Autophagy may promote carcinoma cell invasion and correlate with poor prognosis in cholangiocarcinoma

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Abstract: The role of autophagy in cholangiocarcinoma is poorly understood. This study investigated its involvement in cholangiocarcinoma, focusing on carcinoma cell invasion and prognostic significance using cholangiocarcinoma cell lines, CCKS1 and HuCCT1, and human tissues of hilar and extrahepatic cholangiocarcinoma. Nutrient starvation induced the expression of LC3-II and the formation of LC3 puncta in both CCKS1 and HuCCT1, suggesting the occurrence of autophagy. The induction of autophagy was accompanied by the increased expression of an autophagy-related protein, Ambra1, in the cells. Under starvation conditions, the invasive activity of both cells was significantly increased, and a lysosomal inhibitor, chloroquine, attenuated this increased invasive activity. Transforming growth factor-β1 (TGF-β1), known as an inducer of epithelial-mesenchymal transition (EMT), increased the invasive activity of both cells, and chloroquine also significantly reduced TGF-β1-induced cell invasion. Immunohistochemical staining using cholangiocarcinoma tissues showed that the expression of Ambra1 positively correlated with the expression of Snail, one of the major transcriptional factors of EMT. In addition, overexpression of Ambra1 significantly correlated with lymph node metastasis and poor survival rate of the patients. These results suggest that the occurrence of autophagy may be associated with a malignant phenotype and poor prognosis in cholangiocarcinoma, and autophagy is possibly involved in EMT-related cholangiocarcinoma cell invasion.

Keywords: Cholangiocarcinoma, autophagy, Ambra1, EMT, prognosis

Introduction

Cholangiocarcinoma is a highly malignant neoplasm, the incidence of which is increasing worldwide, accounting for 3% of all gastrointestinal cancers [1]. Local and distant metastasis after radical surgery remains the major cause of mortality in these patients, and adjuvant therapy is often required after surgical resection [1, 2]. To identify novel therapeutic strategies, it is necessary to understand the mechanism underlying cholangiocarcinoma progression, and the identification of prognostic factors that can accurately predict patient outcome would also be useful.

Autophagy is a major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome. In addition to cellular maintenance, autophagy is involved in many physiological and pathological conditions, such as aging, bacterial invasion, neurodegenerative disease, apoptosis and cancer [3, 4]. The role of autophagy seems to differ among various types of cancer. For example, it has been suggested that autophagy prevents tumor initiation of pancreatic ductal adenocarcinoma, while after the establishment of carcinoma, it promotes tumor survival and progression, making it a potential target of therapy for pancreatic cancer [5, 6]. There have been a few reports regarding the role of autophagy in cholangiocarcinoma [7, 8]. In these reported studies, the expression of Beclin1, one of the autophagy-related markers, was mainly analyzed. However, the exact role of autophagy in the progression of cholangiocarcinoma remains to be studied.

Epithelial-mesenchymal transition (EMT) is considered to be critical for invasive and metastatic progression of various cancers. Indeed, our
previous study showed that EMT via transforming growth factor-β1 (TGF-β1)/Snail activation induced invasive growth of cholangiocarcinoma [9]. Recently, it has been shown that autophagy promotes hepatocellular carcinoma cell invasion through the activation of EMT [10].

Activating molecule in Beclin1-regulated autophagy (Ambra1) has been identified as a pivotal factor in regulating autophagy in vertebrates [11-13]. Ambra1 promotes the interaction of Beclin1 with its target lipid kinase, Vps34, thereby mediating the early phase of autophagy (autophagosome nucleation) [14, 15]. The described functional interactions of Ambra1 with the Beclin1 complex suggest that dysregulation of Ambra1 function may lead to oncogenesis [15]. Unlike Beclin1, little is known about the cancer-related role of Ambra1, and the significance of Ambra1 in cholangiocarcinoma has not been examined.

In this study, the role of autophagy in cholangiocarcinoma was investigated, especially focusing on carcinoma cell invasion and prognostic significance in relation to the expression of Ambra1.

Materials and methods

Cell culture

Two cholangiocarcinoma cell lines, CCKS1 and HuCCT1, were used. CCKS1 was established in our laboratory from moderately differentiated adenocarcinoma, and HuCCT1 was obtained from Health Science Research Resources Bank (Osaka, Japan). Cells were maintained in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) and Dulbecco’s modified Eagle’s medium/F-12 (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL) and 1% penicillin streptomycin-glutamine (Gibco-BRL) at 37°C in 5% CO₂.

Quantitative real-time PCR

Total RNA was extracted using an RNA extraction kit (RNeasy mini; Qiagen, Tokyo, Japan) and was used to synthesize cDNA with reverse transcriptase (ReverTra Ace; Toyobo Co., Osaka, Japan). The sequences of the Ambra1 primers used for the PCR analysis were as follows: forward, 5’-AACCCCTCACCTGGAGTTGA-3’; reverse, 5’-CTACCTGTTCCGTTCTCC-3’. Quantitative real-time PCR was performed according to a standard protocol using the SYBR Green PCR Master Mix (Toyobo Co.) and ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Warrington, UK). Fold difference compared with glyceraldehyde-3-phosphate dehydrogenase expression was calculated.

Each experiment was conducted in ten sets.

Western blot analysis

Total proteins were extracted from the cells using T-PER protein extraction reagent (Pierce Chemical, Rockford, IL, USA). The protein was subjected to 10% SDS-polyacrylamide electrophoresis, and then electrophoretically transferred onto a nitrocellulose membrane. This membrane was incubated with primary antibodies against LC3B (1:500, rabbit polyclonal; Cell Signaling Technology, Danvers, MA) and α-tubulin (1:400, TU-01, mouse monoclonal; Zymed, South San Francisco, CA). After washing, the membrane was incubated for 1 hour with horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Danvers, MA) and visualized with a chemiluminescence system. Semiquantitative analysis of the results was performed using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

Invasion assay

The invasion of CCKS1 and HuCCT1 was assayed using a Matrigel invasion chamber (BD Biosciences, Bedford, MA). A total of 5×10⁴ cells were seeded on cell culture inserts, and the effects of Earle’s balanced salt solution (EBSS; Sigma-Aldrich, St. Louis, MO), TGF-β1 (R&D Systems, Minneapolis, MN), and chloroquine (Sigma-Aldrich) were examined. After 24 hours, the cells were fixed in 100% methanol and stained with hematoxylin. Migrated cells were visualized under a microscope, and were quantified by counting the number of cells in ten randomly selected fields.

Immunofluorescence staining

Cultured cells grown in a Lab-Tek chamber (Nalge Nunc International, Naperville, IL) were used. The cells were fixed with 4% paraformaldehyde for 15 minutes, and permeabilized for 3 minutes with 0.1% Triton X-100. After blocking, the cells were incubated overnight at 4°C with primary antibodies against LC3B (1:200, D11,
rabbit monoclonal; Cell Signaling Technology). Color development was performed using the Vector Red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA). Nuclei were stained with 4′,6-diamino-2-phenylindole. The sections were observed under a fluorescence microscope. The number of LC3B dots and total cell number were counted, and the ratio of the number of LC3 dots to the total cell number was calculated.

Case selection of cholangiocarcinoma

A total of 65 cases of cholangiocarcinoma were surveyed. All tumors originated from hilar and extrahepatic bile ducts. Curative surgical resection was performed for all cases, and pathological examination confirmed that they were conventional adenocarcinoma. Clinical and pathological data were obtained through a retrospective review of the medical records. Cases involving relapse, non-curative resection, a history of anticancer treatment before operation, cancerous thrombi in the portal vein, and distant metastasis at the time of operation were excluded. The median follow-up period was 21 months (range 1-122 months). Informed consent was obtained for experimentation with human subjects.

Immunohistochemistry

Immunohistochemical staining was performed using formalin-fixed, paraffin-embedded tissue sections of surgically resected specimens of cholangiocarcinoma. Immunohistochemical staining was performed using primary antibodies against Ambra1 (1:500, rabbit polyclonal; Abcam, Cambridge, MA) and Snail (1:500, rabbit polyclonal; Abcam). After deparaffinization of the sections, antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer, pH 6.0. After blocking endogenous peroxidase, sections were incubated overnight at 4°C with individual primary antibodies. The sections were then incubated with secondary antibodies conjugated to peroxidase-labeled polymer, using a HISTOFINE system (Nichirei, Tokyo, Japan). Color development was performed using 3, 3′-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. Control sections were evaluated by substitution of the primary antibodies with nonimmunized serum, resulting in no signal detection.

Histological evaluation

Immunostained sections were evaluated under a light microscope by two investigators (T.N. and Y.S.) blinded to patient clinicopathological data. Differences in interpretation were resolved by consensual agreement. Both staining intensity and the proportion of stained cells were evaluated according to a previous report, with several modifications [16]. The intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. The percentage of positive cells was scored as follows: 0 (≤ 5%), 1 (6-25%), 2 (26-50%), 3 (51-70%) and 4 (> 70%). The sums of the scores of the intensity of positive cells and the percentage of positive cells were calculated for each case, which was referred to as immunoreactivity scores. The median value of the immunoreactivity score was calculated for 65 cases of cholangiocarcinoma. When the immunoreactivity score exceeded the median value, it was regarded as high expression. An immunoreactivity score of less than the median value was regarded as low expression.

Statistical analysis

Statistical significance was determined using the chi-square test, Fisher’s exact test, and the Mann-Whitney U-test. Overall survival of the patients was calculated from the date of surgery to the date of the last follow-up or patient death. The Kaplan-Meier method was used to estimate overall survival, and survival differences were analyzed by the log-rank test. The confidence interval was determined at the 95% level. Statistical analysis was performed using the JMP 10.0 software package (SAS Institute, Inc., Cary, NC). A P value less than 0.05 was accepted as the level of statistical significance.

Results

Starvation induces autophagy and Ambra1 expression in cholangiocarcinoma

EBSS, a nutrient-free medium, was used for the induction of starvation, and autophagy flux was determined by LC3 turnover assay. This assay measures the amount of LC3-II delivered to the lysosomes by comparing the LC3-II amounts in the presence and absence of a lysosomal inhibitor, chloroquine [17]. Western blot analysis
Autophagy in cholangiocarcinoma

Figure 1. Starvation induces autophagy and Ambra1 expression in cholangiocarcinoma. Cholangiocarcinoma cell lines, CCKS1 and HuCCT1, were incubated in a nutrient-free medium, EBSS. Starvation induced conversion of LC3-I to LC3-II in both cell lines, as determined using LC3 turnover assay (A). Immunofluorescence of LC3 showed that the formation of LC3 puncta was significantly increased in the cells under starvation (B). Quantitative real-time PCR showed that starvation increased the expression of Ambra1 mRNA in CCKS1 and HuCCT1 (C), and similar results were obtained at the protein level using immunohistochemical analysis (D). The results obtained from the cells incubated in EBSS for 6 hours are shown, except for the result of quantitative real-time PCR analysis for CCKS1 (C) and immunohistochemical analysis (D), in which the cells were treated with EBSS for 24 hours. Arrows indicate LC3 puncta (B). *P < 0.01.
Autophagy in cholangiocarcinoma

Figure 2. Autophagy induces cholangiocarcinoma cell invasion. The invasive activity of cholangiocarcinoma cell lines, CCKS1 and HuCCT1, was assayed using the Matrigel invasion chamber. Treatment with EBSS (A) and TGF-β1 (10 ng/ml) (B) for 24 hours significantly increased the number of migrated cells of both cell lines. Chloroquine (10 μM) attenuated the increased invasive activity of CCKS1 and HuCCT1 treated with EBSS (A) and TGF-β1 (B). Representative images of the migrated cells in the invasion chamber are shown in the lower parts of the figures. *P < 0.01; **P < 0.05.

immunofluorescence staining of LC3 showed that nutrient starvation significantly induced the formation of LC3 puncta in both CCKS1 and HuCCT1 (Figure 1B), where the conversion of LC3 fluorescence from a diffuse appearance to puncta reflected autophagosome formation and increased autophagic activity of the cells. The induction of autophagy under starvation conditions was accompanied by the induction of Ambra1 mRNA expression in CCKS1 and HuCCT1 (Figure 1C). Immunostaining showed that the expression of Ambra1 at the protein level in the cells tended to be increased following the treatment with EBSS (Figure 1D).

Autophagy induces cholangiocarcinoma cell invasion

Invasion assay using the Matrigel invasion chamber demonstrated that the invasive activity of CCKS1 and HuCCT1 was significantly increased by the use of EBSS (Figure 2A). Chloroquine attenuated the increased invasive
activity of CCKS1 and HuCCT1 by EBSS, although the difference in CCKS1 was not statistically significant (Figure 2A).

Because TGF-β1 has been shown to increase the invasive activity of cholangiocarcinoma cells via a mechanism involving EMT [9], the correlation of autophagy and EMT was examined. As expected, the invasive activity was increased upon stimulation with TGF-β1 in CCKS1 and HuCCT1 (Figure 2B). Interestingly, chloroquine significantly inhibited TGF-β1-induced cell invasion in both cells (Figure 2B), suggesting possible involvement of autophagy in the process of EMT in cholangiocarcinoma.

Expression of Ambra1 in cholangiocarcinoma

Immunohistochemical staining was performed for tissue sections of hilar and extrahepatic cholangiocarcinoma. In normal bile duct epithelium, the expression of Ambra1 was faint or weak (A). In cholangiocarcinoma, the extent of Ambra1 expression varied from case to case. Representative images of weak (B), moderate (C) and strong (D) expression of Ambra1 are shown. Original magnification: ×200; inset, ×400.

Figure 3. Expression of Ambra1 in cholangiocarcinoma. Immunohistochemical staining was performed for tissue sections of hilar and extrahepatic cholangiocarcinoma. In normal bile duct epithelium, the expression of Ambra1 was faint or weak (A). In cholangiocarcinoma, the extent of Ambra1 expression varied from case to case. Representative images of weak (B), moderate (C) and strong (D) expression of Ambra1 are shown. Original magnification: ×200; inset, ×400.

The expression of Ambra1 positively correlated with the expression of Snail, one of the major transcriptional factors regulating EMT ($P = 0.0315$) (Table 1).

Relationship between Ambra1 expression and clinicopathological features in cholangiocarcinoma

The correlation between Ambra1 expression and clinicopathological parameters was exam-
Autophagy occurs in various types of malignant tumor, such as hepatocellular carcinoma, pancreatic ductal adenocarcinoma and breast cancer [18-23]. It is induced in response to nutrient deprivation, metabolic stress, endoplasmic reticulum stress, radiation and anticancer drugs [3, 4]. Among the various nutrients, nitrogen or amino acid starvation induces the highest levels of autophagy in yeast and cultured mammalian cells, respectively [3]. This study confirmed that autophagy was induced in cholangiocarcinoma cells under nutrient starvation conditions using EBSS that was free of amino acids.

Recently, autophagy has been reported to be involved in the invasion of many types of cancer [10, 19, 24, 25]. The present study revealed that the occurrence of autophagy exacerbated the invasion of cholangiocarcinoma cells. Moreover, pharmacological inhibition of autophagy using chloroquine attenuated the invasive activity of cholangiocarcinoma cells under starvation conditions. Chloroquine also reduced TGF-β1-induced cholangiocarcinoma cell invasion.

In recent years, it has been reported that cancer cell invasion induced by the activation of EMT is associated with autophagy. An in vitro study has shown that autophagy promotes hepatocellular carcinoma cell invasion through the induction of EMT, and that activation of TGF-β/Smad3-dependent signaling plays a key role in regulating autophagy-induced EMT [10]. Our results also suggest a possible link between autophagy and EMT in cholangiocarcinoma, and autophagy may be a therapeutic target for cholangiocarcinoma.

Ambra1 has been identified as a positive regulator of the Beclin1-dependent program of autophagy [11-15]. Ambra1 binds to Beclin1 and stabilizes Beclin1/Vps34 complex, thus potentiating its lipid kinase activity and promot-

| Table 1. Relationship between immunohistochemical expression of Ambra1 and Snail in cholangiocarcinoma |
|---------------------------------|---------------------------------|---------------------------------|
|                        | Ambra1 | P value |
|                        | High    | Low   |
| Snail                |         |       |
| High                  | 28      | 6     | 0.0315 |
| Low                   | 18      | 13    |

A total of 65 cases of cholangiocarcinoma were analyzed.

| Table 2. Relationship between immunohistochemical expression of Ambra1 and clinicopathological features in cholangiocarcinoma |
|-------------------------------------------------|---------------------------------|---------------------------------|
| n                                              | Ambra1                          | P value                        |
| High (%) | Low (%) | Total | 65 | 46 (71) | 19 (29) | 0.0391 |
| Age (years) | Sex | N stage | Tumor size | Tumor grade | Perineural invasion | Venous invasion | Lymphatic invasion |
| < 68 | Male | N0 | ≤ 2.2 cm | Well | Negative | 12 | 9 (75) | 3 (25) | 1.0000 |
| ≥ 68 | Female | N1 | > 2.2 cm | Mod, por | Positive | 53 | 37 (70) | 16 (30) |
| 31 | 19 | 29 | 24 (75) | 9 (27) | 0.7245 |
| 34 | 27 | 17 | 25 (68) | 12 (32) | 0.5122 |

In the whole study population, the 5-year overall survival rate was 32%. The group with high expression of Ambra1 had a significantly poor prognosis (P = 0.0209) compared with the low-expression group, in terms of the 2-year overall survival rate (Figure 4A). The 5-year overall survival rate also tended to be lower in the Ambra1 high-expression group, although the difference was not significant (P = 0.0814) (Figure 4B).

**Discussion**

In the 65 cases (Table 2). Among the parameters examined, a significant correlation was observed between the high expression of Ambra1 and lymph node metastasis (P = 0.0391).

In the whole study population, the 5-year overall survival rate was 32%. The group with high expression of Ambra1 had a significantly poor prognosis (P = 0.0209) compared with the low-expression group, in terms of the 2-year overall survival rate (Figure 4A). The 5-year overall survival rate also tended to be lower in the Ambra1 high-expression group, although the difference was not significant (P = 0.0814) (Figure 4B).
Autophagy in cholangiocarcinoma

Recently, several studies revealed that Ambra1 is not only a cofactor of Beclin1 in promoting its kinase-associated activity, but also acts as a crucial upstream regulator of autophagy initiation [15]. Although our knowledge of the cancer-related role of Ambra1 is limited, it has been shown that increased expression of Ambra1 is significantly associated with poor survival of patients with pancreatic ductal adenocarcinoma [6].

In this study, the expression of Ambra1 was induced in association with the induction of autophagy in cholangiocarcinoma cells, and the overexpression of Ambra1 was significantly correlated with lymph node metastasis and poor survival of patients. The expression of Ambra1 was also associated with the expression of Snail, which further suggests an association between autophagy- and EMT-related signaling pathways.

In summary, this study showed that the occurrence of autophagy might be associated with a malignant phenotype and poor prognosis of cholangiocarcinoma. The overexpression of Ambra1 was involved in this process, and Ambra1 could be a candidate prognostic marker of cholangiocarcinoma. In addition, possible involvement of autophagy in EMT-related cholangiocarcinoma cell invasion was elucidated in this study. Further studies are warranted to address novel therapeutic strategies of cholangiocarcinoma targeting autophagy.

Disclosure of conflict of interest

None.

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References

Autophagy in cholangiocarcinoma


