Abstract. Disturbances in cholesterol homeostasis of the bile duct epithelium, including transport interruption and the hyperaccumulation of intracellular cholesterol can lead to the initiation and progression of cholangiocarcinoma (CCA). Statins, which are lipid-lowering drugs, have been previously documented to exhibit anti-cancer properties. The role of statins in CCA cell cholesterol transport through the expression and function of ATP-binding cassette (ABC) A1 and ABCG1 was investigated in the current study. In four CCA cell lines, ABCA1 and ABCG1 expression was identified. However, neither ABCG5 nor ABCG8 expression was observed. Immunocytochemistry revealed that the expression of ABCA1 was localized in the proximity of the nucleus, while ABCG1 was more dispersed throughout the cytoplasm of KKU-100 cells. A cholesterol efflux assay was performed using bodipy cholesterol, and the translocation of cholesterol via ABCA1 and ABCG1 to Apo-A1 and high density lipoprotein was confirmed, respectively. Simvastatin and atorvastatin demonstrated the inhibitory effects on CCA cell viability. A reduction in intracellular lipid level and a lower expression of ABCA1 and ABCG1 were observed in KKU-100 cells under simvastatin treatment. The pre-exposure of KKU-100 cells to cholesterol diminished the statin effect. Furthermore, when KKU-100 cells were pre-loaded with cholesterol, ABCA1 and ABCG1-mediated exports were unaffected even though they were treated with simvastatin. The results of the current study indicated the limitations of the use of statin in CCA therapy, particularly under hypercholesterolemia conditions.

Introduction

Cholangiocarcinoma (CCA) is a primary adenocarcinoma and malignancy arising from the epithelium of the bile duct. CCA can be classified by its origin into intrahepatic, perihilar, and distal CCA. The pathogenesis of CCA is poorly understood and needs further investigation. Furthermore, the incidence and mortality rate of CCA has increased worldwide over past decades and now accounts for 3% of all gastrointestinal malignancies (1). It is also highly prevalent in Southeast Asia particularly in Thailand due to the endemic parasitic biliary tract infestation (2). Lack of effective biomarkers makes early diagnosis of CCA difficult (3). The occurrence of symptoms may not be apparent until the cancer reaches an advanced stage resulting in severe outcomes (4). Previous studies have reported the association of chronic liver diseases, hepatolithiasis, chronic biliary inflammation and cholestasis with the development of CCA (3). Under liver inflammation, accumulation of cholesterol was observed and caused extreme damage to the cells (5). This indicates that cholesterol plays a significant role in liver diseases.

Bile consisting of cholesterol, phospholipids, bilirubin conjugates, bile salts and toxic substances is secreted from hepatocytes and passes along bile duct through gallbladder or small intestine. Excess cholesterol is excreted via bile and eliminated into feces (6). Some of cholesterol and unconjugated bile acids can passively diffuse into cholangiocytes (7). Cholangiocytes have important roles in modifying and delivering the bile to its destination by secreting bicarbonate and water thus, preventing bile acid diffusion and maintaining osmolality of the cell (7). At the same time, cholangiocytes maintain their cholesterol homeostasis and form junction preventing the hepatic interstitial tissue from these secreted toxic substances and bile (6). When the cholestasis occurred by the bile duct obstruction, this led to overexposure of cholangiocytes to bile lipid contents and toxic substances (8). Oxysterols are found to be increased in bile acids of patients with biliary tract inflammation. They are cholesterol oxidation derivatives in human bile and activators of the hedgehog signaling pathway which associates in cell proliferation, migration, and invasion of CCA (9,10).

Cholesterol transport is a crucial cellular homeostatic mechanism. ATP-binding cassette (ABC) A1 and ABCG1 are well characterized as cholesterol transporters in various cell types. Downregulation of ABCA1 and ABCG1 transporters by simvastatin in cholangiocarcinoma cells

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types (11-14). ABCA1 transports cholesterol and phospholipids to apolipoprotein A-1 (ApoA-1) while ABCG1 transports cholesterol to mature high density lipoprotein (HDL). In this context, ABCA1 malfunction is associated with atherosclerosis. Cholesterol accumulation causes a decrease in ABCA1 level enhancing intracellular cholesterol excess in macrophages. This leads to inflammation and cell apoptosis which eventually resulting in atherosclerosis (15). Furthermore, ABCA1 function is disrupted by epigenetic alteration of promoter hypermethylation in prostate cancer. Decrease in ABCA1 export ability enhances accumulation of intracellular cholesterol. Extended cholesterol pools are consistent with prostate cancer progression and aggressiveness (16). This underlines the importance of ABCA1 and cholesterol in diseases and cancers.

Statins, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are well-known for their LDL cholesterol lowering effects. HMG-CoA reductase is involved in cholesterol synthesis reducing HMG-CoA to mevalonate which is eventually converted into cholesterol. Statins primarily inhibit this process thus lowering cholesterol synthesis to balance cholesterol homeostasis (17). Multiple effects of statins in anti-proliferation and promotion of apoptosis are found to be useful in reducing risk of CCA cancer (18,19). For instance, statins diminished insulin-like growth factor 1 (IGF-1) leading to the reduction of CCA cell cancer (18,19). This underlines the importance of ABCA1 and cholesterol in diseases and cancers.

In this study, the role of statins in the expression and function of major cholesterol transporters, ABCA1 and ABCG1 in CCA cells was investigated. Also, the involvement of Akt pathway in regulation of these transporters was examined. A specific condition of pre-exposure of CCA cells to cholesterol was also set to evaluate the effects of statins on ABCA1 and ABCG1-mediated efflux and its contribution to CCA survival and progression.

**Materials and methods**

**Cell cultures.** CCA cell lines including KKU-100 (JCRB no.1568), KKU-M213 (JCRB no. 1557) (25), HuCCA-1 (JCRB no. 1657) (26) were obtained from JCRB cell bank. RMCCA-1 cell line was cultured in our laboratory (Pubmed 17072981) (27) and HepG2 cells (ATCC no. 8065) were used to represent liver cancer cells. They were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and HAM’s F12 (Invitrogen; Thermo Fisher Scientific, Inc.) used to represent liver cancer cells. They were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and HAM’s F12 (Invitrogen; Thermo Fisher Scientific, Inc.) were dissolved in DMSO (Sigma-Aldrich; Merck KGaA) and dilutions were made for appropriate treatment conditions at 1% final concentration of DMSO. Cholesterol (Santa Cruz Biotechnology, Inc.) was dissolved in chloroform and a desired concentration of cholesterol was left after being evaporated using nitrogen gas. The cholesterol was then resuspended in methyl-β-cyclodextrin (MCD; Sigma-Aldrich; Merck KGaA) at a ratio of 1:4.

**Clonogenic survival assay.** KKU-100 cells were seeded at 700 cells per well in a 6-well plate and incubated for 24 h. Cells were subjected to cholesterol loading for 1 h and statin treatment for 48 h. They were allowed to grow into colonies for 14 days in a complete medium. Cells were then fixed with 4% paraformaldehyde, stained with crystal violet to examine their colony forming ability. Images of colonies were taken. Crystal formation was dissolved with glacial acetic acid before reading at 540 nm using an Infinite M200 PRO microplate reader (Tecan). The optimal statin and cholesterol concentrations were determined from cell viability curve. In subsequent assays, simvastatin at 25-50 µM and cholesterol at 10 µM were used, 1% DMSO in medium and/or MCD was added in a non-treated control cells.

**Oil Red O staining.** After statin and cholesterol treatment, KKU-100 cells were fixed with 4% paraformaldehyde, stained with 0.5% oil red o (Santa Cruz Biotechnology, Inc.) and images were taken using an inverted light microscope (Eclipse TS100; Nikon).

**RNA isolation and gene expression analysis by RT-PCR.** Total RNA isolation was performed using RNeasy plus mini kit (Qiagen) according to the manufacturer’s instructions. Total RNA concentration was determined by measuring a ratio of 260/280 nm absorbance using NanoVue (GE Healthcare Life Sciences). First strand complementary DNA (cDNA) was created from 1 µg total RNA template using RevertAidReverse Transcripase (Thermo Fisher Scientific, Inc.) according to manufacturer’s instructions. One-hundred ng of cDNA was amplified using specific primers and Taq DNA (Vivantis). cDNA was used as a template in PCR analysis to measure the relative expression of ABCA1, ABCG1, ABCG5, and ABCG8 mRNA. The nucleotide sequences of the primers were as follows: hABCA1 (NM_005502.3), hABCA1-F GACGCA AACACAAAAGTGGA, hABCA1-R AACAAGCCATGT TCCCTCAG; hABCG1 (NM_207628.1), hABCG1-F CAG GACCTTTTCATTCGG, hABCG1-R GGCACACCAACTC ACCACTAT; hABCG5 (NM_022436.2), hABCG5-F GGC AGATCATGTCATCTA, hABCG5-R ACATAACCTCC CCCAGAAGA; hABCG8 (NM_022437.2) hABCG8-F ATT TCACAGCCATCGCTAC, hABCG8-R CGAGTGACT GAGGCTTTTCC; hβ-actin (NM_001101.4), hβ-actin-F GCA CAGACCTGCGCCT, hβ-actin-R CTTTGACATGCACGG AG. PCR products were amplified (95°C, 1 min; followed by 40 cycles of 95°C, 45 sec, and 58°C, 45 sec; 72°C, 45 sec) and analyzed on a MC Nexus Gradient PCR cycler (Eppendorf AG). PCR products were loaded onto 2% agarose gel and stained with ethidium bromide. The sequences of the fragments amplified by PCR were confirmed by DNA sequencing. Semi-quantitative mRNA expression of ABCA1, ABCG1,
ABCG5, and ABCG8 was normalized to β-actin mRNA levels using ImageJ program (Version 1.48) to calculate band intensity.

RNA isolation and gene expression analysis by qPCR. Total RNA isolation was conducted and cDNA was created from 1 µg total RNA template as above. A total of 100 ng of cDNA was amplified using above specific primers and SYBR Green real-time PCR master mix (Toyobo) according to manufacturer's instructions to measure the relative expression of ABCA1 and ABCG1 mRNA. PCR products were amplified (95°C, 1 min; followed by 40 cycles of 95°C, 15 sec, and 60°C, 15 sec) and analyzed on a CFX96 Touch real-time PCR cycler (Bio-Rad Laboratories, Inc.). Fluorescence threshold cycles (CT) of each sample were compared and normalized with the CT values of housekeeping gene. The relative expression of ABCA1 and ABCG1 were compared between controls and treatment.

Protein extraction and western blot analysis. Cells were treated with or without simvastatin for 48 h and then lysed in a lysis buffer (10 mM Tris, 150 mM NaCl, 1mM EDTA, 0.5% Triton X-100, and 1 mM PMSF protease inhibitor). Thirty µg proteins were reduced by heating with loading buffer (225 mM Tris, 25% (v/v) glycerol, 0.75 mM bromophenol blue and 10 mM DTT with pH 6.8) at 95°C for 10 min and were separated by discontinuous SDS polyacrylamide gel-electrophoresis (Bio-Rad Laboratories, Inc.). Electrophoresis was performed using a Bio-Rad Mini Trans-Blot Cell at 80 volts (225 mM Tris, 25% (v/v) glycerol, 0.75 mM bromophenol blue and 10 mM DTT with pH 6.8) at 95°C for 10 min and were separated by discontinuous SDS polyacrylamide gel-electrophoresis (Bio-Rad Laboratories, Inc.). Electrophoresis was performed using a Bio-Rad Mini Trans-Blot Cell at 80 volts for 3 h and 8% gels were used for ABCA1, ABCG1, Akt1 and pAkt (ser473). Proteins were transferred to nitrocellulose membranes (HYBOND ECL; GE Healthcare Life Sciences) and pAkt (ser473). Proteins were transferred to nitrocellulose membranes (HYBOND ECL; GE Healthcare Life Sciences) using Bio-Rad Mini Trans-Blot Cell at 90 volts for 2 h after being blocked with 5% skimmed milk in PBS 0.1% Tween-20 for 1.5 h. Mouse monoclonal anti-actin (AC-15; Abcam), mouse monoclonal anti-ABCA1(AB.H10; EMD Millipore), rabbit monoclonal anti-ABCG1 (EP1366Y; Abcam), mouse monoclonal anti-Akt1 (B-1; Santa Cruz Biotechnology, Inc.) and rabbit monoclonal anti-pAkt (ser473)(D9E, Cell Signaling Technology, Inc.) antibodies were added at 4°C for 16 h. Membranes were incubated with HRP-conjugated goat anti-mouse (Invitrogen; Thermo Fisher Scientific, Inc.) and goat anti-rabbit antibody (Invitrogen; Thermo Fisher Scientific, Inc.) diluted in PBS at 25°C for 2 h. They were developed by SureBlue TMB membrane substrate (KPL). Band intensity of TMB color was analyzed by ImageJ program (version 1.48).

Cholesterol efflux assay. Cells were seeded at 2x10^4 cells per well in black 96-well plates and incubated for 24 h. Cells were treated with or without cholesterol for 1 h and replaced with serum-free medium for 24 h before being treated with or without simvastatin. Cells were then replaced with serum-free medium containing labeling media for 1 h. Labeling medium consisted of bodipy cholesterol (Avanti Polar Lipids), MCD, HEPES (Sigma-Aldrich; Merck KGaA), and egg phosphatidylcholine (Avanti Polar Lipids) and prepared as previously described (28). The final concentrations of bodipy cholesterol, egg phosphatidylcholine, and MCD in the labeling medium were 0.025 mM, 0.1 mM, and 10 mM, respectively. Cells were washed with HAM's F12-HEPES and incubated with serum-free medium for 18 h. ApoA-1 and HDL (both from Lee Biosolutions) were added for 6 h at 100 µg/ml and 70 µg/ml, respectively. Cells were dissolved with 1% cholic acid (Sigma-Aldrich; Merck KGaA) in 1N NaOH for 4 h with rocking while the supernatant was collected and centrifuged at 10,000 x g for 5 min. The cholesterol fluorescence intensity value was recorded using a microplate reader (excitation at 482 nm and emission at 515 nm). The % cholesterol efflux = cholesterol efflux intensity/(intracellular cholesterol + cholesterol efflux) x100%.

Immunofluorescence of ABCA1 and ABCG1 transporter. Cells were seeded at 10^4 cells per well in 96-well plates and incubated for 24 h. They were treated with or without cholesterol for 1 h and replaced with serum-free medium for 24 h before being treated with or without simvastatin. Cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% saponin (Sigma-Aldrich; Merck KGaA) in PBS for 15 min. They were then blocked with 2% PBS in PBS for 1 h at 25°C and incubated with mouse monoclonal anti-ABCA1 (Millipore) and rabbit polyclonal anti-ABCG1 (AAS2436C; Antibody Verify) antibodies at 1:50 dilution at 4°C for 16 h. Cells were washed with 0.1% saponin in PBS and incubated with Alexa flour 488-conjugated goat polyclonal anti-mouse (Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa flour 546-conjugated goat polyclonal anti-rabbit (Invitrogen; Thermo Fisher Scientific, Inc.) antibodies for 2 h. They were counterstained with Hoechst (33342; Molecular Probes) for 10 min. Fluorescence intensity was visualized and photographed using an IX83 inverted fluorescence microscope with CellSens software platform (Olympus Cooperation).

Statistical analysis. Quantitative data are presented as mean ± SEM. Statistical significance between groups was analyzed using standard t-tests or two-way ANOVA followed by the Bonferroni test. Significant P-values are indicated within the figure panels. Error bars indicate SEM.

Results

Statin inhibited cell viability and decreased intracellular cholesterol level in KKU-100 cells. The effects of simvastatin, atorvastatin and cholesterol on cell survival and growth of KKU-100 cells were determined by clonogenic assay. These statins caused a concentration-dependent decrease in the colony formation ability of KKU-100 cells. At low concentrations (0.01-10 µM), simvastatin and atorvastatin had minimal effects on colony formation of KKU-100 cells. However, they significantly inhibited KKU-100 cell growth at 100 µM, (P<0.001; Fig. 1A and B). Using the MTT assay, cytotoxic activities of simvastatin and atorvastatin were also observed. Both statins had different levels of cytotoxicity to four CCA cell lines (Fig. S1). Cholesterol increased KKU-100 cell colony formation at 1-10 µM (P<0.001) but decreased it at 100 µM (P<0.001) compared to controls (Fig. 1C).

In order to examine the effect of simvastatin on cell growth and survival in the presence of cholesterol, KKU-100 cells were loaded with 10 µM cholesterol prior to being incubated with simvastatin (25-50 µM) for 48 h. In the absence of cholesterol, KKU-100 cell viability decreased as the concentration
of simvastatin increased from 25-50 µM. In the presence of 10 µM cholesterol, the reduction of cell viability was observed but at lower degree than that without cholesterol (Fig. 1D).

Oil red o staining revealed that without cholesterol, the level of both intracellular lipids and neutral triglycerides declined as KNU-100 cells were exposed to higher concentrations of simvastatin (Fig. 2A and C). Pre-cholesterol loaded KNU-100 cells showed greater intensity of oil red o suggesting elevated levels of intracellular lipids (Fig. 2D). However, with higher concentration of simvastatin, oil red o staining intensity reduced but at lesser extent than those without cholesterol (Fig. 2D and F). In comparison with 0 µM, KNU-100 cell density at 25 and 50 µM simvastatin decreased by 44% (P<0.01) and by 69% (P<0.05), respectively (Fig. 2G). In the presence of 10 µM cholesterol, KNU-100 cell density reduced by 26% (P<0.05) and by 30% (P<0.05), respectively when compared to a control group. (Fig. 2G).

CCA cells expressed ABCA1 and ABCG1 transporters which mediated cholesterol efflux. CCA cell lines were examined for expression of ABCA1, ABCG1, ABCG5 and ABCG8 by RT-PCR. RMCCA1 and KNU-100 cells expressed relatively higher levels of ABCA1 and ABCG1 mRNA than those of HuCCA-1 and KNU-M213 cells (Fig. 3A and C). Neither ABCG5 nor ABCG8 mRNA was present in CCA cell lines. We were able to detect ABCG5 and ABCG8 only in HepG2 cells which were used as a liver cancer cell line (Fig. 3B and D).

Western blot analysis revealed that KNU-100 cells expressed the highest level of ABCA1. The expression level of ABCG1 was higher in HuCCA-1, RMCCA-1 and KNU-100 cells but slightly lower in KNU-M213 cells (Fig. 3E). We conducted cholesterol efflux assay using bodipy cholesterol in KNU-100 cells which expressed highest level of ABCA1 and ABCG1. Baseline condition was set in the absence of cholesterol acceptor by which the constitutive efflux was at 6.00±0.58% (Fig. 4). By adding Apo-A1, cholesterol translocation which was mediated specifically through ABCA1 increased to 11.33±1.86% (P<0.05; Fig. 4). HDL also triggered cholesterol efflux via ABCG1. Export through this mechanism was obvious at 46.33±5.33% (P<0.01) (Fig. 4).
Simvastatin decreased ABCA1 and ABCG1 expression. The effect of simvastatin on ABCA1 and ABCG1 expression was evaluated by real-time PCR and western blot analysis. Compared to control, at 25 and 50 µM of simvastatin treatment, the relative levels of ABCA1 mRNA expression decreased significantly by 72% (P<0.001) and by 91% (P<0.001), respectively (Fig. 5A). Compared to control, at 25 and 50 µM of simvastatin treatment, the relative levels of ABCG1 mRNA expression decreased significantly by 78% (P<0.001) and by 93% (P<0.001), respectively (Fig. 5B). Following exposure of cholesterol at 10 µM, KKU-100 cells showed significantly enhanced ABCA1 and ABCG1 expression at 50% (P<0.001) and 60% (P<0.001), respectively comparing to no cholesterol loaded cells (Fig. 5A and B). However, with 25 and 50 µM simvastatin, expression of ABCA1 and ABCG1 decreased in a concentration-dependent manner (Fig. 5A and B).

Simvastatin also caused a concentration-dependent decrease in the levels of ABCA1 and ABCG1 protein expression in KKU-100 cells. At 25 µM of simvastatin treatment, relative ABCA1 and ABCG1 proteins were reduced by 15% (P<0.05) and by 8%, respectively (Fig. 6D and E). Expression of ABCA1 and ABCG1 protein at 50 µM of simvastatin treatment were drastically decreased by 79% (P<0.001) and by 61% (P<0.01), respectively (Fig. 6D and E). Following exposure of cholesterol at 10 µM, KKU-100 cells showed enhanced ABCA1 and ABCG1 expression at 177% (P<0.001) and 100% (P<0.001), respectively comparing to no cholesterol loaded cells (Fig. 6D and E). However, with 25 and 50 µM simvastatin, expression of ABCA1 and ABCG1 decreased in a concentration-dependent manner (Fig. 6A).

In order to investigate the role of Akt in ABCA1 and ABCG1 expression in KKU-100 cells, Akt and pAkt (ser473

Figure 2. Simvastatin reduced intracellular cholesterol in KKU-100 cells. Light micrographs demonstrated KKU-100 cells that were treated with simvastatin at a variety of concentrations for 48 h. Cells were then fixed and stained with oil red o (dark brown/orange). KKU-100 cells were treated with (A) 0 µM, (B) 25 µM and (C) 50 µM of simvastatin. Cells were pre-loaded with 10 µM cholesterol before being exposed to (D) 0 µM, (E) 25 µM and (F) 50 µM of simvastatin. (G) Semi-quantitative of cell density was analyzed in each condition (mean ± SEM; n=3). Statistical significance was determined using a two-way ANOVA test with Kendall’s tau correlation. *P<0.05; **P<0.01 comparison between 25, 50 and 0 µM groups; ###P<0.001 vs. comparison between pre-cholesterol loaded and no cholesterol loaded cells. Scale bar=50 µm for A-F.
protein expression were evaluated (Fig. 6A). KKU-100 cells constitutively expressed Akt and pAkt (ser473) (Fig. 6B and C). Nonetheless, in the presence of simvastatin, KKU-100 cells retained the levels of those proteins (Fig. 6B and C). Exposures of cholesterol at 10 µM did not noticeably alter the level of Akt and pAkt (ser473) expression (Fig. 6B and C). Among pre-cholesterol loaded KKU-100 cells, levels of those proteins were comparable between simvastatin-treated and non-treated cells suggesting the Akt-independent manner of ABCA1 and ABCG1 expression in KKU-100 cells (Fig. 6B).

Intracellular localization of ABCA1 and ABCG1 in KKU-100 cells. We visualized the intracellular expression of ABCA1 and ABCG1 in KKU-100 cells by immunocytochemistry. The expression of ABCA1 (green) was observed within cytoplasm. Intense staining of ABCA1 was detected particularly in the proximity of the nucleus (Fig. 7A). Similar to ABCA1, ABCG1 expression was recorded in the cytoplasmic region of KKU-100 cells. However, the staining showed a more dispersed pattern
Figure 5. Simvastatin reduced mRNA expression of ABCA1 and ABCG1 in KKU-100 cells. Cells were treated with simvastatin at a variety of concentrations for 48 h. Determination of (A) ABCA1 and (B) ABCG1 mRNA expression was performed using qPCR analysis (mean ± SEM). Statistical significance was determined using a two-way ANOVA analysis with Kendall's tau correlation. **P<0.01; ***P<0.001 comparison between 25, 50 and 0 µM simvastatin and ###P<0.001 comparison between 10 µM pre-cholesterol and no cholesterol loaded cells (0 µM) at each concentration of simvastatin. ABC, ATP-binding cassette; q, quantitative.

Figure 6. Simvastatin reduced ABCA1 and ABCG1, but not Akt1 and pAkt (ser473) in KKU-100 cells. Cells were treated with simvastatin at a variety of concentrations for 48 h. Cells were then evaluated for protein expression of Akt, pAkt (ser473), ABCA1 and ABCG1 were determined using (A) western blot analysis. Band intensity was analyzed and relative expression of (B) pAkt (ser473), (C) Akt, (D) ABCA1 and (E) ABCG1 were calculated (mean ± SEM; n=3). Statistical significance was determined using a two-way ANOVA with Kendall's tau correlation. *P<0.05; **P<0.01; ***P<0.001 compared between 25, 50 and 0 µM simvastatin; ###P<0.001 compared between 10 µM pre-cholesterol and no cholesterol loaded cells (0 µM) at each concentration of simvastatin. ABC, ATP-binding cassette.
of ABCG1 expression (red). This transporter could be detected throughout the cytoplasm and in the submembrane region (Fig. 7B and C). Co-localization of ABCA1 and ABCG1 staining showed overlapping expression of these two transporters (Fig. 7C). Following simvastatin treatment, at 25 µM, KKU-100 cells exhibited lower levels of both ABCA1 and ABCG1 expression (Fig. 7D and F). Simvastatin seemed to act in a concentration-dependent manner as lower expression of these two transporters was observed at higher concentration (50 µM) of simvastatin (Fig. 7G and I).

Pre-exposure to cholesterol minimized simvastatin effect in CCA cells. Following exposure to cholesterol at 10 µM, KKU-100 cells showed enhanced ABCA1 and ABCG1 expression (Fig. 7J and L). With 25 µM simvastatin, KKU-100 cells retained expression of ABCA1 and ABCG1 in pre-cholesterol loaded cells compared with non-loaded cells (Fig. 7M and O). This expression was greatly decreased at greater simvastatin condition (50 µM). However with cholesterol, expressions of both ABCA1 and ABCG1 was more visible (Fig. 7P and R) than those without the pre-exposure to cholesterol (Fig. 7G and I).
Different effect of simvastatin on cholesterol transport in CCA cells. The effect of simvastatin on cholesterol efflux was further examined. Cholesterol export through ABCA1 in simvastatin-treated KKU-100 cells decreased by 60% (P<0.01) and 55% (P<0.05) at 25 and 50 µM simvastatin, respectively comparing to non-treated controls (Fig. 8A). Interestingly, pre-exposure of KKU-100 cells to cholesterol did not change the ABCA1-mediated function, although these cells showed up-regulated ABCA1 expression levels. In simvastatin treatment, efflux to Apo-A1 of pre-cholesterol loaded KKU-100 cells decreased but only at marginal levels (Fig. 8A). For ABCG1 function, simvastatin-treated cells showed similar levels of cholesterol export to HDL to non-treated cells (Fig. 8B). Under stimulation of cholesterol, simvastatin had no considerable effect on this cholesterol efflux compared to controls. ABCG1 knockdown experiment was performed in HuCCA-1 cells using RNA interference. siABCG1 transfection successfully decreased ABCG1 expression level (Fig. S2). However, comparable cholesterol efflux to HDL was found between siABCG1 cells and non-silenced (wild-type) cells (Fig. S3).

Discussion

In our study, cholesterol is found to play an important role in CCA cell viability. We demonstrated that cholesterol (1-10 µM) significantly increased KKU-100 cell viability. Growth promoting effect of cholesterol was also reported in another CCA study (18). Similar to our findings, cholesterol increased prostate cancer cell proliferation (21). In addition, in vivo experiments revealed that high fat diet-fed mice showed enhanced breast tumor size and metastasis compared with normal diet fed mice (29). By increasing intracellular cholesterol viability, this favored cancer cell proliferation because cholesterol is an important component for cell membrane synthesis (30). However, at a very high cholesterol concentration (100 µM), a significant decrease in CCA cell proliferation was found. Studies in gastric carcinoma and neuroblastoma cells also revealed this phenomenon. High cholesterol concentrations caused cytotoxicity which then decreased cell proliferation and induced cell apoptosis (31,32). High accumulation of cholesterol also caused foam cell formation in macrophages leading to their malfunction in preventing the atherosclerosis (33). Exposure to high cholesterol enhanced macrophage cell apoptosis via the mitochondrial Fas signaling pathway (33). These studies emphasize cholesterol as a growth driving substance and its homeostasis is also important in cell survival, growth, proliferation and apoptosis.

Statins have been well studied in terms of their cholesterol lowering ability. They have been widely used to treat hyperlipidemia in humans. Natural cholesterol sources are from plasma cholesterol which is obtained from cholesterol biosynthesis and from intestinal absorption. Statins reduced serum lipids by suppressing cholesterol biosynthesis which eventually caused decrease in cell viability (18,19,34,35). In our experiments, simvastatin reduced intracellular cholesterol level of KKU-100 cells which is similar to other CCA studies that simvastatin suppressed endogenous cholesterol synthesis (18) through the reduced HMG-CoA reductase activity (36). Nonetheless, these effects of simvastatin were prevented in the presence of cholesterol. Cholesterol-loading CCA cells negated the decreased intracellular cholesterol and decreased cell viability under simvastatin (25 µM) exposure. Recent findings elsewhere also showed that pre-cholesterol exposure of various cancer cells including CCA, esophageal adenocarcinoma, macrophages, and T-cell leukemia elevated unesterified cholesterol pools (18,37-41). It is therefore possible that unesterified cholesterol supply resist the effect of simvastatin (18,39,41,42).

In our paper, we confirm that in CCA, ABCA1 and ABCG1 transporters mediate cholesterol efflux to Apo-A1 and HDL, respectively. These transporters are well-known as predominant cholesterol transporters in macrophages and hepatocarcinoma assisting cholesterol export and maintaining cholesterol homeostasis (11-14). ABCA1 is localized in proximity to the nucleus and spread throughout the cytoplasm of KKU-100 cells. In fibroblasts, ABCA1-mediated cellular cholesterol transport also occurs in late endosome (43). Apo-A1 is trafficked via ABCA1 from cell membrane into late endosome in which lipoprotein Apo-A1 would subsequently become nascent HDL (43). ABCG1 is predominantly found perinuclear endosomes and plasma membrane. In ABCG1-transfected HepG2 cells, ABCG1 mediates cholesterol efflux by either vesicular or non-vesicular pathway (44). ABCG1-mediated intracellular cholesterol would be shuttled from late endocytic compartment to the cell membrane in order to deliver...
cholesterol to extracellular HDL. In this study, we could not detect either ABCG5 or ABCG8 expression in our four CCA cell lines used. These transporters are well studied, showing high expression in hepatocarcinoma cells assisting cholesterol export as a part of bile composition (45). Therefore, we speculated that for CCA cells, ABCA1 and ABCG1, but not ABCG5 and ABCG8 potentially play role in cholesterol transport. We demonstrated here in our work that CCA cells constitutively exported cholesterol via ABCG1 to HDL to a greater extent than to Apo-A1 which was mediated through ABCA1. This was similar to cholesterol export levels in macrophages (28,46). This suggests a smaller range of cholesterol translocation ability of ABCA1 compared to ABCG1.

Further examination was carried out to identify the effects of simvastatin on ABCA1 and ABCG1 levels in CCA cell lines. Our experiments are consistent with previous results that ABCA1 and ABCG1 level decreased in simvastatin treatment. The influence of simvastatin on cholesterol homeostasis genes has been shown in a variety of cancer cell lines (37,47). Statin treatment caused reduction in ABCA1 and ABCG1 expression in epithelial colorectal adenocarcinoma cells (47) and macrophages (37). This supported the theory that simvastatin decreased intracellular cholesterol synthesis, thereby limiting mevalonate and sources of oxysterol production. Oxysterols are important for the nuclear receptor, LXR, in maintaining cholesterol homeostasis (48). In macrophages, LXR controls ABCA1 and ABCG1 activation (49). Therefore, the condition which low mevalonate together with oxysterols triggers down-regulation of ABCA1 and ABCG1 expression caused by statins could occur in CCA. Further experiments on the quantification of mevalonate and oxysterols would clarify this point. In this paper, cholesterol efflux to Apo-A1 decreased under simvastatin treatment suggesting a possible role of ABCA1 in KKF-100 cells. Cholesterol efflux to Apo-A1 was reduced in simvastatin treated macrophages (37,39). Our results suggest that the inhibitory effect of simvastatin could be specific to efflux to Apo-A1 but not to HDL, and more apparent in non-cholesterol loaded cells. Cholesterol-loaded cells reversed the effect of simvastatin in cholesterol translocation to Apo-A1. Relevant pathways and ligands such as those in LXR signaling were possibly restored under cholesterol-loaded conditions reversing the effects of simvastatin at certain concentrations (37,39). In contrast, other papers revealed a simvastatin-facilitated increment in ABCA1 levels and function in hepatocarcinoma cells which displayed an atheroprotective effect of simvastatin via peroxisome proliferator-activated receptors (PPARs) (37).

The role of Akt has been reported with cholesterol availability and growth of certain cancer cells (18,22,37). Downregulation of Akt signaling together with decreased proliferation was detected in glioblastoma after statin treatment (22). Inhibition of pAkt by treating with the MK2206 molecule reduced CCA cell growth (50). We speculated that a decrease in CCA cell proliferation under simvastatin would involve the Akt pathway. The levels of Akt and pAkt (serine473) of KKF-100 cells were analyzed. Neither total Akt nor pAkt expression level was affected in these cells under simvastatin treatment. It had similarly shown in pancreatic cancer cells that atorvastatin did not inhibit total Akt levels (51). Also, in the long-term treatment of the non-small lung cancer A549 cell line with atorvastatin, the level of pAkt (serine473) did not alter. As previously indicated by Miraglia and colleagues that a purinergic receptor P2X7 was a specific target of statin and indeed only nuclear level of Akt was downregulated by statin (52), additional analysis of nuclear and cytoplasmic levels of those proteins in KKF-100 cells would clarify the actual Akt expression. The ABCA1 regulation has been reported with an Akt-dependent pathway in macrophages and hepatocytes (23,24). Nonetheless, our results presented otherwise that down-regulation of ABCA1 and ABCG1 was not through the Akt pathway. Disruption in lipid rafts and ABCA1 and ABCG1 transporters could be associated with LXR pathways (49). Further investigation to demonstrate the link between the LXR and ABCA1 and ABCG1 pathways has currently been carried out.

To summarize, we demonstrated that ABCA1 and ABCG1 potentially play roles in cholesterol translocation in CCA cells. Simvastatin decreased CCA cell viability, intracellular cholesterol and ABCA1 and ABCG1 expression by an Akt-independent pathway. However, pre-exposure of KKF-100 cells to cholesterol reduced the effect of statins on cell viability and intracellular cholesterol levels. Cholesterol export via ABCA1 and ABCG1 remained unaffected in cholesterol-loaded KKF-100 cells in the presence of simvastatin. This indicates the limitations of statin treatment in CCA patients suffering with hypercholesterolemia.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

PS, TJ, TK and SK designed the study. PS, SK and RT performed the experiments. PS, TJ, TK, and SK analyzed the data. PS and SK wrote the manuscript. All authors reviewed the manuscript and approved the final version.

Ethics approval and consent to participate

All experimental procedures were performed in compliance with institutional requirements and were approved by the Institutional Ethics Committee of Mahidol University.
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