Chapter 10

Role of Autophagy in Cancer

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1. Introduction

Autophagy is a cellular stress-adaptive process in which double-membrane structures called autophagosomes engage in protein degradation, cellular differentiation, apoptosis and antigen processing, and are recycled to sustain cellular metabolism [1-11]. It is a self-digesting mechanism responsible for removal of long-lived proteins and damaged organelles by lysosomes, and opposing roles in cell death and survival have been described for autophagy.

Autophagy is a multifaceted process, and alterations in autophagic signaling pathways are frequently observed in cancer. Cancer is a disease generated by mutation, selection and genome instability in the resulting tumor tissue, and is considered to be the second leading cause of death in western countries after heart disease [12, 13]. Autophagy can be activated by various stimuli including hypoxia during the tumor formation [14]. One hypothetical mechanism is that autophagy promotes tumor cell survival in response to diverse stresses [15]. Furthermore, autophagy spatially and temporally regulates tumor development by suppressing tumor growth through regulating cell proliferation in the early stages of tumorigenesis [16]. Conversely, when autophagy is reduced, it contributes to tumor formation and growth by the breakdown of tumor cells following autophagy-related cell death, leading to tumor cell survival [17]. There is a controversy about the roles of autophagy in cancer [1, 3, 18]. In this review, we outline the multiple roles of autophagy in cancer, including gene expression, gene mutation, and chemotherapy.

2. Autophagy-related genes in cancer

2.1. ATG genes

Most recently, molecular genetic analyses have focused on the function of autophagy-related gene (*ATG*) products. *ATG* products are implicated in autophagosome formation and associat-

ed pathways. In humans, there are more than 30 known *ATG* genes, some of which have mononucleotide repeats with seven or more nucleotides. Of the many genes associated with autophagy, *ATG* genes are the main regulators and implementers of the autophagy process [19].

Beclin-1 (encoded by *BECN1* gene, a mammalian orthologue of yeast Atg6) protein, a component of PI3-kinase complexes, is a key regulator in the vesicle nucleation process of autophagic programmed cell death [20-22].The role of autophagy in tumor suppression is known to be as a result of allelic loss of the essential autophagy genes. Beclin-1 and Beclin-$1^{+/-}$ mice were shown to be tumor prone, indicating that *BECN1* is a haploinsufficient tumor suppressor gene [20, 21], and allelic deletion and point mutations of *BECN1* gene and loss of Beclin-1 expression is found with high frequency in human breast, ovarian and prostate cancers [22, 23]. Lee et al. detected 11 somatic mutations of the *BECN1* gene, including three missense mutations (N8K, P350R and R389C) in coding sequences and eight mutations in introns [24]. These mutations were observed in five gastric, three colorectal, one lung and one breast carcinoma. However, the expression of Beclin-1 is known to be upregulated in colon and gastric cancers [25]. It also reported that *Atg4C*-deficient mice are prone to tumors [26].

Frameshift mutations of genes with mononucleotide repeats are features of cancers with microsatellite instability (MSI). Mononucleotide repeat frameshift mutations in *ATG* genes are common in gastric and colorectal carcinomas with high MSI, and possibly contribute to cancer development by deregulating the autophagy process. Kang et al. detected truncation mutations of three genes (*ATG2B*; c.3120delA, *ATG5*; c.704delA and *ATG9B*; c.293delC) in high MSI cancers (gastric and colorectal) by single-strand conformation polymorphism analysis [27]. In particular, ATG5 is a protein involved in the early stage of autophagosome formation [18, 28]. ATG5 high expression was altered in prostate cancers and other data showed a low incidence of *ATG5* mutations in gastric hepatocellular, and colorectal cancers with MSI [29, 30]. It is important to identify the expression and mutation status of a gene in cancers to understand its role in cancer development. These frameshift mutations or SNPs in *ATG* genes may alter the autophagic cell death in cancers and might contribute to the pathogenesis of human cancers.

2.2. UVRAG

As an *ATG*-related gene, the ultraviolet (UV) radiation resistance-associated gene (*UVRAG*) was initially identified as a gene that is responsible for the partial complementation of UV sensitivity in xeroderma pigmentosum cells, and binds with Beclin-1/PI3-kinase and Bif-1, a Bax activator to induce autophagy formation and suppress the tumorigenic activity of cancer cells [31, 32]. It has been reported that *UVRAG* exon 8 frameshift mutations containing c.709delA or c.708_709delAA mutations were found in gastric and colorectal cancers with MSI [33, 34].

2.3. IRGM

In the autophagy pathway, the immunity-related guanosine triphosphatase (GTPase) family, M (IRGM), plays a central function and appears to have an important role in the activation of the pathway. *IRGM* is located on chromosome 5q33.1, and its mRNA transcripts can be found

in five different 3′-splicing isoforms [35, 36]. Recent evidence indicates that variants of the *IRGM* locus, especially those in the promoter region, may be correlated with differential expression, and consequently the efficacy of autophagy is affected by alterations in IRGM regulation [36-38]. *IRGM* has two major SNPs (rs13361189 and rs4958847) associated with chronic inflammatory digestive diseases. It is not known exactly why *IRGM* rs4958847 but not rs13361189 polymorphism has reported to influence susceptibility to gastric cancer [39].

2.4. RASSF1

The RAS association domain family 1A (RASSF1A) is one of the most epigenetically silenced elements in human cancers. The tumor suppressor gene, *RASSF1A*, has been reported to play a role in diverse activities including cell cycle regulation, apoptosis and modulation of autophagy or genomic instability [40]. It is also associated with epigenetic silencing of other proteins including that of death-associated protein kinase (DAPK) [41-44]. DAPK is a unique calcium/calmodulin-activated serine/threonine kinase involved in autophagy-related signaling pathways [45-48]. RASSF1A can also promote cell death utilizing the association with the anaphase promoting complex protein cdc20 and the autophagic protein, C19ORF5/MAP1S [49]. Expression of the longer isoform of RASSF1A (39 kDa predicted peptide) is lost or downregulated in many lung tumor lines [50, 51]. Agatheanggelou et al. also reported that *RASSF1A* inactivation by methylation and loss is a critical step in lung cancer [52]. Epidemiological studies have identified an association between the *RASSF1A* A133S polymorphism and cancer risk including breast cancer, lung cancer, and hepatocellular carcinoma [53-57]. Moreover, several studies have shown that expression loss by promoter-specific hypermethylation of *RASSF1A* is one of the most common early events in hepatocellular carcinoma that play important roles in tumorigenesis and metastasis of hepatocellular carcinoma [58, 59]. A133S and S131F polymorphisms resulted in the lost ability of RASSF1A to inhibit growth and cyclin D1 expression, suggesting an important role in tumor suppression [60, 61]. Moreover, Gordon et al. reported that E246K, C65R, R257Q *RASSF1A* polymorphisms were related to tumor suppressor function [62]. Additional evidence suggests that *RASSF1C* may be a tumor suppressor gene in prostate and renal carcinoma cells but not in lung cancer cells [63]. It has reported that the loss of RASSF1C results in the downregulation of proliferation of lung and breast cancer cells, suggesting a prosurvival role for RASSF1C [64-66]. Recently, it has been suggested that a possible pathogenic role for RASSF1C in cancer may exist, as its expression was more than 11-fold greater in pancreatic endocrine tumors than in normal tissue [67].

2.5. NOD2

The nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a member of the Nod-like receptor family and associates with the cell surface membrane. NOD2 activation controls the induction of autophagy, or apoptosis [68-70]. Four major *NOD2* single nucleotide polymorphisms are correlated with increased risk of colorectal cancer, and a possible association of the *NOD2* P268S polymorphism with rectal and gastric cancers has been identified [71-78]. A recent meta-analysis also provided good evidence that *NOD2* R702W, G908R, and most significantly, 3020insC, polymorphisms were associated with increased risk of colorectal

cancer [79]. Other studies also found significant associations with laryngeal, lung, and ovarian cancers [80, 81]. In contrast, Suchy et al. found the association of the TNFα-1,031 T/T genotype and *NOD2* 3020insC polymorphism may act as a modifier to reduce colorectal cancer risk [82]. Further research of *NOD2* polymorphisms and gene–gene interactions will provide a more comprehensive insight into the associations described here.

3. Analysis of autophagy by immunohistochemistry

Recently, the role of autophagy in cancer development and progression has been investigated using immunohistochemistry. Immunohistochemical methods have been developed that supplement the detection of autophagy via genetic analyses. Many antibodies for autophagy detection are routinely used for immunohistochemistry against proteins involved in autophagy pathways [83-86] (Table 1).

Antibody		***ref. No***
LC3 (rabbit polyclonal antibody)		[86]
Source; (1: x, dilution rate)	Medical & Biological Laboratories, Japan	
Antigen retrieval method	Pressure cooker (110C-120C) for 10 min; 10 mM citrate buffer, pH 6.0	
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Invariably granular cytoplasmic staining	
LC3		[100]
Source; (1: x, dilution rate)	Novus Biologicals, USA; (1:400)	
Antigen retrieval method	High temperature and pressure, citrate buffer	
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Cytoplasmic staining	
Beclin-1 (rabbit monoclonal antibody)		[95]
Source; (1: x, dilution rate)	Abcam, UK; (1:100)	
Antigen retrieval method	Microwave oven for 15 min, 10 mM citrate buffer, pH 6	
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Cytoplasmic staining	
Beclin-1 (rabbit polyclonal antibody)		[97]
Source; (1: x, dilution rate)	Abcam, UK; (1:100)	
Antigen retrieval method	Microwave oven, 10 mM citrate buffer, pH 6	

Antibody		ref. No
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Membrane-plasma, cytoplasm and nucleus in the cancer cells and no or modest staining in the adjacent noncancerous tissue	
Beclin-1 (rabbit polyclonal antibody)		[25]
Source; (1: x, dilution rate)	Novus Biologicals, USA	
Antigen retrieval method	Pressure cooker inside a microwave oven at 700 W for 30 min, 10 mM citrate buffer, pH 6.0	
Sample type	Microarray recipient block was constructed containing paraffin-embedded colorectal adenocarcinoma tissue samples from 103 archival patient specimens	
Staining pattern	Cytoplasmic staining	
Beclin-1		[100]
Source; (1: x, dilution rate)	Cell Signaling, USA; (1:100)	
Antigen retrieval method	High temperature and pressure, citrate buffer	
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Cytoplasmic staining	
BIF-1 (mouse monoclonal antibody)		[98]
Source; (1: x, dilution rate)	Imgenex, USA; (1:2500)	
Antigen retrieval method	standard cell conditioning (Ventana Medical Systems, USA)	
Sample type	Formalin-fixed, paraffin-embedded core sections on a tissue array	
Staining pattern	Cytoplasmic staining	
ATG5 (rabbit polyclonal antibody)		[30]
Source; (1: x, dilution rate)	Abcam, UK; (1:800)	
Antigen retrieval method	Pressure cooker inside a microwave oven at 700 W for 30 min, 10 mM citrate buffer, pH 6.0	
Sample type	Formalin-fixed, paraffin-embedded specimens	
Staining pattern	Cytoplasmic and/or nuclear	

Table 1. Immunohistochemical analysis of autophagy-related proteins.

3.1. Proteins involved in autophagy

3.1.1. LC3

Microtubule-associated protein 1 light chain 3 (LC3) is an autophagosomal orthologue of yeast ATG8, with approximately 30% amino acid homology [87, 88]. LC3 is a specific marker of autophagosome formation. LC3-I is localized to the cytoplasm, whereas LC3-II binds to autophagosomes [89].

3.1.2. Beclin-1 (ATG6)

Beclin-1 is a mammalian homolog of the yeast ATG6 protein. The expression of Beclin-1 protein has been reported in tumor tissues such as breast, ovarian, prostate, lung, brain, stomach and colorectum [25, 90]. Beclin-1 was found to be deregulated in human cancers and may play a role in the tumorigenesis and/ or progression of human cancers [21, 91]. It is required for autophagic induction and is a haploinsufficient tumor suppressor.

3.1.3. ATG5

ATG5 is a key regulator of autophagic and apoptotic cell death, and is involved in the early stages of autophagosome formation [18, 28]; binding of ATG5 with ATG12 contributes to autophagosome formation, which sequesters cytoplasmic materials before lysosomal delivery [18]. It is suggested that ATG5 is involved in both apoptotic and autophagic cell death [92].

3.1.4. Bax-interacting factor -1

Bax-interacting factor-1 (Bif-1) protein is a member of the endophilin B family, which plays a critical role in cell death, including autophagy and apoptosis. Loss of Bif-1 suppresses programmed cell death and promotes tumorigenesis [93, 94].

3.1.5. GABARAP

Gamma-aminobutyric acid type A receptor-associated protein (GABARAP) is one of the mammalian homologue of yeast ATG8. It is involved in autophagosome formation during autophagy and was first identified in the brain, but is widely expressed in a variety of normal tissues. Recent reports have suggested that GABARAP is an essential component of autophagic vacuoles in addition to its role as an intracellular trafficking molecule [87,88].

3.2. Expression of autophagy-related proteins in gastrointestinal cancers

Resent reports have demonstrated the expression of autophagy-related proteins in gastrointestinal carcinomas. Chen et al. examined the expression levels of Beclin1 in gastric carcinomas and adjacent normal gastric mucosal tissues by immunohistochemistry. According to their results, high levels of Beclin-1 expression were observed in 90/155 (58.1%) of gastric carcinomas, in 24/60 (40.0%) of adjacent mucosal tissues and in 13/30 (43.3%) of normal gastric mucosa tissues (P=0.036). Decreased expression of Beclin-1 in cancer cells was significantly correlated

with poor differentiation, nodal and distant metastasis, advanced TNM stage, and tumor relapse. More importantly, decreased expression of Beclin-1 was associated with shorter survival as evidenced by univariate and multivariate analysis. Chen et al. concluded that decreased expression of Beclin-1 in gastric carcinoma may be important in the acquisition of a metastatic phenotype, suggesting that decreased Beclin-1 expression, as examined by immunohistochemistry, is an independent biomarker for poor prognosis of patients with gastric carcinoma [95].

In contrast, using a tissue microarray approach, Ahn et al. investigated Beclin-1 protein expression in 103 colorectal and 60 gastric carcinoma tissues by immunohistochemistry. The expression of Beclin-1 was detected in 50/60 (83%) of gastric carcinomas and 98/103 (95%) of colorectal carcinomas. Conversely, the normal mucosal cells of both the stomach and colon showed no or very weak expression of Beclin-1. There was no significant association of Beclin-1 expression with clinicopathological characteristics, including invasion, metastasis and stage. Their data indicate that Beclin-1 inactivation by loss of expression may not occur in colorectal and gastric cancers. Rather, increased expression of Beclin-1 in the malignant colorectal and gastric epithelial cells compared with their normal mucosal epithelial cells suggests that neo-expression of Beclin-1 may play a role in both colorectal and gastric tumorigenesis [25].

An et al. analyzed ATG5 protein expression by immunohistochemistry and *ATG5* somatic mutations by single-strand conformation polymorphism in cancer cells and the normal mucosal cells of gastrointestinal tissues. Their results showed that ATG5 protein was well expressed in normal stomach, colon, and liver epithelial cells, while it was lost in 21/100 (21%) of gastric carcinomas, 22/95 (23%) of colorectal carcinomas, and 5/50 (10%) of hepatocellular carcinomas. Furthermore, such loss of ATG5 expression was observed in the cancers irrespective of the histological subtypes and TNM stages. Also, they found that only 1.5% (2/135) of these cancers harbored *ATG5* mutations. They suggested that loss of ATG5 expression may play a role in the pathogenesis of some gastric and colorectal cancers [30].

Colorectal carcinoma is one of the most common cancers in the world and the incidence rate is rising. Miao et al. performed experiments to investigate a possible correlation between GABARAP expression in colorectal carcinoma and clinicopathological parameters, including patient survival times. Their results showed that the expression of GABARAP protein was significantly higher in colorectal cancers (51.5%) than the adjacent matched non-tumor tissues (33.0%), and overexpression of GABARAP was significantly correlated with a low grade of differentiation and shortened overall survival. They described GABARAP protein expression as a new prognosis marker in colorectal carcinoma [96].

Li et al. analyzed the expression of Beclin-1 protein in stage IIIB colon carcinoma by immunohistochemistry and correlated it with survival. Their results showed Beclin-1 immunostaining was distributed in the plasma membrane, cytoplasm and nuclei of tumor cells in 98/115 cases (85.2%). Modest or no Beclin-1 expression was observed in adjacent non-cancerous tissues. Higher levels of Beclin-1 expression were strongly associated with longer survival. Both univariate analysis and multivariate analysis showed that Beclin-1 expression levels and invasive depth of primary mass (T stage) were independent

prognostic factors. They suggested that Beclin-1 is a favorable prognostic biomarker in locally advanced colon carcinomas [97].

Bif-1 protein plays a critical role in cell death, including autophagy and apoptosis. Coppola et al. examined Bif-1 expression level in colorectal carcinoma using semiquantitative immunohistochemistry and microarray analysis of archival specimens. Bif-1 expression was negative in 23/102 (22.5%) of colorectal carcinomas. Moderate to strong Bif-1 staining was identified in 37/102 (36.3%) of the tumors, and weak staining was noted in 42/102 (41.2%). Moderate to strong Bif-1 immunoreactivity was shown in 26/38 (68.4%) normal colorectal mucosa, and none were negative. In 12/38 (31.6%) cases, the normal colorectal mucosa demonstrated weak Bif-1 stain. The mean staining scores (intensity and percentage of positively stained cells) for colorectal carcinomas and normal colorectal mucosa differed significantly (P=0.0003). The percentage of cases with negative expression also differed significantly between normal colorectal mucosa and colorectal carcinoma (P=0.002). Decreased Bif-1 expression in colorectal carcinomas was confirmed at the mRNA level by microarray analysis. They concluded Bif-1 was downregulated during the transition from normal colorectal mucosa to colorectal adenocarcinoma, a novel finding in agreement with the tumor suppressor function of Bif-1 [98].

LC3 is a one of the most useful markers of autophagy. Yoshioka et al. evaluated LC3 expression in gastrointestinal cancers by immunohistochemistry to elucidate the role of autophagy in human cancer development. LC3 expression was compared with Ki-67 staining and expression of carbonic anhydrase IX, a hypoxic marker. LC3 was expressed in the cytoplasm of cancer cells, but not in non-cancerous epithelial cells. Furthermore, high expression of LC3 was observed in 56/106 (53%) of esophageal, 22/38 (58%) of gastric and 12/19 (63%) of colorectal cancers. The immunoreactive score (intensity and percentage of positively stained cells) of LC3 gradually increased during the early stages of esophageal carcinogenesis in low- and high-grade intraepithelial neoplasia and T1 carcinoma, but did not change in later cancer progression (T2–T4 carcinomas). In early esophageal carcinogenesis, LC3 expression correlated with the Ki-67 labeling index (P=0.0001), but showed no significant association with carbonic anhydrase IX expression. In esophageal cancers, LC3 expression did not correlate with various clinicopathological factors, including survival. LC3 is also upregulated in various gastrointestinal cancers and is partly associated with Ki-67 index. Their results suggest that LC3 expression is advantageous to cancer development, especially in early-phase carcinogenesis. Taken together, these findings suggest that LC3 expression is advantageous to cancer development in early phase of carcinogenesis [99].

Ahn et al. reported that Beclin-1 expression was detected in 95% of colorectal carcinomas examined. In contrast, normal mucosal cells of colon showed no or very weak expression of Beclin-1. There was no significant association of Beclin-1 expression with clinicopathological characteristics, including invasion, metastasis and stage [25].

Guo et al. performed experiments to investigate the utility of Beclin-1 and LC3, in predicting the efficiency of cetuximab in the treatment of advanced colorectal cancer. Their results showed that Beclin-1 and LC3 expression was significantly correlated (r=0.44, P<0.01), and patients with low Beclin-1 expression had longer progression-free survival than those with high Beclin-1 expression [100].

4. Autophagy in cancer chemotherapy

One of the standard modalities for treatment of patients with cancer is chemotherapy. Cytotoxic drug treatment often triggers autophagy, particularly in apoptosis-defective cells, and this excessive cellular damage combined with attempts to remediate that damage through progressive autophagy can promote autophagic cell death [101]. Platinum-containing cisplatin is one of the most extensively used chemotherapeutic agents, and remains the first-line treatment in various types of cancer [102]. Cisplatin-based chemotherapy frequently resulted in acquired resistance of cancer cells. Sirichanchuen et al. indicated that the levels of LC3-related autophagy were significantly lower in cisplatin resistant cells, and autophagosome formation was dramatically reduced in the resistant cells [103]. Patients with low LC3 expression had a higher objective response rate amongst advanced colorectal cancer patients treated with cetuximab-containing chemotherapy [100]. Expression of *ATG5* sensitizes tumor cells to chemotherapy, but its silencing results in resistance to cisplatin therapy combined with AKT inhibitor treatment, thus revealing a key role for autophagy in chemoresistance [92]. Autophagic cell death is activated in cancer cells that are derived from different tissues in response to anticancer therapies [101, 104]. Combination therapy with erlotinib and cisplatin is an effective treatment against erlotinib-resistant cancer by targeting (downregulating) ATG3-mediated autophagy and induction of apoptotic cell death. Autophagy may delay apoptotic cell death caused by DNA-damaging agents and hormonal therapies such as tamoxifen. On the contrary, autophagy has a role as a cell survival pathway. Therefore, autophagy is also induced as a protective and survival mechanism. A major regulator of autophagy is the mammalian target of rapamycin (mTOR) pathway, which consists of two distinct signaling complexes known as mTORC1 and mTORC2 [105]. Thus, results all suggest the role of autophagy in attenuation of chemotherapy-induced cell death or survival.

5. Conclusion

Autophagy is involved in metabolism, cell-death, stress response and carcinogenesis. Several key autophagic mediators containing ATG-related proteins, LC3, Bif-1, GABARAP, UVRAG, IRGM, RASSF1, or NOD2, play pivotal roles in autophagic signaling networks in cancer. By these tumor-suppressive mechanisms in early-stage carcinogenesis, autophagy promotes genomic stability in carcinomas, and possibly contributes to cancer development.

Furthermore, immunohistochemical methods have been developed that supplement the detection of autophagy via genetic analyses. These are especially important since diagnosis of autophagic vacuoles using the classical method of electron microscopy is time-consuming, labor-intensive and costly. Many antibodies for autophagy detection are routinely used for immunohistochemistry. These autophagosomes then fuse with lysosomes to generate autolysosomes. Therefore, LC3 is an efficient and reliable marker for the detection of autophagosome formation.

Autophagy or 'self-eating' is frequently activated in tumor cells treated with chemotherapy. In cancer therapy, adaptive autophagy in cancer cells sustains tumor growth and survival in

the face of the toxicity of cancer therapy. However, in certain circumstances, autophagy mediates the therapeutic effects of some anticancer agents. During tumor development and in cancer therapy, autophagy has been reported to have paradoxical roles in promoting both cell survival and cell death.

Autophagy may play a variety of physiological roles in cancer progression at each stage in various cancers. Further investigations are required to clarify the biological role of autophagy-related proteins so as to estimate their potential value in the diagnosis and treatment of cancer.

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