Curative effect of xanthohumol supplementation during liver fluke-associated cholangiocarcinogenesis: Potential involvement of autophagy

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A B S T R A C T
Xanthohumol (XH), a plant flavonoid, was shown to attenuate cholangiocarcinoma (CCA) development induced by the liver fluke Opisthorchis viverrini (Ov) and N-dinitrosomethylamine (NDMA) in the hamster model. We investigated the possible involvement of autophagy, a self-degrading process dysregulated in cancer, in XH chemotherapeutic effect. During cholangiocarcinogenesis, the expression of LC3 (an autophagic marker) was increased in the precancerous stage and decreased in the cancerous stage. The XH-treated Ov (Ov plus NDMA) group showed retarded progression of CCA along with increased expression of LC3. The possible relation between autophagy and cell death was investigated in cultured human CCA cells. XH induced apoptosis associated with reduced expression of BCL-2 and increased expression of BAX. In parallel, XH induced the autophagy flux, as testified by increased LC3-II and decreased p62, along with induction of BECLIN1 and Vps34. Inhibition of BECLIN1-dependent autophagy greatly limited XH toxicity in CCA cells. These data suggest that XH attenuates the development of CCA through overstimulation of autophagy which then precipitates apoptosis.

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1. Introduction

Xanthohumol (XH) is the principal flavonoid found in the hops plant Humulus lupulus L. (Cannabaceae).1,2 XH has been shown to possess anti-cancer properties,2,3 which have been associated with the abilities to scavenge free radicals1,3 and to induce apoptosis.4-6 XH toxicity has been associated also to induction of autophagy.

Autophagy is a dynamic, self-degradation process in which intracellular proteins and organelles are engulfed within double-membrane autophagosomes that eventually fuse with lysosomes.7 Autophagy has a major role in macromolecular turnover and cell homeostasis and is dysregulated in cancer.12-15 Experimental evidence indicates that autophagy may play a dual-opposite
role in cancer: it prevents and retards carcinogenesis by limiting the accumulation of harmful cellular components, yet it may provide a metabolic advantage to cancer cells experiencing hypoxia, lack of nutrients or exposed to chemotherapeutics. On the other hand, overstimulation of autophagy may precipitate into cell death with the characteristics of autophagy-associated apoptosis or of autophagic cell death. Thus, modulation of autophagy may be curative or not depending on the stage at which it is induced or inhibited.

Cholangiocarcinoma (CCA) is a cancer arising from the biliary duct epithelia. In Southeast Asia CCA is primarily associated with liver fluke Opisthorchis viverrini (Ov) infection. Administration of Ov and N-dinitrosomethylamine (NDMA) at a sub-carcinogenic dose strongly induces CCA development in the hamster. Recently, it has been reported that XH suppresses inflammation and fibrosis resulting in a slow progression of CCA development in this experimental model. XH has been shown to induce apoptosis in CCA cells and to inhibit the growth of CCA xenograft via suppression of the Akt–NF-κB signaling pathway and inhibition of STAT3 expression. These data indicate that XH is a promising chemotherapeutics for CCA treatment. The present study intended to get a deeper insight into the anti-cancer mechanism of XH. Here we show that autophagy contributes to XH anti-cancer activity in CCA by slowing down cancer cell growth and promoting apoptosis.

2. Materials and methods

2.1. Animal model, treatments and histological analyses

CCA carcinogenesis was induced in Syrian golden hamsters by an infection of Ov metacercariae combined with NDMA treatment. The treatment protocol with XH in this experimental model has been reported previously. Animal experiments were approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University (AEKKU 23/2555), Thailand. Syrian golden hamsters ranging from 6 to 8 weeks of age were divided into four groups (eight/group): (i) untreated; (ii) treated with XH; (iii) ON (Ov with NDMA administration); and (iv) KHON (Ov infection and NDMA administration and XH treatment). Overall survival in the subgroups was determined in parallel experiments and plotted in a Kaplan-Meier graph as survival percentage. Hamster subgroups were euthanized and the tumors removed at 60, 90, 120, and 180 days and liver tissue was collected for histology examination by the pathologists. Biliary epithelial alterations were graded as hyperplasia, dysplasia ducts, periductal fibrosis and CCA as described previously.

2.2. Immunohistochemistry

Expression of the autophagy protein LC3 was analyzed in the tumor sections by immunohistochemistry. Sections of liver tissue were deparaffinized and antigen-retrieved in 0.05% Tween20 in 0.1M sodium citrate in microwave, then placed with 0.3% H2O2 to block endogenous peroxidase activity. Non-specific protein binding was blocked by 10% skim milk and the sections were incubated overnight with the primary antibody (rabbit anti-LC3 polyclonal antibody; Abcam; MA, UK; 1:500 dilution). After washing-out, the sections were incubated with peroxidase-conjugated Envision secondary antibodies and peroxidase activity was visualized with a diaminobenzidine solution. Hematoxylin was employed for counterstaining. The staining was observed under a light microscope (Carl Zeiss Axio Scope: A1 microscope) at 400x magnification. LC3 expression in the tissues was semi-quantitatively scored based on the percentages of positive cells as follow: 0% = negative; 1–25% = +1; 26–50% = +2; and >50% = +3. The intensity of LC3 immunostaining was scored as weak = 1, moderate = 2, and strong = 3.

2.3. Cell culture and treatment

The human CCA cell lines K100 and K213, deposited in the Japanese Collection of Research Bioresources (JCBR) Cell Bank, Osaka, Japan (http://cellbank.nibiohn.go.jp/~cellbank/en/search_res_list.cgi) were used in this study. All cell lines were cultured in Ham’s F12 medium and RPMI medium, respectively, supplemented with NaHCO3, 100 units/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum. Cells were maintained in a humidified incubator at 37°C containing 5% CO2. Cells were seeded at a density of 1 x 10^3 cells/ml and left to adhere on plates for 16–18 h (KRU-213) and for 72 h (KRU-100) prior to the start of any treatment (at this time cell density had approximately doubled). Treatments included 30 μM of XH (kindly provided by Hopstein, Mainberge, Germany), 5 μM spautin-1 (SP-1, Sigma–Aldrich, St. Louis, MO, USA), and 30 μM chloroquine (CQ; Sigma–Aldrich, St. Louis, MO, USA). In some cases, cells were exposed to CIQ for 8 h prior to harvesting.

2.4. Western blotting

Protein expression was evaluated by a standard immunoblotting procedure. Briefly, cells were homogenized in RIPA buffer and 20 μg of cell protein was separated by electrophoresis on a 12% polyacrylamide SDS gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% BSA, incubated with primary antibodies and subsequently with a secondary antibody at the indicated dilution. The rabbit anti-LC3 (1:1000), rabbit anti-p62/SQSTM1 (1:500), rabbit anti-Vps34 (1:200) and mouse anti-Beclin1 (1:500) were purchased from Abcam (MA, US). The rabbit anti-BAX (1:500), and rabbit anti-Bcl-2 (1:500) were purchased from Cell Signaling Technology, Inc. USA. Blots were also stained with anti-β-actin antibody (1:20,000, Cell Signaling Technology, Inc. USA) that served as an internal control of total protein loading. Immuno-reactive bands were developed by Enhanced Chemiluminescence Plus solution (ECL; Perkin Elmer, Waltham, MA) with an ImageQuant LAS 4000 (GE Healthcare; Life Sciences). Intensity of the bands was estimated by densitometry (ImageJ software 1.48v; Wayne Rasband, USA). All western blotting experiments were performed in triplicate.

2.5. Immunofluorescence

Subcellular expression and localization of Beclin1, Vps34, LC3 and p62 was assessed by immunofluorescence in CCA cells after fixation with 4% paraformaldehyde. The cells were stained with primary antibodies (same commercial source as for western blotting) against Beclin1 (1:100 dilution), Vps34 (1:100 dilution), LC3 (1:100 dilution) and p62 (1:100 dilution), followed by incubation with secondary antibodies as appropriate. The nuclei were stained with Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, USA). The slides were mounted onto microscope slides using fluorescence mounting SlowFadeTMGold antifade (Invitrogen, Thermo Fisher Scientific, USA). Images were captured with a confocal scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and image analysis was determined using Carl Zeiss ZEN software. At least five fields in each chamber were examined by two independent investigators. Representative images of selected fields are presented. Data were reproduced in at least three independent experiments.

2.6. Colony formation assay

CCA cells were seeded in 6-well plates (200 cells in complete
HAM’s F-12 medium). The cells were treated with 30 μM of XH with or without 5 μM of Spautin-1 (SP-1) for up to 10 days. The medium and substances for treatment were renewed every 3 days. At the end of the treatment, the cells were washed with 1xPBS, fixed with 10% TCA (trichloroacetic acid), stained with a 0.05% crystal violet solution, and washed with tap water until excess dye was removed. The colony number was counted by photometric measurements using the CellCounter software (Nghia, Ho) version 0.2.1. Three independent experiments were performed for each assay condition.

2.7. Cell viability and cell cycle assay

Cell viability after exposure to XH with or without SP-1 was assessed by manual counting using the trypan blue dye to label necrotic cells. The results reflect the average (means ± SD) of four replicates. Cell cycle analysis was performed by flow cytometry.15 CCA cells were stained with propidium iodide (PI, 50 μg/ml final concentration) (Alexis Laboratories, San Diego, CA, USA) and analyzed in a FacScan flow cytometer (Becton Dickinson, USA).

2.8. Statistical analysis

The data are presented as the mean ± SD from at least three independent experiments. The statistical significance of the difference between the control and treated groups was evaluated using the Student’s t-test via SPSS software version 17.0 (SPSS Inc., Chicago, IL). All analyses were two-tailed and P-values less than 0.05 (*) and 0.01 (**) were considered statistically significant.

3. Results

3.1. Effects of XH and tissue expression of LC3 in hamster during CCA development

The outcome on hamster survival in the groups treated with or without ON (Ov plus NDMA; leading to CCA development) and co-treated or not with XH is shown in Fig. 1A. It is apparent that at 180 days XH can save hamster’s survival from ON effect (XHON group). The gross appearance and histopathological changes in hamster liver tissues caused by the combined treatments with ON in the absence or presence of XH has been reported in details in our previous study.24 Based on the morphometric analysis at day 180, the bile ducts in XH-treated and in control hamsters showed a similar morphology with negligible obvious alterations, while in the ON group the alterations of bile duct epithelial tissue increased with negligible obvious alterations, while in the XHON group the alterations of bile duct epithelial tissue increased similar morphology with negligible obvious alterations, while in the ON group the alterations of bile duct epithelial tissue increased similar morphology with negligible obvious alterations, while in the ON group the alterations of bile duct epithelial tissue increased similar morphology with negligible obvious alterations.

3.2. XH activates autophagy in cultured CCA cells

The modulation of autophagy by XH was further investigated in cultured human CCA cells. Two CCA cell lines, KKKU-100 and KKKU-213, were treated with 30 μM of XH for 24 h. Chloroquine (CQ), an inhibitor of autophagosome degradation, was added for 8 h prior to sample collection in order to pause the autophagy flux.22 Western blotting analysis revealed that XH increased the cellular level of LC3-II, which is associated with autophagosomes, and concomitantly reduced the level p62, which reflects the degradation of autophagy substrates (Fig. 2A–C). Chloroquine (CQ), known to interrupt the degradation of autophagosomes,22 caused an accumulation of LC3-II versus its precursor LC3-I and of p62 (Fig. 2A–C).22 The autophagy process induced by XH was further analyzed by immunofluorescence staining (Fig. 2D–E). The treatment of XH plus CQ increased the number of the punctate fluorescent LC3 areas, consistent with the vacuolar localization of LC3. These were observed in the order of XH + CQ > XH > CQ > control. The expression of p62 was decreased in cells exposed to XH and...
accumulated, as expected, when ClQ was added.

3.3. Inhibition of BECLIN1-dependent autophagy attenuates apoptotic cell death induced by XH

Next, we determined whether XH induced BECLIN1-dependent canonical autophagy and whether autophagy served a pro-survival or pro-death function during CCA development. XH strongly induced the expression (Fig. 3A) and co-localization (Fig. 3B) of BECLIN1 and Vps34 (also known as PI3K class III, PI3KC3), which is suggestive of activation of the canonical pathway. As a further proof, we co-treated the cells with Spautin-1 (SP-1), which promotes the proteasome degradation of BECLIN1.29 As shown in Fig. 3, SP-1 effectively reduced the cellular level of BECLIN1 even in the cells exposed to XH. More importantly, SP-1 strongly limited the conversion of LC3-I into LC3-II by XH (Fig. 3A), further confirming that XH induces BECLIN1-dependent autophagy. Taking advantage of this finding, we inhibited autophagy with SP-1 to better assess the pro-survival or pro-death function of autophagy in XH-treated CCA cells. Cell growth and cell death were determined through a clonogenic assay and cell counting. Apoptosis was assessed by determining the SubG1 (hypodiploid) peak via flow cytometry and by western blotting analysis of the expression of BCL-2 and BAX. Data in Fig. 4A–B shows that XH effectively inhibited the growth of CCA cells. Of note, SP-1 partially rescued cell survival in XH-treated CCA colonies. Cell counting data (Fig. 4C–D) confirmed this trend. The number of viable cells was reduced while that of Trypan blue-positive increased in the cultures exposed to XH, and the concomitant treatment with SP-1 attenuated both these effects. Combined with the previous data showing inhibition of autophagy by SP-1 (Fig. 3), these data suggest a pro-death function of XH-induced autophagy. Cytomorphometric analysis of propidium iodide (PI)-stained cells revealed that hypodiploid-subG1 peak was five to eight times higher in XH than in controls (Fig. 4E). The proportion of hypodiploid cells was however much reduced in the population co-treated with XH and SP-1 (histograms in Fig. 4E). Finally, we assayed how the treatments impacted the expression of pro- and anti-apoptotic proteins. XH promoted the aggregation of BAX, and to some extent the degradation of BCL-2, and SP-1 counteracted these effects (Fig. 4F). Taken together, these data show that XH may induce cell death in CCA through a coordinated autophagy-apoptosis pathway.

4. Discussion

Autophagy, the catabolic process of cellular self-digestion, is a housekeeping survival mechanism with a protective function against stress conditions. However, when the severity or duration
of stress increases, it may promote cell death.30 Malfunction of autophagy may contribute to certain diseases, including cancer.31 The ratio of LC3-II to LC3-I is a measure of autophagosome degradation. The actual level of LC3-II and p62 in the cell can be determined by interrupting the autophagosome-lysosome fusion step, referring to autophagy flux.31 In this study, we provide a time profile of the expression of molecules related to autophagy appearing during CCA genesis generated by the combined administration of Ov plus NDMA, including XH treatment. Previous data showed that a combination of Ov infection and NDMA administration synergistically induces fibrosis and CCA development in hamsters in a time-dependent manner.24 The results from the current study show an increased accumulation of LC3-II protein in hyperplastic bile duct epithelial cells at the precancerous stage (60 and 90 days), and a reduction in the cancerous stage (120 and 180 days). These findings indicate that autophagy is activated during the precancerous stage and is suppressed once CCA had developed, suggesting the role of autophagy as a tumor suppressor. Induction of autophagy in CCA carcinogenesis is possibly caused by oxidative stress,42 starvation5 and hypoxia,6 whereas it is suppressed in fully developed CCA possibly due to the activation of the PI3K/AKT pathway.13,14,43 Consistent with this interpretation, XH was shown to induce autophagy in glioma6 and prostate cancer cells6 through the inhibition of Akt/mTOR/SGK and the activation of the MAPK signaling pathways. Another possible cause of autophagy suppression is the inflammation that associates with carcinoma. It is known that pro-inflammatory cytokines released by cancer associated fibroblasts in the tumor microenvironment can inhibit autophagy in cancer cells.6,44 XH could restore autophagy in cancer cells by suppressing the inflammation and the synthesis of pro-inflammatory cytokines in the tumor environment acting in a similar way as Resveratrol, another polyphenol nutraceutical.23 Indeed, XH has been reported to dampen inflammation and fibrosis in liver.16,23 Further supporting this possibility, XH was shown to inhibit the STAT3 inflammatory signaling pathway in CCA.45 XH has thus been claimed to be a potential chemopreventive agent in several types of cancer.24 Particularly, XH was shown to prevent the formation of preneoplastic lesions in livers and colons of rats exposed to chemical carcinogen.46 The possible involvement of autophagy in such cancer preventing effect of XH has not been fully investigated. Here, we found that autophagy was upregulated in the early stage of cholangiocarcinogenesis, while it was down-regulated in frank CCA. XH saved the CCA-bearing animals and this associated with induction of autophagy and reduced progression of hyperplastic nodules to malignant tumors. Interestingly, XH did not raise basal autophagy in normal bile duct epithelium. We further investigated the pro-survival or pro-death function of XH-induced autophagy in cultured human CCA cells. We found that LC3 accumulated while p62 was degraded in CCA cells exposed to XH. Western blotting confirmed the increasing conversion of LC3-I to LC3-II and the decreasing p62 levels associated with the autophagy induction by XH. Our data indicate that the hyper induction of autophagy by XH could lead to apoptosis of CCA cell. In fact, this effect was abolished when autophagy was inhibited by Spautin-1, a chemical that promotes the proteasome degradation of BECLIN-1.19 This observation resembles our previous study where autophagy-dependent apoptosis of CCA cells was obtained with Dihydroartemisinin.41 In that study we showed that both Spautin-1 and direct silencing of the BECN1 gene translation could prevent CCA cell death by Dihydroartemisinin, supporting the view that hyper induction of BECLIN-1-dependent autophagy may be deleterious for cancer cells.

In summary, our findings indicate a biphasic pattern of autophagy regulation in cholangiocarcinogenesis, likely eliciting a preventive effect in the early phase (60–90 days), where it is up-regulated, and being thereafter suppressed and thus unable to avoid the transformation of hyperplastic nodules into cancer (from 120 to 160 days). The ON promoting CCA in association with deregulation of autophagy was attenuated by XH. Our in vitro data demonstrate that autophagy contributes to the anti-cancer ability of XH in CCA, supporting its possible employment for the prevention and treatment of CCA. In this respect, a recent clinical trial has shown that XH can prevent DNA damage in humans naturally exposed to dietary carcinogens.42

**Declarations of interest**

None.
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