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Autophagy : Moving Benchside Promises to Patient Bedsides

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Abstract: Survival rates of patients with metastatic or recurrent cancers have remained virtually unchanged during the past 30 years. This fact makes the need for new therapeutic options even more urgent. An attractive option would be to target autophagy, an essential quality control process that degrades toxic aggregates, damaged organelles, and signaling proteins, and acts as a tumor suppressor pathway of tumor initiation. Conversely, other fascinating observations suggest that autophagy supports cancer progression, relapse, metastasis, dormancy and resistance to therapy. This review provides an overview of the contradictory roles that autophagy plays in cancer initiation and progression and discusses the promises and challenges of current strategies that target autophagy for cancer therapy.

Keywords: Antineoplastic agents/drug effects/autophagy targeted therapy, autophagy addiction/KRAS/BRAF-driven cancers, tumor metabolism, tumor resistance, tumor relapse, tumor metastasis, tumor dormancy, translational medicine.

AUTOPHAGY AND CANCER

On December 1, 2014, a PubMed search of “autophagy and cancer” yielded 5,470 entries, which constitutes 31% of all 17,588 articles published on the topic of “autophagy.” This amount of literature illustrates how autophagy has both positive and negative roles in tumorigenesis: promoting tumor growth or tumor suppression (Fig. 1). Not surprisingly, all the signaling pathways that control cancer development regulate autophagy, which is exploited by both tumor suppressor and oncogenic mechanisms. However, the results of fifteen years of research do not clarify whether cancer therapies can suppress or upregulate autophagy, and whether upregulation of autophagy can favor tumor cell survival or death. The exact role that autophagy plays in cancer is therefore complex and warrants a further unifying model. In the following sections, we provide a detailed discussion of the ways by which autophagy can be both tumorigenic and tumor suppressive.

AUTOPHAGY AT A GLANCE

The beginning of the research on autophagy can be tracked back to 1956 when Sam Clark observed irregularly shaped vacuoles containing amorphous materials and occasional mitochondria [1]. Soon after that, Christian de

Duve established the nomenclature for different lysosomal pathways (Nobel Prize in Physiology or Medicine, 1974).

Autophagy, as suggested by its Greek acronym ‘self-eating’, targets intracellular organelles and constituents for lysosomal degradation. So far, three different types of autophagy have been described; namely, chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. These three types essentially differ in the mechanism by which they deliver substrates (cargo) to the lysosomal lumen. *Chaperone-mediated autophagy* ensures the translocation of a KFERQ-motif bearing protein into the lysosome by the chaperone heat shock protein cognate 70 (HSC70) [2]. *Microautophagy* involves the direct internalization of small cytoplasmic material into the lysosome through invagination of its membrane [3]. *Macroautophagy* delivers proteins and organelles to lysosomes for degradation upon sequestration in a double-membrane vesicle – termed the autophagosome.

The complementarity, speed, specificity, and most importantly the reversibility of these pathways allow for the complete adaptation of a cell to its environment.

Complementarity

All three of these pathways are constitutively active at basal levels to maintain cell homeostasis. In response to environmental stress, such as those encountered in cancer, including nutrient starvation, hypoxia, and other forms of metabolic stress, both macroautophagy and CMA are upregulated, but their activation does not occur simultaneously. In as early as 30 minutes, cells overcome

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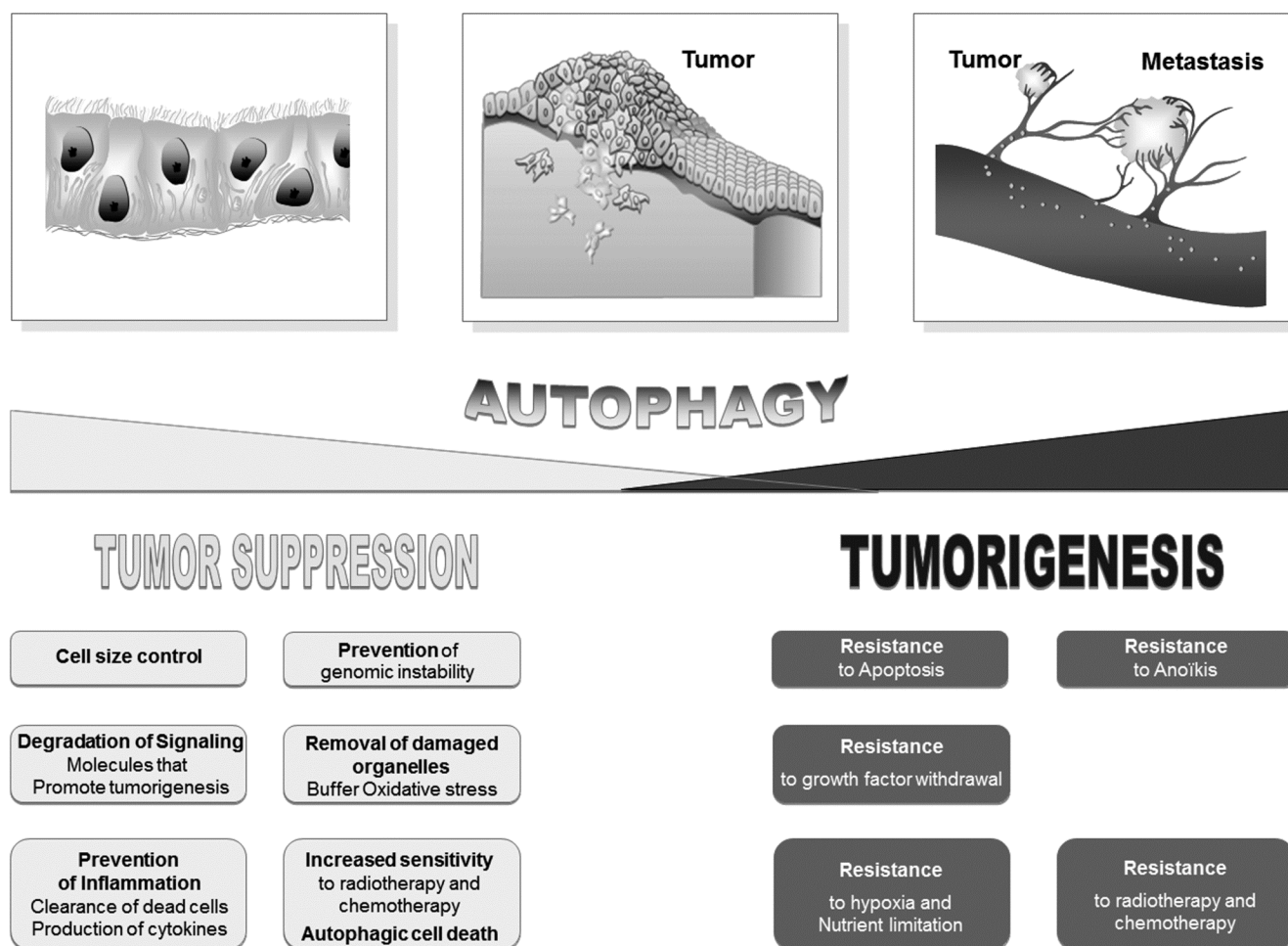


Fig. (1). Positive and negative sides of the autophagic process in promoting and preventing tumorigenesis.

nutrient depletion by dramatically up-regulating autophagy. Even under persistent starvation, cells do not cannibalize themselves. The upregulation of autophagy is transient, reaches maximal activity around the first 4–6 hours, and then gradually declines to basal levels [4]. The decrease in macroautophagy is concomitant with a progressive switch to CMA that may allow the cell to degrade only unwanted proteins for cell survival [2]. In this review, we will focus the discussion on the role of macroautophagy (hereafter referred to as autophagy) in cancer.

Reversibility

It is known that the autophagosomes are formed and degraded within only eight minutes [5]. As a result, the autophagic pathway is highly dynamic, rapidly increased and suppressed once the stress is removed, providing evidence that unlike other stress states, namely apoptosis, necrosis or senescence, autophagy is a reversible phenomenon.

Cargo Specificity

Since its discovery in the 1950s, macroautophagy was first thought to be a bulk, non-selective “self-eating” process. Emerging evidence now suggests that autophagy is a quality control process, which selectively degrades damaged and

unnneeded proteins and organelles that would otherwise unnecessarily accumulate during the life of the cell. Autophagic substrates include organelles such as mitochondria (mitophagy), peroxisomes (pexophagy), large protein aggregates (aggrephagy), and even portions of the nucleus and micronuclei (nucleophagy, chromatophagy). Far from simply being a housecleaner, we as well as others recently provided evidence that autophagy also negatively regulates signaling pathways by degrading kinases, cell-cycle regulators, G-protein and transcription factors [6].

The destiny of the autophagic cargo can be modulated even after it is degraded as part of the adaptive autophagic process. Under most circumstances, nutrients of digested cytoplasmic material are recycled into biosynthetic pathways. However, under metabolic stress, the products of autophagy can be further catabolized to fuel ATP synthesis required for survival.

With all of these features (adaptation, speed, reversibility), autophagy is not only a housekeeping process that suppresses tumor initiation by removing harmful components and the unneeded signaling proteins from the cells, but also the supplier of all intracellular nutrients absolutely required for the survival, proliferation, metastasis, and dormancy of cancer cells.

INSIDE AUTOPHAGY: A TRIO OF VESICLES WITH 36 PLAYERS

The autophagy pathway begins with the formation of a double-membrane compartment, termed the “phagophore” that sequesters a portion of the cytosol. The phagophore expands into a matured vesicle, the “autophagosome”. During the maturation step, the autophagosome acquires an acidic pH and hydrolases by fusing with a lysosome to generate an “autolysosome” where the content is then degraded. The products generated by degradation are then transferred back to the cytosol by permeases in the autolysosomal membrane and recycled into different metabolic pathways (Fig. 2).

At the molecular level, autophagy is orchestrated by a family of 36 genes that were originally identified in yeast and are called autophagy-related genes (Atg), many of which have mammalian orthologues [7].

The nucleation of the phagophore is critically dependent on the class III PI3K (phosphatidylinositol 3-kinase) complex containing an enzyme Vps34, together with Vps15/p150, Becn1 (Beclin-1/Atg6), and Atg14. This PI3K complex catalyzes the production of phosphatidylinositol 3-phosphate [PI3P], thereby generating a signal that recruits several WIPI (WD-repeat domain phosphoinositide-interacting) proteins. WIPI together with ATG2, regulates the trafficking of ATG9 vesicles, the only core ATG protein with a transmembrane domain.

The elongation of the isolation membrane and subsequent closure of the autophagosome require two ubiquitin-like conjugates. Atg12 is conjugated to Atg5 by the sequential activity of Atg7 and Atg10. The resulting Atg5-Atg12 conjugate then associates with Atg16L1, to form a ~800-kDa

multimeric complex (referred to as the Atg16L complex). A fraction of the Atg16L complex localizes to the phagophore and mediates the binding of the Lc3/Atg8-phosphatidylethanolamine conjugate (microtubule-associated protein 1 light chain 3-II, Lc3-II) *via* the activity of Atg4, Atg7 and Atg3. Upon recruitment, the incorporation of Lc3-II to the isolation membrane governs its elongation, curvature, and closure as well as the substrate recruitment into the autophagosome. While the unprocessed form of Lc3 (Lc3-I) is diffusely distributed throughout the cytoplasm, the lipidated form of Lc3 (Lc3-II) specifically accumulates on nascent autophagosome until its degradation and thus represents a marker to monitor autophagy.

The autophagosome eventually seals off and fuses with lysosomes through mechanisms that remain poorly characterized. Upon completion of the autophagosome, the Atg16L complex and most components of the autophagic machinery are released from the membrane. Some regulators of the autophagosome-lysosome fusion include Lc3, the lysosomal proteins Lamp-1 and Lamp-2 (lysosomal-associated membrane protein), the small GTP-binding protein RAB7, the SNARE protein Syntaxin 17 and the AAA-type ATPase SKD1. Autophagosome-lysosome fusion then results in the activation of hydrolases which completely degrade the autophagosomal cargo.

REGULATION OF AUTOPHAGY BY ONCOGENES AND TUMOR SUPPRESSORS

To maintain cellular homeostasis while adapting to environmental conditions, the autophagic flux (from the formation to the degradation of autophagosomes) must be highly dynamic, rapidly increased and suppressed by signaling pathways. Within minutes, reactions of

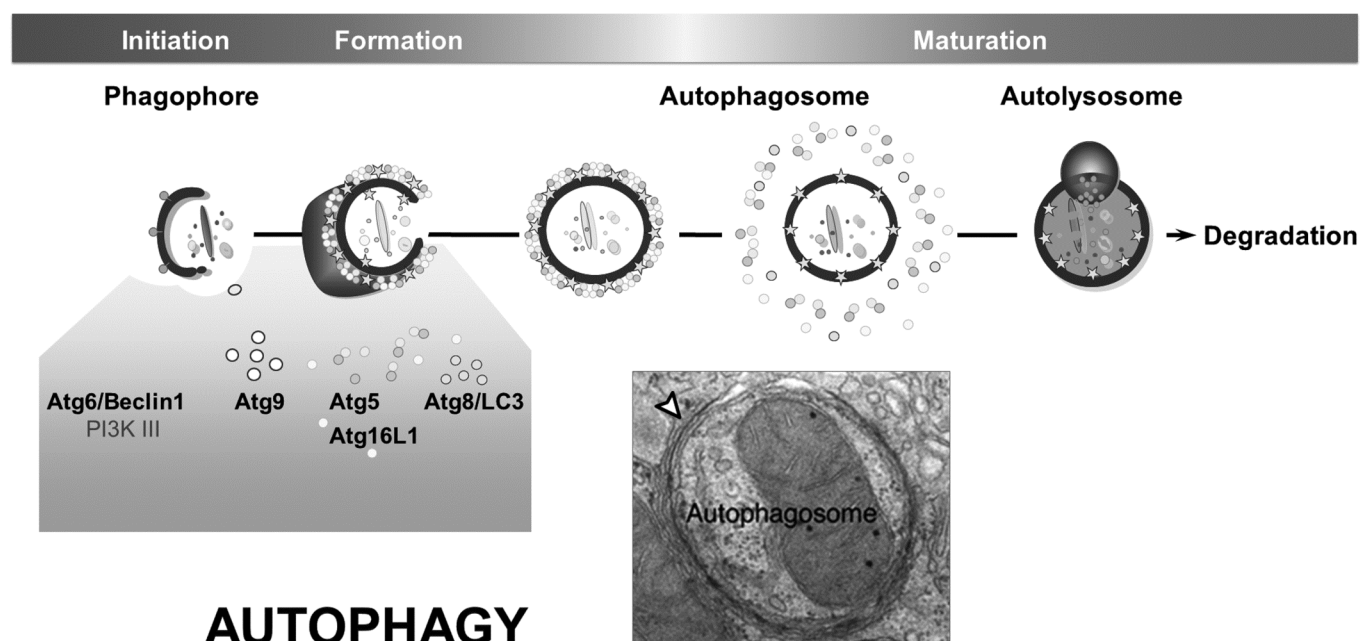


Fig. (2). Autophagy (macroautophagy) pathway. Shown are the vesicular and molecular steps of autophagy, enabling the cell to digest its own cytosol. *Inset:* At ultrastructural level, the double membrane-enclosed autophagosomes sequestering morphologically intact cytoplasm and entire organelles (mitochondria) is a hallmark of autophagy.

phosphorylation, acetylation, ubiquitination, lipidation, and proteolytic cleavage increase the activity of the autophagic machinery [8, 9]. This is followed by a general increase in the expression of autophagy and lysosomal proteins (ATP6V1B2, ATG5, BECN1, LC3...) by the transcription factors Tp53, NFκB, FOXO3a, ATF4 and TFEB [10].

Not surprisingly, the mammalian target of rapamycin (mTOR) kinase, a sensor of the nutritional status of the cell, controls the initiation of autophagy. When nutrients and growth factors are available, mTORC1 inhibits autophagy by phosphorylating and maintaining ULK1 in an inactive state, which is required for the formation of the phagophore. mTOR and its direct regulators are among the most frequently mutated oncogenes and tumor suppressors in cancer. Specifically, tumor suppressors that negatively regulate mTOR, such as TSC1/2, LKB1, PTEN, and AMPK stimulate autophagy, while oncogenes that activate mTOR, such as the tyrosine kinase EGFR, RAS, the class I PI3K, and AKT inhibit autophagy, suggesting that inhibition of autophagy likely contributes to the onset to tumor development [11].

AUTOPHAGY AS A TUMOR SUPPRESSOR PATHWAY

The decade-old discovery that the essential autophagy gene *BECN1* suppresses tumor development has been enthusiastically entertained because of its potential to lead to new therapeutic strategies in the treatment of cancer. The first evidence came from the monoallelic deletion of *BECN1* in 40% - 75% of breast, ovarian, colon, and prostate cancers

[12, 13]. Consistently, allelic loss of *Becn1* was demonstrated in mice to predispose to lymphomas, hepatocellular carcinomas, and lung carcinomas [14, 15]. Similarly, several partners of *Becn1* that positively regulate autophagy, such as *Ambra1* [16, 17], *Bif1/Sh3glb1* [18], and *Uvrag* [19, 20], have been shown to display tumor suppressive effects. Soon thereafter, this tumor suppressor function was extended to *Atg5*, *Atg7*, and *Atg4C* [21-23] (Table 1). It is thus widely accepted that the entire autophagy pathway could suppress tumorigenesis, while a growing list of underlying mechanisms has been proposed.

Mechanisms by which Autophagy Suppresses Tumor Development

Switching off Oncogenic Signaling

The degradation of key signaling proteins is one of the most powerful tumor-suppressive mechanisms by which a cell can control its own growth, survival, and motility. We and others have provided evidence that autophagy limits several key signaling pathways (mTOR, Wnt, NFκB, RHOA) by degrading kinases, G proteins, downstream components, and transcription factors. We therefore propose the term "SIGNALphagy" to indicate a dedicated type of macroautophagy that degrades and thereby maintains the appropriate level of active signaling proteins to achieve tumor suppression [6, 24, 25].

Degrading the SQSTM1 Oncoprotein

Since 1996 [26], SQSTM1 (sequestosome-1; also known as p62) has emerged as a critical oncoprotein involved in a

Table 1. Phenotypes of transgenic mice defective for autophagy (see also table 2).

Gene	Mice	Phenotypes	Refs.
			
<i>Atg4c</i> tumor suppressor	<i>Atg4c</i> $-/-$	↑ carcinogen-induced fibrosarcomas	[23]
<i>Atg5</i> tumor suppressor	mosaic deletion of <i>Atg5</i>	↑ benign liver adenomas	[21]
<i>Atg7</i> tumor suppressor	<i>liver-specific Atg7</i> $-/-$	↑ benign liver adenomas	[21]
<i>Becn1</i> Haploinsufficient tumor suppressor	<i>Becn1</i> $+/-$	↑ lung cancer, lymphoma, hepatocellular carcinoma	[14, 15]
<i>Uvrag</i> tumor suppressor	Ectopic expression Xenograft model	↓ tumorigenicity of HCT116 tumor cells	[19, 20]
<i>Sh3glb1/Bif-1</i> tumor suppressor	<i>Sh3glb1</i> $-/-$	↑ lymphoma, HCC, sarcomas, duodenal adenocarcinomas, small cell lung carcinomas, and esophageal squamous cell carcinomas	[18]
<i>Ambra1</i> tumor suppressor	<i>Ambra1</i> $+/-$	↑ lung carcinomas, liver carcinomas	[16, 17]
<i>Rblcc1/Fip200</i> Protumoral role	Conditional deletion of <i>FIP200</i>	↓ oncogene-driven mammary tumorigenesis Tumor cell glycolysis and proliferation host anti-tumor immune surveillance	[77]

myriad of cellular functions. This multifunctional role of SQSTM1 is explained by its ability to recruit and activate key signaling proteins that control cell survival (NF κ B [27]), nutrient sensing (mTOR [28]), oxidative detoxifying stress (NRF2 [29]), and migration (Twist1 [30]); all crucial events that have a direct impact on cancer development. Not surprisingly, SQSTM1 is essential for *KRASG12D*-induced tumorigenesis of lung and pancreas tumors in mice [27, 31]. In humans, SQSTM1 overexpression was associated with worsening of cancer-specific survival in lung, gastrointestinal, prostate, liver, kidney and breast cancers, suggesting a broader role for SQSTM1 overexpression in cancer progression [32].

Besides these signaling functions, SQSTM1 was the first identified autophagic substrate and the first characterized autophagic receptor [33]. Indeed, the presences of the LIR (LC3-interacting region) and UBA (ubiquitin-associated) domains enable SQSTM1 to serve as an adaptor for selective autophagy of ubiquitinated substrates (misfolded proteins, signaling proteins and damaged organelles). Autophagy therefore mediates the clearance of SQSTM1 and impairment of autophagy results in SQSTM1 accumulation and oncogenesis in multiple cellular settings [21, 34, 35]. Overall, the emerging concept is that autophagy is required to degrade SQSTM1 and thereby suppress the inappropriate activation of oncogenic signaling pathway, which can promote survival and tumorigenesis [35].

Limiting Oxidative Stress and DNA Damage

Given their highly active metabolism, cancer cells have higher levels of Reactive Oxygen Species (ROS) than normal cells. A high ROS level can damage the cell's DNA, and activates signaling pathways, thus stimulating carcinogenesis, cancer initiation, and cancer progression. Mitochondria are the main source of ROS and their ROS production increases as these organelles age or become damaged. As a quality control mechanism, mitophagy is a form of selective autophagy, which degrades compromised mitochondria that would otherwise induce genotoxic stress and DNA mutations [36, 37]. Autophagy is also an integral part of the DNA damage response that favors DNA repair not only by recycling key proteins involved in this process [38], but also by maintaining the pools of ATP and dNTPs [39, 40]. This process is achieved by degrading the damaged DNA (chromatophagy) [41], and ultimately contributes to cell death when hyperactivated [42].

Maintaining Genomic Stability

Genomic instability is present in ~90% of solid human tumors, and associated with tumor progression, aggressiveness, drug resistance and poor patient outcome. Evidence to date suggests that autophagy supports genomic stability by impeding retrotransposon insertions [43] and by controlling cytokinesis [25, 36, 37]. Each time a cell divides, it must duplicate its entire genome, distribute one copy of each chromosome to each mitotic pole, and then split the cytoplasm into two identical daughter cells, during cytokinesis. Recent elegant studies reveal that the autophagy pathway also functions as a "guardian" of cellular genome. Defects of the entire autophagy pathway [irrespective of the defect: i.e., either at the step of autophagosome formation

(*Atg5*^{-/-}, *Becn1*^{-/+}, *ATG7* shRNA), sequestration (*SQSTM1* shRNA) or degradation (*V-ATPase a3*^{-/-})] drive cytokinesis failure, multi-nucleation, and losses/gain of entire chromosomes [25, 36, 37]. Furthermore, activation of autophagy was shown to maintain genome stability in yeast [44] and reduce genomic instability in hepatocellular carcinoma [45]. For faithful inheritance of a diploid genome, we have shown that autophagy is essential to degrade and thereby maintain the appropriate level of the active RHOA GTPase at midbody, a key regulator of the contractile ring that separates the two daughter cells during cytokinesis [25].

Promoting Autophagic Cell Death and Senescence

Although autophagy first serves as a survival mechanism, a sustained autophagy can act as an accomplice that accelerates the mitotic catastrophe or behaves as an actual killer that commits the cell to an autophagic suicide in several eukaryotic models [46, 47]. This non-apoptotic cell death, the type II (autophagic cell death) is defined by enlarged vesicles, and demonstrated dependence on the autophagy machinery [48]. It is noteworthy that the overexpression of the *HRASV12* oncogene induces the demise of several cell types through a type II cell death pathway. Type II cell death pathway depends on the autophagy genes *BECN1* and *ATG5* as well as the BH3-only protein NOXA, which ultimately limits the oncogenic potential of *RAS* [49-50]. This is in line with the observation that autophagy can be induced during the oncogene-induced senescence (OIS) [51] and depending on the level of induction, the RAS-induced autophagy can dictate the cellular response to RAS: to senesce or proliferate [52] (see below). Additionally, in response to chemotherapy, autophagy has also been connected to senescence, but its role remains controversial. Depending on the cancer cell type and the drug, autophagy activation seems to suppress cellular senescence (imatinib, leukemia cells [53]), favor it (microtubule poison, colon cancer cells and melanoma cells [54]; temozolomide in glioma cells [55]; lymphoma cells [56]) or even occur in parallel but in an independent manner [57].

Limiting Inflammation

Chronic inflammation has long been recognized as an important factor for tumorigenesis. The tumor microenvironment, rich in inflammatory cells and pro-inflammatory cytokines supports all stages of tumor development, from the initial growth, the stimulation of angiogenesis and metastasis to the reduced response to therapy [58]. In several models, autophagy impairment has been shown to lead to exacerbated inflammation, and tumor growth [59]. It turns out that autophagy may contribute to tumor suppression by controlling the intensity and duration of the inflammatory responses at multiple levels:

By Promoting the Rapid Removal of Apoptotic Remains

The activation of autophagy in a dying cell transmits two major signals: A signal that reads "eat-me", translated by the presence of phosphatidylserine on the cell surface, and the secretion of the "come-get-me" signal, by lysophosphatidylcholine [60].

By Blocking Cell Necrosis

By blocking cell necrosis and thereby the extracellular release of danger signals, such as HMGB1 and ATP. During cellular stress, HMGB1 moves from the nucleus to the cytosol where it interacts with BECN1 and ATG5 to prevent their cleavage by calpain, allowing autophagy to proceed [61].

By Limiting the Production of the Inflammatory Cytokines

(IL-1 β , IL-18, TNF- α and IL-6) [62-63]. At the inflammatory site the massive release of pro-inflammatory cytokines, reactive oxygen species, and HMGA1 activates autophagy [64]. In turn autophagy limits the activation of the inflammasome (a platform that triggers the maturation of pro-inflammatory cytokines) by inhibiting the release of mitochondrial DNA from damaged mitochondria [65-66] and by ultimately targeting ubiquitinated inflammasome components, as well as pro-IL-1 β , for degradation [67-68]. In parallel, autophagy also dampens the transcription of the pro-inflammatory cytokines through the degradation of SQSTM1 and the key signaling proteins of the NF κ B pathway [6]. Through this arsenal of destruction, autophagy may safeguard against excessive inflammation.

Polemic on the Tumor-suppressive Function of Autophagy

It is important to note that skeptics have correctly pointed out that all the above-discussed mechanisms explaining the tumor suppressive function of autophagy have been mainly reported *in vitro*, and there is little evidence that they actually occur *in vivo*. Perhaps the most puzzling observation is that the *BECN1* gene is located on chromosome 17q21 next to *BRCA1*, a known tumor-suppressor gene. Using the Cancer Genome Atlas (<https://tcga-data.nci.nih.gov/tcga/>), Laddha *et al.* have evidenced that large deletions encompassing both *BRCA1* and *BECN1*, and deletions of only *BRCA1* but not *BECN1*, are found in breast and ovarian cancers, consistent with *BRCA1* loss being a primary driver mutation in these cancers [69].

In response to these concerns, it might be answered that deletions of the essential autophagy genes, *Atg5*, or *Atg7* in mice, similarly to *Becn1*, do produce multiple benign tumors (adenomas, PanIn), but only in the liver [21], in the lung [70-72] and in the pancreas [22] and not in any other tissues (Table 1). In light of these finding, the role of autophagy in cancer needs to be revisited: rather than a general mechanism, these studies support the notion that autophagy may play an important role in tumor suppression but primarily *at early stages and specifically to certain cancer types*. As only benign lesions did emerge and fail to progress, these findings also reveal the Janus-faced nature of autophagy in *tumor progression* (see below).

AUTOPHAGY AS A TUMOR PROMOTER: FUELING TUMOR PROGRESSION

Autophagy “Addiction” of RAS-driven Tumors

The *RAS* GTPases (*HRAS*, *KRAS* and *NRAS*) are the most commonly mutated oncogenes in human cancers, associated with worse prognosis, early metastasis, and resistance to

therapy. Activating *RAS* mutations are highly prevalent in colorectal, pancreatic, and lung cancers (~ 35% of all human cancers). There is currently no effective therapy to treat the *RAS*-driven cancers. Thus, beyond *RAS*, there is clearly an urgent need to identify downstream vulnerabilities directly involved in tumor progression.

Classically, the rapidly proliferating tumor cells are viewed as being critically dependent on a single oncogene that reprograms their metabolism towards aerobic glycolysis and glutaminolysis to generate the “building blocks” that are needed to produce a new cell. However, it is apparent that autophagy degradation also allows the cells to acquire these essential metabolites, particularly under stressful conditions, as found in cancer progression. Supporting this idea, the White [70, 73-74], Debnath [75], and Kimmelman [76] laboratories elegantly demonstrated that i) autophagy is constitutively activated by the *RAS* and *BRAF* oncogenes and ii) the genetic inhibition of autophagy is sufficient to block their tumorigenicity in lung and pancreas, giving rise to benign oncocytomas [70, 72-73, 76] (Table 2). This has led to the notion that cancer cells are “addicted” to autophagy for their progression: the tumor cells that activate autophagy and thereby successfully adapt to their hostile micro-environment, survive and proliferate whereas the losers that do not activate autophagy, die. This autophagy addiction is not limited to the *RAS* pathway, as inactivation of *Fip200* (also known as *Rb1cc1*) in the polyoma middle T (*PyMT*) mammary cancer model impairs breast tumor growth [77], and deletion of *Atg5* or *Atg7* in the liver causes hepatoma formation without progression to hepatocellular carcinoma [21].

Fueling Mitochondrial Metabolism

To support rapid cell growth, cancer cells require an increased levels of ATP and metabolic intermediates to maintain energy status and a higher biosynthesis of macromolecules. Both these metabolic requirements are satisfied by increasing autophagy.

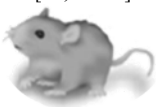
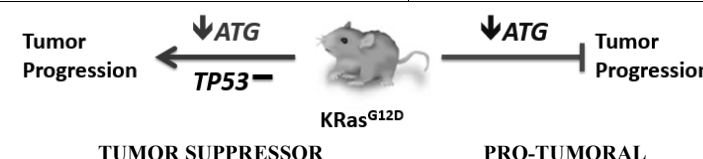
Maintaining a Pool of Functional Mitochondria

Contrary to the conventional wisdom, mitochondria are not defective in tumor cells, and still have an important role in tumorigenesis: Cancer cells are highly dependent on mitochondria to produce the ATP and the tricarboxylic cycle (TCA) cycle intermediates required for biosynthesis of lipids, proteins, and nucleic acids. Remarkably, only autophagy is capable of degrading damaged mitochondria, in a selective process termed mitophagy [78].

Supplying Metabolic Substrates

Mitochondria can generate cellular energy from different fuel sources, including glucose, fatty acids, and amino acids. Through the degradation of proteins and lipids, the upregulation of autophagy supplies the nutrient required to maintain aerobic glycolysis [75, 79], fatty acid oxidation [70, 73] and glutaminolysis [72]. Of note, neoplastic tissues produce high levels of ammonia as a result of an intense flux through glutaminolysis [80]. Ammonia then diffuses to the extracellular space and acts as a signaling molecule that activates autophagy in both the tumor cells and the adjacent stromal cells to optimize *via* autophagy the supply of

Table 2. Summary of the studies showing the two facets of autophagy in *RAS/BRAF*-driven cancers.

Oncogene	Autophagy Function	Models	Ref
HRAS V12 KRAS V12 KRAS G12D	PRO-TUMORAL ↑Proliferation, tumor progression ↑ Mitochondrial respiration (fatty acid oxidation) ↑Glutamine-dependent	Lung Cancer NSCLC Mouse model <i>Atg7</i> [−] , <i>TP53</i> [−]	[70, 73-74] 
KRAS G12D	PRO-TUMORAL	Lung Cancer NSCLC Mouse model <i>Atg5</i> [−]	[71] 
HRAS V12	TUMOR SUPPRESSOR ↑Autophagic cell death	Cells fibroblast	[49-50]
BRAF V600E	TUMOR SUPPRESSOR – Early stages ↓Oxidative stress (NRF2) PRO-TUMORAL – Advanced stages ↑Proliferation, tumor progression ↑ Mitochondrial respiration ↑Glutamine-dependent	Lung Cancer NSCLC Mouse model <i>Atg7</i> [−]	[72] 
HRAS V12 KRAS	PRO-TUMORAL ↑ Glycolysis ↑Transformation (soft agar)	Cells	[75]
KRAS V12	PRO-TUMORAL ↑ATG5 ATG7 ↑Transformation (soft agar, xenograft) ↑ Mitophagy	Liver cancer (HCC) Cells rat2	[79]
KRAS G12D	PRO-TUMORAL <i>independent of p53 status</i>	Pancreas cancer (PDAC) Mouse model patient-derived xenografts, CQ	[22, 76] 
KRAS G12D	DUAL ROLES dependent of p53 status TUMOR SUPPRESSOR – <i>TP53</i> [−] PRO-TUMORAL – <i>TP53</i> ⁺	Lung ADK <i>Atg5</i> [−] Pancreas cancer (PDAC) <i>Atg5</i> [−] , <i>Atg7</i> [−] , HCQ	[71] [147]
			

nutrients [81, 82]. This intricate relationship between metabolism and autophagy is critical for cancer cell growth and survival (as discussed below).

Deletion of *Atg5* or *Atg7* results in the accumulation of damaged mitochondria, which leads to multiple defects in mitochondrial metabolism. Decreased production of TCA cycle intermediates, reduced mitochondrial respiration, and diminished ATP production are among the hallmarks of mitochondrial damage. Impaired production of these metabolic substrates ultimately impedes the growth and the tumorigenesis of cancer cells expressing *RAS/BRAF*, and the *PyMT* oncogenes [70-77].

Increasing Resistance to Apoptosis

Apoptotic cell death is a crucial defense mechanism against malignancy initiated by a diverse range of signals, many of which are autophagy activators. Of note, nutrient deprivation, matrix detachment, and DNA damage play a crucial role in apoptosis and autophagy initiation.

Enabling Survival During Starvation

During tumorigenesis, cancer cells frequently endure limited supply of growth factors, nutrients and oxygen, especially in the inner area of the tumor. Autophagy is then activated and inhibition of autophagy by monoallelic loss

of *Becn1* promotes cell death (necrosis and apoptosis) specifically in the hypoxic regions of tumors [59]. These observations suggest a role for autophagy in promoting tumor cell survival under conditions of metabolic stress.

Enabling Survival During Metastasis

Metastasis is responsible for more than 90% of cancer-related deaths. Disseminated tumor cells circulating throughout the body and metastasizing to a distant organ site, need to overcome anoikis, a form of apoptosis that takes place when cells are detached from the extracellular matrix (ECM). Following ECM detachment, autophagy was found to be robustly activated and essential to protect against anoikis a large array of cancer cell-lines with *RAS*-oncogenic mutations [83, 84]. Besides survival, we have shown that autophagy promotes the migration of lung cancer cells with *KRAS* mutation by degrading RHOA at the lamellipodia [24]. Consistently, there are clinical associations between hyperactive autophagy (*BECN1* overexpression, punctate LC3B expression) and early metastases in melanoma and colorectal cancers [85-87].

Enabling Chemoresistance

Radiation and chemotherapeutic drugs, such as imatinib, paclitaxel, cisplatin, 5-fluorouracil (5-FU), arsenic trioxide (As₂O₃), and TRAIL, induce autophagy along with apoptosis in *in vitro* and *in vivo* models (Table 3, [39, 42, 88-130]). A key contributor to drug and/or radiation resistance in several cancer types (melanoma, brain cancer, gastric cancer, prostate cancer or non-small-cell lung cancer) is undoubtedly autophagy: autophagy precedes apoptosis and helps cancer cells to escape apoptosis by degrading the drug molecules, the mitochondria, and the activated caspase-8 [131, 132]. Inversely, once apoptosis is initiated the caspases 3 and 8 cleave *BECN1*, thus inhibiting autophagy initiation [133, 134]. As a result, all chemo-sensitive cell lines turn out to exhibit apoptosis, whereas cell populations that respond to drugs by inducing autophagy are more drug-resistant and will recover after the withdrawal of the chemotherapeutic agents [135].

Fueling Cancer Stem Cell Dormancy

Tumor dormancy is the leading factor in treatment failure, metastasis, and tumor recurrence even decades after resection of the primary tumor. The tumor microenvironment following metastasis or therapy is not sufficiently protective, allowing a small population of surviving tumor cells, likely the cancer stem cells, to survive by entering a dormant state. Given their dormancy, these tumor cells are resistant to the chemotherapies that exploit the rapid cell division of cancer cells. Since dormancy is a reversible process, cancer cells can resume proliferation when the stressful environment has improved. These surviving cells can therefore become the source of tumor relapse at a later time.

The regulation of cancer dormancy is poorly understood. Recently, transcriptomic analyses reveal that the dormancy of cancer stem cells is characterized by major metabolic changes with an increased autophagy flux [136, 137]. During the dormancy period, *ARHI*/*DIRAS3* is the molecular switch that promotes initiation of autophagosome formation in

cancer cells [138, 139]. Importantly, inhibition of autophagy using chloroquine or silencing (*Atg7* and *Atg12* shRNA) suppresses the increase in ATP levels, impedes cancer cell survival, and reduces tumor regrowth [138, 140], all of which could be partially rescued by the addition of glutamine as an energy source [141]. Once again this supports the notion that autophagy provides nutrients necessary to meet the metabolic needs absolutely required for cancer stem cell dormancy; thus providing the rationale for targeting this pathway to eradicate these aggressive cancer cells.

AUTOPHAGY AS A THERAPEUTIC TARGET

From a therapeutic perspective, the ever-expanding roles of autophagy in tumorigenesis provide the rationale to prioritize this pathway as a new target for drug development. More than 500 patent applications for autophagy regulators have been filed so far. This massive investment has been fueled by the realization that all classes of anticancer treatments including DNA damaging agents, microtubule-targeted drugs, glycolytic inhibitors, death receptor agonists, hormonal agents, anti-angiogenic agents, proteasome inhibitors, histone deacetylase inhibitors, and targeted kinase inhibitors affect autophagy [142] (Table 3). It was originally proposed that autophagic cell death may be part of their cytotoxicity, particularly in apoptotic-defective cancer cells. However, similar to its seemingly contradictory tumor-suppressive and tumor-promoting effects, autophagy is also exploited by dying cancer cells to deal with the cytotoxicity of anticancer agents rather than a cause of cell death [143].

Activators of Autophagy

Clinically available drugs that upregulate autophagy include the inhibitors of receptor tyrosine kinases (for example, EGFR, ERBB2, PDGFR and VEGFR2: imatinib, gefitinib and erlotinib), the inhibitors of PI3K pathway (PIK3CA, Akt) and the inhibitors of mTOR (rapamycin, and its analogues temsirolimus and everolimus). These three groups of oncogenic targets are mechanistically linked because receptor tyrosine kinases activate the PI3K/Akt pathway and then mTORC1, which is a negative regulator of autophagosome formation. Moreover, treatment of cancer cells with the proteasome inhibitor bortezomib can induce the expression of endogenous mTOR inhibitor sestrin-2, while perifosine can induce the degradation of mTORC1 complex components. Likewise, the activation of the PI3K/AKT/mTOR pathway can be suppressed indirectly by the sirtuin activator resveratrol, the glucocorticoid dexamethasone and the anti-diabetic drug metformin. Whatever the underlying mechanism is, these kinase inhibitors and drugs which are currently used in oncology exert their antitumor effect by inducing autophagy (Table 3).

Other autophagy activators directly target *BECN1* itself. A good example comes from BH3 mimetics (ABT-737), which disrupt the interaction between BCL2 proteins and *BECN1* to induce autophagy [118-120]. Direct regulation of *BECN1* is also responsible for autophagy induction by tyrosine kinase inhibitors, such as erlotinib, that inhibits the phosphorylation of *BECN1* by EGFR [144]. Tamoxifen, a well-recognized antitumor drug for breast cancer treatment, can increase the level of *BECN1* to stimulate autophagy

Table 3. Summary of preclinical studies showing enhanced cytotoxic response of chemotherapy in combination with pharmacological modulators of autophagy.

PRECLINICAL STUDIES OF AUTOPHAGY INDUCERS AND AUTOPHAGY BLOCKERS						
Target	Compound	Mechanism	Tumor Type	Autophagy Function	Combination/ Cell Death	Refs.
mTORC1				↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE	AUTOφ ind. + AUTOφ ind.	
	Rapamycin*	mTORC1 inhibitor	Glioma Leukemia (CML)*		RAPA + PI3K inh, LY294002, RAPA + Akt inh, UCN-01 Apoptosis Indep.	[92] [93]
	Temsirolimus*	mTORC1 inhibitor	Mantle cell lymphoma glioma breast cancer		Temsirolimus + HDACi Apoptosis	[94]
	Everolimus*	mTORC1 inhibitor	Leukemia (ALL)		Everolimus + vincristine Apoptosis	[95]
					AUTOφ ind. + AUTOφ block.	
	Perifosine	Degradation of mTOR, raptor, and Akt	Lung cancers		Perifosine + CQ ↑ apoptosis	[96]
PROTEASOME inhibitors	Bortezomib NPI-0052	↓ mTOR pathway ↑ mTOR inhibitor sestrin-2	Multiple myeloma* Prostate cancer		Bortezomib + CQ	[97 , 98 , 99]
PI3KCI	NVP-BEZ235	Dual PI3KCI/Akt inh.	Glioma	↑ Autophagy - ?		[105]
	PI-103	Dual PI3KCI/Akt inh.	Glioblastoma	↑ Autophagy - ?		[106]
	Quercetin	↓ Akt-mTOR pathway	Gastric cancer Colon cells	↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE	Quercetin + CQ ↑ apoptosis	[101]
	Resveratrol	↓ Akt-mTOR pathway	Breast cancer Ovarian cancer	↑ AUTOPHAGY - ANTI-TUMOR PRO-DEATH	↑ Autophagy cell death	[102]
	Curcumin	↓ Akt-mTOR pathway	Leukemia (CML), glioma		↑ Autophagy cell death	[103]
	Dexamethasone	↓ Akt-mTOR pathway	Leukemia (ALL)		↑ Autophagy - Pro-apoptotic	[104]
TYROSINE KINASES Inhibitors	Imatinib (Gleevec)*	KIT, BCR-ABL, PDGFR inh	Gastrointestinal stromal tumor*, leukemia (CML)*	↑ AUTOPHAGY - ANTI-TUMOR PRO-DEATH	↑ Autophagy cell death	[100]
	Linifanib	VEGFR, PDGFR inhibitor ↓ Akt-mTOR pathway	hepatocellular carcinoma (HCC)	↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE	AUTOφ ind. + AUTOφ block. Linifanib + HCQ	[107]
	Erlotinib* Gefitinib	EGFR inh	Glioma* NSCLC* Glioma		AUTOφ ind. + AUTOφ ind. Erlotinib + rapamycin ↑ Autophagic cell death Erlotinib + imatinib	[108] [109] [110]
	Cetuximab		head and neck squamous cell carcinomas (HNSCC)*		Cetuximab +PKI-587 (PI3K/mTOR inhibitor)	[111]

Table 3. contd....

PRECLINICAL STUDIES OF AUTOPHAGY INDUCERS AND AUTOPHAGY BLOCKERS						
Target	Compound	Mechanism	Tumor Type	Autophagy Function	Combination/ Cell Death	Refs.
	trastuzumab	Anti-HER2 antibodies* Inhibition of HER2	Breast cancer*	↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE		[112]
BECN1	Tamoxifen	estrogen receptor antagonist ↑ BECN1	Breast cancer	↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE		[113-114]
	EB1089	Vitamin D analog	Breast cancer	↑ AUTOPHAGY - ANTI-TUMOR PRO-DEATH	↑ Autophagy cell death BECN1 dep.	[115]
	Arsenic trioxide*	↑ BECN1	Leukemia (APL)* Glioma		↑ autophagic cell death ↑ autophagic cell death	[116] [117]
	GX15-070	Bcl2 inhibitor BH3 mimetics	Pancreatic carcinoma Leukemia	↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE	AUTOφ ind. + AUTOφ ind. sorafenib + HDACi + GX15-070 HDACi (vorinostat) + GX15-070	[118] [119]
	ABT-737		Lymphoma, Lung NSCLC		Paclitaxel + ABT-737↑ Autophagy cell death	[120]
DNA-DAMAGING				↑ AUTOPHAGY - PRO-SURVIVAL RESISTANCE	AUTOφ ind. + AUTOφ block.	
	Ionising radiation	DNA damage	Breast cancer		Radiation + 3-MA, CQ	[121]
	Etoposide	DNA damage	Glioma			[39]
	Cisplatin	DNA damage	ovarian cancer, Osteosarcoma		Cisplatin + 3-MA	[122]
	Camptothecin	inhibition of DNA synthesis	Breast cancer		CPT + BafA1 3-MA	[123]
	5-Fluorouracil	Inhibitor of thymidylate synthase	Colon cancer		5-FU + 3-MA	[124]
	Temozolomide*	DNA alkylating agent	Glioblastoma, Metastatic Lymphoma, melanoma*		TMZ + BafA1 : APOPTOSIS TMZ + CQ : APOPTOSIS	[125 , 126] [127-128]
HDAC inhibitors	Vorinostat* Butyrate, SAHA Panobinostat		lymphoma (CTCL)* Multiple cancers, CML Imatinib resistant Lymphoma	↑ autophagic cell death ↑ autophagy - Pro-survival	↑ APOPTOSIS lysosomal membrane permeability	[89] [88] [90]
Metabolism	metformin + 2-deoxyglucose		Prostate cancer			[91]

Abbreviations: AUTOφ ind, autophagy inducer; AUTOφ block, autophagy blocker; BafA1, Bafilomycin A1; CQ, Chloroquine; HCQ, Hydroxychloroquine; 3-MA, 3-methyladenine; 5-FU, 5-fluorouracil; Indep, independent; inh, inhibitor; CPT, Camptothecin; RAPA, rapamycin; *, FDA (FDA, US Food and Drug Administration) approved drugs; HDACi, Histone deacetylase inhibitors; TMZ, Temozolomide;

Acute promyelocytic leukemia (APL); acute lymphoblastic leukemia (ALL), cutaneous T cell lymphoma (CTCL); chronic myeloid leukemia (CML), head and neck squamous cell carcinomas (HNSCC), non-small cell lung cancer (NSCLC).

[113, 114]. As a chemotherapeutic vitamin D analog, EB1089 may trigger and induce BECN1-dependent autophagy in MCF-7 cells [115].

Because *BECN1* and other *ATGs* are haploinsufficient tumor suppressor genes that undergo heterozygous loss in a substantial proportion of human tumors, it might be expected that drugs that may upregulate the remaining copy and thereby restore autophagy back to its physiological levels of activation may suppress initial tumor growth. Despite the excitement surrounding these promises, clinical progress has been uneven. Autophagy activators have been least effective in treating the cancer types that have the highest mortality rates, such as lung, colorectal, pancreatic, skin, and brain cancer.

Why has the clinical application of autophagy activators been so challenging? One reason is that many of these drugs (for instance DNA-damaging drugs) confer resistance by a cytoprotective autophagy [109, 112, 121, 123, 124]. Intuitively, overcoming this resistance will require targeting tumor cells through a cocktail of autophagy activators that efficiently upregulate the autophagic flux to a cytotoxic level when applied as concurrent treatment.

Inhibitors of Autophagy

A general hallmark of *KRAS/BRAF*-driven cancer is the upregulation of basal autophagy, when compared to their normal counterparts. A plethora of evidence suggests that upregulation of autophagy intimately accompanies and allows for all different facets of malignant progression (growth, survival, invasion, and dormancy; section 5). Therefore, autophagy is a central aspect of tumor biology that might be turned into cancer's Achilles heel.

Various autophagy inhibitors have been developed such as the inhibitors of PI3KIII (3-methyladenine, wortmannin, and LY294002 [125]), of microtubule (vinblastine, colchicine [142]), of Vacuolar-ATPase (bafilomycin A1 [145]), of lysosomal proteases (pepstatin A, [146]) and weak bases (chloroquine, hydroxychloroquine, and Monensin) to block the formation of autophagosome, its maturation/fusion with lysosome and then its degradation, respectively.

Currently, there are 40 clinical trials targeting autophagy addiction using primarily chloroquine (CQ) and hydroxychloroquine (HCQ) to kill cancer cells (<http://www.clinicaltrials.gov> Fig. 3). Indeed these antimalarial drugs are already approved for human use, relatively safe, and inexpensive. Both are weak bases that accumulate in the lysosomes causing a rise in lysosomal pH and thus preventing the activity of autolysosomes or the fusion of autophagosomes with lysosomes.

The rationale of combining a chemotherapy and a lysosomal autophagy inhibitor is that the former induces massive autophagic flux and the latter prevents autophagic contents from being degraded, leading to an accumulation of ineffective autophagic vesicles and a burst of ROS, presumably from the damaged mitochondria within autolysosomes. This ROS in turn produces permeabilization of lysosomal membrane, release of cathepsin and activation of apoptotic cell death. A large body of preclinical results

show that the success of CQ as a therapeutic agent lies in its ability to trigger senescence, apoptosis, and autophagic cell death of cancer cells and cancer stem cells.

FUTURE DIRECTIONS: CHALLENGES TOWARDS TRANSLATIONAL INDIVIDUALIZED HEALTH CARE

Despite the great promises of autophagy-based therapies, there are, undoubtedly, many critical issues to address. In particular, it will be important to identify the cancer type that might respond best to these therapies and evaluate the efficacy, safety and long-term outcomes of modulating autophagy.

What are the Best Patient Populations for Autophagy-based Therapies?

The concept of “autophagy addiction” was first suggested for *KRAS*-, and *BRAF*-driven tumors [72, 73]. In transgenic mice, the inhibition of autophagy induces the regression of adenocarcinomas to a more benign oncocytoma, providing a rationale for targeting autophagy to treat these refractory *KRAS*-mutated patients. Importantly, it was demonstrated that autophagy ablation demonstrates efficacy to advanced tumors that have grown to a considerable size in mice [74], with possible extensions in patients receiving therapy. However, at present it is unclear whether all cancer-bearing patients with a mutated *RAS* or activation of the *RAS* pathway will respond similarly to autophagy inhibition. Two recent papers evidenced that inhibition of autophagy (by CQ, *Atg5* or *Atg7* knockout) inhibits the growth of *Kras*-driven tumors in pancreas and lung when the status of *TP53* is wild type but stimulates tumor growth in *Ras* mutant, *TP53* null cells [71, 147] (Table 2). In the near future, these puzzling findings may revolutionize the stratification of patients that will receive autophagy inhibition treatment. However, in the ongoing clinical trials no strategy for patient selection is being pursued.

What are the Main Safety Concerns?

The ultimate goal of any cancer therapy is to robustly target malignant cells while sparing normal cells. Currently, the major concern of using autophagy modulators is their lack of selectivity, targeting both the cancer and the healthy tissue. A recent study by Karsli-Uzunbas and colleagues indicates that the systemic inhibition of autophagy for 5 weeks in mice is not only efficacious in regressing established lung adenocarcinomas to benign oncocytoma but also safe. However, if the inhibition is sustained for 6 to 12 weeks, this strategy is extremely toxic, causing severe liver, muscle and brain degenerations [74]. This suggests that, with proper control of the extent and/or timing of autophagy inhibition, there is a therapeutic window to suppress tumorigenesis while mostly sparing normal tissue.

What are the Best Autophagy-based Therapies?

The diverse outcome of cancer treatments designed to either upregulate or silence autophagy makes autophagy an attractive therapeutic target, but its unpredictable potential might also make such targeted therapy challenging and risky.

Clinical trials (40 studies)

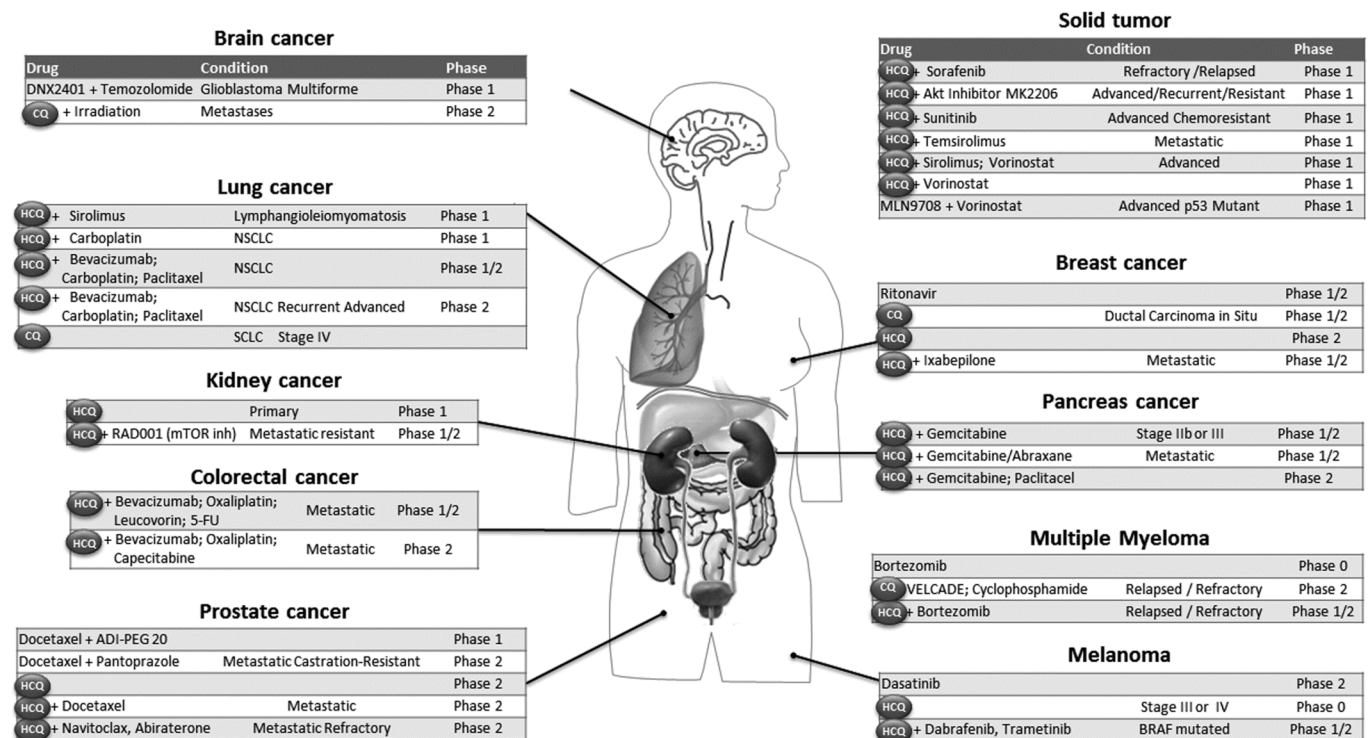


Fig. (3). Summary of 40 clinical trials involving chloroquine and hydroxychloroquine for cancer treatment (data obtained from <http://www.cancer.gov/clinicaltrials>). Abbreviations: CQ, Chloroquine; HCQ, Hydroxychloroquine; 5-FU, 5-fluorouracil; inh, inhibitor; non-small cell lung cancer (NSCLC).

For instance, CQ displays dramatic effects for some drugs/tumor models and modest or no effects in others, even in a panel of *KRAS*-mutated cancer cell lines [148, 149]. Of particular concern, CQ might also exacerbate the progression of established cancers, as suggested by certain studies [24, 147, 150, 151]. Furthermore, the possibility that CQ fulfills an autophagic-independent role cannot be excluded yet [152]. All of the above allows us to stipulate that autophagy-based therapies have to be improved by a specific modulation. Among the druggable targets are the autophagic kinases ULK1/2, and the cysteine protease ATG4. In addition to pharmacological inhibitors, specific gene interference using siRNA or CRISPR/Cas technology against various *ATG* genes may improve the effectiveness of autophagy inhibition.

What are the Best Clinical Markers for Monitoring Autophagy?

Actually, autophagy markers in tumor samples are the detection of autophagic vesicles, the expression of autophagy-related genes, and the degradation of the autophagy substrate SQSTM1. However, it is challenging to predict from these markers whether the drug activates or blocks autophagy and whether autophagy upregulation would be beneficial or detrimental for patients. We have developed methods and guidelines to measure this dynamic process *in vitro* [153], but these approaches are not suitable for application in patient biopsies. For instance, a major

caveat is the difficulty to distinguish by immuno-histochemistry the LC3-I positive aggregates from LC3-II positive “real” autophagosomes. Moreover, autophagosomes represent a mid-point in the dynamic autophagic flux, i.e. rapidly formed and degraded within 8 minutes [5]. Accumulation of autophagosomes can occur throughout induction of autophagy, but can also arise through inhibition of autophagic degradation. As a result, only a few autophagosomes and autolysosomes are observed in normal tissues even under nutrient starvation. Thus, the robust accumulation of LC3 and SQSTM1 spots might be interpreted as an impaired autophagy flux in cancer [25, 154], while recognizing that any delay in the processing/fixation of biospecimens would artificially upregulate autophagy.

CONCLUSION

In conclusion, we are embarking on an exciting journey. All the remarkable studies that have been performed over the past 14 years on the role of autophagy in cancer are culminating in clinical trials. Still in its infancy, the challenge for the cancer research community will now be to identify those patients who are more likely to respond and the combination of autophagy modulators that would be most effective.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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ABBREVIATIONS

ATG	=	autophagy-related
BECN1	=	beclin 1
CQ	=	chloroquine
HCQ	=	Hydroxychloroquine
CMA	=	chaperon-mediated autophagy
LAMP	=	lysosomal-associated membrane protein
LC3 (MAP1LC3)	=	microtubule-associated protein 1 light chain 3
mTOR	=	mammalian target of rapamycin
shRNA	=	short hairpin RNA
SQSTM1	=	sequestosome 1/p62
ROS	=	reactive oxygen species
TCA	=	tricarboxylic acid
ATPase	=	lysosomal V0 subunit A3 H ⁺ transporting
v-ATPase	=	vacuolar-ATPase

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