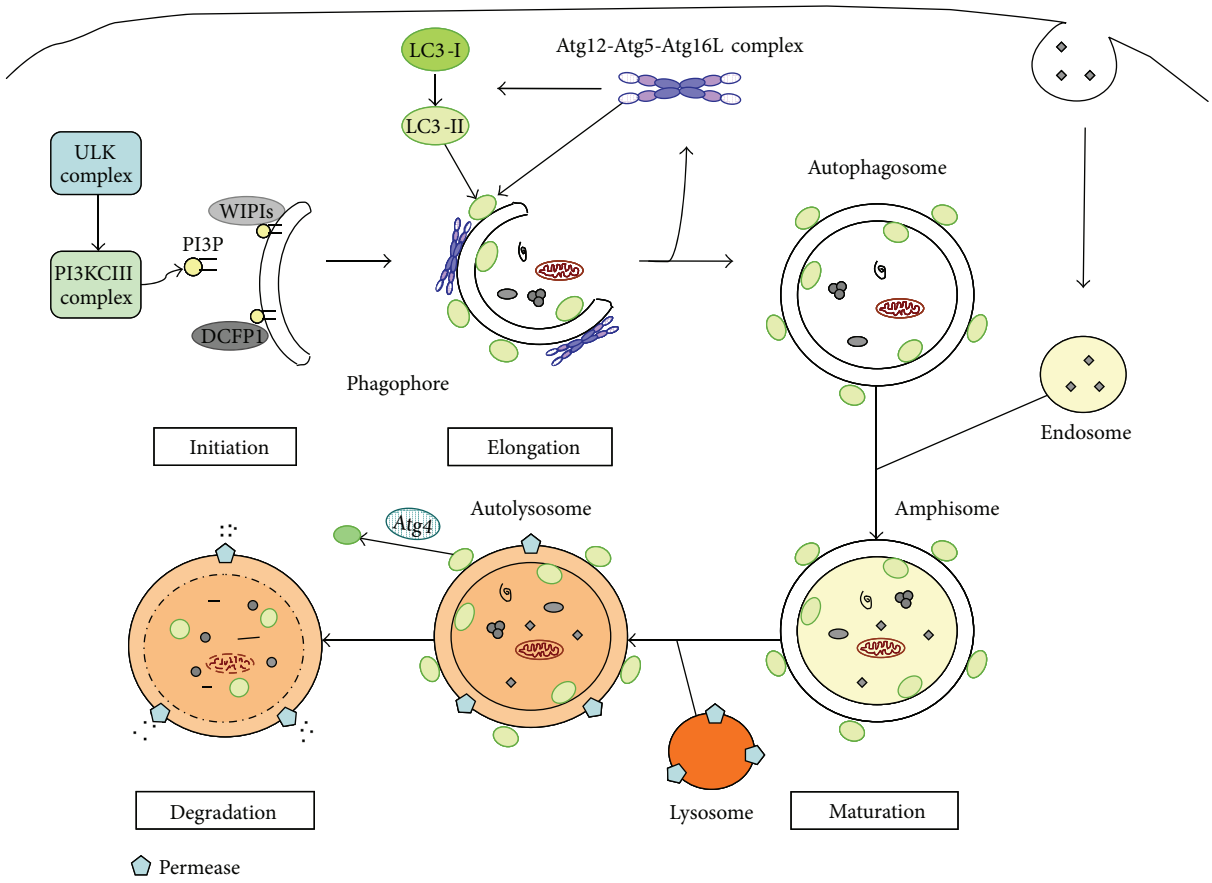

Figure 1: Schematic representation of autophagy. The process begins with the nucleation of the phagophore, followed by its elongation and expansion and its closure to form the double-membrane autophagosome. The autophagosome maturates first through fusion with endosome, producing an amphisome, and then with lysosome to form the final autolysosome, where the inner membrane and the sequestered content are degraded by the lysosomal hydrolases. Finally, the resulting macromolecules are returned to cytoplasm by permeases for reuse. In the figure, the core molecular machinery of autophagy is also illustrated, including the ULK complex that is required for autophagy induction, the PI3KCIII complex and the PI3P interacting proteins, such as WIPIs and DCFP1, which contribute to the phagophore formation and elongation. Also the LC3-II and Atg12-Atg5-Atg16L complexes take part to the elongation step. The Atg12-Atg5-Atg16L complex resides on the outer membrane of the phagophore and dissociates from the completed autophagosome. The LC3-II complex is present on both sides of the phagophore and autophagosome, but it is released by Atg4-mediated deconjugation from the outer membrane after autophagosome maturation.

particularly important for cancer cells that are characterized by high metabolic demand. As a prosurvival mechanism, autophagy may be used by transformed cells to adapt to the tumor microenvironment, which is hypoxic, nutrient limiting and metabolically stressful due to the inadequate blood supply [18]. According to this concept, autophagy is mostly evident in cancer cells localized in the inner, poorly vascularized tumor regions especially during the late stage of carcinogenesis. Cancer cells residing in these tumor regions are generally cells resistant to anticancer treatments [19, 20]. Consistently, in some cancer cells, antineoplastic therapies induce autophagy as a resistance and prosurvival mechanism and, in these cases, genetic or pharmacologic autophagy inhibition can be used to increase the efficacy of the anticancer treatments [21, 22]. The protective role of autophagy has also been evidenced by studies showing an increased activation of programmed cell death pathways when Atg genes are knocked down [23].

Paradoxically, autophagy defects have been found in many human tumors: monoallelic loss of the essential autophagy gene Beclin 1 and decreased levels of the protein have been frequently found in human breast, ovarian, and prostate cancers [24]. In addition molecular analyses of tumors in Beclin 1 heterozygous mice confirmed that Beclin 1 is a haploinsufficient tumor suppressor [25, 26]. Many other Atg genes, such as Atg4, Atg5, Atg7, UVRAG, Bif-1 [27–30], and autophagy regulators, including p53, phosphatase and tensin homolog (PTEN), DAPK [31, 32], have been implicated in tumorigenesis and are considered tumor suppressors. Furthermore, many signaling molecules of the PI3K/Akt/mTOR pathway, a negative regulator of autophagy, have oncogenic properties and the constitutive activation of this pathway is

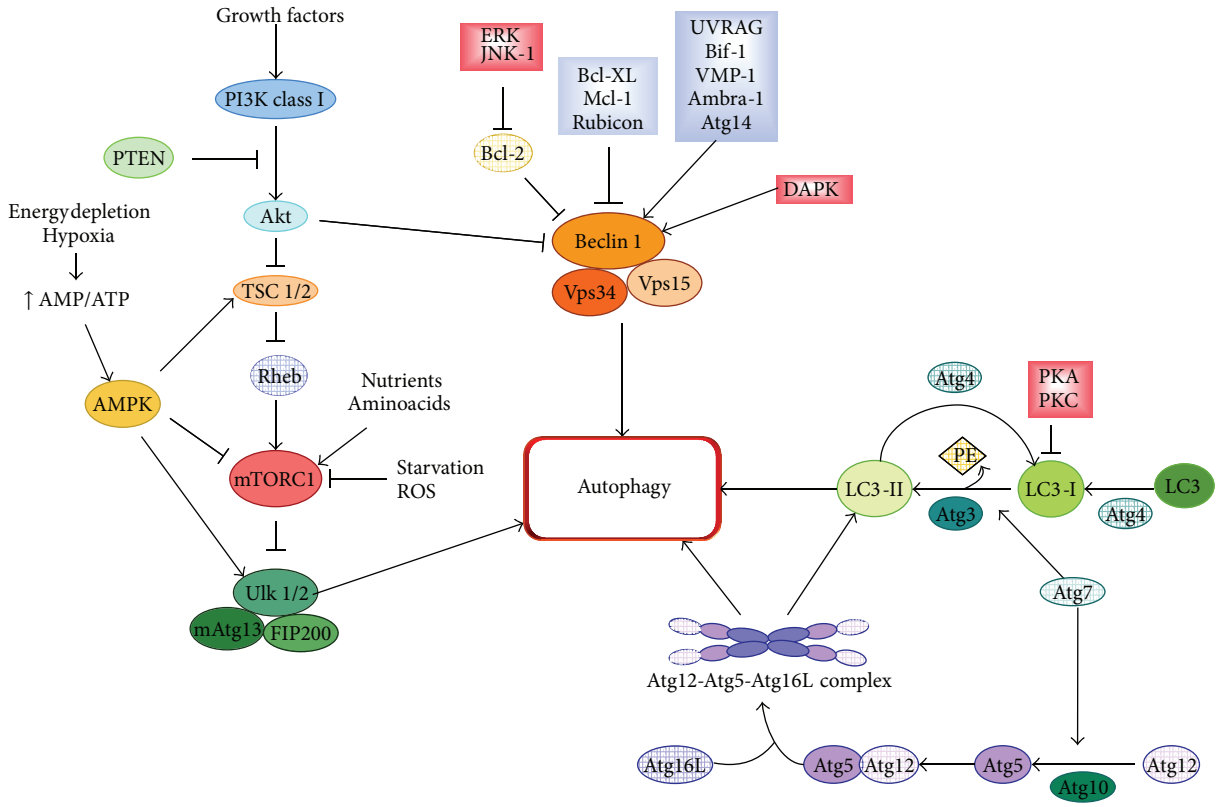

Figure 2: Schematic representation of the molecular regulation of autophagy. Growth factor signalling activates the PI3K/Akt/mTORC1 pathway resulting in autophagy inhibition. mTORC1 is also activated by amino acids and nutrient rich conditions, whereas starvation and oxidative stress induce autophagy via mTORC1 inhibition. Starvation and hypoxia can also induce autophagy through AMPK activation. Beclin 1-Vps34-Vps15 complex (or PI3KCIII complex) is required for the induction of autophagy, and the interaction between its components is regulated by interacting proteins (blue boxes): Rubicon, Mcl-1, and Bcl-XL/Bcl-2 are negative regulators, whereas proteins, such as UVRAG, Atg14, Bif-1, VMP-1, and Ambra-1, through their interaction with Beclin-1 and Vps34, promote the activity of the PI3KCIII complex inducing autophagy. Numerous kinases (red boxes) are involved in autophagy regulation: ERK and JNK-1 can induce autophagy by releasing Bcl-2 inhibition through its phosphorylation; Akt inhibits autophagy via Beclin 1 phosphorylation, whereas DAPK-mediated phosphorylation of Beclin 1 promotes autophagy. Finally, PKA and PKC negatively regulate autophagy acting on LC3. Atg4, Atg3, Atg7 and Atg10 are autophagy-related proteins which mediate the formation of the LC3-II complex and the Atg12-Atg5-Atg16L complex, and they may represent additional control points in the autophagic pathway.

very common in human tumors [22]. The loss of autophagic functions can result in accumulation of protein aggregates and damaged organelles, above all damaged mitochondria, and consequently in reactive oxygen species (ROS) production, which then promotes genome instability furthering oncogenic transformation and cancer progression [28, 33]. This evidence is indicative of the anticancer role of autophagy.

With regard to PCa, studies have indicated that autophagy is compromised in PCa cells: PTEN, the suppressor of the PI3K/Akt/mTOR pathway, is the most frequently deleted tumor suppressor gene in PCa and the PI3K/Akt/mTOR pathway is upregulated in 30–50% of PCa tumors and associated with increasing tumor stage, grade and risk of recurrence [34]. Moreover, a number of Atg genes, such as Beclin 1 and LC3 genes, map to chromosomal loci that are frequently monoallelically deleted in PCa cells [35, 36] and the protein expression of Beclin 1 ad LC3 have been demonstrated to be lower in prostate adenocarcinoma than in prostate benign hyperplasia [37]. Nevertheless, a recent study has demonstrated that about 35% of PCa shows an over-expression of key autophagy proteins (LC3 and p62) directly related to a high Gleason score, indicating that autophagy signaling may be important for cell survival in high-grade PCa [38].

The response of cancer cells to the autophagic stimulus can trigger cell death or cell survival depending on the integration of complex signaling pathways not yet completely clarified. Due to this two-faced role, a better understanding of the regulation and modulation of the autophagic pathway might provide new insights into cancer treatment and prevention. If the prosurvival role of cancer cell autophagy is generally accepted, intense investigations are needed to understand whether the autophagy-associated cancer cell death, induced by some drugs and natural compounds, may be exploited as a promising strategy for cancer therapy.

Table 1: Functional status of autophagy induced by different natural compounds.

	In vitro/*in vivo* system	Dose	Mechanism	Effects on cell fate	Reference
Sulphoraphane	LNCaP and PC-3 cell lines; TRAMP mice	20–40 μM; 1 mg	Mitochondria-derived ROS	Prosurvival	[39–41]
Benzyl isothiocyanate	22Rv1 and PC-3 cell lines	20 μM	mTOR	Prosurvival	[42]
Phenethyl isothiocyanate	LNCaP and PC-3 cell lines; PC-3 xenograft models; TRAMP mice	2.5–5 μM; 9 μmol; 3 μmol/g	ROS production, Akt/mTOR	Prodeath	[43–45]
Resveratrol	DU145 cell line	50 μM	SIRT1/S6K/mTOR	Prodeath	[46]
Polyphenon E	PNT1a cell line	35 μg/mL		Prosurvival	[47]
Curcumin	22Rv1 cell line	20 μM		Prodeath	[48]
Gossypol	CL-1 and PC-3 cell lines and PC-3 xenograft models	10 μM	Bcl-2-Beclin 1	Prodeath	[49, 50]
Apogossypolone	LNCaP and PC-3 cell lines	10 mg/L		Prosurvival	[51]
Ascorbate	PC-3 cell line	5 mM	ROS production	Prodeath	[52]
Vitamin K3/vitamin C	PC-3 cell line	3 μM vit. K3 + 0.4 mM vit. C	ROS production	Prosurvival	[53]
Rottlerin	Human PCa stem cells	0.5–1-2 μM	AMPK, PI3K/Akt/mTOR, Bcl-2-Beclin 1	Prodeath	[54]
Piperine	LNCaP and PC-3 cell lines	160 μM			[55]
Piperlongumine	PC-3 cell line	10 μM	ROS production, Akt/mTOR	Prosurvival	[56]
Ursolic acid	PC-3 cell line	40 μM	Akt/mTOR	Prosurvival	[57]
Marchantin M	PC-3 cell line	10 μM	ER stress, PI3K/Akt/mTOR pathway	Prodeath	[58]
Monascuspiloin	PC-3 cell line; PC-3 xenograft models	50 μM; 40–120 mg/kg	AMPK	Prodeath	[59, 60]

3. Natural Compounds Inducing Autophagy in PCa

Natural products are receiving increasing attention for the prevention and/or treatment of cancer because of their promising efficacy and low toxicity to normal tissue. Therefore there is a great interest in identifying new natural products active against PCa and in understanding the mechanisms of action of these compounds to exploit their properties in the development of new therapeutic or preventive treatments. Since autophagy may become a new therapeutic target for PCa treatment, in this section we will report the evidence on natural compounds able to modulate autophagy influencing PCa cell fate (Table 1).

3.1. Isothiocyanates. Isothiocyanates are a family of compounds derived from the myrosinase-mediated hydrolysis of glucosinolates contained in cruciferous vegetables. High intake of cruciferous vegetables may be associated with reduced risk of aggressive PCa [61, 62], and isothiocyanates are believed to be responsible for the anticancer effects of these vegetables [63]. Sulphoraphane [1-isothiocyanato-4-(methylsulfinyl)-butane] (SFN), the most studied isothiocyanate, was firstly identified as a chemopreventive agent able to both inhibit Phase 2 detoxification enzymes and induce Phase 1 enzymes involved in carcinogen activation [64, 65]. SFN, as well as other naturally occurring isothiocyanates, can also block cancer development by causing cell cycle arrest and apoptosis induction in cancer cells. SFN, benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and allyl isothiocyanate are all isothiocyanates having antitumor effects on PCa both *in vitro* and *in vivo*, without affecting normal prostate epithelial cells [66–70]; moreover, most of these isothiocyanates are able to induce autophagy in PCa cells as well as in breast, colon, and pancreatic cancer models [39–45, 71–74].

In PCa cell lines, SFN inhibited cell proliferation by causing G2-M phase cell cycle arrest and caspase-dependent apoptosis [75–78]. Orally administration of this isothiocyanate reduced prostate tumor growth and pulmonary metastasis in transgenic adenocarcinoma of mouse prostate (TRAMP) mice without causing any side effects [67] and

retarded the growth of PC-3 xenografts in nude mice [78, 79]. Recently, SFN-induced autophagy in PCa cells has been reported both *in vitro* and *in vivo*. Human PCa cells (LNCaP and PC-3) treated with SFN at a dose of 40 μM exhibited the hallmarks of autophagy including the formation of AVOs, the processing and the punctuate localization of LC3 [39]. Autophagy occurred before the onset of apoptosis, and presumably the sequestration of mitochondria by autophagosomes was the cause of a delayed release of cytochrome *c* and activation of intrinsic caspase cascade. This evidence, showing autophagy as a protective mechanism against apoptosis induced by SFN, was confirmed by pharmacologic inhibition of autophagy using 10 mM 3-methyladenine (3-MA), which augmented SFN-induced apoptotic cell death [39]. SFN (20 μM) caused ROS generation due to the inhibition of mitochondrial respiratory chain enzymes, and these mitochondria-derived ROS initiated the apoptotic cell death and the earlier protective autophagic response in PCa cell lines [41, 80]. Autophagy induction by SFN was also observed *in vivo*: in TRAMP models, a 5-week cotreatment with SFN (1 mg by oral intubation 3 times per week) and the autophagy inhibitor chloroquine (1.2 mg by intraperitoneal injection 3 times per week) resulted in a reduction of poorly differentiated prostate tumors and lymph node metastasis compared to the untreated control group and the group treated with SFN alone. In addition, the TUNEL-positive apoptotic bodies were significantly increased by the combination of SFN with chloroquine [40]. This evidence confirms *in vivo* the cytoprotective function of SFN-induced autophagy in PCa.

Another isothiocyanate able to induce autophagy in human cancer cells is BITC [42, 71]. In PC-3 cells, BITC induced Bcl-XL phosphorylation, cell cycle arrest and subsequent apoptosis [68], and in DU145 human PCa cells, BITC was shown to induce ROS production triggering the activation of the apoptotic pathway [81]. Though in these studies BITC-mediated autophagy induction was not investigated, both Bcl-XL phosphorylation and ROS production may stimulate autophagy [10, 33]. A recent study specifically examined BITC ability to induce autophagy in human hormone-sensitive (22Rv1) and -refractory (PC-3) PCa cell lines. BITC was shown to inhibit mTOR signaling triggering autophagy in a dose- and time-dependent manner [42]. Combination of 20 μM BITC and 1 mM 3-MA significantly increased BITC-induced apoptotic cell death in either 22Rv1 or PC-3 cells, showing that BITC-induced autophagy represented an early protective response, as also observed for SFN-treated PCa cells [39, 42].

PEITC is another naturally occurring isothiocyanate that has received increasing attention due to its cancer chemopreventive effects. *In vitro*, PEITC suppressed growth of PCa cells (PC-3, LNCaP and DU145) through induction of G2-M phase cell cycle arrest and apoptotis [44, 82–86]. Moreover, PEITC oral administration retarded the growth of PCa xenografts in nude mice, reduced the incidence of poorly differentiated tumors and increased the TUNEL-positive apoptotic bodies in PC-3 xenografts and TRAMP mice [44, 45, 83, 84]. Hallmarks of autophagy have been characterized *in vitro* and *in vivo*: PCa cells (LNCaP and PC-3), but not normal prostate epithelial cell line (PrEC), treated with 2.5–5 μM PEITC exhibited significant accumulation of AVOs and enhanced processing and punctuate localization of LC3 [43, 44, 87]; increased expression and cleavage of LC3 were also revealed in tumor sections from mice with PC-3 xenografts gavaged with 9 μM PEITC and from TRAMP mice fed 3 μmol PEITC/g diet [44, 45]. Both PEITC-induced autophagy and apoptosis in LNCaP and PC-3 cell lines were strongly dependent on Atg5 protein level [44], thus proving an interrelation between the two pathways activated by PEITC treatment. PEITC, as well as other isothiocyanates, induced mitochondria-derived oxidative stress in LNCaP and PC-3 cells, and the generated ROS played a critical role in the initiation of apoptosis by induction of Bax mitochondrial translocation and cytosolic release of cytochrome *c* [43]. Nevertheless, differently from SFN and BITC, PEITC induced an autophagic process that was only partially dependent upon ROS production [43]. The treatment with 5 μM PEITC resulted in the suppression of Akt/mTOR. However, overexpression of positive regulators of mTOR, Akt or Rheb, conferred only a partial protection against PEITC-mediated autophagy [44], suggesting the potential involvement of other mechanisms in the activation of the autophagic response evoked by PEITC. Remarkably, cotreatment with the autophagy inhibitor 3-MA (4 mM) or knockdown of Atg5 protein attenuated the apoptotic DNA fragmentation and the activation of caspase 3, thus suggesting that PEITC-mediated autophagy contributed to the promotion of apoptotic and nonapoptotic cell death [44].

3.2. Polyphenols. Polyphenols constitute one of the largest and ubiquitous group of phytochemicals: flavonoids and phenolic acids represent the most common ones in food. Epidemiological evidence suggests lower PCa risk in populations with higher consumption of major polyphenols [88, 89]. Several naturally occurring polyphenols, including resveratrol, green tea catechins and curcumin, are currently being studied for their potential role in PCa prevention and treatment. These compounds can induce both apoptotic and autophagic cell death in various type of cancers [90].

Resveratrol (3,5,40-trihydroxystilbene) is a natural non-flavonoid polyphenolic compound present in grape skin, red wine and nuts. After Jang et al. reported for the first time in 1997 the ability of resveratrol to inhibit the carcinogenic process at multiple stages, including initiation, promotion and progression [91], subsequent studies have focused on its potential chemopreventive function in many different animal models of carcinogenesis [92]. Resveratrol has been reported to have antiproliferative and proapoptotic effects on PCa cell lines [93]. Relatively few *in vivo* studies however have investigated and confirmed the effects of resveratrol on PCa prevention and treatment [92]. Nevertheless, there is no evidence from human clinical trials for resveratrol as an effective supplement for prevention and treatment of prostate diseases [94]. Resveratrol ability to induce autophagy in different cancer cell lines as either a prosurvival or a prodeath mechanism [90, 95, 96]. With regard to PCa, Li et al. showed that, in DU145 cells, resveratrol (50 μM for 24 h) induced a significant increase in autophagy leading to nonapoptotic programmed cell death. Conversely, androgen-responsive

LNCaP and androgen-independent C42B cells resulted relatively resistant to resveratrol treatment. The data suggested that SIRT1, a NAD-dependent histone deacetylase belonging to the family of sirtuins, could act as a positive regulator of autophagy in DU145 cells triggering the dephosphorylation of S6K, one of the key effectors of mTOR [46]. Consistently with the *in vitro* findings, treatment of 4- or 5-week-old PTEN knockout mice with resveratrol for 14 weeks was associated with reduction in the prostatic levels of mTORC1 activity and increased expression of SIRT1, supporting that the SIRT1/S6K pathway could play an important role in autophagy induced by resveratrol in PCa [46].

Green tea catechins have antitumoral and chemopreventive properties demonstrated by *in vitro* and *in vivo* studies [97–99]. Of all the catechins found in green tea, (-)-epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active. In PCa cells green tea catechins, and especially EGCG, are able to modulate a plethora of cell signaling pathways crucial for cancer cell transformation and survival [97, 100–102]. Chemopreventive and chemotherapeutic effects of these polyphenols have been observed in preclinical models of PCa, including both genetic and xenograft models [103–105]. In addition, there have been 5 intervention studies evaluating the effect of green tea intake on the change in risk markers of PCa: among them, there was only one randomized, double-blind, placebo-controlled trial demonstrating the efficacy of green tea supplementation on PCa incidence [106–111]. Green tea catechins on cancer. The regulation of autophagy by EGCG seems to be dependent on concentration, cell types and stress conditions [112]. Our data reported that two prostate epithelial cell lines, PNT1a and PC-3, mimicking initial and advanced stages of PCa respectively, responded differently to the treatment with Polyphenon E, a standardized green tea extract. The treatment of PNT1a cells with 35 μg/mL Polyphenon E for 24 h triggered the activation of caspases committing cells to anoikis, while 145 μg/mL Polyphenon E caused PC-3 cell death through a caspase-independent necroptotic event. Autophagy was transiently activated only in PNT1a cells between 6 and 12 h of treatment as a survival response to overcome Polyphenon E-induced ER stress [47].

Curcumin is a polyphenolic compound isolated from the rhizomes of *Curcuma longa*, exhibiting anti-inflammatory, anticancer and antioxidant activities based on its chemical features and its ability to interact with multiple signaling molecules [113]. Curcumin exerts a cytotoxic and cytostatic action in many transformed cells, prevents carcinogen-induced cancer in rodents and inhibits the growth of human tumors in xenograft or orthotransplanted animal models, either as single treatment or in combination with chemotherapeutic drugs or radiation [114]. Curcumin and its derivatives have been described to inhibit different signaling pathways in cancer resulting in apoptotsis [115, 116] or in caspase-independent cell death mechanisms, like autophagy [117–120]. Curcumin-induced autophagy is generally described as a prodeath signal [119, 121, 122], however it has recently been demonstrated to exert a prosurvival and prodifferentiation role in tumor initiating cells [123] and to precede or accompany a senescence/quiescence-promoting effect in cancer cells [124–126]. Curcumin affected cell proliferation of androgen-sensitive (22Rv1), but not of androgen-independent (DU145 and PC-3) PCa cells, through the induction of G2 cell cycle arrest and modulation of Wingless (Wnt/β-catenin) signaling pathway. The reduction of cell viability observed after curcumin treatment (20 μM for 24 h) in 22Rv1 cells was linked to autophagy induction as demonstrated by the appearance of LC3-II form and the decrease of Bcl-XL expression [48]. Bcl-XL is an antiapoptotic protein, but also an antiautophagic protein via its inhibitory interaction with Beclin 1 [9, 10]. This highlights the complex interrelationship existing between autophagy and the apoptotic cell death pathway.

Gossypol is a natural polyphenolic compound isolated from cottonseeds that acts as a BH3-mimetic small molecule pan-inhibitor of antiapoptotic Bcl-2 family members including Bcl-2, Bcl-XL and Mcl-1 [127]. Treatment with gossypol led to inhibition of cell viability and induction of apoptosis in different kinds of PCa cells and significantly inhibited angiogenesis and PCa xenografts growth [128, 129]. Unfortunately, only limited efficacy was proved in clinical trials [130, 131]. Gossypol has been reported to induce Beclin 1-dependent or -independent autophagy, with a prosurvival or a prodeath effect depending on the cancer cell type [127]. Lian et al. investigated *in vitro* and *in vivo* the mechanism leading to gossypol-induced cell death in human PCa cells expressing different levels of Bcl-2. Gossypol (10 μM) preferentially induced apoptosis in PCa cells with low Bcl-2 (LNCaP, DU145 and C4-2B), whereas an autophagic cell death was observed in apoptosis-resistant, androgen independent cells with high Bcl-2 (PC-3 and CL-1) [49]. Functioning as a pan-Bcl-2 inhibitor, gossypol down-regulated Bcl-2, Bcl-XL and Mcl-1. Thus, gossypol triggered autophagy mainly via inhibition of the interaction between Beclin 1 and Bcl-2/Bcl-XL [49, 50]. *In vivo* evidence confirmed that gossypol inhibited CL-1 and PC-3 xenografts tumor growth by autophagy induction [49]. Also apogossypolone, a semi-synthesized derivative of gossypol, at a concentration of 10 mg/L, was able to provoke an early activation of the autophagic pathway in both PC-3 and LNCaP cells. However, in this case, autophagy acted as a protective response against apoptosis induction [51].

3.3. Vitamins. In recent years, various reports have shown that vitamins, such as vitamin C and vitamin K, exhibit antioncogenic effects [132, 133]. In various cancer cell lines, autophagy has been evidenced to be evoked as a response to vitamin K or ascorbic acid treatment [52, 53, 134–140]. Autophagy triggered by vitamins has mainly been described as an alternative caspase-independent cell death pathway that supports apoptosis [134–138]. Nevertheless, autophagy has also been characterized as a prosurvival response against apoptosis in human hepatoma cells treated with vitamin K3 (a synthetic version of vitamin K) [139] and in glioblastoma cells treated with ascorbic acid [140]. In PCa, autophagy induced by vitamins can have both a prodeath and a prosurvival function depending on doses and treatment conditions [52, 53].

The treatment of different types of PCa cell lines, including androgen-dependent (LNCaP), androgen-sensitive (22Rv1) and androgen-independent cells (PC-3 and C4-2),

with ascorbate (0–20 mM) for 2 h demonstrated that ascorbic acid, at concentrations clinically achievable with pharmacological intravenous infusion, could induce H_2O_2-dependent cytotoxicity [52, 141]. Increased conversion of LC3-I to LC3-II, punctuated pattern of GFP-LC3 signal and transmission electron microscope observations of autophagosome structures were demonstrated after exposure of PC-3 cells to 5 mM ascorbate for 6 h, evidencing the activation of the autophagic pathway [52]. Inhibition of ascorbate-induced autophagy by 3-MA treatment increased cell viability, and knockdown of Bif-1, a positive mediator of autophagy, resulted in PC-3 cell resistance to ascorbate-induced cell death, thus suggesting a prodeath role for autophagy [52].

Vitamin K3 (menadione, 2-methyl-1,4-naphthoquinone) is a synthetic derivative of vitamin K1 that has been demonstrated to exhibit anticancer activity in human cancer cell lines and to potentiate the cytotoxic effects of several chemotherapeutic agents [132]. The combination of vitamin K3 and vitamin C has shown synergistic antitumour activity against PCa *in vitro* [142–144] and *in vivo* [145, 146]. In PC-3 cells the combination of subtoxic concentrations of vitamin K3 (3 μM) and ascorbic acid (0.4 mM) caused autophagy activation, which acted as a protective mechanism induced by oxidative stress [53]. In this conditions, autophagy could be overcome by the coadministration of subtoxic doses of the redox-silent vitamin E analogue α-tocopheryl succinate (30 μM), which acted as a ROS scavenger [53]. The triple combination treatment (vitamin K3, ascorbic acid and α-tocopheryl succinate) was associated with synergistic/additive cytotoxic effects on PC-3 cell line and PC-3 xenografts in nude mice [53, 144], supporting that the inclusion of α-tocopheryl succinate in the combinatorial treatment could result in the overcoming of the prosurvival responses to ascorbic acid/vitamin K3 treatment.

3.4. Emerging Natural Compounds Able to Induce Autophagy in PCa. Since autophagy is involved in carcinogenesis, the ability of newly discovered or confirmed anticancer natural compounds to modulate this cellular pathway in PCa cells is receiving increasing attention, as supported by recent evidence.

Rottlerin is a natural plant polyphenol, isolated from *Mallotus philippinensis* (Euphorbiaceae), with demonstrated anticancer activity: this active compound is able to affect several cell pathways involved in survival, apoptosis, autophagy and invasion [147]. Recent data reported that in human PCa stem cells, rottlerin (0.5–2 μM) induced autophagy in a dose-dependent manner by activating AMPK pathway, inhibiting the PI3K/Akt/mTOR pathway and decreasing Bcl-2 and Bcl-XL protein levels [54]. Rottlerin-induced autophagy could be characterized as a prodeath pathway linked to apoptotic cell death. In effect, the administration of autophagy inhibitors (3-MA, chloroquine or bafilomicyn A1) caused the suppression of both autophagy and apoptosis in PCa stem cells treated with rottlerin [54].

Piperine and piperlongumine, two alkaloids present in black (*Piper nigrum* Linn) and long (*Piper longum* Linn) peppers, have been recently reported to mediate antitumoral effects on human PCa cells *in vitro* and *in vivo* [55, 56, 148–150], and autophagy was one of the mechanism triggered by this active compounds [56, 151]. In particular, piperlongumine (10 μM) was shown to induce autophagy in PC-3 cells by down-regulating the Akt/mTOR signaling pathway. In this case, autophagy was ROS-dependent, as cotreatment with the antioxidant N-Acetyl-L-Cysteine reversed piperlongumine effects [56]. Concomitant treatment with piperlongumine and chloroquine enhanced cell death in PC-3 cell lines and reduced growth of xenograft tumors in immunodeficient mice, demonstrating a prosurvival function of piperlongumine-mediated autophagy [56].

Ursolic acid is a natural pentacyclic triterpenoid isolated from plants and medicinal herbs and has many biological functions, including antitumor activities on PCa cells [152, 153]. In PC-3 cells, the treatment with 40 μM ursolic acid for 24 h caused an early activation of autophagy via the disruption of the PI3K/Akt/mTOR pathway [57]. Ursolic acid-induced autophagy represented an early protective mechanism to allow cell escape from apoptosis [57]. Autophagy induced by ursolic acid has also been evidenced in human breast, colorectal and cervical cancer with conflicting effects on cell survival [154–156].

Marchantin M, a macrocyclic bisbibenzyl extracted from *Asterella angusta*, has anti-inflammatory and cytotoxic effects on PCa cells [58, 157, 158]. Marchantin M-triggered proteasome inhibition and ER stress, as well as suppression of the PI3K/Akt/mTOR pathway, contributed to autophagy induction in PC-3 cells [58]. Autophagy was shown to be implicated in marchantin M-mediated cell death, as demonstrated by the almost complete restoration of PC-3 cell viability after the combined treatment with pan-caspase and autophagy inhibitors [58].

Red yeast rice, produced by the fermentation of rice with fungus of the *Monascus* species, is a traditional Asian food spice that has also medicinal uses due to the anti-inflammatory, antioxidative and antitumor properties of its metabolites [159, 160]. One of these metabolites is monascuspiloin, which is able to inhibit the growth of hormone-sensitive (LNCaP) and hormone-insensitive (PC-3) PCa cells. Monascuspiloin (50 μM for 12 h) induced apoptosis in both PCa cells, but preferentially in LNCaP cells, whereas the induction of the autophagic pathway via AMPK activation prevailed in PC-3 cells [59]. Autophagy induced by monascuspiloin represented a prodeath mechanism that sustained apoptosis [59]. Hallmarks of autophagy, including high expression of LC3-II, Atg5, Atg12 and Beclin 1, were also confirmed in PC-3 xenograft tumors in nude mice treated with 40–120 mg/kg monascuspiloin [59]. Furthermore, monascuspiloin was shown to sensitize PC-3 cells and PC-3 xenografts to ionizing radiation through inducing ER stress and autophagy [60].

4. Conclusion

Autophagy has a controversial and quite complicated role in PCa tumorigenesis. It can act as a tumor suppressor during the early stages of carcinogenesis, but it can also be used by transformed cells as a survival mechanism to overcome

the stresses imposed during tumor growth. The prosurvival role of autophagy is responsible, at least in part, for the adaptive response of PCa cells to various anticancer therapies, including radiation therapy and conventional DNA damaging chemotherapy. The prodeath function of autophagy, at the moment poorly characterized, could be attributed to two separate functions: the proapoptosis function of autophagy and the induction of autophagic cell death, without the involvement of apoptosis machinery.

On one hand, the synergism between autophagy inhibitors and conventional chemotherapeutic drugs is attracting more and more attention since this strategy could overcome resistance, indeed increasing and maximizing the clinical effectiveness of PCa therapy. On the other hand, the induction of autophagic cell death could represent a promising strategy to trigger an alternative type of programmed cell death in cancer cells that have acquired resistance to apoptosis.

Many published studies, especially those based on a morphology-based definition of autophagic cell death, fail to establish the causative role of autophagy in the cell death process. It is urgently required a joint effort of many researchers to understand whether and when autophagy is a real independent cell killer and an accomplice of apoptosis, or a passive bystander effect that occurs concomitantly with cell death.

The studies summarized in this review suggest that many natural compounds induced autophagy by specifically downregulating the Akt/mTOR pathway, thus indicating that autophagy may induce cell death through a specific molecular commitment. It is noteworthy that the Akt/mTOR pathway, frequently upregulated in PCa, contributes to the disease development and progression also through an extensive crosstalk with many other signaling pathways involved in cell survival, apoptosis, growth and differentiation. Since mTOR activity can be directly or indirectly modulated by a number of upstream signaling pathways, it is mandatory to uncover the mechanisms through which these natural compounds inhibit the Akt/mTOR pathway and impact on the cell fate.

In addition, a better understanding of the molecular effectors that interconnect autophagy to programmed cell death is urgently required to look at many natural compounds as a "sustainable" hope for therapeutic anticancer strategy. There is a strong need of well designed clinical studies to answer to the question whether natural substances may have a relevant role in PCa therapeutic managing. Developing methods and techniques useful to monitor the role of autophagy *in vivo* will be fundamental to reach the target.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Elaborating the Role of Natural Products-Induced Autophagy in Cancer Treatment: Achievements and Artifacts in the State of the Art

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Autophagy is a homeostatic process that is highly conserved across different types of mammalian cells. Autophagy is able to relieve tumor cell from nutrient and oxidative stress during the rapid expansion of cancer. Excessive and sustained autophagy may lead to cell death and tumor shrinkage. It was shown in literature that many anticancer natural compounds and extracts could initiate autophagy in tumor cells. As summarized in this review, the tumor suppressive action of natural products-induced autophagy may lead to cell senescence, provoke apoptosis-independent cell death, and complement apoptotic cell death by robust or target-specific mechanisms. In some cases, natural products-induced autophagy could protect tumor cells from apoptotic death. Technical variations in detecting autophagy affect data quality, and study focus should be made on elaborating the role of autophagy in deciding cell fate. In vivo study monitoring of autophagy in cancer treatment is expected to be the future direction. The clinical-relevant action of autophagy-inducing natural products should be highlighted in future study. As natural products are an important resource in discovery of lead compound of anticancer drug, study on the role of autophagy in tumor suppressive effect of natural products continues to be necessary and emerging.

1. Introduction

Accumulating studies have revealed the role of autophagy as an important cellular homeostatic process. Autophagy, derived from Greek meaning self-eating, is a self-degradative approach to clear intracellular organelles and proteins [1]. It was first coined by Deter and de Duve in 1960s, with observation that subcellular organelles like mitochondria could be degraded in lysosome of perfused rat liver [2]. Physiologically, autophagy is highly conserved across types of mammalian cells and works in housekeeping manner to scavenge misfolded proteins and damaged organelles, as well as infections [3]. This process is critical for energy balance and genome stability of cells. Autophagy could recycle the nonessential long-term proteins to generate energy in response to nutrient deficiency in starved cells [4]. In general speaking, autophagy is a machinery of cell survival that serves to conquer different types of cellular stress, which may subsequently result in cell death. However, studies in recent years rediscovered autophagy as a mechanism of nonapoptotic cell death, as sustained autophagy may excessively degenerate intracellular structures, and cells undergoing uncontrolled autophagy eventually vanish [5]. It is a morphological definition and no conclusive evidence of specific mechanisms underlying autophagy-induced cell death could be observed [6]. Aberrancy or deficiency of autophagy was observed in various kinds of human disorders such as cardiomyopathy, diabetes, neurodegeneration, autoimmune diseases, infections, liver diseases, and cancers.

It was particularly found that the role of autophagy in human cancer is complicated. On one hand, autophagy may be critical in removing stress induced by infection, hypoxia, nutrient deprivation, and metabolic damage. Autophagy-deficient cells are much susceptible to stress and damage to cell genome, leading to easier tumorigenesis in vivo. It was found that chemical carcinogen-induced hepatocarcinoma

could be suppressed by enhanced autophagy which removed aggresome and damaged organelles that could lead to DNA double strand break and genome instability [7], while mice with Beclin-1 knockout are tumor prone [8]. Overexpression of Beclin-1 in mice could develop excessive autophagy to prevent tumor development [9]. On the other hand, activation of autophagy in tumor cells is found to promote tumor development. As tumor cells expand so quickly that nutrients and oxygen could not be sufficiently supplied, tumor cells may undergo serious starvation that causes cell stress. Autophagy in tumor cells is therefore carried out to maintain cell survival by complementing energy supply to conquer nutrient deficiency, and it could remove the damaged organelles and proteins under hypoxia condition [10]. It was observed that inhibition of autophagy led to tumor regression and extended survival of xenografted mice of pancreatic cancer model [11]. However, autophagy may contribute to tumor cell death, in a robust or target-specific way, in cytotoxic agents-treated cancer cells [12]. In recent years, accumulating studies have shown that natural compounds and extracts isolated from medical plants with anticancer property could regulate autophagy in human cancer cells. Phenols, alkaloids, flavones, and organic acids are reported to be autophagy regulators, and it was shown that autophagy may play either cytoprotective or cytotoxic role in natural products-treated cancer cells. With a great interest of finding lead compounds of potential anticancer drug from natural compounds, whose mechanism of action involves autophagy regulation, we summarized the recent advance in studying natural autophagy regulators and discussed the achievements and artifact of the state of the art.

2. Natural Products-Induced Autophagy in Cancer

Data was retrieved from publications regarding the regulatory action of natural products on autophagy in cancer cells. Interestingly, almost all the literature refers to the property of natural products in inducing autophagy in cancer. Few studies reported inhibition of autophagy by natural products. Kallifatidis et al. reported that a marine natural compound manzamine A could suppress autophagy in pancreatic cancer cells. Manzamine A could block the fusion of autophagosome and lysosome and abrogate autophagosome turnover [13]. Despite the fact that manzamine A exhibits potent antitumor activity, no direct experimental evidence could show that inhibition of autophagy could contribute to tumor suppression. A recent study revealed that oblongifolin C from *Garcinia yunnanensis* Hu could suppress autophagy and enhance the antitumor effect of nutrient deprivation. As cancer cell develops autophagy as adaptive mechanism towards nutrient deprivation, inhibition of autophagy may lead to death of the cells [14]. However, studies reporting natural products-induced autophagy elaborated more details on the role of autophagy in mediating inhibition of cancer by these compounds (Tables 1 and 2).

2.1. Natural Product-Induced Autophagy as a Tumor Suppressor

2.1.1. Autophagy That May Result in Cancer Cell Senescence. Previous studies have revealed that natural products may induce autophagy and cell cycle arrest in cancer cells without significant presentation of cell death. Natural compound isolated from Radix Ophiopogon Japonicus named ophiopogonin B could induce both G0/G1 cell cycle arrest and autophagy in lung cancer cells, without significant apoptosis observed [15]. Sustained arrest of cell phase contributes to cell senescence, during which proliferation rate of cancer cells reduces and motility and invasiveness might be retarded. Pedro and colleagues reported that seven natural prenylated flavones may initiate autophagy in ER positive breast cancer cells while DNA synthesis was suppressed. The proliferation of cancer cells was reduced, though there was no evidence that could directly link up autophagy initiation with proliferation inhibition [16]. Kaushik et al. showed that natural compound honokiol may cause proliferation inhibition which is associated with autophagy and cell cycle arrest [17]. Evidence showing interaction between autophagy and cell cycle arrest is not very clear though some efforts have been made and found that redistribution of cell cycle phase may result from autophagy initiation. Ko et al. showed that autophagy induction may be associated with proliferation inhibition by natural product in cancer cells. In colon cancer cells treated with dimethyl cardamonin isolated from *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae), presence of autophagy inhibitor 3-MA or siRNA against Beclin-1 or Atg5 could restore proliferation of tumor cells [18]. A natural compound penta-1,2,3,4,6-O-galloyl-beta-D-glucose (PGG) may in parallel induce both autophagy and cell cycle arrest. Inhibition of autophagy by the presence of chemical inhibitor or RNA interference in PGG-treated cancer cells results in reentering of cell cycle, suggesting the role of autophagy in mediating cancer cell senescence [19]. However, we cannot simply rule out the possibility that autophagy may be induced due to arrest of cell cycle. Law showed that Alisol B isolated from *Alisma orientale* could initiate cell cycle redistribution and autophagy. Blockade of autophagy in Alisol B-treated cancer cells may result in unfolded protein accumulation-related endoplasmic reticulum stress [20], which indicated that autophagy was initiated to scavenge misfolded protein in Alisol B-treated cancer cells. The accumulation of misfolded protein might be associated with abnormal arrest of cell cycle and aberrant distribution of protein expression profile. In this case, induction of autophagy might be initiated to maintain cell under cell phase arrest conditions by clearing cellular stress. This was further evidenced by the observation that a natural compound Cucurbitacin B could initiate autophagy to conquer nutrient stress induced by DNA damage-associated G2/M cell cycle arrest [21]. As retrieving molecules that could specifically facilitate reentering of cell cycle are hardly obtained, evidence of cell cycle arrest-induced autophagy may not be straightforward and further investigation is still required.

Table 1: Natural products that induce tumor suppressing autophagy.

Compound	Plant of origin	Pathway involved	Reference
2-Methoxyestradiol	*Brassica oleracea* var. botrytis	N.A.	[22]
Akebia saponin PA	*Dipsacus asperoides*	JNK, caspase-3 activation↑	[23]
Alisol B	*Alisma orientale*	CaMKK-AMPK-mTOR↑	[20]
Baicalin	*Scutellaria baicalensis* Georgi	Akt↓	[24]
Berberine	Coptidis Rhizoma	Akt/mTOR, CD147↓; Beclin-1, p38 MAPK↑	[25, 26]
Bufalin	Toad venom	ROS, JNK, Atg, Beclin-1, TNF, Atg8↑	[27, 28]
Caffeine	Coffee beans	Erk1/2↑; P70S6K, S6, 4E-BP1, Akt↓	[29]
Celastrol	*Tripterygium wilfordii*	LC3-II, MAPK, Beclin-1↑; Akt, mTOR, S6K, MEK↓	[30, 31]
Coibamide A	Marine cyanobacterium	N.A.	[32]
Crotoxin	South American rattlesnake	N.A.	[33]
Curcumin	*Curcuma longa*	Erk1/2, Beclin-1, LC3-II, AMPK↑; Akt/mTOR/p70S6K↓	[34–38]
Dimethyl cardamonin	*Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae)	N.A.	[18]
Evodiamine	*Evodia rutaecarpa*	Bcl-2/Beclin-1↓	[39]
Fangchinoline	Radix Stephaniae tetrandrae	AMPK, sestrin2, p53 translocation↑	[40]
Flavokawain B	Alpha pricei Hayata	N.A.	[41]
Furanodiene	Rhizoma curcumae	N.A.	[42]
Gambogenic acid	Gamboge	Beclin-1↑	[43]
Honokiol	Officinal Magnolia Bark	Beclin-1↑; Akt/mTOR↓	[17, 44]
Indirubin	*Isatis indigotica* Fort.	N.A.	[45]
Jia-Wei-Xiao-Yao-San	N.A.	N.A.	[46]
KIOM-C	N.A.	JNK, ROS, CHOP↑	[47]
Liensinine, isoliensinine, dauricine, cepharanthine	N.A.	AMPK↑; mTOR↓	[48]
Magnolol	Officinal Magnolia Bark	Bax/Bcl-2 ratio↑	[49]
Matrine	Radix Sophorae flavescentis	Endosome/lysosome pH value↑	[50]
Neferine	*Nelumbo nucifera*	ROS↑; GAH, PI3K/Akt/mTOR↓	[51]
Neoalbaconol	*Albatrellus confluens*	PDK1, PI3K, Akt, HK2↓, glucose consumption, ATP↓	[52]
Nexrutine	*Phellodendron amurense*	N.A.	[53]
Oblongifolin C	*Garcinia yunnanensis* Hu	Lysosome cathepsin↓	[14]
Oleifolioside B	*Dendropanax morbifera* Leveille	Atg3, LC3-II↑; Nrf2↓	[54]
Ophiopogonin B	Radix Ophiopogon Japonicus	pAkt/mTOR/p70S6K↓	[15]
Oyaksungisan (OY)	N.A.	JNK↑	[55]
Penta-1,2,3,4,6-O-galloyl-beta-D-glucose (PGG)	N.A.	Unfolded protein response, MAPK8/9/10↑	[19]
Pheophorbide-a	*Scutellaria barbata*	Erk↓	[56]
Piperlongumine	*Piper longum* L.	ROS, p38 MAPK↑	[57]
Plumbagin	*Plumbago zeylanica*	PI3K/Akt/mTOR↓	[58]
Riccardin D	*Dumortiera hirsuta*	Caspases cleavage↑	[59]
Rottlerin	*Mallotus philippinensis*	N.A.	[60]

Table 1: Continued.

Compound	Plant of origin	Pathway involved	Reference
Saikosaponin-D	N.A.	ER stresses, unfolded protein response, cytosolic calcium, AMPK↑; sarcoplasmic/endoplasmic reticulum Ca(2+) ATPase pump↓	[61]
Samsoeum	N.A.	AMPK, Erk, Beclin-1, LC3-II↑; Akt, mTOR↓	[62]
Solanum nigrum leaves extract	N.A.	N.A.	[63]
Stellettin A	*Geodia japonica*	ER stresses↑	[64]
Tanshinone IIA	Salviae miltiorrhizae	Erk↑	[65]
Tetrandrine	Radix Stephaniae tetrandrae	N.A.	[66]
Timosaponin AIII	*Anemarrhena asphodeloides*	AMPK↑; XIAP, mTOR↓	[67]
Triterpenes	*Ganoderma lucidum*	Beclin-1↑; p38 MAPK↓	[68]
Ursolic acid	*Bupleurum falcatum* L. (Umbelliferae)	JNK↑	[69]
Viriditoxin	Jellyfish *Nemopilema nomurai*	LC3-II, Atg5, Atg7, Beclin-1↑	[70]
Vitexin 6	*Byrsonima crassifolia*	N.A.	[71]
Voacamine	*Peschiera fuchsiaefolia*	N.A.	[72]
Weikang Keli	Root of *Codonopsis pilosula*, Rhizoma Atractylodis Macrocephalae, Rhizoma Curcumae Aeruginosae, Rhizoma Pinelliae, *Actinidia chinensis* Planch, and *Rhodiola rosea*	N.A.	[73]

Table 2: Natural compounds that induce cytoprotective autophagy.

Compound	Plant of origin	Pathways involved	Reference
α-Eleostearic acid	*Momordica charantia*	Akt↓, Erk1/2↑	[74]
Anthricin	*Anthriscus sylvestris* (L.) Hoffm.	Akt/mTOR↓	[75]
Arenobufagin	Toad venom	PI3K/Akt/mTOR↓	[76]
β-Elemene	Zedoary	Atg5↑, Akt/mTOR↓, Erk1/2↓	[77–79]
Bufalin	Toad venom	LC3-II↑, Beclin-1↑, Atg7↑, Atg12↑, AMPK↑, mTOR↓	[80, 81]
Crotoxin	South American rattlesnake	Not applicable (N.A.)	[33]
Cucurbitacin I	*Cucumis sativus* L.	Beclin-1↑, Beclin-1/Bcl-2 interaction, HIF-1α↓	[82]
Dioscin	Soybean	N.A.	[83]
Englerin A	*Phyllanthus engleri*	Akt↓, Erk↓, AMPK↑	[84]
Gossypol	Cotton seeds	Interaction between Bcl-2 and Beclin-1↓	[85]
Isobavachalcone	*Fructus psoraleae*	N.A.	[86]
Mollugin	*Rubia cordifolia* L.	Erk↑ PI3K/Akt/mTOR↓	[87]
Parthenolide	Feverfew	AMPK↑	[88]
Physalin A	*Physalis alkekengi* L. var. *franchetii* (Mast.) Makino	Beclin-1↑	[89]
Resveratrol	Japanese knotweed	Erk1/2↑, p38 MAPK↑, Atg5↑, Beclin-1↑, LC3-II↑, Akt/mTOR↓,	[90–92]
Sesbania grandiflora flowers	N.A.	N.A.	[93]
Wogonin	*Scutellaria baicalensis*	mTOR↓, Raf/Erk1/2↓	[94]
Zearalenone	*Fusarium graminearum*	LC3-II, Beclin-1	[95]

2.1.2. Autophagy as an Exclusive Mechanism of Cell Death. Although many studies have reported that autophagy may be initiated for killing the cells, there is lack of evidence that can be markers and mechanisms of presence of autophagic cell death (ACD). The existence of ACD is therefore under constant criticism, as autophagy is generally observed in dying cells. In this case, autophagy in dying cells may be considered as an automatically raised mechanism for cell survival, even though it is not effective enough to overcome the cellular stress-induced death [5]. Hau et al. reported that coibamide A, a compound isolated from marine Cynabacterium could initiate both apoptosis and autophagy while autophagy may not be essential for cell death or survival, as initiation of autophagy likely occurred in dying cells in response to coibamide A treatment [32]. However, existence of ACD cannot be simply ruled out as many studies have shown that natural products could lead to cell death exclusively with presence of autophagy. Aoki et al. showed that curcumin could initiate autophagy in cancer cells while no apoptosis could be detected, and induction of autophagy is associated with cell death [34]. However, it is contradicting to observe that suppression of autophagy in curcumin-treated cell initiated apoptosis and enhanced cytotoxicity. Autophagy is therefore likely to be a combined mechanism of both cell death and survival. In another study, Meschini and colleagues found that voacamine from *Peschiera fuchsiaefolia* induced tumor cell death dependent on autophagy but not apoptosis [72]. Similar observation was got in other natural products-treated cancer cells including bufalin [28] and triterpenes [68]. The criteria to rule out the presence of apoptosis are pivotal for identifying autophagy as an exclusive mechanism of inducing cell death. Liu and colleagues reported that stellettin A from *Geodia japonica* could induce autophagy as they could observe neither the altered expression of apoptosis marker Bcl-2 nor the appearance of apoptotic nuclei [64]. However, it is not sufficient to conclude with such simple observation on Bcl-2 expression as apoptosis can be initiated by many other pathways. Study from our group introduced a variety of markers to exclude the presence of apoptosis in fangchinoline-treated hepatocellular carcinoma cells, including presentation of Annexin V on dying cells, fragmentation of cell DNA, and cleavage of caspases [40]. Further evidence of nonapoptotic death was obtained to observe that fangchinoline induced cell death was not attenuated by caspase inhibitor. Xie et al. reported in similar way that bufalin-induced colon cancer cell death is independent of caspase activation [27]. As caspases are much exclusively necessary to apoptotic cell death, natural product may have differential mechanisms in inducing cell death in caspase-3-proficient and caspase-3-deficient MCF-7 cells. Rottlerin could induce both apoptosis and autophagy in caspase-3-proficient cells, while in caspase-3-deficient cells, it can only initiate autophagy that was associated with cell death [60]. Furthermore, the exclusiveness of autophagy as mechanism of cell death may be determined in the presence of caspase inhibitor. Wang et al. measured autophagy induced by piperlongumine from *Piper longum* L. in the presence of caspase and necrosis inhibitors. It was found that cell death was attenuated when autophagy inhibitor was added to piperlongumine-treated cancer cells [57]. Observation of this study may confirm that cell death caused by piperlongumine resulted from autophagy induction. Nonetheless, in some cases, apoptosis still could be activated to induce caspases independently [96]. To gain more reliable conclusion, Wong et al. examined autophagic cell death induced by natural compound saikosaponin-D in apoptosis-defective cells [61]. The use of apoptosis-defective cells might be quite self-explanatory but authentication and identification of the cell lines are critical for data interpretation.

2.1.3. Autophagy That Plays a Supportive Role in Apoptosis. As autophagy is usually observed in dying cells, argument was raised to criticize the role of autophagy in promoting cell death, which claimed that autophagy might be an accompanying event that occurs after initiation of apoptosis. Despite the fact that the argument appears to be reasonable since autophagy could be regarded as a mechanism to scavenge dysfunctional organelles and proteins during apoptosis, some studies indeed observed that autophagy may contribute to supporting apoptosis. It was massively considered that autophagy may play a supportive role in inducing cell death though Xavier and colleagues found that, in ursolic acid-induced cell death, apoptosis only contributes to a small proportion while autophagic cell death may be the mass [69]. Saiki et al. showed that, in autophagy-deficient cells, induction of apoptosis by caffeine was attenuated [29]. In most literature apoptosis was found to take the leading role. Xiao et al. found that natural compound curcumin could induce both apoptosis and autophagy, while inhibition of autophagy with small molecule inhibitor 3-MA results in reduced cell death [38]. As a simply obtained autophagy inhibitor, 3-MA was widely used in the studies which would like to distinguish autophagy cell death from apoptosis. Natural compound riccardin D was reported to cause cancer cell death with both apoptosis and autophagy, which could be reduced by presence of 3-MA [97]. A herbal formula Oyaksungisan was found to initiate autophagic cell death while cotreatment of 3-MA rescued the tumor cell [55]. Although these studies may preliminarily try to distinguish cell death induced by autophagy from apoptosis, the use of 3-MA is under criticism as it was found that 3-MA could also block apoptosis. This may lead to overestimation on proportion of cell death induced by autophagy. Another autophagy inhibitor bafilomycin A1 was introduced in some other studies to gain more accurate results. Bafilomycin A1 retards the fusing of autophagosome and lysosome and as a result abrogates autophagy process. Jia et al. used bafilomycin A1 to inhibit autophagy induced by curcumin in leukemia cells and showed that cell death was attenuated in the presence of bafilomycin A1. The results are more compelling but still cannot rule out any nonspecific action of bafilomycin A1, which survives the cells. A better understanding may be obtained if genetic suppression of autophagy-related proteins such as Atg5 could be introduced. Qiu et al. inhibit autophagy both pharmacologically and genetically to show that autophagy contributed to tetrandrine-induced cell death in human cancer [66]. Berberine was shown to induce both autophagy and apoptosis in hepatoma [26], and

study from our laboratory measured the proportion that autophagy contributes to cell death induced by berberine. By introducing 3-MA and RNA interference against Atg5, we found that autophagy may contribute to about 10% of the total cell death in berberine's action [25]. This solution seems much reliable, despite the fact that some recent studies also found an autophagy-independent function of Atg5 in triggering death of cell [98]. Knockdown of one more essential protein in autophagy pathway may possibly improve accuracy in technical way. Solution may be also available to examine the time course of autophagy in dying cells. Evodiamine from *Evodia rutaecarpa* could trigger cell death with both autophagy and apoptosis, while the presence of 3-MA reduced the number of dead cells [39]. The induction of apoptosis and autophagy looks parallel in time course, which indicates that autophagy might not just serve as a scavenger after apoptotic cell death is initiated by evodiamine.

The other way by which autophagy may support apoptosis in dying cells with apoptosis is much interesting. Xu et al. examined the relationship between autophagy and apoptosis induced by *Akebia* saponins PA from *Dipsacus asperoides*. When AGS cells were cotreated with autophagy inhibitor bafilomycin A1, the *Akebia* saponins A1-triggered caspase-3-dependent apoptosis was decreased; however, autophagy was not affected in cells with caspase-3 inhibitor [23]. Induction of apoptosis in this case was possibly autophagy-dependent. Similar observation was obtained in bufalin-treated liver cancer cells [99]. Since apoptosis is regarded to be critically controlled but autophagy is generally much more robust process, it is not likely that less fine-tuned autophagy could particularly target proteins that tightly regulate apoptosis pathways. However, recent studies have revealed autophagy may mediate degradation of some particular proteins. This kind of autophagy requires binding of chaperons to help recognition of autophagosome to the targeted protein and therefore was given a term as chaperon-mediated autophagy (CMA) [100]. The mechanism of natural products-induced CMA is not fully addressed; however, study from our laboratory showed that, in timosaponin AIII-induced apoptosis, autophagy may be essential to cause degradation of intracellular inhibitor of apoptosis, XIAP protein. Suppression of XIAP was sufficient to trigger apoptosis in hepatocellular carcinoma. And while autophagy was reduced, apoptosis could not be programmed and timosaponin AIII-treated cells would undergo necrosis instead [67].

2.2. A Protective Role of Natural Products-Induced Autophagy on Cancer Cells. The protective effect of autophagy in natural products-induced cancer cell death could be much more straightforward as the process was naturally regarded as cellular stress clearance. Table 2 listed out compounds that have been reported to initiate a protective autophagy in cancer cells, among which resveratrol is most frequently studied. Resveratrol was found to trigger cytoprotective autophagy in both glioma and melanoma cells [90–92]. Retrieved data reveal that cytoprotective autophagy initiated by natural products was always present with apoptosis. This is not surprising since apoptosis and autophagy may be regulated by some common proteins and signaling. The antiapoptotic Bcl-2 protein is the typical one whose expression may be reduced to trigger apoptosis. Moreover, absence of Bcl-2 could release Beclin-1 to initiate autophagosome formation and as a result both apoptosis and autophagy are induced [101]. The herbal extract Samsoeum (SSE) was shown to trigger both apoptosis and autophagy in cancer cells with reduced expression of Bcl-2 while with an increase in Beclin-1 [62]. Similar observation was obtained in cells treated with methanolic fraction of bitter melon extract [102]. The Akt/mTOR pathway, which is aberrantly activated in cancer, may be another molecular mechanism of crosstalk interconnecting apoptosis and autophagy [103]. While inhibition of Akt triggers cell apoptosis and reduces survival, the subsequent mTOR suppression could initiate autophagy in cells [104]. In this case, natural inhibitors of Akt pathway may be able to induce both apoptosis and autophagy in one cell (Table 2). Data interpretation is much easier as it is not quite difficult to identify the protective role of autophagy technically; however, quantitative measurement on the protection is still problematic, since till now no inhibitor could specifically and efficiently block autophagy in cells. This may lead to either overestimating or underestimating the action of cytoprotective autophagy.

3. Discussion

3.1. Monitoring a Real Autophagy: A Technical Issue. As a dynamic intracellular process, autophagy is composed of an onset activity, during which autophagosome is formed and fused with lysosome, and an offset activity that facilitates degradation of autophagosome-lysosome complex (also called autolysosome) [105]. Although some major events could be monitored as biomarkers of autophagy, it is far from being accurate to draw any conclusion with only one or two events observed. We are quite sure that the criteria are improving with time; as a result, conclusion from some early literature reporting natural products-induced autophagy in cancer is thought to be too hasty as only least markers were monitored. For example, it was found that flavokawain B from Alpha pricei Hayata could increase LC3-EGFP expression in HCT116 cells [41], but it could possibly happen when lysosome function was simply blocked out, which resulted in increase of both cellular and membrane forms of LC3. This was quite often observed in some early studies of other natural products including honokiol from *Magnolia officinalis* [44], celastrol [30], and tanshinone IIA [65]. As onset of intracellular acidic compartments is a marker of autophagy, Rasul and colleagues monitored magnolol-induced autophagy by staining the acidic vesicular organelle with acridine orange [49]. While increase of acidic particles is usually observed during induction of autophagy, the lysosome protease activity may be regulated independently to autophagy process. In this case, it is still preliminary to draw a conclusion that it was a real autophagy induced in magnolol-treated cells. In some cases, it was found that natural products may initiate the onset of autophagy but block the offset process. Rasul et al. mentioned that matrine may trigger conversion of LC3 to form autophagosome; however, the degradation of autophagosome was impaired as protease activity of lysosome was blocked

due to elevated lysosomal pH values in matrine-treated cells [49]. Therefore, conclusion could not be simply drawn by only determining the conversion of LC3. Markers that monitor the offset of autophagy, such as degradation of p62/SQSTM1, should be employed in parallel. The turnover of p62/SQSTM1 is massively operated by lysosome during autophagy; as a result, reduced expression of p62/SQSTM1 may be monitored as a marker of autolysosome degradation [106]. It was found that oblongifolin C from *Garcinia yunnanensis* Hu could induce the number of autophagosomes; however, blockade of lysosome function was found and p62/SQSTM1 turnover was impaired. In this case, whether oblongifolin C could initiate autophagy remains uncertain and requires further investigation [14]. Mei et al. considered autophagy in such a case was aberrant, as study from the research group showed that gambogenic acid could induce accumulation of autophagosome but disrupt p62/SQSTM1 degradation [43]. In addition, natural products-stimulated autophagy should be blocked when pharmacological or genetic inhibitors are present. Practice was made by Lin et al. to monitor baicalin-induced autophagy in bladder cancer cells. Besides autophagy markers were monitored, and cotreatment of 3-MA reduced autophagy initiation by baicalin [24]. Presence of 3-MA helps much in identification of autophagy triggered by natural products, though it could be observed that long-term treatment of 3-MA with natural products such as celstrol controversially increased formation of autophagosome [31]. Genetic inhibition using RNA interference against genes essential for autophagy induction, like Atg5, Atg7, and Atg12, may be required in parallel to confirm a real autophagy is induced. Guidelines for autophagy monitoring and data interpretation are available, which provide comprehensive and restricted methods in evaluating autophagy [107]. It is quite good if all the studies on natural products could strictly follow the guidelines, but the least requirements in identifying autophagy triggered by natural products could be the following: (i) formation of autophagosome: this includes conversion of LC3 from cytoplasmic form to membrane form and increase of autophagic flux; (ii) degradation of autolysosome: it could be illustrated as elevated acidic compartments and turnover of p62/SQSTM1; (iii) induction of autophagy by natural products could be blocked by the presence of pharmacological inhibitors or RNA interference.

3.2. Does Origin of Cancer Cells Matter? As a cellular response mechanism to internal and external stress, autophagy is regarded as robust but highly conserved across different types of cells including tumor cells [108]. The regulation of natural products on autophagy in different cancer cell lines originated from various tumor tissues should be consistent in this regard. Curcumin was reported to initiate autophagy in tumor cells from a variety of cancers including glioma [34], myeloid leukemia [35], glioblastoma [36], uterine leiomyosarcoma [37], and lung carcinoma [38]. It was observed that curcumin could also induce autophagy in colon cancer stem cells, indicating curcumin-provoked autophagy is highly consistent and conserved in nondifferentiated cells as well as well differentiated tumor cells [109]. However, the role of autophagy in determining fate of cancer cells treated with natural products may vary across different types of tumor. It was noticed that bufalin from toad venom is a potent stimulator of autophagy in various types of cancers; nonetheless, bufalin-induced autophagy may play opposite roles in regulating cell death according to results reported by different research groups. It may be due to the technical variations; however, we cannot rule out the influence from genetic variations across different tumor cells, especially when apoptosis is stimulated along with autophagy in natural products-treated cells. Compared with autophagy, apoptosis is under quite restricted control that involves a series of molecules with proapoptotic or antiapoptotic functions. These proteins may be aberrantly expressed due to mutation on cell genome, which results in acquired resistance against acquired apoptosis in cancer cells [110]. Variation of genetic mutations across cancers with different origins leads to various response to natural stimulators of apoptosis. In this case, the role of autophagy in determining cell fate may differ accordingly. The way of apoptosis resistance in influence of the outcome of autophagy may be too complicated to well illustrate currently; however, it was found that, in extreme condition when apoptosis is totally defective, natural products-induced autophagy is mostly contributing to death of tumor cells. It was also noticed that, in noncancerous cells that are sensitive to apoptosis stimulators, acquired autophagy always plays a cytoprotective role to prevent cell death. Taken together, it may indicate the pivot of switch between cytoprotective and cytotoxic role of autophagy may locate at the center of the scale of resistance against apoptosis.

3.3. Is Focus on Signaling Pathways That Important? Retrieved studies have revealed that a single natural product may regulate multiple signaling pathways in cancer cells that could mediate onset of autophagy. Expression of molecules that are directly involved in autophagosome formation, such as Beclin-1, Atg5, Atg7, Atg12, and LC3, was altered upon exposure of the compounds as reported by some studies; however, literature majorly focused on signal transduction through various pathways of kinase like Akt/mTOR, Erk1/2, p38 MAPK, AMPK, and JNK. It was shown that some of these pathways might be responsible for the induction of autophagy. Kim et al. found that blockade of JNK pathway by specific inhibitor SP600125, Samsoeum (SEE), extract-triggered autophagy could be attenuated via suppression on expression of autophagy-related proteins Beclin-1 and LC3 [62]. Nonetheless, in most of studies, it is still difficult to clarify if the pathways are actually responsible for autophagy initiation or just a bypass mechanism that occurs in parallel with autophagy. A herbal extract named KIOM-C was found to activate JNK, which seemed to play a role in generating cellular oxidative and ER stresses in KIOM-C-treated cells [47]. Autophagy was observed in KIOM-C-treated cells; however, it was likely that the initiation of autophagic flux was a spontaneous response to oxidative and ER stress in cancer cells, as there was no evidence to show that autophagy was involved in KIOM-C-induced cell death. Autophagy might be the consequence of stress and was independent of the action of KIOM-C. Neoalbaconol from *Albatrellus confluens* could block the consumption of glucose and ATP generation

of cancer cells, as reported by Deng and colleagues, and was able to initiate autophagy. Autophagy is a spontaneous response to energy deprivation in cancer cells [52]. Therefore, signaling transduction induced by natural products is not likely sufficient for autophagy induction, while in cells where autophagy is not responsible for cell death or survival upon natural compounds treatment, autophagy may be regarded as a consequence of changes on other cellular activities induced by natural product treatment [32].

However, study on signaling pathway involved in natural product-induced autophagy may still have significance in elaborating the action of mechanism. For instance, it was found, in different studies, bufalin from Chan-su exhibits both tumor suppressive and cytoprotective autophagy in human cancer cells. The controversial actions of bufalin may be due to the differential effect in inducing changes of signaling pathways. When bufalin was given at lower dose, it may cause endoplasmic reticulum stress, which would initiate autophagy to dispose misfolded proteins, and bufalin-induced autophagy at this dose may elicit protective role to prevent cancer cells from ER stress-induced apoptosis [28, 111]; while the dose goes higher, autophagy may be excessively induced, and cancer cell would undergo autophagic cell death upon treatment of higher dose of bufalin [27, 80]. The differential activation of ER stress-related signaling may mediate switch of the role of autophagy from cytoprotection to cytotoxicity and PERK/eIF2α/CHOP was responsible for the interplay between apoptosis and autophagy in bufalin-treated cancer cells [81]. Similar conclusion could be obtained from studies on resveratrol-induced autophagy in cancer cells. Collectively, study on signaling pathway fosters great significance in elaborating the role and action of autophagy. However, as the technical inconsistence was wildly observed, conclusion derived from these investigations may not be convincing enough to illustrate the exact relationship between action of autophagy and the involved signaling pathway. More comprehensive and consistent studies are therefore expected.

3.4. Cytotoxic or Cytoprotective? The Cellular Mechanism. Autophagy could be either tumor promoting or tumor suppressive. The tumor suppressive effect may be much more clear, as autophagy may facilitate cell cycle arrest and apoptosis or directly induce cell death. The cellular events in cytoprotective action, in which autophagy helps cancer cell to overcome various cellular stresses, are not clearly elucidated in most of the publications. Recycling of nonessential proteins and organelles to overcome the lack of substrates of metabolism that are required for survival of cancer cells under nutrient deprivation may be possibly one of the major cellular events that fosters the cytoprotective action of autophagy. In some of the studies, researchers observed natural products could suppress mTOR pathway, which was considered as a major mechanism in inducing autophagy. In fact, mTOR signaling mediated translational control of protein synthesis and high activity of mTOR signaling in cancer cells was found in different types of cancer cells. mTOR inhibition is in this case not just the way mediating activation of autophagy but an indicator that cancer cell is trying to overcome nutrient deprivation by restricting nascent protein synthesis. Autophagy induction may subsequently contribute to overcoming nutrient deprivation by future recycling the existing proteins. However, due to technical limit in most of the published studies, direct evidence of natural product-induced autophagy in facilitating recycling of cellular substrates in nutrient-depriving cancer cells is not yet available. Moreover, lack of nutrient and oxygen supply, as well as cytotoxic agent treatment, may lead to damage of proteins and organelles in cancer cells, which brings stress to endoplasmic reticulum and mitochondria. The malfunction of endoplasmic reticulum and mitochondria results in release of proapoptotic factors and causes subsequent cancer cell death. The induction of autophagy by natural products may collectively dispose the damaged proteins and organelles and therefore normalize endoplasmic reticulum and mitochondria function. It was recently shown that blockade of autophagy in bufalin-treated HCC cell could potentiate apoptosis, indicating that bufalin-induced autophagy may be involved in normalizing endoplasmic reticulum [111]. Unfortunately, direct evidence in describing the role of natural product-induced autophagy to stabilize endoplasmic reticulum and mitochondria in cancer cells is not yet available in most of published studies. It is expected in future studies researchers can seek to explore the cellular events occurring in natural product-induced autophagy, which makes elucidation on action of autophagy in a more mechanistic way.

3.5. Crosstalk with Apoptosis by Natural Product-Induced Autophagy. As a homeostatic process that responds to cellular stress, autophagy is essential in preventing stress-induced DNA damage and genomic instability [112]. Despite the fact that some previous studies have shown that disruption of autophagy may lead to tumorigenesis, the induction of autophagy in cancer cell may play more complicated roles. While autophagy may counteract the natural product-induced cell death by removing misfolded proteins and damaged organelle to attenuate cellular stress [113], it was also noticed that autophagy initiation may contribute to apoptosis induced by natural products, as evidenced by reduced antitumor action of the compounds when autophagy was blocked. In this case, natural product-induced autophagy is considered a double-edged sword in determining cell fate of human cancers, and interplay between autophagy and apoptosis is recently highlighted in this field of study. Several proteins and signaling pathway may act as scaffold in mediating the crosstalk of autophagy and apoptosis, including p53, Bcl-2 family, DAPK, and JNK [114]. It was particularly noticed that Bcl-2 protein is extensively studied in natural product-induced autophagy and apoptosis. Bcl-2 may interact with both autophagy inducer Beclin-1 and proapoptotic factor Bax to block their function. A series of natural products, such as curcumin, berberine, and evodiamine, was shown to block the interaction between Bcl-2 and beclin-1/Bax and therefore results in autophagy and apoptosis initiation. In addition, activation of JNK pathway was widely observed across various studies that focused on anticancer natural products. As a stress responding pathway, JNK could disrupt

the inhibition of Beclin-1 by phosphorylating BIM or Bcl-2. This not only activates beclin-1-dependent autophagy, but also promotes apoptosis by releasing proapoptotic proteins due to the inactivation of Bcl-2. The role of p53 and DAPK in natural product-induced autophagy is not fully revealed, but Wang et al. found that fangchinoline could lead to nuclear translocation of p53, which subsequently activated sestrin2 transcription and initiated autophagic cell death in HCC [40]. These studies revealed that the interplay molecules, which mediate both autophagy and apoptosis activation, may be effective targets in developing cancer killing agents, though the function of autophagy induced still requires critical examination case by case.

4. Direction of Future Study

4.1. In Vivo Monitoring of Autophagy. The property that natural products could suppress cancers in animal models is quite important for evaluating the druggability of the compounds. More and more autophagy-inducing natural compounds exhibit potent inhibition on tumor growth in vivo. However, although the role of autophagy in determining cancer cell fate could be elaborated on in vitro platform, so far no study could provide compelling evidence showing that in vivo tumor inhibition of natural products involves its regulation on autophagy. This may be due to lack of useful in vivo animal models in which autophagy could be selectively monitored. He and Klionsky reported in vivo monitoring of autophagy in a transgenic GFP-LC3 zebrafish line [115]. Induction of autophagy in zebrafish could be viewed by microscopy in real-time scale. As the approach is simple and noninvasive, the zebrafish model may be quite suitable for high throughput screening of natural products that could initiate autophagy in vivo. However, the model is not disease-related in nature. Studies by Tian et al. developed transgenic mice line expressing GFP-LC3, and the role of autophagy in the pathologic progress of neurological diseases could be monitored by live imaging technology [116, 117]. This successfully established GFP-LC3 transgenic mice which may be applied to elaborate the role of autophagy in carcinogenesis; however, monitor of tumor growth over inner organ may not be available without invading operation. Orthotropic implantation of tumor cells expressing firefly luciferase has been established by our colleagues, to provide a real-time, noninvasive monitoring system on in vivo growth of tumor [118]. Based on this animal model, a dual-bioluminescent reporting system could be taken into consideration in which autophagy as well as tumor growth would be monitored. The Renilla luciferase reporter is the alternative to firefly system and is able to yield reliable results. A previous study has constructed a Renilla luciferase vector-expressing rat LC3 (pRL-rLC3) which demonstrated the possibility and reliability of using pRL-rLC3 to monitor autophagy induction in cell model [119]. Quenching of Renilla luciferin signal would be therefore observed if a treatment could induce autophagy. This hypothesized model may be suitable for monitoring the role of autophagy in the suppression of tumor growth by natural products.

4.2. The Synergistic Action of Autophagy-Inducing Natural Products. In recent years, many reviews have summarized the synergistic action of natural product in cancer treatment. Many autophagy-inducing natural products, including berberine, curcumin, and resveratrol, could enhance the therapeutic effect of other agents, and this synergistic effect is correlated with induction of autophagy. Peng et al. found that berberine could enhance tumor-killing action of irradiation by inducing autophagic cell death [120]; curcumin was found to sensitize cancer cells towards treatment of 7-deoxypancratistatin, a novel chemotherapeutic agent [121]; resveratrol-initiated autophagy enhances the cytotoxicity of arsenic oxide on primitive leukemic progenitors, indicating a positive role of autophagy induction in the combination treatment of arsenic oxide and resveratrol against leukemia [122]. The enhancing action of natural products-induced autophagy could also be observed in some herbal extracts including *Koelreuteria henryi* Dummer and *Emblica officinalis* [123, 124]. These studies have shed light on elucidating the mechanistic role of autophagy in mediating the synergistic action of natural products in cancer treatment. In fact, there are still a lot of autophagy-inducing natural products which have been revealed for their capacity of enhancing antitumor action of chemotherapy and radiotherapy; however, the role of autophagy has not yet been fully elucidated. Future study is highly expected to focus on this property of natural products and hopefully the contribution of autophagy in combination therapy could be illustrated in more mechanistic and clinical-relevant approaches.

4.3. The Clinical Significance of Autophagy. The ultimate goal of mentioned studies remains to discover autophagy-inducing natural products with significant clinical efficacy in cancer treatment. However, rare published study has focused on clinical trials of autophagy-inducing natural products. In fact, the clinical role of autophagy in cancer treatment is not yet fully elucidated. However, from the data we collected, it is easy to find some compounds may have been used in some countries as anticancer agents. For example, bufalin injection has been used clinically in some parts of China and has shown some therapeutic action in restricting tumor progression in cancer patients. A phase I pilot study showed that bufalin treatment in HCC patients leads to no significant dose-limiting toxicity and improvement of quality of life [125]. Some natural products may exhibit potential of clinical use; for instance, resveratrol, a natural compound universally present in edible and medical plants, has been considered chemopreventive in some previous studies. Phase I clinical trials have been conducted in healthy volunteers, and it was found that consumption of resveratrol did not cause serious adverse effects [126]. However, resveratrol can regulate carcinogen-metabolizing enzymes in human subjects, which can be the mechanism of its chemopreventive action as well as the cause of potential herbal-drug interaction [127]. Clinical study showed that resveratrol alone or in combination with chemotherapeutic agents has beneficial effect on cancer patients [128, 129]. This observation sheds light on the clinical use of resveratrol in treating cancer. Unfortunately, no available information has been disclosed

to define the relationship between autophagy and the clinical action of these natural products.

5. Conclusion

As a conclusion, recent studies have remarked that autophagy in human cancer cells could be initiated by natural compounds and extracts isolated from anticancer medical plants. Induction of autophagy by natural products may contribute to its tumor suppressive effect by causing cell senescence, inducing apoptosis-independent death, and provoking apoptotic death. Natural products-induced autophagy can also be cytoprotective and cause resistance of cancer cells against death. Higher technical requirement on monitoring autophagy in natural products-treated cancer cells is required to improve study quality in this field. In vivo action of autophagy in mediating tumor regression may be necessary to explore in the future, and significance of synergistic action and clinical relevancy in future studies should be highlighted. Studies in this field will shed light on the development of lead compounds of anticancer drug from autophagy-inducing natural products.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Gene Network Exploration of Crosstalk between Apoptosis and Autophagy in Chronic Myelogenous Leukemia

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Background. Gene expression levels change to adapt the stress, such as starvation, toxin, and radiation. The changes are signals transmitted through molecular interactions, eventually leading to two cellular fates, apoptosis and autophagy. Due to genetic variations, the signals may not be effectively transmitted to modulate apoptotic and autophagic responses. Such aberrant modulation may lead to carcinogenesis and drug resistance. The balance between apoptosis and autophagy becomes very crucial in coping with the stress. Though there have been evidences illustrating the apoptosis-autophagy interplay, the underlying mechanism and the participation of the regulators including transcription factors (TFs) and microRNAs (miRNAs) remain unclear. *Results*. Gene network is a graphical illustration for exploring the functional linkages and the potential coordinate regulations of genes. Microarray dataset for the study of chronic myeloid leukemia was obtained from Gene Expression Omnibus. The expression profiles of those genes related to apoptosis and autophagy, including MCL1, BCL2, ATG, beclin-1, BAX, BAK, E2F, cMYC, PI3K, AKT, BAD, and LC3, were extracted from the dataset to construct the gene networks. *Conclusion*. The network analysis of these genes explored the underlying mechanisms and the roles of TFs and miRNAs for the crosstalk between apoptosis and autophagy.

1. Introduction

Apoptosis is a kind of programmed cell death, which plays a very important role in maintaining the adult tissue homeostasis and supporting the embryonic tissue remodeling [1]. Besides the proper cell development, external factors, such as nutrient deprivation, toxin, hypoxia, and radiation, trigger the mechanism of apoptosis by inducing cellular stress and subsequent signal transmission through molecular interactions. B-cell CLL/lymphoma 2 (BCL2) homologues have been extensively studied and experimentally validated as the key antiapoptotic and proapoptotic regulators that control the outer membrane permeability or integrity of mitochondria for the release of cytochrome c [2]. Among the antiapoptotic BCL2 homologues, BCL2 and BCL-XL can inhibit the formation of cytochrome c/Apaf-1/caspase-9 apoptosome by binding their unique BH4 domain to the C terminal of apoptotic peptidase activating factor 1 (Apaf-1) [3, 4]. The myeloid cell leukemia 1 (MCL1) is another anti-apoptotic BCL2 homologue whose degradation in response to the stress through translation inhibition enhances the activation of apoptosis [2]. However, the whole apoptotic process cannot be controlled tightly by the high responsiveness of MCL1 without the commitment of the downstream proapoptotic regulators, such as BCL2-associated X protein (BAX) and BCL2-antagonist/killer (BAK). As proapoptotic BCL2 homologues, BAX and BAK form homooligomers within the mitochondrial membrane and breach its integrity, activating the caspases and apoptosis. These negative and positive regulations of apoptosis stop the division of damaged cells selectively and control a viable cell number to reduce the burden of nutritional supply.

Autophagy is a catabolic process responding to the stress induced by the above-mentioned external factors. Different from apoptosis, autophagy helps the cells to survive and maintain their functions by eliminating the damaged organelles and recycling the obsolete cytosol. These damaged or obsolete materials are contained by autophagosome and then fuse with a lysosome for bulk degradation. The autophagosome is double-membrane vesicle regulated by a set of autophagy-related (ATG) genes and nucleated by a protein complex of beclin-1 and phosphatidylinositol 3-kinase (PI3K) [5]. BCL2, as mentioned above, an antiapoptotic regulator, can also inhibit autophagy by binding to beclin-1 at the endoplasmic reticulum and its dissociation with beclin-1 is required for inducing autophagy [6]. In the same family, MCL1 regulates autophagy through its degradation under stress and interaction with beclin-1 on mitochondria [2]. However, the degradation of MCL1 or the inhibition of BCL2 is not decisive to activate autophagy without the fusion between autophagosomes and lysosomes regulated by lysosomal inhibitors and dissociation with ATG proteins [5, 6]. Thus, the interactions between the upstream and downstream molecules, such as beclin-1 and ATG, are also critical for the activation of autophagy.

The relationship between apoptosis and autophagy depends on the cellular context. As the mechanisms of apoptotic and autophagic responses share common pathways but mutually inhibit each other, the cells may adapt to the stress with a combination of these responses or in a mutually exclusive manner. The apoptotic response can be postponed or transformed to the autophagic one when the essential apoptotic proteins, such as BAX and BAK, are removed or inhibited [5]. Also, the long-lived differentiated nerve cells are more susceptible to autophagy than apoptosis to maintain homeostasis under stress [2]. Conversely, the inhibition of autophagy by the deletion of beclin-1 drives the cells towards apoptosis. The cells undergo apoptosis when beclin-1, ATG, or PI3K is inactivated to block the autophagy at an early stage, or when the lysosomal protein LAMP2 is depleted to block the fusion of autophagosomes and lysosomes at the late stage [5]. These evidences support the polarization between apoptosis and autophagy. On the other hand, apoptosis and autophagy share the common inducers, which are BCL2 homology-3 (BH3) only proteins, and the common stress mediators, including reactive oxygen species (ROS), free Ca^{2+} ions, and ceramide, as well as transcription factor p53, in their pathways. Thus, the concurrent triggers of both processes are allowed [5]. Therefore, the cell survival and death have to be balanced to maintain the normal cell functions and suppress carcinogenesis. The participation of transcription factors (TFs) and microRNAs (miRNAs) is crucial to tune the interplay by imposing changes in the expression of genes related to apoptosis and autophagy.

Transcriptional regulation is a kind of molecular interactions where the TF coded by a gene binds to a specific site in the $5'$ untranslated region (UTR) of the target gene to regulate its expression [7]. As the change in the expression of a TF could be relayed to its target gene through such protein-DNA binding, transcriptional regulation may account for the coexpression of TF and target gene [8, 9]. By the same principle, a gene pair may exhibit correlated expression profiles when these two genes are concurrently regulated by a common TF [8]. The translation process of a gene is regulated by miRNAs, noncoding transcripts of approximately 21 nucleotides long. Through the imperfect base pairing with a binding site in the $3'$ UTR of mRNA, miRNA regulates the expression of the target gene or destabilizes its mRNA [10]. It was shown that most of the miRNA-mRNA pairs exhibit highly correlated expression profiles, though both negative and positive correlations [11]. It is straightforward to anticipate the coexpression of two target genes, which are concurrently regulated by a common miRNA.

In a bioinformatics study, the gene-gene interactions controlling the human T helper cell differentiation process were identified by coexpression network but many of which would not be detected using differential expression [12]. Coexpressed genes tend to participate in the same regulatory and signaling circuits, forming complexes, pathways, and network modules [9, 13–16]. Further, strong coexpression was proved to cohere with higher gene ontology (GO) similarity and protein-protein interaction than that of random gene pairs [12].

This study adopted a gene network analysis approach based on coexpression measure. Correlation coefficient is a scale-invariant statistic that can be applied to measure the gene coexpression [17]. Two genes are linked if their correlation exceeds a specific threshold. Some existing approaches attempted to optimize the threshold with respect to the statistical significance of correlation or the network complexity, but not to the overall coexpression profiles of the disease and the normal states [12, 18]. Underlying mechanisms of gene interaction can be deciphered by contrasting the coexpression networks of the disease and the normal groups.

Chronic myelogenous leukemia (CML) is considered as the disease of interest for gene network analysis. The disrupted and invoked gene connections in CML represent the impaired mechanism when compared with the healthy individuals. In CML, a number of mitogenic signaling pathways, such as mitogen-activated protein kinase (MAPK) pathway and janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, are activated so that the pluripotent hematopoietic stem cells aberrantly proliferate and differentiate to granulocytes in the blood [19]. The reciprocal translocation between chromosomes 9 and 22 results in the oncoprotein expressed from the BCR-ABL fusion oncogene and triggers the mitogenic signaling pathways. Apoptosis and adhesion properties of hematopoietic progenitors are deregulated by BCR-ABL, leading to massive leaving of immature progenitors from bone marrows [23]. The participation of autophagy and the transcriptional and posttranscriptional regulations in the molecular mechanism altering the progenitor cell functions is still unclear. This study is aimed to compare the CML patients with the healthy individuals in terms of the balance control between apoptosis and autophagy and identify the roles of TFs and miRNAs in the control mechanism. The gene networks provide global interactomic information to facilitate deeper understanding of carcinogenesis and identification of efficacious drug targets.

Table 1: List of selected genes, relevance, and supporting references.

HUGO gene symbol	Relevance	References
MCL1; BCL2; BAD	Apoptosis; autophagy; CML	[2, 19, 20]
BAX; BAK1	Apoptosis	[1–3, 6]
E2F1; E2F2; E2F3; MYC	Apoptosis; autophagy; cell cycle; CML	[3, 6, 19, 21, 22]
PIK3R2; PIK3R3; PIK3R5; AKT1; AKT2; AKT3	Apoptosis; cell cycle; CML	[19, 23–25]
ATG5; ATG7; ATG12; MAP1LC3B; BECN1	Autophagy	[2, 5, 6, 26]

2. Methods

2.1. CML Dataset. The microarray dataset, analyzed in this work, was provided by a study comparing the normal and the CML hematopoietic stem/progenitor cells in gene expression [27]. The study recruited eight (Philadelphia) Ph+ CML patients and collected their peripheral blood. The bone marrows of four healthy donors were purchased from private sectors. The CD34+ cells were selected and sorted to G0 and G1/S/G2/M fractions. There were eventually 24 samples (16 CML and 8 normal) after the sorting. Total RNA was isolated from the cells of each sample, labeled, and hybridized to Human Genome U133 plus 2.0 arrays. The dataset has been deposited on the Gene Expression Omnibus (GEO) under the Accession number GSE24739 for public access.

Based on the relevance to apoptosis, autophagy, cell cycle, and CML, twenty genes were selected for the coexpression network analysis. Table 1 shows the human genome organization (HUGO) gene symbols of the selected genes and the corresponding references supporting the relevance. This work considered a small portion of the related genes because it is aimed to differentiate the disease from the normal patterns of functional linkages between the key mediators and markers in molecular level.

2.2. Coexpression Measure. Coexpression between two genes can be quantified by a measure evaluating how similar their expression patterns are across the biological samples. The scale invariant property of Pearson correlation coefficient makes it a suitable choice for measuring the similarity between the expression patterns [13, 17]. Let x_i and x_j and $\mathrm{cor}(x_i, x_j)$ be the expression profiles of the ith and jth genes extracted from the expression matrix and the Pearson correlation coefficient between them. The coexpression level, $C(i, j)$, was defined as in the formula (1) [17]:

$$C(i, j) = \left|\mathrm{cor}\left(x_i, x_j\right)\right|. \tag{1}$$

The absolute value was taken because the coexpression measure will output a scalar in the range from 0 to 1 where a high output indicated a strong biological relationship in either positive or negative direction and a low output indicated a weak biological relationship. Such implementation ensured that the inhibiting molecular interactions, such as degradation of MCL1 by beta-TrCP [28], can be detected using this measure. The coexpression level was denoted by $C_d(i, j)$ if two expression profiles were extracted across samples of the disease (CML) group and $C_n(i, j)$ for the normal group.

2.3. Threshold Selection. In this study, a network presented genes as nodes and connected them with undirected edges if their coexpression levels exceeded a particular threshold value [12, 17]. In order to obtain two gene networks that characterized and differentiated the disease and the normal states, an optimal threshold of coexpression level was identified to classify the gene pairs in the disease and the normal states into strong and weak coexpression classes so that the classes were best associated with the groups. Two-sample Kolmogorov-Smirnov (KS) test was a good choice because it was sensitive to the differences in the distributions of two samples, that is, C_d and C_n in this case, and gave a threshold value, at which the deviation between the cumulative distribution functions of C_d and C_n was the maximum [29]. Let F_d, F_n, and D be the cumulative distribution functions (CDFs) of C_d and C_n and the maximum deviation, respectively. The value of D was given by the following formula (2):

$$D = \max_C \left|F_d(C) - F_n(C)\right|. \tag{2}$$

Note that the inequalities inside the CDFs were inverted because our interest focused on the strong coexpression (Formula (3)). The optimal threshold (C) represented a coexpression level, at which F_d and F_n were extremely deviated. After the optimal threshold was identified, the gene pairs can be bisected into two coexpression classes. Chi-square test was also used to verify the association between the coexpression class and the disease. Consider

$$\begin{aligned} F_d(C) &= \mathrm{Prob}\left(C_d \geq C\right), \\ F_n(C) &= \mathrm{Prob}\left(C_n \geq C\right). \end{aligned} \tag{3}$$

2.4. Gene Network Construction. For clearer illustration of gene network, the identified gene pairs were classified into common, normal-specific, and disease-specific connections. The common connections were defined as the strongly coexpressed pairs shared by both the disease and the normal groups. The disease-specific connections, that is, CML-specific, were the strongly coexpressed pairs in the disease group with the common connections removed. The normal-specific connections were the strongly coexpressed pairs in the normal group with the common connections removed. Each type of connections can form a coexpression network

having a particular biological meaning. The normal-specific connections were the potential molecular interactions maintaining physiological balance in healthy individuals. The disease-specific connections represented the characteristics of the disease.

A coexpression network consisted of genes connected by edges. Pajek was used to analyze and visualize the coexpression networks because it supports the global and local views of networks with various abstraction, visualization, and algorithmic tools [30]. Further, the coexpression levels were transformed and input along with the gene pairs to Pajek so that their values can be reflected by the edge thicknesses in the network. To display edges with thickness from 1 to 6 points, the coexpression levels between the threshold value and the maximum value were linearly transformed to the range from 1 to 6. Thus, the thicker edges could catch more attention in the visualization.

2.5. Identification of Regulatory Signatures. Composite regulatory signature database (CRSD) is a bioinformatic web-based resource, which integrates UniGene, mature miRNAs, putative promoter, TRANSFAC, pathway, GO, miRNA regulatory signature (MRS), and TF regulatory signature (TRS) databases to facilitate the comprehensive analysis of gene regulation networks [31]. MRS is defined as a set of interactions between a miRNA and a group of genes with its putative targets in the $3'$ UTR. TRS is defined as a set of interactions between a TF and a group of genes with its putative binding sites in the $5'$ UTR. Combining MRSs and TRSs of a common group of genes yields the composite regulatory signature (CRS). CRSD was used to query the MRS, TRS, or CRS for the strongly coexpressed gene pairs. The identified signatures can help to explore how the TFs and miRNAs drive the normal-specific, disease-specific, and common gene coexpression patterns.

3. Results

3.1. Thresholds of Coexpression Levels. Among 20 genes considered in this work (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/459840), the coexpression levels of 190 gene pairs were computed for the normal group and the CML group independently. Gene pairs can be dichotomized into strong and weak coexpression classes, which characterized the corresponding groups. The threshold for the dichotomy was determined by two-sample KS test. The CDFs for the normal and the CML groups were numerically evaluated at every possible threshold value from 0 to 1 (Figure 1). It was found that the evaluated cumulative fractions were optimally deviated by D, 0.2789, at the coexpression level C, 0.4233 (optimal threshold). The KS test indicated that the two distributions were significantly different (P value < 0.01 for the statistic $D = 0.2789$). The contingency table of the gene pair counts at the optimal threshold is shown in Table 2. At the optimal threshold, the dichotomy of gene pairs was significantly associated with the disease as the Chi-square statistic was 31.4957 (P value < 0.01). The differential coexpression distribution suggested

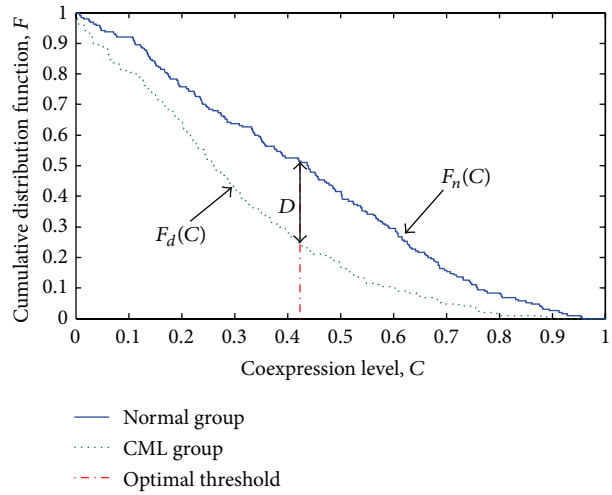

Figure 1: Cumulative distribution functions of coexpression levels for the normal and the CML groups with the candidate thresholds from 0 to 1.

Table 2: Contingency table of gene pair counts at the optimal threshold.

Class	CML	Normal
Strong coexpression	45	98
Weak coexpression	145	92

that the genes related to apoptosis and autophagy, in overall, exhibited more robust functional links in the normal group than the CML group.

3.2. Gene Networks. The CML and the normal groups shared 27 common strongly coexpressed gene pairs according to the optimal threshold. After removing the common gene pairs from the strong coexpression class, the normal-specific gene pairs comprised 71 pairs and the CML-specific comprised 18 pairs. The coexpression networks for the normal-specific, CML-specific, and common gene pairs were constructed as shown in Figure 2.

3.3. Regulatory Signatures. By querying the regulatory signatures in CRSD, the miRNAs and TFs predicted to target the normal-specific and the CML-specific gene pairs were identified. To maintain the significance of the identified regulatory signatures, a miRNA or TF was selected for further investigation if it targeted no less than four gene pairs. It was found that each of hsa-miR-504 and hsa-miR-125a concurrently regulates the expression of four genes in the normal-specific coexpression network, forming two MRSs. As the two MRSs shared two common genes, BAK1 and BCL2, they were combined to form the normal-specific MRS network (Figure 3(a)). It was also found that each of zinc finger protein (AP-4) and E2F concurrently regulates the expression of five genes, and vitamin D (1,25-dihydroxyvitamin D3) receptor (VDR) concurrently regulates the expression of four genes in the normal-specific coexpression network. As the three TRSs

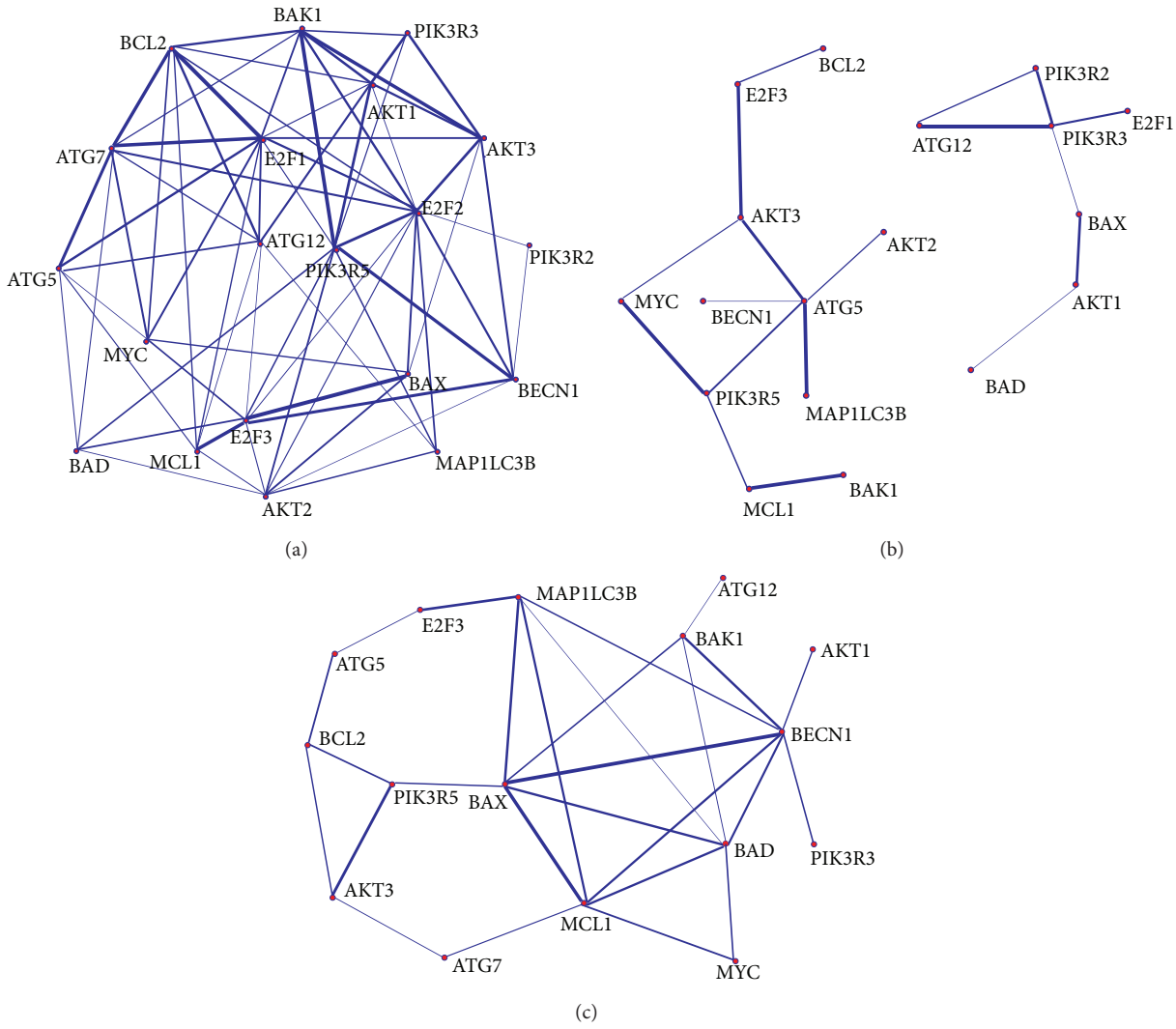

FIGURE 2: Coexpression networks (Using Pajek). The nodes represent genes. The edges indicate the strong correlation between nodes. The edge thickness reflects the coexpression levels. (a) Normal-specific coexpression network. (b) CML-specific coexpression network. (c) Common coexpression network.

shared E2F2 as the common target gene, they were combined to form the normal-specific TRS network (Figure 3(b)). The connections coincided in the normal-specific MRS and TRS networks were the triangle linking E2F2, BAK1, and PIK3R5. These all three connections had strong coexpression levels and formed the normal-specific CRS network with AP-4 and hsa-miR-125a (Figure 3(c)).

In the CML-specific network, each of the identified miRNAs and TFs targets not more than two gene pairs. The MRS and TRS were not considered for further investigation because they were not so informative to suggest the concurrent regulations. Instead, it is interesting to note that E2F3 was linked to v-akt murine thymoma viral oncogene homolog 3 (AKT3) directly and v-myc avian myelocytomatosis viral oncogene homolog (MYC) indirectly, and these three genes are the predicted targets of hsa-miR-15a, hsa-miR-15b, hsa-miR-34c, and hsa-miR-342. Further, E2F3 and BCL2 were found to be strongly coexpressed, which are the predicted targets of E2F1:DP-2 and E2F4:DP-2. Among these genes, E2F3 is predicted to be coordinately regulated by four miRNAs and two TFs. These connections were combined to form the CML-specific E2F3 regulatory signature (E2F3-RS) network (Figure 3(d)).

4. Discussion

4.1. Disease-Associated Coexpression Threshold. Protein encoded by a gene performs its functions through the molecular interactions with that of the other genes. Without considering its functional partners, the expression level and differential expression of a gene are not informative enough to indicate whether it performs its known functions. Coexpression level between two genes quantifies the extent, in which the change in expression level of a gene coincides with that of the other. There may not be a coexpression threshold that can indicate the molecular interactions of two genes, but

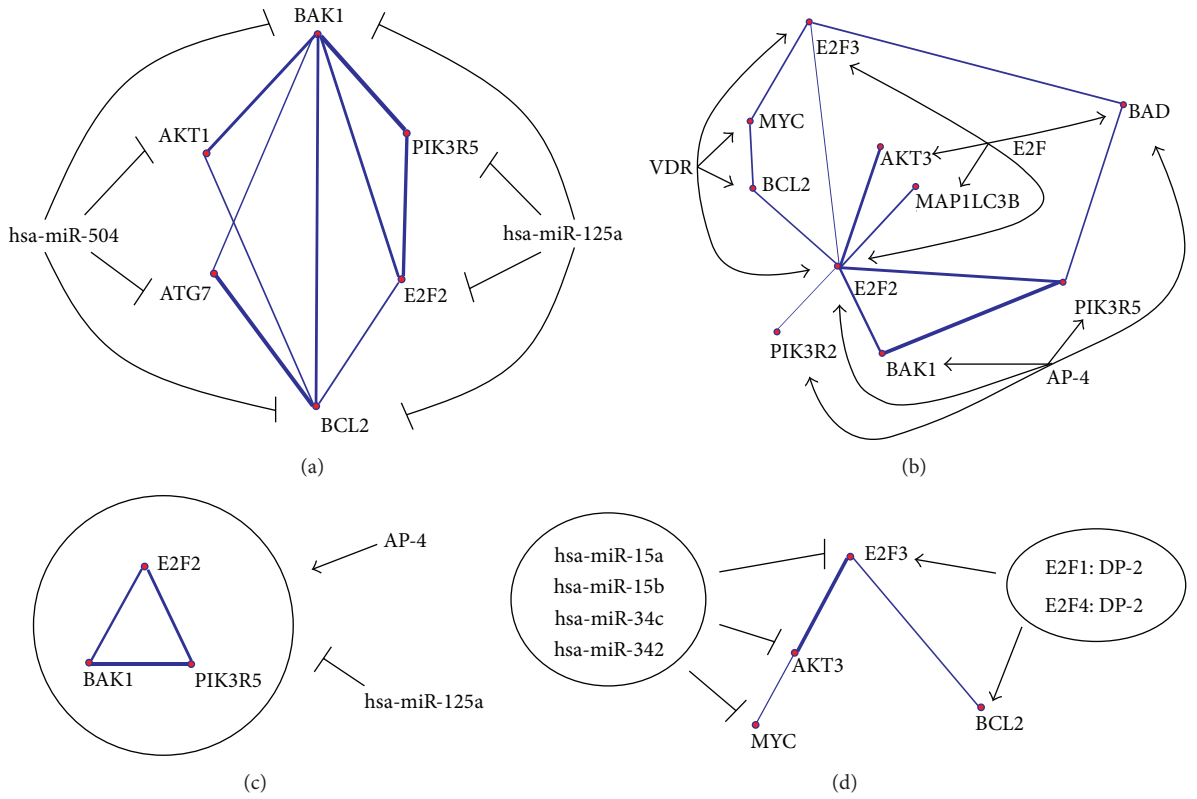

Figure 3: Regulatory signature (RS) networks. (a) Normal-specific MRS network. (b) Normal-specific TRS network. (c) Normal-specific CRS network. (d) CML-specific E2F3-RS network.

a threshold exists for identifying the strongly coexpressed gene pairs to optimally differentiate the normal and the disease groups in terms of the functional linkages. In our study, more strongly coexpressed gene pairs were found in the normal group than the CML group (Table 2). It implied that many functional links between genes, which may react to the external factors to further maintain the proper cellular functions or the tissue homeostasis in the normal group, were impaired in the CML group. The impaired connections may provide useful information for understanding the underlying molecular interaction mechanism and exploring the novel drug targets of CML.

4.2. Functional Coexpression Patterns. Genes highly connected with other genes act as the hubs for relaying the adaptive changes in gene expression through the molecular interactions. In the normal-specific network (Figure 2(a)), E2F1, E2F2, and E2F3 established, respectively, 11, 13, and 9 connections with other genes, implicating their central roles in the proper regulation of apoptosis, autophagy, and cell cycle. E2F1, E2F2, and E2F3 activate the cell cycle progression and drive the cells from quiescent (G0) into synthesis (S) phase [32]. The interplay of E2F1, E2F2, and E2F3 with other genes in the network maintained the balance between cell death, survival, and proliferation. Three remarkable coexpression patterns connected by E2F1, E2F2, and E2F3 were found in the normal-specific network and further discussed as follows.

In the normal-specific network, E2F2 was connected with BAK1, PIK3R5, and AKT3 in the high coexpression levels (correlation coefficients were 0.701, 0.778, and −0.756, resp.). BAK1 is a proapoptotic molecule. The positive correlation implied that when E2F2 was upregulated to speed up the cell cycle for the cellular proliferation, BAK1 may respond with the upregulation to promote apoptosis to control the cell number. We also revealed that PIK3R5 was an essential hub with 10 connections in the network. PIK3R5 is a regulatory subunit, which combines with a catalytic subunit to form the class I PI 3-kinase (PI3K). Since the PI3K pathway contributes to antiapoptosis and cell survival, E2F2 and PIK3R5 may be activated by RAS to promote the proliferation in the normal group [33]. In the common coexpression network (Figure 2(c)), PIK3R5 and AKT3 were strongly coexpressed in both two groups, but the correlation coefficients for the normal and the CML groups were of opposite signs (normal: −0.802; CML: 0.757). It implicated that the activation of AKT3 by PI3K may be retarded by the 3′-phosphoinositide phosphatase (PTEN) in the normal group so that the growth signal could not be relayed to mTOR signaling pathway and thus autophagy was possibly allowed [6, 34]. In contrast, the PI3K/AKT signaling may repress autophagic response in the CML group so that the damaged organelles could not be degraded. The negative correlation between E2F2 and AKT3 implied that the proper autophagy could be maintained during the cell cycle progression in the normal group. From

the above observations, we can hypothesize that E2F2 favors both apoptosis and autophagy.

The normal-specific network showed that E2F1 was connected with ATG5, ATG7, ATG12, BCL2, and MYC in the high coexpression levels (correlation coefficients were −0.708, 0.883, −0.627, 0.954, and 0.642, resp.). ATG5 and ATG12 are involved in the vesicle elongation of the autophagy, while ATG7 helps the covalent conjugation of ATG5 and ATG12 [5]. During the cell proliferation, autophagy may be partially inhibited, as ATG5 and ATG12 are downregulated at the early stage of autophagy, but their covalent conjugation is readily facilitated by the upregulation of ATG7. BCL2 inhibited the apoptosis during cell cycle progression as its expression was positively correlated with E2F1 in the normal group. The positive correlation between E2F1 and MYC is supported by the mutual induction of gene expression [35]. MYC is a proapoptotic molecule. The antiapoptotic and proapoptotic responses of BCL2 and MYC coexisted in the normal-specific network because they can activate different pathways. BCL2 regulates the release of cytochrome c and caspase activation and then inhibits apoptosis [1–4]. MYC triggers the p53 signaling pathway to induce cell death when DNA damage happens [36]. It is hypothesized that E2F1 linking to various genes can promote and inhibit apoptosis through different pathways and partially links to autophagy.

It was shown in the normal-specific network that MCL1, BAX, and beclin-1 (BECN1) were connected with E2F3 in the high coexpression levels (correlation coefficients were −0.876, −0.957, and −0.804, resp.). BAX and BECN1 can promote apoptosis and autophagy, respectively, [1, 3, 5]. The negative correlation implied that both apoptosis and autophagy were inhibited when E2F3 was upregulated during the cell proliferation. MCL1 expression was also negatively correlated with E2F3. The hematopoietic cells may not be so sensitive to the stress as MCL1, a stress sensor, was downregulated during the cell cycle progression. Conversely, these three genes were upregulated to promote autophagy and apoptosis when the cells were situated in the G0 phase. In contrast, MCL1, BAX, and BECN1 were strongly coexpressed with each other without the participation of E2F3 in the common coexpression network (correlation coefficients of BECN1 and MCL1, BAX and MCL1, and BAX and BECN1 were 0.607, 0.831, and 0.895, resp.). The results demonstrated the persistent interplay between apoptosis and autophagy. In all, we can hypothesize that E2F3 opposes against both apoptosis and autophagy.

In the CML-specific network (Figure 2(b)), E2F3 and AKT3 were connected with a positive correlation (0.660). Though E2F3 opposed autophagy again as in the normal group, AKT3 responded to oncogenic or endoplasmic reticulum stress that were different from the stress detected by MCL1 [34].

4.3. Regulatory Mechanisms. The hsa-miR-504 and hsa-miR-125a MRSs shared the BAK1 and BCL2 connection as a common link in the normal-specific MRS network (Figure 3(a)). The balance between the proapoptotic and antiapoptotic properties of BAK1 and BCL2 was supported by their positive correlation (0.696), which may be induced by the coordinate regulation of hsa-miR-504 and hsa-miR-125a.

Three normal-specific TRSs were observed where VDR, E2F, and AP-4 are predicted as the TFs (Figure 3(b)). Again, E2F2 was the hub at the center of the TRSs and concurrently targeted by the three TFs. The E2F TRS concurred with the autoregulatory mechanism of E2F family proteins in cell cycle regulation. It was illustrated that the gene silencing of AP-4 is able to trigger apoptosis [37]. Apoptotic regulatory roles of AP-4 were further justified by the fact that the genes related to apoptosis, BAK1, BAD, PIK3R2, and PIK3R5, are predicted to be its targets.

It was straightforward to observe a motif shared by the MRS and TRS networks, that is, the CRS consisting of E2F2, BAK1, and PIK3R5 (Figure 3(c)). The correlation coefficients between them were positive and high (E2F2 and BAK1: 0.701; E2F2 and PIK3R5: 0.778; BAK1 and PIK3R5: 0.913). Through the coregulation by AP-4 and hsa-miR-125a, these three genes established a tight balance between cell death and survival when the cell proliferation was activated.

In Figure 3(d), E2F3 is predicted as the common target of four miRNAs and two TFs. The TFs and miRNAs were found to be counteracted to control the expression level of E2F3. It was proved that the deletion of miR-15 was frequently found in chronic lymphocytic leukemia (CLL) [38]. It is anticipated that the overexpression of E2F3 caused by the deletion of miR-15 may induce myeloid malignancy. Further, the miRNAs, including miR-15a, downregulated both E2F3 and AKT3 and maintained their strong coexpression in the CML group. These evidences further justify that E2F3-AKT3 connection may be CML-specific.

5. Conclusion

Gene network analysis helps us to explore the gene connectivity and the potential functional linkages. This work adopted an approach for identifying the gene pairs with strong coexpression classified by a disease-associated threshold. CML was the disease of interest in this work. The normal-specific network illustrated the gene connections found in the proper cellular regulation but not in cancer molecular mechanism. As the key transcription factors of cell cycle regulation, E2F1, E2F2, and E2F3, acted as the hubs for the normal-specific connections. E2F1 was associated with antiapoptosis and proapoptosis through different pathways but partially associated with autophagy. E2F2 was linked with the promotion of apoptosis and autophagy, while E2F3 exhibited opposition to apoptosis and autophagy. In the CML-specific network, the link between E2F3 and AKT3 demonstrated a possible cellular response to oncogenic stress in the proliferation of hematopoietic cells. It is important to note that E2F3 and AKT3 are both the predicted targets of miR-15, whose deletion was proved to be associated with cancer. The coregulations of genes by miRNAs and TFs were indicated by the MRS, TRS, and CRS. The central role of E2F2 was further confirmed by the normal-specific TRS network. In the normal-specific MRS network, the apoptotic balance was strengthened by the coregulation of BAK1 and BCL2 by

miRNAs. As a normal-specific CRS, the E2F2-BAK1-PIK3R5 motif may constitute the core mechanism controlling the cell cycle progression, apoptosis, and autophagy, which requires further investigation in the future experimental studies.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Review Article

Calcium Homeostasis and ER Stress in Control of Autophagy in Cancer Cells

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Autophagy is a basic catabolic process, serving as an internal engine during responses to various cellular stresses. As regards cancer, autophagy may play a tumor suppressive role by preserving cellular integrity during tumor development and by possible contribution to cell death. However, autophagy may also exert oncogenic effects by promoting tumor cell survival and preventing cell death, for example, upon anticancer treatment. The major factors influencing autophagy are Ca^{2+} homeostasis perturbation and starvation. Several Ca^{2+} channels like voltage-gated T- and L-type channels, IP3 receptors, or CRAC are involved in autophagy regulation. Glucose transporters, mainly from GLUT family, which are often upregulated in cancer, are also prominent targets for autophagy induction. Signals from both Ca^{2+} perturbations and glucose transport blockage might be integrated at UPR and ER stress activation. Molecular pathways such as IRE 1-JNK-Bcl-2, PERK-eIF2α-ATF4, or ATF6-XBP 1-ATG are related to autophagy induced through ER stress. Moreover ER molecular chaperones such as GRP78/BiP and transcription factors like CHOP participate in regulation of ER stress-mediated autophagy. Autophagy modulation might be promising in anticancer therapies; however, it is a context-dependent matter whether inhibition or activation of autophagy leads to tumor cell death.

1. The Outline of Autophagy

Autophagy pathway is unique and is characterized by the appearance of double- or multiple-membrane cytoplasmic vesicles which absorb the bulk of cytoplasm and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum to be destroyed by the lysosomal system of the same cell [1, 2]. Autophagy begins with enwrapping the cytoplasmic constituents by membrane, which originates from ER, Golgi apparatus or is formed de novo through nucleation, assembly, and elongation of small membrane structures. Closure of these membranes results in the formation of double-membrane structure called autophagosome. In the next step autophagosome fuses with late endosome (multivesicular body) or directly with lysosome and generates amphisome or autolysosome, respectively. Subsequently, amphisome as transient form also fuses with lysosome. Finally, the lysosomal hydrolases degrade the cytoplasm-derived contents inside the autolysosome together with its inner membrane [3]. Autophagosomal membrane formation requires a multiprotein complex that consists of Beclin 1 (Atg6), class III PI3K (Vps34), and p150 myristoylated protein kinase [1] as well as Vps15, UVRAG, Bif1, and Ambra 1 [4]. Another complex involved in autophagosome formation is focal adhesion kinase (FAK) family interacting protein of 200 kDa, Unc-51-like kinase 1/2, autophagy-related gene 1/13 (FIP200-ULK1/2/Atg1/13) [5]. Further elongation is mediated by two ubiquitin-like conjugation systems based on conversion of microtubule-associated protein 1 light chain 3 (MAP LC3) from the free form (LC3 I) to the lipid-conjugated membrane-bound form (LC3 II) [6]. Although autophagy is thought to be mainly nonselective degradation mechanism,

latest studies report the presence of specific receptors and other selective adaptor proteins sensitive to organelle injury or aggregated proteins [3, 7].

2. The Role of Autophagy in Cancer Cells

Autophagy is the major cellular route for degrading long-lived proteins and cytoplasmic organelles to provide the energy required for minimal cell functioning when nutrients are scarce or not available [1, 2]. The catabolic advantage of increased autophagy may be also critical in various stress conditions, for example, hypoxia, growth factor deprivation, starvation, ER stress, ROS accumulation, protein aggregation, and numerous anticancer treatments. Therefore, autophagy in mammalian cells serves as an adaptive mechanism and is activated when cell is prone to die, to recycle amino acids and macromolecules necessary for cell survival. The role of autophagy in cancer cells, however, is still under investigation. It seems that autophagy function depends on several factors, for example, step of tumor formation, tissue origin, and gene mutations existing in specific cancer type. Some cancer types like human pancreatic cancers with constitutive Ras activation have elevated levels of autophagy that contributes to their growth and survival [8]. Conversely, other tumor types like human breast, ovarian, and prostate cancers have allelic deletions of the essential autophagy regulator Beclin 1, indicating that decreased autophagy may promote tumor development [9]. Overall the significance of autophagy in tumors can be distinguished into two functions. Firstly, autophagy can play a tumor suppressor role by maintaining cellular fidelity and, if necessary, contributing to cell death execution. Secondly, autophagy may exert oncogenic effects by promoting tumor cell survival and preventing cell death. Both roles of autophagy in tumor development will be discussed in this review.

Autophagy can play protective roles in early stages of cancer development by eliminating aggregated proteins or damaged organelles, preserving cells from further damages [2, 10]. Moreover oncosuppressive function of autophagy is manifested by limiting chromosomal instability, reducing oxidative stress, preventing intratumoral necrosis and local inflammation, and supplying nucleotides for DNA replication and repair [10, 11]. Several proteins involved in autophagy regulation are actually described as oncosuppressors. For instance, the UVRAG protein, monoallelically deleted at high frequency in human colon cancers, interacts with Beclin 1 to form a class III PI3-K signaling complex, the initial step in autophagosome formation [12]. Another function of autophagy in preventing of tumor progression is its ability to regulate cell death processes. It is known that autophagy and apoptosis share some regulatory pathways including proteins such as Bcl-2, Bcl-X_L, cFLIP, caspase 3, tBid, Bad, and PUMA [8, 13]. In many cases execution of apoptosis even depends on autophagy. During the DNA damage, expression of macroautophagy regulator DRAM-1 is required for p53 mediated apoptosis [14]. In NIH 3T3 spontaneously immortalized cells inhibition of B-Raf by UI-152 was specifically cytotoxic to v-Ha-Ras-transformed cells and evoked both autophagy and apoptosis. Inhibition of autophagy by 3-MA did not rescue transformed cells from cell death indicating the cooperation between autophagy and apoptosis pathways. Another example is autophagy and apoptosis induction upon carnosol treatment both *in vitro* and *in vivo* in triple-negative breast cancer (TNBC) [15]. Surprisingly, autophagy in these studies was Beclin 1-independent, which, according to the authors, might be responsible for death-stimulating effect of autophagy. Autophagy might also participate in necrotic type of cell death [13]. In pancreatic cancer cells PANC-1, a derivative of allocolchicine, Green 1 [(S)-3,8,9,10-tetramethoxyallocolchicine], caused necrotic cell death that was autophagy-dependent [16]. These processes occurred selectively in cancer cells and autophagy was induced in response to elevated ROS levels after Green 1 administration. Furthermore, many authors even refer to autophagic cell death or type II programmed cell death. In apoptosis deficient tumor cells, autophagy is induced to maintain cell metabolism and viability during nutrient starvation and protect cells from necrosis. Ultimately, if the nutrient deprivation persists, prolonged autophagy may lead to autophagic cell death [17]. Xiong et al. [18] reported 5-FU (5-fluorouracil) induced autophagic cell death in Bax and PUMA deficient HCT116 colon cancer cells which were apoptosis defective. Furthermore, autophagy inhibition by 3-MA resulted in decreased cell death rate [18]. In hepatocellular carcinoma cell lines HepG2 and HuH-7 and line xenografts treated with cannabinoids (Δ9-tetrahydrocannabinol, JWH-015) autophagy was mediated by CaMKKβ-AMPK and led to apoptosis. In these studies, blockage of autophagy also impeded apoptosis [19]. These results suggest autophagy to be supporting or alternative to apoptotic cell death pathway.

In established tumors, autophagy may conversely exert an oncogenic effect by preventing tumor cell death. Autophagy can limit the cytotoxicity of tumor necrosis factor superfamily (TRAIL), can suppress p53 response induced by DNA damage, and can sustain mitochondrial metabolism and stress tolerance [10, 20]. Thus, inhibition of autophagy often sensitizes cancer cells to apoptotic, necrotic, or necroptotic cell death [8, 21]. It was shown that in the presence of a potent chemotherapeutic agent, cisplatin, esophageal cancer cell line EC9706 induced autophagy through class III PI3-kinase pathway. Although cell growth was effectively inhibited in time- and dose-dependent manner, additional treatment with autophagy inhibitor 3-MA potentiated cell growth inhibition and induced apoptosis [22]. Under stress conditions, DNA repairing enzyme, PARP-1, massively synthesizes poly-ADP-ribose and this causes the decrease in cellular NAD^+ and ATP levels [23]. Insufficient ATP production to maintain plasma membrane integrity may induce metabolic catastrophe and cell lysis [17]. A rapid drop in ATP is also a feature of proceeding necrosis that can be abolished by providing necessary sources for ATP synthesis by autophagy. However, tumor cells have commonly inactive mechanisms of apoptosis induction and constitutively active PI3-K pathway, being responsible for cell growth and proliferation. These types of tumors cannot induce autophagy (active mTOR) in response to metabolic stress, which may lead to necrotic cell death [17].

Taken together, autophagy is currently considered as a possibly important mechanism to be used in anticancer therapy. However, possible role of autophagy in both oncogenesis as a survival promoting factor and tumor prevention as a death inducing factor should be seriously considered.

3. Molecular Pathways Related to Autophagy Induction in Cancer Cells

There are several known pathways leading to autophagy activation in cancer cells. Beclin 1 has been first identified as a Bcl-2 interacting protein [12]. Originally the contribution of the Bcl-2 family in tumorigenesis was limited to modulate apoptosis but recently there are also evidences to their function in control of metabolic processes including autophagy. In fact, the role of antiapoptotic factors like Bcl-2, Bcl-X_L, and Bcl-w is to suppress autophagy, mainly by interacting with Beclin 1 [12]. Accordingly, the inhibition of Bcl-2 was shown to induce autophagy in multiple tumors [12, 24]. Furthermore, proapoptotic BH3-only proteins such as Bad, Bik, and BNIP3L are also described as autophagy inducers, acting by releasing Beclin 1 from the inhibitory action of Bcl-2 protein [12]. Bcl-2 is mainly located on mitochondrial, nuclear, and ER membranes and can affect autophagy in at least two ways: by directly binding to Beclin 1 or possibly by binding to IP3-R and regulating the Ca^{2+} level in ER [25].

The main molecular mechanism of autophagy occurs via repressed activity of mTORC1, which consists of serine/threonine protein kinase, mammalian target of rapamycin (mTOR), its regulatory associated partner, Raptor, PRAS40, and mLST8 [26]. Direct target of mTORC1 inhibition is ULK complex; however, its phosphorylation might be regulated also independently of mTORC1 activity [3]. The mTORC1 activity is regulated by distinct molecular pathways. It might be activated via class I PI3-K-Akt pathway which is sensitive to growth factors and transduces signal to activate cell growth and proliferation [17, 27] or by Raf-1-MEK1/2-ERK1/2 pathway, which contribute to amino acid depletion-induced autophagy [2, 28]. Nutrient deprivation and calcium homeostasis disturbances can influence mTORC1 activity as well. AMP-activated protein kinase (AMPK) is a mediator of autophagy in such circumstances. It might be activated either by increased AMP/ATP ratio inside the cell during starvation or possibly by CaMKKβ-dependent pathway in response to elevated calcium levels in cytoplasm [6]. Finally, when mTORC1 complex is inhibited, it relocates from lysosomes that proceeds autophagy and allows nuclear translocation of dephosphorylated transcription factor EB (TFEB), which in turn activates the *Atg* genes [3]. Both starvation and calcium perturbations may lead to activation of UPR cascade and ER stress. This review focuses mostly on involvement of calcium homeostasis and glucose deprivation in ER stress-mediated autophagy induction in cancer cells (Figure 1).

4. Involvement of Ca^{2+} Homeostasis in Autophagy Induction

Calcium homeostasis is regulated by several calcium channels. Plasma membrane calcium ATPases (PMCA) are located in plasma membrane and actively pump Ca^{2+} outside the cell. Ca^{2+} release-activated Ca^{2+} channels (CRACs) are also located in plasma membrane and mediate the store-operated calcium channel entry (SOCE) [29]. CRACs are activated by Ca^{2+} released from ER by channels formed by IP3-R and RyR receptors. Ca^{2+} redundancy in cytoplasm is actively transported backward into ER by sarco-/endoplasmic reticular calcium ATPase (SERCA), which is a membrane pump located in ER. Several voltage-dependent calcium channels are also reported to participate in calcium homeostasis regulation. Specific voltage values are required to activate dedicated types of calcium channels: L, N, P, Q, R, and T.

Ca^{2+} is one of the most important regulators of cell survival/death processes. As a second messenger, Ca^{2+} is able to activate or inactivate various regulatory proteins such as enzymes, transcriptional factors, or molecular chaperones. It was shown previously by several authors that the disorder of calcium homeostasis can evoke different types of cell death in cancer cells. There are evidences that β-lapachone induces μ-calpain-mediated and is independent of caspase activity cell death in MCF-7 cells [30]. Pajak and Orzechowski [31] reported the proapoptotic effect of calcium ion chelators EGTA and EDTA in COLO 205 adenocarcinoma cells. Høyer-Hansen and Jäättelä [25] observed autophagic cell death induced by factors increasing in different manners the cytosolic Ca^{2+} such as vitamin D3 and its chemotherapeutic analog EB 1089, ATP, ionomycin, and thapsigargin in MCF-7 breast cancer cells. In Ca^{2+}-dependent induction of autophagy, Ca^{2+} released from intracellular stores or fluxed from extracellular space via distinct calcium channels activates CaMKKβ, which mediates AMPK-dependent inhibition of mTORC1 [6].

One of the best investigated mechanisms of autophagy induction, involving modulation of calcium channels activity, is IP3-R-Beclin 1-Bcl-2 pathway [4]. This pathway is mTOR-dependent and mediated through ER stress and UPR activation. IP3-R, the receptor for major cellular second messenger IP3, is known for regulating apoptosis signaling, although its inhibition is also described as an event leading to macroautophagy induction [4]. Beclin 1 promotes autophagosome formation by interacting with class III PI3-K, p150 myristoylated kinase, and other proteins. Creation of autophagy promoting complex can be abolished by competitive Beclin 1 interaction with IP3-R or Bcl-2. In fact, the application of xestospongin B, the antagonist of IP3-R, caused autophagy due to disruption of IP3-R and Beclin 1 complex. Moreover, the activity of this complex might be increased or inhibited by overexpression or knockdown of Bcl-2 which can be ectopically expressed in ER and interact with IP3-R-Beclin 1 [4]. Thus, when IP3-R serves as a scaffold protein, lowering its level may trigger autophagy [4, 32]. Inhibition of inositol monophosphatase by lithium chloride (LiCl) also evoked IP3-R-dependent autophagy but this process was mTOR independent [33]. Indeed, Ca^{2+} released to the cytoplasm through IP3-R is reported to play role in both inhibition and activation of autophagy, probably dependently on cellular state [32]. In autophagy induced by ATP, P2 purinoreceptors were stimulated to generate IP3

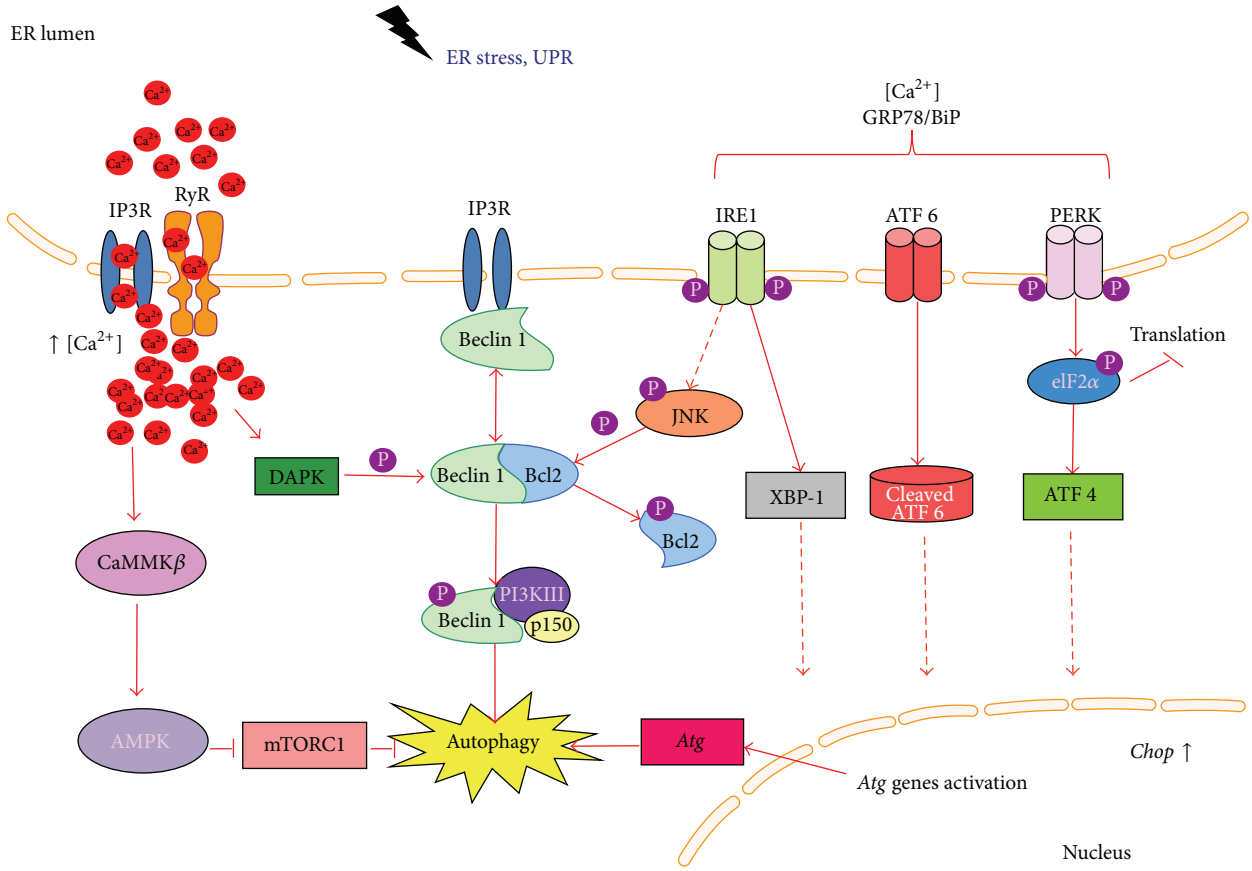

FIGURE 1: Autophagy mediated via ER stress and UPR activation. The figure represents proposed scheme for autophagy modulation in cancer cells through ER stress and UPR activation.

that triggers the release of Ca^{2+} from the ER through IP3-R [25]. Similarly, rapamycin treatment in HeLa cells increased ER Ca^{2+} store content and resulted in potentiated release through the IP3-R [32]. Furthermore, intracellular Ca^{2+} signal was essential for mTOR-dependent autophagy. It is possible though both IP3-R inhibition and activation can evoke autophagy via distinct signaling pathways. While IP3-R Ca^{2+} signaling-dependent autophagy leads to mTORC1 inhibition, it should be studied whether the blockage of IP3-R induces ER stress and UPR or if it promotes autophagy independently of these mechanisms [25].

Inhibition of voltage-dependent calcium channel T or L is also reported to evoke autophagy in cancer cells. Long-lasting, high voltage-activated (L-type) calcium channels and transient, low voltage-activated (T-type) calcium channels are often overexpressed in various cancers [34–36]. Verapamil, the L-type calcium channel blocker, induced autophagy-like process in human adenocarcinoma COLO 205 cells as judged by ultrastructural studies [37]. The other L-type calcium channel blocker, nifedipine, decreased proliferation and migration and evoked autophagy in endometrial carcinoma HEC-1A cells. Moreover, suppression of autophagy by 3-MA led to apoptosis induction [21]. As calcium inward currents regulators, T-type calcium channels play also role in cells proliferation and differentiation processes. Zhang et al. [38] described retarded proliferation and migration upon endostatin in human glioblastoma U87 cells. The mechanism of action in endostatin-treated cells occurred via blockage of T-type, but not L-type calcium currents. Furthermore, T-type calcium channel blocker, [3-(1,1′-biphenyl-4-yl)-2-(1-methyl-5-dimethylaminopentylamino)-3,4-dihydroquinazolin-4-yl]-N-benzylacetamide 2 hydrochloride (KYS 05090), induced autophagy and apoptosis-mediated cell death in human lung adenocarcinoma A549 cell line. These processes were inhibited either by bafilomycin A, a potent autophagy inhibitor, or by inhibitor of caspase 3, zVAD-fmk [35]. Regardless of decreasing intracellular Ca^{2+} levels, KYS 05090 inhibited also glucose uptake and elevated ROS generation [35].

Voltage-gated calcium channels inhibitors are known for blockage of calcium entry inside the cell; however some of them are reported to modulate autophagy in noninvolving calcium channels pathway. For instance, tetrandrine is reported to be a lysosomal deacidification agent, able to block autophagy flux at the step of lysosomal degradation. Furthermore tetrandrine induced apoptosis in PC3 cells and tumor xenografts, due to reduced glucose uptake [39]. Also verapamil and its derivatives were reported to evoke

cytotoxicity on various cancer cells via distinct mechanisms including reversing multidrug resistance and inhibition of glucose import [40–42].

CRAC is a major channel contributing to changes in intracellular Ca^{2+} concentration. It consists of structural proteins from ORAI family (ORAI 1, ORAI 2, and ORAI 3) and Ca^{2+} sensor stromal interaction molecule (STIM). CRAC activation resulting in SOCE plays critical role in physiological processes of immune system cells such as T lymphocytes [43, 44]. CRAC is also important for cancer cells maintenance, usage of CRAC blockers, as well as ORAI 1 silencing, markedly inhibited cell proliferation of glioblastoma cell lines C6 (rat) and U251 (human) [45]. In HeLa cells targeting the ORAI 1 and calcium-transporting ATPase type 2C member 1 (ATP2C1) by the microRNA miR-519 resulted in their downregulation and subsequent intracellular Ca^{2+} elevation. Furthermore p21 level was increased by activation of CaMKKβ and GSK3β. Altogether this resulted in cell growth arrest, autophagic phenotype, and increased cell survival [29].

5. Glucose Metabolism during Autophagy

Most malignant cells use glycolysis as a major pathway for ATP generation and since this process is inefficient, cancer cells exhibit abnormally high glycolytic rates to maintain ATP homeostasis [46]. Glycolysis is triggered by mutations in several oncogenes like Ras, Akt, and Myc [9, 17]. This feature of cancer cells and other immortalized cell lines is called "Warburg effect" and is mainly manifested by high glucose uptake followed by intensive lactic acid formation in the cytosol. High lactate production is desired as acidic environment favors the tumor growth [47]. In fact, tumor cells which are highly dependent on glycolysis are more sensitive to basic nutrient deprivation such as glucose [48]. In 4T1 cancer cells, glucose deprivation and blockage of lactic acidosis caused cell death within 24 hours [46]. Limited glucose access may also lead to autophagy induction. In poor nutrient conditions the mTORC1 is suppressed by AMPK and autophagy provides cells with new energy sources. In PC3 and LNCaP prostate cancer cell lines therapeutic starvation with 2DG evoked autophagy judged by presence of autophagic hallmarks such as higher expression of Beclin 1 and LC3 II, together with its membrane translocation [49]. Another example is autophagy induced by diindolylmethane (DIM) in ovarian cancer cells, mediated by ER stress and AMPK activation [50]. Activation of AMPK in pancreatic adenocarcinoma PANC-1 cell line after cannabinoids treatment was mediated by ROS elevation which led to inhibition of Akt/c-Myc pathway and consequently Krebs cycle and glycolysis downregulation. Disturbance in PANC-1 metabolism caused strong induction of autophagy and limitation in cell growth [48].

Transport of glucose across plasma membrane is a first rate-limiting step for glucose metabolism and is mediated by dedicated protein group named as GLUTs [51]. Upregulation of specific glucose transporters may play a key role in exaggerated glucose uptake in malignant cells, which is required to maintain high rate of glycolysis. GLUT 1, which is involved in glucose uptake in the basic and stress state, is expressed at elevated levels in almost all human cancers including brain, breast, head and neck, bladder, renal, colorectal, lung, and ovarian cancers [51]. Krzeslak et al. [52] reported the involvement of GLUT 1 and GLUT 3 in glucose transport in endometrial and breast carcinoma cells. Lately, GLUT 12, physiologically expressed in the insulin-sensitive tissues such as skeletal muscle, heart, and fat, has been localized in human breast and prostate cancer both intracellularly and at the plasma membrane. Moreover, this can be an example of reactivation of a gene, expressed in the embryo and downregulated in most adult tissues in nonpathological conditions [51]. Besides the GLUT family, other proteins like SGLT1 or IGFs and their receptors (IGF-Rs) are engaged in glucose transport and are often upregulated in cancer cells [53, 54]. SGLT-1 was overexpressed in colorectal cancer tissue together with EGFR and these were correlated with higher clinical stages of tumor disease [53]. IGF-I/IGF-IR is a protein that promotes cell survival, proliferation, and differentiation, whose overexpression is associated with many types of cancer including lung cancer, neuroblastoma, cervical, breast, and colon cancer [54, 55]. Moreover, IGF-I was shown to significantly increase the initial rate of glucose uptake by HT29-D4 colon cancer cells [56]. On the contrary, in Caco-2 intestinal adenocarcinoma cell line, transport of glucose occurred via an Na^+/glucose transporter, independently of insulin and IGF-1 receptors [57]. Restriction of glucose uptake may affect the metabolism of malignant cells and limit significantly tumor growth by inducing cell death. In fact, downregulation of IGF-I/IGF-IR signaling can inhibit tumorigenesis, reverse the transformed phenotype, and induce apoptosis [55]. Next, in MM.1S myeloma cell line the decrease in glucose consumption stimulated by purine analog 8-aminoadenosine application exerted energetic stress and activation of autophagy, which played role in cell redundancy to the treatment. Cotreatment by 8-aminoadenosine and autophagy inhibitors stimulated apoptosis induction, although the effect was reversible by pretreatment with metformin and overexpression of GLUT1 [58].

Glucose transporters might be influenced by distinct factors during tumor progression. Tolerance of tumor cells to nutrient deprivation depends on deregulation of both oncogenes and oncosuppressors. Cav1 is a growth suppressor protein, although its level is often elevated in advanced cancer, suggesting the oncogenic switch to the role in growth progression. In various human colorectal tissues and cancer cell lines, inhibition of Cav1 triggered AMPK-mediated autophagy with p53-dependent G1 cell-cycle arrest. Moreover, glucose uptake, lactate accumulation, and ATP levels were reduced. Thus, overexpression of Cav1 in tested human colon cancers is thought to be responsible for their upregulated metabolism via stimulation of *Glut 3* gene expression [59, 60]. Another oncosuppressor involved in glucose metabolism regulation is HIPK2, whose activation upon several cellular stresses causes cell death [61]. In human RKO colon cancer cells harboring wt-HIPK2 ($HIPK2^+/^+$), cell death was induced, mainly due to c-Jun NH2-terminal

kinase (JNK) activation upon glucose starvation. In contrast the same conditions did not induce cell death in siHIPK2, which exhibited upregulated glycolytic activity and autophagy. Although targeting glycolysis by 2-DG or siGlut-1 does not induce siHIPK2 cell death under glucose starvation, this was achieved by zinc supplementation that reversed p53 misfolding and inhibited HIF-1 activity. The cytotoxic effect in siHIPK2 RKO cells was potentiated by inhibiting autophagy, which played role in establishing tumor survival under glucose deprivation [61].

Also ER stress can affect glucose metabolism in cancer cells. In IL-3-dependent $Bak^{-/-}/Bax^{-/-}$ hematopoietic cells resistant to apoptosis, exposure to tunicamycin resulted in decreased cell surface GLUT 1 level and impaired Akt signaling, which was probably a reason for observed reduced glucose uptake and lactate production and fall in mitochondrial potential and ATP level disturbance. In the absence of apoptosis, tunicamycin evoked autophagy, which might be important for cell survival [62].

6. ER Stress

Endoplasmic reticulum (ER) facilitates the proper folding of the synthesized proteins and serves as a Ca^{2+} store inside the cell. ER stress appears in response to different physiological and pathological conditions, for example, when the aggregation of prone proteins occurs, glucose starvation causes limited protein glycosylation, or during hypoxia when the formation of disulfide bonds is reduced [25]. In such situations a specific nuclear signaling pathway called UPR is activated and results in reduced global protein synthesis and increased production of proteins required for proper folding at ER such as chaperones. Meister et al. [63] reported enhanced ER stress followed by cell death in myeloma cells after combined treatment with verapamil and proteasome inhibitor, bortezomib. When ER stress is being prolonged and misfolded/unfolded proteins exceed the capacity of the proteasome degradation system, it might trigger autophagy [25]. In mammalian cells UPR can be mediated by activation of different stress transducers like PERK, ATF6, or IRE1, which sense the level of unfolded proteins in ER lumen and pass the signal to cytoplasm and nucleus. Activation of PERK leads to phosphorylation of the α subunit of the eukaryotic initiation factor (elF2α), which inhibits the assembly of the 80S ribosome and inhibits protein synthesis while autophagy is induced [64]. Activation of IRE1 and ATF6 promotes the transcription of UPR target genes [65]. ER stress leads also to Ca^{2+} releasing from the ER to the cytosol, which results in activation of numerous kinases and proteases involved in autophagy, such as CaMKKβ or DAPK. Both of them stimulate disruption of Beclin 1 inhibitory complexes (Beclin 1-IP3-R or Beclin 1-Bcl-2). Furthermore, CaMKKβ is also an upstream activator of AMPK, which inhibits mTORC1 [6, 66]. Beclin 1 can be activated also through IRE1-JNK pathway [67].

In cancer cells, levels of misfolded proteins and ER stress are often increased because of gene mutations and stressful microenvironment. Furthermore, ER stress is frequently a cellular response to anticancer treatment. Salazar et al. [68] reported autophagy and cell death induction upon Δ^9-tetrahydrocannabinol (THC) treatment in human glioma cells and tumor xenografts. Moreover inhibition of autophagy prevented cell death; also, autophagy deficient tumors were resistant to THC growth-inhibiting action. THC caused ceramide accumulation and elF2α phosphorylation leading to ER stress induction. Consequently autophagy occurred through tribbles homolog 3- (TRB3-) dependent inhibition of the Akt-mTORC1 pathway [68].

It is known that ER stress-induced autophagy depends strongly on Ca^{2+} homeostasis. Studies conducted with thapsigargin or tunicamycin, inhibitors of ER Ca^{2+}-ATPase, revealed that in IRE1-deficient MEFs or MEFs treated with JNK inhibitor, autophagy induced by ER stress, was inhibited, indicating that the IRE1-JNK pathway is required for autophagy activation [65]. In HCT 116 colon cancer cells inhibition of proteasome activity by MG132 resulted in increased cytoplasmic Ca^{2+} levels, ER stress, and autophagy induction. BAPTA-AM treatment overcame Ca^{2+} elevation, ER stress, and cellular vacuolization but did not prevent from MG132-induced apoptosis [69]. These results indicate that changes in calcium homeostasis often trigger ER stress leading to UPR mediated autophagy.

It seems that distinct cellular pathways inducing autophagy might be integrated in some points. For instance, starvation-induced autophagy may also depend on IP3-R-mediated Ca^{2+} signaling [32]. ER stress-mediated autophagic pathways also integrate various cellular stresses. Ca^{2+} perturbations as well as glucose starvation may lead to similar cellular response as ER stress and UPR activation.

During maturation the majority of proteins require Ca^{2+} to proper folding. It is also known that 80% of proteins synthesized in rough ER undergo glycosylation [70] and highly depend on glucose availability. Treatment with glucose analog, 2-DG, is known to induce ER stress-mediated autophagy in pancreatic, melanoma, and breast cancer cell lines. Further, 2-DG not only blocked glycolysis, thereby lowering levels of ATP, but also impaired glycosylation, as addition of exogenous mannose was able to reverse ER stress and autophagy [71]. In turn, in HT-29 human colonic carcinoma cells, high rate of N-glycosylated proteins substituted with ER glycans is degraded in autophagy pathway. Inhibition of ER glucosidases stabilized freshly synthesized N-linked glycoproteins and inhibited their degradation via macroautophagy. In this case autophagy seems to be a selective process connected with ER-associated quality control of synthesized N-glycoproteins [72].

7. ER Molecular Chaperones and Transcription Factors in Autophagy Regulation

N-Glycosylation of newly synthesized proteins takes place in ER lumen and ER-associated molecular chaperones are involved in the quality control of this process. Firstly, during folding, polypeptides are recognized by ER chaperones

such as 78 kDa glucose-regulated protein GRP78/BiP, calnexin/calreticulin [72], and proteins involved in the disulphide bond formation like PDI [73]. Interaction with molecular chaperones lasts till they are exported to Golgi apparatus to undergo further proceedings. The misfolded/unfolded proteins are directed to ER-associated degradation (ERAD) system where they are degraded via ER-attached proteasomes [72]. ATF4, ATF6, and XBP-1 (transcription factor targeted by IRE 1) upregulate ER chaperones, folding enzymes, and protein degradation molecules, which either prevent the aggregation of unfolded proteins or assist in their degradation [74]. In HT-29 human colon cancer cells ER stress was induced by compound K. This process was mediated via increased expression of ER chaperone GRP78/BiP and proapoptotic protein-CHOP, possibly as a consequence of PERK and IRE 1 phosphorylation and ATF 6 cleavage to active form [75].

The main UPR-upregulated protein is GRP78/BiP. It is involved not only in proteins proper folding but also in transport of proteins across ER membrane, regulation of proliferation, tumor progression, angiogenesis, autophagy, chemosensitivity, and apoptosis [73, 76, 77]. Levels of GRP78/BiP mRNA and protein are modulated by glucose availability and Ca^{2+} concentration [78–80]. Under physiological conditions GRP78/BiP is attached to ATF6, IRE1, and PERK protein, residing in ER membrane. When quantity of misfolded/unfolded proteins exceeds the ER lumen capacity, GRP78/BiP dissociates from its binding partners so they have ability of autophosphorylation and activate the UPR reaction [81]. Elevated level of GRP78/BiP was observed in human nasopharyngeal carcinoma cells during ER stress-mediated autophagy induced after DDP, 2-DG, ionizing irradiation, and tunicamycin treatment. Autophagy was activated as a protective mechanism; therefore, using 3-MA contributed to apoptosis induction [82]. GRP78/BiP is also a key player in autophagy induced in tumors from $BRAFV^{600E}$ melanoma patients treated either with B-Raf inhibitor or with combined B-Raf and MEK inhibition. Furthermore, autophagy level was significantly higher in B-Raf inhibition-resistant tumors. Induced autophagy is mediated via mutated B-Raf bounding to GRP78/BiP allowing the subsequent PERK phosphorylation. This data provides the possible mechanism of B-Raf mutation-driven myeloma tumors resistance to B-Raf inhibition therapy [83]. Moreover Li et al. [74] showed that GRP78/BiP is required for UPR activation and following autophagy in HeLa cancer cells. Inhibition of GRP78/BiP by siRNA resulted in blockage of autophagosome formation upon ER stress or nutrition starvation. Impaired autophagy was recovered after simultaneous knockdown of GRP78 and XBP-1, which are known to regulate ER functions [74]. However, Bennett et al. [81] reported androgen receptor-mediated temporary upregulation of GRP78/BiP in prostate cancer LNCaP cell line upon chronic serum starvation, which contributed to ER stability and the delay in onset of autophagy and cell death execution.

Other ER stress-upregulated protein is CHOP, a transcription factor implicated in the control of translation and apoptosis, downstream target of PERK and ATF4 [74]. Induced by UPR, CHOP protein may also contribute to autophagy induction by downregulating the Bcl-2 expression [25, 73]. It is also known that ATF4 and CHOP activate expression of two genes essential for autophagy: ATF4 binds to the promoter of *Map1lc3B*, while CHOP activates the transcription of *Atg5* [84].

Hsp27 is stress-activated multifunctional chaperone that inhibits treatment-induced apoptosis and causes therapy resistance. It is expressed mainly in cytoplasm but occurs also in ER and nucleus. Hsp27 is present in many cancer types, for example, in castration-resistant prostate cancer (CRPC). Using OGX-427, a second-generation antisense inhibitor of Hsp27, Kumano et al. [85] confirmed that Hsp27 reduced proteasome inhibitor-induced ER stress and accumulation of misfolded/ubiquitinated protein levels, mainly by increasing proteasome activity and/or stabilization client-protein complexes. However, inhibition of Hsp27 led to suppression of ubiquitin-proteasome system and activation of ER stress and UPR. Moreover, Hsp27 knockdown induced cytoprotective autophagy, yet combined inhibition of Hsp27 and autophagy further disrupted proteostasis and caused apoptosis in prostate cancer cells [85].

CLU is a heterodimeric, highly conserved, disulfide-bond glycoprotein. During maturation secretory form of CLU (sCLU) undergoes heavy glycosylation which contributes to its cytoprotective role and possibly protects from its aggregation [86], whereas nonglycosylated nuclear CLU (nCLU) plays role in apoptosis induction [87]. Because CLU maturation is complex and highly depends on processing in ER lumen and in Golgi apparatus, CLU seems to be extremely sensitive to ER stress. Lately, Kang et al. [88] reported other nonglycosylated variants of CLU accumulated in ER, confirming the role of N-glycosylation in preventing from terminal misfolding of CLU protein. Moreover N-glycan deficient CLU induced cytotoxicity may be a reason for disease pathogeneses associated with chronic ER stress [88]. CLU function is also often associated with intracellular Ca^{2+} homeostasis. It is assumed that in Ca^{2+} deficiency CLU translocates to nucleus to intermediate apoptosis induction, while in elevated levels of Ca^{2+} in cytosol it is mainly secreted to play cytoprotective role [89]. Little is known about CLU role in autophagy, although its involvement in carcinogenesis, tumor survival, regulation of adhesion, cell cycle, and apoptosis has been widely described [87, 90, 91]. Increased levels of CLU have been reported in several malignancies, including breast, colon, lung, and prostate cancers [92, 93]. CLU function as an extracellular and intracellular chaperone during ER stress was also reported. Wyatt et al. [94] described CLU to form stable complexes with misfolded client proteins and target them to lysosomal degradation both *in vitro* and *in vivo*. Balantinou et al. [95] confirmed that CLU is degraded by both proteasome and lysosome systems. In TRAMP mouse model of prostate cancer, phenethyl isothiocyanate treatment inhibited carcinogenesis through autophagy induction and decreased level of CLU protein. CLU was then identified as a potential plasma biomarker of phenethyl isothiocyanate-induced chemopreventive activity [92].

8. Conclusions and Current Perspectives in Anticancer Therapy

Primary function of autophagy process is the maintenance of cellular energetic status while coping with numerous affecting stresses. During tumor development autophagy is often used to clear defective proteins and organelles. Moreover, tumor cells take an advantage of autophagy and maintain cell survival in response to anticancer treatments [8]. Numerous studies were undertaken to evoke molecular mechanisms leading to autophagy induction in cancer cells as well as its downstream consequences including contribution to various cell death processes. It is known that autophagy might be modulated by changes in ion homeostasis and metabolic perturbations, which may alter intracellular biochemical reactions and proceed in further induction of cellular stress signaling pathways. Ca^{2+} ion channels and glucose transporters can mediate signal transduction from extracellular to intracellular space and therefore participate in ER stress induction, which is one of possible ways to evoke autophagy. This opens the possibility of using Ca^{2+} ion channels and glucose transporters modulators in control of autophagy for therapeutic purposes. In mice with acute pancreatitis the utilization of 2APB, an antagonist of IP3-R, extenuated pathological changes, possibly via blockage of autophagy [96]. In turn, glucose analog, 2-DG, responsible for autophagy induction upon inhibition of glycolysis and insufficient ATP levels, is reported to evoke cell death when combined with antimycin A or metformin in various cancer cell types. Moreover combination of 2-DG and docetaxel or radiotherapy is currently on trials for therapy for glioblastoma, lung, and breast cancers [97]. Research is being undertaken on establishing new therapy based on autophagy activation via, for example, ER stress induction (sorafenib), mTOR inhibition (aurora kinase A), or AMPK activation (atorvastatin) in various cancer types [28]. Zhao et al. [98] showed in preclinical studies on nasopharyngeal carcinoma (NPC) model, that upon AKT inhibition by MK-2206, autophagy, but not apoptosis, was induced in CNE-2 cell line. Moreover MK-2206 inhibited the growth of tumor while CNE-2 cells were implanted into nude mice.

Modulation of autophagy seems to be promising in anticancer therapy; however, it should be further investigated whether therapeutic induction of autophagy might potentiate chemoresistance in tumor cells or contributes to cell death. The role of autophagy in cancer is complex and especially depends on cancer type and stage of development. In a mouse model of non-small-cell lung cancer (NSCLC) *Atg7* deletion did not affect tumor formation caused by kRas activation and p53 deletion. However, in mice with established tumors, deletion of *Atg7* blocked tumor progression and led to tumor cell death, before normal tissue destruction [99]. These findings indicate that depletion of autophagy might be a useful tool in providing the time frame for destructing tumor tissue. Autophagy impairment might also play a beneficial role in response to ionizing radiation (IR). Deletion of *Atg5* sensitized human and mouse cancer cell lines to induced cell death and inhibited *in vivo* tumor growth in immunodeficient mice. However, in immunocompetent mice, autophagy depletion decreased therapeutic effect of IR [100], indicating a context-dependent matter in autophagy modulation. Recently Rosenfeldt et al. [101]. showed in a humanized genetically modified mouse model of pancreatic ductal adenocarcinoma (PDAC) that autophagy role in tumor development depends on the status of the tumor suppressor p53. In mice bearing oncogenic kRas, deletion of *Atg5* and *Atg7* inhibited tumor development. On the contrary, in mice containing oncogenic kRas but lacking p53, deletion of autophagy genes accelerated tumor formation. It is also proved that in this case blocking of autophagy with hydroxychloroquine contributed to tumor progression. These results are important in view of prospective anticancer treatment targeting autophagy. In particular, many current clinical trials cover therapies combining chloroquine or hydroxychloroquine with classical chemotherapy agents such as DDP or bortezomib [28]. Chloroquine was also shown to induce cell death in mice glioblastoma model in combined treatment with mTOR inhibitor, CC214-2 [102].

Autophagy ability to perform detoxification seems to occur frequently after anticancer drugs application. Therefore autophagy modulation might be a prospective tool in anticancer therapy, although further studies are of a high importance to uncover the circumstances when autophagy inhibition or activation may be implemented into clinic.

Abbreviations

2DG: 2-Deoxy-D-glucose
3-MA: 3-Methyladenine
5-FU: 5-Fluorouracil
Ambra 1: Activating molecule in Beclin 1 regulated autophagy
AMPK: AMP-activated protein kinase
ATF 4: Activating transcription factor 4
ATF 6: Activating transcription factor 6
Atg: Autophagy-related gene
ATP2C1: Calcium-transporting ATPase type 2C member 1
Bad: Bcl-2 associated death
Bax: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma 2
Bcl-X_L: B-cell lymphoma extra large
Bif1: B1/BAX-interacting factor 1
Bif-1: Bax interacting factor 1
CaMKKβ: Ca^{2+}/calmodulin-dependent kinase β
Cav1: Caveolin 1
cFLIP: Cellular FLICE-like inhibitory protein
CHOP: C/EBP-homologous protein
CLU: Clusterin
CRAC: Ca^{2+} release-activated Ca^{2+} channel
DAPK: Death-associated protein kinase
DDP: Cis-diamminedichloroplatinum (II)
DRAM: Damage regulated autophagy modulator
EGFR: Epidermal growth factor receptor

[2pt]elF2α: Eukaryotic initiation factor
ER: Endoplasmic reticulum
ERK1/2: Extracellular signal-regulated kinases 1/2
FIP200: Focal adhesion kinase (FAK) family interacting protein of 200 kDa
GLUT: Glucose transporter
HIF-1: Hypoxia-inducible factor 1
HIPK2: Homeodomain-interacting protein kinase 2
Hsp27: Heat shock protein 27
IGFs: Insulin-like growth factors
IP3-R: Inositol triphosphate receptor
IRE1: Inositol-requiring enzyme 1
JNK: c-Jun NH2-terminal kinase
MAP LC3: Microtubule-associated protein 1 light chain 3
MEK1/2: Mitogen-activated protein kinase 1/2
mTOR: Mammalian target of rapamycin kinase
mTORC1: Mammalian target of rapamycin complex 1
PARP-1: Poly[ADP-ribose]polymerase-1
PDI: Protein disulfide isomerase
PERK: RNA-dependent protein ER kinase
PI3-K: Phosphoinositide 3-kinase
PMCA: Plasma membrane calcium ATPase
PUMA: p53 upregulated modulator of apoptosis
Raf-1: Ras protooncogene serine/threonine protein kinase
ROS: Reactive oxygen species
RyR: Ryanodine receptor
SERCA: Sarco-/endoplasmic reticular calcium ATPase
SGLT: Na^+-dependent glucose cotransporter
SOCE: Store-operated calcium channel entry
TFEB: Transcription factor EB
ULK 1/2: Unc-51-like kinase 1/2
UPR: Unfolded protein response
UVRAG: Ultraviolet irradiation resistance-associated gene product
XBP-1: X-box binding protein 1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Nucleofection of Rat Pheochromocytoma PC-12 Cells with Human Mutated Beta-Amyloid Precursor Protein Gene (*APP-sw*) Leads to Reduced Viability, Autophagy-Like Process, and Increased Expression and Secretion of Beta Amyloid

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Pheochromocytoma PC-12 cells are immune to physiological stimuli directed to evoke programmed cell death. Besides, metabolic inhibitors are incapable of sensitizing PC-12 cells to extrinsic or intrinsic apoptosis unless they are used in toxic concentrations. Surprisingly, these cells become receptive to cell deletion after human *APP-sw* gene expression. We observed reduced cell viability in *GFP* vector + *APP-sw*-nucleofected cells (drop by 36%) but not in *GFP* vector − or *GFP* vector + *APP-wt*-nucleofected cells. Lower viability was accompanied by higher expression of Aβ 1-16 and elevated secretion of Aβ 1-40 (in average 53.58 pg/mL). At the ultrastructural level autophagy-like process was demonstrated to occur in *APP-sw*-nucleofected cells with numerous autophagosomes and multivesicular bodies but without autolysosomes. Human *APP-sw* gene is harmful to PC-12 cells and cells are additionally driven to incomplete autophagy-like process. When stimulated by TRAIL or nystatin, CLU protein expression accompanies early phase of autophagy.

1. Introduction

Pheochromocytoma (PCC) is a rare neuroendocrine tumor located in adrenal medulla which secretes massive quantities of catecholamines with malignant hypertension as the fatal outcome. The treatment of choice is surgery associated with high-risk complications (refractory hypertension). In laboratory conditions, pheochromocytoma cells undergo differentiation to neural cells upon treatment with nerve growth factor (NGF) [1]. Alternatively, pheochromocytoma cells could be manipulated with selected gene insertions/silencing leading to additional phenotypic modifications (neural) that cease disproportionate endocrine activity. Accordingly, rat pheochromocytoma cells (PC-12 cell line) are frequently used in *in vitro* studies as a cellular model of neurodegenerative diseases. Alzheimer's disease (AD) is the most prevalent neurodegenerative disease. The hallmark is the extracellular deposition beta amyloid (Aβ) accompanied by dementia and progressive loss of cognitive processes. Familial form (early onset associated with mutations in beta-amyloid precursor protein gene, *APP*) is rare, whereas sporadic form prevails (late onset, 95% of cases). Notably, the molecular mechanisms of neuronal decay in hippocampus and prefrontal cortex in AD remain ambiguous despite extensive studies carried out for more than a century [2]. Formation of Aβ is associated with the activity of membrane bound γ-secretase complex including catalytic role of presenilins (PS-1 and PS-2). This complex attacks remaining of APP just after proteolytic α-secretase (nonfibrillogenic) or β-secretase (fibrillogenic) processing. Shorter isoforms of APP (695 amino acids and less) are specific substrates for α-secretase (ADAM10) while others (751 amino acids and more) are specific for β-secretase

(BACE1). Protein products of mutated *APP* gene (amino acid substitutions) are main targets of BACE1 regardless of APP length [3]. Key players in Aβ formation are located in lipid rafts (LR), nanodomains formed by deposition of cholesterol in membrane lipid bilayers [4, 5]. Lipid rafts are cognate platforms for several signaling pathways including death receptor ligands (DRL) widely known in etiology of AD [6]. Brain is unique in both highly self-sufficient cholesterol (CHOL) metabolism, macroautophagy (autophagy), and DRL (TNF-α, TRAIL) secretion by microglia [7] as either process is independent from peripheral regulation. Thus, autophagy in neurons is constitutive rather than cellular adaptation to nutritional stress as brain is specially protected tissue with constant supply of nutrients even under starvation [8]. Aging is known to differently affect brain CHOL metabolism, autophagy, and DRL activity. Age-dependent onset of neurodegenerative diseases most likely correlates with age-dependent increase of brain cholesterol and DRL secretion [9] as well as age-dependent decline of autophagic activity. The former is associated with altered CHOL metabolism and the latter with unknown changes in control of autophagy and DRL [10]. With the progress of aging, the average concentrations of DRL become elevated [11]. Clusterin protein (CLU) is the rare extracellular ATP-independent chaperone present in both body fluids and tissues where it acts either as cytoprotective (glycosylated-secreted form) or cytotoxic (unglycosylated-intracellular form) factor [12–15]. CLU is upregulated in the brains of individuals affected by neurodegenerative diseases [16, 17], via most likely outcome of ER stress-induced calcium depletion and debilitated N-glycosylation resulted in intracellular accumulation of N-glycan deficient proteins [18]. Although neurodegeneration accompanying cell death mechanism (apoptosis, necrosis, necroptosis (aponecrosis), paraptosis (cell death type 3), or autophagic cell death) is still a matter of debate, ER stress and unfolded protein response (UPR) are frequently associated with impaired autophagy [19, 20].

Autophagy starts when isolation double membrane bilayer (phagophore probably starts off in endoplasmic reticulum) appears in the cytosol, where it engulfs cytoplasmic constituents including organelles. Elongation of the isolation membrane around the region of cytoplasm and closure of the inner and outer bilayers of the isolation membrane leads to formation of double membrane autophagosome. To digest the contents, autophagosome must fuse with lysosome to create autolysosome. Before fusion with lysosome, it may also mingle with endosome to form amphisome. In neurons, autophagosomes are located in the cytoplasm but lysosomes are restricted mainly to juxtanuclear region. Apparently, autophagosomes produced in dendrites or neurites have to be transported to the lysosomes in the cell body by retrograde movement along microtubules. Young neurons achieve this task relatively easily but it is not the case for old neurons. Without efficient autophagy, neurons accumulate ubiquitinated protein aggregates and degenerate [20]. Autophagy can be monitored by targeting proteins present in the outer membrane of autophagic vacuoles. MAP LC3 (microtubule-associated protein 1 light chain 3/MAP1LC3) a small subunit of MAP-1A/MAP-1B is subject of subsequent processing by Atg4B protease while the processed form (LC3-I) is further modified by Atg7 and Atg3 to become coupled with phosphatidylethanolamine (PE), an important component of inner leaflet of lipid rafts. Thus, the PE-conjugated form, designated as LC3-II, is available for embedment with outer and inner membrane of autophagosome [21]. Initiation step in autophagy (selection and sequestration) is the translocation of Beclin 1 (mammalian ortholog of yeast Atg6) and VPS34 assembled in multiprotein complex to preautophagosomal structures (PAS) [22]. Once substrates (proteins and/or organelles) for autophagy are bound to specific receptors (p62, NBR1, NDP52, optineurin (OPTN), histone deacetylase 6 (HDAC6), or NIX) they can join LC3-II and Atg12-Atg5 to become core of future autophagosome [23]. Phagophore elongates with subsequent closure which permits isolation of the cargo trapped in autophagic vacuole surrounded by double membrane [24]. The last step, process of substrate digestion, requires lysosome degradative machinery, and hence autophagosomes fuse with lysosome for the execution phase of autophagy. The principal role of autophagy is to accomplish cellular needs to cope with various stressors such as deficit of essential amino acids or ATP or both, lack of growth factor signals, oxygen debt, build-up of damaged proteins, or endoplasmic reticulum stress [25]. The fundamental task in cellular homeostasis is played by lysosomes. Whatsoever, macroautophagy, mitophagy, or chaperone-mediated autophagy the final common path is lysosomal digestion of sequestered substrates. Obviously, lysosome is the target and stand organelle for primary regulator (mTOR complex I, TORC1) which antagonizes release of amino acids. When TORC1 becomes inactive, the lysosomes encourage autophagy through control of substrate sequestration. Although regulatory network of autophagy is complex and far from complete, the outline of basic processes at the molecular level is generally well known.

In this experiment, we hypothesized that mutated *APP* gene (*APP-sw*) might be helpful in making pheochromocytoma cells less viable and more susceptible to cell death triggering mechanisms. Foundations of assumption were based on results acquired from former study pointing to higher mortality of PC-12 cells transfected with the mutated *APP* gene [26–28]. Nevertheless, up to date the research was forced to study apoptosis and/or necrosis. Our preliminary observations with transmission electron microscopy revealed that PC-12 cells nucleofected with mutated *APP-sw* gene show symptoms of extensive autophagy-like process which is frequently observed in neurodegenerative diseases. The novelty of this study is that it put concern on the process of autophagy widely believed to be survival mechanism, even though its anomalous course leads to cell deletion (cell death type 2). As APP processing is often located in lipid rafts reliant on cholesterol, we tested statins and cholesterol chelator MβCD. Additionally, pheochromocytoma cells are immune to death receptor ligands [29] so alternate use of TNF-α and TRAIL was justified with regard to cell viability, APP processing, and molecular markers of autophagy. Intracellular CLU expression was monitored to check if the protein is accumulated in transgene-bearing cells.

Overall, this study should shed more light on the molecular mechanisms of cell death related to tumor cell differentiation and changes observed in cellular models of neurodegenerative diseases.

2. Materials and Methods

2.1. Materials. Media (Dulbecco's modified Eagle's medium (DMEM) low glucose (5.5 mM), F-12K medium, Kaighn's Modification of Ham's F-12 medium with Glutamax), PBS (including Ca^{2+} and Mg^{2+}), antibiotics, heat inactivated sera (fetal bovine serum (FBS) and horse serum (HS)) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Nerve growth factor (NGF), tumor necrosis factor alpha (TNF-α), and tumor necrosis factor alpha-related apoptosis inducing ligand (TRAIL) (Sigma Aldrich Chemical Co., St. Louis, MO, USA) were dissolved according to manufacturer. Metabolic inhibitors (atorvastatin: ATOR, simvastatin: SIM, nystatin: NY, methyl-beta-cyclodextrin: MβCD, rapamycin: RAPA, and chloroquine: CHLOR) if necessary were dissolved in DMSO. All other reagents were cell culture tested, of high purity, and unless otherwise stated they were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Plastics, including BioCoat Collagen IV coated Cellware, were from Becton Dickinson (BD Biosciences, Franklin Lakes, NJ, USA) and tubes for deep freezing from Nunclon (Nunc, Roskilde, Denmark) while syringe filters were purchased from Corning-Costar Inc. (Cambridge, MA, USA).

2.2. Rat Pheochromocytoma PC-12 Cell Cultures and Treatments. The rat PC-12 cell line was obtained from European Collection of Animal Cell Cultures (ECAAC). Cells were initially suspended in growth media (GM) containing DMEM with Glutamax supplemented with 10% (v/v) fetal bovine serum (FBS) + 5% (v/v) horse serum (HS), pen : strep (penicillin : streptomycin solution, 50 IU/mL/50 μg/mL), gentamicin sulfate 20 μg/mL, and Fungizone (amphotericin B, 1 μg/mL) and plated onto plastic noncoated culture flasks. G-418 (400 μg/mL) and ampicillin (100 μg/mL) were additionally present in GM for growth and selection of positively transfected PC-12 cells. They were cultured at 37°C in a humidified 5% CO_2 and 95% air in incubator. After reaching 70–80% confluence, PC-12 cell suspensions were subcultured, and the same quantity of cells was seeded onto 60 mm Petri dishes, 96-multiwell plates (Becton Dickinson, BD Biosciences, Franklin Lakes, NJ, USA) depending on the experimental protocol. For differentiating and differentiation states when PC-12 cells reached 80% confluence, growth medium was switched to differentiation medium (DM) containing DMEM with Glutamax supplemented with 4% (v/v) fetal bovine serum (FBS) + 2% (v/v) horse serum (HS) + NGF (50 ng/mL) and the same antibiotic : antimycotic mixture. Cells were allowed to differentiate for 48 h and the DM was replaced by freshly prepared serum-free reference medium (RM) containing 0.1% BSA (w/v) + NGF (50 ng/mL) with or without experimental factors for another 48 h (48 h/48 h). When the experimental factors were dissolved in DMSO, the equivalent volume of vehicle (0.1% v/v) was added to the control cells. If necessary the same quantity of differentiated cells with neural phenotype was removed from the culture plates using 0.5% (w/v) trypsin-EDTA (harvesting), centrifuged in DM at 200 ×g for 5 min, media were aspirated, and cell pellets were resuspended in RM. Media were changed every other day. Cell monolayers were harvested for Western blots or PCR. Floating dead cells were removed during media change or washed with PBS and were not included in these experiments.

2.3. Cloning and Expression Vectors of the Homo sapiens Beta-Amyloid Precursor Protein Gene 1-695 (Transcript Variant 3, APP-wt), Swedish Mutation in Homo sapiens Beta-Amyloid Precursor Protein Gene APP-KM670/671NL Double Mutation in the APP Gene Resulting in Amino Acid Substitutions of Lys to Asn (Codon 670) and Met to Leu (671), and PrecisionShuttle Mammalian Vector with C-Terminal Tag GFP (pCMV6-ACGFP). Cloning and expression of the *Homo sapiens* beta-amyloid precursor protein gene 1-695 (transcript variant 3, *APP-wt*), Swedish mutation in *Homo sapiens* Aβ precursor protein gene *APP-KM670/671NL* double mutation in the *APP* gene resulting in amino acid substitutions of Lys to Asn (codon 670) and Met to Leu (671), and PrecisionShuttle mammalian vector with C-terminal tag GFP (*pCMV6-ACGFP*) carrying resistance genes to antibiotics (Neo^r and Amp^r) were ordered in OriGene Technologies, Inc., Rockville, MD, USA, and purchased from STI Bartosz Czajkowski (Poznań, Poland). Both genes (*APP-wt* and *APP-sw*) were expressed under *GFP* promoter (Supplementary material Figure 1 available online at http://dx.doi.org/10.1155/2014/746092). Mock-nucleofected cells (reference, "M") underwent complete procedure except for no vector was added prior to nucleofection.

2.4. Transfection of PC-12 Cells with the Homo sapiens Beta-Amyloid Precursor Protein Gene 1-695 (Transcript Variant 3, APP-wt), Swedish Mutation in Homo sapiens Beta-Amyloid Precursor Protein Gene APP-KM670/671NL Double Mutation in the APP Gene Resulting in Amino Acid Substitutions of Lys to Asn (Codon 670) and Met to Leu (671), and PrecisionShuttle Mammalian Vector with C-Terminal Tag GFP (pCMV6-ACGFP). The procedure for PC-12 cell transfection was based on the method described by the manufacturer (Amaxa Cell Line Nucleofector Kit V, Lonza Cologne AG, Cologne, Germany). Program was used for Nucleofector 4D. In short, 6-multiwell plates were prepared by filling appropriate number of wells with 1.4 mL of media (Ham's F-12 with 15% HS + 2.5% FCS with Glutamax, 1.5 g/L sodium bicarbonate without antibiotics). Plates were preincubated/equilibrated in a humidified 37°C/5% CO_2 incubator. To obtain single cell suspension the PC-12 clusters were passed through a 22-gauge needle (10–15 times). The required number of cells (2×10^6 cells per sample) was centrifuged in sterile Eppendorf tubes (1.5 mL) of molecular purity at 200 ×g for 10 minutes at room temperature. Supernatant was removed completely, and cell pellets were resuspended carefully in 100 μL room-temperature complete nucleofector solution. An aliquot of 100 μL of cell suspension was mixed with

2 μg DNA of appropriate vector. Then, cell/DNA suspension was transferred into certified cuvette and closed with a cap and after selection the appropriate program nucleofection was performed. The cuvettes were left for 15 min in room temperature; afterwards 500 μL of the preequilibrated culture medium was added to the cuvette and samples were gently transferred into the prepared 6-well plate (final volume 1.9 mL media per well). Cells were analyzed 24 hours after nucleofection using light (A) and fluorescence microscopy (B) (supplementary material Figure 2). Average transfection efficiency of PC-12 cells after 24 hours after nucleofection was analyzed by flow cytometry. Viability was measured by using MTT assay. One day after nucleofection, cells were used to carry out the experiments.

2.5. Determination of Cell Viability. Cell viability was based on the ability of cells grown on 96-multiwell plates to convert soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] into an insoluble purple formazan reaction product with minor modifications to protocol described [30]. Briefly, cells were uniformly seeded in 96-multiwell flat bottomed plates and grown in GM. Confluent cultures were washed with PBS and then exposed to DM for 48 h. Afterwards, DM was replaced by RM with or without experimental factors for another 48 h. Relative viability (percentages of mean control value) was evaluated. To do this, media were removed and cells were washed with PBS and were further incubated with MTT for 1 h at 37°C in a humidified 5% CO_2 and 95% air in incubator. Next, MTT solution was removed and water insoluble formazan was immediately dissolved in DMSO. Alternatively, cell viability was determined on the basis of modified lysosomal uptake of neutral red dye [31]. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes, whereas nonviable cells will not take up the dye. PC-12 cells were grown in 96-multiwell flat-bottomed plates in GM. After reaching confluence, cells were switched into postmitotic status by incubation in DM (differentiation) for 48 h. Next, wells were immersed with RM (CTRL) and experimental media for another 48 h (percentages of control value). For the additional 1 h of incubation, these media were replaced by 50 μL neutral red (NR) reagent (5 mg/mL in PBS, sterilized by filtration). After incubation, the medium was aspirated and cells were washed with PBS. Cell monolayers were allowed to dry at ambient temperature, and neutral red accumulated within lysosomes of living cells was dissolved by addition of 100 μL DMSO (70% in H_2O).

The absorbances for MTT and neutral red were measured at 490 and 550 nm, respectively, with ELISA reader type Infinite 1000 (TECAN, Austria). Relative percentages (versus nontreated controls) of viable cells were measured by MTT conversion into purple formazan and accumulation of neutral red in intact lysosomes, respectively.

2.6. Determination of Apoptosis and Necrosis. To evaluate apoptosis (percent YO-PRO-1 positive cells) and necrosis (percent propidium iodide positive cells) the cells were seeded at black 96-well multiplates with transparent bottom (BD Biosciences, Franklin Lakes, NJ, USA). Confluent cultures were washed with PBS and then exposed to DM for 48 h. Afterwards, DM was replaced by RM with or without experimental factors for another 48 h. Relative percentages of apoptosis and necrosis (versus nontreated controls) were measured according to the method adapted from Plantin-Carrenard et al. [32]. To do this, media were removed, cells were washed with PBS and were further incubated with YO-PRO-1 (1 μM), and propidium iodide (10 μg/mL, PI) dissolved in PBS for 0.5 h at 4°C on ice in the dark. After incubation, YO-PRO-1 and PI accumulated in apoptotic and necrotic cells, respectively. The validity of the method was verified by observations in fluorescent microscope (Olympus IX71 Series, Osaka, Japan). For YO-PRO-1, the fluorescence was measured using the optimum wavelengths of 485 nm (λ_{ex}) and 530 nm (λ_{em}). Simultaneously, the fluorescence of PI bound to nucleic acids was measured using the optimum wavelengths of 590 nm (λ_{ex}) and 630 nm (λ_{em}).

2.7. Antibodies, Immunoblotting, and Microscopic Imaging. For analysis of protein expression, 30 μg of protein isolated from whole-cell lysates and wide-range molecular weight standards (Precision Plus Protein Kaleidoscope, Bio-Rad Polska, Warsaw, Poland) was electrophoresed on a 7.5, 10, or 12% acrylamide SDS-PAGE gels and immunoblotted onto polyvinylidene difluoride Immun-Blot PVDF membranes (Bio-Rad Polska, Warsaw, Poland). The membranes were blocked for 1 h in room temperature either with 5% nonfat dry milk (NFDM) in TBST (NaCl 137 mM, KCl 2.7 mM, and Tris base 19 mM) or in 5% BSA in TBST (depending on the antibody used). Cells were cultured with or without experimental factors indicated in figure legends, harvested, washed, and lysed with RIPA lysis buffer (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was added. To lyse the cell pellets, cells were broken up by repetitive triturating with the syringe with attached needle (21 G, 0.8 mm diameter). Cell suspension was then left on ice (4°C) for 30 min and centrifuged for another 5 min (4°C, 8,000 ×g). Next, viscous solution was divided into smaller volumes and transferred to fresh Eppendorf tubes and stored at −80°C until being used. For protein quantification in the whole-cell lysates, a protein-dye-binding method [33] with commercial reagent was used (Bio-Rad Laboratories, Hercules, CA, USA).

Antibodies against listed proteins were used: actin, clusterin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), APP, sAPPα, beta amyloid 1-16 (6E10) (Covance, Inc., NY, USA), APP (Merck Millipore, Darmstadt, Germany), MAP LC3 (Novus Biologicals, Cambridge, UK), and VPS34 (Thermo Fisher Scientific, Pierce Biotechnology, IL, USA). Working antibody concentrations (from 1 : 200 to 1 : 2000) varied depending on the protein detected and were applied according to the manufacturer's recommendation. Secondary polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against respective species and conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence assay (Amersham International, Aylesbury, UK). After exposure and processing the films were scanned and analyzed using Kodak EDAS 290/Kodak 1D 3.5 system.

Morphological changes and cell survival were monitored under an inverted phase-contrast and fluorescent microscope, respectively (Olympus CK40, model ICD703WP, and Olympus IX71 Series, Osaka, Japan). The formation of neural cells was monitored by obtaining photographs using digital camera (supplementary material Figure 2, Olympus Camera, Tokyo, Japan).

Demonstration of the presence and intracellular location of certain modifications (autophagosomes, autophagic vacuoles, and multivesicular and multilamellar bodies) was based on ultrastructural studies (transmission electron microscopy (TEM)). Cells were fixed in 2.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Cells were washed with the same buffer and postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h. Next, cells were dehydrated in a graded ethanol alcohol series and embedded in Epon 812. Ultrathin sections were mounted on copper grids, air-dried, and stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. The sections were examined and photographed with a JEOL JEM 1011 electron microscope (Jeol, Tokyo, Japan).

2.8. Cellular Aβ 1-40 Assays. Production of Aβ 1-40 was measured in PC-12-transfected cells expressing wild-type human *APP* (*APP-wt*, W), Swedish mutated APP (*APP-sw*, S), empty vector (*GFP* only, G), and complementary DNA (cDNA). Cells were seeded overnight at 3×10^4 cells per well in a 96-multiwell plate. Cells were incubated in DM for 48 h and washed with PBS, and fresh RM media were added for another 48 h with or without experimental factors. Next, cellular media were harvested and assayed for the presence of Aβ 1-40 with an Aβ 1-40 homogenous time resolved fluorescence (HTRF) assay (CisBio) according to manufacturer's instructions. For all cell types (W, S, and G), Aβ 1-40 values were normalized for cell viability, as determined with the MTT assay.

2.9. Statistical Analysis. Each experiment was repeated at least three times. The data are expressed as the means ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Kruskal-Wallis, Tukey's, Newman-Keuls, or Benferroni multiple range test. Regression analysis (linear) was carried out to draw appropriate standard curves. P values of less than 0.05 were considered statistically significant. Statistical differences from nontreated control cells were indicated by different lower case letters ($P < 0.05$, bar charts). Statistical analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Viability of Nucleofected PC-12 Cells Is Markedly Reduced by APP (APP-sw) but It Is Not Affected by TNF-α, TRAIL, Atorvastatin, Simvastatin, Nystatin, or MβCD. Nucleofected PC-12 cells (*GFP* vector, *GFP* vector + *APP-wt,* or *GFP* vector + *APP-sw*) after two days of differentiation in DM were left untreated or treated with TNF-α (10 ng/mL), TRAIL (10 ng/mL), atorvastatin (5 μM), simvastatin (5 μM), nystatin (1 μM), or MβCD (0.2 μM) for another two days in RM. Cell viability (MTT and NR assays) expressed as percent of control value (untreated cells) was significantly reduced after *APP-sw* cell nucleofection (Figures 1(a) and 1(b), supplementary material Figure 4). No effect of the above-mentioned experimental factors was observed irrespectively to nucleofection (*GFP* vector, *GFP* vector + *APP-wt,* or *GFP* vector + *APP-sw*) ($P > 0.05$). In turn, percentage of YO-PRO-1 and PI-positive cells in relation to nontreated cells (% control) did not differ markedly between the transgene or type of treatment (data not shown). It points to other than apoptosis and necrosis cell death mechanism responsible for lower cell viability after *APP-sw* cell nucleofection.

3.2. Production of β-Amyloid 1-40 Is Markedly Elevated in GFP Vector + APP-sw Nucleofected Cells Comparing to GFP Vector and GFP Vector + APP-wt-Nucleofected Cells. The concentration of immunoreactive β-amyloid 1-40 (nonfibrillogenic form) in supernatants of *GFP* vector + *APP-sw*-nucleofected cells collected from 96-multiwell plates used to determine cell viability increased significantly to 53.58 pg/mL (Figures 1(c) and 1(d)). No changes in β-amyloid 1-40 concentration were found in supernatants collected from remaining untreated cell cultures (*GFP* vector and *GFP* vector + *APP-wt*, $P > 0.05$). Neither administration of TNF-α, TRAIL, atorvastatin, simvastatin, nystatin, or MβCD led to significant elevation of β-amyloid 1-40 concentration in supernatants collected from *GFP* vector + *APP-sw* nucleofected cells as the observed increase was at the cut-off line for HTRF method (according to manufacturer β-amyloid 1-40 detected in supernatants at concentration of 30 pg/mL and below has to be neglected Figures 1(c) and 1(d)).

3.3. GFP Vector + APP-sw-Nucleofected PC-12 Cells Show Symptoms of Excessive Formation of Autophagosomes and Multivesicular Bodies but Not Autolysosomes. Ultrastructural examination of PC-12-nucleofected cells (*GFP* vector, *GFP* vector + *APP-wt*) with TEM revealed little evidence of autophagy. As shown in respective micrographs, the symptoms of autophagy-like process were observed regardless of nucleofection (*GFP* vector, *GFP* vector + *APP-wt,* or *GFP* vector + *APP-sw*); however, the intensity of autophagic phenotype was considerably advanced in *GFP* vector + *APP-sw*-nucleofected PC-12 cells (Figure 2). There is scarce evidence for ER abnormalities in micrographs representing *APP-sw*-nucleofected PC-12 cells (polysomes and reticular tubules are regularly distributed). Nevertheless, as some tubules seem to be distended we suggest that it may represent phagophores in the elongation phase.

3.4. Expression of Clusterin Protein Is Not Affected by Nucleofection (GFP Vector, GFP Vector + APP-wt, or GFP Vector + APP-sw) Although It Is Markedly Increased after TRAIL or Nystatin Administration; Additional Treatment with Rapamycin (1 μM) or Chloroquine (30 μM) for 1 Hour Revealed the Highest Expression of MAP LC3-I, LC3-II,

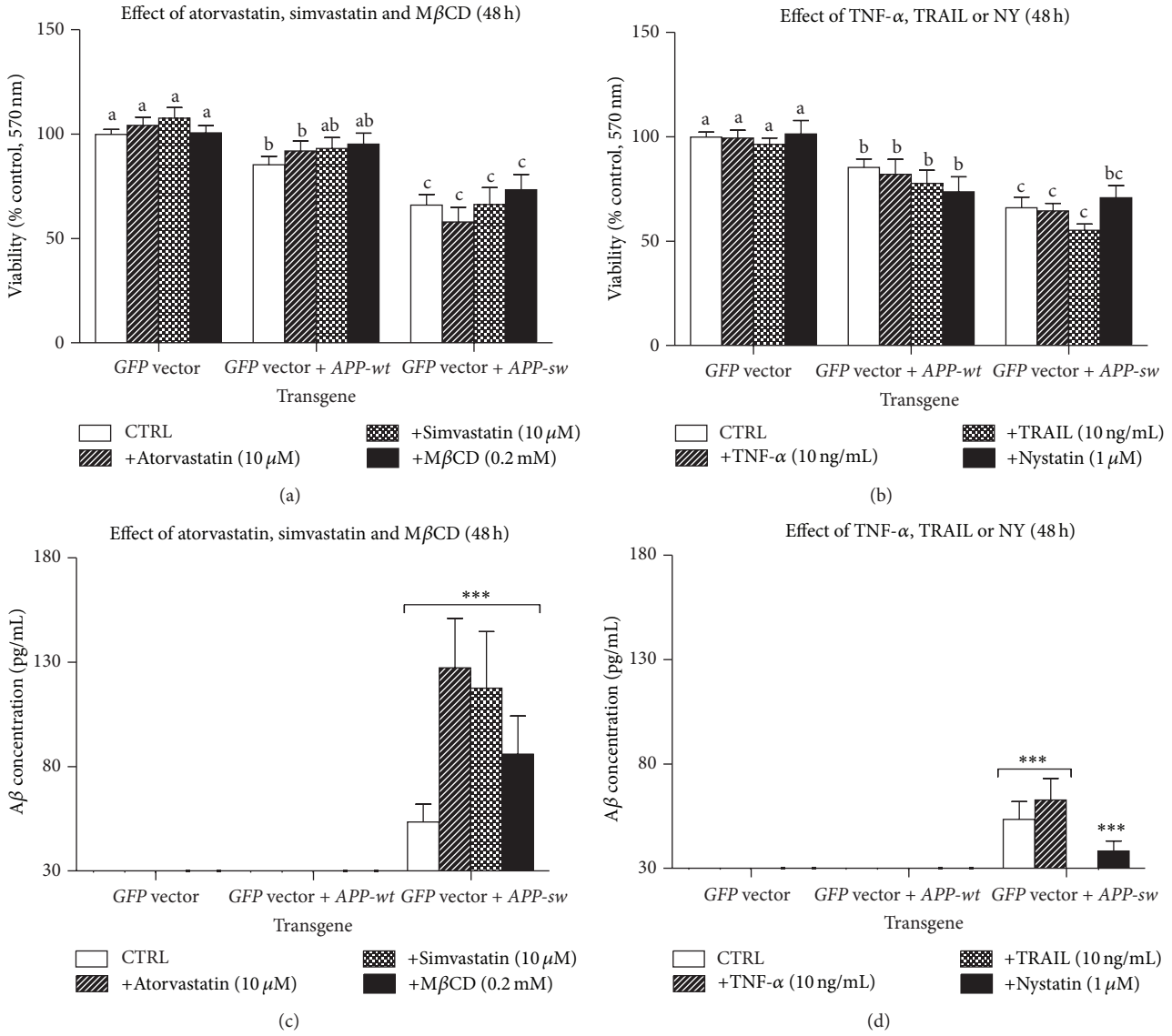

Figure 1: Bar charts (means ± SEM) represent cell viability (MTT assay, upper panel) or Aβ 1-40 concentration in supernatants (HTRF assay, lower panel) normalized for cell viability as determined with the MTT assay measured in the same wells of multiwell plates. Values are expressed as % of control (untreated PC-12 cells nucleofected with *GFP*, or *GFP* + *APP-wt*, or *GFP* + *APP-sw*). ((a)–(c)) The effect of atorvastatin (10 μM), simvastatin (10 μM), or MβCD (0.2 mM); ((b)–(d)) the effect of TNF-α (10 ng/mL), TRAIL (10 ng/mL), or nystatin (1 μM). Different lower case letters indicate statistically significant differences between means ($P < 0.05$).

and VPS34 Proteins in GFP Vector + APP-sw-Nucleofected Cells. Immunoblotting carried out with PVDF membranes loaded with proteins separated by SDS-PAGE from six subsequent nucleofections (1-6) unraveled that *APP* genes had no significant effect on the expression of secretory clusterin (sCLU, Figure 3(a)). Expression of clusterin protein rose, however, after treatment with TRAIL or nystatin (Figure 3(b)). The sAPPα protein (α-secretase product) was exclusively observed in *GFP* vector + *APP-wt*-nucleofected cells, except lane 5 representing mixture of *GFP* vector + *APP-wt* plus *GFP* vector + *APP-sw*-nucleofected cells (positive control) (Figure 3(a)). In turn, total APP protein expression (unprocessed and processed form) was exclusively detected in *APP* gene-nucleofected cells (*GFP* vector + *APP-wt* and *GFP* vector + *APP-sw*, Figure 3(a)). In contrast, the expression of immunoreactive β amyloid 1-16 (any form) was absent in *GFP* vector and *GFP* vector + *APP-wt* and found solely in *GFP* vector + *APP-sw*-nucleofected cells (Figure 3(a)). Markers of autophagy (VPS34, MAP LC3-II, and Beclin 1) were also monitored with WB. Functionally active MAP LC3-II (LC3-II) was detected in *GFP* vector, *GFP* vector + *APP-wt*-, and *GFP* vector + *APP-sw*-nucleofected cells (Figure 3(a)). Interestingly, expressions of early autophagy marker VPS34 (class III phosphoinositide 3-kinase, PI3K III) and LC3-II were significantly elevated after TRAIL, nystatin, simvastatin, or MβCD administration (Figure 3(b)).

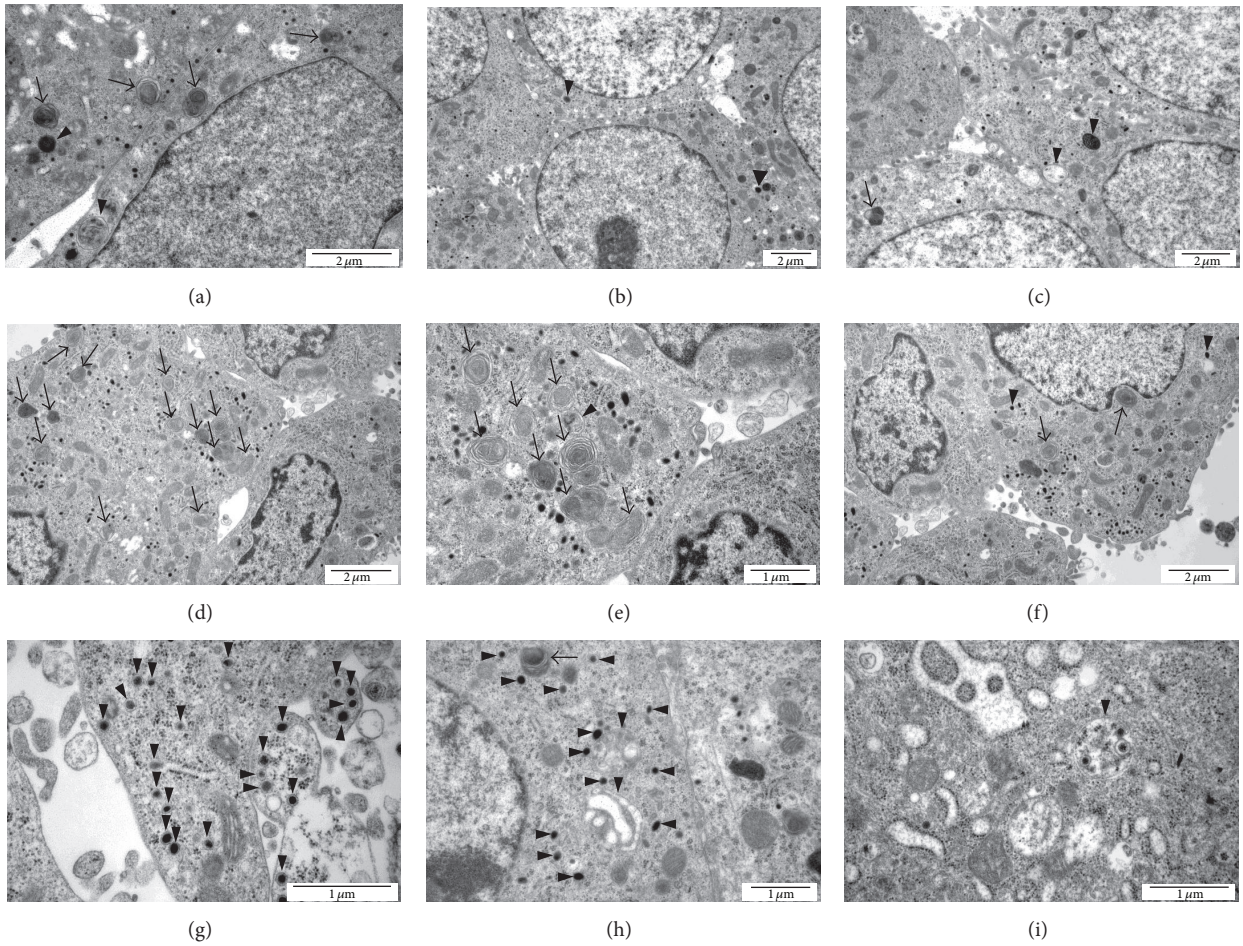

Figure 2: Micrographs (TEM) show ultrastructure of PC-12 cells nucleofected with *GFP* ((a)–(c)), or *GFP* + *APP-wt* ((d)–(f)), or *GFP* + *APP-sw* ((g)–(i)). Symptoms of autophagy: black arrows indicate multilamellar bodies and black arrowheads indicate autophagosomes, multivesicular bodies, and lysosomes. Abnormal autophagy in *GFP* + *APP-sw*-nucleofected cells is manifested by buildup of autophagosomes with double membrane (g) and multivesicular bodies (h); large autophagosome with double membrane contains smaller autophagosomes (i) and there is scarce evidence for single-membrane autolysosomes. Bars represent 100 μm.

TNF-α and atorvastatin apparently diminished clusterin and LC3-II expressions, even though they did not affect VPS34 expression levels (Figure 3(b)). No effect of treatment was found in the expression of Beclin 1, protein important at the sequestration stage of autophagy (Figure 3(b)). To make the issue of autophagy clearer, additional "flux" experiment was performed. After the experiment ended, the cells were additionally treated with rapamycin (1 μM) or chloroquine (30 μM) for 1 hour. As it is presented in supplementary material Figure 5, the highest expression of LC3-I, LC3-II, and VPS34 proteins was observed in *GFP* vector + *APP-sw*-nucleofected cells.

4. Discussion

Incompetent autophagy causes decline of cell viability resulting from accumulation of nonfunctional organelles, proteins, and protein aggregates. Progressive changes inevitably end up in cell death but the precise link mechanisms and type of cell death beneath are unknown. We could not demonstrate that reduced viability of nucleofected cells was caused by apoptotic and/or necrotic cell death as percent of YO-PRO-1 and PI-positive cells did not differ significantly irrespectively of transgene or treatment (data not shown). Discussion about any other types of cell death (necroptosis and/or paraptosis) that could account for drop in percentage of viable cells in this experiment would be highly speculative. The paradox of autophagy is that it is essential for keeping cellular homeostasis, and consequently any disruption of this homeostasis results in severe effects [34]. Autophagy impairment as a method of tumor cell elimination provides strong rationale for developing strategies other than apoptosis induction. Lysosomal membrane permeabilization (LMP) which is known to occur in lysosome storage diseases (LSD) represents large group of disturbances where autophagy went wrong. LMP is frequently observed in AD where it is associated with elevated lysosomal pH [35]. Definitely, nucleofection of PC-12 with mutated *APP* gene was confirmed at genomic (PCR) and translational levels (WB) (Figure 3(a), supplementary material Figure 3). It led to significant drop of

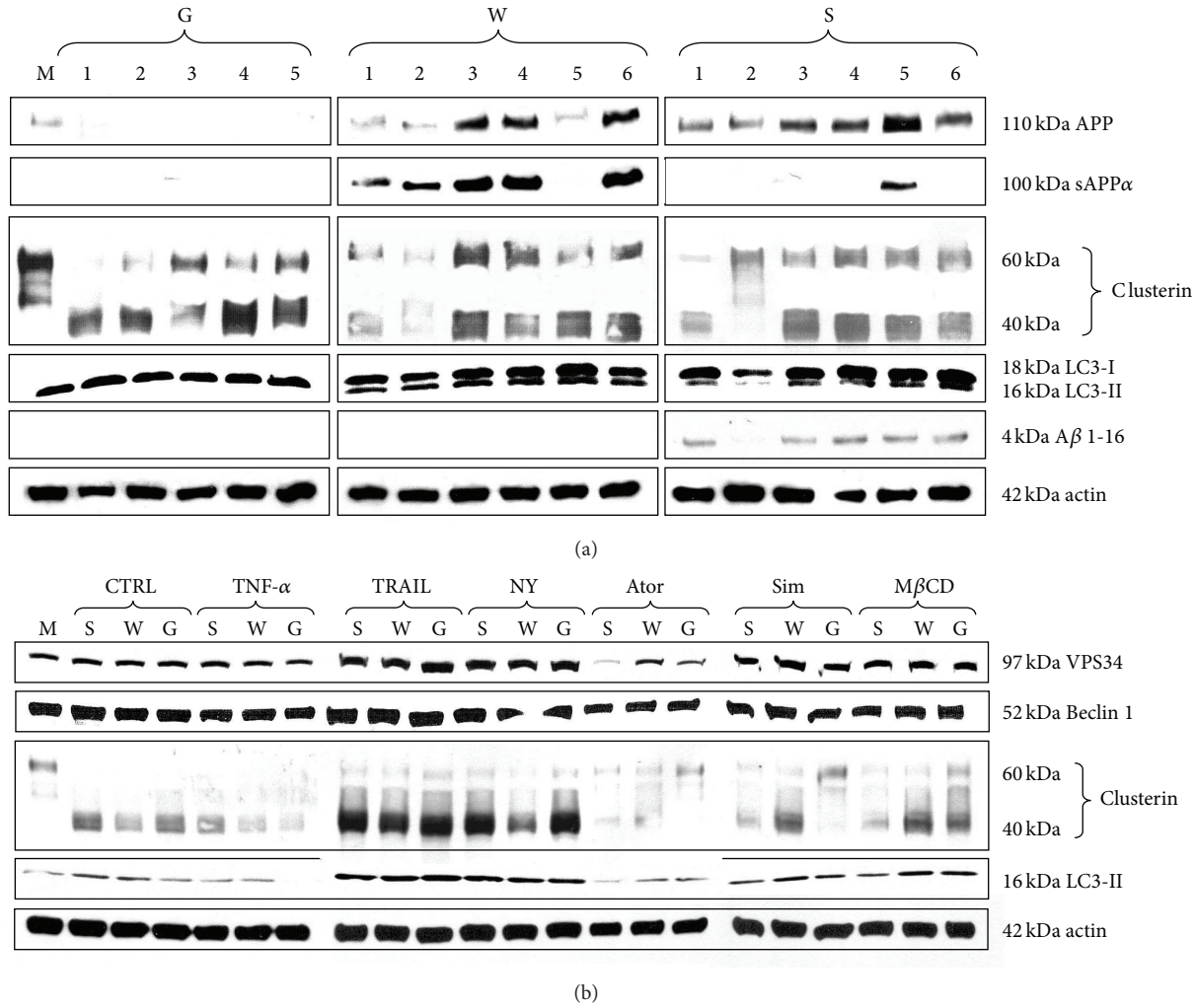

Figure 3: Analysis of protein expression. Letter "M" indicates mock-nucleofected cells, while G, W, S stand for PC-12 cells nucleofected with *GFP* vector (G), or *GFP* vector + *APP-wt* (W), or *GFP* vector + *APP-sw* (S), respectively. (a) Identification of APP, sAPPα, clusterin, LC3-II, Aβ 1-16, and beta-actin in whole cell lysates isolated from six subsequent nucleofections; (b) identification of VPS34, Beclin 1, clusterin, LC3-II, and beta-actin in whole cell lysates isolated from cells nucleofected with *GFP* vector (G), or *GFP* vector + *APP-wt* (W), or *GFP* vector + *APP-sw* (S).

cell viability (by 36%) accompanied by prevalent autophagy-like process. Possible ultrastructural modifications of ER including excessive formation of phagophores might result from the expression of mutated form of APP and impaired lysosome fusion with autophagosomes. Why the *APP-sw*-nucleofected PC-12 cells have nonfunctional modifications in cellular organelle essential for autophagy is not known, although numerous papers emphasize the importance of this process in pathogenesis of Alzheimer's disease [19, 20]. Moreover, the autophagy could not reverse lower cell viability suggesting that it could go awry. Cautious analysis of micrographs obtained in TEM provided evidence of extensive formation of autophagosomes as well as numerous multivesicular bodies with little presence of autolysosomes in *APP-sw*-nucleofected PC-12 cells (Figures 2(g)–2(i)). Neither experimental factor affected cell viability confirming extreme resistance of PC-12 cells to death receptor ligands (TNF-α, TRAIL) or cholesterol inhibitors (atorvastatin, simvastatin, nystatin, and MβCD), (Figure 1(a)). Previously, statins were reported to induce autophagy and inhibit viability of cancer cells [36–38]. Apparently, in our study lower cell viability had predominantly something to do with changes induced by the expression of *APP-sw* gene ($P < 0.001$ by two-way analysis of variance). Actually, significant rise of human Aβ 1-40 secretion was solely noticed in *APP-sw*-nucleofected PC-12 cells (from undetectable levels to 53.83 pg/mL, $P < 0.05$). Elevated concentration of Aβ in supernatants collected from *APP-sw*-nucleofected PC-12 cell cultures was substantiated by the results of WB. The expression of Aβ 1-16 peptide (any form: Aβ 1-40, Aβ 1-42, and Aβ 1-43) was detected in *APP-sw*-nucleofected but not in *GFP* vector- or *GFP* vector + *APP-wt*-nucleofected cell cultures (Figure 3(a)). It is important to stress that each form of Aβ evokes distinct effects in affected cells. In general, AD pathology and neuronal death are

associated with excessive production of Aβ, but mainly Aβ 1-42 peptide is severely fibrillogenic (the source of fibrilles and senile plaques). In turn, the expression of sAPPα, the protein product of α-secretase which rules out the formation of Aβ, was found entirely in *GFP* vector + *APP-wt*-nucleofected cell cultures (Figure 3(a)). The latter observation authenticates APP695 as highly specific substrate for α-secretase activity. Finally, total APP expression levels (both processed and unprocessed form) measured by immunoblotting showed this protein in *GFP* vector + *APP-wt*- and *GFP* vector + *APP-sw*-nucleofected cells only (Figure 3(a)). These results are consistent with a common view addressing importance of Aβ in etiology and pathogenesis of AD and also advocate assumption that Aβ is harmful to PC-12 cells. How does Aβ affect autophagy is not known at present and needs additional scrutiny. Expression of clusterin (CLU) protein which represents unique ATP-independent extracellular chaperone was measured to find out if this protein is affected by nucleofection, treatment, and autophagy. No changes were detected irrespectively to type of nucleofection in six subsequent nucleofections (Figure 3(a)). The expression of protein markers of initial steps of autophagy (VPS34 and LC3-I, LC3-II, and Beclin 1) did not differ between transgenes or the type of treatment (Figures 3(a) and 3(b)) but the image markedly changed after final 1-hour treatment with rapamycin (1 mM, RAPA) or chloroquine (30 mM, CHLOR). This "flux" experiment demonstrated that autophagy was incomplete and has the highest rate in the *GFP* vector + *APP-sw*-nucleofected cells as autophagy inhibitor at the level of autolysosome formation (CHLOR) led to the accumulation of LC3-I/ LC3-II (supplementary material Figure 5). Additionally, autophagy stimulator rapamycin was unable to reverse the effect of chloroquine. Thus, it is essential to monitor autophagy with including stimulators/inhibitors (RAPA, CHLOR). The use of inhibitor (CHLOR) revealed that lack of differences in the expression of VPS34 and LC3-I, LC3-II, and Beclin 1 between the *GFP* vector and *GFP* vector + *APP-wt*-nucleofected cells was probably due to lysosome-dependent degradation. As mentioned earlier the LC3-I is activated by APG7L/ATG7, transferred to ATG3, and conjugated to phospholipid (PE) to form LC3-II. It should be stressed that soluble form of LC3-II is not observed in PC-12 cells and thus LC3-II bands shown in the respective immunoblots represent LC3-II anchored to phagophores. However, similarly to VPS34 and LC3-II, CLU expression levels increased in response to TRAIL or nystatin treatment pointing to possible involvement of this protein in early phase of autophagy. Given that TRAIL or nystatin administration was able to increase expression of incompletely glycosylated 40 and 60 kDa CLU variants (Figure 3(b)) in either type of transgene bearing cells, it remains unclear whether this effect is directly linked to autophagy. It is not possible that clusterin simply is induced by TRAIL and NY in all types of cells and has nothing to do, at least here, with autophagy. As far as we know, this is first report showing that VPS34, LC3-II, and CLU are elevated in parallel when PC-12 cells are challenged with TRAIL or nystatin. No obvious link between these proteins and clusterin was demonstrated so far.

5. Conclusions

Pheochromocytoma PC-12 cells are completely resistant to treatment with death receptor ligands (even two days with 100 ng/mL of TNF-α or TRAIL combined with 1 nM of actinomycin D had no effect on cell viability, data not shown). Nonetheless, cell viability dropped significantly in *GFP* vector + *APP-sw*-nucleofected cells but not in *GFP* vector- or *GFP* vector + *APP-wt*-nucleofected cells. At the same time just *GFP* vector + *APP-sw*-nucleofected cells expressed Aβ 1-16 and secreted Aβ 1-40. At the same time profound autophagy-like process occurred with numerous autophagosomes and multivesicular bodies but with scarce evidence of autolysosomes. Summing up, human *APP-sw* gene is apparently destructive to PC-12 cells as cells are driven to incomplete autophagy-like process. It seems that CLU protein accompanies early phase of autophagy (isolation and sequestration).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Combined Epidermal Growth Factor Receptor and Beclin1 Autophagic Protein Expression Analysis Identifies Different Clinical Presentations, Responses to Chemo- and Radiotherapy, and Prognosis in Glioblastoma

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Dysregulated EGFR in glioblastoma may inactivate the key autophagy protein Beclin1. Each of high EGFR and low Beclin1 protein expression, independently, has been associated with tumor progression and poor prognosis. High (H) compared to low (L) expression of EGFR and Beclin1 is here correlated with main clinical data in 117 patients after chemo- and radiotherapy. H-EGFR correlated with low Karnofsky performance and worse neurological performance status, higher incidence of synchronous multifocality, poor radiological evidence of response, shorter progression disease-free (PDFS), and overall survival (OS). H-Beclin1 cases showed better Karnofsky performance status, higher incidence of objective response, longer PDFS, and OS. A mutual strengthening effect emerges in correlative power of stratified L-EGFR and H-Beclin1 expression with incidence of radiological response after treatment, unifocal disease, and better prognosis, thus identifying an even longer OS group (30 months median OS compared to 18 months in L-EGFR, 15 months in H-Beclin1, and 11 months in all GBs) ($P = 0.0001$). Combined L-EGFR + H-Beclin1 expression may represent a biomarker in identifying relatively favorable clinical presentations and prognosis, thus envisaging possible EGFR/Beclin1-targeted therapies.

1. Introduction

Glioblastoma (GB) is the most frequent primary brain neoplasm and one of the most lethal tumors. Standard treatment is the maximal safe surgical resection followed by adjuvant radiotherapy (RT) and chemotherapy (CHT) [1]. Despite this multimodal treatment, prognosis remains poor, with less than 5% of the patients alive beyond five years from diagnosis [2]. Extensive multiplatform genomic characterization is increasing our understanding of the molecular bases of GB and is leading to the discovery of promising novel therapeutic targets, although efficient new treatments are still not available [3–5].

Epidermal growth factor receptor (EGFR) gene mutations, amplification and overexpression, EGFR protein overexpression, and PI3K-Akt-mTOR-EGFR pathway dysregulation are hallmarks of GB, usually related to an aggressive phenotype [6] and characterize the most frequent GB molecular subtype showing the classical expression profile [7].

The PI3K-Akt-mTOR signaling pathway, driven or not by EGFR activation, negatively regulates autophagy [8]. Autophagy is a degrading, self-eating cellular process involved in an array of physiological and paraphysiological cellular functions [9]. Its relevance is emerging also in cancer, in which, based on cell context, tumor type, and stage, autophagy may play different roles [10]. While autophagy halts tumor initiation, in advanced cancer it can either promote tumor progression, allowing cell survival, or lead to cell death [11]. Autophagy-related death, also known as "type II programmed cell death," has been recognized as a major type of nonapoptotic death in GB, both *in vivo* [12] and *in vitro* [13], being induced by RT and CHT [14, 15]. Thus, novel autophagy-based GB treatment approaches may be envisaged. Therapeutic perspectives also derive from the complex crosstalk between autophagy genes and apoptotic and other types of cell death [16, 17]. The autophagic gene *Beclin1* and its complex with either Bcl2 or Vps34, the Class III PIK involved in autophagosome initiation, are key determinants of autophagy and cell fate [18].

Beclin1 also binds EGFR [19] and EGFR is able to directly regulate Beclin1 and autophagy also in an mTOR-independent manner [19]. EGFR promotes tumor growth and cell motility [20] and has been associated with a poor clinical GB outcome and unfavorable GB presentation [21]. Beclin1 expression, instead, decreases with tumor progression [22, 23], and we observed also that it is positively correlated with a better GB patient clinical outcome [24]. In a recent study, we found that the modulation of autophagy and EGFR expression has an impact on GB cell migration activity and response to radiation treatment [25].

We are not aware of previous studies correlating EGFR and Beclin1 expression with clinical features in GB. Here, we retrospectively analyzed the potential relevance of concomitant Beclin1 and EGFR protein expression and examined their colocalization in a series of patients affected by GB, aiming at investigating clinical implications of the patterns of their expression in GB tissue, out of a patient series undergoing postoperative CHT-RT.

2. Materials and Methods

2.1. Ethics Approval. This study was approved by both the Institutional Review Board and Ethics Committee of the University Hospital of Siena, and all the patients had provided signed informed consent before any treatment.

2.2. Patients. We retrospectively reviewed the medical records of patients affected by GB (Grade IV-WHO Classification [26]) submitted to the Radiation Oncology Unit of the University Hospital of Siena for postoperative adjuvant CHT-RT from February 2002 to November 2013. Patients who had undergone a full-course RT and CHT (i.e., standard RT and concurrent and sequential temozolomide (TMZ) administration) were included in the study, whereas patients enrolled in clinical trials with experimental RT/CHT with antiangiogenetic, anti-EGFR, and any other targeted therapy were excluded.

Patients were referred, treated, and followed up on a three-month interval basis after therapy in our unit. All clinical and pathological data were recorded, including extent of surgery and histological diagnosis, clinical examination, blood counts and chemistry, Karnofsky performance status (KPS), neurological performance status (NPS), chest X-ray, and pre- and postoperative and follow-up magnetic resonance imaging (MRI).

2.3. Postoperative Treatment. RT and TMZ CHT schedules were adopted as previously described, according to a protocol-driven schedule [27]. Briefly, RT consisted of a 54–60 Gy total dose administered with three-dimensional conformal irradiation in all cases with a unifocal presentation. The planning target volume (PTV) [28] included residual tumor mass and postoperative cavity with a 2-3 cm margin. Suitable patients with small lesions received a boost dosage up to 70 Gy, limited to the gross tumor volume (GTV) [28] if no obvious progression or relevant toxicity occurred during the previous irradiation course. Patients with multifocal lesions were submitted to whole brain irradiation, up to 50 Gy. Five weekly 1.8–2 Gy sessions were administered during the entire RT course, in all cases. All patients received TMZ concurrent with RT (75 mg/mq/die) up to a maximum of 7 weeks, and most of them also received sequential TMZ CHT (150–200 mg/mq for 5 days, every 28 days), unless tumor progression or relevant toxicity occurred. The patients included in this evaluation completed at least 80% of the planned treatment.

2.4. Follow-Up. After treatment, all patients were included in a follow-up program. General and neurological examinations, with blood counts and chemistry, were performed every three months, as previously outlined.

2.5. Clinical Variables Included in the Study. Age: A cut-off value of 50 years (≤50 y, >50 y) was established according to literature [2].

KPS: Two categories were considered, 100–80%; ≤70%, after surgery.

NPS: Patients were assigned to five categories after surgery (1: no neurological impairment; 2: some neurological impairment; 3: moderate impairment; 4: major functional impairment; 5: no conscious response), according to the Medical Research Council Brain Tumor Working Party [29].

MRI disease presentation (unifocal versus synchronous multifocal disease) was assessed at the preoperative MRI examination. Multifocality consisted of at least two lesions at the gadolinium-enhanced T1 sequence, separated by a distance of at least 1 cm.

After RT and concurrent TMZ administration, MRI was repeated, in order to assess the subsequent tumor volume evolution with respect to pre-RT status, the first time at 2-3

weeks after completion, then on a 3-month basis, and in any case of suspicion of tumor progression on clinical grounds.

Radiological response (RR) was so detected and classified into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) at the first MRI examination. Objective response is defined as OR = CR + PR. RR was assessed using either MacDonald's criteria [30]or response assessment in neurooncology (RANO) criteria [31], respectively, before and after 2010.

2.6. Molecular Determination of the Methylation Status of the MGMT Gene Promoter. MGMT gene promoter methylation was assessed by methylation-specific polymerase chain reaction (PCR). Briefly, genomic DNA was extracted from paraffin-embedded tumor sections and treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (HISS Diagnostics, GmbH, Freiburg, Germany). Primer sequences were used to detect methylated and unmethylated MGMT promoter sequences. PCR products were separated on 2% agarose gel. A glioma cell line, with a completely methylated MGMT promoter, and peripheral blood mononucleated cells served as positive and negative control samples, respectively.

2.7. EGFR and Beclin1 Immunohistochemistry. In each case, 3 μm thick sections were cut from paraffin blocks of 10% formalin-fixed tumor fragments and processed for immunohistochemistry. Briefly, after deparaffinization and rehydration, before applying the anti-EGFR mouse monoclonal (clone EGFR.25, ready to use, and catalogue number: RTU-EGFR-384, Novocastra, Milan, Italy) or the anti-Beclin1 rabbit polyclonal (gene ID: 8678, amino acids 329–345, diluted 1 : 200, and catalogue number: B6186, Sigma-Aldrich, Milan) primary antibodies, sections were pretreated either with Pronase XIV of *Streptomyces griseus* (Bio-Optica, Milan, Italy) at 37°C for 10 minutes or with WCAP citrate buffer pH 6.0 (Bio-Optica), for 40 min at 98.5°C, respectively.

The evaluation of the signal was performed by Ultra-Vision LP Large Volume Detection System HRP Polymer (Bio-Optica, Milan), with the diaminobenzidine chromogen (Dako) for 8 min. Sections were then counterstained with Meyer's hematoxylin. In all cases, negative controls were performed by repeating the procedure and omitting the primary antibody.

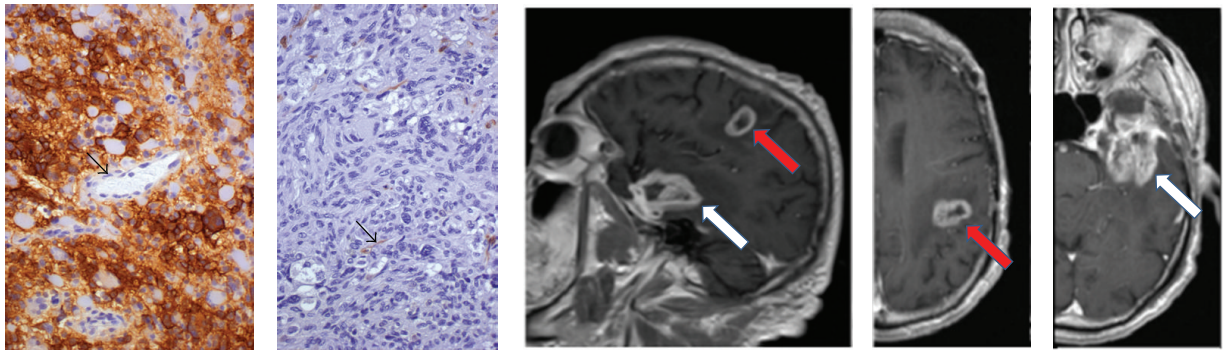
2.8. Assessment of Immunostaining. Staining was independently evaluated by two of the authors (CM, MAGMB), at medium resolution (20x objective, eye piece 1.25) all throughout tumor sections. EGFR membranous and/or cytoplasmic and Beclin1 cytoplasmic immunoreactivity scored 0 if negative and from 2 to 5, if positive, on the basis of both the stain's intensity (1: weak, 2: moderate, and 3: strong) and the percentage of positive cells (1: ≤50%, 2: >50%). We considered scores 0–2 as a low (L) and scores 3–5 as a high (H) protein expression, respectively.

2.9. Double EGFR-Beclin1 Immunofluorescence Stain. In order to colocalize EGFR and Beclin1 protein in tumor cells, in representative cases of each group of low or high protein expressing GBs, a double immunofluorescence stain was performed. Briefly, 4 μm thick sections were deparaffinized in xylene and rehydrated in graded ethanol solutions (100%, 95%, 80%, and 70%), 5 minutes each, and washed in dH_2O. Then, antigen retrieval was obtained by incubation with 10 mM sodium citrate buffer (pH 6.0) at a subboiling temperature for 20 min. Sections were then cooled for 10 min, washed in phosphate-buffered saline (PBS), and incubated overnight at 4°C with the following antibodies: mouse anti-EGFR (Undiluted, Novocastra); rabbit anti-Beclin1 (diluted 1 : 200, Sigma-Aldrich). The slides were washed three times with PBS and incubated with the secondary antibody fluorochrome conjugate (goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 568) for 1 hour at room temperature in the dark. The nuclei were counterstained by incubating the sections for 10 min with 4′,6-diamidino-2-phenylindole (DAPI). Slides were washed in PBS and mounted with Antifade. In each case, a negative control was generated by omitting the primary antibody. Images were acquired and analyzed with a microscope Leica AF CTR6500HS (Microsystems).

2.10. Western Blotting. Tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors, following standard procedures. After protein determination, using BioRad protein assay (BioRad, Milan, Italy), equal amounts of proteins (40 μg) were resolved on 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (BioRad). Membranes were blocked with 3% nonfat milk (BioRad) in PBS Tween 0.05% (PBST) and incubated overnight at 4°C with the following antibodies: anti-EGFR (undiluted, Novocastra), anti-Beclin1 (1 : 270, Sigma-Aldrich), and anti-β-actin (1 : 500, catalogue number: 04-1116, MERCK Millipore Corporation, Billerica, MA, US) diluted with 3% nonfat milk in PBST. Membranes were washed three times in PBS Tween 0.1% and incubated with specific secondary antibodies diluted with 3% nonfat milk in PBST (goat anti-rabbit IgG (H + L)-HRP conjugate, diluted 1 : 10000, catalogue number: 172-1019, BioRad; goat anti-mouse IgG (H + L)-HRP conjugate, diluted 1 : 5000, catalogue number: 172-1011, BioRad) for 1 h at RT. The membranes were incubated with ECL reagents (BioRad) for 1 min and then were developed on Hyperfilm ECL (Amersham GE Healthcare, 28906835).

Images of the bands were digitized and the densitometry was performed using the open source image processing program ImageJ (http://imagej.nih.gov/ij/); β-actin bands were used for normalization.

2.11. Statistical Analysis. We performed a correlation analysis using Spearman's rho two-tailed correlation test between clinical parameters and L- and H-EGFR/Beclin1-expression groups. Overall and progressive disease-free survival (OS and PDFS, resp.) were calculated with the Kaplan-Meier method. The univariate survival analysis was used to identify prognostic parameters as follows: clinical factors (age, KPS, NPS, and synchronous multifocality), treatment-related factors (extent of surgery, RT dose, sequential TMZ, and radiological response from MRI scans), and biological factors (EGFR/Beclin1 protein expression).



(a) (b) (c)

Figure 1: Patient with H-EGFR (a) and L-Beclin1 (b) pattern of protein expression in a GB. The arrows indicate the endothelium of vessels, negative for EGFR (a) and positive for Beclin1 (b) Immunohistochemistry, diaminobenzidine, original magnification ×200. The MRI T1-sequence with gadolinium (c) shows a multifocal GB with a left temporal (white arrow) and a parietooccipital lesion (red arrow).

We used the log-rank test to assess the significance of survival differences for the considered parameters (P values ≤ 0.05 were considered as statistically significant). We also performed a multivariate analysis (Cox regression) to quantify the relationship between survival and potential predictors, in order to identify a subgroup of independent factors significantly related to survival. All statistical analyses were performed with the SPSS 15.0 software package for Windows.

3. Results

3.1. Patients and Postoperative Treatment. One hundred and seventeen patients were included in this study, all planned for RT and concurrent TMZ. At surgery, 23 had a gross tumor resection (GTR) (19.7%) and 94 (80.3%) a biopsy or a subtotal tumor resection (B-STR), respectively. Median age was 62 years (range 26–83), 15 patients (12.8%) were ≤ 50 y, and the other 102 were >50 y (87.2%). The KPS score was 100–80 in 91 patients (77.8%) and ≤ 70 in 26 (22.2%). The NPS score, at admission, was 1-2 in 61 patients (52.1%), 3 in 34 patients (29.1%), and 4 in 22 patients (18.8%). Preoperative MRI showed a single tumor in 98 out of 117 patients (83.8%) and a multifocal presentation in 16 patients (16.2%) (Figure 1(c)).

Forty-three patients (36.6%) received a RT dose of <54 Gy (due to a multifocal presentation or constraints, such as critical structures very close to the tumor), 47 (33.8%) a dose of 54–60 Gy, and 27 (23.7%) a boost up to 70 Gy, according to the aforementioned protocol. Ninety-one (77.6%) patients completed the full-course of TMZ concurrently to RT; out of them, sequential TMZ was then administered in 76 patients (64.9%), until the events of tumor progression or severe toxicity (64.9%), whereas 41 (35.1%) patients did not have this treatment scheduled, due to early tumor progression or toxicity at the end of the concurrent RT and TMZ administration.

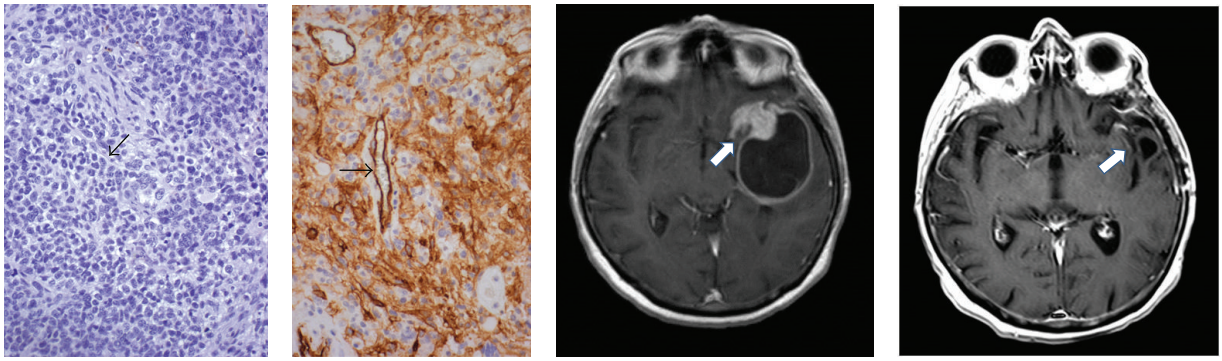
3.2. Results of Treatment. After RT and concurrent TMZ, the RR demonstrated that 19 patients (16.2%) had a CR, 26 (22.2%) a PR (thus an OR was achieved in 45 patients, 38.5%), and 25 (21.4%) a SD, whereas 47 (40.2%) experienced a PR at the post-RT MRI controls. The median OS was 11 months; 1-year and 2-year OS values were, respectively, 47.8% and 25.4%. The median PDFS was 10 months (39.6% at 1 year and 27.0% at 2 years, resp.).

3.3. Methylation Status of MGMT Gene Promoter. MGMT methylation status was assessed in 83 patients: the MGMT promoter was unmethylated in 45 cases (54.2%) and methylated in 38 (45.8%).

3.4. EGFR and Beclin1 Immunohistochemistry. EGFR membranous and/or cytoplasmic positivity was observed in most GBs, while few nuclei were decorated by EGFR in a minority of cases. Some cases were completely negative for EGFR. EGFR staining was not observed in vessel endothelia or in normal/reactive glia (Figures 1(a), 2(a), and 3(a)). On the other hand, Beclin1 stained normal cells and was heterogeneous in GBs, with a higher number of cases negative or lowly expressing the protein; a pin point cytoplasmic staining and variable nuclear immunopositivity were observed (Figures 1(b), 2(b), and 3(b)). In several cases, a heterogeneous expression of both proteins was observed in limited areas.

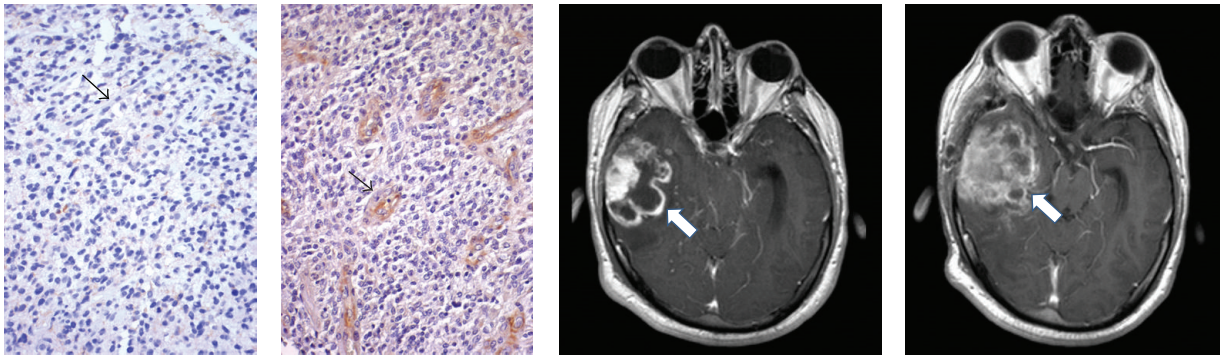
3.5. Assessment of Immunostaining Patterns and Double EGFR-Beclin1 Immunofluorescence Stain. There were 68 cases (58%) expressing H-EGFR; 49 (41.7%) expressing L-EGFR; 59 (50.4%) expressing L-Beclin1, and 58 (49.6%) expressing H-Beclin1.

Overall, two main immunoreactivity patterns were observable: H-EGFR/L-Beclin1 (34 cases, 29.1%; Figures 1, 4(a), 4(b), and 4(c)) and L-EGFR/H-Beclin1 (24 cases, 20.5%; Figures 2, 4(d), 4(e), and 4(f)), the former being the dominant pattern in the majority of cases, although there was a large stain heterogeneity. In fact, there were also cases either highly (H-EGFR/H-Beclin1; Figures 4(g), 4(h), and 4(i)) or lowly expressing (L-EGFR/L-Beclin1; Figures 3, 4(j), 4(k), and 4(l)) the two proteins. Furthermore, several cases showed heterogeneous expression of both proteins in limited areas (Figure 5). EGFR and Beclin1 protein expressions were mutually exclusive in large areas in many cases. This was more



(a) (b) (c) (d)

Figure 2: L-EGFR (a) and H-Beclin1 (b) patterns of protein expression in a GB. The arrows indicate the endothelium of vessels, negative for EGFR (a) and positive for Beclin1 (b) Immunohistochemistry, diaminobenzidine, original magnification ×200. (c) MRI T1-sequence with gadolinium shows a left temporal glioblastoma (white arrow) before treatment. (d) Radiological complete response (white arrow) after treatment (MRI at 4 months after adjuvant RT-CHT treatment).



(a) (b) (c) (d)

Figure 3: Patient with L-EGFR (a) and L-Beclin1 (b) pattern of protein expression in GB. The arrows indicate the endothelium of vessels, negative for EGFR (a) and positive for Beclin1 (b) Immunohistochemistry, diaminobenzidine, original magnification ×200. (c) MRI T1-sequence with gadolinium shows a right temporal lesion (white arrow) before treatment. (d) Radiological progression (white arrow) after treatment (MRI at 6 months after adjuvant RT-CHT treatment).

evidenced by the double EGFR-Beclin1 immunofluorescence stain (Figure 5).

3.6. Western Blotting. Specific EGFR (170 kDa) and Beclin1 (60 kDa) bands were detected and high versus low protein expression was confirmed in several cases, representative of H-EGFR/L-Beclin1, L-EGFR/H-Beclin1, H-EGFR/H-Beclin1, and L-EGFR/L-Becin1 groups of patients (Figure 6).

3.7. Statistics

3.7.1. Univariate Analysis. Distribution of EGFR and Beclin1 protein expression according to age, KPS, NPS, RMN disease presentation, extent of surgery, data regarding local tumor control, and significant P values are shown in Table 1.

PDFS and OS were negatively correlated with age (P = 0.035 and 0.04, resp.), KPS (P = 0.001), NPS (P = 0.0001), synchronous multifocality at preoperative MRI (P = 0.001), extent of surgical resection (P = 0.0001 and 0.001, resp.), radiation dose (P = 0.04 and 0.033, resp.), and sequential TMZ (P = 0.0001 and 0.001, resp.) (Table 2).

EGFR and Beclin1 expressions were not correlated with each other. H-EGFR significantly correlated with low KPS (P = 0.03), a worse NPS class (P = 0.002), a synchronous multifocal presentation (P = 0.01) (Figure 1(c)), a worse RR (P = 0.013), a shorter PDFS (P = 0.002), and OS (P = 0.004). H-EGFR versus L-EGFR patients had, in fact, a worse median PDFS (5 months versus 14 months) and OS (9 months versus 18 months) (Figure 7).

H-Beclin1 was instead positively correlated with a better KPS (P = 0.009), a higher OR (P = 0.002), and a better PDFS and OS (P = 0.001).

H-Beclin1 patients had a median OS of 15 months, compared to 5 months for the L-Beclin1 group (Figure 8). Clustering L-EGFR and H-Beclin1 expression resulted in a stronger correlation with a better RR (P = 0.001) (Figures 2(c) and 2(d)) than other patterns of expression (Figures 3(c) and 3(d)) and absence of MRI multifocality of disease at onset (P = 0.002). In particular, no multifocal disease was found in this subgroup of patients.

Clustering EGFR expression and Beclin1, we found that H-Beclin1/L-EGFR had a significantly better prognosis, with

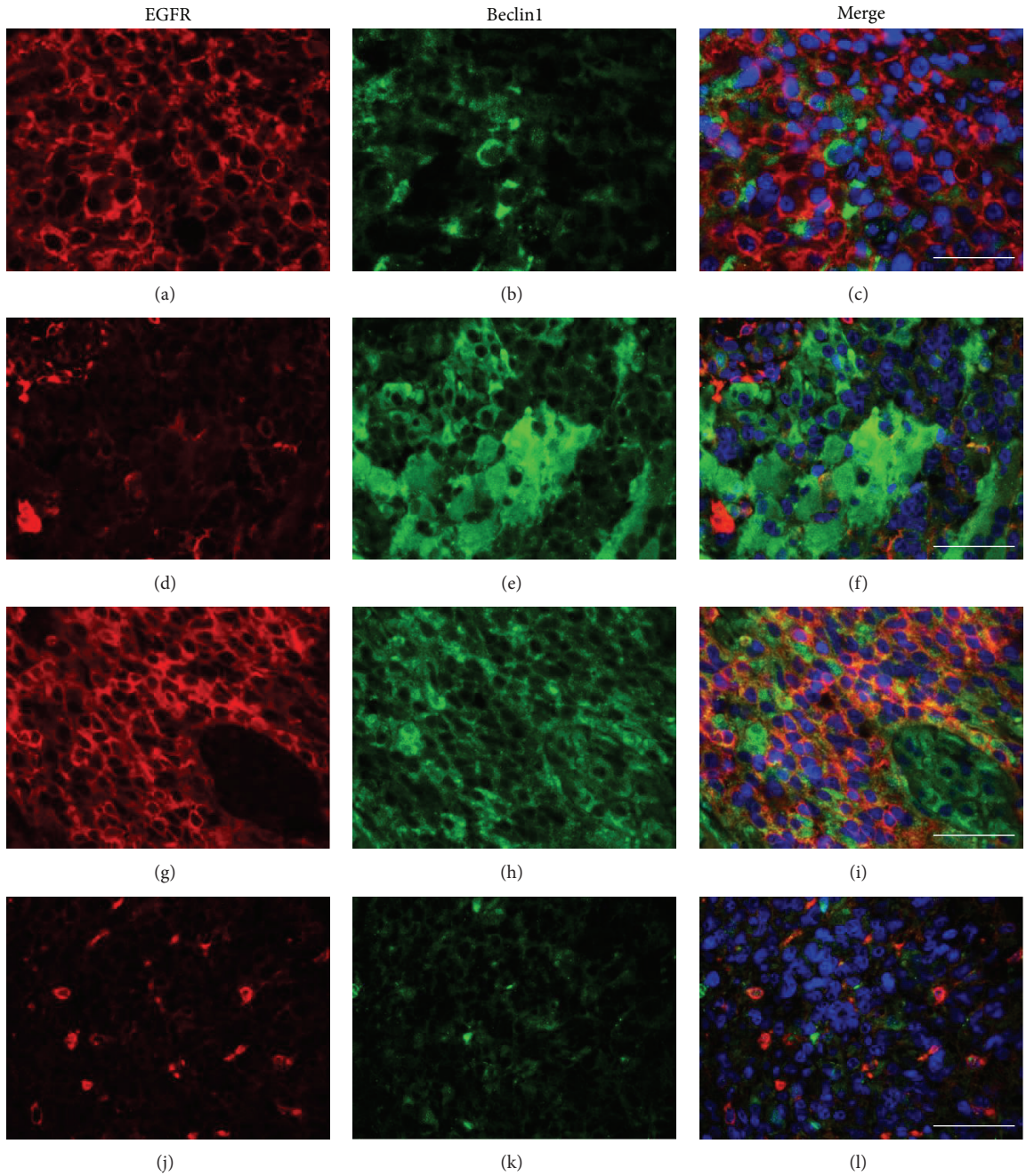


FIGURE 4: Double immunofluorescence staining for EGFR (a, d, g, and j; red stain) and Beclin1 (b, e, h, and k; green stain). Representative GBs showing H-EGFR + L-Beclin1 (a, b, and c), L-EGFR + H-Beclin1 (d, e, and f), H-EGFR + H-Beclin1 (g, h, and i), and L-EGFR + L-Beclin1 (j, k, and l) patterns of protein expression. Nuclei are marked by staining with 4,6-diamidino-2-phenylindole (DAPI) and appear blue in the merged pictures (c, f, i, and l). Original magnification ×650. Scale bar = 50 μm.

a median survival of 30 months, compared to 18 months of L-EGFR, 15 months of H-Beclin1, and 11 months of all GBs (Figure 9).

3.7.2. Cox Regression Proportional Hazards Regression Multivariate Analysis. Given the dependency of the clinical/therapeutic factors on the relationship with EGFR and Beclin1 expression (Table 1), the multivariate analysis of survival factors evidenced that H-EGFR (HR: 2.21; 95% CI: 1.404–3.481; $P = 0.001$), L-Beclin1 (HR: 1,898; 95% CI: 1.244–2.896; $P = 0.003$), and B-STR (HR: 3,119; CI: 1.711–5.669; $P = 0.0001$) were independently associated with a shorter survival.

4. Discussion

Glioblastoma has a poor prognosis. Patients under current therapies have a median survival of approximately one year

Table 1: Distribution of patient in each category, on account of clinical parameters and in each L/H-EGFR and L/H-Beclin1 protein expression group. Significant ($P < 0.05$) correlations with clinical parameters are also given for each EGFR and Beclin1 expression group, and in L-EGFR + H-Beclin1 versus all the other expression groups in regard of both these proteins. Objective Response (OR) was considered Complete Radiological Response plus Partial Radiological Response.

	L-EGFR N° pts	H-EGFR N° pts	P-value
KPS			
100–80	43	48	
<70	6	20	0.03
NPS			
class 1	13	7	
class 2	20	21	
class 3	10	24	
class 4	6	16	0.002
Syncronous Multifocality			
Yes	3	16	
No	46	52	0.01
Radiological Response			
Complete Response	10	9	
Partial Response (OR)	17 (27)	9 (18)	
Stable Disease	10	15	0.013
Progressive Disease	12	35	
	H-Beclin1 N° pts	L-Beclin1 N° pts	P-value
KPS			
100–80	51	40	
<70	7	19	0.009
Radiological Response			
Complete Response	14	5	
Partial sdResponse (OR)	19 (33)	7 (12)	
Stable Disease	14	11	0.009
Progressive Disease	11	36	
	L-EGFR + H-Beclin1	All the other GBs	P-value
KPS			
100–80	24	67	
<70	0	26	0.03
NPS			
class 1	9	11	
class 2	9	32	
class 3	5	29	
class 4	1	21	0.002
Syncronous Multifocality			
Yes	0	19	
No	24	74	0.002
Radiological Response			
Complete Response	13	6	
Partial Response (OR)	8 (21)	18 (24)	
Stable Disease	3	22	
Progressive Disease	0	47	0.013

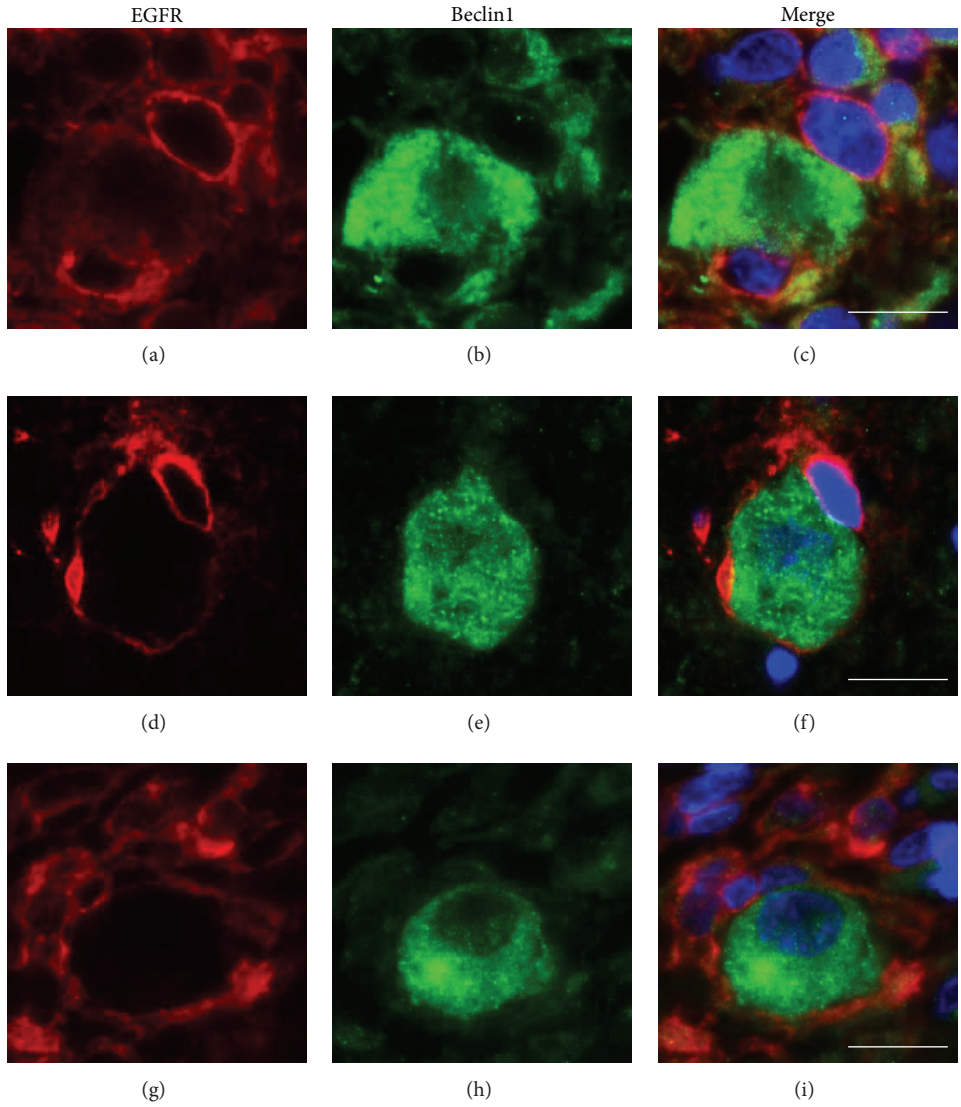


Figure 5: Double immunofluorescence staining. An admixture of H-EGFR (a, d, and g; red stain) and H-Beclin1 (b, e, and h; green stain) positive GB cells is observable in areas showing a heterogeneous pattern of protein expression. The merged pictures (c, f, and i), however, show that the positivity is mutually exclusive; a pin point cytoplasmic positivity for Beclin1 is evident in these enlarged details of Figure 2. Scale bar = 25 μm.

after diagnosis, and it rarely exceeds 15 months for patients enrolled in clinical trials [5]. An increased efficacy of the standard of care was recently achieved, in fact, with TMZ concomitant and adjuvant in respect to radiotherapy [32, 33]. However, response to therapy and prognosis highly depends on both clinical and molecular determinants. Clinical trials as well as retrospective case series analyses outline the prognostic profile of subgroups of patients with a more favorable outcome, based on classical factors (i.e., young age, a good KPS and NPS, circumscribed neoplasms arising in noneloquent areas allowing for a gross total resection, and a high RT dose, i.e., 60 Gy [2, 34]), which were all associated with a better prognosis also in our series, novel chemoradiotherapeutic approaches [33], and molecular biomarkers (i.e., themethylation of O6-methylguanine-DNA methyltransferase (MGMT) gene promoter [32] and mutations of *isocitrate dehydrogenase (IDH1)* gene [35]). We also found a significant correlation of MGMT methylation with a longer survival, although the analysis was not conducted in all the cases. The network of prognostic factors is continuously growing and large-scale genome analyses are further subgrouping molecular subtypes of GB associated with different prognosis [3, 7]. However, to date, among molecular biomarkers, only the MGMT promoter methylation status predictive biomarker has an undoubtedly high impact on clinical practice, being used for stratification of RT and TMZ-CHT treatment regimes, leading to a limited but significant improvement of survival [32, 34].

Therefore, further molecular prognostic biomarkers and targets for identifying patients at a higher prognostic risk are needed, for enlarging the horizon of future individualized therapies.

Table 2: Clinical (Age = age at diagnosis, NPS = Neurological Performance Status, KPS = Karnofsky Performance Status), treatment (GTR = Macroscopic Gross Total Resection, B/STR = Biopsy or Sub-Total Tumor Resection, Dose RT = total dose for radiotherapy treatment, Sequential CHT = TMZ, sequentially administered after the RT-TMZ concomitant course), and biological (EGFR expression and Beclin-1 expression) prognostic factors (Kaplan-Meier method, Survival Analysis).

	n° pts	Progression Disease Free Survival Median (months)	P-value	Overall Survival Median (months)	P-value
Age					
>50	102	7	0.035	11	0.04
<50	15	17		18	
NPS					
class 1	20	10		15	
class 2	41	4	0.0001	18	0.0001
class 3	34	3		11	
class 4	22	1		5	
KPS					
100–80	91	9	0.001	15	0.0001
<70	26	2		5	
Extent of Surgery					
GTR	23	17	0.003	30	0.001
B/STR	94	5		10	
Dose RT					
<54 Gy	36	3	0.04	5	0.033
54–60 Gy	57	7		18	
>60 Gy	24	10		16	
Sequential CHT					
yes	61	9	0.0001	15	0.0001
no	56	1		5	
Synchronous Multifocality					
yes	19	4	0.002	7	0.001
no	98	10		15	
MGMT status					
Methylated	38	20	0.002	22	0.003
Unmethylated	45	4		5	
EGFR expression					
Low	49	14	0.002	18	0.004
High	68	5		9	
Beclin-1 expression					
High	58	12	0.001	15	0.001
Low	59	4		5	
EGFR and Beclin1 co-expression					
L-EGFR H-Beclin1	24	22	0.001	30	0.001
Others	93	8		11	

Our study suggests that the combined evaluation of EGFR and Beclin1 autophagic protein expression in tumor tissue sections could add valuable information to the prognostic molecular profile of GB.

In our experience, in fact, multivariate analysis indicated that, combining the two variables, that is, L-EGFR and H-Beclin1 expression, a subgroup of patients (20.4%) with a more favorable prognosis could be identified. This subgroup

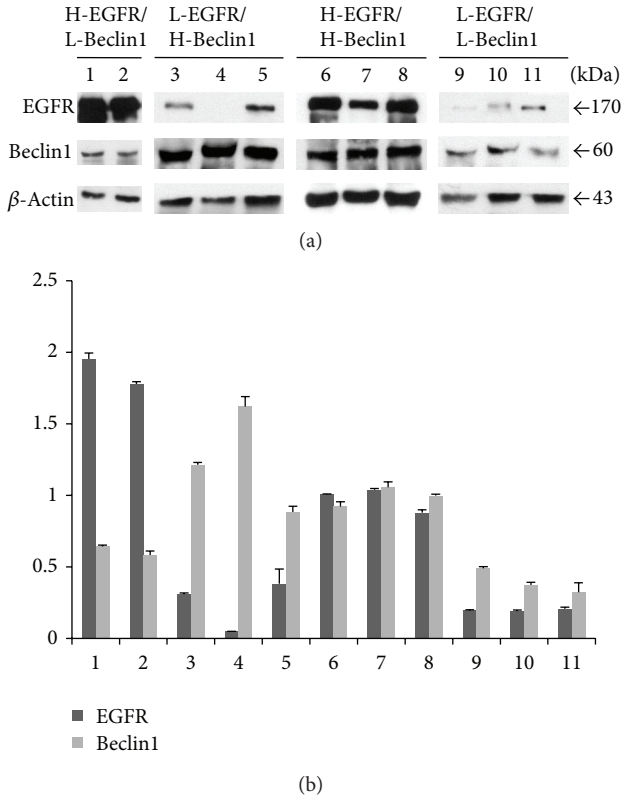


Figure 6: (a) Western blotting for EGFR (170 kDa) and Beclin1 (60 kDa) in representative H-EGFR/L-Beclin1 (lanes 1 and 2), L-EGFR/H-Beclin1 (lanes 3–5), H-EGFR/H-Beclin1 (lanes 6–8), and L-EGFR/L-Beclin1 (lanes 9–11) GBs. (b) Up- or downregulation of either EGFR or Beclin1 is quantified by densitometric data analysis, which shows the relative expression of EGFR and Beclin1 after normalization to the β-actin bands. Data are reported as means ± S.E. of three densitometric analyses of the same sample.

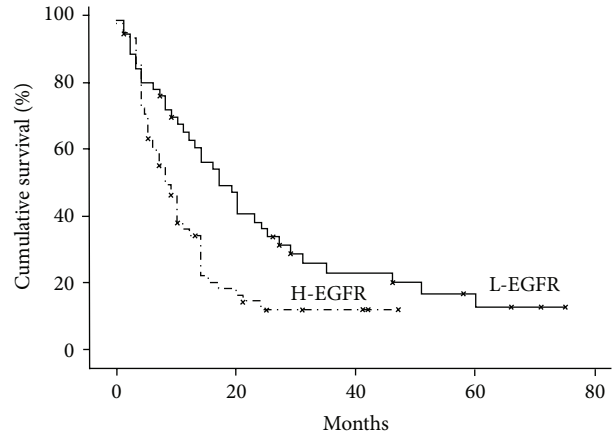


Figure 7: Kaplan-Meier survival curves for EGFR expression levels (P value < 0.05).

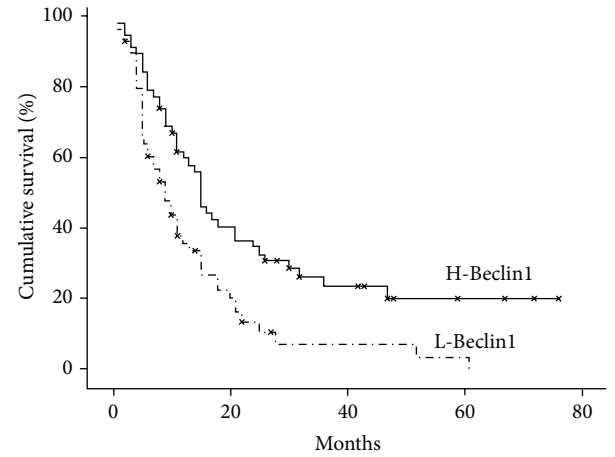


Figure 8: Kaplan-Meier survival curves for Beclin1 expression levels (P value < 0.05).

of GB patients reached a median survival of 30 months compared to 11 months of all the 117 cases, 18 months of L-EGFR, and 15 months of H-Beclin1 protein expressing GB subgroups. Furthermore, EGFR/Beclin1 protein expression identified subgroups of GB patients with a different clinical presentation, in terms of clinical and neurological patient status and multifocality of lesions and edema, and with MRI radiological evidence of response to therapy, in terms of ORs. The worst clinical set was associated with a H-EGFR and the best with a L-EGFR + H-Beclin1 protein expression profile. In the latter group, in particular, in no case was there multifocality of the neoplasm at onset.

EGFR protein overexpression and gene mutation/amplification are known drivers of gliomagenesis and GB aggressiveness, activating signaling cascades that trigger tumor cell proliferation and invasiveness, angiogenesis, and suppressing apoptotic cell death [20, 36–39], largely contributing to the high RT-CHT therapy resistant GB phenotype [6, 39, 40]. EGFR alterations are found in most GBs and characterize the most frequent molecular classic subtype [3], thus being an ideal targetable molecule. However, the use of monoclonal antibodies for therapy has not yielded promising results to date in clinical trials [41]. Novel strategies are intended to target the key phosphorylated kinases and/or altered metabolic pathways downstream EGFR [41]. Autophagy is one of the altered metabolic pathways inhibited by EGFR, which acts via mTOR [8] or by direct inhibition of Beclin1, a cytoplasmic protein that induces autophagy by binding to Vps34-Vps15 core [8, 18, 19, 42]. It is known that this catabolic process acts as a tumor suppressor in the early phases of carcinogenesis, while, in advanced neoplasms, depending on tumor cell context and type, it may either promote or inhibit cancer progression and therapy resistance, being also able to induce an autophagy-related or type II programmed tumor cell death [11]. The authors [22], as others [23], found that Beclin1 was underexpressed in most GBs: this was associated with a decreased apoptosis and negatively impacted on prognosis [24]. The overall negative impact of activated EGFR on autophagy could partly explain the alternate, almost mutually exclusive expression of EGFR and Beclin1 that we observed in most GBs. By examining the colocalization of the two proteins with a double immunofluorescence

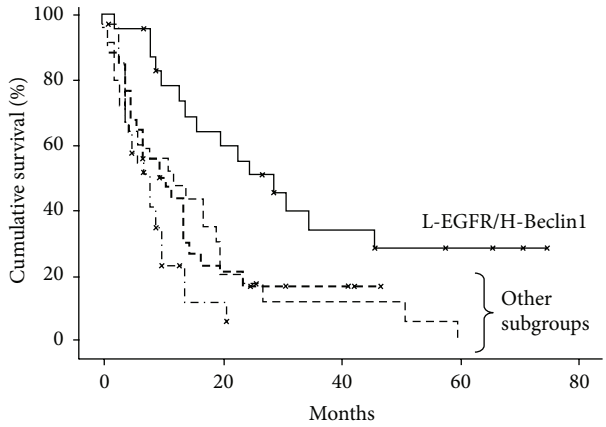


Figure 9: Kaplan-Meier survival curves for EGFR and Beclin1 coexpression (P value < 0.05).

stain, in areas showing heterogenous protein expression, cells positive for EGFR were negative for Beclin1 and vice versa.

EGFR's direct inhibition of Beclin1 and/or the existence of mutated forms of Beclin1 recently observed in human nonsmall cell lung carcinoma cells [19], in which EGFR was found colocalized with Beclin1 in autophagic vacuoles, support the coexpression of the two proteins that we also observed in GB cells. The distribution of the two proteins was, in fact, heterogenous in many cases, although subgroups of GBs could be identified based on the dominant protein expression pattern. Other unknown-to-us factors may occur in cases in which both proteins were negative. Synchronous multifocality of newly diagnosed GBs and GB aggressiveness are partly linked to the invasion ability of neoplastic cells, resulting from an orchestrated activation of cell migration, in which EGFR plays a pivotal role [38, 43–45].

Low expression of Beclin1 was also related to distant metastasis risk in breast cancer [46] and recently we demonstrated that the combined silencing of EGFR and induction of autophagy by rapamycin has additive effects both in increasing radiation sensitivity and in inhibiting cell migration ability, in U373 and T98G GB cells [25]. Therefore, both L-EGFR and H-Beclin1 might have interacted in contributing to the minor aggressiveness, the higher MRI evaluated response to therapy, and the lack of multifocal presentation, which we observed in the GB group with a more favorable prognosis, whereas the leading EGFR ability on cellular invasiveness may partly justify why H-EGFR patients showed a significantly higher multifocal presentation, besides a more frequent progression of the disease at imaging after treatment and a poorer prognosis.

MGMT methylation was positively correlated with prognosis. However, it was not correlated with either EGFR or Beclin1 expression, further supporting their independent role as prognosis biomarkers, despite the fact that MGMT analysis was not performed in all our GB cases.

5. Conclusion

Our results provide a preliminary assessment of the role of EGFR and Beclin1, extrapolated from a series of GB patients treated according to a prospectively definite protocol. Some correlations, in terms of clinical features at referral, imaging data, response to RT-TMZ treatment, and survival, can be established with EGFR and Beclin1 expression. In particular, some parameters of aggressiveness in GBs, such as multifocality (probably related to tumor cell invasiveness) and the type of response to postoperative treatment, deserve further study with regard to these relationships, in our opinion. It is noteworthy that a combined L-EGFR and H-Beclin1 GB profile seems to identify, in our observations, a subgroup of long-surviving patients. This new disclosure might contribute to the other available data suitable for prognostic stratification of GB patients and also envisage future implications for targeted therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Tumor Suppression and Promotion by Autophagy

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Autophagy is a highly regulated catabolic process that involves lysosomal degradation of proteins and organelles, mostly mitochondria, for the maintenance of cellular homeostasis and reduction of metabolic stress. Problems in the execution of this process are linked to different pathological conditions, such as neurodegeneration, aging, and cancer. Many of the proteins that regulate autophagy are either oncogenes or tumor suppressor proteins. Specifically, tumor suppressor genes that negatively regulate mTOR, such as PTEN, AMPK, LKB1, and TSC1/2 stimulate autophagy while, conversely, oncogenes that activate mTOR, such as class I PI3K, Ras, Rheb, and AKT, inhibit autophagy, suggesting that autophagy is a tumor suppressor mechanism. Consistent with this hypothesis, the inhibition of autophagy promotes oxidative stress, genomic instability, and tumorigenesis. Nevertheless, autophagy also functions as a cytoprotective mechanism under stress conditions, including hypoxia and nutrient starvation, that promotes tumor growth and resistance to chemotherapy in established tumors. Here, in this brief review, we will focus the discussion on this ambiguous role of autophagy in the development and progression of cancer.

1. Introduction

According to the World Health Organization (WHO), noncommunicable diseases (NCDs) or chronic diseases (CDs), such as cardiovascular diseases, cancer, diabetes, and chronic respiratory diseases, are the leading causes of global mortality. Moreover, because average life-expectation is increasing, their incidence is on the rise and approaching epidemic proportions. The resulting public health burden is spiraling out of control and doing so at an accelerated rate particularly among lower income countries [1]. Despite this rather bleak outlook, the good news is that the impact of these diseases could be significantly reduced and considerable suffering avoided by changes in lifestyle to reduce associated risk factors and by the implementation of easy measures for early detection and timely treatment. Specifically, NCDs could be avoided to a considerable extent by reducing four main behavioral risk factors: tobacco use, physical inactivity, harmful use of alcohol, and unhealthy diet. Of interest, particularly in the context of this review series, the latter three risk factors result in a chronic systemic imbalance between caloric intake and consumption, thereby positioning metabolic alterations at the core of chronic disease development. Importantly, although perhaps not as immediately obvious as for diabetes and obesity, cancer is no exception in this respect.

Cancer, a group of diseases generally characterized by abnormal and uncontrolled growth of a population of cells (tumor cells), which eventually invade tissues and form metastases, is one of the leading causes of death worldwide. The latest cancer statistics according to GLOBOCAN 2012

(http://globocan.iarc.fr/Default.aspx) reveal that the global burden of cancer increased in 2012 to 14.1 million new cases and 8.2 million deaths, up from 12.7 million and 7.6 million, respectively, in 2008. Furthermore, these figures are expected to continue increasing to a worrisome 26.4 million new cases and 17 million cancer-related deaths by 2030. In the more developed world (MDW) cancers of the lung, breast, prostate, and colon are the most prevalent types encountered. In contrast, in less developed world (LDW), stomach, liver, oral cavity, and cervical cancers are a more significant concern. These notable differences can be attributed to variations in lifestyles and habits. However, patterns are gradually changing in the LDW and beginning to resemble those of the MDW due to the aging of the population, as well as the acquisition of similar lifestyles and associated risk factors [1, 2].

Thus, despite the many scientific and technological advances that have been developed since the "War on Cancer" was declared by Richard Nixon in 1971, cancer not only remains one of the leading causes of morbidity and mortality worldwide, but it is in fact predicted to become the leading cause of human demise in the coming 20–30 years. In large part, the complexity associated with successful treatment is directly linked to the incredible variety of molecular changes implicated in disease development. The cancer hallmarks defined by Hanahan and Weinberg [3, 4] helped enormously in identifying the general nature of the changes that are required to convert normal cells into tumor cells (transformation). Amongst these, metabolic changes, including the famous Warburg effect, are now recognized as crucial to the development of the transformed phenotype. Bearing this in mind, it should come as no surprise that processes that facilitate cell survival under conditions of metabolic stress are likely to be important in the development of tumors. In this context, we will focus our discussion here on how an evolutionarily ancient response to cellular stress, coined autophagy, may contribute to the pathogenesis of a wide range of cancers. A better understanding of the role of autophagy in tumorigenesis may open up opportunities for more successful treatment of the disease.

2. Autophagy: General Aspects and Regulation

Autophagy is a crucial biological process for the survival of unicellular and multicellular eukaryotic organisms under conditions of nutrient deprivation that participates in the maintenance of cellular homeostasis by controlling the quality of proteins and cytoplasmic organelles. The term autophagy ("self-eating") was introduced by Christian De Duve in the decade of the sixties, based on the observation, by transmission electron microscopy, of double membrane vacuoles containing cytoplasmic material [5]. Nowadays, autophagy is defined as a cellular pathway by which cytoplasmic macromolecules and organelles are delivered to the lysosomes for degradation [6].

At least three different forms of autophagy have been identified to date [6], macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). These differ with respect to their function and the mode of delivery of the cargo to the lysosomes. In this review, we will focus the discussion on macroautophagy (hereafter referred to as autophagy) and its role in cancer. During macroautophagy, the cargo is sequestered within a *de novo* formed double membrane vesicle, the autophagosome, which fuses with the lysosome to generate autolysosomes, in which lysosomal enzymes degrade the vesicle content. Not surprisingly, autophagy represents an important catabolic mechanism that cancer cells activate in response to cellular stress and/or increased metabolic demands imposed by rapid cell proliferation. In this scenario, autophagy should favor tumor cell survival. Interestingly, however, autophagy also acts as a tumor suppressor mechanism by preventing the accumulation of damaged organelles and proteins. Here, we will discuss our current understanding of the apparently contradictory role that autophagy plays in cancer development and progression.

The autophagosome is the double membrane vesicle that represents the morphological hallmark of autophagy. Autophagosomes originate from the phagophore, an isolation membrane that most likely derives from the endoplasmic reticulum (ER) [7, 8]. However, the source of the membrane still remains a matter of debate and recent findings indicate that both the ER and mitochondria may provide the membranes required [9, 10]. The phagophore then expands and surrounds the material destined for degradation and finally forms the characteristic double membrane vesicle, known as autophagosome. The mature autophagosome then fuses with the lysosome generating the autolysosomes, where the internal membrane and material enclosed in the autolysosome are degraded by the activity of the lysosomal hydrolases and acidification of the luminal microenvironment. The degradation products generated by autophagy are then transferred back to the cytosol by permeases in the autolysosomal membrane and recycled into different metabolic pathways.

The molecular execution of the autophagic pathway—generation, maturation and degradation of the autophagosomes—requires the participation of specific autophagy-related (ATG) proteins [11] that were first described in yeast before orthologs in higher eukaryotes were identified. The ATG proteins organize into multiprotein complexes that function in a nonredundant manner in the different steps of the process. Thus, although many ATGs exist, inhibition of just one ATG suffices to block execution of the autophagic cascade.

In mammalian cells, nucleation of the phagophore is regulated by a protein serine/threonine kinase complex that responds to the mammalian target of rapamycin (mTOR), a key regulator of the autophagic pathway, which shuts down autophagy in the presence of nutrients and growth factors [12]. Phagophore nucleation [7, 13] is regulated by the balance between class I and class III phosphatidylinositol 3-kinase (PI3K) enzymatic activities [14]. The active enzyme VPS34, a class III PI3K, together with the counterparts of yeast Vps15 and Vps30/Atg6, identified in mammals as p150 and Beclin-1, and ATG14 form a PI3K complex that catalyzes the production of phosphatidylinositol-3-phosphate, thereby generating a signal to initiate the recruitment of effectors proteins, such as double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins [15–18]. The elongation of the isolation membrane

and subsequent closure of the autophagosome require the formation of two ubiquitin-like conjugates. First, ATG12 is conjugated to ATG5 by the sequential activity of ATG7 and ATG10. The resulting ATG5-ATG12 complex interacts with ATG16L, which then oligomerizes to form the ATG16L complex [19]. Second, LC3 (the mammalian homologue of yeast Atg8) is cleaved by the protease ATG4 and then conjugated to the lipid phosphatidylethanolamine via the activity of ATG7 and ATG3 [19, 20]. While the unprocessed form of LC3 (LC3I) is diffusely distributed throughout the cytoplasm, the lipidated form of LC3 (LC3II) specifically accumulates on nascent autophagosomes and thus represents a marker to monitor autophagy [21]. The autophagosome eventually seals off and fuses with lysosomes through mechanisms that remain poorly characterized in mammalian cells [11]. Some regulators of the autophagosome-lysosome fusion process include LC3, the lysosomal proteins LAMP-1 and LAMP-2, the small GTP-binding protein RAB7, and the AAA-type ATPase SKD1 [22–24]. Autophagosome-lysosome fusion then results in the activation of the hydrolases which completely degrade the autophagosomal cargo.

Different signaling mechanisms are known to modulate autophagy in mammalian cells [25]. The best characterized pathways are those that modulate autophagy in response to nutritional changes and, as previously mentioned, mTOR is critical for sensing the nutritional status of the cell and regulating the initiation of autophagy [12]. In higher eukaryotes, mTOR can be found in at least two distinct multiprotein complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [26, 27]. The former is considered the principal regulator of autophagy [28]. When nutrients and growth factors are available, mTORC1 inhibits autophagy by phosphorylating and maintaining in an inactive state ULK1, which is required for the formation of the phagophore [29, 30]. As indicated (see Figure 1), mTOR activity is controlled by different signaling pathways triggered via cues from the extracellular and intracellular microenvironment.

AMP-activated protein kinase (AMPK), another sensor of the cellular energy status, responds to decreases in ATP/AMP ratios [31]. In conditions of nutrient deprivation, AMPK directly phosphorylates and inhibits mTOR (not shown in Figure 1). The ensuing reduction in mTOR activity decreases ULK1 phosphorylation and promotes autophagosome formation [31, 32]. Moreover, AMPK also directly activates autophagy by phosphorylation of TSC2 [33, 34]. In summary, autophagy is a highly regulated process that involves a large number of modulators and the complexity of these events will increase as novel components continue to be identified in both mTOR dependent and independent pathways [35].

3. Control of Autophagy by Oncogenes and Tumor Suppressors

Most of the proteins that participate in the regulation of autophagy are either tumor suppressor proteins or oncogenes. Perhaps not surprisingly, mechanisms involved in the regulation of autophagy largely overlap with signaling pathways implicated in the control of cancer. Thus, tumor suppressor genes that negatively regulate mTOR, such as PTEN, AMPK, LKB1, and TSC1/2 stimulate autophagy while, conversely, oncogenes that activate mTOR, such as class I PI3K, Ras, RHEB, and AKT, inhibit autophagy [51] (see Table 1). In the following paragraphs, the role of Beclin-1, DAPK, Bcl2/Bcl-XL, and mTOR will be discussed briefly (see Figure 1).

Consistent with this view, Beclin-1, which is part of the class III PI3K complex that promotes autophagy, functions as a tumor suppressor in mammalian cells. Interestingly, monoallelic mutations in the *beclin-1* gene are frequently observed in prostate, ovarian, and breast cancers in humans. In addition, studies in mice have demonstrated that the animals are more sensitive to spontaneous tumor development when *beclin-1* is monoallelically disrupted. These observations provide direct evidence for a role of *beclin-1* as a haploinsufficient tumour suppressor gene implicated in the pathogenesis of several human cancers [41, 52–54]. Additionally, the death-associated protein kinase, DAPK, a protein that phosphorylates Beclin-1 thereby disrupting Beclin-1/BCL-2 complex and favoring autophagy, is another inducer of autophagy that is commonly silenced in different types of human cancers by methylation [55].

BCL-2 and BCL-XL are antiapoptotic members of the BCL-2 family that modulate cell death in an autophagy-independent manner and are overexpressed in several hematological malignancies [56]. There, BCL-2 and BCL-XL suppress cell death and promote survival and growth of cancer cells by suppression of BAK/BAX-dependent pore formation during mitochondrial outer membrane permeabilization (MOMP) [57]. In addition to the role of BCL-2 and BCL-XL in the inhibition of apoptosis, they have also been implicated in oncogenesis as negative regulators of autophagy. Although these proteins do not directly participate in mTOR signaling, they can interact with the Beclin-1 BH3 domain and sequester Beclin-1 into an inactive complex in the ER [58, 59].

The protein kinase mTOR is the major negative regulator of autophagy [60]. This kinase participates in multiple signaling pathways that regulate cell growth, especially downstream of growth factor receptors with tyrosine kinase activity. Interestingly, both the constitutive activation of these receptors, as well as activating mutations of downstream elements in these pathways (Ras, PI3-K, AKT, and PDK-1) or mutations that inactivate negative regulators (TSC1/2, LKB1, and PTEN) are common in the development of cancer [36, 38, 47, 48, 50], suggesting that inhibition of autophagy likely contributes to the onset to tumor development.

4. Autophagy as a Tumor Suppressor Mechanism

The first data pointing towards the possible tumor suppressor role of autophagy were obtained in studies of Beclin-1. Monoallelic loss of the *beclin-1* gene on chromosome 17q21 has been reported in 40% to 75% of human breast, ovary, and prostate tumors, suggesting that autophagy represents a tumor suppressor mechanism [41]. Also, a reduction in Beclin-1 protein levels has been observed in various brain cancers [61]. Accordingly, Beclin-$1^{+/-}$ mice have a high incidence of spontaneous tumors, especially lymphoma and

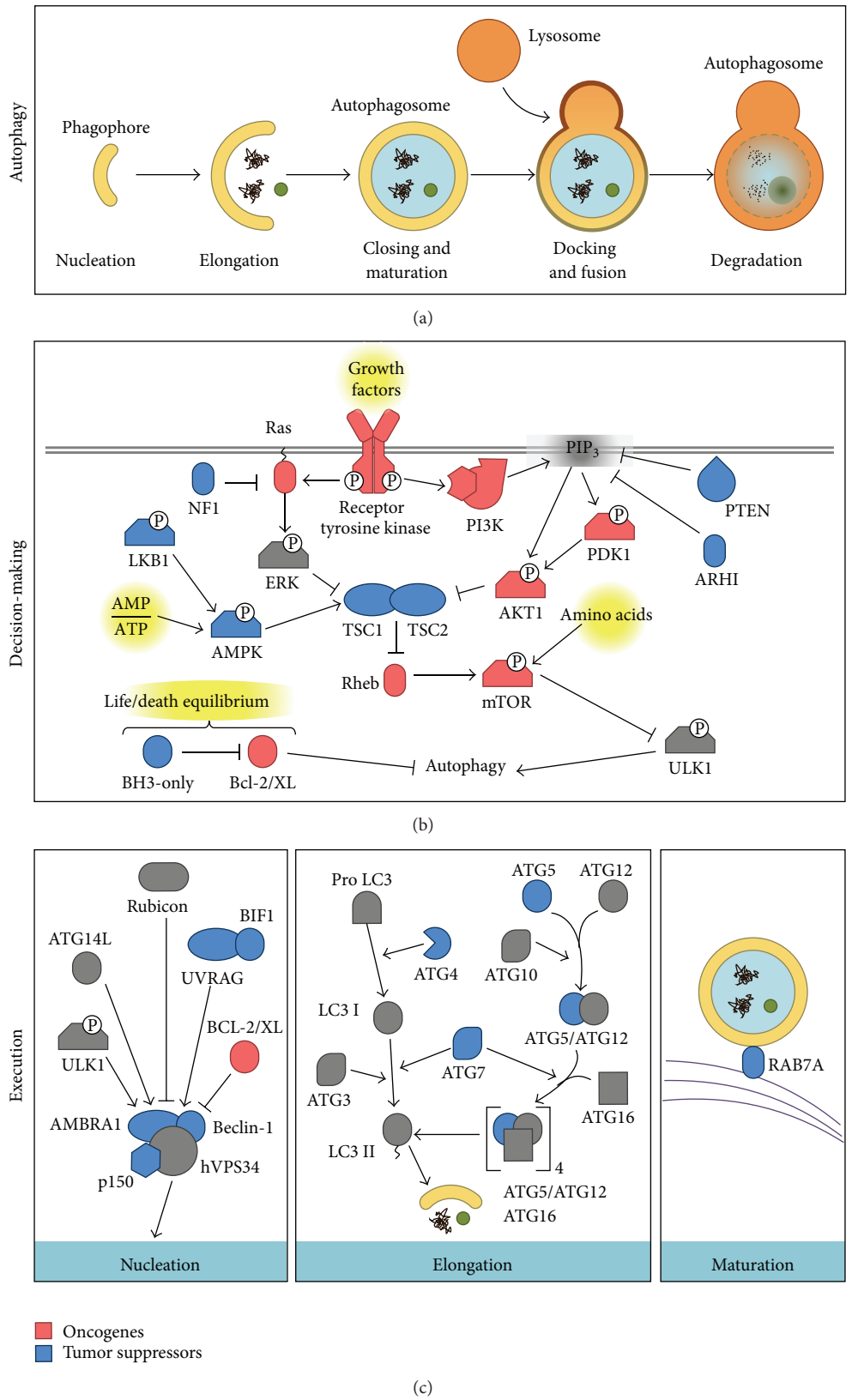

Figure 1: Phases of autophagy and its regulation by oncogenes and tumor suppressors. In (a), the five stages of autophagy are summarized. In (b), inhibition of autophagy by oncogenes (in red) and activation by tumor suppressors (in blue) is shown. Finally, (c) summarizes details of the complex regulation and interplay between different proteins in each stage of autophagy (see text for more details).

Table 1: Summary of oncogenes and tumor suppressors involved in autophagy regulation.

Oncogenes	Role in autophagy	Evidences of oncogenesis	Reference
AKT1	Upstream inhibitor of autophagy via mTOR activation	Gain-of-function mutations in several cancer types	[36]
BCL-2, BCL-XL	Sequester Beclin-1 into inactive complexes	Overexpressed in several cancer types	[37]
PI3K	Upstream inhibitors of autophagy via AKT1 activation	Gain-of-function mutations in many cancer types	[36, 38]
Ras	Upstream inhibitors of autophagy via mTOR activation	Hyperactivated in several cancer types	[37]
Tumor suppressors	Role in autophagy	Evidences of tumor suppression	
ATG4	Converts LC3 into LC3 I during stress conditions	Mutations in ATG4C increase susceptibility to carcinogens	[39]
ARHI/DIRAS3, PTEN	Relieve autophagy inhibition mediated by PI3K-AKT1	Downregulated in ovarian cancer	[36, 40]
Beclin-1, p150	Required in the nucleation complex for autophagy initiation	Deleted in breast, ovarian, and prostate cancer	[41]
BH3-only proteins	Relieve autophagy inhibition mediated by BCL-2/BCL-XL	Mutated or silenced in many cancer types	[42–44]
UVRAG, BIF1	Positive regulator of the nucleation complex	Deleted or downregulated in colorectal cancer	[45]
DAPK1	Relieve autophagy inhibition mediated by BCL-2/BCL-XL	Silenced in many tumor types	[46]
LKB1/STK11	Promotes autophagy via AMPK activation	Mutated in Peutz-Jeghers syndrome and non-small cell lung carcinomas	[47, 48]
NF1	Relieve autophagy inhibition mediated by Ras	Mutated in neurofibromatosis, juvenile myelomonocytic leukemia	[37]
RAB7A	Modulates endosomal trafficking involved in autophagosome maturation	Rearranged in leukemia, deleted in solid tumors	[49]
TSC1, TSC2	Stimulate Rheb GTPase, thus inhibiting the PI3K-AKT1-mTOR pathway	Mutated in TSC	[50]

hepatocellular carcinoma. Furthermore, the evidence provided suggests that Beclin-1 functions as a haploinsufficient tumor suppressor gene, given that the tumors continued to express Beclin-1 [53, 54]. Moreover, immortalized breast epithelial cells with a monoallelic deletion of Beclin-1 form tumors more rapidly after inoculation into nude mice [62]. More recently, phosphorylation of Beclin-1 on multiple tyrosine residues in an EGFR-dependent manner was found to decrease the activity of the Beclin-1/PI3KC3 complex and therefore decreased autophagy in non-small-cell lung carcinoma cells (NSCLC) and that this effect was reduced in the presence of an inhibitor of EGFR kinase activity. Alternatively, the expression of a tyrosine phosphomimetic mutant of Beclin-1 reduces autophagy and increases tumor growth [63]. Similarly, several proteins that interact with Beclin-1 and positively regulate autophagy, such as AMBRA 1 [64], BIF-1 [45], and UVRAG [65], have been shown to display antiproliferative or tumor suppressor effects. However, a complication here is that all these proteins have other functions that are independent of their role in autophagy, for example in regulating the endocytic pathway [66]. Moreover, the Beclin-1/PI3KC3 complex also controls the ubiquitination and degradation of p53 by regulating the stability and activity of the deubiquitinating enzymes USP13 and USP10 [67]. Given these additional functions, the contribution of such autophagy-independent mechanisms to the observed tumor suppressor phenotype cannot be excluded.

In agreement with the tumor suppressor hypothesis, the generation of knockout mice for specific genes involved in autophagy (ATGs) has shown that defects in specific regulators of this process are associated with the development of a tumorigenic phenotype. Because systemic deletion of *Atg*3, *Atg*5, *Atg*7, *Atg*9, or *Atg*16L1 causes neonatal death [68–72], long-term effects of the inhibition of autophagy could not be assessed until mice with systemic mosaic Atg5 deletion were generated. In this background, systemic mosaic *Atg5* deletion or liver-specific deletion of *Atg7* results in mice that spontaneously develop benign liver adenomas [73]. While these data suggest that defects in autophagy promote the development of benign tumors in this tissue, they also indicate that, in the absence of autophagy, progression to a malignant phenotype is prevented. Similarly, Strohecker et al. showed that the deletion of *Atg7* in mice expressing an activating mutation of B-Raf ($Braf^{V600E/+}$) promotes early tumor development in the lung but also inhibits the progression to a more malignant phenotype and increases mouse survival [74]. Additional autophagy-promoting factors that have tumor suppressor functions are Atg4C and RAB7A. For animals deficient in

Atg4C, increased susceptibility to the development of fibrosarcomas induced by chemical carcinogens was detected [39]. RAB7A has been shown to prevent growth factor-independent survival by inhibiting cell-autonomous nutrient transporter expression and the *RAB7A* gene is frequently rearranged in different types of leukemia [49, 75].

Despite this evidence that favors a role for autophagy in tumor suppression, some more recent findings concerning Beclin-1 contrast with the previous interpretation of data. The *beclin-1* gene lies close to *BRCA1* on chromosome 17q21 raising the specter that the relevance of the loss of Beclin-1 in ovarian, breast, and prostate cancer may have been overinterpreted. Indeed, deletions encompassing both genes (*BRCA1* and *beclin-1*) and deletions of only *BRCA1* but not *beclin-1* were found in breast and ovarian cancers, which is consistent with *BRCA1* loss representing the primary driver mutation in these cancers. Furthermore, no evidence for *beclin-1* mutations or loss have been detected in any other cancer, which questions whether *beclin-1* is indeed a tumor suppressor in various human cancers [76]. Taken together, the evidence presented supports the hypothesis that autophagy may play an important role in tumor suppression at early stages. However, the findings discussed also reveal the potentially dual nature of this process in tumor development and progression.

4.1. Mechanisms Involved in Tumor Suppression by Autophagy

4.1.1. Oxidative Stress and Genomic Instability. One of the most important connections between autophagy and tumor suppression is via the regulation of reactive oxygen species (ROS). Increased ROS production accelerates mutagenesis, increasing the activation of oncogenes, thus stimulating carcinogenesis [77, 78]. Mitochondria are considered the main source of intracellular ROS and their production increases as these organelles age or become damaged [79]. In this context, autophagy helps to avoid damage through selective degradation of defective mitochondria, a process known as mitophagy. Consequently, inhibition of autophagy facilitates genomic instability by promoting the activation of oncogenes [62, 80] and genotoxic effects observed in autophagy-defective cells seem to be dependent on ROS generation [81]. Thus, the selective removal of potentially damaged mitochondria (mitophagy) reduces excessive ROS production and thereby limits tumor-promoting effects dependent on the production of such species [82]. Accordingly, inhibition of autophagy in different models leads to accumulation of defective mitochondria [69, 73, 74, 83–85].

Autophagy also permits the degradation of protein aggregates. Defects in the autophagic process have been associated with the accumulation of protein aggregates and the autophagy substrate p62/SQSTM1. Such events are associated with increased production of ROS, ER stress, and activation of the DNA damage response [81]. The p62 protein is a selective autophagy substrate that accumulates when autophagy is reduced. This scaffolding protein contains a PB1 domain that permits protein oligomerization, an UBA domain required for binding to polyubiquitinated proteins and an LIR domain (LC3-interacting region) necessary for association with LC3. For these reasons, p62 favors selective degradation of both polyubiquitinated proteins and organelles (i.e., mitochondria) [86, 87]. Interestingly, p62 levels are commonly elevated in human tumors. In addition, tumorigenic development observed in autophagy-deficient cells is reversed by genetic inactivation of p62 in various models, suggesting that the accumulation of p62 promotes tumor formation in this context [73, 81, 83, 88]. Moreover, p62 accumulation stabilizes and activates the transcription via NRF-2, by binding to Keap-1, the main negative regulator of NRF-2. In doing so, antioxidant defense is upregulated and may contribute to tumor development [88–90]. Specifically, overexpression of p62 and activation of NRF-2 are critical for anchorage-independent growth observed in hepatocellular carcinoma cells [88].

4.1.2. Inflammation and Necrosis. The tumor microenvironment is defined by complex interactions between various cell types that coexist within tumors (tumor and stromal cells) and crosstalk between these cells regulates both tumor growth and progression. In this context, it is important to note that both inflammatory cells and cytokines are extremely relevant because a proinflammatory environment promotes proliferation and survival of malignant cells, stimulates angiogenesis, metastasis, and modifies the response to drugs [91]. In different models, autophagy inhibition in apoptosis-deficient tumor cells has been shown to promote necrotic cell death, local inflammation, and tumor growth [92]. These results suggest that autophagy may contribute to tumor suppression by restricting tumor necrosis and local inflammation [60]. The anti-inflammatory effect of autophagy has been suggested to be linked to the removal of cell corpses [93] because of findings in $Atg5^{-/-}$ embryonic stem cells, where defects in the clearance of apoptotic bodies during embryonic development are observed [94]. Moreover, a complex connection between autophagy and different aspects of the immune response has been noted, which could contribute to the tumor suppressor role of autophagy, as has been reviewed elsewhere [95].

4.1.3. Autophagic Cell Death and Senescence. Although autophagy is primarily considered a mechanism that permits survival under stress conditions, some reports indicate that, under specific conditions, an increase in autophagic flux may cause cell death due to autophagy and explain in part the tumor suppressor effects [96]. The findings of Pattingre et al. revealed that the expression of a mutant Beclin-1, unable to interact with BCL-2, induced autophagy to a greater extent than wild-type Beclin-1, and unlike the latter, it promoted cell death [58]. More recently, studies in an ovarian cancer cell line showed that ectopic expression of Ras induces autophagic cell death through the upregulation of Beclin-1 and Noxa, a BH3-only protein, which ultimately limits the oncogenic potential of Ras [97]. Similarly, Zhao and colleagues demonstrated that the transcription factor FoxO1 promotes autophagy in a manner independent of its transcriptional activity and induces autophagic cell death in tumor cells, suppressing tumor growth of xenografts in nude mice. These

results suggest that autophagy promoted by cytosolic FoxO1 is a tumor suppressor mechanism [98, 99].

Another controversial mechanism that may potentially explain the tumor suppressor activity of autophagy is its role in senescence. Young et al. [100] showed that autophagy is activated during senescence induced by the oncogene Ras in fibroblasts and that inhibition of autophagy in this context delays but does not abrogate the development of the oncogene-mediated senescence phenotype. This finding is important because senescence represents a major intrinsic barrier to malignant transformation [101] although this barrier function may only be transient. In studies of senescence induced by chemotherapy in breast and colon cancer cell lines, autophagy and senescence occur in parallel but not in an interdependent manner. In fact, senescence was only transiently subdued and subsequently recovered despite prolonged inhibition of autophagy [102]. Similar results have been obtained in different experimental settings and are discussed in [103].

5. Autophagy as a Promoter of Tumor Growth

5.1. Autophagy Promotes Cell Survival under Conditions of Stress. The notion that autophagy represents a mechanism that promotes tumor growth is based on the necessity of tumoral cells to adapt to ischemia in an environment that is hypoxic, as well as growth factor and nutrient deprived. Consistent with this conundrum, autophagy is activated in hypoxic regions of tumors and inhibition of autophagy by monoallelic deletion of *beclin-1* (*Bcn1*$^{+/-}$) promotes cell death specifically in those regions. These observations suggest a role for autophagy in promoting survival of tumor cells under conditions of metabolic stress [92].

Furthermore, tumor cells generally have high proliferation rates, which translate into higher bioenergetic and biosynthetic needs than is the case for non-transformed cells. These requirements can be satisfied by increasing autophagy as a mechanism that permits obtaining both ATP and metabolic intermediates [66]. Importantly, for tumor cells in which the oncogene Ras is activated, high levels of basal autophagy and dependence on this mechanism for survival are observed [83, 85]. For these reasons, autophagy is thought to promote tumor cell survival by increasing stress tolerance and providing a pathway that permits obtaining the nutrients necessary to meet the enhanced energetic requirements of these cells [66].

5.2. Ras-Dependent Tumor Progression and Autophagy Addiction. Small GTPases of the Ras family are involved in signaling pathways important for proliferation, cell survival, and metabolism. RAS-activating mutations are present in 33% of all human cancers, whereby mutations in *KRas* are most prevalent and linked to the development of some of the most lethal cancers, including those of the lung, colon, and pancreas [104, 105]. In human pancreatic cancer, enhanced levels of active autophagy and LC3 correlate with poor patient prognosis [106]. *In vitro* studies shown in several cell lines with Ras-activating mutations revealed that these cells exhibit high levels of basal autophagy and marked autophagy-dependent survival under conditions of nutrient deprivation. Moreover, silencing of proteins involved in autophagy promotes the accumulation of dysfunctional mitochondria, low oxygen consumption, and decreased cell growth [83, 85].

In vivo studies confirm the aforementioned results. In a KRas-driven pancreatic cancer model, inhibition of autophagy by *Atg5* deletion, decreased the capacity of preneoplastic lesions to progress to invasive cancer, in a manner that was independent of the p53 status [107]. Rosenfeldt et al. reported similar results using a mouse model of humanized pancreatic cancer, but they demonstrated that p53 deletion precludes tumor progression promoted by autophagy. Therefore, the role of autophagy in pancreatic cancer progression may depend on the presence of p53 [108]. In a model of KRas-dependent NSCLC, the inhibition of autophagy through inducible *Atg7* deletion leads to abnormal accumulation of mitochondria and decreases in proliferation and necrotic cell death, which in combination translated into reduced tumor burden. Moreover, in this same study, the absence of Atg7 resulted in progression of Ras-induced adenomas and adenocarcinomas to oncocytomas, benign tumors characterized by accumulation of dysfunctional mitochondria [84]. In studies following up on the role of the Ras pathway in tumor promotion and its dependence on autophagy, Strohecker et al. investigated whether pulmonar carcinogenesis driven by an activating B-Raf mutation was dependent on autophagy. *Atg7* deletion increased oxidative stress and enhanced tumor growth at early stages, but promoted abnormal mitochondria accumulation, proliferation defects, a decrease in tumor burden, and increased survival of animals in more advanced stages of tumorigenesis. Furthermore, when cell lines derived from these tumors were supplemented with glutamine, nutrient deprivation-induced cell death was prevented, suggesting that metabolic stress due to mitochondrial dysfunction may be related to the sensitivity of autophagy-deficient cells to nutrient deprivation [74]. Finally, an *in vitro* study demonstrated that adhesion-independent growth promoted by Ras was dependent on autophagy. Specifically, upon inhibition of autophagy in different cell lines with Ras-activating mutations, the ability of cells to grow in an anchorage-independent manner was lost. These observations underscore the importance of autophagy in maintaining glycolytic activity, which facilitates Ras-mediated anchorage-independent cell growth [109].

5.3. Mechanisms Involved in Autophagy Addiction in Ras-Driven Tumors. The dependence on autophagy in Ras-/Raf-driven tumoral cells is explained by the decrease in the acetyl-CoA pool necessary to fuel the tricarboxylic acid (TCA) cycle. Ras activation modulates mitochondrial metabolism by inducing hypoxia-inducible factor (HIF)-1α-dependent expression of lactate dehydrogenase (LDH) expression [110], which converts pyruvate to lactate resulting in reduced acetyl-CoA synthesis and, hence, acetyl-CoA depletion. In addition, the Raf/Erk pathway promotes inactivation of LKB1 and subsequently AMPK, thereby preventing β-oxidation [111] and decreasing the available mitochondrial acetyl-CoA pool. Due to acetyl-CoA depletion, Ras/Raf-driven

tumors require autophagy in order to obtain TCA cycle intermediates. These in turn promote mitochondrial activity, which provides reductive equivalents necessary for oxidative phosphorylation and mitochondrial respiration. Moreover, in Ras-driven tumors, autophagy inhibition promotes the accumulation of dysfunctional mitochondria. This kind of cancer requires autophagy to maintain a pool of functional mitochondria necessary for enhanced energetic requirements of tumoral cells [83, 85].

Beyond the requirement of autophagy for survival of Ras-driven cancer cells, Ras activation also promotes cell signaling events involved in the induction of autophagy by the upregulation of Noxa and Beclin-1 expression [97]. Furthermore, Ras can directly stimulate BNIP3 expression through activation of the Ras/Raf/Erk pathway or indirectly through HIF-1α induction [112–114].

5.4. Autophagy in Ras-Independent Tumor Progression. The role of autophagy also has been studied in different contexts that are independent of Ras. For instance, in a model of breast cancer driven by the PyMT oncogene, the inhibition of autophagy by FIP200 deletion suppresses mammary tumor initiation and progression. Here, FIP200 ablation increased the number of mitochondria with abnormal morphology in tumor cells and reduced significantly proliferation, but it did not affect apoptosis of mammary tumor cells [115]. Although PyMT requires Ras activation to initiate cell transformation, PI3-kinase and Src activation are also involved [116]. Thus, it would be interesting to determine whether these kinases contribute to dependence on autophagy for cell proliferation. Another study employed a *Palb2* knockout model specific to epithelial breast cells to determine the role of autophagy in breast cancer progression. PALB2 is a protein that cooperates with BRCA1 and BRCA2 in DNA repair via homolog recombination and helps maintain genomic stability. *Palb2* knockout mice develop breast adenocarcinoma when p53 is mutated. Partial inhibition of autophagy by monoallelic loss of Beclin-1 ($Bcn1^{+/-}$) increased apoptosis and delayed tumor growth in a manner dependent on p53. The authors proposed that autophagy promotes tumor growth by p53 suppression when DNA is damaged [117]. These studies indicate that autophagy can promote tumor progression in a manner independent of Ras activation and that autophagy could be a more general mechanism involved in cancer cell survival and tumor progression.

In summary, current evidence points towards autophagy as a mechanism that ensures adequate mitochondrial metabolism in Ras-driven cancers by supplying mitochondrial intermediates via the degradation of macromolecules under basal and starvation conditions [118]. Particularly, Ras-driven tumorigenesis appears to be "addicted to autophagy" for metabolic support and maintenance of rapid tumor growth. All these data explain why autophagy is required in Ras-driven cancers to promote tumor cell survival and tumor progression. Interestingly, however, some more recent evidence indicates that autophagy is also important for tumoral cell survival of other cancers, independent of the Ras activation status.

6. Caveolin-1, a Connection to Autophagy?

Caveolin-1 (CAV1) is a scaffolding protein that is essential for caveolae formation, is expressed in a wide variety of tissues, and is involved in many biological processes, including cholesterol homeostasis, vesicular transport, and signal transduction. Moreover, similar to autophagy, CAV1 plays a dual role in cancer, functioning both as a tumor suppressor and promoter of tumor metastasis [119–121]. Although, E-cadherin has been identified as important in determining CAV1 function in this context [122–125], the molecular mechanisms explaining such ambiguous behavior remain largely undefined.

Given the parallels between the roles of CAV1 and autophagy in cancer, it is intriguing to speculate that there might be a connection between the two. Indeed, Martinez-Outschoorn et al. demonstrated, using a coculture system, that CAV1 is degraded via lysosomes in stromal fibroblasts subjected to hypoxia and that this correlated with increased levels of autophagic markers such as LC3, ATG16L, BNIP3, BNIP3L, HIF-1α, and NF-kB. Moreover, knockdown of CAV1 in stromal fibroblasts was sufficient to induce the upregulation of lysosomal and autophagic markers, suggesting that the loss of CAV1 in the stromal compartment induces autophagy [126]. Also, loss of CAV1 leads to metabolic reprogramming of stromal cells to support the growth of adjacent tumor cells by delivering energy-rich metabolites and essential building blocks [127]. Consistent with the notion that CAV1 is a negative regulator of autophagy, CAV1 depletion in HCT116 colorectal cancer cells was shown to reduce glucose uptake and ATP production, which then triggered autophagy via activation of AMPK-p53 signaling [128]. Moreover, both *in vitro* cell growth and *in vivo* xenograft tumor growth were attenuated to a greater extent by CAV1 depletion in $p53^{+/+}$ than in $p53^{-/-}$ cells [128].

An inverse relationship between autophagy and CAV1 has also been observed in models of nontransformed cells. For instance, metabolomic profiling of endothelial cell lysates following transfection with si-CAV1 or si-control resulted in marked increases in dipeptide levels for the CAV1 knockdown cells, which was attributed to an increase in autophagy [129]. To corroborate these results, the authors evaluated the processing of LC3 I to LC3 II by western blotting and showed that siRNA-mediated CAV1 knockdown led to an increase in the presence of the autophagy marker LC3-II. Also, treatment with the lysosomal inhibitor bafilomycin A1 markedly increased LC3-II levels, indicating that reduced CAV1 expression leads to an increase in autophagy flux [129]. Recently, CAV1 was also shown to regulate autophagy in cigarette smoking-induced injury of lung epithelium [130]. Specifically, CAV1 depletion increased basal and starvation-induced levels of ATG12-ATG5 and autophagy. Biochemical analysis revealed that CAV1 interacted with ATG5, ATG12, and the active ATG12-ATG5 complex to suppress autophagy in lung epithelial cells, thereby providing new insights as to how CAV1 modulates autophagy in this model [130]. However, details of the molecular mechanisms by which CAV1 regulates autophagy in cancer cells remain to be determined. A rather speculative idea is that the dual role

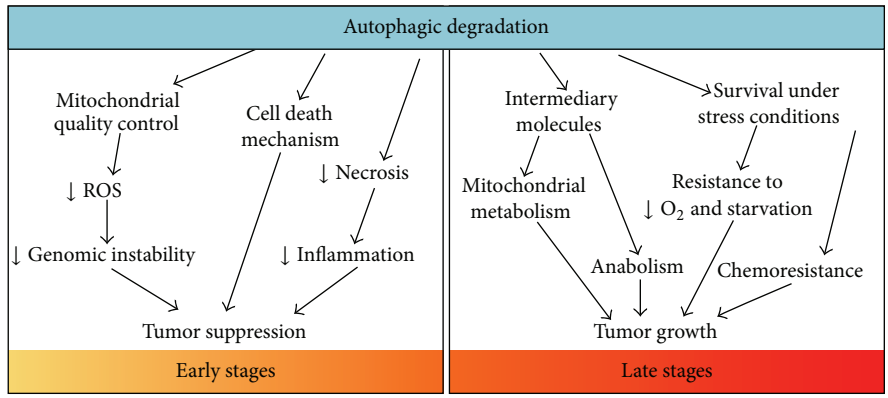

Figure 2: The two facets of autophagy in cancer. At early stages, autophagy acts as a tumor suppressor mechanism by enhancing the degradation of damaged proteins and organelles, mostly mitochondria. In doing so, autophagy acts as a quality control system that decreases ROS production and genomic instability. Moreover, autophagy prevents necrotic cell death in apoptosis-defective cells, thereby reducing local inflammation and tumor growth. Also, autophagy may serve (in some cases) as a mechanism that leads to cell death. On the other hand, at later stages of tumor development, activation of autophagy supplies tumor cells under metabolic stress conditions with nutrients and also maintains mitochondrial metabolism by providing metabolic intermediates, which promote cell survival and tumor growth. Finally, autophagy acts as a mechanism that promotes resistance to cancer therapy.

of CAV1 in cancer may be linked to its participation in the control of autophagy. However, further experimentation is required to corroborate this intriguing hypothesis.

In summary, CAV1, a membrane protein typically implicated in the formation of cell surface structures like caveolae and regulation of signalling, also plays a dual role in cancer, functioning as a tumor suppressor at early stages and a tumor promoter later on. The future will reveal how the seemingly opposing roles of autophagy in tumor development and progression are controlled, and to what extent the ambiguous role of CAV1 in cancer may be linked to the control of autophagy.

7. Conclusions

Autophagy is an evolutionarily conserved mechanism that developed in eukaryotes to ensure protein and organelle homeostasis. A hallmark of cancer cells is their increased proliferation and as a consequence their demand for energy equivalents and specific metabolites, which can be provided by autophagy. In this context, autophagy favors tumor cell development, adaptation, and progression, and particularly some oncogene-driven tumors are "addicted" to autophagy in this respect. However, autophagy also appears to have a tumor suppressor function early in cancer development by eliminating damaged mitochondria and reducing ROS-mediated genotoxic damage (see Figure 2). Accordingly, pharmacological modulation of autophagy in established tumors may represent an important anticancer therapy, as is supported by the use of autophagy inhibitors (chloroquine or hydroxychloroquine) in a large number of clinical trials and currently as a treatment for various kinds of cancers that are generally very aggressive or resistant to therapy (see Table 2).

Alternatively, considering the potential tumor suppressor role of autophagy in early stages of cancer development, one may speculate that stimulation of this process could be useful as a preventive mechanism against the development of cancer. Consistent with this notion, caloric restriction has been shown to prolong life span and reduce cancer incidence in several animal models [131]. Also, treatments with metformin, an activator of the AMPK pathway that stimulates autophagy, are associated with lower risk of different kinds of cancers [132].

Clearly, the role of autophagy in cancer depends on many factors like tissue type, tumor stage, and the type of oncogenic mutation involved. Because of these dramatic differences, more research is required to understand the role of autophagy in cancer biology and how we may harness such knowledge to improve cancer therapies and patient survival.

Abbreviations

AMBRA1: Autophagy/Beclin-1 regulator 1
AMPK: AMP-activated protein kinase
ATG: Autophagy-related
BCL-2: B-cell lymphoma 2
BCL-XL: B-cell lymphoma-extra large
BIF-1: BAX-interacting factor 1
BNIP3: BCL2/adenovirus E1B 19 kDa interacting protein 3
BNIP3L: BCL2/adenovirus E1B 19 kDa interacting protein 3-like
BRCA1: Breast cancer 1, early onset
BRCA2: Breast cancer 2, early onset
ER: Endoplasmic reticulum
FIP200: FAK family-interacting protein of 200 kDa
FoxO: Forkhead box O
LAMP: Lysosomal-associated membrane protein
LC3: Microtubule-associated protein 1 light chain 3 (homolog of yeast Atg8)
LKB1: Liver kinase B1
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

Table 2: Summary of clinical trials involving autophagy inhibitors (chloroquine or hydroxychloroquine) for cancer treatment (data obtained from http://www.cancer.gov/clinicaltrials).

Cancer type	Therapy	Phase	Status	Protocol ID
Relapsed and refractory multiple myeloma	Cyclophosphamide and pulse dexamethasone with **hydroxychloroquine** or rapamycin	0	Completed	NCT01396200
Glioblastoma multiforme	**Hydroxychloroquine**, radiation, and temozolimide	I, II	Closed	NCT00486603
Pancreas adenocarcinoma	**Hydroxychloroquine**, gemcitabine	I, II	Closed	NCT01128296
Prostate cancer	**Hydroxychloroquine** after prostate cancer treatment	II	Closed	NCT00726596
Non-small cell lung cancer	Erlotinib with or without **hydroxychloroquine**	II	Closed	NCT00977470
Metastatic pancreatic cancer	**Hydroxychloroquine** after prostate cancer treatment	II	Closed	NCT01273805
Relapsed and refractory multiple myeloma	**Chloroquine**, bortezomib, and cyclophosphamide	II	Closed	NCT01438177
Advanced solid tumors irresponsive to chemotherapy	**Hydroxychloroquine**, sunitinib	I	Closed	NCT00813423
B-cell chronic lymphocytic leukemia	**Hydroxychloroquine**	II	Temporarily Closed	NCT00771056
Surgery removable Stage III or Stage IV melanoma	**Hydroxychloroquine**	0	Temporarily Closed	NCT00962845
Relapsed and refractory multiple myeloma	**Hydroxychloroquine**, bortezomib	I, II	Active	NCT00568880
Lung cancer	**Hydroxychloroquine**, gefitinib	I, II	Active	NCT00809237
Ductal carcinoma in situ	**Chloroquine**	I, II	Active	NCT01023477
Colorectal cancer	**Hydroxychloroquine**, folinic acid, 5-fluorouracil, oxaliplatin, and bevacizumab	I, II	Active	NCT01206530
Pancreatic cancer	**Hydroxychloroquine**, protein-bound paclitaxel, and gemcitabine	I, II	Active	NCT01506973
Previously treated renal cell carcinoma	**Hydroxychloroquine**, everolimus	I, II	Active	NCT01510119
Renal cell carcinoma	**Hydroxychloroquine**, aldesleukin	I, II	Active	NCT01550367
Unresectable hepatocellular carcinoma	**Hydroxychloroquine**, transarterial chemoembolization (TACE)	I, II	Active	NCT02013778
Metastatic colorectal cancer	**Hydroxychloroquine**, capecitabine, oxaliplatin, and bevacizumab	II	Active	NCT01006369
Chronic myeloid leukemia	Imatinib mesylate with or without **hydroxychloroquine**	II	Active	NCT01227135
Breast cancer	**Hydroxychloroquine**	II	Active	NCT01292408
Advanced or metastatic breast cancer	**Chloroquine**, taxane	II	Active	NCT01446016
Resectable pancreatic cancer	**Hydroxychloroquine**, capecitabine, and radiation	II	Active	NCT01494155
High grade gliomas	**Hydroxychloroquine**, radiation	II	Active	NCT01602588
Advanced/recurrent non-small cell lung cancer	**Hydroxychloroquine**, paclitaxel, carboplatin, and bevacizumab	II	Active	NCT01649947
Progressive metastatic castrate refractory prostate cancer	Navitoclax, abiraterone acetate with or without **hydroxychloroquine**	II	Active	NCT01828476
Soft tissue sarcoma	**Hydroxychloroquine**, rapamycin	II	Active	NCT01842594
Potentially resectable pancreatic cancer	Protein-bound paclitaxel, gemcitabine with or without **hydroxychloroquine**	II	Active	NCT01978184
Metastatic or unresectable solid tumors	**Hydroxychloroquine**, temozolomide	I	Active	NCT00714181
Irresponsive metastatic solid tumors	**Hydroxychloroquine**, temsirolimus	I	Active	NCT00909831

Table 2: Continued.

Cancer type	Therapy	Phase	Status	Protocol ID
Stage IV small cell lung cancer	**Chloroquine**	I	Active	NCT00969306
Advanced solid tumors	**Hydroxychloroquine**, vorinostat	I	Active	NCT01023737
Primary renal cell carcinoma	**Hydroxychloroquine** before surgery	I	Active	NCT01144169
Advanced cancer	**Hydroxychloroquine** sirolimus, or vorinostat	I	Active	NCT01266057
Solid tumors	**Hydroxychloroquine**, radiation	I	Active	NCT01417403
Melanoma	**Chloroquine**, radiation, DT01	I	Active	NCT01469455
Advanced solid tumors, melanoma, prostate, or kidney cancer	**Hydroxychloroquine**, Akt inhibitor MK2206	I	Active	NCT01480154
Stages I–III small cell lung cancer	**Chloroquine**, radiation	I	Active	NCT01575782
Refractory or relapsed solid tumors	**Hydroxychloroquine**, sorafenib	I	Active	NCT01634893
Lymphangioleiomyomatosis in women	**Hydroxychloroquine** sirolimus	I	Active	NCT01687179
Relapsed or refractory multiple myeloma	**Hydroxychloroquine**, cyclophosphamide, dexamethasone, and sirolimus	I	Active	NCT01689987
Nonresectable pancreatic adenocarcinoma	**Chloroquine**, gemcitabine	I	Active	NCT01777477
BRAF mutant metastatic melanoma	**Hydroxychloroquine**, vemurafenib	I	Active	NCT01897116
Advanced solid tumors	**Chloroquine**, carboplatin, and gemcitabine	I	Active	NCT02071537
Brain metastasis	**Chloroquine**, radiation	0	Active	NCT01727531

NRF-2: Nuclear factor erythroid 2-related factor 2
PALB2: Partner and localizer of BRCA2
PI3KC3/VSP34: Phosphatidylinositol 3-kinase, catalytic subunit type 3
PI3K: Phosphatidylinositol 3-kinase
PTEN: Phosphatase and tensin homolog
PyMT: Polyoma middle T-antigen
ULK: Unc-51 like autophagy activating kinase
UVRAG: Ultraviolet radiation resistance-associated gene.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Importance of Autophagy Regulation in Breast Cancer Development and Treatment

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Breast cancer (BC) is a potentially life-threatening malignant tumor that still causes high mortality among women. One of the mechanisms through which cancer development could be controlled is autophagy. This process exerts different effects during the stages of cancer initiation and progression due to the occurring superimposition of signaling pathways of autophagy and carcinogenesis. Chronic inhibition of autophagy or autophagy deficiency promotes cancer, due to instability of the genome and defective cell growth and as a result of cell stress. However, increased induction of autophagy can become a mechanism which allows tumor cells to survive the conditions of hypoxia, acidosis, or chemotherapy. Therefore, in the development of cancer, autophagy is regarded as a double-edged sword. Determination of the molecular mechanisms underlying autophagy regulation and its role in tumorigenesis is an essential component of modern anticancer strategies. Results of scientific studies show that inhibition of autophagy may enhance the effectiveness of currently used anticancer drugs and other therapies (like radiotherapy). However, in some cases, the promotion of autophagy can induce death and, hence, elimination of the cancer cells and reduction of tumor size. This review summarizes the current knowledge on autophagy regulation in BC and up-to-date anticancer strategies correlated with autophagy.

1. Introduction

Breast cancer (BC) is the most common and fatal cancer in women worldwide, despite decreasing mortality rates that result mostly from efficient screening strategies [1, 2]. It has been estimated that approximately 1.3 million females develop BC each year, with around 465.000 expected to succumb to the disease [3, 4]. BC is ranked in the second place in mortality among cancer types [5], causing death of about 350,000 women in both developed and developing countries every year [6]. More than 90% of lethality in patients is caused by metastasis, and the occurrence of distant metastases (distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung, and liver) severely limits the prognosis [7–9]. The 5-year survival rate for patients with BC drops sharply from 98% for individuals with localized disease to 23% for those with metastatic disease [10]. Many factors are involved in the pathogenesis and progression of BC, including genetic, biological, and environmental factors, as well as lifestyle [6]. It has been estimated that 75% of women with sporadic invasive BC have no known epidemiological risk factors [11]. On the other hand, *BCL-2* protooncogene is overexpressed in half of all human malignancies and more than 60% of BC and is thought to exert its oncogenic role by preventing cells from undergoing apoptosis [12].

Recent studies demonstrated association between autophagy and cancer. Autophagy is a genetically regulated process, controlled by a group of evolutionarily conserved genes called *ATG* (autophagy-related genes). At least 11 *ATG* genes identified in yeast have orthologs in mammals. Autophagy initially was identified as a cell survival mechanism to protect cells from nutrient deprivation. It maintains proteins and organelles turnover and by that ensures homeostasis. Removing excess or damaged intracellular components in response to stress, as well as microbes, allows cells to restrain damage, including alternations in the genome (genome instability), and subsequent inflammation. Suppressing of genome instability limits initiation and progression of cancer. In certain

developmental conditions, in cell's response to metabolic stress, but also under cytotoxic stimuli, autophagy results in a form of cell death described as type II programmed cell death [13]. This could be seen also, for example, in cells expressing BCL-2 or Bcl-xl or lacking both Bax and Bak proteins.

The role of autophagy in tumorigenesis and treatment responsiveness is complicated and context-dependent and presumably differs in different stages of cancer development. At the initial stages of cancer development, autophagy may represent a protective response thanks to its catabolic roles, by degrading and/or recycling cell components, like damaged organelles and misfolded proteins [14–16]. Autophagy may hinder proliferation of cells with cancer-linked mutations. It can also limit propagation of this type of mutations, thus suppressing tumorigenesis by facilitating senescence (biological aging). However, once a tumor develops, the cancer cells can utilize autophagy for their own cytoprotection. Autophagy can increase oxidative stress, thereby promoting genome instability and malignant transformation [15–18], and cancer cells can use enhanced autophagy to survive under metabolic and therapeutic stress [16]. Additionally, it has been suggested that metastatic cancer cells may escape from anoikis (process of apoptosis induced by lack of correct cell-extracellular matrix attachment) via the induction of autophagy [19, 20].

Current BC therapy depends on the type and stage of the BC and traditionally consists of a multivariate approach including surgery, hormone therapy, systemic chemotherapy, radiotherapy, and molecular targeted therapy [2, 9]. Despite treatment, the majority of breast cancers are incurable and ultimately claim the life of the patient with complications and development of chemoresistance [9]. The pharmacological or genetic inhibition of autophagy is studied and correlated with sensitization of cancer cells to the lethal effects of various cancer therapies including chemotherapy, radiotherapy, and targeted therapies, suggesting that suppression of the autophagic pathway could represent a valuable strategy for cancer treatment. Continuing progress in this field will be critical for developing new cancer therapies and improving those already in use [21, 22].

2. Autophagy Outline

Currently, over 35 proteins are believed to be essential for autophagy occurrence and progression [23]. Biological and morphological changes have been observed under the influence of autophagic pathway [24]. Under stress conditions autophagy is induced to keep the balance in cells [25]. Cellular stress can be caused by a variety of chemical and physical agents, like nutrient starvation, proinflammatory state, hypoxia, oxidants, infectious agents, and xenobiotics [26, 27].

The complete macroautophagic (referred to hereafter as autophagy) flow is generally divided into the following stages: induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, degradation, and recycling [26, 28, 29]. Basal levels of macroautophagy are kept in check by mTORC1 (mammalian target of rapamycin complex 1) phosphorylation of *ATG13* (autophagy-related gene 13) and ULK1 (uncoordinated 51-like kinase 1/*ATG1*) or ULK2. It inhibits ULK1 phosphorylation of FIP200 (focal adhesion kinase interacting protein of 200 kD/*ATG17*) [30, 31]. The mTORC1 complex is an important component of a network that senses the nutrient state of the cell and accordingly maintains homeostasis by controlling the levels of anabolism and catabolism. High levels of amino acids maintain mTORC1 in an active state by enhancing its binding to regulatory proteins Rag and Rheb (Ras homolog enriched in brain) GTPases (guanosine triphosphatases) [32]. Insulin and IGF1 (insulin-like growth factor 1) indirectly induce mTORC1 activity by stimulating class 1 PI3K (phosphoinositol 3-kinase) production of PIP3 (PtdIns(3,4,5)P3), which induces Akt kinase (protein kinase B) at the plasma membrane, which in turn activates mTORC1 by inhibiting TSC (tuberous sclerosis complex) proteins 1/2, thereby relieving their repression of Rheb [28]. Low glucose levels or high levels of AMP (adenosine 5′-monophosphate), which indicate low cellular energy status or stress, activate AMPK (AMP-activated protein kinase), which inhibits mTORC1 and stimulates macroautophagy [26, 27, 33, 34].

Vesicle nucleation is the initial step in which proteins and lipids are recruited for construction of the autophagosomal membrane. In mammalian cells, this process is initiated by activation of the class III PI3K/Beclin-1 complex, including the core members hVps34/PIK3C3, Beclin-1, and p150. Numerous additional binding partners of this complex function as either positive or negative regulators and include BAX-interacting factor-1 (BIF-1), ATG14L, UVRAG (UV irradiation resistance-associated gene), Ambra1 (activating molecule in Beclin-1-regulated autophagy protein 1), and Rubicon [23, 26, 29, 35, 36]. Additionally, Ambra1 is required for the interaction of Beclin-1 with PI3KC3/Vps34 [36]. Interaction of UVRAG with Beclin-1 leads to increased PI3KC3/Vps34 kinase activity, which results in increased autophagic initiation. UVRAG is also involved in the interaction of Bif with Beclin-1, which facilitates the activation of PI3KC3/Vps34. Rubicon (RUN domain Beclin-1-interacting cysteine-rich-containing protein) has also been shown to negatively regulate autophagy [37, 38]. The regulation of the maturation process of the autophagosome is multifactorial and involves Rab GTPase, SNAREs (soluble N-ethylmaleimide-sensitive fusion attachment protein receptors), and ESCRT (endosomal sorting complexes required for transport) proteins, molecules of the acidic lysosomal compartment (e.g., v-ATPase, LAMP proteins-lysosome-associated membrane glycoproteins, lysosomal carriers, and hydrolases), and Beclin-1 [39].

3. Autophagy Regulation in Breast Tumors

Autophagy seems to have different roles in cancer cells. Autophagy plays a complex role in tumor initiation and progression. It protects against the deleterious effects of reactive oxygen species in the cells, which may lead to mutations in DNA, and promotes cell transformation [40]. Autophagy has been shown to be required for the transformation of mouse embryonic fibroblasts by the Ras oncogene and this effect is linked to its role in nutrients recycling, such as glucose uptake

and increased glycolytic flux [41, 42]. During the later stages of *in vivo* tumor formation, autophagy is necessary for cancer cells survival in hypoxia conditions before the vascularization of tumor takes place [40, 42]. In fully transformed cancer cells it appears to function as a tumor suppressor, as defective autophagy is associated with malignant transformation and carcinogenesis. However, in normal cells and in some cancer cells the induction of autophagy is associated with cell death [12]. Studies have shown that cancer cells express lower levels of the autophagy-related proteins LC3-II and Beclin-1 than normal epithelial cells and that while heterozygous disruption of *BECN1* promotes tumorigenesis, the overexpression inhibits tumorigenesis, supporting the assertion that defective autophagy or inhibition of autophagy plays a role in malignant transformation [12].

Regulation of autophagy in tumors is governed by principles similar to normal cells, only in a much more complicated manner, given the frequently observed abnormal PI3K activation in cancer and the multitude of interactions between the PI3K/Akt/mTOR pathway and other cell signaling cascades, often also deregulated in tumor cells [35]. A deregulated PI3K/Akt/mTOR axis not only suppresses autophagy but also induces protein translation, cell growth, and proliferation, a major driving force in tumorigenesis. Tumors with high metabolic demands, with constitutively active PI3K mutations, PTEN loss, or Akt activation, would be expected to be dependent on autophagy for energy homeostasis and survival. Suppression of autophagy by the PI3K signaling cascade presents a disadvantage for rapidly proliferating tumor cells and leads to the prediction that compensatory mechanisms (like deregulated apoptosis and/or metabolism) might be concurrently activated to prevent the negative implications of defective autophagy on tumor cell survival. Ras/Raf/ERK pathway is among the most commonly deregulated pathways identified in tumors, as indicated by frequently observed activating mutations in Ras or B-Raf oncogenes [13].

Many proteins and active factors correlated with autophagy are reported to be associated with human cancers [43]. Autophagic cell death has been described, for example, in antiestrogen-treated cultured human mammary carcinoma MCF-7 cells [44]. The role of autophagy might be different in certain stages and aspects of tumor development. Various tumor suppressors (e.g., PTEN, TSC1/2, p53, and DAPK) are autophagy inducers, whereas some inhibitors of autophagy (e.g., Akt and Ras) possess oncogenic activity [45]. Studies of Kadota et al. [46] and Kim et al. [47] showed that the more advanced stages of breast cancer overexpress several other oncogenic and signaling proteins, such as IGF-1R, Cyclin D1, c-myc, pERK, Stat3, and Pak4, some of which are known activators of Akt-mTOR pathway. ERK activity has also been associated with autophagy and autophagic cell death in many cellular models in response to different stresses [13] and also in TNF-α (tumor necrosis factor-alpha) treatment in MCF-7 cells. Several other autophagy regulators in cancer cells, like mitogen-activated kinases (BNIP3) [48] and HSpin 1 (human homologue of the *Drosophila* spin gene product) [49], play a critical role.

PTEN has a stimulatory effect on autophagy, by down-regulating PI3K/Akt signaling. It is a critical regulator of the PI3K pathway [39], which selectively hydrolyzes PIP3 to PIP2, and inhibits the activation of Akt/PKB. Akt inhibition leads to suppression of mTOR signaling and the induction of autophagy [39]. Unlike the Ras/Raf/Erk and PI3K pathways, AMPK (AMP-activated protein kinase) pathway has a negative effect on mTOR signaling and promotes autophagy. Upon starvation and activation of calcium signaling, AMPK phosphorylates and activates TSC2, which inhibits mTOR signaling.

EI24/PIG8 (etoposide induced gene) can be mentioned as a critical factor of autophagic degradation, which remains under control of p53 [50, 51]. p53 is well known as a critical tumor suppressor, which protects organism by initiation of cell-cycle arrest, removal of cells with incurred DNA damage, senescence, and apoptosis. *TP53* is the most commonly deleted or mutated gene in human cancers [52]. p53 target genes, effectors of p53 apoptotic response, are currently widely studied. The human EI24 genomic locus is on chromosome 11, in region frequently altered in cancers, and was reported to be mutated in aggressive breast cancers. Furthermore, since EI24/PIG8 (induced by p53) is also known as important apoptotic effector [50, 52], this role may contribute to tumor suppression. Loss of EI24/PIG8 was associated with tumor invasiveness but not with the development of the primary tumor [52].

3.1. Beclin-1. The most important evidence linking dysfunctional autophagy and cancer comes from studies demonstrating that the inhibition of autophagy in mice, by disruption of *BECN1*, increases cellular proliferation, increases the frequency of spontaneous malignancies (i.e., lung cancer, liver cancer, and lymphomas) as well as mammary hyperplasia, and accelerates the development of carcinogen-induced pre-malignant lesions [12].

FISH analysis of human breast cancer cell lines using the Beclin-1-containing PAC 452O8 as a probe revealed that 9 out of 22 cell lines had allelic Beclin-1 deletions [35]. Ectopic expression of Beclin-1 restores full autophagy potential in MCF-7 cells, which are tetraploid but have three Beclin-1 copies, and slows cell proliferation *in vitro* and in xenograft tumors *in vivo*. Monoallelic deletion of *BECN1* has been detected in 40–75% cases of human breast, ovarian, and prostate tumors [15, 27, 53], and thus Beclin-1 is considered as a tumor suppressor gene [54]. The aberrant expression of Beclin-1 in many kinds of tumor tissues correlates with poor prognosis [15]. Accordingly, heterozygous deletion of *BECN1* in mice (*BECN1*+/−) resulted in increased incidence of spontaneous tumors [54]. Many breast carcinoma cell lines, although polyploidal for chromosome 17 (*BECN1* gene is placed in 17q21 loci), exhibit deletions of one or more *BECN1* alleles, and human breast tumors show decreased Beclin-1 levels compared to normal adjacent tissue. Restoration of Beclin-1 and autophagy in MCF-7 cells is associated with inhibition of MCF-7-induced tumorigenesis in nude mice [54]. *BECN1*+/− mice do not have increased incidence of mammary tumors but rather are susceptible to lymphomas and carcinomas of the lung and liver after long latency. Tumors forming in *BECN1*+/− mice express wild-type *BECN1*

mRNA and protein, indicating that Beclin-1 is a haploinsufficient tumor suppressor [35, 36, 53]. Furthermore, Beclin-1 and EI24 alter expression of several autophagy proteins, such as ATG5 and UVRAG [15].

One of the latest factors correlating autophagy and BC is adipokines, autocrine, endocrine, and paracrine-acting bioactive molecules [55]. The adipokine secreted in greatest abundance is adiponectin (AdipoQ). The prevalent evidence indicates that low levels of AdipoQ in the circulation indicate poorer BC risk and prognosis. AdipoQ in breast tissue has a direct anticarcinogenic effect at the site of tumor growth. AdipoQ is potentially able to regulate autophagy through AMPK, whose activation has been observed in breast cancer cells [55, 56]. Liu and colleagues [57] observed that AdipoQ caused upregulation of autophagy in MDA-MB-231 cells *in vitro* and *in vivo*.

3.2. MicroRNA. MicroRNAs (miRNAs) are small RNA molecules. Unregulated miRNAs of lymphoma, prostate, lung, and breast cancer have been also detected in blood plasma and serum; circulating miRNAs are currently assessed as proxy biomarkers for BC [58]. There are many pieces of evidence that miRNAs can influence autophagy process in BC cells in many points. MiR-20a, miR-101, miR-106a/b, and miR-885-3p have been reported to have direct possibility to target ULK1/2 [59]. Conserved and predicted binding sites for miR-885-3p exist in *MATG13*, *ATG9A*, and *ATG2B* [29]. MiR-155 might target multiple players in mTOR signaling, including Rheb, RICTOR (RPTOR independent companion of mTOR), and RPS6KB2 (ribosomal protein S6 kinase). MiR-30a and miR-519a can directly target Beclin-1 in the autophagic flow, causing its negative regulation, thereby resulting in decreased autophagic activity. This negative regulation of Beclin-1 expression by miR-30a was shown in the *in vitro* study by Zhu et al. [60] on human breast cancer cell lines MDA-MB-468 and MCF-7. Treatment of tumor cells with the mimic of miR-30a decreased the expression of Beclin-1 mRNA and protein, whereas administration of the miR-30a antagomir increased Beclin-1 levels. Furthermore, high expression of miR-30a blunted the activation of autophagy induced by rapamycin [60]. MiR-376b also regulates Beclin-1, and it can also directly target *ATG4C* [29, 61, 62] in MCF-7 cells, because the antagomir-mediated inactivation of the endogenous miR-376b results in an increased level of ATG4C and Beclin-1 [26, 61]. The direct regulation of UVRAG is modulated by miR-374a and miR-630 [63]. The tumor suppressive miR-101 could act as a potent inhibitor of basal, etoposide-induced, and rapamycin-induced autophagy in MCF-7 cells. Frankel et al. [64] used 4-hydroxytamoxifen (4-OHT) to induce cell death in breast-cancer-derived MCF-7 cells (a stimulus to which they are usually resistant), synergistically with miR-101 as an autophagy inhibitor. The miR-101-mediated inhibition of autophagy sensitized breast cancer cells to 4-hydroxytamoxifen-induced apoptotic cell death, and thus miR-101 was suggested to modulate the chemosensitivity of cancer cells [29]. Three components, including STMN1 (stathmin1), RAB5A (Ras-related protein 5A), and ATG4D, have been identified as targets of miR-101, among which the overexpression of STMN1 could partially rescue cells from miR-101-mediated inhibition of autophagy, indicating a functional importance for this target. RAB5A and STMN1 had previously uncertain roles in autophagy. RAB5A has been shown to regulate ATG5–ATG12 conjugation in the autophagosome completion, while STMN1 destabilizes microtubules and plays an important role in cell-cycle regulation [65]. Most likely, at least in breast cancer cells, elevated levels of autophagy, due to the progressive loss of miR-101, have the potential to trigger cancer cell survival [29, 64]. MiR-221/222 might inhibit the cell-cycle inhibitor, p27Kip1, a downstream modulator of PI3KCI/Akt, leading to autophagic cell death in HER2/neu-positive primary human breast carcinoma MCF-7 cells, whereas the ectopic expression of miR-221/222 renders the parental MCF-7 cells resistant to tamoxifen [66, 67].

4. Anticancer Therapies Correlated with Autophagy

4.1. Cytoprotective and Nonprotective Forms of Autophagy. A number of antineoplastic therapies, including radiation therapy, chemotherapy (e.g., doxorubicin, temozolomide, and etoposide), histone deacetylase inhibitors, arsenic trioxide, TNFα, IFNγ, imatinib, rapamycin, and antiestrogen hormonal therapy (e.g., tamoxifen), have been shown to induce autophagy as a protective and prosurvival mechanism in human cancer cell lines [12, 68, 69]. In fact, the therapeutic efficacy of these agents can be increased if autophagy is inhibited [12, 70]. The evidence suggests that autophagy leads to cell death in response to several compounds, including rottlerin, cytosine arabinoside, etoposide, and staurosporine, as well as growth-factor deprivation. A link between autophagy and related autophagic cell death has been demonstrated using pharmacological inhibitors (e.g., 3-MA (3-methyl adenine), CQ (chloroquine), bafilomycin A1, or ammonium chloride) and genetic silencing or knockdown (silencing of *ATG5*, *ATG7*, *ATG12*, and *BECN1*) approaches for suppression of autophagy. For example, the knockdown of *ATG5* or *BECN1* in cancer cells containing defects in apoptosis leads to a marked reduction in autophagic cell death (as well as autophagic response) in response to cell death stimuli with no signs of apoptosis [12, 71]. This is connected with *cytoprotective form of autophagy* [72].

Autophagy has also been shown to protect against cellular stress induced by the chemotherapeutic drugs used in cancer treatment (*nonprotective autophagy*) [72]. Furthermore, because autophagy is frequently upregulated in tumors in response to therapy, it may protect the tumors against therapy-induced apoptosis [73]. Huang et al. studied the effect of *PTTG1* inhibition on tumor growth and metastasis. *PTTG1*/securin is an oncogene that is highly expressed in various tumors and has been correlated with tumor invasiveness and poor prognosis. Huang et al. [68] reported that inhibition of *PTTG1* suppressed tumor growth and metastasis *in vitro* and *in vivo*. The group also investigated autophagy induced during radiation in human breast cancer cells expressing

different levels of PTTG1 by measuring the expression of MAP1LC3-I and MAP1LC3-II [68]. The results revealed that radiation increased the ratio of MAP1LC3-II/MAP1LC3-I in MDA-MB-231-2A cells (*PTTG1*-knockdown MDA-MB-231 cells) and MCF-7 cells (low *PTTG1* expression), but not in the parental MDA-MB-231 cells, suggesting that radiation induced autophagy in *PTTG1*-depleted cancer cells. These data suggest that autophagy promotes cell survival and plays a decisive role in choosing between radiation-induced senescence and apoptosis. Inhibition of autophagy by 3-MA resulted in the MDA-MB-231-2A cells undergoing apoptosis instead of radiation-induced senescence; cells undergoing apoptosis could have enhanced radiosensitivity. It appears that apoptosis is a more efficient way to trigger cell death, as inhibition of autophagy and senescence by treatment with 3-MA and bafilomycin A1 enhanced cell death [68].

Gewirtz [72] reported that ionizing radiation could promote autophagy in BC cells in cell culture, but autophagy inhibition did not alter sensitivity to radiation. Furthermore, they showed that chloroquine did not sensitize (4T1) murine breast tumor cells to radiation in an immunocompetent animal model. Based on the results obtained it was impossible to determine whether radiation promoted autophagy or the chloroquine actually effectively inhibited autophagy in the tumor-bearing animals. Supposedly, the lack of sensitization could be related to the findings [23] that autophagy inhibition interferes with the immune system's capability to recognize the tumor undergoing a stress response.

Such disclosures have led to several clinical trials involving the use of inhibitors of the autophagy flux ("autophagic flux" represents the synthesis of autophagosomes, transportation of different substrates, and degradation of autophagy inside the lysosome) as a combination therapy [74], to improve the efficacy of radiotherapy in BC patients. For example, hydroxychloroquine (HCQ), an autophagy inhibitor that is currently in phase I and phase II clinical trials, has been used in combination with several chemo- and radiotherapies [28, 68, 75]. HCQ is a less toxic version of CQ and the best autophagy inhibitor currently commercially available for clinical trials [76]. Currently, there are 52 clinical trials of HCQ listed on the United States Government website (clinicaltrials.gov), of which 32 are cancer studies. There are 48 results for HCQ in cancer therapy (2 for breast cancer) of HCQ in combination with a range of chemotherapeutic agents.

Irradiated cancer cells can induce damage in neighboring unirradiated cells by intracellular gap-junction communication or signals that are released outside of the cells [77]. Huang et al. [68] have indicated that radiation-induced senescent MDA-MB-231-2A cells secrete multiple cytokines and chemokines, including CSF2 (colony stimulating factor, expressed in the highest level), CXCL1 (C-X-C motif ligand 1), IL6 (interleukin 6), and IL8 (interleukin 8). These factors are involved in multiple functions during cancer progression. Autophagy inhibition in MDAMB-231-2A cells significantly decreased the release of CSF2, suggesting that autophagy plays an important role in promoting the secretion of SASPs (senescence-associated secretory phenotypes). In support of this notion, it has been reported that inhibition of autophagy delays the secretion of several senescence-associated cytokines, such as IL6 and IL8 [78].

4.2. Cytotoxic and Cytostatic Autophagy. The next form of autophagy, which should be taken under consideration in the field of cancer treatment, is *cytotoxic autophagy*. For example, Bristol et al. [79] have reported that vitamin D (or the vitamin D analog, EB 1089) can be combined with radiation to promote a cytotoxic form of autophagy in breast tumor cells (MCF-7 and ZR-75). Other research groups also showed that the generation of *cytotoxic autophagy* may either lead independently to cells death or act as a precursor to apoptosis [80]. Functionally, *cytotoxic autophagy* is associated with a reduction in the number of viable cells and/or reduced clonogenic survival upon treatment [81]. Gewirtz [81] identified an additional form of autophagy, termed *cytostatic autophagy*, in nonsmall cell lung cancer cells (A549 and H460), which was induced in similar conditions to the ones previously described in regard to breast tumor cells. Similarly to the impact on *cytotoxic autophagy* in breast tumor cells, pharmacologic inhibition of autophagy with either chloroquine or 3-MA protected the cells from the sensitization to radiation by vitamin D or EB 1089. What distinguishes cytostatic autophagy from the cytoprotective form is the failure to detect evidence of cell killing reported in the breast tumor cells [81]. The group of both Gewirtz [81] and Kroemer had demonstrated cytoprotective autophagy by radiation alone [82], but the addition of vitamin D or EB 1089 converted cytoprotective autophagy to cytostatic autophagy.

As Ras/Raf/ERK pathway belongs to the most commonly deregulated pathways identified in tumors, it is currently the target of new antitumor strategies, based on the inhibition of upstream ERK regulators. However, because ERK activation is implicated in DNA-damaging agent-induced cell death, inhibiting ERK activity in combination therapy with classical antitumor compounds might affect the efficiency of such compounds. For example, in MCF-7 human breast adenocarcinoma cell line such combined therapies with doxorubicin [83], tamoxifen [84], taxol [85], or Δ Raf1 [86] and TNFα [87] were used. Targeting autophagy was used to circumvent TRAIL-resistance in tumors with apoptosis defects; knockdown of autophagy, in combination with tamoxifen or 4-hydroxy-tamoxifen (4-OH-T), resulted in decreased cell viability of two human hormone-dependent breast cancer cell lines, ER-positive MCF-7 [70, 88] and T-47D cells [89]. Tamoxifen, the most commonly used antiestrogen, exerts its pharmacological action by binding to estrogen receptor alpha (ERα) and blocking the growth promoting action of the estrogen bound receptor in BC cells. However, the development of antiestrogen resistance has become a major impediment in the treatment of ER-positive BC. Samaddar et al. [89] had reported that autophagy plays a critical role in the development of antiestrogen resistance, and overexpression of Beclin-1 downregulated estrogenic signaling and growth response [90].

4.3. Silencing Autophagic Genes. Genetic approaches could be represented by some studies using gene silencing to receive therapeutic effect via cell death induction. For example, the *BCL-2* protooncogene is overexpressed in half of all human malignancies and more than 60% of BC. It exerts its oncogenic role by preventing cells from undergoing apoptosis. *BCL-2* overexpression not only leads to the resistance of cancer cells towards chemotherapy, radiation, and hormone therapy but also causes an aggressive tumor phenotype in patients with a variety of cancers [12]. Recent findings suggested that silencing *BCL-2* expression by siRNA in MCF-7 cells led to significant autophagic, not apoptotic, cell death [39, 71]. It has been demonstrated that the knockdown of autophagy genes (e.g., *ATG5* and *BCN1*) significantly inhibited both autophagy and cell death induced by *BCL-2* siRNA after a long-term treatment of up to seven days [71]. MCF-7 cells are known to be caspase 3-deficient, providing a higher threshold for the induction of apoptosis, potentially rendering the autophagic cell death pathway more important. Furthermore, about 45–75% of tumor tissues from BC patients do not have detectable caspase 3 expression [91]. Additionally, Akar et al. [71] reported that doxorubicin predominantly induced autophagy at low doses and apoptosis at high doses. Furthermore, the combination of *BCL-2* siRNA treatment with a low dose of doxorubicin enhanced the autophagic response, tumor growth inhibition, and cell death. It was the first evidence that targeted silencing of *BCL-2* induces autophagic cell death in BC cells, which constitutes a good beginning for further research on this type of alternative therapeutic strategies.

4.4. Pharmacological Approach to the Antitumor Autophagic Therapies. Studies on the role of autophagy in tumor development and progression have led to a subsequent progress in pharmacological approach to the antitumor autophagic therapies, which aim to activate or inhibit autophagy. Many drugs and compounds that modulate autophagy are currently receiving considerable attention [15, 43]. These include, for example, autophagy inducers such as mTORC1 inhibitor rapamycin [15] and its analogs that are called rapalogs, such as Everolimus (RAD001), which are also often used as tools to study autophagy process [28, 74]. Everolimus was shown to enhance the sensitivity of tumors to radiation by induction of autophagy [28].

Also natural products are considered as potential anticancer candidates, being direct or indirect sources of new chemotherapy adjuvants to enhance the efficacy of chemotherapy and/or to ameliorate its side effects [92]. Lu et al. [92] have used Cyclovirobuxine D (CVB-D), an alkaloid component isolated from the roots of *Buxus microphylla* var. *sinica* (recently used for the cardiovascular diseases treatment) as a potential inducer of autophagy in MCF-7 cell line. After exposure of the breast cancer cells to CVB-D (10 mM) for 24 h, an induction of cell death was noted in a concentration- and time-dependent manner. It was accompanied with parallel increase in the level of autophagy with accumulation of autophagosomes (upregulated LC3II). Then to explore whether autophagy was involved in the cytotoxicity of CVB-D, 3-MA was used. The results of Western blotting and MDC staining showed that autophagy promoted by CVB-D was significantly attenuated by 3-MA [92].

Another team, Lu et al. [93], was working on the sesquiterpene lactone, PTL (bioactive component in feverfew, used for fever, migraine, and arthritis). They have reported that PTL increased ROS level, activated AMPK, and induced autophagy in MCF-7 cells and that inhibition of AMPK or autophagy significantly potentiated PTL-induced apoptosis in MCF-7 breast cancer cells. Moreover, PTL showed similar effects on other BC cell lines, MDA-MB-231 and T47D, and again usage of 3-MA suppressed PTL-induced autophagy.

The more challenging issue is the monitoring of autophagic activity in humans, in tissue and blood samples. It seems to be more important to measure autophagic flux than autophagosome number. However, till now, measurement of autophagic flux in paraffin-embedded tissue samples has been unsuccessful, and even the detection of endogenous LC3-II (commonly used marker for autophagosomes) is problematic in tissue sections [15].

5. Concluding Remarks

There has been a tremendous amount of progress in our understanding of the role of autophagy in cancer. Overall, the data support a dynamic role of autophagy in cancer, both as a tumor suppressor early in progression and later as a protumorigenic process, critical for tumor maintenance and therapeutic resistance. The specification of the autophagic cargo in tumors with increased autophagy is important for understanding the changes in metabolism between normal and malignant cells. Undoubtedly, progress in genomics, proteomics, and metabolomics will be helpful in this scope. Currently, the molecular mechanisms underlying the regulation of autophagy and the role of autophagy in cancer cells are not fully understood but are progressively revealed. Defects in apoptosis lead to increased resistance to chemotherapy, radiotherapy, and other therapies. Therefore, induction of autophagic cell death may be an ideal approach in these resistant cancers therapies. Continued progress in this field will be critical in developing new cancer therapies and improving those already in use.

As was presented in this review, most experiments regarding BC are carried out on cell lines, *in vitro*. Autophagy inhibition by HCQ in combination with chemotherapy is currently being evaluated in multiple ongoing clinical trials in patients with solid tumors, but we should take into account that autophagic effect is context-dependent. While tumor cell susceptibility to autophagy may depend on tumor genotype and the therapeutic agents utilized, data are very limited and it remains unclear whether such new strategies will be clinically beneficial.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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