Unravelling the multifaceted roles of Atg proteins to improve cancer therapy

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Received 3 September 2013; revision accepted 4 November 2013

Abstract

Autophagy follows a lysosomal degradation pathway in which a cell digests its own components. It is highly regulated by a limited number of autophagy-related genes (Atg) and the proteins they encode, that are crucial for cells to undergo the process *via* modulating autophagosome formation. Recently, accumulating evidence has revealed the core molecular machinery of autophagy; however, intricate relationships between autophagy and cancer remain an enigma. Several studies have shown that Atgs can play an important role in carcinogenesis, by which Atgs may modulate a series of oncogenic and tumour suppressive pathways, implicating microRNA (miRNA) involvement. In this review, we will present the key role of Atgs in deciding the fate of cancer cells, discuss some representative Atgs and their proteins such as ULK, Beclin-1, and Atg8/LC3-Atg4, which can also be regulated by miRNAs. Thus, Atgs can be considered to be targets for cancer treatment, which may illuminate the future of cancer therapy.

Introduction

Autophagy, a well-conserved self-digestion process, can degrade intracellular materials within the lysosome/vacuole, which could be potentially deleterious proteins and organelles (1). At least three forms of autophagy have been identified: chaperone-mediated autophagy, microautophagy and macroautophagy, differing in their physiological functions and the mode of cargo delivery to the lysosome (2). Macroautophagy (hereafter referred to as autophagy) is the main catabolic mechanism, highly reg-

ulated by a limited number of autophagy-related genes (Atg) and the proteins they encode, which were originally found in yeast as a model to study autophagy in eukaryotic cells (3).

So far, the process of autophagy has been divided into five stages, including induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, then followed by degradation and recycling (4). Induction of autophagy is initiated by the proteins of the ULK complex, composed of the mammalian Atg1 homologues ULK1 or ULK2, Atg13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) and Atg101 (5). Vesicle nucleation then occurs, initiated by activation of the class III PI3K/Beclin-1 complex to recruit proteins and lipids for construction of the autophagosomal membrane (6). Ubiquitin-like protein conjugation is required for vesicle elongation and autophagosome completion, mediated by proteins Atg3, Atg5, Atg7, LC3, Atg10, Atg12 and Atg16L, to fully encapsulate the cytosolic cargo (7). Recruitment of cargo from the cytoplasm requires autophagy receptors such as p62, which serves as a proteotoxic stress sensor, promoting segregation and degradation of misfolded proteins by autophagy, thereby mediating cross-talk between the ubiquitin–proteasome system and autophagy. Finally, docking and fusion are required for the disassembly of Atg protein complexes from mature autophagosomes, regulated by Atg2, Atg9 and Atg18 (8). Fusion of the autophagosome with endolysosomal compartments may lead to breakdown of cargoes by acidic hydrolases, resulting in further degradation and recycling.

Over 30 kinds of Atg have been identified with their respective unique roles in autophagosome promotion, with astonishingly numerous links to several pathological processes, especially cancer. On the one hand, ability of autophagy to recycle nutrients, maintain cell energy homeostasis and degrade toxic cytoplasmic constituents can help keep cells alive during conditions of nutrient and growth factor deprivation (9). On the other hand, if

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the cell stress leads to continuous or excessively induced autophagy, cell death ensues. Under this circumstance, autophagy plays a death-promoting role, type II programmed cell death (type II PCD) compared to apoptosis (type I PCD) (10). However, these paradoxical studies are often confusing; depending on different cell types, Atg family members in autophagy seem to be able play either an oncogenic or a tumour suppressive role for regulation of core pathways, which seal the distinctive fate of cancer cells.

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs of ~22 nucleotides, and are involved in modulation of Atgs in autophagic pathways in different cancers (11). Also, a number of studies has shown that some small molecule drugs can target Atgs in autophagy, to treat cancers. Thus, in this review, we focus on the Janus role of some representative Atgs in autophagy, oncogenic and tumour suppressive autophagic signalling pathways involved with miRNAs, as well as relevant autophagy-modulated drugs, in cancer therapy.

Atg proteins in cancer initiation and progression

ULK complex

Unc-51-like kinases 1 and 2 (ULK1 and ULK2) have been known as homologues of Atg1, which are evolutionarily conserved and negatively regulated by rapamycin (12,13). Domain structure of both ULK1 and ULK2 reveals unique features at the N-terminal kinase domain, the middle proline/serine-rich domain and at the C-terminal domain (14).

ULK1/2 complex can orchestrate initiation of autophagy in the following ways: (i) ULK1 and ULK2 are involved in recruitment of other Atg proteins (such as microtubule LC3 and Atg16), to the autophagosome formation site, or in re-localization of membrane protein Atg9 during the autophagy process; (ii) ULK1/2 is regulated through interaction with mTOR and their binding partners such as mAtg13 and FIP200b (15,16). ULK1 may also contribute to inhibition of mTORC1 by hindrance of substrate docking to Raptor, forming negative feedback on activation of autophagy to maintain mTORC1 inhibition (17). Moreover, adenosine monophosphate-activated protein kinase (AMPK), a downstream regulator of the mammalian target of rapamycin (mTOR), can phosphorylate ULK1 and suppress mTOR activity, thereby mTOR may in turn inhibit ULK1 (18–20) (Fig. 1).

In addition, both ULK1 and ULK2 have been confirmed as being able to perform significant differences in various cancers. The relationship between ULK1 expression and clinicopathological characteristics has

Figure 1. ULK complex.

been investigated in hepatocellular carcinoma (HCC). ULK1 can be expressed in HCC as well as cirrhotic and inflammatory tissues adjacent to HCC, and high expression level of ULK1 is related to tumour size and poor survival time in patients with HCC (21). These findings may provide evidence that ULK1 can play an important role during the progression of HCC, suggesting it to be a potential prognostic biomarker for HCC patients. Also, ULK1 is crucial in development of breast cancer. Expression of ULK1 is negatively correlated with tumour size, lymph node status and pathological stage. Furthermore, log-rank testing has shown that patients with lower levels of ULK1 have significantly shorter cancer-related survival time. Thus, reduced expression of ULK1 enhances progression of breast cancer, together with closely related decreasing autophagic capacity; thus, ULK1 may be used as a novel prognostic biomarker for breast cancer (22). Additionally, clinical and prognostic significance of ULK1 in oesophageal squamous cell carcinoma (ESCC) has been reported. ULK1 protein level only is up-regulated in ESCC samples compared to normal oesophageal cells and tissues, and protein stabilization of ULK1 is higher in ESCC cells. In this condition, ULK1 may be a novel and clinically useful biomarker for ESCC patients too, as it can play an important role during progression of ESCC (23).

Contemporaneously, miRNAs have been found to be involved in regulation of the ULK1/2 complex during cancer progression. miR-290-295 can induce down-regulation of ULK1, resulting in inhibition of autophagic cell death induced by glucose starvation. Similar effects have been observed after knockdown of ULK1 in B16F1 melanoma cells. Together, the evidence reveals that miR-290-295 may confer a survival advantage on melanoma cells by down-regulating ULK1 to inhibit autophagic cell death (24). In addition, two members of the miR-17 family, miR-20a and miR-106b, can participate in regulating leucine deprivation-induced autophagy by suppressing ULK1 expression in C2C12 myoblasts. Treatment of C2C12 cells with miR-20a or miR-106b

mimic may lower endogenous ULK1 protein levels. Furthermore, inhibition of ULK1 expression by miR-20a or miR-106b mimic blunts activation of autophagy induced by leucine deprivation, while suppression of endogenous miR-20a or miR-106b by specific antagomir in C2C12 cells, results in normal autophagic activity (25). However, as far as we currently know, there is no specific drug that targets the ULK1/2 complex directly or indirectly in treating cancer. With the emerging crucial biomarker role of ULK1/2 in different cancers such as HCC and ESCC, it can be assumed that in the future the ULK1/2 complex will be able to be used in cancer treatment, but this awaits further development.

Beclin-1 interactome

Beclin-1 (the mammalian orthologue of yeast Atg6) has been well characterized as playing a pivotal role in autophagy (26). So far, it has been reported to contain three identifiable domains, namely a Bcl-2 homology (BH3) motif, a coiled-coil domain (CCD) and an evolutionarily conserved domain (ECD) (27). Beclin-1 can regulate autophagy by directly combining with PI3KCIII/Vps34. Other co-factors include Vps15, Atg14L/Barkor, UV-radiation resistance-associated gene (*UVRAG*), Bax-interacting factor-1 (Bif-1), Rubicon, Ambra1, high mobility group box 1 (HMGB1), Survivin, Akt and Bcl-2/Bcl-X_L. Atg14L/Barkor can direct complex I composed of Atg14L, Beclin-1, PI3KCIII/Vps34 and Vps15 to the phagophore assembly site (PAS) or endoplasmic reticulum (ER) (28). *UVRAG* protein can interact with Beclin-1, PI3KCIII/Vps34 and Vps15 *via* its CCD to generate complex II. Bif-1 is another positive mediator, interacting with Beclin-1 through *UVRAG* to regulate autophagy (28). Furthermore, Ambra1 can promote Beclin-1 interaction with its target PI3KCIII/Vps34 (29). HMGB1, a chromatin-associated nuclear protein, disrupts interaction between Beclin-1 and its negative regulator Bcl-2 (30). Moreover, interaction of Beclin-1 with Survivin can regulate sensitivity of human glioma cells to TRAIL, presenting a possible mechanism for regulating crosstalk between apoptosis and autophagy (31). BH3 motifs are necessary to bind to Bcl-2, Bcl-X_L and Mcl-1, which may contribute to association of Beclin-1 with the BC groove of Bcl-2. By interacting with Beclin-1, Bcl-2 can block its interaction with PI3KCIII/Vps34 and reduce PI3KCIII activity, thereby negatively regulating autophagy (32). Bcl-X_L and Mcl-1 can inhibit Beclin-1 activity by stabilizing its homo-dimerization (33). In addition, Bcl-2 can directly bind a pool of Ambra1 at the mitochondria, which prevents Ambra1 from associating with Beclin-1 (29). Unlike *UVRAG* or ATG14L, Rubicon can inhibit kinase activity of PI3K-

CIII/Vps34 and block maturation of autophagosomes (Fig. 2).

Furthermore, *Beclin-1* is a haploinsufficient tumour suppressive gene that is either monoallelically deleted (75% of ovarian, 50% of breast and 40% of prostate cancers) or shows reduced expression in several types of cancer. The link between *Beclin-1*-mediated autophagy and malignant behaviour of lymphomas has been minutely analysed, revealing that *Beclin-1* can be a valuable independent prognostic factor (34). In ovarian carcinomas, a highly significant association between low expression *Beclin-1* and reduced patient survival has been evaluated. Additionally, lower expression of Beclin-1 has been shown to be inversely correlated with altered expression of Bcl-X_L in an ovarian carcinoma cohort, and combined analysis showed that a low Beclin-1/high Bcl-X_L group had the lowest survival level (35). Thus, Beclin-1 expression can serve as a tool to identify ovarian carcinoma patients and predict patient survival with increased expression of Bcl-X_L. In addition, a significant association has been found between expression of Beclin-1 and pT stages of urothelial tumours. Level of Beclin-1 expression was inversely correlated with histological grade and pT stage, while Bcl-2 expression level was positively correlated with histological grade and pT stage (36). Accordingly, Beclin-1 low-expression and Bcl-2 over-expression are important in progression and aggressiveness of urothelial tumours. In laryngeal squamous cell carcinoma (LSCC), expression of Beclin-1 in tumour tissues has been found to be significantly lower than that in non-tumour tissues, and reduced Beclin-1 expression was significantly correlated with lymph node metastases (37). Further analysis has confirmed that lymph node metastases and Beclin-1 expression are statistically significant. During autophagy initiation and autophagosome formation, Beclin-1 binds to LC3I, which is then converted to LC3II and interacts with the ubiquitin-binding protein p62/sequestosome 1 (SQSTM1).

Figure 2. Beclin-1 interactome.

In breast and colon carcinomas, expressions of Beclin-1, LC3I, LC3II and SQSTM1 are differentially expressed; however, in breast cancers, all of them can be prognostic markers while in colon carcinomas, only Beclin-1 illustrates prognostic effects (38,39). Malignant breast tissue can contain a rare population of multipotent cells with the capacity to self-renew; these are known as cancer stem cells (CSCs). Expression of Beclin-1 is higher in mammospheres established from human breast cancers or breast cancer cell lines (MCF-7 and BT474) than in parental adherent cells, and autophagic flux is more robust in mammospheres (40). Furthermore, Beclin-1 can increase in HCC, while its reduction can be considered as a predictor of disease-free survival. Autophagy defects synergized with altered apoptotic activities might facilitate malignant tumour cell differentiation, resulting in a more aggressive HCC phenotype (41). Moreover, expression of a Beclin-1 mutant resistant to Akt-mediated phosphorylation can increase autophagy, reduce anchorage-independent growth and inhibit Akt-driven carcinogenesis. Akt-mediated phosphorylation of Beclin-1 may enhance its interactions with 14-3-3 proteins and vimentin intermediate filament proteins. It can interact with 14-3-3 through its binding sites S234 and S295, which are negatively regulated by starvation and Akt inhibition. Thus, Akt signalling, intermediate filaments and 14-3-3 proteins may be mechanistically linked to autophagy inhibition and carcinogenesis through regulation of the Beclin-1 complex (42).

miRNAs also may represent a crucial mechanism for regulating Beclin-1 expression and autophagy. miR-30a can bind to Beclin-1, and inhibition of Beclin-1 expression by the miR-30a mimic blunts activation of autophagy induced by Rapamycin (43). Thus, Beclin-1 can be a potential target for miR-30a, which negatively regulates its expression, leading to reduced autophagic activity. Moreover, Beclin-1 can be regarded as a cellular target of miR-376b. Indeed, on miR-376b overexpression, both mRNA and protein levels of Beclin-1 can decrease. Also, miR-376b target sequences are present in the 3'-untranslated region (3' UTR) of Beclin-1 mRNA and introduction of mutations may abolish their miR-376b responsiveness. Antagomir-mediated inactivation of endogenous miR-376b can lead to a rise in level of Beclin-1 (44). These miRNAs may provide a new perspective concerning their potentials as biomarkers in different cancers *via* targeting Beclin-1-related autophagic signalling pathways, or be investigated for potential gene therapy for cancer treatment.

Hitherto, some anti-tumour agents have been reported to regulate Beclin-1 activity for modulation of autophagy in current cancer therapy. Tamoxifen, a well-

recognized anti-tumour drug for breast cancer treatment, can cause increased synthesis of Beclin-1, leading to stimulation of autophagy (45). BH3 mimetic ABT-737 specifically reduces interaction between Bcl-2 and Bcl-X_L with the BH3 motif of Beclin-1, then stimulating the Beclin-1 dependent activation of PI3KCIII (46). In addition, as a chemotherapeutic vitamin D analogue, EB1089 can trigger and induce Beclin-1-dependent autophagy in MCF-7 cells (47). Spautin-1, a potent small molecule, is able to promote degradation of PI3KCIII/Vps34 complexes by inhibiting two ubiquitin-specific peptidases, USP10 and USP13, targeting the Beclin-1 subunit of Vps3 complexes in cancer (48). Xestosping B can disrupt the molecular complex formed by Ins (1, 4, 5) P3 receptor and Beclin-1 through an indirect link, established by Bcl-2 (49). Everolimus, known as an mTORC1 inhibitor, also increases Beclin-1 expression to induce autophagy in leukaemia cells (50). Interestingly, Beclin-1 can intervene at every major autophagic step by its interactions, including with autophagic inducers and autophagic inhibitors. Thus, the best hope for a novel therapeutic strategy would lie in discovering candidate anti-tumour drugs directly and systematically targeting the Beclin-1 network.

Atg8/LC3-Atg4 signalling

Atg8-PE, serving as a good marker for detection of membrane structures during autophagy, can trace its whole process, including formation of the autophagosome and its fusion with lysosomes/vacuoles (51). Atg8 and its mammalian homologues including LC3, are ubiquitin-like proteins that are synthesized as precursors with additional sequences at their C termini, which are processed by the cysteine protease Atg4 (52). The resulting C-terminal glycine-exposed form of Atg8 is activated by Atg7 (E1 enzyme), transferred to Atg3 (E2 enzyme) and finally covalently linked to an amino group of PE (53). LC3-I can mediate tethering and hemifusion of membranes, and PE converts LC3-I into a membrane bound, thereby autophagosome-associated form of LC3-I (54). Once autophagosome expansion completes, LC3-I detaches from PE with the assistance of Atg4 (55). Atg4 is a cysteine protease of the C54 family, with four homologues including Atg4A, Atg4B, Atg4C and Atg4D in humans, functioning as a deconjugating enzyme as well as regulating level of free Atg8 (56). In mammals, unclosed isolation membranes with abnormal morphology accumulate when knockout of Atg3 or overexpression of an Atg4 dominant-negative mutant blocks lipidation of Atg8 homologues, which suggests that lipidation of Atg8 homologues is important for normal development of the isolation membrane (Fig. 3).

Figure 3. Atg4-LC3 signalling.

Figure 4. Targeting Atg protein network for cancer therapy.

In early oesophageal carcinogenesis, expression of LC3 may correlate with Ki-67 labelling index, but shows no significant association with carbonic anhydrase (CA) IX, a marker of hypoxia, while LC3 is up-regulated in various gastrointestinal cancers, which is partly associated with Ki-67 index. Thus, LC3 expression is advantageous to cancer development, specially in early-phase carcinogenesis (57). Moreover, in human nasopharyngeal carcinoma cells, activation of the JNK pathway can also be assessed under the context of ceramide-induced autophagy. Inhibition of the JNK pathway may block ceramide-induced autophagy and up-regulation of LC3 expression (58). Immunohistochemical localization of LC3 in granular cell tumours and schwannomas reveals increased autophagic activity in some schwannomas, further reinforcing the close relationship between the granular cell tumour and the schwannoma (59). p53-induced nuclear protein 1, known as TP53INP1, is a tumour suppressor down-reg-

ulated in cancers. It can interact with Atg8-family proteins to induce autophagy-dependent cell death. TP53INP1-LC3 interaction occurs *via* a functional LC3-interacting region (LIR), and inactivating mutations of this sequence may abolish TP53INP1-LC3 interaction, re-localize TP53INP1 in autophagosomes and reduce TP53INP1 ability to trigger cell death (60). In addition, interference expression of LC3 may lead to proteotoxicity in cancer cells and enhance caspase-8 oligomerization activation by interacting with ubiquitin-binding proteins SQSTM1/p62 and LC3 (61). Concerning all these together, targeting or modifying the LC3 interacting region enhances LC3 recognition to selectively degrade certain key carcinogenesis proteins (Fig. 4).

miR-204 also, has been demonstrated to be an miRNA regulator of the vesicle elongation process, and its role in autophagy regulation has been further confirmed in renal clear cell carcinoma (RCC) by its direct regulation

of the main LC3 homologue, LC3B (62). Atg8/LC3 may also be a potential cancer therapeutic target. Elevated expression of mitotic kinase aurora kinase A (AURKA) as a negative autophagic regulator in cancer cells is clinically associated with adverse prognosis. Chemical inhibition of AURKA by its small molecule inhibitor VX-680 increases the level of LC3-II and number of autophagosomes, along with reduced level of SQSTM1 (63). However, with little literature focusing on Atg8/LC3-Atg4 signalling as target for cancer treatment, we can regard Atg8/LC3 and Atg4 as emerging potential focal points in our study of future cancer therapy.

Other Atg proteins

As formation of autophagosome requires multiple related proteins of the Atg family, targeting different periods could bring different outcomes. Besides those above-mentioned proteins, other Atg proteins also play important roles in autophagy and cancer. Mononucleotide repeats in Atg2B, Atg5 and Atg12 are common in autophagosome formation, which can be altered by frame-shift mutations in gastric and colon carcinomas with high micro-satellite instability (MSI), suggesting that these mutations may contribute to cancer development by deregulating the autophagic process (64). In addition, in prostate cancer cells, Atg5 may exert unusual expression in cytoplasm compared to normal prostate cells (65). Atg12, synthesized as a C-terminal glycine-exposed form, is activated by Atg7 (E1 enzyme) then transferred to Atg10 (E2 enzyme), finally forming a conjugate with the sole target protein Atg5 (66). The Atg12–Atg5 conjugate interacts with Atg16 to form a complex with a 2:2:2 stoichiometry *via* homodimerization of Atg16/Atg16L1. In mammals, Atg12–Atg5–Atg16L1 predominantly localizes on the outer surface of isolation membranes but dissociates from it immediately before or after completion of autophagosome formation (67). Atg16L1 is essential for autophagosome formation, with significantly high expression levels in oral squamous cell carcinoma (68). Also, both Atg10 (an E2-like enzyme that achieves Atg12–Atg5 conjugation in an E3-enzyme-independent manner) and Atg12 (a ubiquitin-like protein required for autophagy conjugated to Atg3 and Atg5) have noticeable effects on cancer cells (69). Furthermore, Atg9-containing structures are recruited to the PAS, and finally, Atg9 localizes on the outer membrane of the autophagosome (70). Then, Atg9 translocates from PAS to cytoplasmic compartments by retrograde transport, which depends on Atg1 complex, Atg2–Atg18 complex and PI3KCI complex.

Conclusions

As an evolutionarily conserved lysosomal degradation process, autophagy plays the Janus role by regulating oncogenes and also tumour suppressors, which are implicated in autophagically relevant pathways, to jointly seal the fate of cancer cells. Autophagy-related genes, known as Atgs orchestrate the formation of autophagosomes during the process of autophagy. After numbers of years of study, the molecular details of autophagosome formation mediated by core Atg proteins have been unveiled, and so far, 35 Atgs have been identified with their respective unique roles in promoting autophagosomes. Moreover, mounting evidence has demonstrated that some Atgs such as ULK1/2, Beclin-1 and Atg8/LC3-Atg4 can play their key roles at different stages of cancer. In addition, ULK complex and Beclin-1 have also been shown to be regulated in miRNA involvement. These oncogenic and tumour suppressive autophagic pathways involved in miRNA regulation could be integrated into the “dynamic” autophagy network, as they play distinctive roles in survival and death at each cancer stage. Thus, unravelling the roles of miRNAs is crucial to understanding the importance of miRNA regulation of autophagy in cancer. Lastly, it has been revealed that Atgs may orchestrate drug resistance in cancer, and thus the Atgs protein family can be used to treat cancer from bench to clinic. Thus, the best hope for cancer therapeutics may lie in discovering drugs targeting oncogenic or tumour-suppressive autophagic pathways and even the entire autophagic network, rather than the individual gene or protein (Fig. 4).

Acknowledgements

We appreciate the financial support of National 973 Basic Research Program of China (no. 2010CB529900), Key Projects of the National Science and Technology Pillar Program (no. 2012BAI30B02), National Natural Science Foundation of China (no. 81260628, 81202403, 81303270 and 81172374), West China Hospital-Chengdu Science and Technology Department Translational Medicine Innovation Foundation (No. ZH13039), and Sichuan Provincial Science and Technology Department Application Infrastructure Plan (no. 2013JY0154) and Autonomous Region of Xinjiang Project of Science and Technology (No. 201191259).

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