Abstract. STAT3 plays a significant role in the development of cholangiocarcinoma (CCA) associated with the liver fluke (Opisthorchis viverrini; Ov). Xanthohumol (XN), a prenylated flavonoid extracted from hops, has known anticancer activity and could potentially target STAT3. The present study determined the effect of XN on STAT3, as well as ascertained its usefulness against CCA. The CCA cell proliferation at 20 µM and 50 µM of XN was shown to inhibited, while 20 µM partially inhibited IL-6-induced STAT3 activation. At 50 µM, the inhibition was complete. The reduction in STAT3 activity at 20 and 50 µM was associated with a significant reduction of CCA cell growth and apoptosis. We also found that the administration of 50 µM XN orally in drinking water to nude mice inoculated with CCA led to a reduction in tumor growth in comparison with controls. In addition, apoptosis of cancer cells increased although there was no visible toxicity. The present study shows that XN can inhibit STAT3 activation both in vivo and in vitro due to suppression of the Akt-NFκB signaling pathway. XN should be considered as a possible therapeutic agent against CCA.

Introduction

Cholangiocarcinoma (CCA) or bile duct epithelial cancer associated with the liver fluke (Opisthorchis viverrini; Ov) infection is the most common cancer in northeast Thailand (1-3). CCA is a slow progression cancer with no specific symptoms and most CCA patients usually present with the advanced incurable stage. Surgical restriction is the best treatment regimen for CCA (4,5). However, not all CCA patients are good candidates for curative surgery and complete surgical restriction is often followed by local recurrence with a less than satisfactory 5-year survival rate (6,7). Therefore, the identification of putative therapeutic targets and/or potential anticancer agents against this malignancy is urgently needed.

A signal transducer and activator of transcription (STATs) family of protein kinases play roles in the immune response mechanism, inflammation and cellular development (8,9). Conversely, abnormal activation of STATs has been shown to be involved in the genesis and progression of several types of cancers as well as CCA (10-12). We have previously reported the involvement of protein kinases in CCA development and they represent promising targets for CCA treatment (13,14). Among the kinases, the STAT protein family particularly STAT3 was defined as the major STAT which played a role in inflammation that contributed to CCA carcinogenesis and progression, and was associated with poor prognosis of CCA (15). Therefore, STAT3 could be a potential molecular target for CCA prevention and treatment.

During the past decade, the strategy for cancer prevention and treatment of the identification and characterizations of dietary phytochemicals that are capable of blocking or reversing carcinogenesis as well as possessing anticancer properties has received increased research focus (16-19). Xanthohumol (XN) has been identified and suggested to possess chemopreventive and anticancer properties in every step of carcinogenesis. XN can potently inhibit pro-carcinogen activating and detoxifying enzymes as well as exhibiting antioxidant and free-radical scavenging activity (20). This compound also has an anti-inflammatory activity by abrogating the expression of several inflammatory genes, such as cyclo-oxygenase (COX-1, COX-2) and inducible nitric oxide synthase (iNOS) and it can inhibit cancer cell growth as well as tumor angiogenesis via the suppression of Akt and NFκB activation (21-24). In previous studies, the anticancer potential of XN has been demonstrated in several types of cancer.
However, an inhibitory effect of XN on STAT3 and CCA development has not been reported. Therefore, the present study explored the effects of XN on STAT3 as well as CCA development in both an in vitro and a CCA xenograft model. Results obtained may assist in evaluating whether STAT3 is a potential target for CCA treatment and provide data regarding the effectiveness of XN against CCA.

Materials and methods

Cell culture. Human CCA cells, M214 and M139 were cultured and maintained as previously described (13).

Antibodies and reagents. Antibodies for western blotting were as follows: anti-phospho-STAT3 (Cambridge, UK), anti-phospho-STAT3, phospho-Akt, total Akt (Cell Signaling Technology, Danvers, MA, USA), anti-p65 NfκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Recombinant human IL-6 was commercially available and purchased from R&D Systems, Minneapolis, MN, USA. XN was kindly provided by Hopstein, Mainberge, Germany.

Western blot analysis. Western blot analysis was performed as previously described (15).

Cell proliferation assay. M214 and M139 CCA cells (2x10^4/100 µl) were seeded into 96-well plates and incubated overnight at 37°C and 5% CO_2. Then, XN at designated concentrations was added and incubated for 24, 48 and 72 h. Cell proliferation assay was performed using sulforhodamine B (SRB; Sigma-Aldrich, St. Louis, MO, USA) as previously described (25).

For the XN suppressed IL-6-induced STAT3 activation experiment, cell proliferation was determined by trypan blue exclusion assay. Cells were treated with 10 ng/ml recombinant human IL-6 concomitant with the indicated concentration of XN (0, 10, 20 and 50 µM) for 24 h after that cell was trypsinized and the viable cells were counted in a cell counting chamber under a light microscope. The experiment was carried out in duplicate.

Animal study. Six-week-old female BALB/cAJcl-nu/nu mice were purchased from CLEA Japan (Tokyo, Japan). Animals were housed under specific pathogen-free conditions at the animal center, Institute of Medical Science, The University of Tokyo. All animal experiments were performed according to institutional guidelines. Mice were subcutaneously injected with 2x10^6 cells of KKU-M214 at both flanks. One week after tumors were visible, animals were divided into two groups; the control group was provided with a vehicle (0.5% ethanol) whereas treatment groups were administrated 20 and 50 µM of XN in drinking water for 30 days. Drinking water solutions were secured in the amber bottles to prevent degradation and renewed on a daily basis. Mice were determined for water consumption every other day, and body weight and tumor volume were measured twice a week. The tumor volume was calculated by the formula: 0.5 x width^2 x length and tumor growth was indicated by relative tumor volume (tumor volume normalized with tumor volume day 0).

**Immunohistochemistry detection of Ki67 proliferation marker.** Immunostaining of Ki67, proliferation marker was performed on paraffin-embedded nude mouse tumor tissues to determine the antiproliferative effect of XN in a CCA animal model. Nude mouse tissue sections were deparaffinized in xylene followed by rehydration in a series of different ethanol concentrations. Then, the antigen was retrieved using Tris-EDTA buffer, pH 8.8 in pressure cooker and 0.3% H_2O_2 was used to block endogenous peroxidase activity for 30 min with agitation. Nonspecific binding was blocked by 10% skim milk in phosphate-buffered saline (PBS) for 30 min. Sections were incubated with the anti-Ki67 antibody at 4°C overnight in a moisture chamber.

Sections were then incubated with peroxidase-conjugated EnVision™ secondary antibody (Dako, Denmark) followed by washing with working PBS for 5 min, three times. After that the color was developed with 0.1% diaminobenzidine tetrahydrochloride solution for 5 min and followed by counterstaining with Mayer's hematoxylin. Sections were observed under a light microscope (Carl Zeiss, Germany). Ki67-positive cells of each tumor section was counted in at least five of the x200 power fields.

**Apoptosis assay.** Histologic analysis of DNA fragmentation was used to identify apoptotic cells in the paraffin sections of CCA nude mouse tissues. In situ terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out using the In situ Cell Death Detection kit, POD (Roche). TUNEL-positive cells were quantified in at least five of the x200 power fields of randomly selected tissue sections.

**Statistical analysis.** Results from cell proliferation, Ki67 staining analysis, apoptosis assay and animal experiments are represented as mean ± SD, statistical significance was addressed by independent samples t-test and a two-way ANOVA (GraphPad Prism 5 software). P-value of <0.05 was considered to indicate a statistically significant result.

**Results**

**Antiproliferative effect of XN on CCA cells.** The effects of XN on the growth of CCA cells were determined in human CCA cell lines established from primary tumors of Ov-associated CCA patients namely, KKU-M214 and KKU-M139. The results showed that XN inhibited CCA cell growth which occurred in a dose- and time-dependent manner. A 20 µM concentration of XN significantly inhibited CCA cell growth at 24, 48 and 72 h (P<0.05) in both KKU-M214 and KKU-M139 cell lines (Fig. 1A and B). Moreover, a 50 µM concentration of XN significantly inhibited CCA cell growth at 48 and 72 h (P<0.05) when compared to control cells (Fig. 1A and B). Low concentrations of XN caused no evidence or significant effects on cell growth inhibition even at long exposure times.

**Effects of XN on IL-6 induces STAT3 activation and CCA cell growth.** We then evaluated whether inhibiting STAT3 activation leads to growth inhibition as well as apoptosis induction in CCA cells. CCA cells were exposed to XN upon stimulation with IL-6. The results showed that a low concentration of XN (10 µM) caused an elevation of STAT3 activation while...
XN at 20 μM concentration partially inhibited STAT3 activation. A 50 μM concentration of XN, however, completely inhibited STAT3 activity (Fig. 2A). In addition, abrogation of STAT3 activation by XN at 20 and 50 μM concentrations was associated with a significant reduction of M214 and M139 CCA cell growth which was concordant with decreasing expression of cell cycle controlling proteins, cyclin D1 and CDK4 (Fig. 2B and C).

Apoptosis induction of XN in CCA cells. To investigate if suppression of STAT3 activation by XN inhibited CCA growth resulted from apoptosis induction, we examined the expression of anti-apoptosis protein, Bcl-2 as well as BAX, pro-apoptotic protein. The results demonstrated that decreasing protein levels of Bcl-2 was seen in M214 and M139 CCA cells after treatment with XN whereas BAX protein expression was increased (Fig. 3).

Antitumor activity of XN in CCA inoculated mice. To evaluate an in vivo anticancer activity of XN, KKM-M214 CCA cells were subcutaneously inoculated into athymic BALB/c nude mice, then the mice were administrated with 0.5% DMSO or XN at 0, 2.5, 5, 10, 20, or 50 μM concentrations for 7 days after inoculation.
ethanol (as control) or 20 and 50 µM concentrations of XN in drinking water for 30 days and tumor growth was determined. The results showed that 50 µM concentrations of XN significantly suppressed the rate of tumor growth when compared with control mice at day 23 (Fig. 4A). A 20 µM concentration of XN, however, had no effect on the inhibition of tumor growth (Fig. 4A). No side-effects were observed during the treatment. Histological features of internal organs including liver, spleen and kidney indicated an absence of toxicity (data not shown). Mice treated with XN had similar body weight and water intake rate as the control mice (Fig. 4B and C).

**XN inhibits STAT3 activation and tumor cell proliferation, but induces apoptosis in the CCA mouse model.** As shown in the *in vitro* results, we found that XN can inhibit STAT3 activation as well as CCA cell growth and survival. Moreover, the inhibitory growth effect of XN was observed for XN 50 µM concentrations in treated CCA xenograft. Thus, we investigated whether the observed effects were due to an inhibitory effect of XN on STAT3 activation. We demonstrated that STAT3 activation was reduced in tumor tissues of XN 50 µM concentrations in treated mice when compared to control mice (Fig. 5A). The effects of XN on tumor cell proliferation inhibition and apoptosis induction were further evaluated. Immunostaining of Ki67 proliferation marker was performed to confirm antiproliferation activity of XN (Fig. 5B). Ki67 nuclei stained tumor cells of XN 50 µM concentrations treated mice was significantly decreased when compared to control group (Fig. 5C). Moreover, the apoptosis induction activity of XN was detected by immunohistochemistry of TUNEL (Fig. 5D). Cell apoptosis was significantly higher in XN-treated tumors than in the control group (Fig. 5E).

**Molecular mechanisms by which XN inhibits STAT3 activation in CCA.** The above data revealed an inhibitory effect of XN on ethanol (as control) or 20 and 50 µM concentrations of XN in drinking water for 30 days and tumor growth was determined. The results showed that 50 µM concentrations of XN significantly suppressed the rate of tumor growth when compared with control mice at day 23 (Fig. 4A). A 20 µM concentration of XN, however, had no effect on the inhibition of tumor growth (Fig. 4A). No side-effects were observed during the treatment. Histological features of internal organs including liver, spleen and kidney indicated an absence of toxicity (data not shown). Mice treated with XN had similar body weight and water intake rate as the control mice (Fig. 4B and C).

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**Molecular mechanisms by which XN inhibits STAT3 activation in CCA.** The above data revealed an inhibitory effect of XN on
STAT3 activation both in vitro and in vivo. Thus, we explored the molecular mechanisms by which XN could inhibit STAT3 activation in CCA. We focused on Akt and NFκB signaling as molecular targets of XN as there is mounting evidence that support the association between Akt, NFκB and STAT3 activation (26-29).

Our results showed that XN also suppresses Akt activation as well as the nuclear translocation activity of p65 NFκB in both IL-6-induced CCA cells (Fig. 6A and B) and the CCA xenograft model (Fig. 6C and D). This suggests that the mechanisms by which XN suppresses STAT3 activation in CCA resulted from the inhibition of Akt-NFκB signaling.

**Discussion**

STAT3 is a protein kinase, which plays various roles as a signal messenger and as a transcription factor. STAT3 signaling can be triggered by inflammatory cytokines, growth factors and hormones, particularly IL-6 (8,9). Stat3 knockout mice and tissue-specific gene deletions revealed the critical roles of STAT3 in the regulation of epithelial cell apoptosis, involution in skin remodeling, keratinocyte migration, macrophage inactivation, and reduction of T-helper cell responses to IL-6 (30-32). Thus indicating diverse functions of STAT3 both in the immune response and cellular development.

Conversely, sustained activation of STAT3 is implicated in malignant transformation. Various studies have demonstrated that constitutive STAT3 signaling was required for oncogenic transformation (33-35). In CCA, several studies have investigated whether STAT3 acts as a critical molecule in carcinogenesis and progression of CCA (11,36-38).

Our previous study showed that the activation of the STAT protein family occurred in both CCA cells and tissues (13). Moreover, we demonstrated that among the members of the STAT protein, STAT3 expression was associated with shorter survival of CCA patients as well as prominently activating chronic inflammatory CCA carcinogenesis in a hamster model and CCA cell lines (15). Furthermore, we showed that LPS-induced macrophage conditioned media, which contains several inflammatory cytokines including IL-6 (39), can mediate STAT3 activation in CCA cells (15). Hence, STAT3 is the major STAT that is involved in inflammation contributing to CCA carcinogenesis and progression, and may serve as a molecular marker for CCA poor prognosis. Therefore, targeting STAT3 could be beneficial for CCA prevention and treatment. In the present study, we aimed to inhibit STAT3 activation using a potential anti-inflammatory agent, xanthohumol (XN) in order to evaluate whether STAT3 could be a promising target for XN resulting in the inhibition of CCA growth.
XN, prenylated chalcone which can be isolated from the hop plant (*Humulus lupulus* L.), has been identified and reported as an anti-inflammatory and chemopreventive agent (20,40). XN provides anti-inflammation and antitumor potential by interfering with molecules which are recognized as key mediators in inflammation associated carcinogenesis and progression including iNOS, COX2, NFκB and Akt. Previous studies on Kaposi's sarcoma, hematopoietic cancer, prostate cancer, and breast cancer, have demonstrated apoptosis induction and an anti-angiogenic effect of XN through Akt and NFκB signaling inhibition (21,22,24,41,42). Recently, we demonstrated the inhibitory effect of XN on COX activity which leads to decreased PGE2 production as well as CCA cell migration inhibition (43), suggesting a potential chemopreventive and anticancer activity of XN against cancers including CCA.

The present study showed that XN can inhibit CCA cell proliferation in a dose- and time-dependent manner. Moreover, this is the first time that an inhibitory effect of XN on STAT3 activation has been demonstrated. We revealed that XN at 20 µM concentration could partially suppress IL-6-induced STAT3 activation in CCA cells and a complete inhibitory effect was seen at 50 µM concentration. In addition, our results revealed that inhibition of STAT3 activation by XN was associated with not only growth inhibition but also apoptosis induction of CCA cells. Abrogation of STAT3 activation by XN caused significant reduction of CCA cell growth and concurrently suppressed the expression of the growth-related gene, cyclin D1, which is a specific target gene of STAT3 (44) as well as CDK4, its partner protein. We also found that suppression of STAT3 activation by XN was correlated with CCA cell apoptosis as indicated by downregulation of the anti-apoptotic protein Bcl-2, which is a STAT3 target gene (45) while increasing of the pro-apoptotic protein expression BAX was seen.

Based on the *in vitro* results, we next investigated the inhibitory effects of XN on STAT3 activation and CCA development in a nude mouse model. Our results showed that oral administration of XN at 50 µM concentrations to CCA-inoculated mice attenuated tumor growth without noticeable toxicity. Conversely, a 20 µM XN concentration had no effect on tumor growth suppression. This result was similar to *in vitro* results which showed that low concentrations of XN (2.5, 5 and 10 µM) cannot inhibit CCA cell growth, however, it induced CCA cell growth as well as STAT3 activation when compared to control group. This may result from the compensatory signaling mechanisms of cancer cells that can overcome an inhibitory effect of low concentration of XN which can lead to an increase of STAT3 activation as well as tumor proliferation. This phenomenon can be explained by the acquired resistance mechanism of cancer when blocked by inhibitor treatment. When signaling is inhibited by the inhibitor, the signaling loop is disrupted which causes upregulation or increased activation of target molecules that mediate signaling redundancy, which is the compensatory signaling mechanism in cancer treatment (46).

Our *in vivo* results showed that tumor tissues from XN-treated mice exhibited reduced STAT3 activation as well as suppressed tumor proliferation and increased apoptosis
induction. These findings suggest that STAT3 is a promising target of XN and reveal, antitumor activity of XN against CCA growth and survival.

Furthermore, we explored the molecular mechanisms by which XN inhibits STAT3 activation in CCA. Results showed that XN provided anticancer activities via the suppression of Akt and NFκB, the molecules that are involved in the proliferation, survival and angiogenesis of tumor cells. Moreover, the interconnection between Akt-NFκB and STAT3 signaling has been described (26-29). Our results showed a decreased activation of Akt and NFκB after treatment with XN in both the IL-6-induced CCA cells and the CCA inoculated mice. Therefore, the possible mechanisms by which XN suppresses STAT3 activation in CCA could be due to Akt-NFκB signaling inhibition.

In conclusion, we have shown that XN can inhibit STAT3 activation in human CCA cell lines as well as CCA inoculated mice. Moreover, XN can effectively suppress the growth of tumor and induce apoptosis in CCA cells and tumor inoculated mice without any noticeable side-effects. This is the first time that STAT3 has been demonstrated as a potential target of XN. Moreover, our results have shown the potential efficacy of XN for CCA treatment. The above knowledge can provide the basis to develop new therapeutic strategies for CCA using XN alone and/or combined with conventional chemotherapy drugs to improve the efficacy of CCA treatment.

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