Down-regulated Expression of ATG5 in Colorectal Cancer

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Abstract. *The role of autophagy in tumor development is paradoxical. Although some genetic evidence has indicated that autophagy has as a tumor suppressor function, it also provides some advantages to tumors under metabolic stress conditions. Autophagy is regulated by several autophagy-related gene (ATG) proteins. In mammals, 16 different ATG genes have been identified. To investigate the clinicopathological role of ATG5 in colorectal cancer, we firstly investigated its expression in patients with sporadic colorectal cancer. Expression analysis revealed ATG5 to be strongly down-regulated in colorectal cancer (38/40 patients). Interestingly, immunohistochemical analysis of colorectal cancer tissues indicated that increased ATG5 expression is associated with lymphovascular invasion (p=0.035). The findings in our limited clinical cohort indicate that ATG5 could be a potential prognostic or diagnostic biomarker.*

Autophagy is a lysosome-dependent degradative process that maintains cellular homeostasis. Autophagy has been implicated in the pathogenesis of many diseases, such as neurodegenerative diseases, infections, atherosclerosis, myopathies and cancer, as well as in response to treatment (1). However, the precise molecular mechanisms that regulate autophagy in these diseases have not been fully elucidated. The role of autophagy in cancer is complex and is likely to be dependent on the tumor type, stage, and the genetic context. In the past decade, a number of studies have highlighted the role of autophagy in cancer and proposed that defective autophagy is an additional hallmark of cancer (2, 3). Autophagy removes damaged molecules or organelles and limits cell growth, as well as causes genomic instability. Thus, defective autophagy can accelerate tumorigenesis (4). Indeed, some autophagy regulators including Beclin-1/ATG6 (autophagy related gene 6) and BIF-1 (Bax interacting factor 1) display loss of heterozygosity (LOH) in cancer. *Beclin-1*$^{+/-}$ mice develop various types of cancer (5). The mosaic deletion of *ATG5* causes the development of multiple liver tumors in mice (6). In addition, *ATG4C*-deficient mice exhibit increased susceptibility to fibrosarcoma (7). In another study p62/Sequestosome1-deficient mice were protected against Ras (rat sarcoma)-induced lung carcinomas (8). Moreover, defective autophagy promotes tumor growth by enhancing necrosis-dependent inflammation (9). Additionally, overexpression of Beclin-1 or UVRAC (UV radiation resistance-associated gene) represses tumor growth, suggesting that autophagy is a tumor suppressive process. Despite these tumor-suppressive functions, autophagy also contributes to tumor development (10). For example, autophagy promotes tumor cell survival in hypoxic and nutrient-limited regions during tumorigenesis (11). Autophagy inhibition *via* chemical or genetic means induced apoptotic cell death of cancer cells. In addition, combination chemotherapy including autophagy inhibitors suppressed tumor growth and triggered cell death more strongly than did single-agent chemotherapy (4).

To date, more than 30 autophagy-specific regulatory genes have been indentified in yeast, including 16 with mammalian homologes. Among them, ATG5, a key regulator of autophagosome formation, is one of the highly investigated ATGs. ATG5 conjugates with ATG12 through an ubiquitin-like system. Besides autophagy activation, ATG5 is also involved in cell death progression. Ectopic expression of ATG5 sensitizes cells to apoptotic stimuli including anticancer agents (12). During cell death, calpain can cleave ATG5 and truncated ATG5 then induces mitochondria-dependent apoptosis (13). Thus, ATG5 may be a key regulator of the switch between autophagy and apoptosis.

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Key Words: ATG5, autophagy, colorectal cancer, tumor suppressor, LVI.

Table I. *Clinicopathological features of 124 colorectal cancer patients.*

Parameter	No.
Age, (years mean±SD)	58±12
Male/female	60/64
Pre-operative serum CEA, ng/ml, mean±SD	47.2±384.1
Tumor site[a], right/left/rectum	27/35/62
Growth type, expanding/infiltrative	101/23
AJCC stage[b], I/II/III/IV	11/38/59/16
T category, 1/2/3/4	1/16/99/8
N category, 0/1/2	50/43/31
M category, 0/1	108/16
Grade, W/M/P	20/91/13
Lymphovascular invasion+patients	60
Patients with synchronous adenoma	30

CEA, Serum carcinoembryonic antigen; W/M/P, well-differentiated/ moderately-differentiated/poorly-differentiated + mucinous; [a]right, cecum – splenic flexure of the transverse colon; left, descending colon – sigmoid of colon; [b]cancer staging according to the American Joint Committee on Cancer (7th ed., 2010).

However, the role of ATG5 in cancer progression is poorly understood. In this study, we examined the expression pattern of ATG5 in colorectal carcinomas and compared its expression in patients according to their clinicopathological features.

Materials and Methods

Patients and tumor samples. For the initial screening of ATG5 expression, 40 colorectal cancer tissues were randomly chosen from the archival samples of the Asan Medical Center (Between June 1999 and May 2003, Seoul, Korea). Subsequently for the clinical validation, a total of 124 patients with sporadic colorectal cancer whose tumor samples were available, including 119 patients who underwent curative operation (R0 resection) and five patients who underwent relatively curative operation (R1 resection) (Table I) were consecutively recruited. The exclusion criteria included hereditary non-polyposis colorectal cancer, and familial adenomatous polyposis and pre-operative chemoradiation therapy. Recurrences, including regional and distant metastases, occurred in 22 out of 124 patients (17.7%) who underwent curative operations during a mean follow-up time of 58 months (range=3-123 months). All patients provided written informed consent, and the study protocol was approved by the Institutional Review Board, in accordance with the Declaration of Helsinki.

Cell lines and reagents. FHC, a colorectal cell line, was obtained from the American Type Culture Collection (ATCC, Manassas VA, USA). CCD841 cells were kindly provided by Dr. S.Y. Rha (Yonsei University, Seoul, Korea). The AMC5 cell line was established in our laboratory (14) and all other colorectal cancer cells were obtained from ATCC, and cultured at 37°C in an incubator with 5% CO_2 and maintained as recommended by the ATCC in RPMI 1640, containing 10% FBS and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

Western blotting. All tissues and cells were extracted in 2× Laemmli sample buffer [62.5 mM Tris-HCl, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue (BioRad, Hercules, CA, USA)], separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes. After blocking with skimmed milk in TBST (10 mM Tris-HCl, 0.1 M $NaCl_2$, 0.1% Tween 20; pH 7.4), membranes were incubated with specific primary antibodies [anti-ATG5 and anti-ATG7 were from Abcam (Cat.# ab54033 and ab53255, Cambridge, UK)]; anti-ATG6 was purchased from Cell Signaling Technology (Cat.# 3738, Beverly, MA, USA); anti-Actin antibody was obtained from Millipore (Cat.# MAB1501, Temecula, CA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL, USA).

Immunohistochemical (IHC) staining. Tissue array blocks prepared using a precision instrument (Beecher Instruments, Sun Prairie, WI, USA) were subjected to immune staining based on the labeled streptavidin–biotin method by using a Dako LSAB kit (Dako, Carpinteria, CA, USA) and monoclonal antibodies against ATG5 (Medical & Biological Laboratories, Nagoya, Japan). Negative staining (–) was mainly categorized into two groups: weak staining (≤10%) and no staining. All samples displaying negative immune staining were repeatedly assayed.

Statistical analysis. Immunohistochemical findings were compared according to the clinicopathological features of the patients and incidence recurrence by cross-table analysis using the Fisher's exact test with two-sided verification. The primary endpoints were recurrence, overall survival (OS) and disease-free survival (DFS). The survival rates were compared using the Kaplan–Meier method with a log-rank test. The significance level was 5% for each analysis, and all calculations were carried out using the SPSS software (ver.19; SPSS Inc., Chicago, IL, USA).

Results

ATG5 is strongly down-regulated in colorectal cancer. To investigate the role of ATG5 in colorectal cancer, we first examined its expression by western blot analysis. The tumor tissues and their surrounding normal tissues were obtained at the time of surgery. Unlike ATG6 and ATG7, the results indicated that ATG5 is strongly down-regulated in tumors compared to its expression in the adjacent normal mucosa: among 40 patients with colorectal cancer, ATG5 expression was decreased in 38 cases (38/40, 95%) (Figure 1A and 1B). Interestingly, ATG5-12 conjugated form was also more reduced in colorectal cancer than the adjacent normal mucosa (data not shown). Next, we extended the expression analysis to colorectal cell lines. Consistent with the tissue results, ATG5 was weakly expressed in all cancer cell lines examined excluding DLD1 cells, compared with its expression in normal colorectal cell lines (Figure 1C). These results indicate that ATG5 is strongly down-regulated in colorectal cancer.

ATG5 expression is associated with tumor progression and invasion. We further investigated the role of ATG5 expression in tumor tissues. To examine the
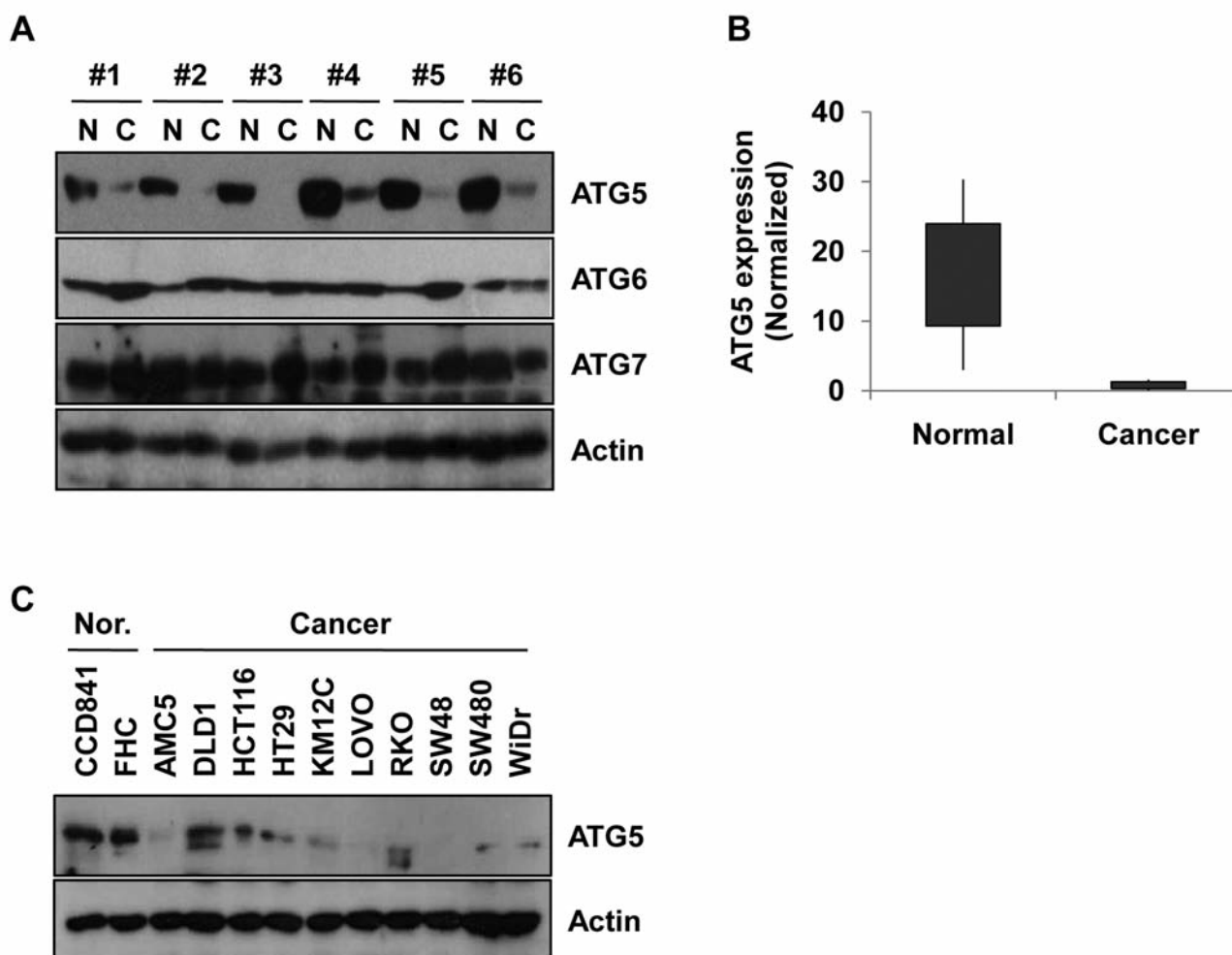
Figure 1. *Autophagy-related gene 5 (ATG5) is down-regulated in colorectal cancer. The expression of ATG5 was assessed by western blotting with an ATG5 antibody in colorectal tissues (T, tumor tissue; N, corresponding normal tissue) (A) and in several colorectal cell lines (Cancer) and normal cells (Nor) (C). Actin was used as a loading control. The expression levels of ATG5 in normal and cancer tissues were quantified by densitometric image scanning of the western blots. The expression ratio was normalized by actin expression (n=40) (B).*

clinicopathological features of the patients, an IHC assay was performed using a colorectal cancer tissue array (Figure 2 and Table I). Negative staining (–) was categorized into two groups: weak staining (≤10%) and no immunoreactivity. Positive staining (+) was identified by more than 10% positivity. Interestingly, the IHC assay revealed that the tumors of approximately 80% of patients exhibited ATG5 expression (102/124 patients). The correlation of ATG5 protein expression with the clinicopathological features of the patients is presented in Table II. Patients with ATG5 expression in tumors were at least two-fold more likely to exhibit lymphovascular invasion (LVI) than those without expression ($p=0.035$). These results indicate that high ATG5 expression is associated with LVI in colorectal cancer.

ATG5 expression is not associated with the OS and DFS of patients. The 5-year DFS and OS rates did not differ in relation to the ATG5 expression (ATG5 – *vs.* +: DFS, 72.7% *vs.* 71.6%, $p=0.816$; OS, 72.7% *vs.* 72%, $p=0.966$) (data not shown).

Discussion

Autophagy appears to paradoxically play dual functions in tumor development. Inhibition of autuophagy sensitizes tumor cells to anticancer chemotherapy, whereas its activation helps cancer cells survive under stress conditions such as hypoxia, suggesting that autophagy may positively regulate tumor development (2, 4, 15, 16). By contrast, the down-regulation of autophagy is also associated with tumorigenesis. The haplo-insufficiency of *ATG6*, a key regulator of autophagy, promotes
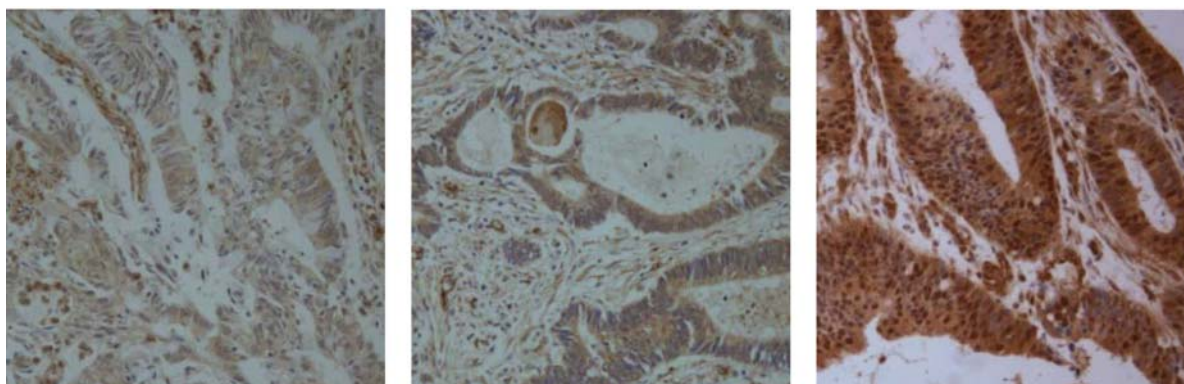
Figure 2. *The expression pattern of Autophagy-related gene 5 (ATG5) in colorectal cancer. Cytoplasmic expression of ATG5 in colorectal cancer tissues was examined by immunohistochemical (IHC) analysis on tissue microarrays (×200). The images show examples of IHC staining with less than 10% positivity (left), between 10 and 50% positivity (middle), and more than 50% positivity (right).*

the spontaneous development of malignancies (5). Moreover, the expression patterns of ATG6 in various types of cancer, including breast, brain, liver, gastric colorectal, ovarian, lymphoma, and gastric cancer, have been widely examined. In mammals, more than 16 ATG genes have been identified. Most studies related of ATG and cancer focused on ATG6 expression. However, the role of ATG5, another key regulator of autophagy, has been poorly investigated in cancer. Recent emerging evidence showed that ATG5 deficiency is involved in tumorigenesis in mice (6). In the current study, we examined the expression of ATG5 in colorectal cancer. Colorectal cancer is the second most prevalent type of cancer and the third leading cause of cancer-related death worldwide. Similarly to ATG6, ATG5 was frequently and strongly down-regulated in tissue samples from patients with colorectal cancer. In addition to colorectal cancer tissues, ATG5 expression was also highly down-regulated in colon cancer cell lines, implying that ATG5 may function as a tumor suppressor. In fact, ectopic expression of ATG5 inhibits tumor cell proliferation and sensitizes cancer cells to anticancer drugs (13).

Differential gene expression can be regulated at several levels, transcriptionally and translationally. Both DNA and protein modifications also control differential gene expression. For example, ATG6 is deregulated in various types of cancer *via* LOH on the chromosome and by aberrant DNA methylation (17, 18). Thus, we also investigated the *ATG5* locus. Interestingly, the *ATG5* gene maps to chromosome locus 6q21, which is commonly deleted in human cancer. Deletions of the 6q21-q22 region have been detected in several malignancies, such as melanoma, gastric carcinoma and B-cell lymphoma (19-21). Since ATG5 expression was down-regulated in colorectal cancer, we also examined LOH of the 6q21 locus in several colorectal cancer patients' samples. However, we did not observe any significant alterations of the 6q21 locus in this study. More precise investigations are

needed to elucidate the possibility of *ATG5* LOH in colorectal cancer because colorectal carcinomas are highly heterogeneous. Epigenetic regulation is another possible mechanism of *ATG5* down-regulation. The expression of a number of tumor suppressor genes is decreased by epigenetic modifications (22). In an *in silico* analysis, we detected high CpG content upstream of the *ATG5* promoter region. CpG islands are frequently methylated to selectively suppress tumor-suppressor genes in cancer. Thus, we plan to examine this issue in a future investigation.

Table II. *Autophagy-related gene 5 (ATG5) protein expression relative to the clinicopathological feature of the patients.*

Parameters	ATG5		
	−	+	p-Value
Age, years ≤/>50	6/16	27/75	1
Male/female	10/12	50/52	0.817
CEA, ≤/>6 ng/ml	18/4	69/33	0.212
Site, R/L+rectum	4/18	23/79	0.781
T[a], 1+2/3+4	3/19	14/88	1
N[a], 0/1+2	11/11	39/63	0.344
M[a], 0/1	19/3	89/13	1
Growth, E/I	18/4	83/19	1
Grade, W+M/P	17/5	94/8	0.054
LVI, −/+	16/6	48/54	**0.035**
Adenoma, −/+	16/6	72/30	1
Recurrence, −/+	20/2	82/20	0.36

CEA, Serum carcinoembryonic antigen; R/L, right (cecum – splenic flexure of the transverse colon)/left (descending colon – sigmoid of colon); E/I, expanding/infiltrative; W/M/P, well-differentiated/moderately differentiated/poorly differentiated+mucinous; LVI, lymphovascular invasion. Bold font, $p<0.05$. [a]Cancer staging according to the American Joint Committee on Cancer (7th ed., 2010).

Recently, Comb *et al*. reported that ATG5 expression is regulated by IκB kinase (IKK) (23). The authors indicated that on starvation, the expression of autophagic genes was not increased in IKK-deficient cells. IKK activates nuclear translocation of NF-κB, which leads to the activation of oncogenic target genes. NF-κB plays multiple and complex roles in tumor development (24). By contrast, autophagic genes are controlled independently of NF-κB (22). Thus, the transcriptional regulation mechanism by which IKK controls *ATG5* gene expression in colorectal cancer remains to be elucidated.

Tissue-based IHC assays have been utilized for many years to assess tumor features. Although ATG5 protein expression was down-regulated in colorectal cancer, our intensive IHC analysis indicated that ATG5 expression in tumors was closely associated with LVI which is an established risk factor for recurrence and survival outcome. However, there was no significant correlation between the ATG5 expression and other parameters such as age, sex, tumor stage, and tumor site in colorectal cancer. The association of ATG5 expression with LVI may be related to the tumor-containing nodes or intravascular tumor aggregates being connected to systemic lymph nodes *via* lymphovascular channels (25, 26). A previous study reported that severe tumor budding was associated with pelvic lymph node metastasis in early-rectal cancer (27). Additionally, in the late stage of tumor development, tumor cells adapt to stressful situations. Autophagy could promote metastasis by promoting tumor cell fitness in response to environmental stresses such as hypoxia or detachment from the extracellular matrix (28). Although LVI is considered an indicator of poor survival, ATG5 expression was not correlated with OS or DFS in our study. In colorectal cancer, it is widely accepted that genetic alterations such as mutations in *APC*, *RAS*, and *p53* are required for tumor development. Additionally, assays based on the molecular detection of these genetic changes have revealed the utility of these mutations as potential diagnostic, prognostic or predictive biomarkers for colorectal cancer (29). However, the associations of these mutations with autophagy or *ATG* genes are largely unknown. Thus, further clinical and pre-clinical investigations are necessary to understand the role of *ATG5* in colorectal cancer.

In conclusion, our limited clinical cohort and biological analyses indicate that the role of ATG5 in colorectal cancer requires further investigation before it can be suggested as a possible prognostic marker or target in colorectal cancer.

Acknowlededgments

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