Immunohistochemical Expression of MAP1LC3A and MAP1LC3B Protein in Breast Carcinoma Tissues

Ekhlas Qaid Gazem Othman,¹ Gurjeet Kaur,¹ Ahmad Faisal Mutee,¹
Tengku Sifzizul Tengku Muhammad,^{2,3} and Mei Lan Tan^{1,2*}

¹Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia

²Malaysian Institute of Pharmaceuticals and Nutraceuticals, Ministry of Science, Technology and Innovation, Universiti Sains Malaysia, Pulau Pinang, Malaysia

³Faculty of Science and Technology, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu, Malaysia

Autophagy is a protein degradation process within the cell and its deregulation has been linked to various diseases and the formation of cancer. One of the important proteins involved in the autophagy process is microtubule-associated protein 1 light chain 3 (MAP1LC3). The aims of this study were to determine the MAP1LC3A and MAP1LC3B protein expression in both normal and cancer breast tissues and to determine the relationship between the expression of these proteins and type of tissues. Immunohistochemistry assessments were carried out on tissue microarrays consisting of breast tissues. MAP1LC3A expression was detected in 52/56 of normal breast tissue cores and 65/67 of breast cancer tissue cores.

MAP1LC3B expression was detected in 55/56 of normal breast tissue cores and 67/67 of breast cancer tissue cores. MAP1LC3A and MAP1LC3B protein are expressed in the majority of normal and cancer breast tissues. A large number of MAP1LC3A and MAP1LC3B positive breast cancer tissues cores have high proportion of stained cells (81–100%) as compared with normal breast tissues. However, a significantly higher number of breast cancer tissues were found to express the MAP1LC3A protein with strong immunoreactivity as compared with the normal tissues, suggesting that MAP1LC3A may play a role in breast cancer development. *J. Clin. Lab. Anal.* 23:249–258, 2009.

© 2009 Wiley-Liss, Inc.

Key words: MAP1LC3; autophagy; breast cancer; immunohistochemistry

INTRODUCTION

Autophagy is a degradation and recycling process of cellular constituents and it plays an important role in the bioenergetic management of starvation. It is a mechanism highly conserved from yeast to mammalian cells (1). It is responsible for the turnover of unnecessary or dysfunctional organelles and proteins, such as damaged mitochondria (2). Briefly, the autophagy process begins with the formation of a pre-autophagosomal structure of unknown origin, which gives rise to an isolation membrane or phagophore (3). The isolation membrane engulfs cytosolic components, which further elongates to form the autophagosome, surrounding the protein concern. The autophagosome, which is a double membrane-bounded structure undergoes maturation and fuses with both endosomal and lysosomal vesicles (1,3). In mammalian autophagy, LC3 protein, a

mammalian homolog of the yeast Atg8p, is used as an index of autophagosome formation. Following the synthesis as Pro-LC3, C-terminal fragment is cleaved off by hAtg4Bp to yield a cytosolic precursor form, LC3-I (soluble, unlipidated). LC3-I subsequently conjugates with a phospholipid via a ubiquitylation-like system through hAtg7p and hAtg3p, to form the active LC3-II (lipidated, membrane bounded), which is then targeted to the early autophagosome membrane (4).

*Correspondence to: Mei Lan Tan, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Lot 1-8, Persiaran Seksyen 4/1, Bandar Putra Bertam, Kepala Batas, Pulau Pinang, Malaysia. E-mail: tanml@usm.my

Grant sponsors: Sciencefund (MOSTI); IPPT (USM).

Received 15 January 2009; Accepted 4 March 2009

DOI 10.1002/jcla.20309

Published online in Wiley InterScience (www.interscience.wiley.com).

LC3-II is localized to pre-autophagosomes and autophagosomes, making this protein an autophagosomal marker (5). Following the fusion of autophagosomes with lysosomes, intra autophagosomal LC3-II is rapidly degraded by lysosomal hydrolytic enzymes (6).

There are three known human isoforms of microtubule-associated protein 1 light chain 3 (MAP1LC3; A, B, and C), which show different patterns of expression in different human tissues. Although MAP1LC3A and MAP1LC3B are most abundantly expressed in the heart, brain, skeletal muscle, and testis, MAP1LC3C expression is much lower in all tissues (7). MAP1LC3B is least expressed in the liver, where MAP1LC3A is most plentiful. MAP1LC3B is expressed highly in peripheral blood leukocytes in which MAP1LC3A mRNA is absent (7). Although it is known that the increase in LC3B-II levels, but not in LC3A-II, correlated with elevated levels of autophagic vesicles monitored either by electron microscopy or GFP-LC3 transfection in response to autophagy-inducing stress in most studies (8), Wu and co-workers revealed that both LC3A and LC3B are associated with the autophagic membranes in rats (9).

Autophagic process plays a role in both survival and pathological cell death (2). As autophagy can provide essential nutrients necessary for cells to survive, it is conceivable that autophagy may be used to provide nutrients to other cells in multicellular organisms. This could be an economical way for the remaining cells to reuse the components of organs or tissues that are removed during tissue recasting and organ morphogenesis (10). However, there is also evidence that if the process is left unabated or if over-stimulated, autophagy can progress to cell death, which is often referred to as autophagic cell death (11). As autophagy is under the control of several tumor suppressor proteins (e.g., Beclin-1 and PTEN), it has been hypothesized that a decrease in autophagy is associated with tumor progression (12). Stimulation of autophagy in cancer cells is also observed in response to anti-cancer treatments (13–15). There are reports suggesting the participation of autophagic cell death in various diseases. Cells with autophagic features are found in the lesions of some neurodegenerative diseases, such as Parkinson disease and Alzheimer disease as well as in some forms of myopathy (16,17). Blocking of autophagic maturation is linked to Danon disease and neuronal ceroid lipofuscinosis disease, which suggests that inhibition of autophagy may lead to diseases (18).

The role of autophagy in cancer remains debatable as experimental evidence supports a role for both cancer suppression and development. Autophagic degradation is found to be reduced in breast cancer cells when compared with their normal counterparts (19). Many breast cancer cell lines exhibit deletion of one or more

beclin-1 alleles and human breast tumors showed decreased beclin-1 levels compared with normal adjacent tissues (19). It was also hypothesized that autophagy defects could compromise the ability of breast tumors to cope with metabolic stress resulting in genome damage and instability, which may accelerate tumor progression (20). So far, the implications on defective autophagy in breast tumorigenesis were mainly carried out as in-vitro experiments using breast carcinoma cell lines, such as MCF-7 and iMECs (19,20). However, the exact role of autophagy in breast cancer development and in response to therapy and how the autophagy process can be manipulated to improve anti-cancer therapeutics remain unclear (21).

Using immunohistochemistry method to assess proteins involved in the autophagic process may offer an attractive method for paraffin-embedded clinical tissues. Therefore, this study attempts to determine the protein expression of MAP1LC3A and MAP1LC3B using anti-LC3 antibodies by standard immunohistochemistry method in human breast tissues.

MATERIALS AND METHODS

Tissue Microarrays and Antibodies

Paraffin-embedded tissue microarray slides consisting of breast cancer tissues (BR961, BR541) and normal breast tissues (BRN801) were purchased from Biomax (Rockville, MD). The microarrays were provided with data on patient age, tumor receptor status (ER, PR, and c-erb-2), clinical stage, and grade. Human brain slides as positive controls were purchased from Abcam (Cambridge, MA). As for negative control, rabbit IgG (Sigma Aldrich, St. Louis, MO) was used instead of primary antibody. Cleaved-APG8b and APG8a (MAP1LC3A and MAP1LC3B) antibody purified rabbit polyclonal antibody was obtained from Abgent (San Diego, CA).

Immunohistochemistry Detection in Tissues

Briefly, deparaffinization of the tissue microarray slides and control tissue sections was carried out by heat treatment at 60°C for 30 min. This was followed by rehydration through a series of graded alcohol to water. Endogenous peroxidase activity was blocked by incubating the sections in two changes of 3% (v/v) H₂O₂ in phosphate-buffered saline (PBS) (pH 7.4) at room temperature. Tris-EDTA acid buffer, pH 9.0 at 1 mM was used for antigen retrieval according to standard procedures. Immunohistochemistry procedures and antibody titration was carried out using Dako REAL™ EnVision™ Detection System, (Dako, Glostrup, Denmark). Briefly, the slides were rinsed with PBS for 5 min, and nonspecific antibody binding was

blocked by incubation of the tissue array slides with horse serum before proceeding to the primary antibodies. Immunostaining was visualized using DAB chromogen (Dako). Positive staining to MAP1LC3A and MAP1LC3B was recognized under light microscope as a diffuse cytoplasmic brown color stain. The optimized dilution for both MAP1LC3A and MAP1LC3B antibodies was determined at 1:100.

Assessments of MAP1LC3A and MAP1LC3B Immunostaining and Statistical Analysis

Assessments were carried out by an independent pathologist and a researcher. A consensus opinion was arrived on the percentage of cells stained and intensity of staining. The percentage of positively stained cells was categorized into five groups, namely; 1–20%, 21–40%, 41–60%, 61–80%, and 81–100%. A minimum of 100 cells were counted each time and the average counts were recorded. The staining intensity was graded as absent (0), weak (1+), moderate (2+), or strong (3+). Data were recorded and statistically analyzed using Fisher's Exact test to determine the relationship between staining intensity and staining proportion of these proteins (MAP1LC3A and MAP1LC3B) and type of tissues. The relationship between the protein expression and clinicopathological variables of the tumors was also analyzed. Statistical analysis of the data was performed using SPSS Software (Version 15).

RESULTS

In this study, MAP1LC3A and MAP1LC3B protein expression was successfully evaluated using immunohistochemistry method in a total of 56 of 84 cases of normal breast tissues (missing data/empty cores for 28 cases) and 67 of 71 cases of breast carcinoma tissues (missing data/empty cores for 4 cases). The tissue microarray core details, clinicopathological variables and results of the MAP1LC3A and MAP1LC3B protein expression in breast cancer and normal breast tissues are shown in Tables 1 and 2.

The age distribution of the breast cancer cases ranged from 18 to 75 years (mean age, 46.9 years). Majority of the cases were categorized in the 30–50 years age group. However, not all cases were supplemented with complete clinicopathological data (Table 1). 39 of 67 cases have their tumor grade determined. There was 1 case (2.5%) with grade 0 cancer, 3 cases (7.6%) with grade I, 1 case (2.5%) with grade I–II, 8 cases (20.5%) with grade II, 10 cases (25.6%) with grade II–III, and 16 cases (41.0%) with grade III cancer. Most cases (59 cores) were consisting of invasive ductal carcinoma (88.0%), 3 cases (4.5%) of ductal carcinoma in situ,

3 cases (4.5%) of medullary carcinoma, and 2 cases (2.9%) of tubular carcinoma (Table 1).

In normal breast tissues, 4 cases (7.1%) showed absent staining and 52 (92.9%) cases showed positive staining for MAP1LC3A (Table 3). Of the positive cases, 31 (55.4%) showed weak staining intensity, 13 (23.2%) moderate and 8 (14.3%) strong. In breast cancer tissues, 2 cases (3.0%) showed absent staining and 65 (97.0%) cases showed positive staining for MAP1LC3A. In these positively stained cases, 16 (23.9%) expressed the protein weakly, 28 (41.8%) moderately, and 21 (31.3%) strongly. On the other hand, MAP1LC3B protein was present in 55 cases (98.2%) of the normal breast tissues and absent in 1 case (1.8%). In the positively stained normal tissues, 1 case (1.8%) showed weak staining intensity, 20 cases (35.7%) moderate, and 34 cases (60.7%) strong. In breast cancer tissues, all were stained positive for the protein, 7 cases (10.4%) showed weak staining intensity, 33 cases (49.3%) moderate, and 27 cases (40.3%) strong (Table 3). Representative images from tissue cores expressing both proteins of varying staining intensity are shown in Figure 1 (breast cancer tissues) and Figure 2 (normal breast tissues).

Fisher's Exact test showed that there was significant relationship between both MAP1LC3A and MAP1LC3B staining intensity and type of tissues ($P = 0.001$; $P = 0.024$, respectively). A significantly higher number of breast cancer tissue cores were found to have moderate to strong immunoreactivity toward MAP1LC3A protein as compared with normal breast cores (Table 3). On the other hand, more cancer tissues were found to express MAP1LC3B weakly and moderately as compared with the normal tissues.

The proportion of MAP1LC3 indicates the percentage of cells expressing the protein in each core. In normal breast tissue, the proportion of positively stained cells (MAP1LC3A) was between 1–20% in 11 cases, 21–40% in 2 cases, 41–60% in 14 cases, 61–80% in 10 cases, and 81–100% in 15 cases (Table 4). In breast cancer tissues, the proportion of positively stained cells (MAP1LC3A) was between 41–60% in 1 case, 61–80% in 1 case, and 81–100% in 63 cases (Table 3). On the other hand, the proportion of positively stained cells (MAP1LC3B) in normal breast tissue was between 1–20% in 1 case, 21–40% in 3 cases, 41–60% in 6 cases, 61–80% in 11 cases, and 81–100% in 34 cases (Table 3). In breast cancer tissues, the proportion of cells expressing MAP1LC3B was between 61–80% in 1 case and 81–100% in 66 cases. Fisher's Exact test showed that there was significant relationship between MAP1LC3A and MAP1LC3B staining proportion and type of tissues ($P < 0.001$; $P < 0.001$). A significant higher number of the breast cancer tissues were found to express both

TABLE 1. The Relationship Between Clinicopathological Variables and MAP1LC3 (A and B) Protein Expression in Breast Cancer Tissues (BRN961, BR451)

No	Age	Pathology diagnosis	Grade	ER	PR	HER2	LC3A (intensity)	LC3B (intensity)	LC3A (%)	LC3B (%)
1	58	IDC	I-II	0	0	+++	++	+++	100	100
2	53	IDC	II	0	0	+	++	++	100	100
3	55	IDC	II	+++	0	++	++	++	100	100
4	44	IDC	II-III	0	0	0	0	+++	0	100
5	34	IDC	II-III	+	+	0	+++	+++	100	100
6	72	IDC	III	0	0	+++	++	++	100	100
7	41	IDC	II-III	+	0	+++	++	+++	100	100
8	18	IDC	III	0	0	+	++	+++	100	100
9	31	IDC	III	0	0	+++	+++	++	100	100
10	54	IDC	II-III	0	0	+++	+++	+++	100	100
11	44	IDC	II	+	++	0~+	++	+++	100	100
12	58	IDC	III	+	0	++	+	+++	100	100
13	75	IDC	III	+~+++	++~+++	0	+++	+++	100	100
14	43	IDC	II-III	0	0	0~+	+	+++	100	100
15	43	IDC	III	0	0	0	++	+++	100	100
16	37	IDC	III	0	+~++	+++	++	++	100	100
17	50	IDC	II-III	0	0	0	+	++	100	100
18	46	IDC	II-III	+	0	+	++	++	100	100
19	40	IDC	II-III	+	+~++	0	+	++	100	100
20	60	IDC	II	0	0	+++	+	+++	100	100
21	35	IDC	III	++	++	+++	++	+++	100	100
22	37	IDC	II-III	+	0	0	+++	+++	100	100
23	58	IDC	II-III	++	+	+	+++	+++	100	100
24	49	IDC	III	0	0	+++	+++	+++	100	100
25	52	IDC	III	+	++~+++	+++	+++	++	100	100
26	53	IDC	III	+	+	0	+	++	100	100
27	55	IDC	-	0	-	-	++	++	100	80
28	46	IDC	-	+	-	-	++	++	100	100
29	54	IDC	-	+	-	-	++	++	100	100
30	47	IDC	-	+	-	-	+++	++	100	100
31	43	IDC	-	0	-	-	++	++	100	100
32	35	IDC	-	+	-	-	+	+	100	100
33	47	IDC	-	0	-	-	++	++	100	100
34	53	IDC	-	-	-	-	+++	++	100	100
35	51	IDC	-	+	-	-	+++	+	100	100
36	42	IDC	-	-	-	-	+++	+++	100	100
37	52	IDC	-	-	-	-	++	+++	100	100
38	41	IDC	-	+	-	-	+	++	100	100
39	59	IDC	-	+	-	-	+	++	100	100
40	36	IDC	-	0	-	-	++	+++	100	100
41	44	IDC	-	+	-	-	+++	+++	100	100
42	35	IDC	-	-	-	-	++	++	100	100
43	38	IDC	-	0	-	-	+++	+++	100	100
44	59	IDC	-	+	-	-	+++	+++	100	100
45	37	IDC	-	0	-	-	+++	+++	100	100
46	48	IDC	-	-	-	-	++	++	50	100
47	65	IDC	-	+	-	-	++	+	100	100
48	47	IDC	-	0	-	-	++	+	100	100
49	57	IDC	-	+	-	-	+	+	100	100
50	61	IDC	III	-	-	-	+	+	100	100
51	47	IDC	0	-	-	-	+	++	100	100
52	64	IDC	II	-	-	-	+++	+++	100	100
53	42	IDC	II	-	-	-	+	++	100	100
54	28	IDC	II	-	-	-	+++	++	100	100
55	53	IDC	III	-	-	-	+	+	100	100
56	74	IDC	II	-	-	-	+++	+++	100	100
57	43	IDC	III	-	-	-	++	++	100	100
58	47	IDC	III	-	-	-	+	++	100	100

TABLE 1. Continued

No	Age	Pathology diagnosis	Grade	ER	PR	HER2	LC3A (intensity)	LC3B (intensity)	LC3A (%)	LC3B (%)
59	29	IDC	III	—	—	—	+	++	70	100
60	52	DCIS	I	+++	+	++	++	+++	100	100
61	28	DCIS	I	0	0	+++	++	+++	100	100
62	41	DCIS	I	+	+++	+	++	++	100	100
63	30	MC	—	0	0	0	+++	++	100	100
64	40	MC	—	0	0	0	++	++	100	100
65	52	MC	—	+~++	0	0~+	++	++	100	100
66	61	TC	—	++~+++	++	+~++	0	++	100	100
67	30	TC	—	0	++	+	+++	++	100	100

+, weak stained; ++, moderate stained; +++, strong stained cytoplasm; 0, negative stain; —, not supplied by the manufacturer; %, the percentage of cells expressing the protein in each core; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; MC, Medullary carcinoma; TC, tubular carcinoma. The clinical data presented in the table were provided by Biomax ([http:// www.biomax.us/](http://www.biomax.us/)).

MAP1LC3 proteins in larger proportion (81–100%) (Table 4).

Grade 0, grade I, or grade I–II tumors were found to express the MAP1LC3A protein moderately (Table 1). The majority of the cases with grade II–III tumors exhibited stronger immune reactivity for the protein. On the other hand, grade I–II tumors exhibited moderate to strong immune reactivity for the MAP1LC3B protein. However, the majority of the grade III tumors showed moderate immune reactivity toward the protein. In general, the expression of MAP1LC3A and MAP1LC3B protein were moderate to strong in the high-grade tumors (grade II, grade II–III, and grade III). However, the association was not found to be significant. Fisher's Exact test also revealed no significant relationship between MAP1LC3 expression and clinical stage, age group and receptor status (intensity and proportion).

DISCUSSION

Autophagy is a protein degradation system within the cell and it is widely postulated that the autophagic pathway is deregulated in tumor cells. Cell lines derived from hepatic, pancreatic, and breast carcinoma exhibit low autophagic activity, as compared with normal cells from the same origin (19,22). Autophagic capacity is known to increase during premalignant stages of pancreatic carcinogenesis, and then decreases during the transition of pancreatic adenoma into adenocarcinoma, suggesting again that decreased autophagic activity is necessary and compatible with malignant stages of pancreatic cancer (23–25). A decrease in autophagic capacity is also observed during animal experimental carcinogenesis, where cells from preneoplastic liver nodules, or primary hepatocellular carcinomas induced by chemical carcinogens showed a decreased autophagic capacity as compared with normal cells from the liver (24–27). Therefore, there are good

reasons to suggest that monitoring and manipulation of autophagy may provide useful ways to increase the efficacy of cancer treatments, prevent cancer development, and limit tumor progression.

MAP1LC3 is cytoplasmically localized. It is cleaved, lipidated, and localized exclusively to autophagosomes during autophagy. Although MAP1LC3-II (lipidated form) is currently a reliable marker for the presence of autophagosomes (28), it will be interesting to see the protein expression of both forms in cancer tissues. This is a first attempt to analyze the presence of two isoforms of MAP1LC3 protein in breast cancers using tissue microarrays and immunohistochemical techniques. The antibodies detect the nonlipidated (LC3-I) and lipidated (LC3-II) forms of LC3 protein. Some researchers have even suggested that anti-LC3 antibodies may provide a useful tool for monitoring the induction of autophagy in cancer cells and tissues (29). Both antibodies produced diffused cytoplasmic stain in positive controls and in breast tissues expressing the isoforms, which was in agreement with published studies using similar methods (29,30). There was no punctuated appearance seen using normal light microscopy magnification (often referred to LC3-II in GFP-based experiments).

In general, more than 92% of both normal and cancer tissues expressed the MAP1LC3A and MAP1LC3B isoforms, the differences mainly focused on the abundance or intensity and percentage of cells expressing the protein. The majority of the normal breast tissue cores showed weak expression for MAP1LC3A (31 cores, 55%), whereas the majority of the breast cancer tissue (49 cores, 73%) showed moderate to strong expression, respectively. On the other hand, a higher number of breast cancer tissues (40 cores, 59%) showed weak to moderate expression of MAP1LC3B protein as compared with the normal tissues (21 cores, 38%). However, there were more normal tissues (34 cores, 61%) expressing MAP1LC3B in stronger intensity as

TABLE 2. MAP1LC3 (A and B) Protein Expression in Normal Breast Tissues (BRN801)

No	Age	LC3A (Intensity)	LC3B (Intensity)	LC3A (%)	LC3B (%)
1	41	—	+++	—	100
2	58	+++	+++	100	100
3	24	+++	+++	100	100
4	43	++	+++	100	100
5	47	+++	+++	100	100
6	43	+++	+++	100	100
7	53	+++	+++	100	80
9	57	+++	+++	100	100
10	50	+	++	<10	100
11	46	+	++	30	50
12	61	++	+++	50	100
13	50	+	+++	50	100
14	50	+	++	50	100
15	41	++	++	100	100
16	42	++	+++	100	100
17	52	+++	+++	100	100
18	40	++	+++	100	100
19	43	++	+++	80	100
20	45	+	+++	50	100
21	40	+	++	20	50
22	44	0	+	0	100
23	46	+	+++	50	100
24	49	++	+++	100	100
25	50	++	+++	50	100
26	46	++	+++	70	70
27	52	+	+++	80	100
28	42	+	++	50	100
29	40	+	++	50	100
30	37	++	+++	100	100
31	53	+	++	20	70
32	45	0	++	0	100
33	49	+	+++	50	100
34	60	+	++	20	50
35	51	+	++	20	100
36	43	0	0	0	0
37	69	+	++	40	100
38	51	++	+++	80	100
39	38	++	+++	70	100
40	45	+	+++	10	100
41	44	0	++	0	50
42	37	+	+++	50	70
43	41	+	+++	20	100
44	60	+	++	20	40
45	48	+	++	50	90
46	56	+	+++	70	100
47	52	+	+++	70	80
48	47	+	++	20	80
49	43	+	++	20	30
50	60	+	++	10	50
51	53	+	+++	70	80
52	51	+	++	50	70
53	18	+	+++	100	80
54	40	+	+++	100	80
55	50	++	+++	70	50
56	40	+	++	50	30
57	43	+++	++	100	100

+, weak stained; ++, moderate stained; +++, strong stained cytoplasm; 0, negative stain; %, the percentage of cells expressing the protein in each core. The clinical data (age) presented in the table were provided by Biomax (<http://www.biomax.us/>).

compared with the cancer tissues (27 cores, 40%). Fisher's Exact tests revealed that there was a significant association between MAP1LC3 expression and type of tissue ($P < 0.05$, respectively) (Table 3). The MAP1LC3A isoform was found to be expressed abundantly in a higher number of breast cancer tissues. By contrast, a significantly higher number of normal tissues are expressing the MAP1LC3B protein strongly as compared with the cancer tissues. There were also a significantly higher number of MAP1LC3A and MAP1LC3B positive breast cancer tissues (97 and 99%, respectively), which showed a high percentage of stained cells (81–100%) as compared to the normal tissues. This study revealed that both normal and cancer breast tissues expressed MAP1LC3A and B isoforms. However, MAP1LC3A appeared to be predominantly expressed in breast cancer tissues, suggesting that MAP1LC3A may play a role in breast cancer development or pathogenesis.

Although the immunohistochemistry procedure detects both isoforms, LC3-I and LC3-II, the differences in abundance of both protein detected in normal and breast cancer tissues may provide an indirect clue on the autophagy activities in these tissues. Thus, an increased expression of MAP1LC3 protein in breast cancer tissues may indirectly suggest an increased autophagy activity.

Studies in human lung carcinoma cells revealed that the autophagic pathway in response to nutrient deprivation is not downregulated when compared with their normal counterparts (31). The human colon cancer cells are found to be able to survive for a long periods in absence of nutrients, have a high rate of autophagy activity (32). Studies in colorectal cancer cells revealed that these cancerous cells harbor functional autophagic machinery functions to prolong cell survival during shortages of nutrients (33). One recent study by Fuji and coworkers has also showed that strong LC3 expression in the peripheral area of pancreatic cancer tissue is correlated with poor outcome and short disease-free period (30). The authors have concluded that activated autophagy associated with pancreatic cancer cells may be a response to factors in the cancer microenvironment, such as hypoxia and poor nutrient supply (30).

Researchers support the divergent nature of autophagy in both tumor suppression and tumor progression (34). It is suggested that if cells cannot activate autophagy, protein synthesis predominates over protein degradation and cellular growth continues (typical characteristic of tumor cells). By contrast, autophagy could be activated in more advanced stages of cancer to guarantee survival of cancer cells under extreme conditions, such as the restricted access of cells located in the inner areas of solid tumors to nutrients (35). For example, in solid tumors, during the early stages of

TABLE 3. Immunohistochemical Expression of MAP1LC3 Protein in Breast Cancer and Normal Tissues (Staining Intensity)

Tissue arrays	Sample size	Protein expression	Staining intensity				Fisher's Exact Test <i>P</i> -value
			0	+	++	+++	
Normal breast tissues	56	MAP1LC3A	4 (7%)	31 (56%)	13 (23%)	8 (14%)	0.001
Breast cancer tissues	67	MAP1LC3A	2 (3%)	16 (24%)	28 (42%)	21 (31%)	
Normal breast tissues	56	MAP1LC3B	1 (2%)	1 (2%)	20 (35%)	34 (61%)	0.024
Breast cancer tissues	67	MAP1LC3B	0	7 (10%)	33 (50%)	27 (40%)	

(-) absent staining; (+) weak staining; (++) moderate staining; (+++) strong staining.

Fig. 1. Representative immunohistochemical staining of MAP1LC3A and MAP1LC3B in breast cancer tissues. (A–C) weak, moderate and strong expression for MAP1LC3A. (D–F) weak, moderate, and strong expression for MAP1LC3B (200× magnification).

development, blockage of autophagy maintains continuous growth. As the tumor mass grows, internal cells will probably switch to a catabolic state to guarantee their survival, although cells in the periphery close to

blood vessels can maintain the anabolic state. Therefore, although autophagy is suppressed during the early stages of tumor progression, it seems to be up-regulated during later stages as a protective mechanism against

Fig. 2. Representative immunohistochemical staining of MAP1LC3A and MAP1LC3B in normal breast tissues. (A–C) weak, moderate, and strong expression for MAP1LC3A. (D–F) weak, moderate, and strong expression for MAP1LC3B (200 × magnification).

TABLE 4. Immunohistochemical Expression of MAP1LC3 Protein in Breast Cancer and Normal Tissues (Proportion Staining)

Tissue arrays	Positive cores	Protein expression	Average percentage of stained cells (100 cells/count)					Fisher's Exact Test <i>P</i> -value
			1–20%	21–40%	41–60%	61–80%	81–100%	
Normal	52	MAP1LC3A	11	2	14	10	15	<0.001
Breast	65	MAP1LC3A	0	0	1	1	63	
Normal	55	MAP1LC3B	1	3	6	11	34	<0.001
Breast	67	MAP1LC3B	0	0	0	1	66	

stress conditions such as low oxygen and/or nutrient levels (36).

Autophagy is known to promote the survival of normal cells during nutrient starvation. Similarly, autophagy might enhance the survival of rapidly

growing cancer cells that have outgrown their vascular supply and face oxygen shortage or metabolic stress. Tumor cells may make use of the process to survive the shortage of energy and nutrients. Autophagy may also promote the survival of cancer cells by targeting

damaged mitochondria and other organelles for lysosomal degradation, thereby buffering oxidative stress that can be triggered by activated cancer-causing genes or by cancer treatments (37). Recent studies have revealed that both oxygen and glucose supply is usually insufficient for the aggressively proliferating cancer cells in locally advanced cancers (38–40). Tumor hypoxia has been used as a marker for poor prognosis and how cancer cells become more malignant in limited oxygen supply environment is poorly understood (41). Therefore, an increase in the autophagic activity in some cancer cells may contribute to survival in harsh microenvironments in which cancers are known to progress (30). Suppression of autophagy may contribute to the initial rapid growth of tumors; however, in more advanced stages of cancer, autophagy may be required to provide essential nutrients to the cells in the inner part of a solid tumor that do not have direct access to the circulation (42).

Therefore, exploring the yet unknown relationship between deregulated autophagy and other breast cancer promoting functions may provide valuable insight into the pathogenesis of breast cancer and may have significant prognostic and therapeutic implications for breast cancer patients (20). A better understanding of mechanisms underlying autophagy will be very helpful to develop pharmacological strategies to modulate the altered autophagy responses according to pathological situations ranging from cancer to other diseases. In conclusion, this preliminary study revealed that MAP1LC3 protein, as a protein involved in the autophagy process is present in both normal and breast cancer tissues. Interestingly, MAP1LC3A protein expression occurred in greater abundance and in a larger percentage of cells in breast cancer tissues as compared with the normal tissues, suggesting that MAP1LC3A isoform may play a role in breast cancer pathogenesis.

ACKNOWLEDGMENTS

This work was supported in part by the Sciencefund (MOSTI) and IPPT (USM). The authors are also thankful to the staffs at Pusat Klinik IPPT for their technical assistance.

REFERENCES

- Ferraro E, Cecconi F. Autophagic and apoptotic response to stress signals in mammalian cells. *Arch Biochem Biophys* 2007;462: 210–219.
- Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 2004;6:463–477.
- Suzuki K, Ohsumi Y. Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. *FEBS Lett* 2007;581: 2156–2161.
- Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 2004;117:2805–2812.
- Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000;19:5720–5728.
- Kabeya Y, Kamada Y, Baba M, Takikawa H, Sasaki M, Ohsumi Y. Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Mol Biol Cell* 2005;16:2544–2553.
- He H, Dang Y, Dai F, et al. Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. *J Biol Chem* 2003;278:29278–29287.
- Klionsky DJ, Abeliovich H, Agostinis P, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008;4:151–175.
- Wu J, Dang Y, Su W, et al. Molecular cloning and characterization of rat LC3A and LC3B—two novel markers of autophagosome. *Biochem Biophys Res Commun* 2006;339: 437–442.
- Maruyama R, Goto K, Takemura G, et al. Morphological and biochemical characterization of basal and starvation-induced autophagy in isolated adult rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2008.
- White E. Autophagic cell death unraveled: Pharmacological inhibition of apoptosis and autophagy enables necrosis. *Autophagy* 2008;4:399–401.
- Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol* 2004;36:2445–2462.
- Bursch W, Hohegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci* 2000;113:1189–1198.
- Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol* 2002;157:455–468.
- Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61:439–444.
- Bahr BA, Bendiske J. The neuropathogenic contributions of lysosomal dysfunction. *J Neurochem* 2002;83:481–489.
- Kaneda D, Sugie K, Yamamoto A, et al. A novel form of autophagic vacuolar myopathy with late-onset and multiorgan involvement. *Neurology* 2003;61:128–131.
- Eskelinen EL. Maturation of autophagic vacuoles in Mammalian cells. *Autophagy* 2005;1:1–10.
- Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402: 672–676.
- Karantza-Wadsworth V, White E. Role of autophagy in breast cancer. *Autophagy* 2007;3:610–613.
- Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer* 2005;5:726–734.
- Kisen GO, Tessitore L, Costelli P, et al. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis* 1993;14:2501–2505.
- Toth S, Nagy K, Palfia Z, Rez G. Changes in cellular autophagic capacity during azaserine-initiated pancreatic carcinogenesis. *Acta Biol Hung* 2001;52:393–401.
- Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 2004;23:2891–2906.

25. Toth S, Nagy K, Palfia Z, Rez G. Cellular autophagic capacity changes during azaserine-induced tumour progression in the rat pancreas. Up-regulation in all premalignant stages and down-regulation with loss of cycloheximide sensitivity of segregation along with malignant transformation. *Cell Tissue Res* 2002;309:409–416.
26. Schwarze PE, Seglen PO. Reduced autophagic activity, improved protein balance and enhanced in vitro survival of hepatocytes isolated from carcinogen-treated rats. *Exp Cell Res* 1985;157:15–28.
27. Canuto RA, Tessitore L, Muzio G, Autelli R, Baccino FM. Tissue protein turnover during liver carcinogenesis. *Carcinogenesis* 1993;14:2581–2587.
28. Mizushima N. Methods for monitoring autophagy. *Int J Biochem Cell Biol* 2004;36:2491–2502.
29. Aoki H, Kondo Y, Aldape K, et al. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. *Autophagy* 2008;4:467–475.
30. Fujii S, Mitsunaga S, Yamazaki M, et al. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. *Cancer Sci* 2008;99:1813–1819.
31. Lee HK, Jones RT, Myers RA, Marzella L. Regulation of protein degradation in normal and transformed human bronchial epithelial cells in culture. *Arch Biochem Biophys* 1992;296:271–278.
32. Hourii JJ, Ogier-Denis E, De Stefanis D, et al. Differentiation-dependent autophagy controls the fate of newly synthesized N-linked glycoproteins in the colon adenocarcinoma HT-29 cell line. *Biochem J* 1995;309:521–527.
33. Sato K, Tsuchihara K, Fujii S, et al. Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res* 2007;67:9677–9684.
34. Bialik S, Kimchi A. Autophagy and tumor suppression: Recent advances in understanding the link between autophagic cell death pathways and tumor development. *Adv Exp Med Biol* 2008;615:177–200.
35. Cuervo AM. Autophagy: in sickness and in health. *Trends Cell Biol* 2004;14:70–77.
36. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: A novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. *Oncologist* 2007;12:1395–1403.
37. Levine B. Cell biology: Autophagy and cancer. *Nature* 2007;446:745–747.
38. Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002;2:38–47.
39. Jain RK. Molecular regulation of vessel maturation. *Nat Med* 2003;9:685–693.
40. Vaupel P, Thews O, Hoeckel M. Treatment resistance of solid tumors: Role of hypoxia and anemia. *Med Oncol* 2001;18:243–259.
41. Kitano M, Kudo M, Maekawa K, et al. Dynamic imaging of pancreatic diseases by contrast enhanced coded phase inversion harmonic ultrasonography. *Gut* 2004;53:854–859.
42. Ogier-Denis E, Codogno P. Autophagy: A barrier or an adaptive response to cancer. *Biochim Biophys Acta* 2003;1603:113–128.