รายงานฉบับสมบูรณ์

โครงการ “บทบาทของออกซีสเตอรอลและโปรตีนขนส่งออกซีสเตอรอลในกระบวนการเกิดมะเร็งท่อน้ำดีเพื่อใช้เป็นเป้าหมายในการป้องกันมะเร็ง”

โดย

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มหาวิทยาลัยขอนแก่น

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ออกซิสเตอรอล (oxysterol) คืออนุพันธ์ของคลอเรสเตอรอลที่เกิดจากกระบวนการ oxidation วิถีการสร้างออกซิสเตอรอล มี 2 วิธีคือการสร้างโดยเอนไซม์ cytochrome P450 (CYP) ซึ่งเป็นวิถีหลักในการสร้างกรดน้ำดี (bile acid) และการสร้างโดยการทำปฏิกิริยาของอนุมูลอิสระชนิด reactive oxygen/nitrogen species ในสภาวะปกติ ออกซิสเตอรอล มีบทบาทสำคัญที่เกี่ยวข้องกับกระบวนการควบคุมการสร้างและสลายคลอเรสเตอรอลในเนื้อเยื่อหลายชนิดในทางตรงกันข้าม ออกซิสเตอรอลยังมีบทบาทที่เกี่ยวข้องกับภาวะความผิดปกติหลายชนิดเช่น โรคหลอดเลือดหัวใจอุดตัน อัลไซเมอร์ และมะเร็งหลายชนิด บทบาทของออกซิสเตอรอล ในกระบวนการก่อมะเร็งตั้งแต่ขั้นเริ่มต้นจนถึงขั้นก้าวหน้าจึงเป็นที่น่าสนใจศึกษา โดยผู้วิจัยได้ตั้งสมมติฐานว่า ในสภาวะการติดเชื้อเรื้อรังของพยาธิใบไม้ตับ ในท่อทางเดินน้ำดีส่งผลให้เกิดการสร้างออกซิสเตอรอล ทั้งชนิดและปริมาณที่ผิดปกติ ซึ่งออกซิสเตอรอลที่เกิดขึ้นนี้อาจมีบทบาทในการกระตุ้นให้เกิดมะเร็งท่อน้ำดี (cholangiocarcinoma, CCA)

ผลการศึกษาชนิดและปริมาณของออกซิสเตอรอล ในชิ้นเนื้อมะเร็งของหนูแฮมสเตอร์ที่ถูกเหนี่ยวนำให้เป็น CCA ด้วยพยาธิ *Opisthorchis viverrini* และสาร *N*-nitrosodimethylamine พบปริมาณของ ออกซิสเตอรอล ชนิด 3-keto-cholest-4-ene (3K4) and cholestan-3β, 5α, 6β-triol (Triol) เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับชิ้นเนื้อตับจากหนูปกติ นอกจากนี้ยังได้ทำการศึกษากลไกของ Triol และ 3K4 ที่เกี่ยวกับการส่งเสริมให้เกิด CCA ในเซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดี (MMNK-1 cell line) ผลการศึกษาพบว่า Triol และ 3K4 กระตุ้นให้เกิดการตายแบบ apoptosis โดยอาศัยกลไกผ่านทางไมโทคอนเดรีย (mitochondria-dependent mechanism) นอกจากนี้Triol และ3K4ยังกระตุ้นให้เกิดการทำลายดีเอ็นเอ โดยพบว่ามีปริมาณของ etheno adducts ได้แก่ 1,N^6-etheno-2'-deoxyadenosine, 3,N^4-etheno-2'-deoxycytidine และ 8-oxo-7,8-dihydro-2'-deoxyguanosine สูงขึ้น ผู้วิจัยยังได้ทำการกระตุ้นเซลล์ MMNK-1 ให้ดื้อต่อการตายโดยกระตุ้นด้วย ออกซิสเตอรอล ชนิด Triol โดยวิธี long term culture จนได้ Triol-resistant cell ที่ดื้อต่อกระบวนการ apoptosis เมื่อกระตุ้นด้วย H_2O_2 การศึกษากลไกของ Triol-resistant cell ที่เกี่ยวข้องกับการดื้อต่อ apoptosis และมีผลต่อการเกิด CCA พบว่า ใน Triol-resistant cell มีการกระตุ้นกลไกที่เกี่ยวข้องกับการอยู่รอดของเซลล์ เช่น มีการกระตุ้นให้เกิดการ phosphorylation ของ p38 mitogen-activated protein kinase α (p38-α), extracellular signal-regulated kinase (ERK1/2), oncogenic kinase ชนิด Src, Lyn, Yes และ Fak และ oncogenic transcription factors, cAMP response element-binding (CREB), and c-Jun (S63) นอกจากนี้ยังพบการเพิ่มขึ้นของการแสดงออกของโปรตีนที่เกี่ยวข้องกับการต้าน apoptosis (anti-apoptosis) เช่น Bcl-2, Bcl-x, cIAP-2 และ survivin สรุปได้ว่าใน Triol-resistant cell มีการกระตุ้น receptor tyrosine kinase pathway ส่งผลให้เกิดการแสดงออกของโปรตีนที่เกี่ยวข้องกับการดื้อต่อ apoptosis อย่างชัดเจน

การศึกษาชนิดและปริมาณของออกซิสเตอรอลในเนื้อเยื่อมะเร็งท่อน้ำดีของผู้ป่วย พบออกซิสเตอรอลจำนวน 7 ชนิด คือ 7-keto-cholesta-3,5-diene (7KD), 7β-hydroxycholesterol (7β-OH), 5,6-epoxycholesterol (Epoxy), 7-ketocholesterol (7-keto), 24-hydroxycholesterol (24-OH), Triol และ 3K4 โดยปริมาณของ 7β-OH, 3K4 และ epoxy ในเนื้อเยื่อ CCA สูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับเนื้อเยื่อตับปกติ ปริมาณของ 7KD, Triol และ 24-OH ใน CCA ยังสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับมะเร็งตับ (hepatocellular carcinoma, HCC) และตับปกติ นอกจากนี้ยังพบปริมาณของ 7β-OH, Epoxy, Triol และ 7-keto ในน้ำดีจากถุงน้ำดีของผู้ป่วย CCA และ HCC สูงขึ้นเมื่อเปรียบเทียบกับผู้ป่วย benign biliary disease แต่ไม่พบความแตกต่างของปริมาณออกซิสเตอรอลรูปใดๆ ระหว่างผู้ป่วย CCA ที่มีและไม่มีภาวะท่อทางเดินน้ำดีอุดตัน เมื่อศึกษาเอนไซม์ CYP7A1 และ CYP39A1 ซึ่งมีบทบาทในการเมทาบอลิซึมของ 24-OH ในตับ พบการการลดลงของระดับ mRNA ของยีน CYP7A1 และ CYP39A1 ผลการทดลองนี้แสดงให้เห็นว่าการลดการแสดงออกของเอนไซม์ CYP7A1 และ CYP39A1 น่าจะเกี่ยวข้องกับปริมาณของ 24-OH ที่เพิ่มขึ้นในผู้ป่วย CCA ซึ่งยังคงต้องศึกษาสาเหตุของการเปลี่ยนแปลงการแสดงออกนี้ต่อไป

เพื่อให้ทราบถึงความสัมพันธ์ของการแสดงออกระหว่างออกซิสเตอรอล กับ oxysterol binding proteins (OSBPs) เราพบการแสดงออกที่ลดลงของ OSBP1 และ OSBPL8 mRNA ในชิ้นเนื้อมะเร็งของผู้ป่วย CCA และ HCC ในขณะที่มีการเพิ่มสูงขึ้นของ OSBP2 และ OSBPL7 ในชิ้นเนื้อมะเร็งของผู้ป่วย CCA เมื่อเปรียบเทียบกับเนื้อตับปกติ นอกจากนี้ผู้วิจัยยังพบความสัมพันธ์อย่างมีนัยสำคัญ (r=0.57, P<0.0001) ของระดับ 24-OH และ การแสดงออกของ OSBP2ในตับของผู้ป่วย CCA และระดับของ 7-keto และ 7KD กับ การแสดงออกของ OSBPL5 ทั้งนี้ยังต้องมีการศึกษากลไกการทำงานร่วมกันของ ออกซิสเตอรอล และ OSBP ต่อไป

ผู้วิจัยยังได้ทำการศึกษาชนิดและปริมาณของกรดน้ำดี (bile acids) ในน้ำดีจากถุงน้ำดี พบว่าในผู้ป่วย CCA มีปริมาณ total bile acid, total conjugated bile acid และ total primary bile acid เพิ่มสูงขึ้นเมื่อเปรียบเทียบกับผู้ป่วย benign biliary disease ปริมาณกรดน้ำดีชนิด cholic acid, chenodeoxycholic acid และ deoxycholic acid มีระดับเพิ่มสูงขึ้นในน้ำดีของผู้ป่วย CCA อาจกล่าวได้ว่า ภาวะท่อทางเดินน้ำดีอุดตันและความสามารถในการเมทาบอลิซึมของเซลล์อาจมีผลต่อปริมาณของกรดน้ำดี

ดังนั้นในการศึกษานี้ได้ชี้ให้เห็นถึงชนิดและปริมาณของออกซิสเตอรอล ที่เปลี่ยนแปลงไปที่อาจเกี่ยวข้องกับกระบวนการเกิดมะเร็งท่อน้ำดีทั้งในสัตว์ทดลองและในมนุษย์นอกจากนี้ยังได้แสดงถึงกลไกของออกซิสเตอรอล ที่เกี่ยวข้องกับการเกิดมะเร็งซึ่งจะนำไปสู่การใช้เป็นกลไกที่จะนำไปสู่การป้องกันหรือยับยั้งการเกิดมะเร็งท่อน้ำดีต่อไป

ABSTRACT

Oxysterols are oxidation products of cholesterol that are generated by enzymatic reactions mediated by cytochrome P450 family enzymes or by non-enzymatic reactions involving reactive oxygen and nitrogen species. Oxysterols play various regulatory roles in normal cellular processes such as cholesterol homeostasis. Pathological effects of oxysterols have also been described, including atherosclerosis, neurological disease, and cancer. The molecular mechanisms whereby oxysterols contribute to the initiation and progression of cancer are an area of active investigation. We hypothesized that biliary oxysterols resulting from liver fluke infection participate in cholangiocarcinogenesis. To prove the hypothesis, the study was performed in both in animal and human models.

Using gas chromatography/mass spectrometry, we identified oxysterols in liver tissue of cholangiocarcinoma (CCA)-induced hamsters by administration with *Opisthorchis viverrini* and *N*-nitrosodimethylamine. Certain oxysterols including 3-keto-cholest-4-ene (3K4) and cholestan-3β, 5α, 6β-triol (Triol) were found at significantly higher levels in the livers of CCA hamsters. We therefore investigated the effects of Triol and 3K4 on induction of cholangiocarcinogenesis using an *in vitro* MMNK-1 culture model. Cytotoxicity and apoptosis studies showed that Triol- and 3K4-treated cells caused apoptosis via a mitochondrial-dependent mechanism as shown by western blot analysis. Interestingly, Triol and 3K4 also induced formation of the DNA adducts 1,N^6-etheno-2'-deoxyadenosine, 3,N^4-etheno-2'-deoxycytidine and 8-oxo-7,8-dihydro-2'-deoxyguanosine in cholangiocytes. In order to determine whether oxysterol -induced cell death is involved in the development of cholangiocarcinoma, we established oxysterol -resistant MMNK-1 cells by long term culturing with Triol. Triol-resistant cells could resist apoptosis induced by H_2O_2, a known apoptosis inducer. To investigate the mechanisms whereby oxysterol -resistant cells may be involved in cholangiocarcinogenesis, we performed protein array analysis for phosphorylated kinases and apoptosis-related proteins. Kinase phosphorylation array showed increased induction of mitogen-activated protein kinases such as p38 mitogen-activated protein kinase α (p38-α) and extracellular signal-regulated kinase (ERK1/2) in Triol-resistant cells. Phosphorylation of oncogenic tyrosine kinases such as Src, Lyn, Yes, and Fak were also elevated. Moreover, the oncogenic transcription factors cAMP response element-binding (CREB) and c-Jun (S63) were activated in resistant cells. Additionally, we found the increased expression of anti-apoptosis proteins including Bcl-2, Bcl-x, cIAP-2 and survivin. Conversely, we found reduced expression of the death receptor Fas in Triol-resistant cells compared to controls. Thus, activation of down-stream effectors that play role in receptor tyrosine kinase signaling, results in regulation of gene expression of apoptosis-related proteins. These enhance cellular survival and evasion of apoptosis.

We profiled oxysterols in human bile and normal and malignant hepatic tissue using gas chromatography and mass spectrometry. Seven oxysterols, 7-keto-cholesta-3,5-diene (7KD), 7β hydroxycholesterol (7β-OH), 5,6-epoxycholesterol (Epoxy), 7-ketocholesterol (7-keto), 24-hydroxycholesterol (24-OH), Triol and 3K4 were identified in normal human liver and cancer tissue including CCA and HCC. Oxysterols accumulated in the cancer groups compared to normal liver. Similar oxysterol species were found in human gallbladder bile. High levels of oxysterols were found in the bile of CCA patients compared to the bile of patients with benign biliary disease. No significant differences were observed in oxysterol levels

between samples from CCA patients with low and high level of total serum bilirubin. Interestingly, we found high levels of 24-OH in CCA tumor tissue and bile compared to cadaveric donors and HCC. Here we demonstrated low mRNA expression of CYP7A1 and CYP39A1 enzymes in CCA resulting in accumulation of 24-OH in tumor tissue. Thus, CYP expression and its role in 24-OH metabolization in CCA tissue need further investigation.

We studied the correlation between certain types of oxyterols and their binding proteins (OSBPs). Relative OSBP1 and OSBPL8 gene expression in CCA tumor tissue and HCC was significantly decreased (P<0.05), whereas, relative OSBPL7 and OSBP2 gene expression in CCA tumor tissue and HCC was increased compared to normal liver from cadaveric donors (although this difference was not statistically significant). Correlation between OSBP expression and oxysterol levels in liver tissue was also found. A significant correlation between OSBP2 expression and 24-OH level was found (r=0.57, P<0.0001). Moreover, OSBPL5 expression was correlated with 7-keto, Epoxy and 7KD levels.

Major oxysterols were also investigated in human gallbladder bile. Elevated levels of total bile acids, conjugated bile acids and primary bile acids were found in samples from CCA patients, suggesting that bile duct obstruction may contribute to alterations in bile acid concentration. Levels of cholic acid, chenodeoxycholic acid and deoxycholic acid were increased in cancer groups. Correlation of total bile acids, cholic acid and chenodeoxycholic with cholesterol, bilirubin and ALP concentrations was found. These findings suggest a different pattern of bile acid concentration in patients with hepatobiliary cancer compared to patients with benign biliary disease. Alteration of bile acid concentration may be a reflection of the disease process.

In conclusion, our results suggest that certain oxysterols are involved in cholangiocarcinegenesis. Oxysterols may enhance carcinogenesis , thereby providing a rational strategy for the chemoprevention of CCA.

EXECUTIVE SUMMARY

ผลงานวิจัยนี้เป็นการแสดงให้เห็นถึงบทบาทของสารกลุ่มออกซิสเตอรอลในการก่อมะเร็งท่อน้ำดี (cholangiocarcinoma, CCA) ที่สัมพันธ์กับการติดเชื้อพยาธิใบไม้ตับ ซึ่งบทบาทของสารกลุ่มลิพิดต่อการเกิดมะเร็งยังมีผู้ศึกษาไม่มากนัก และในความเป็นจริงลิพิดมีบทบาทไม่น้อยไปกว่ากลุ่มโปรตีน/เอ็นไซม์ คาร์โบไฮเดรต หรือกรดนิวคลิอิค หากเรามีองค์ความรู้เพียงพอในเรื่องนี้ก็จะช่วยให้การวินิจฉัยหรือการรักษาโรค CCA ได้มีประสิทธิภาพมากขึ้น ผลการศึกษาที่น่าสนใจจากงานวิจัยเรื่องนี้คือ ในภาวะความเครียดอ๊อกซิเดชั่นอันเกิดจากการติดเชื้อพยาธิใบไม้ตับที่ทำให้มีการอักเสบเรื้อรัง และการเป็นมะเร็งท่อน้ำดี ทำให้เกิดการสร้างสารกลุ่มออกซิสเตอรอลที่มีบทบาททางพยาธิวิทยาได้ทั้งแบบไม่ใช้เอ็นไซม์โดยผ่านทางการทำปฏิกิริยาของอนุมูลอิสระชนิด reactive oxygen/nitrogen species ได้แก่ 3-keto-cholest-4-ene (3K4) and cholestan-3β, 5α, 6β-triol (Triol) ทั้ง 3K4 และ Triol ถูกจัดอยู่ในกลุ่ม carcinogenic oxysterols และแบบใช้เอนไซม์ cytochrome P450 (CYP) ได้แก่ 24-hydroxycholesterol (24-OH) ดังรายละเอียดพอสังเขปดังนี้

เราได้ตรวจพบปริมาณที่เพิ่มขึ้นของ 3K4 และ Triol ในกระบวนการก่อมะเร็งท่อน้ำดีของหนูแฮมสเตอร์และในเนื้อเยื่อ CCA ของผู้ป่วย จากนั้นได้พิสูจน์ว่าสารทั้งสองเป็น carcinogenic oxysterol โดยใช้เซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดี (MMNK-1 cell line) ผลการศึกษาพบว่า Triol และ 3K4 กระตุ้นให้เกิดการตายแบบ apoptosis โดยอาศัยกลไกผ่านทางไมโทคอนเดรีย นอกจากนี้Triol และ3K4ยังกระตุ้นให้เกิดการทำลายดีเอ็นเอ โดยพบว่ามีปริมาณของ etheno adducts และ 8-oxo-7,8-dihydro-2'-deoxyguanosine สูงขึ้น ผู้วิจัยยังได้ทำการกระตุ้นเซลล์ MMNK-1 ให้ดื้อต่อการตายด้วย Triol โดยวิธี long term culture จนได้ Triol-resistant cell ที่ดื้อต่อกระบวนการ apoptosis เมื่อกระตุ้นด้วย H_2O_2 พบว่า ใน Triol-resistant cell มีการกระตุ้นกลไกที่เกี่ยวข้องกับการอยู่รอดของเซลล์ และพบการเพิ่มขึ้นของการแสดงออกของโปรตีนที่เกี่ยวข้องกับการต้าน apoptosis สรุปได้ว่าทั้ง 3K4 และ Triol ทำให้เซลล์บางเซลล์พัฒนาตัวเองจนรอดจาก apoptosis แต่ในขณะเดียวกันก็เกิดการกลายพันธุ์ และเซลล์ชุดนี้หากมีการแบ่งเซลล์ก็จะเกิด clonal expansion เข้ากระบวนการก่อมะเร็งต่อไป ผลงานชุดนี้ได้ตีพิมพ์ทั้งหมด 3 เรื่อง ได้แก่

1. Jusakul A, Yongvanit P, Loilome W, Namwat N, Kuver R. Mechanisms of oxysterol-induced carcinogenesis. Lipids Health Dis. 2011 Mar 9;10:44.
2. Jusakul A, Loilome W, Namwat N, Haigh WG, Kuver R, Dechakhamphu S, Sukontawarin P, Pinlaor S, Lee S. P., Yongvanit P. Liver fluke-induced hepatic oxysterols stimulate DNA damage and apoptosis in cultured human cholangiocytes. Mutation Research. 2012; 731: 48-57.
3. Jusakul A, Loilome W, Namwat N, Techasen A, Kuver R, Ioannou GN, Savard C, Haigh WG, Yongvanit P. Anti-apoptotic phenotypes of cholestan-3β, 5α, 6β-triol-resistant human cholangiocytes: characteristics contributing to the genesis of cholangiocarcinoma. J Steroid Biochem Mol Biol. 2013 Nov;138:368-75.

การศึกษาชนิดและปริมาณของออกซิสเตอรอล ในเนื้อเยื่อมะเร็งท่อน้ำดีของผู้ป่วยพบออกซิสเตอรอลจำนวน 7 ชนิด คือ 7-keto-cholesta-3, 5-diene (7KD), 7β-hydroxycholesterol (7β-OH), 5, 6-epoxycholesterol (Epoxy), 7-ketocholesterol (7-keto), 24-hydroxycholesterol (24-OH), Triol และ 3K4 ปริมาณของ 7β-OH, 3K4 และ epoxy ในเนื้อเยื่อ CCA สูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับเนื้อเยื่อตับปกติ นอกจากนี้ ปริมาณของ 7KD, Triol และ ใน CCA สูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับตับปกติและมะเร็งตับ (hepatocarcinoma, HCC) การตรวจพบ carcinogenic oxysterols (Triol และ 3K4) ในผู้ป่วย CCA เป็นการยืนยันถึงบทบาทการก่อโรคของสารนี้ในมนุษย์ และเป็นครั้งแรกที่มีการค้นพบระดับสาร 24-OH สูงขึ้นในเนื้อเยื่อมะเร็ง เพราะก่อนหน้านี้มีรายงานเฉพาะในโรคเกี่ยวกับสมอง เช่น อัลไซเมอร์ โดยซึ่งมีบทบาทในกระบวนการอักเสบและภาวะเครียดอ๊อกซิเดชั่น

(Jusakul A, Haigh WG, Khuntikeo N, Loilome W, Namwat N, Pairojkul P, Khenjunta C, Savard C, Ioannou G, Kuver R, Yongvanit P. Differential profile of hepatobiliary oxysterols in subjects with and without cholangiocarcinoma. Will be submitted to *Steroids*.)

เมื่อศึกษาในเชิงลึกถึงเอนไซม์ที่เมแทบอไลส์ 24-OH ก็พบการลดลงของระดับ mRNA และโปรตีนของยีน CYP7A1 และ CYP39A1 ซึ่งเอนไซม์ทั้งสอง มีบทบาทในการสลาย 24-OH ในตับ ยังพบการลดลงของ ซึ่งเป็น transcription factor ของ CYP39A1 การลดลงของระดับ RUNX2 และ CYP39A1 ยังสัมพันธ์กับการแพร่ลุกลามของ CCA และระดับของ 24-OH ที่เพิ่มขึ้นยังทำให้เพิ่มภาวะเครียดอ๊อกซิเดชั่นอีกด้วย (Khenjanta C, Thanan R, Jusakul A, Techasen A, Jamnongkan W, Namwat N, Loilome W, Pairojkul C, Yongvanit P. Association of CYP39A1, RUNX2 and oxidized alpha-1 antitrypsin expression in relation to cholangiocarcinoma progression. Asian Pac J Cancer Prev. 2014;15(23):10187-92.) และเพื่อให้ทราบถึงความสัมพันธ์ของการแสดงออกระหว่างออกซิสเตอรอล กับ oxysterol binding proteins (OSBPs) เราพบการแสดงออกที่เพิ่มขึ้นของ OSBP2 และ OSBPL7 mRNA ในชิ้นเนื้อมะเร็งของผู้ป่วย CCA เมื่อเปรียบเทียบกับเนื้อตับปกติ นอกจากนี้ผู้วิจัยยังพบความสัมพันธ์อย่างมีนัยสำคัญ ($r=0.57$, $P<0.0001$) ของระดับ 24-OH และ การแสดงออกของOSBP2ในตับของผู้ป่วย CCA และระดับของ 7-keto และ 7KD กับการแสดงออกของ OSBPL5 ทั้งนี้ยังต้องมีการศึกษากลไกการทำงานร่วมกันของออกซิสเตอรอล และ OSBP ต่อไป (Loilome W, Wechagama P, Namwat N, Jusakul A, Sripa B, Miwa M, Kuver R, Yongvanit P. Expression of oxysterol binding protein isoforms in opisthorchiasis-associated cholangiocarcinoma: a potential molecular marker for tumor metastasis. Parasitol Int. 2012 Mar;61(1):136-9.)

ผู้วิจัยยังได้ทำการศึกษาชนิดและปริมาณของกรดน้ำดีในน้ำดีจากถุงน้ำดี พบว่าในผู้ป่วย CCA มีปริมาณ total bile acid, total conjugated bile acid และ total primary bile acid เพิ่มสูงขึ้นเมื่อเปรียบเทียบกับผู้ป่วย benign biliary disease ปริมาณกรดน้ำดีชนิด cholic acid, chenodeoxycholic acid และ deoxycholic acid มีระดับเพิ่มสูงขึ้นในน้ำดีของผู้ป่วย CCA อาจกล่าวได้ว่า ภาวะท่อทางเดินน้ำดีอุดตันและความสามารถในการเมทาบอลิซึมของเซลล์อาจมีผลต่อปริมาณของ bile acids (Jusakul A, Khuntikeo N, Haigh WG, Kuver R, Ioannou GN, Loilome W, Namwat N, Bhudhisawasdi V, Pugkhem A, Pairojkul C, Yongvanit P. Identification of biliary bile acids in patients with benign biliary diseases, hepatocellular carcinoma and cholangiocarcinoma. Asian Pac J Cancer Prev. 2012;13 Suppl: 77-82.)

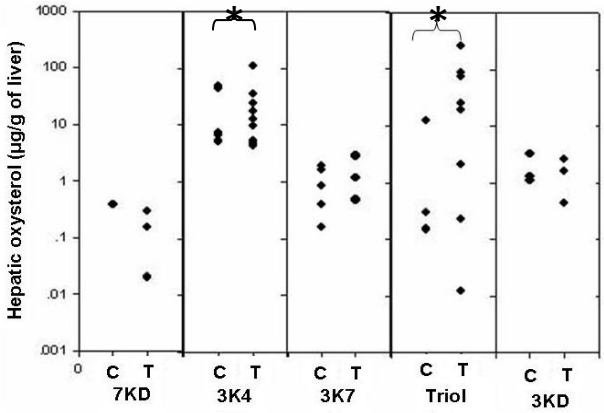
ผลการศึกษาในครั้งนี้ได้เป็นการเปิดประเด็นด้านบทบาทของลิพิดและน้ำดีของกระบวนการก่อมะเร็งท่อน้ำดีที่มีสาเหตุมาจากการติดเชื้อพยาธิใบไม้ตับที่ไม่เคยมีใครศึกษามาก่อน จึงเป็นการสร้างองค์ความรู้ที่มีนัยสำคัญเป็นอย่างมาก เนื่องจากไม่เคยมีใครเคยตอบคำถามได้ว่าน้ำดีมีบทบาทใดในโรคมะเร็งชนิดนี้ ทั้งๆที่พยาธิใบไม้ตับต้องอาศัยอยู่ในน้ำดี และมะเร็งชนิดนี้เกิดที่เซลล์บุผิวของท่อน้ำดี นอกจากองค์ประกอบของน้ำดีของผู้ป่วยที่แตกต่างคนปกติแล้ว ทั้งนี้เพราะลิพิดเป็นสารชีวโมเลกุลที่วิเคราะห์ได้ยาก ไม่เหมือนเทคนิคทาง Molecular Biology ที่ทำกันได้โดยทั่วไป ผลการศึกษาที่มีนัยสำคัญคือเราสามารถพิสูจน์ได้ว่าองค์ประกอบในน้ำดีของผู้ป่วยมะเร็งท่อน้ำดีนั้นมีทั้ง carcinogenic oxysterols (2K4 & triol) ที่ได้พิสูจน์ต่อไปได้ว่ามีบทบาทในกระบวนการก่อมะเร็งได้ รวมถึงได้ค้นพบ pathological oxysterol (24OH-chol) ที่ยังไม่เคยมีการรายงานในโรคมะเร็งชนิดอื่นมาก่อน องค์ความรู้พื้นฐานที่สำคัญนี้กำลังได้รับการศึกษาต่อยอดเพื่อใช้ประโยชน์ทั้งการใช้เป็นตัวบ่งชี้ชีวภาพและการใช้สารยับยั้งเพื่อป้องกันการเกิดและความรุนแรงของโรค โครงการวิจัยนี้มีผลงานตีพิมพ์ในวารสารระดับนานาชาติรวม 7 เรื่อง (6 เรื่องตีพิมพ์แล้ว และอีก 1 เรื่องกำลังจะส่งตีพิมพ์) มีการนำเสนอผลงานในที่ประชุมวิชาการแบบปากเปล่า 7 เรื่อง และแบบโปสเตอร์ 5 เรื่อง

เนื้อหางานวิจัย

I <u>การศึกษาชนิดและบทบาทของออกซิสเตอรอล ในกระบวนการก่อมะเร็งท่อน้ำดี</u>

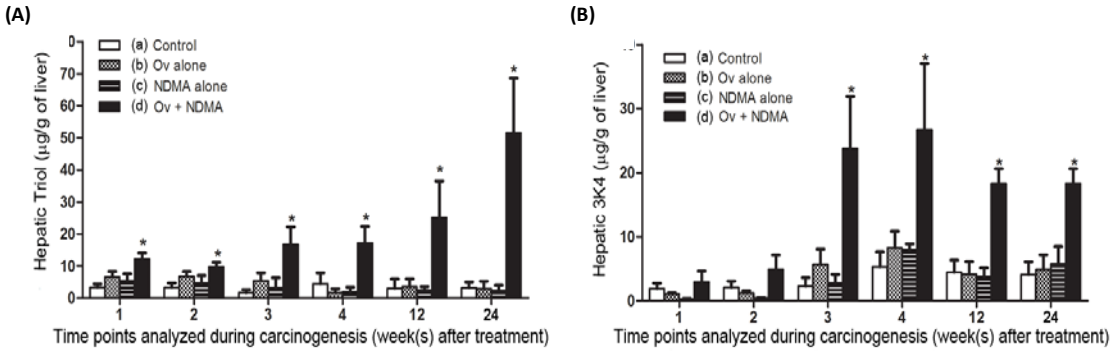
1. การศึกษาชนิดของออกซิสเตอรอล ในชิ้นเนื้อมะเร็งท่อน้ำดีของหนูแฮมสเตอร์ด้วยวิธี GC/MS

จากการทดลองตรวจหาออกซิสเตอรอล จากชิ้นเนื้อมะเร็งในหนูแฮมสเตอร์ที่ถูกกระตุ้นให้เป็นมะเร็งท่อน้ำดีด้วยพยาธิ *Opisthorchis viverrini* (Ov) และสาร *N*-nitrosodimethylamine (NDMA) ด้วยวิธี GC/MS พบ ออกซิส-เตอรอล 5 ชนิดดังนี้ 7-keto-cholesta-3,5-diene (7KD), 3-keto-cholest-4-ene (3K4), 3-keto-cholest-7-ene (3K7), cholestane-3,5,6-triol (Triol) และ 3-keto-cholesta-4,6-diene (3KD)ดังแสดงในรูปที่ 1



รูปที่ 1: ระดับของออกซิสเตอรอล ชนิดต่างๆในชิ้นเนื้อมะเร็งของหนูแฮมสเตอร์ที่ถูกกระตุ้นให้เป็นมะเร็งท่อน้ำดีโดยการติดเชื้อพยาธิใบไม้ตับ (*Opisthorchis vivierrini*) ร่วมกับการได้รับสารก่อมะเร็ง *N*-nitrosodimethylamine (NDMA) (C= control, T= tumor tissue)

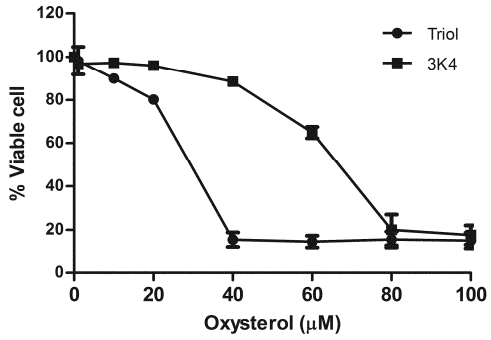
เมื่อติดตามดูระดับของออกซิสเตอรอล ตามระยะเวลาของการก่อมะเร็ง พบว่ามีออกซิสเตอรอล 2 ชนิด คือ cholestane-3,5,6-triol (Triol) และ 3-keto-cholest-4-ene (3K4) ที่มีปริมาณสูงอย่างมีนัยสำคัญทางสถิติตั้งแต่สัปดาห์ที่ 1 และ 3 ตามลำดับ เมื่อเปรียบเทียบกับระดับออกซิสเตอรอล ในชิ้นเนื้อตับจากหนูปกติ (รูปที่ 2) ซึ่งระดับของออก-ซิสเตอรอล ทั้งสองชนิดยังคงสูงมากในสัปดาห์ที่ 24 ซึ่งเป็นช่วงที่เกิดมะเร็งแล้ว ดังนั้นผู้วิจัยจึงได้ตั้งสมมติฐานว่าออก-ซิสเตอรอล ชนิด 3K4 และ Triol น่าจะมีบทบาทในการกระตุ้นในเกิดมะเร็งท่อน้ำดี



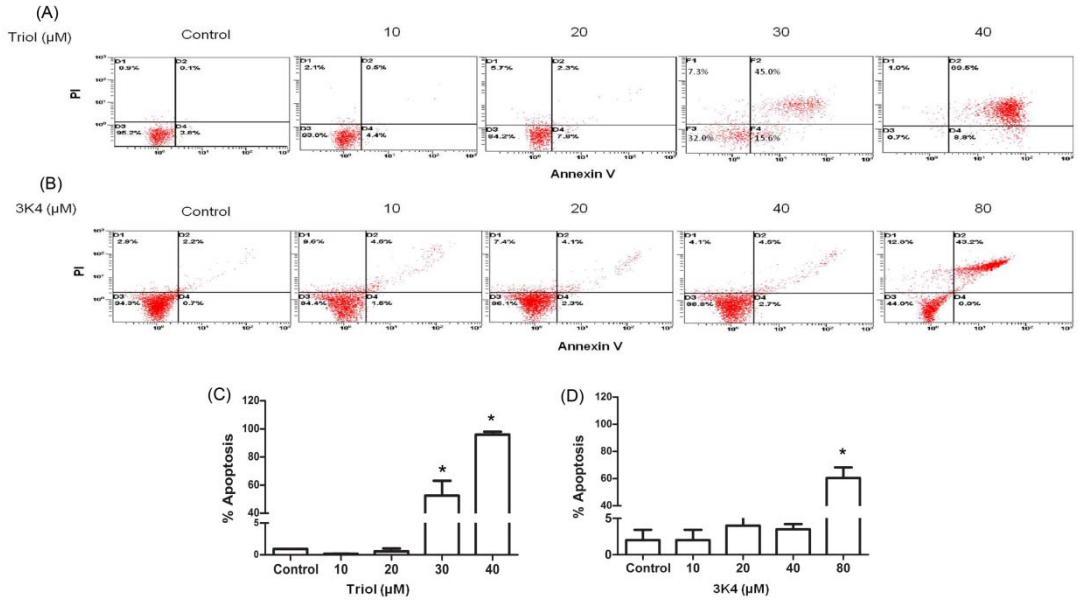
รูปที่ 2 ระดับของออกซิสเตอรอล ชนิด Triol (A) และ 3K4 (B) ในชิ้นเนื้อตับของหนูแฮมสเตอร์ปกติ (a), หนูที่ถูกกระตุ้นด้วย *Opisthorchis vivierrini* (b), *N*-nitrosodimethylamine (c) และ *Opisthorchis vivierrini*+ *N*-nitrosodimethylamine (d) ตามลำดับ (* หมายถึงระดับของ ออกซิสเตอรอล แตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

2. การศึกษากลไกการกระตุ้นกระบวนการ apoptosis ในเซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดีปกติ (MMNK-1) ของออกซิสเตอรอล

จากผลการทดลองในข้อ 1 ผู้วิจัยจึงได้ทำการศึกษาบทบาทของ 3K4 และ Triol ที่มีผลต่อการเจริญของเซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดีปกติ (MMNK-1) จากผลการศึกษาโดยวิธี Sulphorhodamine B assay (รูปที่ 3) และ การย้อมเซลล์ด้วย Annexin V และ Propidium iodide (รูปที่ 3) พบว่า 3K4 และ Triol สามารถกระตุ้นให้เกิดการตายของเซลล์ MMNK-1 โดยวิธี apoptosis ดังนั้นจึงเป็นที่มาของการศึกษากลไกของออกซิสเตอรอล ที่ใช้การการกระตุ้นกระบวนการ apoptosis ในเซลล์ MMNK-1



รูปที่ 3 กราฟแสดงปริมาณ viable cell ที่เกิดจากการกระตุ้นด้วย Triol และ 3K4 ในเซลล์ MMNK-1 เป็นเวลา 48 ชั่วโมง โดยวิธี Sulphorhodamine B assay ผลการศึกษาแสดงปริมาณของเซลล์เป็นลดลงหลังการกระตุ้นด้วย Triol และ 3K4 โดยมี IC50 = 27.5 ± 3.0 และ 63.3 ± 5.8 μM ตามลำดับ

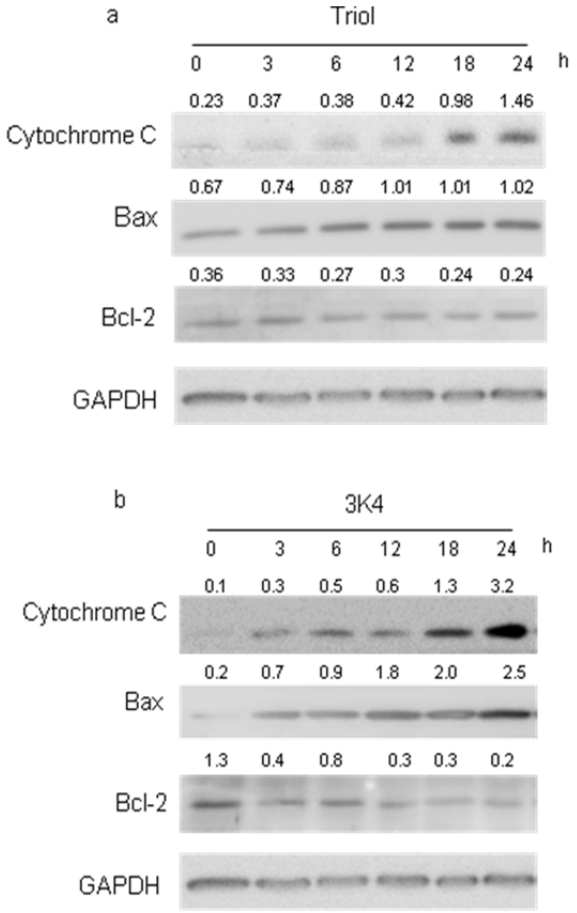


รูปที่ 4 แสดงปริมาณ apoptosis cell ที่เพิ่มขึ้นหลังจากการกระตุ้นเซลล์ MMNK-1 ด้วย Triol (A) และ 3K4 (B) เป็นเวลา 48 ชั่วโมง ศึกษาโดยการย้อมด้วย Annexin V และ Propidium iodide และวัดผลโดยใช้ Flow cytometer, ภาพ (C) และ (D) เป็นกราฟแสดงจำนวน apoptosis cell (%) ที่เพิ่มขึ้น หลังจากการกระตุ้นเซลล์ MMNK-1 ด้วย Triol และ 3K4 ตามลำดับ

นอกจากนี้ผู้วิจัยจึงได้ทำการศึกษาการแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการ apoptosis ที่เกิดจากการกระตุ้นเซลล์เพาะเลี้ยงชนิด MMNK-1 ด้วย Triol และ 3K4 โดยวิธี Western blot จากผลการทดลองดังที่แสดงในรูปที่ 5 พบว่า

1. เซลล์ที่ถูกกระตุ้นด้วย Triol หรือ 3K4 มีระดับของโปรตีน cytochrome C และ Bax ที่เพิ่มสูงขึ้นตามระยะเวลาของการกระตุ้นเมื่อเปรียบเทียบกับเซลล์ในกลุ่มควบคุม

2. เซลล์ที่ถูกกระตุ้นด้วย Triol หรือ 3K4 มีระดับของของโปรตีน Bcl-2 ที่ลดลงตามระยะเวลาของการกระตุ้นเมื่อเปรียบเทียบกับเซลล์ในกลุ่มควบคุม



รูปที่ 5 ผลของการกระตุ้นเซลล์ MMNK-1 ด้วย Triol (a) และ 3K4 (b) ที่มีต่อการแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการ apoptosis (Cytochrome C, Bax and Bcl-2) ศึกษาโดยวิธี Western blot

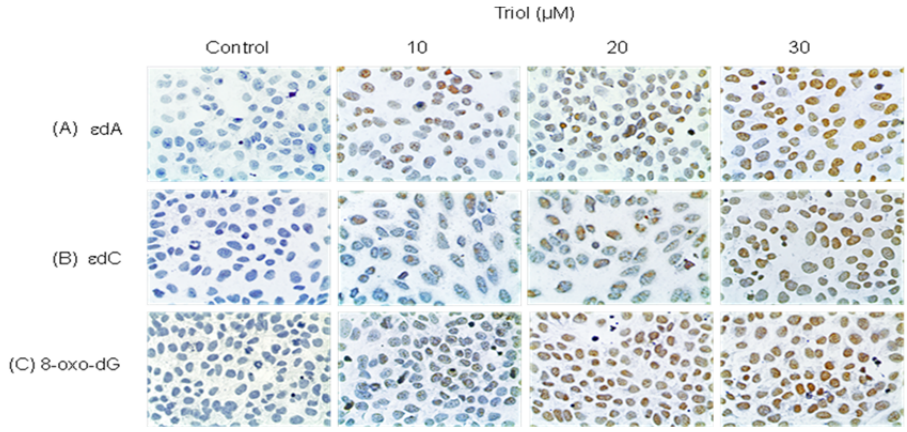
จากผลการทดลองข้างต้น สรุปได้ว่า Triol และ 3K4 มีบทบาทในการกระตุ้นให้เกินการตายแบบ apoptosis โดยอาศัยกลไกที่เกี่ยวข้องกับไมโทคอนเดรีย เนื่องจาก Triol และ 3K4 สามารถกระตุ้นให้เกิดการหลั่ง cytochrome C ออกจากไมโทคอนเดรีย และมีการแสดงออกของโปรตีน Bcl-2 ซึ่งเป็น anti-apoptotic protein รวมถึงระดับของโปรตีน Bax ซึ่งเป็น pro-apoptotic protein ที่สูงขึ้น

3. การศึกษาบทบาทของออกซิสเตอรอลที่มีต่อการเกิด DNA damage

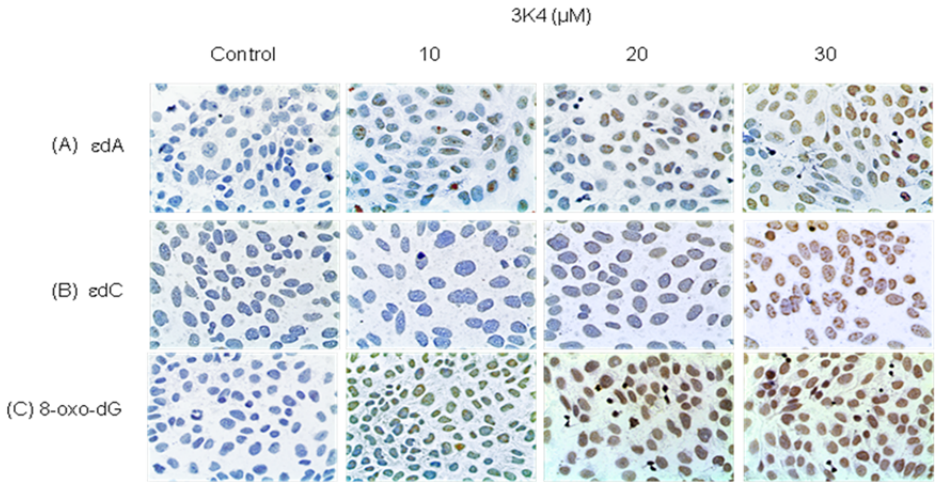
เพื่อทดสอบว่าออกซิสเตอรอล มีบทบาทในการกระตุ้นให้เกิดมะเร็งท่อน้ำดีหรือไม่ ผู้วิจัยจึงได้ทำการศึกษาความสามารถของออกซิสเตอรอล ที่เกี่ยวข้องกับการทำลาย DNA ซึ่งจะนำไปสู่กระบวนการ Mutation และทำให้เซลล์กลายเป็นเซลล์มะเร็งในที่สุด

จากการศึกษาด้วยวิธี Immunocytochemistry ผู้วิจัยได้ทำการศึกษาบทบาทของ Triol และ 3K4 ที่เกี่ยวข้องกับการทำลาย DNA ในเซลล์ MMNK-1 โดยศึกษาการกระตุ้นให้เกิดการสร้างเบสที่ผิดปกติ บน DNA (DNA adduct) ผู้วิจัยได้ทำการศึกษา DNA adduct 3 ชนิด ดังนี้ 1,N^6-etheno-2'-deoxyadenosine (ƐdA), EM-C-1 against 3,N^4-etheno-2'-deoxycytidine (ƐdC) และ 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)

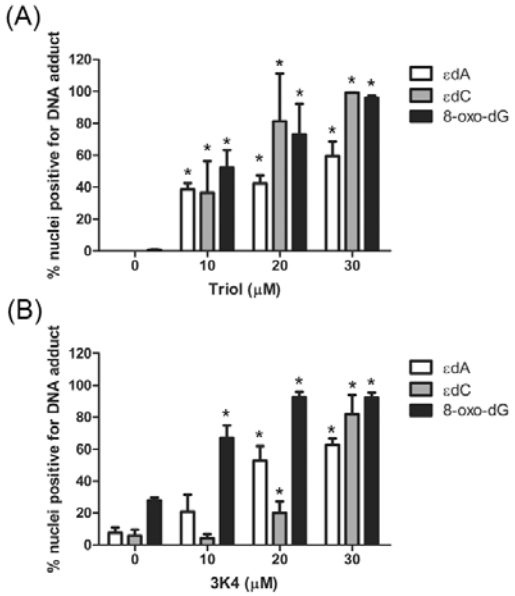
จากผลการทดลองพบว่าเมื่อกระตุ้นเซลล์ MMNK-1 ด้วยออกซิสเตอรอลทั้งสองชนิด พบว่ามีระดับการสร้าง DNA adduct ทั้งสามชนิดที่เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับเซลล์ที่ไม่ได้ถูกกระตุ้น (รูปที่ 6,7,8)



รูปที่ 6 ผลการย้อม DNA adducts ด้วยวิธี Immunocytochemistry ในเซลล์ MMNK-1 ที่ถูกกระตุ้นด้วย Triol ความเข้มข้น 10-30 uM เป็นเวลา 48 ชั่วโมง



รูปที่ 7 ผลการย้อม DNA adducts ด้วยวิธี Immunocytochemistry ในเซลล์ MMNK-1 ที่ถูกกระตุ้นด้วย 3K4 ความเข้มข้น 10-30 uM เป็นเวลา 48 ชั่วโมง



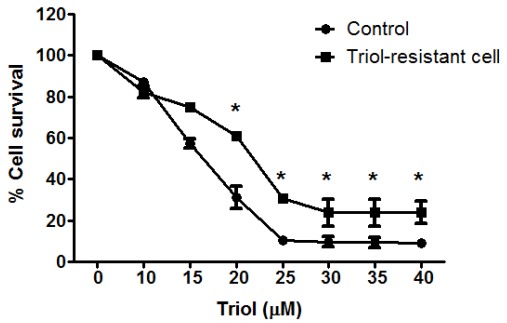
รูปที่ 8 กราฟแสดงผลการย้อม DNA adducts ด้วยวิธี Immunocytochemistry ในเซลล์ MMNK-1 ที่ถูกกระตุ้นด้วย Triol (A) และ 3K4 (B) เป็นเวลา 48 ชั่วโมง (* หมายถึงระดับของ DNA adducts แตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ)

จากผลการทดลองทั้งหมดจึงสรุปได้ว่า Triol และ 3K4 มีบทบาทให้การกระตุ้นให้เกิดการสร้าง DNA ที่ผิดปกติในเซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดี ซึ่งกระบวนการนี้จะนำไปสู่การเกิดการกลายพันธุ์และกลายเป็นมะเร็งในที่สุด

4. การศึกษากระบวนการที่เซลล์ดื้อต่อการกระตุ้นให้เกิดการตายด้วย ออกซิสเตอรอล

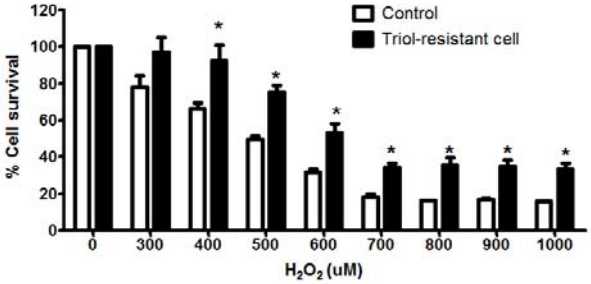
ถึงแม้ว่าจากผลการศึกษาที่ผ่านมาของผู้วิจัยพบว่าออกซิสเตอรอล ชนิด Triol และ 3K4 จะมีบทบาทในการกระตุ้นให้เซลล์มะเร็งท่อน้ำดีเกิดการตายแบบ apoptosis แต่ในระบบร่างกายเซลล์มะเร็งท่อน้ำดีจะถูกกระตุ้นด้วยสารต่างๆรวมถึงออกซิสเตอรอล ที่เป็นหนึ่งในส่วนประกอบของน้ำดีอยู่เป็นระยะเวลานาน ผู้วิจัยจึงมีสมมติฐานว่า ในสภาวะที่มีการอักเสบของเซลล์เยื่อบุมะเร็งท่อน้ำดีอันเนื่องมาจากการติดพยาธิใบไม้ในตับ ทำให้เกิดการสร้าง ออกซิส-เตอรอล ที่ผิดปกติ การอักเสบที่มีระยะเวลานานจะทำให้เซลล์เยื่อบุท่อน้ำดีต้องสัมผัสกับสารที่มีฤทธิ์เป็น carcinogen รวมถึงออกซิสเตอรอลอยู่ตลอดเวลา ดังนั้นผู้วิจัยจึงได้ทำการทดลองกระตุ้นเซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดี (MMNK-1) ด้วยออกซิสเตอรอลแบบต่อเนื่องเพื่อให้ได้เซลล์ที่ดื้อต่อการตายหลังจากการกระตุ้น และศึกษากลไกที่อาจเกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดี

จากการกระตุ้น เซลล์เพาะเลี้ยง MMNK-1 ด้วย Triol ความเข้มข้นต่ำๆ เป็นระยะเวลานาน ทำให้ได้เซลล์ที่ดื้อการกระตุ้น (Triol-resistant cell) โดย Triol-resistant cell มี IC50 เพิ่มขึ้นจาก control (22.7±1.1 uM และ 16.7±0.5 uM ตามลำดับ) (รูปที่ 9)



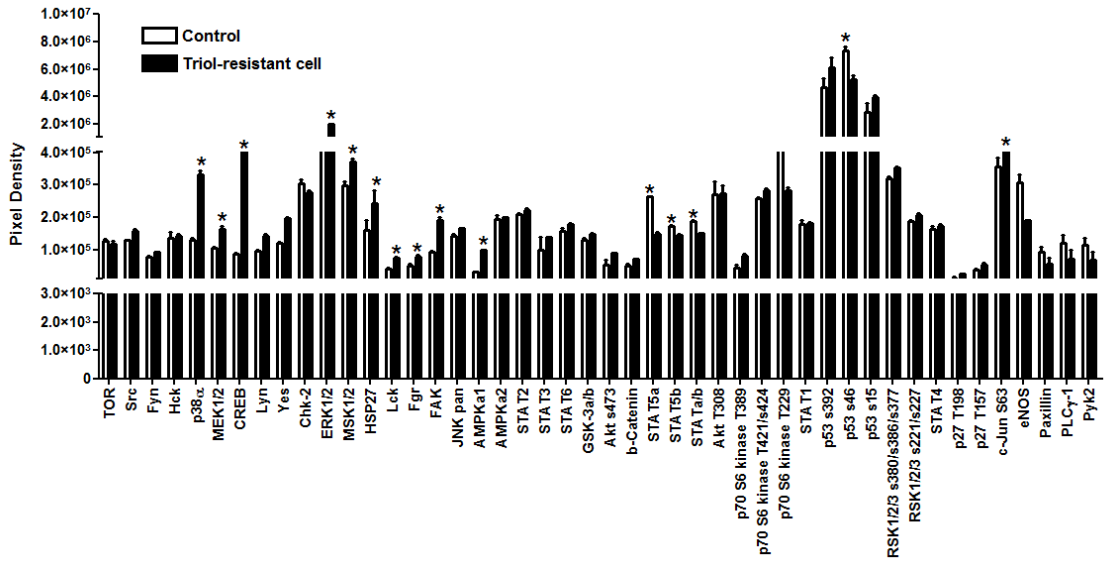
รูปที่ 9 กราฟแสดงผลการกระตุ้นเซลล์เพาะเลี้ยง MMNK-1 ด้วย Triol ความเข้มข้นตั้งแต่ 0-40 uM เป็นเวลา 48 ชั่วโมง โดย % cell survival หลังการกระตุ้นของ Triol-resistant cell เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติตั้งแต่กระตุ้นด้วย 20-40 uM Triol เมื่อเปรียบเทียบกับเซลล์ control (* หมายถึง ระดับของ % cell survival แตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

นอกจากนี้ยังพบว่า Triol-resistant cell ยังดื้อต่อการกระตุ้นให้เกิดการตายแบบ apoptosis โดยกระตุ้นด้วย H_2O_2 เมื่อเปรียบเทียบกับเซลล์กลุ่ม control (รูปที่ 10)

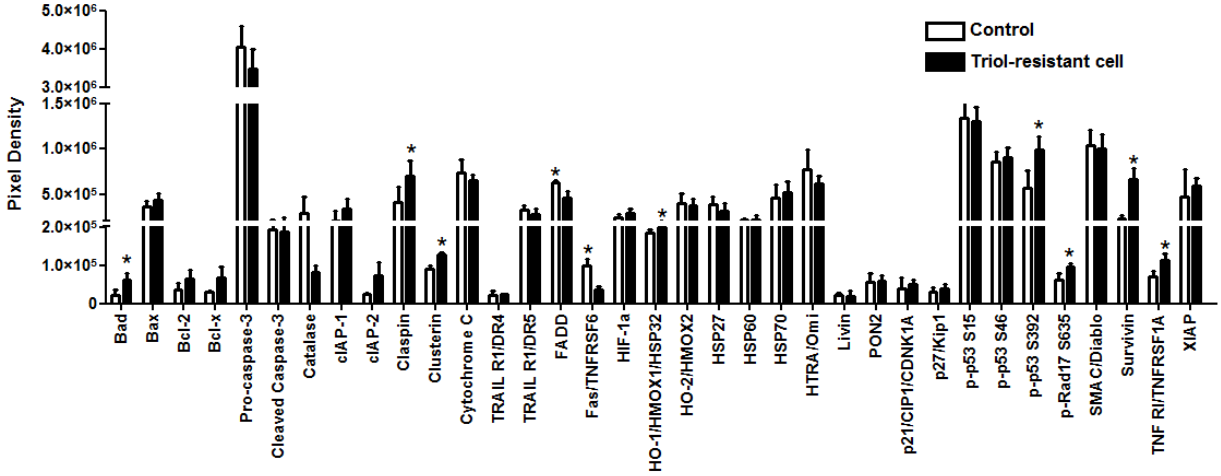


รูปที่ 10 กราฟแสดงผลการกระตุ้นเซลล์เพาะเลี้ยง MMNK-1 ด้วย H_2O_2 ความเข้มข้นตั้งแต่ 0-1000 uM เป็นเวลา 48 ชั่วโมง โดยจำนวนร้อยละของ cell survival หลังการกระตุ้นของ Triol-resistant cell เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อกระตุ้นด้วยความเข้มข้น H_2O_2 ที่ 400-1000 uM เมื่อเปรียบเทียบกับเซลล์ควบคุม (* หมายถึง ระดับของ % cell survival แตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

ดังนั้นผู้วิจัยจึงได้ทำการศึกษากลไกที่เกี่ยวข้องต่อการดื้อต่อการกระตุ้นให้เกิดการตายแบบ apoptosis ซึ่งอาจเกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดี โดยใช้เทคนิค Protein array ซึ่งได้ทำการศึกษาทั้งโปรตีนที่เกี่ยวข้องกับ Signaling transduction pathway และ apoptosis pathway ซึ่งจากการทดลองพบว่า ใน Triol-resistant cell มีการกระตุ้นกลไกที่เกี่ยวข้องกับการอยู่รอดของเซลล์ เช่น มีการกระตุ้นให้เกิดการ phosphorylation ของ p38 mitogen-activated protein kinase α (p38-α), extracellular signal-regulated kinase (ERK1/2), oncogenic kinase ชนิด Src, Lyn, Yes และ Fak และ oncogenic transcription factors, cAMP response element-binding (CREB), and c-Jun (S63) (รูปที่ 11)นอกจากนี้ยังพบการเพิ่มขึ้นของการแสดงออกของโปรตีนที่เกี่ยวข้องกับการต้าน apoptosis (anti-apoptosis) เช่น Bcl-2, Bcl-x, cIAP-2 และ survivin (รูปที่ 12) สรุปได้ว่าใน Triol-resistant cell มีการกระตุ้น receptor tyrosine kinase pathway ส่งผลให้เกิดการแสดงออกของโปรตีนที่เกี่ยวข้องกับการดื้อต่อ apoptosis อย่างชัดเจน

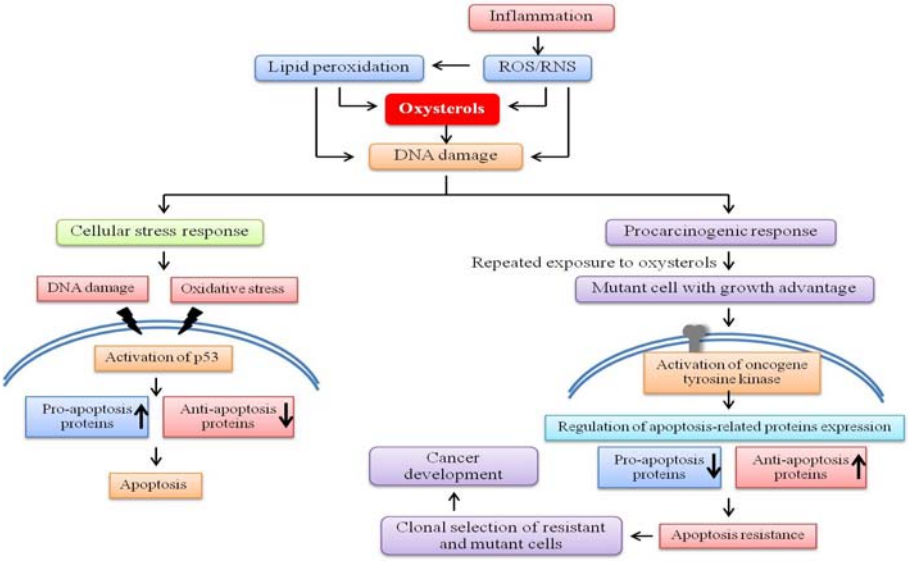


รูปที่ 11 กราฟแสดงผล Phosphorylated-Kinase protein array



รูปที่ 12 กราฟแสดงผลการกระตุ้นเซลล์เพาะเลี้ยง MMNK-1 ด้วย H_2O_2 ความเข้มข้นตั้งแต่ 0-1000 uM เป็นเวลา 48 ชั่วโมง โดย % cell survival หลังการกระตุ้นของ Triol-resistant cell เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติตั้งแต่กระตุ้นด้วย 400-1000 uM H_2O_2 เมื่อเปรียบเทียบกับเซลล์ control (* หมายถึง ระดับของ % cell survival แตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

ดังนั้นจึงสรุปได้ว่าในสภาวะที่เซลล์เพาะเลี้ยงเยื่อบุท่อน้ำดีได้รับการกระตุ้นด้วยออกซิสเตอรอล เป็นเวลานาน ส่งผลให้เซลล์มีการปรับตัวจนดื้อต่อการตายแบบ apoptosis ทั้งนี้การที่เซลล์รอดจากการกระตุ้นให้เกิดการตาย ประกอบกับภาวะที่มีการกระตุ้นให้เกิด DNA damage อยู่เป็นเวลานานอาจส่งผลให้เกิดมะเร็งท่อน้ำดีต่อไปได้ (รูปที่ 13)

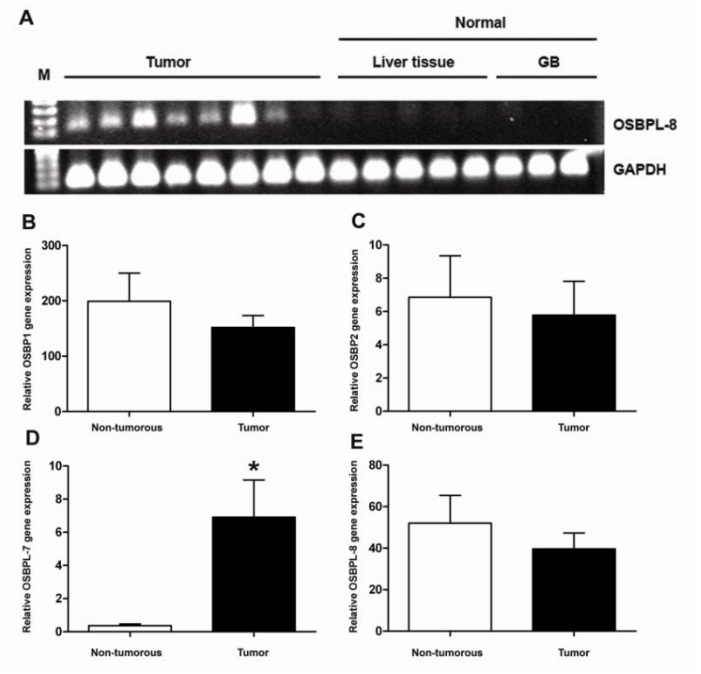


รูปที่ 13 Proposed scheme แสดงกลไกของ oxysterol ในกระบวนการก่อมะเร็งท่อน้ำดี

II <u>การศึกษาชนิด โปรตีนที่จับ และบทบาทของ ออกซิสเตอรอล ในเนื้อเยื่อมะเร็งของผู้ป่วยมะเร็งท่อน้ำดี</u>

1 การแสดงออกของ oxysterol binding protein (OSBP) ในเนื้อเยื่อมะเร็งท่อน้ำดีในสัตว์ทดลองและเนื้อเยื่อตับของผู้ป่วยโรคมะเร็งท่อน้ำดี โดยผลการศึกษาแบ่งออกเป็น 2 ส่วนคือ

1.1 การแสดงออกของ oxysterol binding protein (OSBP) mRNA ในเนื้อเยื่อมะเร็งท่อน้ำดีในสัตว์ทดลอง โดยวิธี RT-PCR โดยการศึกษาในครั้งนี้ได้ตรวจวัดการแสดงออกของยีน OSBP ที่เป็น isoform ชนิด OSBPL-8 ทั้งนี้เพราะมีผลจากการศึกษาก่อนหน้านี้ว่ามีการแสดงออกที่เพิ่มมากขึ้นในเนื้อเยื่อมะเร็งท่อน้ำดีของหนูแฮมสเตอร์ เมื่อเปรียบเทียบกับเนื้อเยื่อตับและเยื่อถุงน้ำดี (gall bladder epithelial) ปกติ โดยผลการศึกษานี้เป็นการตรวจยืนยันด้วยเทคนิค RT-PCR อีกครั้ง ซึ่งพบว่าให้ผลสอดคล้องกันคือ มีการแสดงออกที่เพิ่มมากขึ้นในเนื้อเยื่อมะเร็งท่อน้ำดีของหนูแฮมสเตอร์ เมื่อเปรียบเทียบกับเนื้อเยื่อตับและเยื่อถุงน้ำดี (gall bladder epithelial) ปกติ และพบว่าเมื่อนำเนื้อเยื่อตับของหนูที่ถูกกระตุ้นให้เป็นมะเร็งท่อน้ำดีในระยะต่างๆ เริ่มจาก 1,2,3,4,12 สัปดาห์หลังการกระตุ้นด้วยเชื้อพยาธิใบไม้ตับและสารก่อมะเร็งมาตรวจสอบ พบว่าไม่พบมีการแสดงออกของจีน OSBPL-8 ในเนื้อเยื่อดังกล่าว (รูปที่ 14A) แสดงให้เห็นว่ายีน OSBPL-8 แสดงออกเฉพาะในเนื้อเยื่อมะเร็งเท่านั้น

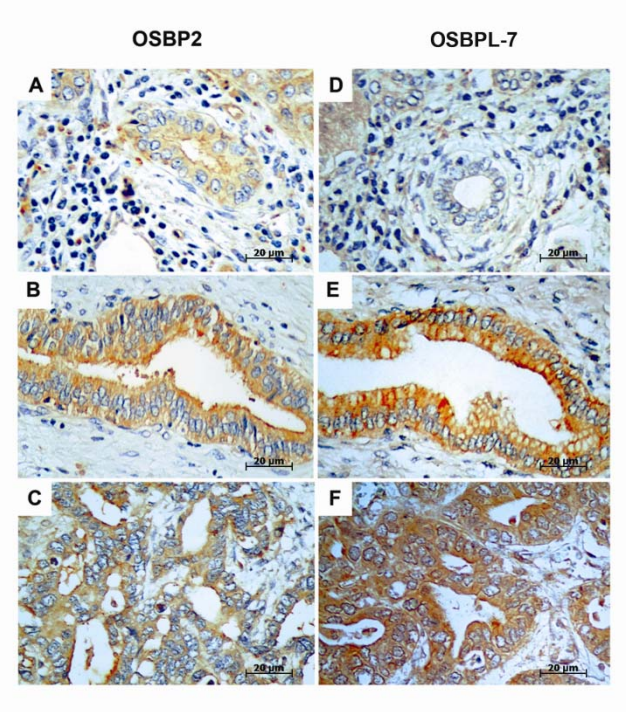


รูปที่ 14 การแสดงออกของ OSBPL-8 ในเนื้อเยื่อมะเร็งของหนูแฮมสเตอร์เปรียบเทียบกับเนื้อเยื่อตับและเซลล์เยื่อบุถุงน้ำดีปกติ (A) และการแสดงออกของ OSBP1, OSBP2, OSBPL-7 และ OSBPL-8 ในเนื้อเยื่อมะเร็งท่อน้ำดีและเนื้อเยื่อตับที่อยู่ข้างเคียงของคนไข้มะเร็งท่อน้ำดี (14B – 14E ตามลำดับ)

1.2 การแสดงออกของ oxysterol binding protein isoforms ในระดับ mRNA ในเนื้อเยื่อมะเร็งของผู้ป่วยมะเร็งท่อน้ำดีโดยวิธี real time RT-PCR ซึ่งในการศึกษาครั้งนี้ได้ตรวจวัดระดับการแสดงออกของ oxysterol binding protein isoforms จำนวน 4 ชนิดซึ่งมีรายงานว่ามีการแสดงออกที่สูงขึ้นในเนื้อเยื่อมะเร็งหลายชนิดได้แก่ OSBP1 (รูปที่ 14B), OSBP2 (รูปที่ 14C), OSBPL-7 (รูปที่ 14D) และ OSBPL-8 (รูปที่ 14E) ซึ่งพบว่ามีการแสดงออกที่สูงขึ้นของ oxysterol binding protein ทั้ง 4 isoforms ในเนื้อเยื่อมะเร็งและเนื้อเยื่อตับที่อยู่ติดกับมะเร็ง (adjacent tissue) ของคนไข้รายเดียวกัน โดยเฉพาะ OSBPL-7 ที่พบว่ามีระดับการแสดงออกที่สูงในเนื้อเยื่อมะเร็งมากกว่าเนื้อเยื่อตับที่อยู่ข้างเคียงอย่างมีนัยสำคัญทางสถิติ

2 การศึกษาตำแหน่งการแสดงออกของ oxysterol binding protein isoforms ในเนื้อเยื่อมะเร็งของผู้ป่วยมะเร็งท่อน้ำดีโดยวิธี immunohistochemistry

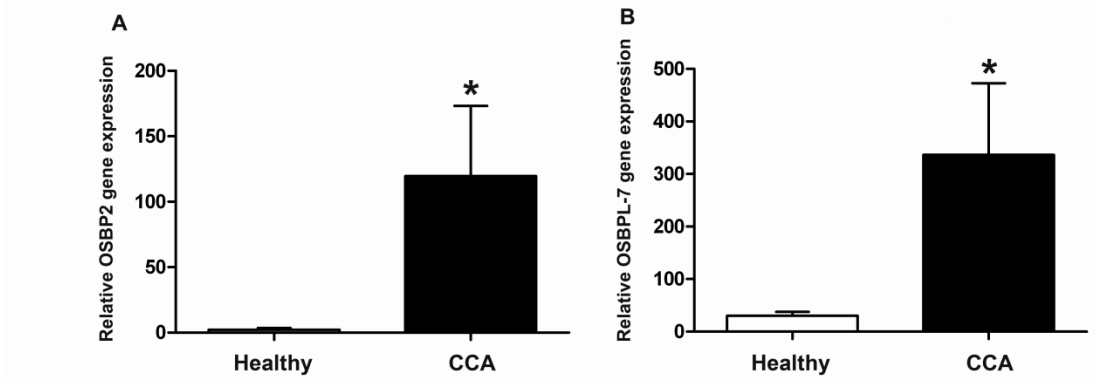
พบว่าการศึกษาในส่วนนี้ให้ผลสอดคล้องกับการตรวจวัดระดับการแสดงออกของ mRNA กล่าวคือโปรตีน OSBP2 มีการแสดงออกที่สูงในเซลล์ตับ เซลล์เยื่อบุทางเดินน้ำดีปกติ เซลล์เยื่อบุทางเดินน้ำดีที่มีกำลังมีการเปลี่ยนแปลงจากเซลล์ปกติได้แก่ hyperplastic cell และ dysplastic cell รวมถึงเซลล์มะเร็ง ในขณะที่โปรตีน OSBPL-7 พบว่ามีการแสดงออกเฉพาะในเซลล์เยื่อบุท่อน้ำดีที่กำลังมีการเปลี่ยนแปลงไปเป็นเซลล์มะเร็ง (hyperplastic cell และ dysplastic cell) และเซลล์มะเร็งตามลำดับ (รูปที่ 15) ที่น่าสนใจก็คือไม่พบการแสดงออกของโปรตีนทั้ง 2 ชนิดในเซลล์เม็ดเลือดขาวที่อยู่ในเนื้อเยื่อมะเร็ง ซึ่งนำไปสู่สมมุติฐานว่าสามารถใช้ oxysterol binding protein ชนิด OSBP2 และ OSBPL-7 เป็นตัวบ่งชี้ชีวภาพที่แสดงถึงการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีในกระแสเลือดได้หรือไม่



รูปที่ 15 แสดงตำแหน่งการแสดงออกของโปรตีน OSBP2 และ OSBPL-7 ในเนื้อเยื่อตับของผู้ป่วยมะเร็งท่อน้ำดีในระยะต่างๆของการเปลี่ยนแปลงจากเซลล์เยื่อบุมะเร็งท่อน้ำดีปกติ (A และ D) ไปเป็น hyperplastic cell (B และ E) และเซลล์มะเร็ง (C และ F) ตามลำดับ

3 การตรวจแสดงออกของ oxysterol binding protein ในระดับ mRNA ในกระแสเลือดของคนผู้ป่วยมะเร็งท่อน้ำดี โดยวิธี real time RT-PCR

ผลการศึกษาพบว่าระดับการแสดงออกของ OSBP2 และ OSBPL-7 ในเซลล์ที่แยกได้จากกระแสเลือดของผู้ป่วยมะเร็งท่อน้ำดีสูงกว่าเซลล์ที่แยกได้จากกระแสเลือดของคนปกติอย่างมีนัยสำคัญทางสถิติ (รูปที่ 17) และระดับของ oxysterol binding protein ที่ตรวจพบน่าจะมาจากเซลล์มะเร็งท่อน้ำดีที่มีการแพร่ลุกลามมายังกระแสเลือดไปยังอวัยวะต่างๆ เพราะจากผลของการย้อม immunohistochemistry ไม่พบการแสดงออกของ oxysterol binding protein ในเซลล์เม็ดเลือดขาว (รูปที่ 16) ผลการศึกษานี้แสดงให้เห็นว่ามีความเป็นไปได้ที่จะใช้ oxysterol binding protein เป็นตัวบ่งชี้ชีวภาพที่แสดงถึงการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีในกระแสเลือด



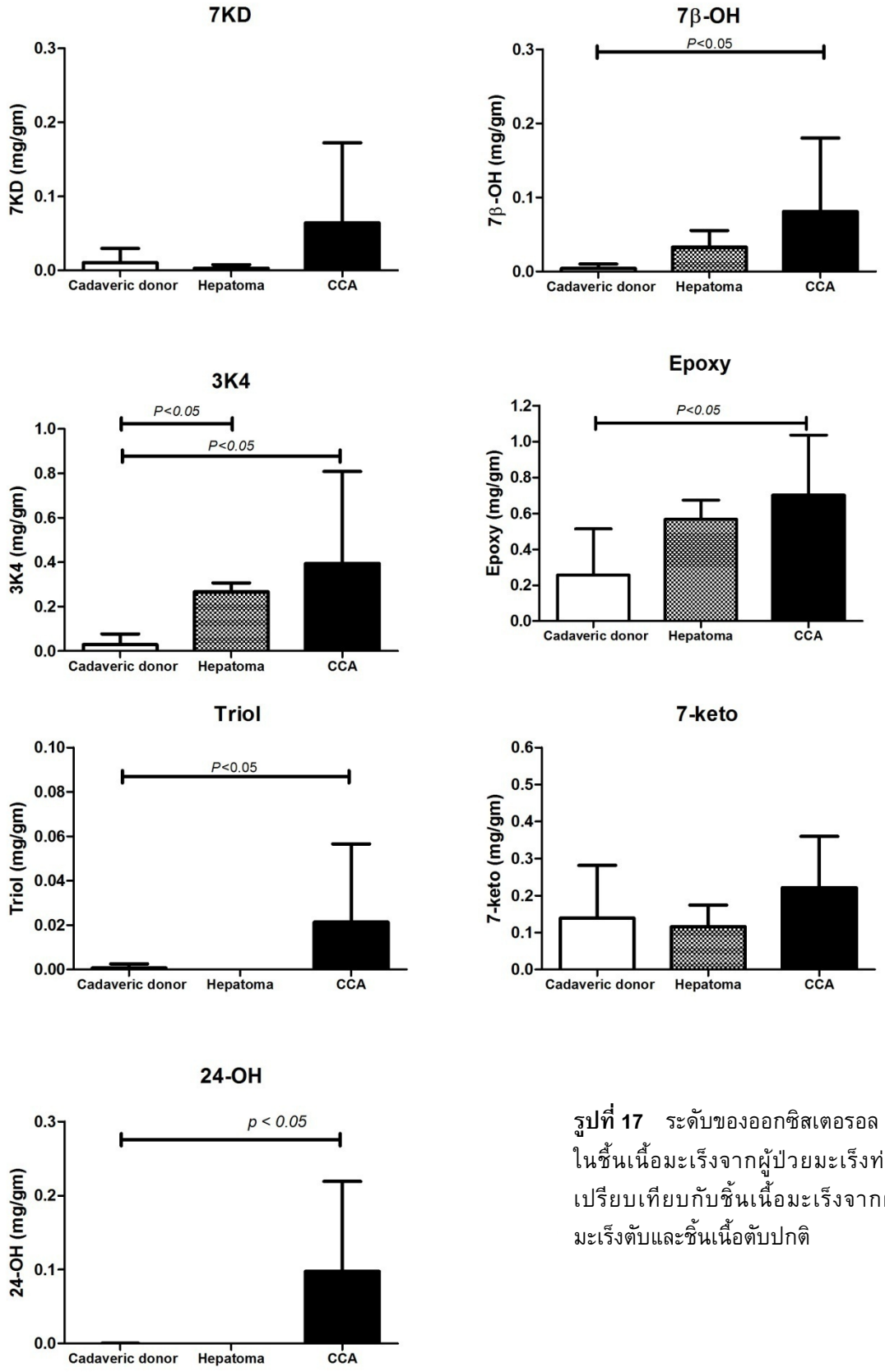
รูปที่ 16 แสดงระดับการแสดงออกของ OSBP2 (A) และ OSBPL-7 ในเซลล์ที่แยกจากกระแสเลือดของคนไข้มะเร็งท่อน้ำดีเปรียบเทียบกับเซลล์ที่แยกได้จากกระแสเลือดของคนปกติ

4 การวิเคราะห์หาชนิดของออกซิสเตอรอล ในน้ำดีและชิ้นเนื้อมะเร็งของผู้ป่วยมะเร็งท่อน้ำดีโดยใช้วิธี Gas chromatography และ Mass spectrometry

เพื่อศึกษาชนิดและปริมาณของออกซิสเตอรอล ที่อาจเกี่ยวข้องกับกระบวนการกระตุ้นให้เกิดมะเร็งท่อน้ำดีในจึงได้มีการเก็บตัวอย่างชิ้นเนื้อมะเร็งและน้ำดีจากผู้ป่วยมะเร็งท่อน้ำดีที่เข้ารับการผ่าตัดที่โรงพยาบาลศรีนครินทร์ จังหวัดขอนแก่น ซึ่งมีจำนวนชิ้นเนื้อมะเร็งทั้งหมดจำนวน 45 ตัวอย่าง และมีจำนวนน้ำดีทั้งหมด 86 ตัวอย่าง มาทำการศึกษาชนิดและปริมาณของ ออกซิสเตอรอล โดยใช้วิธี Gas chromatography และ Mass spectrometry ซึ่งในขั้นตอนการตรวจวัดและวิเคราะห์นี้ได้มาทำการศึกษาวิจัย ที่ University of Washington ประเทศสหรัฐอเมริกา ซึ่งตรวจวิเคราะห์พบ Oxysterol ทั้งหมด 7 ชนิด ดังนี้

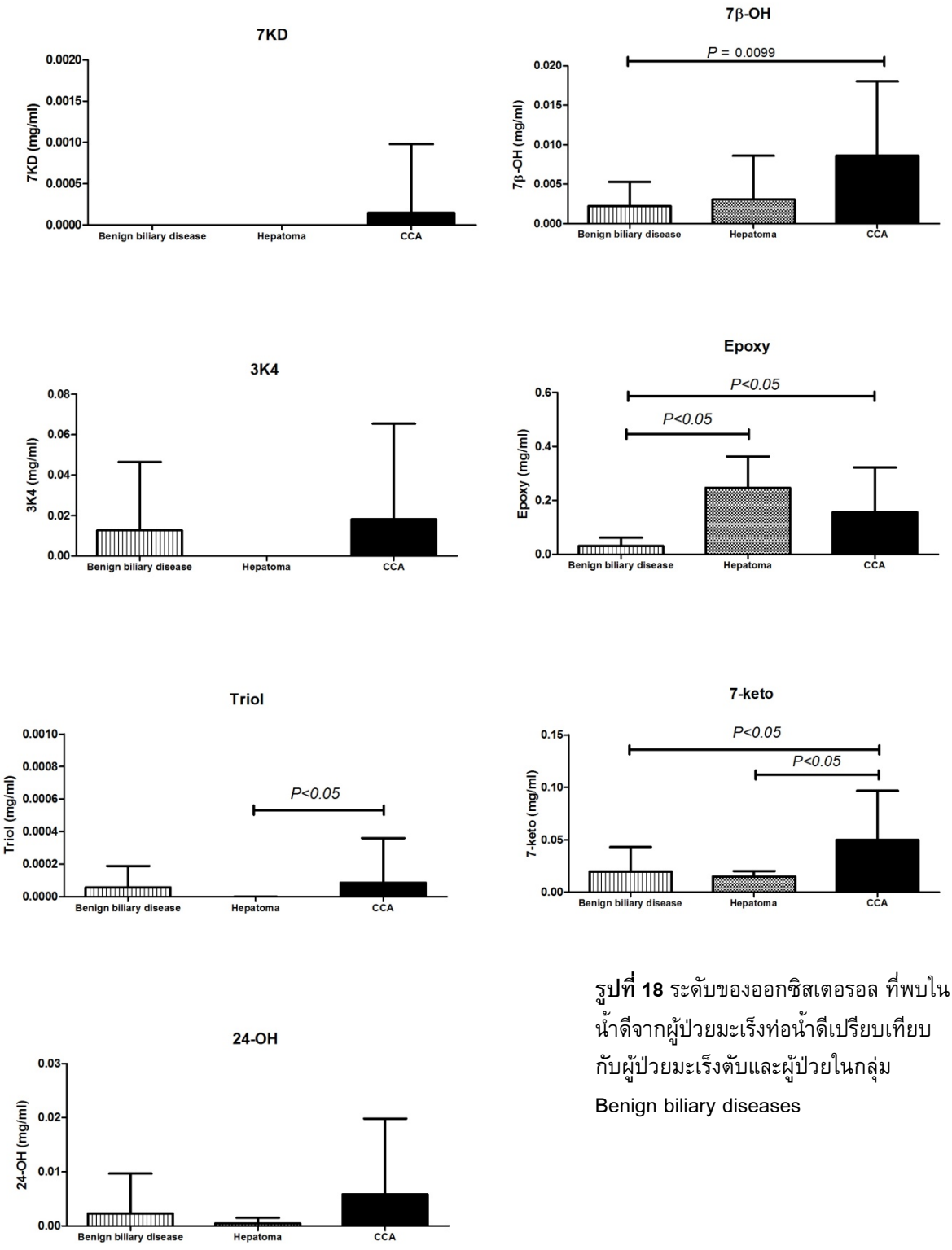
1. 7-keto-cholesta-3,5-diene (7KD)
2. 7beta –hydroxycholesterol (7β-OH)
3. 5,6 epoxycholesterol (Epoxy)
4. 3-keto-cholest-4-ene (3K4)
5. cholestane-3,5,6-triol (Triol)
6. 7ketocholesterol (7-keto)
7. 24-hydroxycholeasterol (24-OH)

ซึ่งเมื่อทำการเปรียบเทียบปริมาณออกซิสเตอรอล ที่ตรวจพบได้จากชิ้นเนื้อมะเร็งท่อน้ำดี กับ มะเร็งตับ (hepatoma) และชิ้นเนื้อตับปกติจาก Cadaveric donor พบว่าปริมาณของออกซิสเตอรอล ชนิด Triol, 3K4, 7β-OH, และ 24-OH ในผู้ป่วยมะเร็งท่อน้ำดีสูงขึ้นเมื่อเปรียบเทียบกับปริมาณที่พบในชิ้นเนื้อจากผู้ป่วย hepatoma และ ชิ้นเนื้อตับปกติ อย่างมีนัยสำคัญทางสถิติ ซึ่งออกซิสเตอรอล ชนิด Triol และ 3K4 เป็นชนิดเดียวกับที่พบว่ามีปริมาณเพิ่มขึ้นในแฮมสเตอร์ที่ถูกกระตุ้นในเป็นมะเร็งท่อน้ำดี เมื่อเปรียบเทียบกับหนูปกติ (รูปที่ 17)



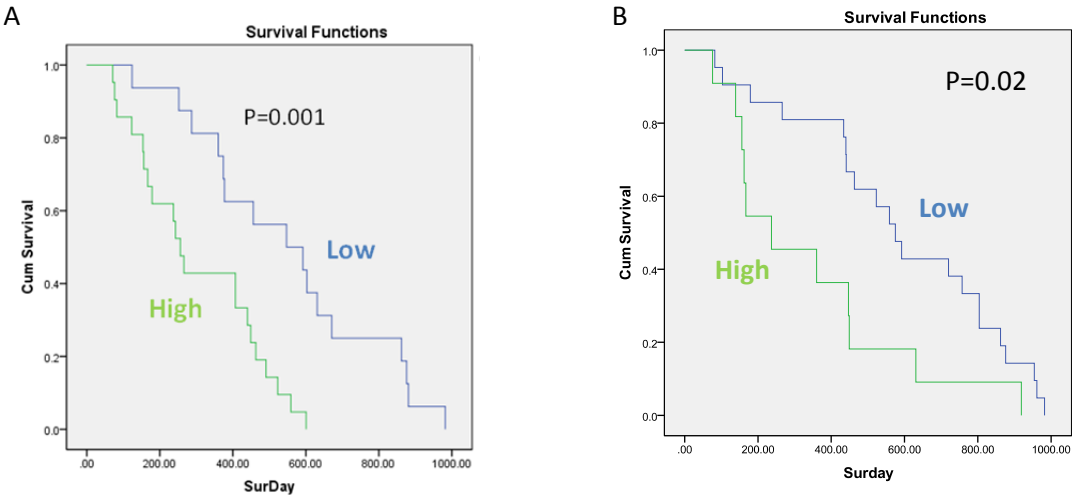
รูปที่ 17 ระดับของออกซิสเตอรอล ที่พบในชิ้นเนื้อมะเร็งจากผู้ป่วยมะเร็งท่อน้ำดี เปรียบเทียบกับชิ้นเนื้อมะเร็งจากผู้ป่วยมะเร็งตับและชิ้นเนื้อตับปกติ

การวิเคราะห์ปริมาณออกซิสเตอรอล ที่ตรวจพบในน้ำดีจากถุงน้ำดีของผู้ป่วยมะเร็งท่อน้ำดี ก็ได้ผลเช่นเดียวกับเนื้อเยื่อมะเร็ง กล่าวคือมีปริมาณของออกซิสเตอรอล ชนิด Triol, 3K4, 7β-OH, และ 24-OH สูงขึ้นเมื่อเปรียบเทียบกับปริมาณที่ตรวจพบได้ในน้ำดี จากผู้ป่วย hepatoma และ ผู้ป่วยกลุ่ม benign biliary disease (รูปที่ 18)



รูปที่ 18 ระดับของออกซิสเตอรอล ที่พบในน้ำดีจากผู้ป่วยมะเร็งท่อน้ำดีเปรียบเทียบกับผู้ป่วยมะเร็งตับและผู้ป่วยในกลุ่ม Benign biliary diseases

ทั้งนี้มีรายงานว่าออกซิสเตอรอล ที่ตรวจพบในชิ้นเนื้อมะเร็งและน้ำดีหลายชนิดมีส่วนเกี่ยวข้องกับการเกิดโรคและภาวะความผิดปกติที่เกี่ยวข้องกับกระบวนการอักเสบหลายชนิด เช่น 7β-OH ที่มีระดับเพิ่มสูงขึ้นในซีรัมของผู้ป่วย Atherosclerosis, ระดับของ 3K4 ที่ตรวจพบในนิ่วในถุงน้ำดี รวมถึง 24-OH ที่เพิ่มสูงขึ้นในซีรัมของผู้ป่วย Alzheimer's นอกจากนี้ยังพบว่าระดับของ 24-OH ที่ตรวจพบในชิ้นเนื้อมะเร็งและในน้ำดีของผู้ป่วยมะเร็งท่อน้ำดียังมีความสัมพันธ์กับระยะรอดชีพของผู้ป่วยอีกด้วย โดยผู้ป่วยที่มีระดับของ 24-OH ต่ำจะมีระยะการรอดชีพสูง (รูปที่ 19)



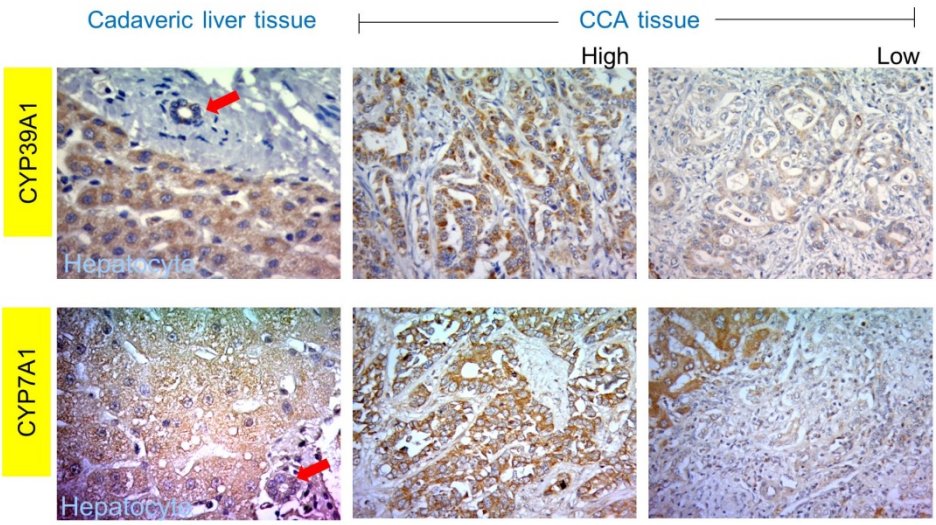
รูปที่ 19 กราฟแสดงความสัมพันธ์ระหว่างระดับ 24-OH ในชิ้นเนื้อมะเร็งท่อน้ำดี (A) และน้ำดี (B) กับระยะการรอดชีพของผู้ป่วยมะเร็งท่อน้ำดี ผู้ป่วยที่มีระดับ 24-OH ต่ำ (Low) จะมีระยะการรอดชีพสูงกว่า ผู้ป่วยที่มีระดับ 24-OH สูง (High) อย่างมีนัยสำคัญทางสถิติ

จากผลการทดลองข้างต้นสรุปได้ว่าออกซิสเตอรอลบางชนิด อาจมีผลต่อกระบวนการก่อมะเร็งท่อน้ำดีที่สัมพันธ์กับการอักเสบซึ่งนำไปสู่การทดลองเพื่อหากลไกของออกซิสเตอรอล ที่เกี่ยวข้องกับกระบวนการกระตุ้นให้เกิดมะเร็งท่อน้ำดีต่อไป

5 การวิเคราะห์การแสดงออกของเอนไซม์ CYP39A1 และ CYP7A1 ในชิ้นเนื้อมะเร็งท่อน้ำดี

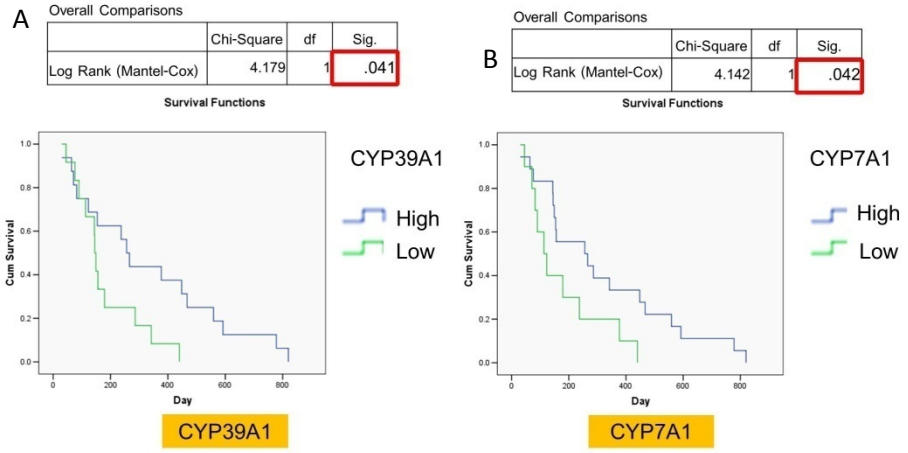
ทั้งนี้เนื่องจาก 24-OH เป็นออกซิสเตอรอล ที่ถูกสร้างจากเอนไซม์ Cytochrome P450 (CYP) 46 ที่อยู่ในเซลล์สมอง โดย 24-OH เกี่ยวข้องกับการควบคุมระดับคลอเรสเตอรอลในสมอง ซึ่ง 24-OH จะถูกขับออกจากเซลล์สมองสู่กระแสเลือดเข้าสู่ตับและจะถูกเมทาบอไลส์ด้วยเอนไซม์สองชนิดคือ CYP39A1 และ CYP7A1 ก่อนจะถูกกำจัดออกจากร่างกายในรูปกรดน้ำดีต่อไป มีรายงานว่า พบระดับ 24-OH เพิ่มสูงขึ้นในซีรัมของผู้ป่วย Alzheimer's ถึงแม้จะมีการรายงานผลของ 24-OH ที่เกี่ยวข้องกับการเกิดโรคในระบบประสาทแต่ยังไม่มีการรายงานระดับของ 24-OH ในระบบท่อทางเดินน้ำดีมาก่อน ดังนั้นผู้วิจัยจึงได้ทำการศึกษาการแสดงออกของโปรตีนที่เกี่ยวข้องกับกระบวนการเมทาบอลิซึมของ 24-OH คือเอนไซม์ CYP39A1 และ CYP7A1 ซึ่งอาจมีผลต่อการเปลี่ยนแปลงของระดับ 24-OH และอาจเกี่ยวข้องกับกระบวนการเกิดมะเร็งท่อน้ำดี

ผลการศึกษาการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ในชิ้นเนื้อมะเร็งท่อน้ำดีจำนวน 28 รายพบว่า โปรตีนทั้งสองชนิดมีระดับการแสดงออกที่สูงขึ้นในชิ้นเนื้อมะเร็งท่อน้ำดีเมื่อเปรียบเทียบกับท่อน้ำดีปกติจาก Cadaveric donor (รูปที่ 20) นอกจากนี้ยังพบว่าในจำนวนชิ้นเนื้อ 28 ราย มี 16 ตัวอย่างที่มีการแสดงออกของ โปรตีน CYP39A1 แบบ High expression และ 12 ตัวอย่างที่มีการแสดงออกแบบ Low expression เมื่อเปรียบเทียบระดับการแสดงออกในชิ้นเนื้อมะเร็ง ในขณะที่ มี 18 ตัวอย่างที่มีการแสดงออกของ โปรตีน CYP7A1 แบบ High expression และ 12 ตัวอย่างที่มีการแสดงออกแบบ Low expression เมื่อเปรียบเทียบระดับการแสดงออกในชิ้นเนื้อมะเร็ง



รูปที่ 20 การศึกษาการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ในชิ้นเนื้อมะเร็งท่อน้ำดี และชิ้นเนื้อตับปกติ (Cadaveric liver tissue) ด้วยวิธี Immunohistochemistry ผลการศึกษาพบว่า พบว่า โปรตีนทั้งสองชนิดมีระดับการแสดงออกที่สูงขึ้นในชิ้นเนื้อมะเร็งท่อน้ำดีเมื่อเปรียบเทียบกับท่อน้ำดีปกติจาก ลูกศรสีแดงแสดงถึงท่อน้ำดีปกติ

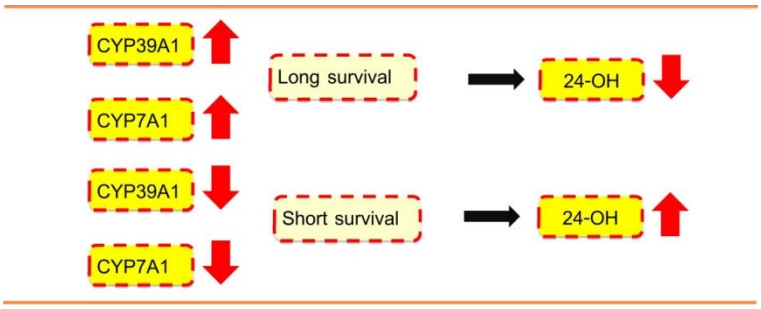
ผู้วิจัยยังพบว่าระดับการแสดงออกของโปรตีนทั้งสองในชิ้นเนื้อมะเร็งท่อน้ำดียังมีความสัมพันธ์กับระยะการรอดชีพของผู้ป่วยมะเร็งท่อน้ำดีอีกด้วย โดยผู้ป่วยที่มีระดับการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 สูง จะมีระยะการรอดชีพยาวกว่าผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ต่ำอย่างมีนัยสำคัญทางสถิติ (รูปที่ 21)



รูปที่ 21 กราฟแสดงความสัมพันธ์ระหว่างระดับการแสดงออกของโปรตีน CYP39A1 (A) และ CYP7A1 (B) กับระยะการรอดชีพของผู้ป่วยมะเร็งท่อน้ำดี ผู้ป่วยที่มีระดับการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 สูง (High) จะมีระยะการรอดชีพยาวกว่าผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ต่ำ (Low) อย่างมีนัยสำคัญทางสถิติ

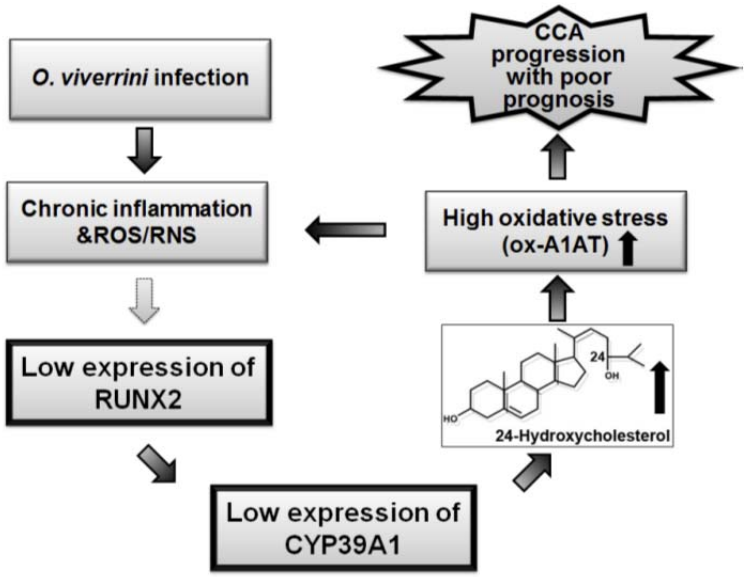
จากผลการทดลองข้างต้นสรุปได้ว่าในผู้ป่วยมะเร็งท่อน้ำดีพบการเปลี่ยนแปลงการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 เมื่อเปรียบเทียบกับชิ้นเนื้อตับปกติ โดยในผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 สูง จะมีระยะการรอดชีพดีกว่า ผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ต่ำ ซึ่งการแสดงออกของโปรตีนทั้งสองชนิดและความสัมพันธ์กับระยะการรอดชีพ ยังสัมพันธ์กับระดับของ 24-OH ด้วย ดังแสดง

ในรูปที่ 22 ผู้วิจัยจึงได้ตั้งสมมติฐานเพื่อทำการศึกษากลไกที่ทำให้เกิดการเปลี่ยนแปลงของระดับของโปรตีนทั้งสองในผู้ป่วยมะเร็งท่อน้ำดีต่อไป



รูปที่ 22 รูปภาพแสดงความสัมพันธ์ระหว่างการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 และ ระดับ 24-OH กับระยะการรอดชีพของผู้ป่วยมะเร็งท่อน้ำดี โดยผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 สูง ซึ่งทำให้มีระดับ 24-OH ในชิ้นเนื้อต่ำจะมีระยะการรอดชีพที่ดีกว่า ผู้ป่วยที่มีการแสดงออกของโปรตีน CYP39A1 และ CYP7A1 ต่ำ และ ระดับ 24-OH สูง

นอกจากนี้เรายังได้ตรวจโปรตีน RUNX2 ซึ่งเป็น transcription factor ของ CYP39A1 ก็ได้พบความสัมพันธ์ในเชิงบวกระหว่างระดับโปรตีนทั้งสอง และหากผู้ป่วย CCA คนใดมีการแสดงออกของทั้ง CYP39A1 และ RUNX2 ต่ำก็จะมีอัตราการรอดชีพที่ต่ำด้วย และการทดสอบบทบาทของ 24-OH ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี พบว่า 24-OH สามารถเหนี่ยวนำให้เกิดภาวะเครียดออกซิเจนได้ ดังสรุปผลความเชื่อมต่อของกลไกที่ทำงานร่วมกันระหว่าง RUNX2 และ CYP39A1 ที่ทำให้ระดับ 24-OH สูงขึ้นในเนื้อเยื่อ CCA ที่ส่งผลให้มีภาวะเครียดออกซิเจนในเซลล์เพิ่ม และการพยากรณ์โรคที่ไม่ดี ดังแสดงในรูปที่ 23



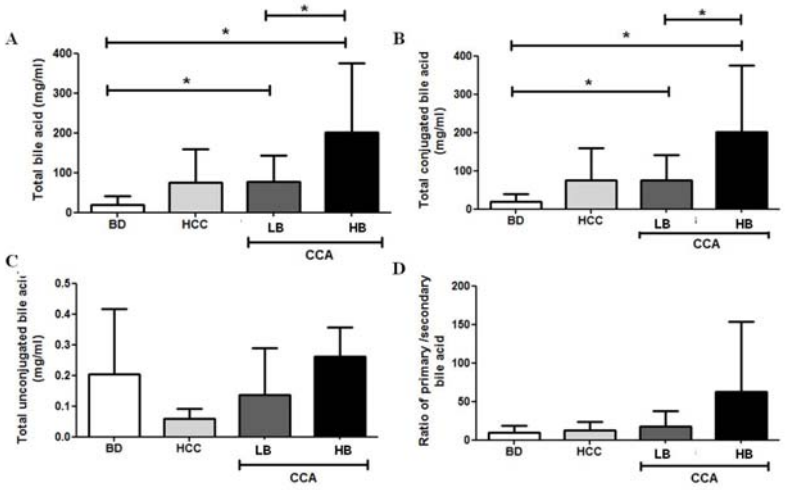
รูปที่ 23 แสดงกลไกความเชื่อมต่อของการทำงานร่วมกันระหว่าง RUNX2 และ CYP39A1 ที่ทำให้ระดับ 24-OH สูงขึ้นในเนื้อเยื่อ CCA ที่ส่งผลให้มีภาวะเครียดออกซิเจนในเซลล์เพิ่มและการพยากรณ์โรคที่ไม่ดี

III <u>การวิเคราะห์ชนิดของกรดน้ำดีในผู้ป่วยมะเร็งท่อน้ำดี</u>

กรดน้ำดี (bile acid) เป็นออกซิสเตอรอลชนิดหนึ่ง ที่เป็นองค์ประกอบหลักในน้ำดีซึ่งมีหน้าที่เกี่ยวข้องกับการย่อยไขมัน ในปัจจุบันมีรายงานว่ากรดน้ำดีหลายชนิดอาจมีส่วนเกี่ยวข้องกับกระบวนการเกิดมะเร็งท่อน้ำดีได้ แต่ยังไม่มีรายงานการศึกษาทั้งปริมาณและชนิดของกรดน้ำดีที่เปลี่ยนแปลงซึ่งอาจส่งผลต่อการเจริญเติบโตที่ผิดปกติของเซลล์เยื่อบุท่อน้ำดีได้ ผู้วิจัยจึงได้ทำการศึกษาชนิดและปริมาณของกรดน้ำดีในน้ำดีจากถุงน้ำดี โดยเทคนิค HPLC ซึ่งได้ทำการวิเคราะห์กรดน้ำดีในผู้ป่วยมะเร็งท่อน้ำดี จำนวน 37 ราย, ผู้ป่วยมะเร็งตับ จำนวน 5 ราย และ ผู้ป่วย benign biliary diseases จำนวน 9 ราย ทั้งนี้ผู้ป่วยมะเร็งท่อน้ำดีถูกแบ่งออกเป็น 2 กลุ่มโดยอาศัยระดับของ bilirubin ใน serum เป็นเกณฑ์ คือผู้ป่วยที่มีระดับ bilirubin $\leq$ 2 mg/dl (LB) และ $>$ 2 mg/dl (HB) เพื่อเป็นตัวบ่งชี้ว่าภาวะการอุดตันของท่อทางเดินน้ำดีมีผลต่อการเปลี่ยนแปลงของระดับกรดน้ำดีหรือไม่

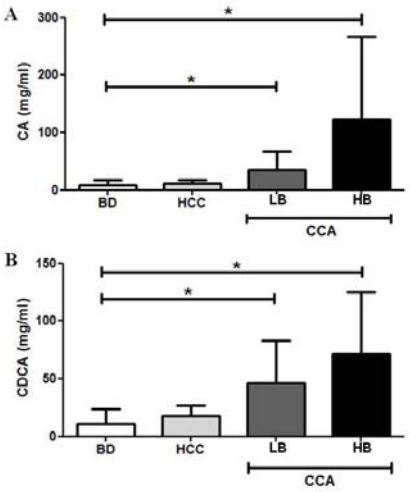
ผู้วิจัยได้ทำการวิเคราะห์ปริมาณกรดน้ำดีทั้งกลุ่มที่เป็น primary bile acids ที่ถูกสร้างในตับ และ secondary bile acid ที่ถูกสร้างโดยแบคทีเรียในลำไส้ โดยได้ทำการศึกษากรดน้ำดีทั้งในรูปที่เป็น unconjugated bile acid และ conjugated bile acid ชนิด taurine และ glycine ดังนั้นจึงมีกรดน้ำดีที่วิเคราะห์ดังนี้ tauroursodeoxycholic acid (TUDC), glycoursocholic acid (GUDC), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), glycolithocholic acid (GLC), free ursodeoxycholic acid (UDCA), free lithocholic acid (LCA), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDCA), free cholic acid (CA), free chenodeoxycholic acid (CDCA) และ free deoxycholic acid (DCA)

ผลการศึกษาพบว่าในผู้ป่วย CCA มีปริมาณ total bile acid และ total conjugated bile acid เพิ่มสูงขึ้นเมื่อเปรียบเทียบกับผู้ป่วย benign biliary disease (รูปที่ 24) นอกจากนี้ยังระดับของ total bile acid และ total conjugated bile acid ยังเพิ่มสูงขึ้นในผู้ป่วย CCA ระดับ bilirubin $>$ 2 mg/dl เมื่อเปรียบเทียบกับผู้ป่วย CCA ที่มี ระดับ bilirubin $\leq$ 2 mg/dl



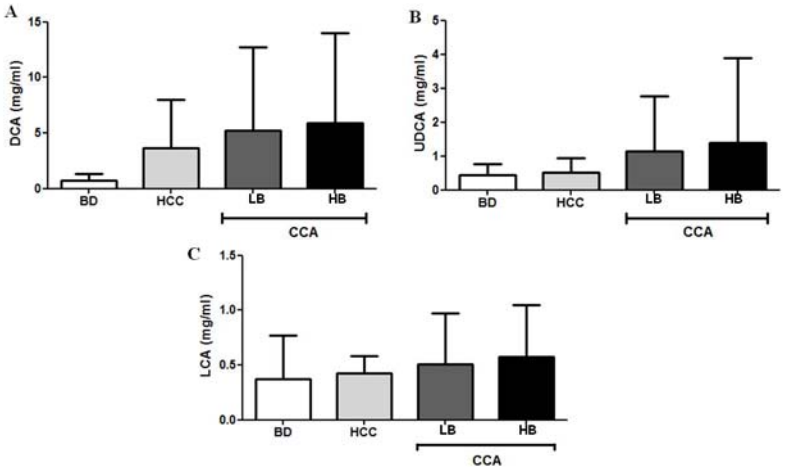
รูปที่ 24 กราฟแสดงปริมาณ Total bile acid (A), Total conjugated bile acid (B), Total unconjugated bile acid (C) และ Ratio of primary bile acid/secondary bile acid (D) ในน้ำดีจากถุงน้ำดีของผู้ป่วย benign biliary disease (BD), Hepatocellular carcinoma (HCC) และ ผู้ป่วย Cholangiocarcinoma (CCA) ที่มีระดับ bilirubin $\leq$ 2 mg/dl (LB) และ $>$ 2 mg/dl (HB) (* หมายถึงระดับแตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

นอกจากนี้ยังพบว่าระดับของ primary bile acids ชนิด CA และ CDCA สูงขึ้นในผู้ป่วย CCA ทั้งในกลุ่ม LB และ HB เมื่อเปรียบเทียบกับผู้ป่วยในกลุ่ม benign biliary disease อย่างมีนัยสำคัญทางสถิติ (รูปที่ 25)



รูปที่ 25 กราฟแสดงปริมาณ Cholic acid (A) และ Chenodeoxycholic acid (B) ในน้ำดีจากถุงน้ำดีของผู้ป่วย benign biliary disease (BD), Hepatocellular carcinoma (HCC) และ ผู้ป่วย Cholangiocarcinoma (CCA) ที่มีระดับ bilirubin $\leq$ 2 mg/dl (LB) และ $>$ 2 mg/dl (HB) (* หมายถึงระดับแตกต่างกันอย่างมีนัยสำคัญทางสถิติ)

ในขณะที่ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของปริมาณกรดน้ำดีที่เป็น secondary bile acid ชนิด DCA, LCA และ UDCA (รูปที่ 26)



รูปที่ 26 กราฟแสดงปริมาณ Deoxycholic acid (A), Ursodeoxycholic acid (B) และ Lithocholic acid ในน้ำดีจากถุงน้ำดีของผู้ป่วย benign biliary disease (BD), Hepatocellular carcinoma (HCC) และ ผู้ป่วย Cholangiocarcinoma (CCA) ที่มีระดับ bilirubin $\leq$ 2 mg/dl (LB) และ $>$ 2 mg/dl (HB)

เมื่อทำการหาความสัมพันธ์ระหว่างระดับของกรดน้ำดีชนิดต่างๆ กับ biochemical parameter ในซีรัมของผู้ป่วย CCA พบว่าระดับของ total bile acid, total primary bile acid และ primary bile acid ชนิด CA และ CDCA มีความสัมพันธ์กับระดับของ bilirubin และ ALP ซึ่งค่าทั้งสองสามารถบ่งชี้ถึงภาวการณ์อุดตันของท่อทางเดินน้ำดีได้ (ตารางที่ 2)

ตารางที่ 2 แสดงความสัมพันธ์ระหว่างระดับของกรดน้ำดีกับ biochemical parameter ในซีรัมของผู้ป่วย CCA

		Cholesterol	Total bilirubin	Direct bilirubin	ALT	AST	ALP
Total bile acid	Pearson Correlation	.460**	.514**	.536**			.510**
	P value	.004	.001	.001	ns	ns	.001
Total primary bile acid	Pearson Correlation	.456**	.531**	.554**			.543**
	P value	.005	.001	.000	ns	ns	.001
Total secondary bile acid	Pearson Correlation						
	P value	ns	ns	ns	ns	ns	ns
Total CA	Pearson Correlation	.402*	.456**	.477**			.643**
	P value	.014	.005	.003	ns	ns	.000
Total CDCA	Pearson Correlation	.404*	.490**	.509**			
	P value	.013	.002	.001	ns	ns	ns
Total DCA	Pearson Correlation						
	P value	ns	ns	ns	ns	ns	ns
Total UDCA	Pearson Correlation				.455**		
	P value	ns	ns	ns	.005	ns	ns
Total LCA	Pearson Correlation						
	P value	ns	ns	ns	ns	ns	ns

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

Cholic acid (CA), Chenodeoxycholic acid (CDCA), Deoxycholic acid (DCA), Ursodeoxycholic acid (UDCA), Lithocholic acid (LCA), Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST)

จากผลการทดลองดังกล่าวจึงสรุปได้ว่าในผู้ป่วยมะเร็งท่อน้ำดีมีการเปลี่ยนแปลงระดับของกรดน้ำดีเมื่อเปรียบเทียบกับผู้ป่วย benign biliary disease นอกจากนี้ระดับของกรดน้ำดีที่เปลี่ยนแปลงอาจมีผลมาจากภาวะการอุดตันของท่อทางเดินน้ำดีร่วมด้วย ผลการศึกษาครั้งนี้สามารถนำไปใช้เป็นข้อมูลอ้างอิงถึงระดับของกรดน้ำดีแต่ละชนิดที่เปลี่ยนแปลงไปซึ่งจะนำไปสู่การศึกษากลไกของกรดน้ำดีที่อาจเกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดีต่อไป

Output ที่ได้จากโครงการ

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LIPIDS IN HEALTH AND DISEASE

REVIEW Open Access

Mechanisms of oxysterol-induced carcinogenesis

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Abstract

Oxysterols are oxidation products of cholesterol that are generated by enzymatic reactions mediated by cytochrome P450 family enzymes or by non-enzymatic reactions involving reactive oxygen and nitrogen species. Oxysterols play various regulatory roles in normal cellular processes such as cholesterol homeostasis by acting as intermediates in cholesterol catabolism. Pathological effects of oxysterols have also been described, and various reports have implicated oxysterols in several disease states, including atherosclerosis, neurological disease, and cancer. Numerous studies show that oxysterols are associated with various types of cancer, including cancers of the colon, lung, skin, breast and bile ducts. The molecular mechanisms whereby oxysterols contribute to the initiation and progression of cancer are an area of active investigation. This review focuses on the current state of knowledge regarding the role of oxysterols in carcinogenesis. Mutagenicity of oxysterols has been described in both nuclear and mitochondrial DNA. Certain oxysterols such as cholesterol-epoxide and cholestanetriol have been shown to be mutagenic and genotoxic. Oxysterols possess pro-oxidative and pro-inflammatory properties that can contribute to carcinogenesis. Oxysterols can induce the production of inflammatory cytokines such as interleukin-8 and interleukin-1β. Certain oxysterols are also involved in the induction of cyclo-oxygenase-2 expression. Inflammatory effects can also be mediated through the activation of liver-X-receptor, a nuclear receptor for oxysterols. Thus, several distinct molecular mechanisms have been described showing that oxysterols contribute to the initiation and progression of cancers arising in various organ systems.

Introduction

Oxysterols are derived from either enzymatic or non-enzymatic oxidation of cholesterol [1,2]. The chemical structures of oxysterols vary depending upon the number and position of oxygenated functional groups, and include keto-, hydroxyperoxy-, and epoxy forms (Figure 1). Enzymatic pathways of oxysterol production mainly involve cytochrome P450 family enzymes. Examples include 27-hydroxylase (CYP27A1), the key enzyme in the alternative bile acid synthesis pathway which leads to the production of 27-hydroxycholesterol [3,4]; and 7α-hydroxylase (CYP7A), the rate limiting enzyme in the classical bile acid synthesis pathway that leads to the production of 7α-hydroxycholesterol [3,5]. Certain oxysterols are produced by non-enzymatic oxidation (or auto-oxidation), a process that involves reactive oxygen and nitrogen species (ROS, RNS). Examples of ROS that participate in non-enzymatic oxidation of cholesterol include hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) [6,7]. 7α-hydroperoxycholesterol is an example of a product of non-enzymatic oxidation of cholesterol which can be further oxidized by non-enzymatic and enzymatic means. Examples of oxysterols generated by this mechanism are 7-ketocholesterol and 7α/β-hydroxycholesterol.

Numerous *in vivo* and *in vitro* studies have described biological effects of oxysterols [5]. Oxysterols can act as intermediates in cholesterol catabolism, especially in bile acid synthesis and are therefore involved in the elimination of excess cholesterol from the body. As oxysterols possess more hydrophilic moieties compared to the parent cholesterol molecule, they can pass more easily through cell membranes. This can occur because polar moieties inserting into hydrophobic regions of cell membranes result in redistribution of the sterol in conjunction with local re-ordering of the acyl chains [3,8,9]. Oxysterols are also able to regulate key enzymes in cholesterol turnover such as 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase [10], the rate-limiting enzyme in cholesterol synthesis.

Pathogenic effects of oxysterols has been described predominantly in cardiovascular diseases [6,11,12] and

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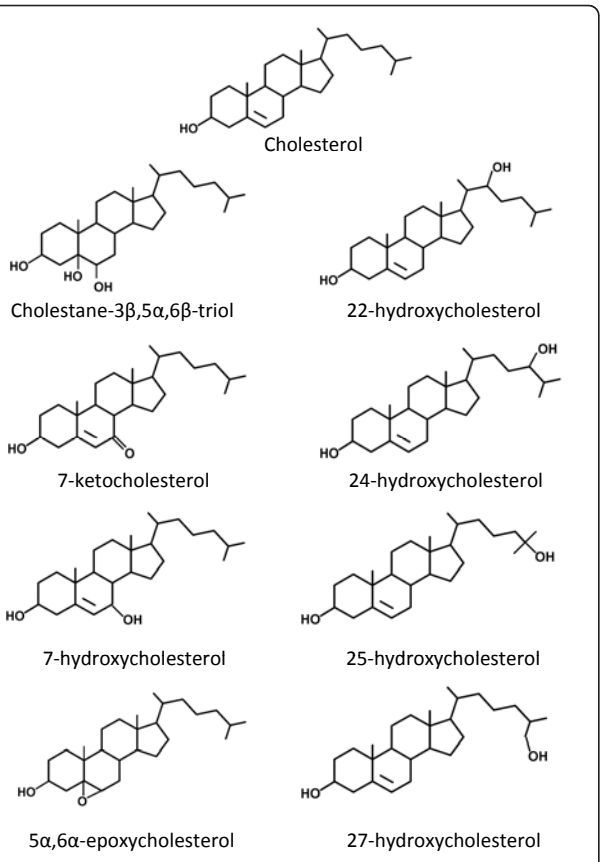

Figure 1 Structure of cholesterol and several common oxysterols.

degenerative diseases such as age-related macular degeneration [13], Alzheimer's disease [14,15] and osteoporosis [16]. Several reports report a role for oxysterols in neurological disease (summarized in Table 1) [17-20].

The effects of cholesterol oxidation products on carcinogenesis was first reported by Bischoff and Rupp in 1946 [21]. Ovariectomized mice that received 10 mg of crude progesterone in sesame oil showed a higher incidence of cancer than mice that received purified progesterone. Cholesterol oxidation products were found in mice that received the crude progesterone extract. This study provided circumstantial evidence that carcinogens may be produced endogenously from cholesterol. Subsequent studies have demonstrated the carcinogenic potential of specific oxysterols. For example, 6β-hydroperoxy-4-cholestane-3-one injected subcutaneously led to the development of local sarcomas in mice [22]. Cholesterol-5α, 6α-epoxide also induced local sarcomas [23].

Role of oxysterols in tissue-specific carcinogenesis

Oxysterols and lung cancer

An epidemiological study described the relationship between lung cancer risk and oxysterols. Using a nested case-control study design, smoking-independent associations between cholesterol oxidation and lung cancer risk were investigated. Six oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, cholesterol-α-epoxide, cholesterol-β-epoxide, cholestanetriol, 7-ketocholesterol and 7-keto-pregnenolone), were measured in plasma of 20 lung cancer patients. The results showed slightly increased levels of all oxysterols compared with control with the exception of 7α-hydroxycholesterol and cholesterol-α-epoxide. Additionally, a risk analysis showed no statistical significant link between oxysterol concentrations and lung cancer development. When adjusted for participation in sports, however, the association between 7β-hydroxycholesterol levels and lung cancer was statistically significant. The data suggest that plasma levels of 7β-hydroxycholesterol are a smoking-independent predictor and biomarker for lung cancer risk [24]. Increased levels of 7β-hydroxycholesterol may be caused by lipid peroxidation, oxysterol dietary intake and individual differences in the metabolism of oxysterols.

Several reports describe the anti-cancer effects of 7β-hydroxycholesterol in lung cancer and various other types of cancer [25-28]. 7β-hydroxycholesterol mediated a cytotoxic effect on human NCI-H460 lung cancer cells through the induction of apoptosis via caspase activation [29].

Role of oxysterols in colon carcinogenesis

Several reports have provided evidence that oxysterols contribute to gastrointestinal cancer development. Animal fat oxidation products, including oxysterols, have been linked to colon carcinogenesis. One possible mechanism for this effect is via upregulation of expression of transforming growth factor-β1 (TGF-β1) in macrophages and fibroblasts, cells which are present in

Table 1 Oxysterol disease associations

Oxysterols	Cancer	Atherosclerosis	Neurological diseases	References
5,6-cholesterolepoxide	√	√		[84,85]
Cholestane-3β,5α,6β-triol	√	√		[17,86,87]
25-hydroxycholesterol			√	[88]
24-hydroxycholesterol			√	[89-91]
7-Hydroperoxy-cholesterols		√		[92]
7-hydroxycholesterol		√		[93]

the tumor microenvironment [30]. TGF-β1 is normally involved in promoting differentiation and apoptosis in intestinal epithelial cells. Colon cancer cells demonstrate decreased expression of TGF-β1 type I and/or type II receptors [31]. Thus, colon cancer cells are less susceptible to the growth regulatory effects of TGF-β1. An increased concentration of TGF-β1 is thought to be involved in a selective elimination of neoplastic cells susceptible to the pro-apoptotic effects of this cytokine, thereby indirectly allowing the clonal expansion of TGF-β1-insensitive neoplastic cells [32,33]. Consequently, disruption of TGF-β1 growth inhibition would support uncontrolled cell proliferation and transformation. Such alterations in TGF-β1 receptor expression have been described in cancers of the colon [34], prostate [35], breast [36] and lung [37]. An oxysterol mixture composed of 7α-hydroxycholesterol (43%), 5α, 6α-epoxycholesterol (32%) and 5β, 6β-epoxycholesterol (6%) induced apoptosis in the human colon adenocarcinoma cell line CaCo-2. The mitochondrial pathway of apoptosis was upregulated in these cells after stimulation by oxysterols as shown by a decrease in mitochondrial membrane potential, release of cytochrome c, and activation of caspase-3. The mechanism of oxysterol-induced upregulation of apoptosis in differentiated CaCo-2 cells involved increased production of ROS by NADPH oxidase activation [38]. Excessive levels of ROS and pro-inflammatory cytokines likely contribute to impairment of enteric mucosal function [39].

Patients with ulcerative colitis are at risk for developing colon cancer. These patients excrete high levels of cholesterol, coprostanol and cholestane-3β-5α-6β triol (cholestanetriol) in feces compared to patients with other digestive diseases and healthy controls [40]. However, the carcinogenic effect of oxysterols has not been demonstrated in rat colons. No colonic tumors were induced by sterols including cholesterol epoxide, cholestanetriol and cholesterol in standard and germ free female rats. Only lithocolic acid, a bile acid produced by bacterial oxidation of chenodeoxycholic acid in the colon, induced colon cancer in this model [41].

Role of oxysterols in cholangiocarcinogenesis

Oxysterols may also play a role in the development and progression of cholangiocarcinoma (CCA), a malignancy arising from intra- and extra-hepatic bile duct epithelial cells or cholangiocytes. A risk factor for the development of CCA in Thailand is chronic infection with the liver fluke, *Opisthorchis viverrini* [42,43]. Infection leads to accumulation of nitric oxide (NO) and oxygen radicals in inflamed tissue resulting in direct and indirect DNA damage both in animal and human studies [44-47]. We found significantly increased levels of 4-cholesten-3-one and cholestanetriol in a hamster model of CCA induced by liver fluke infection combined with *N*-nitrosodimethylamine treatment (unpublished data). Interleukin 6 (IL-6) is a potent mitogen for cholangiocytes and cholangiocarcinoma cells [48-50]. The role of oxysterols in mediating tumor formation in cholangiocytes was reported by Yoon et. al.[51]. These investigators examined the effect of 22(R)-hydroxycholesterol (22-HC) on cyclooxygenase-2 (COX-2) expression and found that this oxysterol stabilized the level of COX-2 mRNA via a p38-mitogen activated protein kinase (MAPK)-dependent mechanism leading to COX-2 protein accumulation in a CCA cell line. This induction of COX-2 could promote cellular replication and inhibit apoptosis [52] and thus contribute to the progression of CCA. In addition to induction of COX-2 expression, bile acids also enhance cellular levels of a potent anti-apoptotic protein, myeloid cell leukemia protein 1 (Mcl-1) [53]. Given the important role of apoptosis inhibition in carcinogenesis and the dominant anti-apoptotic effects of Mcl-1 in CCA cells, this observation may be important in understanding the tropism displayed by bile acid-induced CCA. Indeed, CCA appears to have co-opted the toxic bile milieu to promote growth and survival. Alteration of bile composition during chronic inflammation warrants further investigation as a mechanism of biliary tract carcinogenesis. This observation suggests that these tumors may not only have developed mechanisms to survive the toxic constituents in bile but may actually use bile to promote growth and survival.

Oxysterols were found in infected human hepatic bile, with 7α-hydroxycholesterol and 7β-hydroxycholesterol present at significantly higher concentrations in infected than in uninfected bile. The levels of 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholestanetriol, 7-α-hydroxy-4-cholestane-3-one and 7-ketocholesterol were also positively correlated with C-reactive protein (CRP) levels. These findings suggest that infection of biliary cells may be linked to the synthesis of oxysterols and consequently participate in the development of biliary diseases [54,55].

Mechanisms of oxysterols in carcinogenesis

Mutagenicity of oxysterols

The mutagenic effects of oxysterols were first described in1979. Smith *et al.* [56] reported this effect in air-aged samples of cholesterol on five strains of *Salmonella typhimurium* using the Ames method. Exposure of pure cholesterol to heating in air at 70°C or to 60°C or γ-radiation resulted in the genesis of mutagenic compounds. No mutagenic effect of cholesterol-α-epoxide and cholesterol-β-epoxide was shown, similar to cholesterol. In a hamster model, cholesterol-α-epoxide showed a weak mutagenic effect. V79 Chinese hamster lung fibroblasts treated with cholesterol-α-epoxide induced

8-azaguanine-resistant mutants at frequencies that were 4.6 to 11.8-fold higher than the spontaneous mutation rate in rats. Cholesterol-α-epoxide accumulates in cells and can transform into cholestanetriol which possesses greater cytotoxicity and acts as a potent inhibitor of DNA synthesis. This modification causes a decrease in the mutagenic effects of cholesterol-α-epoxide. Thus the mutagenicity of cholesterol-α-epoxide in V79 Chinese hamster lung fibroblast cells may depend on the ability of cell to metabolize cholesterol-epoxide to cholestanetriol via epoxide hydrolase activity.

The genotoxicity of cholestanetriol using the Ames method and the generation of chromosomal aberrations has been demonstrated. Cholestanetriol, but not 7-ketocholesterol and cholesterol-5α-6α-epoxide, exhibited genotoxicity as determined by the rate of bacterial revertants in the absence of the metabolic activating enzyme (S9). In addition, cholestanetriol induced chromosomal aberrations in Chinese hamster ovary cells (CHO-K1) in the presence and absence of the S9 fraction. However, the degree of abnormalities in chromosomal structure was reduced with S9 treatment when compared to no treatment. Anti-oxidant enzymes including catalase and superoxide dismutase can inhibit cholestanetriol-induced increases in bacterial revertant rates and chromosome aberration in CHO-K1 cells. ROS production also increased in cholestanetriol-treated cells. These results suggest that genotoxicity of cholestanetriol may involve ROS generation [57]. Moreover, the effect of oxysterols on DNA mutation was reported by Gramajo et al.[58]. 7-ketocholesterol-treated culture significantly increased the degree of mitochondrial DNA (mtDNA) damage in the human RPE cell line (ARPE-19). Various studies also provide evidence that oxysterols enhance the production of ROS/RNS [57-62]. Oxysterols might enhance the production of ROS/RNS through alterations in the mitochondrial electron transport chain [59].

Role of oxysterols in oxidative stress and inflammation

Numerous studies have found a role for oxysterols in mediating pro-oxidative and pro-inflammatory processes in age-related degenerative diseases such as Alzheimer's disease and atherosclerosis [6,13,63,64]. The pro-oxidative and pro-inflammatory effect of 7α-, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α-6α-epoxide, cholesterol-β-epoxide, 22R-, 22S-, 25- and 27-hydroxycholesterol were studied in U937 human promonocytic leukemia cells. 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-β-epoxide increased the production of singlet oxygen. The highest capacity to stimulate IL-8 secretion and enhance IL-8 mRNA levels was observed in cells treated with 7β-hydroxycholesterol and 25-hydroxycholesterol [18]. Oxysterols that were oxidized at C7 enhanced the production of free radicals [62,65,66]. 7-ketocholesterol caused a decrease in cellular glutathione content, oxidation of polyunsaturated fatty acids and enhanced the production of ROS [62]. Moreover, 7-ketocholesterol enhanced IL-1β secretion in endothelial cells and participated in the recruitment of monocytes and T lymphocytes.

The cytotoxicity of 7-ketocholesterol and 7β-hydroxycholesterol involves induction of oxidative stress and down-regulation of anti-oxidative mechanisms [62]. These effects can be inhibited by anti-oxidant compounds such as lycopene, carotene, folate, glutathione, N-acetylcysteine and resveratrol [64,65,67-70]. Notably, the pro-oxidative and cytotoxic effects may occur in the absence of activation of NADPH oxidase, as shown in J774A.1 macrophage cells [60,71]. NADPH oxidase plays a key role in oxysterol-induced ROS production in a human colon adenocarcinoma cell line [38].

Pro-inflammatory effects of oxysterols have also been described. 7-ketocholesterol, 7β-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol and cholestanetriol have the capability to induce IL-8 production. The pro-inflammatory effects of 25-hydroxycholesterol was increased in hypoxic culture conditions and potentiated lipopolysaccharide (LPS)-induced IL-1β secretion [72]. In U937 and THP-1 cells, 7β-hydroxycholesterol and 25-hydroxycholesterol were involved in the recruitment of immunocompetent cells via MCP-1, MIP-1β, TNF-α, IL-1β and IL-8 [73]. In addition, certain oxysterols are involved in the induction of COX-2 expression which correlates with CRP levels [51,55].

Pro-inflammatory effects of oxysterols also act at the transcriptional level. This can involve the activation of the liver-X-receptor (LXR) which is a nuclear receptor for certain oxysterols [74]. Pro-inflammatory oxysterols that exert their effect through LXR include 22R-hydroxycholesterol and 25-hydroxycholesterol but not 7β-hydroxycholesterol [19]. 7β-hydroxycholesterol appears to exert its pro-inflammatory effects at translational and post-translational levels. In 7β-hydroxycholesterol-treated U937 and THP-1 cells, increased IL-8 secretion was found. This activation is associated with activation of the MEK/ERK1/2 signaling pathway [73]. The anti-inflammatory effects of oxysterols were also described in human macrophages incubated with 7-ketocholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol before exposure to LPS-induced TNF-α secretion [75]. The 25-hydroxycholesterol which was produced from the stimulation of macrophage toll-like receptors (TLRs) can suppress IL-2-mediated stimulation of B cell proliferation and block IgA class switch recombination, resulting in decreased IgA production [76].

Oxysterol signaling pathways

Oxysterols may exert their cellular effects by binding to specific proteins and activating signaling cascades. In human breast cancer (MCF-7) cells, Silva *et al.* [77]

found that oxysterols in the lipid fraction isolated from human osteoblast-like MG63 cell-conditioned medium (MG63CM) induced MCF-7 cell migration to bone tissue. Interestingly, insulin-like growth factor 1 increased the expression of oxysterol binding protein-related protein 7 (ORP7) in MCF-7 cells. Binding between these ligands and receptors altered the organization of lipid microdomains in cell membranes. Several oxysterol-binding proteins such as ORP7 and ORP 3 were recently reported to play a role in cell adhesion and migration through R-Ras, a small GTPase [78]. ORP3 is abundantly expressed in leukocytes and epithelial cells, and abnormally high expression levels are found in leukemias and solid tumors [79]. Oxysterol-binding protein-like-8 is overexpressed in hamster induced cholangiocarcinoma [80].

Oxysterols may act as secondary messengers in signaling transduction pathways. 22R-hydroxycholesterol stabilizes COX-2 mRNA via the 38-MAPK pathway in human cholangiocytes [51]. Hepatocellular carcinoma cells HepG2 and Huh7 senesce via alteration of p38 MAPK when treated with 5,6 secosterol [81]. Moreover, bile acids, which can be considered a type of oxysterol in bile, are known to activate various signaling cascades. Bile acids can stimulate cell proliferation by activation of the phosphatidylinositol-3-kinase (PI3-K) pathway via a number of mitogen receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) [82]. This activation is mediated by TGF-α, which is a ligand for EGFR. Bile acids that transactivate EGFR through a TGF-α dependent mechanism can be blocked by matrix metalloproteinase (MMP) inhibitors. This suggests that bile acids may activate the release of TGF-α through the induction of MMP. The simplest interpretation of this finding is that bile acids induce MMP activity by generating soluble TFG-α leading to activation of EGFR (Figure 2). Rats with obstructed bile ducts demonstrate increased cholangiocyte proliferation and secretion [83].

Conclusions

This review focuses on the mechanisms that may be operative in oxysterol-induced or associated carcinogenesis. *In vitro* and *in vivo* studies have demonstrated an association between different types of oxysterols and the development and progression of cancer of the colon, lung, breast and bile ducts. Oxysterols may play critical roles in multiple stages of carcinogenesis (Figure 3). First, they may be involved in tumor initiation by enhancing the production of ROS/RNS. Second, tumor promotion may be enhanced by oxysterols through upregulated expression of proteins such as COX-2 leading to the alteration of cellular phenotypes. In addition,

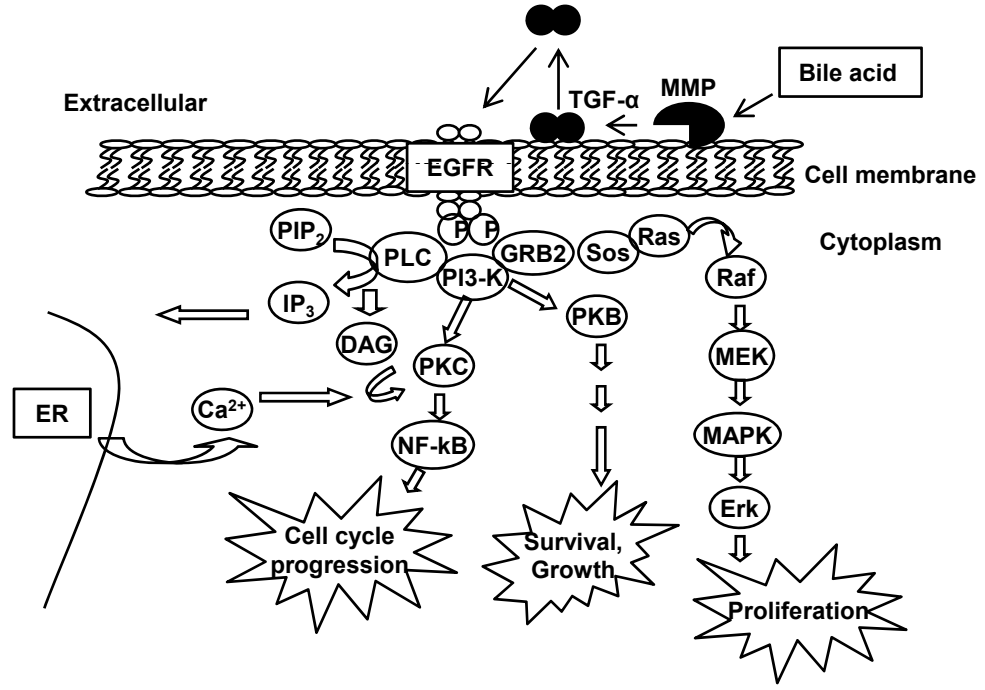

Figure 2 Bile acids transactivate EGFR in cholangiocytes. Bile acids induce a signaling transduction cascade through EGFR. Bile acids can stimulate Src kinase activity, enhance MMP activity and generate TGF-α. Soluble TGF-α acts as the ligand for activation of EGFR. This activation induces signaling molecules such as protein kinase C (PKC), PI3-K and MAPK resulting in alterations in the cell cycle, cell growth, and cell survival (adapted from [53].

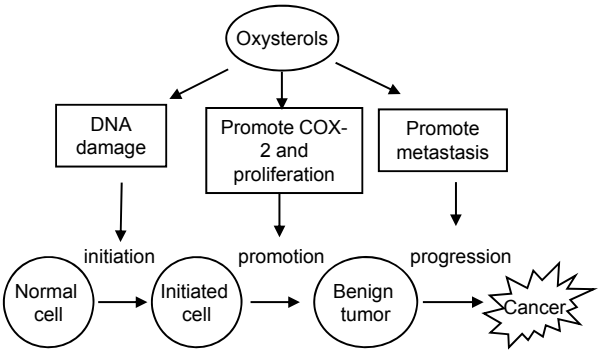

Figure 3 Effects of oxysterols on carcinogenesis. Oxysterols exert their effect on three stages of carcinogenesis by induction of DNA damage, enhancing production of COX-2 and stimulation of tumor cell migration [51,57,77].

certain oxysterols can support cancer progression through the induction of migration. Oxysterols may exert their effect by binding to specific proteins and activating signaling cascades. Further investigations of oxysterols and their impact on carcinogenesis are required for developing improved strategies for the prevention and treatment of various types of cancer.

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Authors' contributions
AJ wrote the manuscript. WL, PY, NN, and RK reviewed and revised the manuscript. All authors approved the final version of the manuscript.

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Expression of oxysterol binding protein isoforms in opisthorchiasis-associated cholangiocarcinoma: A potential molecular marker for tumor metastasis

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ABSTRACT

Oxysterols are oxygenated derivatives of cholesterol generated by enzymatic reactions mediated by cytochrome P450 family enzymes or by inflammation-associated non-enzymatic reactions. Oxysterol binding proteins (OSBPs) are cytosolic high affinity receptors for oxysterols. We previously found that OSBPL-8 is upregulated in liver fluke (*Opisthorchis viverrini*)-induced hamster cholangiocarcinoma (CCA). Our aims were to determine the expression patterns of OSBP isoforms in human CCA tissues and to evaluate whether OSBPs could be used as molecular markers for the identification of blood-borne CCA metastasis. Expression levels of OSBP1, OSBP2, OSBPL-7 and OSBPL-8 in CCA tissues were detected using qRT-PCR and immunohistochemistry. Expression of OSBPs at mRNA level in the blood of CCA patients was also investigated. We confirmed increased expression of OSBPL-8 in *O. viverrini* -induced hamster CCA tissues. Moreover, increased expression of OSBP1, OSBP2, OSBPL-7 and OSBPL-8 was seen in human CCA tissues. Notably, a significant increased level of OSBPL-7 mRNA was observed in tumor compared to non-tumor liver tissue. Immunohistochemistry supported the mRNA results, in that OSBPL-7 protein was over-expressed in cancer cells and hepatocytes but not in normal biliary cells and surrounding inflammatory cells. Interestingly, in our preliminary results, significantly higher levels of OSBP2 and OSBPL-7 mRNA were seen in blood samples from CCA patients than in healthy controls. These results suggest that OSBP2 and OSBPL-7 might serve as molecular markers for the identification of CCA metastasis in the bloodstream.
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1. Introduction

Liver fluke (*Opisthorchis viverrini*) infection has long been implicated in the genesis of cholangiocarcinoma (CCA) [1–3], a highly malignant tumor originating from bile duct epithelial cells. CCA is a major public health problem in northeast Thailand where its incidence is the highest in the world [4]. CCA is a slowly progressive disease and hence is difficult to diagnose until advanced or metastatic, at which stage the prognosis is poor. Therefore, it is necessary to find markers for early stage tumors and metastases in order to improve diagnosis and treatment. Serum markers such as CA19-9 and CA125 have been suggested for CCA diagnosis [5–7]. However, a useful genetic marker for CCA metastasis has not yet been described.

Alterations in expression of oxysterol binding proteins (OSBPs) have been described in various cancers and might serve as potential markers for solid tumor dissemination and poor prognosis [8,9]. OSBPL-7 is involved in breast cancer metastasis [10]. Oxysterol binding protein-related protein 5 expression was related to invasion and poor prognosis in pancreatic cancer patients [11,12]. Over-expression of oxysterol binding protein like-8 (OSBPL-8) was reported by our group in *O. viverrini* and *N*-nitrosodimethylamine (NDMA)-induced hamster CCA [13].

The aims of this study were to determine the patterns of expression of OSBP isoforms, including OSBP1, OSBP2, OSBPL-7 and OSBPL-8, in human CCA tissues; and to evaluate whether OSBPs can be used as molecular markers for CCA dissemination in the bloodstream.

2. Materials and methods

2.1. Animals, patients and blood samples

Hamsters were induced to develop CCA by combined treatment with *O. viverrini* and NDMA. All animal studies conformed to the guidelines of the National Committee of Animal Ethics, Faculty of Medicine, Khon Kaen University (KKU) as previously described [13]. Forty-nine frozen and paraffin embedded archival specimens of CCA

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Table 1
Hamster and human oxysterol binding protein gene primers.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Hamster-OSBPL-8	AGGCTATTCCCCAGAAC	AGACTCAATGGAACTTTGAA
Hamster-GAPDH	AGAAGACTGTGGATGGCCCC	TGACCTTGCCCACAGCCTT
Human-OSBP1	AAGAAGAGAGAAGCGGAAGC	TGGTAACAGGGTCCTTCTTC
Human-OSBP2	GAGGCCAATACCGAGAAG	CAGCCTCTTCTCAAACCAC
Human-OSBPL-7	CAAAGTCATGGAGGAAAACA	GCCTCCAGTAGGTATTGTTG
Human-OSBPL-8	CTGGGAGACATTCAGAGTTC	CGGTGTGCTTGAAACTAAA
Human-GAPDH	TCATCAGCAATGCCTCCTGCA	TGGGTGGCAGTGATGGCA

from patients were selected from the biological sample bank of the Liver Fluke and Cholangiocarcinoma Research Center, KKU. Moreover, eight blood samples from CCA patients and four from healthy donors were included. Informed consent was obtained from each subject before surgery. The Human Research Ethics Committee of KKU approved the research protocol (HE531163).

2.2. RNA extraction, cDNA synthesis and measurement of mRNA expression levels by qRT-PCR

Total RNA and cDNA synthesis were carried out as previously described [13]. mRNA levels of hamster OSBPL-8 and human OSBP isoforms (OSBP1, OSBP2, OSBPL-7, OSBPL-8) and GAPDH were measured using a SYBR Green assay [13]. PCR primers for each OSBP isoform and GAPDH were designed using Vector NTI software (InforMax, Inc., Frederick, MD, USA). The detail of primer sequences for oxysterol binding protein genes is shown in Table 1. Data were analyzed using ABI PRISM 7500 system SDS software. Relative quantification of gene expression was determined using the comparative cycle threshold method [14], with GAPDH as the endogenous control. Levels of mRNA were expressed as the ratio of target cDNA to GAPDH.

2.3. Immunohistochemical staining

Immunohistochemical detection of OSBP2 and OSBP7 was carried out as previously described [15]. In brief, sections were incubated with goat polyclonal anti-OSBP2 antibody (1:100, Abcam, Cambridge, UK) and goat polyclonal anti-OSBPL-7 antibody (1:100, Abcam), followed by peroxidase-conjugated Envision™ (Dako, Carpinteria, CA, USA) secondary antibodies. Sections were stained with diaminobenzidine tetra hydrochloride, counterstained with Mayer's hematoxylin and examined with light microscopy.

2.4. Statistical analysis

Gene expression data were analyzed using gene expression analysis software. The Mann–Whitney *U* test was used to compare the differences in OSBP isoforms expressed among unpaired sample groups. Results were considered significant at $P \leq 0.05$.

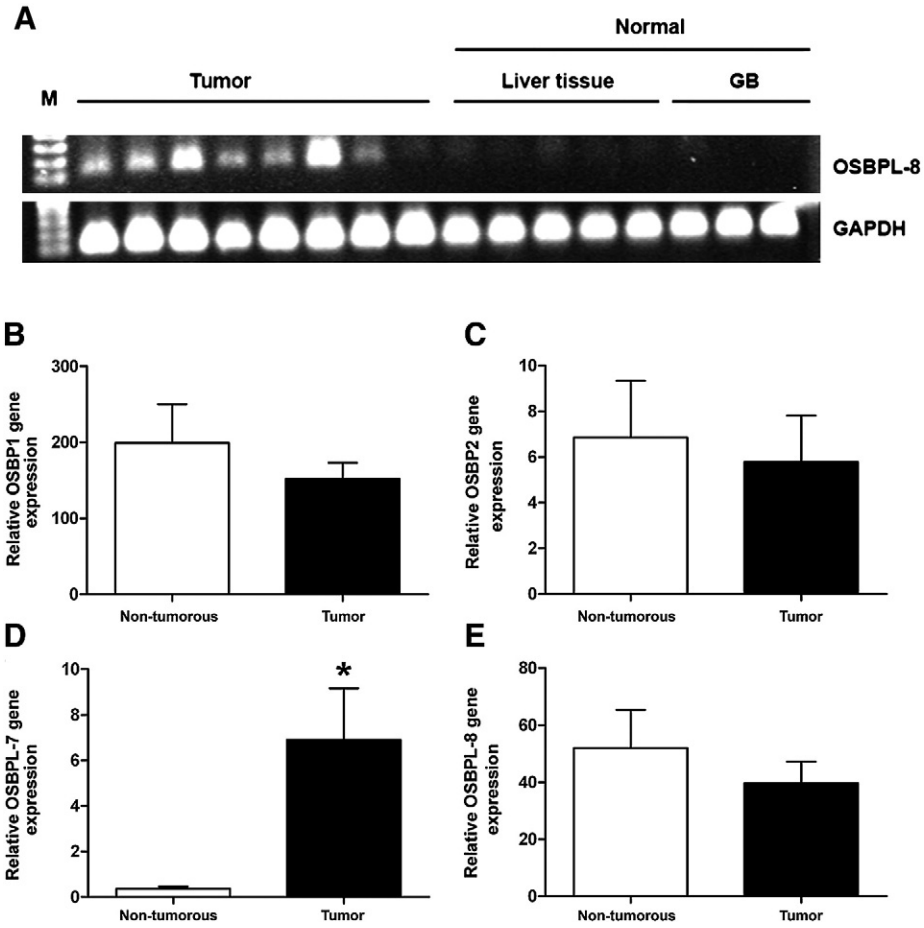

Fig. 1. Expression of hamster OSBPL-8 mRNA in *O. viverrini*-induced CCA tissues compared with normal liver tissues and normal gall bladder epithelial cells (GB), respectively. (A) The relative gene expression of OSBP1 (B), OSBP2 (C), OSBPL-7 (D) and OSBPL-8 (E) in human CCA tissues compared with non-tumor tissues. Data are expressed as the median ± S.E. *$P<0.05$ compared with adjacent non-tumor tissues.

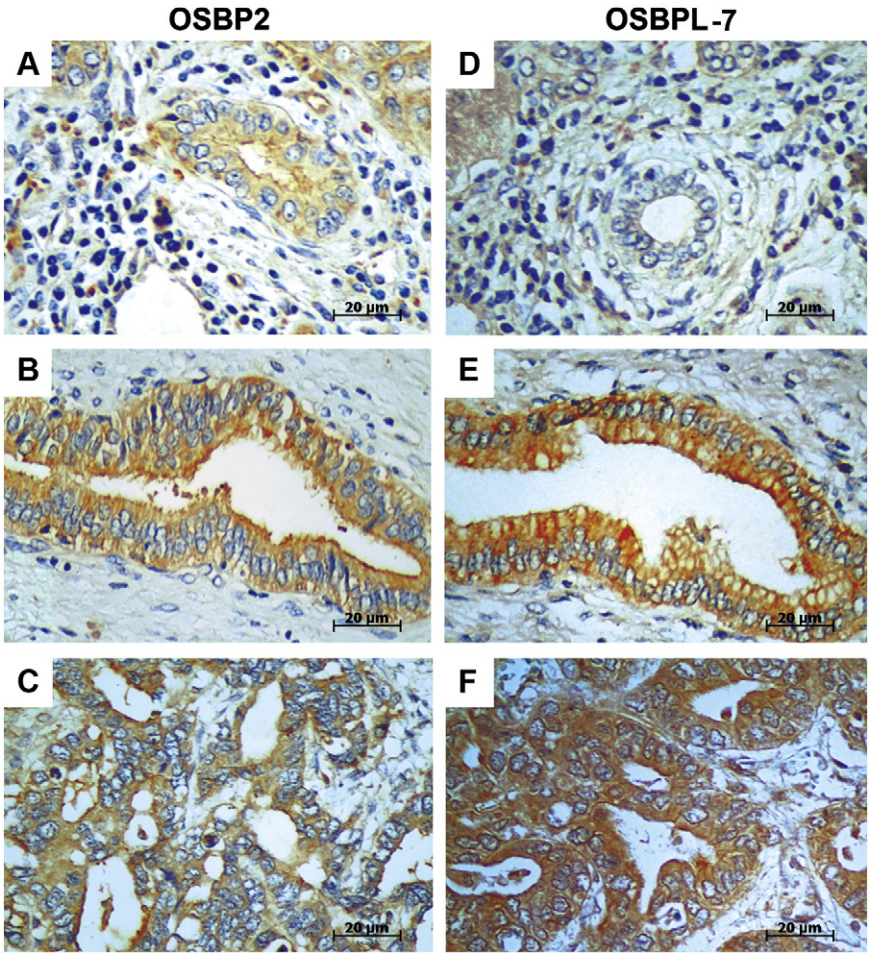

Fig. 2. The pattern of expression of OSBP2 and OSBPL-7 proteins in human CCA tissues by immunohistochemical staining, with different stages of bile duct cell transformation including normal (A, D), hyperplasia (B, E) and cancer (C, F). (40× magnification).

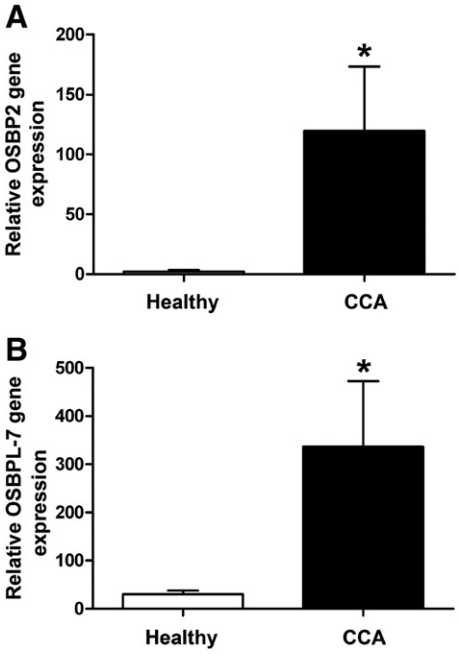

Fig. 3. Expression of OSBP2 and OSBPL-7 mRNA in blood samples from CCA patients compared to healthy controls. Data are expressed as the median ± S.E. *$P<0.05$ compared to healthy controls.

3. Results

3.1. Patient characteristics

Of the 49 intrahepatic CCA patients studied, 71.8% were male (male to a female ratio 2.5:1). The mean age was 56.9 ± 9.0 (range, 39–74 years). The histopathological type of CCA was classified as poorly differentiated (10.9%), moderately differentiated (26.1%), well differentiated (39.1%) and papillary (23.9%).

3.2. mRNA expression of OSBPL-8 in hamster CCA tissue

We confirmed the expression of OSBPL-8 in *O. viverrini*-induced hamster CCA. OSBPL-8 expression was up-regulated in 7 of 8 hamster CCA tissues at week 24, but not in normal liver or in normal gallbladder epithelial cells (Fig. 2A). Moreover, OSBPL-8 mRNA was undetectable at weeks 1 to 12 in Ov-treated hamsters and in control animals (data not shown).

3.3. mRNA expression of OSBP isoforms in human CCA tissue

mRNA expression of OSBP isoforms was compared between human CCA tumor tissue and adjacent non-tumor liver tissue by qRT-PCR. High levels of expression of OSBP1, OSBP2, OSBPL-7 and OSBPL-8 mRNA were seen in human CCA tissues (Fig. 1B, C, D and E). Moreover, significantly increased OSBPL-7 mRNA was observed in tumor compared to non-tumor liver tissue (Fig. 1D).

3.4. Immunohistochemical localization of OSBP2 and OSBPL-7

Immunohistochemical staining confirmed the mRNA findings: OSBPL-7 protein was overexpressed in tumor tissue and hepatocytes, but was absent or minimally expressed in normal biliary epithelial cells and surrounding inflammatory cells (Fig. 2D, E and F). OSBP2 protein was expressed in normal bile ducts, tumor tissue, and hepatocytes surrounding tumor tissue, but absent or minimal expression was observed in inflammatory cells (Fig. 2A, B and C).

3.5. OSBP2 and OSBPL-7 as molecular markers for CCA metastasis

We determined whether OSBP2 and OSBPL-7 could serve as markers for metastasis of CCA. Blood samples from 8 CCA patients collected before any antitumor treatment or surgery and 4 healthy controls were used. Significantly higher levels of both OSBP2 and OSBPL-7 were found in the blood of CCA patients than in healthy controls (Fig. 3).

4. Discussion

We previously identified increased expression of OSBPL-8 in *O. viverrini*-induced hamster CCA using fluorescent differential display [13]. In the present study, we have confirmed that OSBPL-8 is increased during Ov-induced cholangiocarcinogenesis. However, OSBPL-8 expression is undetectable during the early time points. This indicates that OSBPL-8 does not play a role in the early stages of *O. viverrini*-induced cholangiocarcinogenesis.

We also determined the expression of OSBP isoforms in human CCA tissues. OSBP isoforms of interest included OSBP1, which was the first isoform described [16]; OSBP2, reported to be overexpressed in various solid tumors and a potential molecular marker for hematogenous dissemination [8,9]; OSBPL-7, reported to be involved in breast cancer metastasis [10], and OSBPL-8, differentially expressed in the CCA hamster model as described above [13]. We found that all OSBP isoforms included in this study were up-regulated in human CCA tissues. Furthermore, a significant difference in mRNA expression of OSBPL-7 was found in CCA tissue compared to adjacent non-tumor tissue. Immunohistochemistry staining corroborated the mRNA results: high expression of OSBPL-7 was seen in tumor tissues and hepatocytes but not in normal biliary cells. A similar expression pattern was seen with OSBP2 except that normal bile duct epithelial cells also expressed this isoform. Immunoreactive staining of OSBP2 and OSBPL-7 was absent in inflammatory cells in CCA tissues. This finding supports previous work showing that OSBP2 could be a potential molecular marker for hematogenous dissemination of solid tumors [8].

Our findings suggest that OSBP2 and OSBPL-7, the two isoforms which have been reported to be associated with cancer metastasis [8,10], could be used as markers for detecting CCA cells circulating in the bloodstream. Expression levels of OSBP2 and OSBPL-7 in blood from CCA patients are significantly higher than in the blood of healthy controls. Low levels of these markers in peripheral blood from controls suggest that the qRT-PCR assay detects circulating tumor cells, although this finding will need verification.

A limitation of this study is the small number of CCA cases. Moreover, the detection of neoplastic cells in the peripheral circulation in CCA patients has never been documented. Nevertheless, this is the first report describing a means of potentially detecting circulating CCA cells. Further studies should be undertaken to understand how OSBP expression and its ligand (oxysterols) are regulated by *O. viverrini* infection.

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Liver fluke-induced hepatic oxysterols stimulate DNA damage and apoptosis in cultured human cholangiocytes

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ABSTRACT

Oxysterols are cholesterol oxidation products that are generated by enzymatic reactions through cytochrome P450 family enzymes or by non-enzymatic reactions involving reactive oxygen and nitrogen species. Oxysterols have been identified in bile in the setting of chronic inflammation, suggesting that biliary epithelial cells are chronically exposed to these compounds in certain clinical settings. We hypothesized that biliary oxysterols resulting from liver fluke infection participate in cholangiocarcinogenesis. Using gas chromatography/mass spectrometry, we identified oxysterols in livers from hamsters infected with *Opisthorchis viverrini* that develop cholangiocarcinoma. Five oxysterols were found: 7-keto-cholesta-3,5-diene (7KD), 3-keto-cholest-4-ene (3K4), 3-keto-cholest-7-ene (3K7), 3-keto-cholesta-4,6-diene (3KD), and cholestan-3β,5α,6β-triol (Triol). Triol and 3K4 were found at significantly higher levels in the livers of hamsters with *O. viverrini*-induced cholangiocarcinoma. We therefore investigated the effects of Triol and 3K4 on induction of cholangiocarcinogenesis using an *in vitro* human cholangiocyte culture model. Triol- and 3K4-treated cells underwent apoptosis. Western blot analysis showed significantly increased levels of Bax and decreased levels of Bcl-2 in these cells. Increased cytochrome c release from mitochondria was found following treatment with Triol and 3K4. Triol and 3K4 also induced formation of the DNA adducts 1,N^6-etheno-2′-deoxyadenosine, 3,N^4-etheno-2′-deoxycytidine and 8-oxo-7,8-dihydro-2′-deoxyguanosine in cholangiocytes. The data suggest that Triol and 3K4 cause DNA damage via oxidative stress. Chronic liver fluke infection increases production of the oxysterols Triol and 3K4 in the setting of chronic inflammation in the biliary system. These oxysterols induce apoptosis and DNA damage in cholangiocytes. Insufficient and impaired DNA repair of such mutated cells may enhance clonal expansion and further drive the change in cellular phenotype from normal to malignant.

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1. Introduction

Oxysterols are the oxygenated derivatives of cholesterol derived from either enzymatic or non-enzymatic reactions [1,2]. The production of oxysterols through the enzymatic pathway involves CYP450 family enzymes; this mechanism is integral to the conversion of cholesterol into bile acids [3]. In contrast, non-enzymatic mechanisms involve reactive oxygen and nitrogen species (ROS/RNS) [4,5]. Numerous studies describe biological effects of oxysterols in areas such as cholesterol catabolism and cholesterol homeostasis [6,7]. Pathological effects of oxysterols have also been described, including in cardiovascular disease [5], Alzheimer's disease [8], osteoporosis [9] and cancer [10–13]. Several studies describe the effects of oxysterols on various types of cancer, including those of the lung [14], breast [12], colon [15], and bile ducts [16]. Oxysterols may contribute to tumor initiation, promotion and progression [17]. Oxysterols including Triol induced increased numbers of revertants in *Salmonella typhimurium* and increased the frequency of chromosomal aberrations in CHO cells through the induction of ROS production

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[18]. 7-Ketocholesterol could damage mitochondrial DNA [19]. Oxysterols also have pro-oxidative and pro-inflammatory effects. 7α-, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α-6α-epoxide, cholesterol-5β-6β-epoxide, 22R-, 22S, 25- and 27-hydroxycholesterol act as pro-oxidative and pro-inflammatory molecules in U937 human promonocytic leukemia cells, and induce pro-inflammatory cytokines such as interleukin-8 (IL-8) and IL-1β [20,21]. Osteoblast-derived oxysterols trigger the migration of breast cancer cells toward bone [12]. Carcinogenic effects of oxysterol have been recently reviewed by Jusakul et al. [17].

Several reports show a role for oxysterols in the development of cholangiocarcinoma (CCA) [16,22], a malignant tumor of bile duct epithelium. Chronic inflammation caused by *Opisthorchis viverrini* (Ov) infection can induce the expression of inducible nitric oxide synthase in bile duct epithelia and can subsequently cause nitrative and oxidative damage to DNA, suggesting a potential role for Ov infection in CCA development [23,24]. The expression of cytosolic oxysterol-binding protein is increased in Ov-induced hamster CCA [22]. Certain oxysterols can stabilize cyclooxygenase-2 (COX-2) mRNA via a p38 MAPK-dependent mechanism resulting in COX-2 protein accumulation in CCA cells [16]. Increased concentrations of oxysterols have been reported in infected human hepatic bile [25], suggesting that biliary infection is involved in the biogenesis of oxysterols in bile through the production of reactive oxygen species by activated leukocytes [25,26].

Taken together, this body of evidence suggests that overproduction of oxysterols during chronic biliary inflammation contributes to the genesis and progression of CCA. In the present work, we determined which types of oxysterols are involved in cholangiocarcinogenesis and what roles they play using *in vivo* and *in vitro* methods.

2. Materials and methods

2.1. Chemicals

Triol and 3K4 were purchased from Steraloids Inc. (RI, USA). Monoclonal antibodies EM-A-1 against 1,N^6-etheno-2′-deoxyadenosine (εdA) and EM-C-1 against 3,N^4-etheno-2′-deoxycytidine (εdC) (cell culture supernatant) were obtained from Dr. Petra Lorenz (University of Essen, Essen, Germany). Details of the production of monoclonal antibodies and their specificity were previously reported [27,28]. 8-Oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) were purchased from Japan Institute for the Control of Aging (Fukuroi, Japan). RNaseA and Proteinase K were obtained from Qiagen (Hilden, Germany). Anti-mouse antibody Envision system conjugated with HRP was purchased from Dako (Glostrup, Denmark). Peroxidase Substrate Kit, 3,3′-diaminobenzidine (DAB) was purchased from Vector Laboratories (CA, USA).

2.2. Animal protocol and tumor induction

The animal experimental protocol was conducted according to guidelines of the National Committee of Animal Ethics, Faculty of Medicine, Khon Kaen University, Thailand. Animal induction was done according to Loilome et al. [22]. In brief, Syrian golden hamsters (Animal Unit, Faculty of Medicine, Khon Kaen University) aged 6–8 weeks were divided into 4 groups: (a) Untreated controls; (b) Ov-infected; (c) *N*-nitrosodimethylamine (NDMA)-treated; and (d) Ov plus NDMA-treated. Fifty Ov metacercariae were injected into each hamster via intragastric intubation, and NDMA was administered in drinking water (12.5 ppm) provided *ad libitum* for 2 months. Animals were sacrificed on weeks 1, 2, 3, 4, 12 and 24 after treatment. Liver tissue was collected, then immediately snap frozen in liquid nitrogen and stored at −80 °C until use. Histological changes in hamster livers were studied on paraffin-embedded sections using standard hematoxylin and eosin staining as described previously [29].

2.3. Oxysterol analysis in hamster livers

Oxysterol concentrations in hamster livers were analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously [30] with modifications. All samples were processed under nitrogen and in the presence of butylated hydroxytoluene (BHT) with careful control of temperature and pH to prevent generation of oxysterols by autooxidation. In brief, the lipid fraction of liver tissue was extracted with chloroform/methanol 2:1 containing 100 ppm BHT and cholestane as an internal standard, at room temperature. After the removal of the lower (chloroform) layer which was evaporated under nitrogen at 40 °C, the residues were saponified at room temperature overnight with 2 N methanolic potassium hydroxide. Nonsaponifiable lipids were extracted twice with diethyl ether; the combined extracts were then washed with distilled water. The ether was evaporated and the residue converted to the TMSi derivative with BSTFA/pyridine. The solvent was evaporated and the sample was taken up in 200 μl of hexane. One μl of sample was injected into a Shimadzu QP2010 plus with 60 m DB-5 column. Oven temperature was 225 °C for 2 min, then 10 °C/min to 290 °C and 2 °C/min to 320 °C with 15 min at final temperature. Sterol derivatives were eluted at 19–25 min, and spectra were identified by comparison with authentic standard (Steraloids Inc, RI, USA) and with the NIST library of spectra and the Wiley Registry of Mass Spectra, 7th edition.

2.4. Cell culture

The immortalized human cholangiocyte MMNK-1 cell line, transduced with SV40T and hTERT, was generated and supplied by Professor Naoya Kobayashi (Okayama University, Japan) Cells were cultured in Ham F'12 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

2.5. Cytotoxicity assay

MMNK-1cells (2×10^3/100 μl) were seeded into 96-well plates and incubated overnight at 37 °C and 5% CO_2. Cells were treated with Triol and 3K4 at designated concentrations for 48 h. Triol and 3K4 were dissolved in 100% ethanol and added to culture media at different concentrations; the final concentration of ethanol was 0.5%. The number of viable cells was determined using sulforhodamine B (SRB, Sigma-Aldrich, MO, USA) as described previously [31]. Briefly, cells were fixed in 10% cold trichloroacetic acid for 1 h at 4 °C and stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 min. Excess dye was washed with 1% acetic acid and stained cells were solubilized with 200 μl of 10 mM unbuffered Tris-base. The absorbance was measured with a microplate reader (Sunrise, TECAN Trading, Switzerland) at 540 nm. The results were expressed as a percentage of cell viability relative to control (cells treated with 0.5% ethanol). The concentration that inhibits cell growth to 50% of control (IC_{50}) was calculated by plotting the percentage of cell growth inhibition against oxysterol concentration.

2.6. GC/MS detection of oxysterol uptake

The incorporation of oxysterols into MMNK-1 cells was determined by GC/MS. Briefly, 1×10^6 cells were treated with 20 μM Triol or 20 μM 3K4 for 0, 3, 6, 12, 24 and 48 h. At the end of treatment, cells were trypsinized and washed with media. Cell pellets were collected and the lipid fraction extracted with chloroform/methanol 2:1 containing 100 ppm BHT and cholestane as an internal standard at room temperature. The extraction and GC/MS procedures were performed as described above. The percentage of intracellular oxysterol was shown relative to the level of oxysterol added in media before treatment.

2.7. Detection of apoptotic cells with flow cytometry using annexin V and propidium iodide (PI) staining

10^6 cells were seeded in 100 mm culture dishes overnight. Cells were then treated with Triol and 3K4 at designated concentrations for 48 h, trypsinized and washed with ice-cold PBS. Cell pellets were resuspended in 100 μl annexin-V-FLUOS labeling solution (Roche, Penzberg, Germany) and incubated at room temperature for 15 min. At least 10,000 cells were counted and analyzed with a flow cytometer (Cytomic FC500, Beckman Coulter, USA).

2.8. DNA fragmentation assay

Briefly, 10^6 cells were lysed twice with 1 ml lysis buffer (1% (w/v) Triton X-100, 0.32 M saccharose, 5 mM $MgCl_2$, 10 mM Tris–HCl) and centrifuged for 20 s at 10,000 × *g*. Cell pellets were collected and treated with 200 μl of enzyme reaction solution (1% (w/v) SDS, 5 mM EDTA–Na_2, 10 mM Tris–HCl) and 8 μl of 1 mg/ml of RNaseA for 20 min at 37 °C. Subsequently, 10 μl of 10 mg/ml Proteinase K was added to the sample and incubated for 1 h at 37 °C. Then, 300 μl of 7.6 M NaI solution (7.6 M NaI, 20 mM EDTA–Na_2, 40 mM Tris–HCl) and 500 μl of isopropanol were added to the sample. After centrifugation, the pellet was mixed with 40% isopropanol, washed with 70% ethanol and centrifuged. The pellet was dried and resuspended with 100 μl of TE buffer (10 mM Tris–HCl and 1 mM EDTA) and loaded onto 1% agarose gel in 1× TBE buffer (89 mM Tris-base, 89 mM boric acid, and 2 mM EDTA). Electrophoresis was done at 50 V for 1 h and the gel then stained with ethidium bromide for 15 min. Visualization was by Imagequant 400 (GE Healthcare Bio-Science, UK).

2.9. Western blot analysis

Cells were treated with 30 μM Triol or 80 μM 3K4 for 3, 6, 12, 18 and 24 h then 30 μg of whole cell lysate protein was separated by 10% SDS-polyacrylamide electrophoresis and blotted to PVDF membrane (GE Healthcare Bio-Science, UK). Blots were probed with 1:100 goat anti-Bax antibody (BD Biosciences, USA), 1:100 rabbit anti-Bcl-2 (C21) antibody (Santa Cruz Biotechnology, USA), 1:2000 mouse

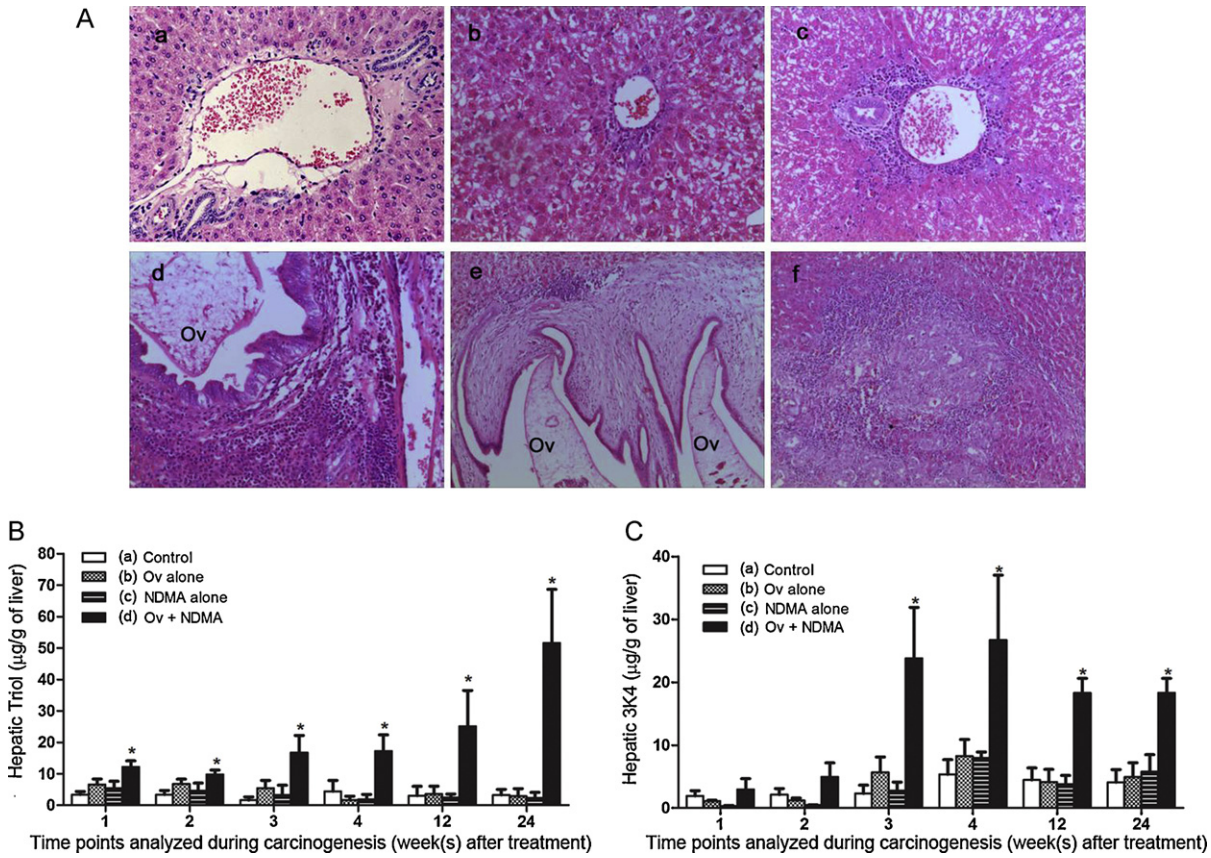

Fig. 1. (A) Representative figures of inflammatory response in livers of control group (a), NDMA-treated group (b), Ov-treated group (c), and Ov plus NDMA-treated group (d) at week 3; whereas (e) and (f) are Ov- and Ov plus NDMA-treated groups at week 24, respectively. Ov indicates localization of *O. viverrini*. Original magnifications 20×. Levels of hepatic Triol (B) and 3K4 (C) in liver of Ov-treated hamsters with (d) or without (b) NDMA treatment, NDMA treatment (c) and untreated control (a). Kruskal–Wallis one way analysis was used to test for differences in each treatment group. *Significant ($P < 0.05$) for each treatment group versus untreated control.

anti-cytochrome c antibody (Santa Cruz Biotechnology, USA), or 1:2000 rabbit anti-glyceraldehydes 3-phosphate dehydrogenase (GAPDH) antibody (Invitrogen, CA, USA). Detection was performed using a chemiluminescence western blotting system (ECL, GE Healthcare Bio-Science, UK). Signals were analyzed using Imagequant 400 (GE Healthcare Bio-Science, UK) and quantified using Imagequant TL v2005 (GE Healthcare Bio-Science, UK).

2.10. Immunocytochemical detection of DNA adducts

Staining of DNA adducts including εdA, εdC and 8-oxodG was performed as previously described [32]. Briefly, MMNK-1 cells were treated with Triol or 3K4 at the designated concentrations in slide chambers (Lab-Tek II chamber slide, Nalgene Nunc, USA). Slides were fixed with acetone for 10 min at −20 °C, then dipped in PBS for 10 min and the endogenous peroxidases quenched with 0.3% H_2O_2 in absolute methanol for 10 min. Slides were incubated in 5 μg/ml proteinase K in double distilled H_2O at room temperature for 10 min then washed with PBS and incubated with 10 μg/ml RNaseA in 50 mM Tris-base (heated for 10 min at 80 °C before use) at 37 °C for 30 min and washed with PBS. Slides were treated with 1.5 N HCl for 5 min at room temperature, rinsed with PBS and double distilled water. The pH was neutralized with 50 mM Tris-base (pH 7.4) at room temperature then washed with double distilled water and PBS. Non-specific binding sites were blocked with 10% skim milk (w/v), 0.05% Tween-20 (v/v) and 0.05% Triton X-100 (v/v) for 20 min at room temperature. Slides were incubated with primary monoclonal antibody EM-A-1 against εdA and EMC-1 against εdC at 1:10 dilution, and primary monoclonal antibody 8-oxo-dG at 1:200 dilution at 4 °C overnight. Detection of εdA and εdC was by incubation with anti-mouse Envision system conjugated with HRP (Dako, Glostrup, Denmark). For 8-oxo-dG detection, slides were incubated with rabbit anti-mouse (1:400 in PBS) for 3 h. Visualization was with DAB. After stopping the reaction, counterstaining was performed with hematoxylin. Slides were dehydrated by increasing the concentration of ethanol and mounted with Kaiser's glycerin. Results were analyzed by counting more than 500 cells.

2.11. Statistical analysis

The results were presented as a mean ± SD for at least three individual experiments. The level of oxysterols in hamster tissue was statistically analyzed using Kruskal–Wallis one-way analysis. Other data were compared using Student's *t*-test. *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Oxysterol levels in Ov-induced CCA hamster liver

Histopathological changes in hamster liver tissues are shown in Fig. 1A. There was no pathological change observed in the control group, whereas the livers of the NDMA-treated group developed mild periductal inflammation and a low degree of bile ductule proliferation and cholangiofibrosis, which was seen at week 12. In the Ov-treated group, the early pathological changes consisted of acute inflammatory changes observed around the bile ducts where liver flukes reside. The highest degree of inflammation was observed at week 3. As liver flukes developed into adult worms, they induced hyperplasia of the bile duct epithelia. In the chronic phase, a decrease in the number of inflammatory cells and the thickness of fibrosis were observed. In the Ov plus NDMA-treated group, most of the liver alterations were similar to those found in the Ov infected alone group, with the exception of a greater degree of inflammation. In addition, cholangiocarcinoma was observed at week 24, with inflammatory cells still observed in areas with tumor. Alterations in Triol and 3K4 levels during cholangiocarcinogenesis in hamsters are shown in Fig. 1B and C, respectively. Besides 3K4 and Triol, several other oxysterols were found in hamster livers, specifically 7KD, 3K7 and 3KD. There were no significant changes in these oxysterols in all treatment groups and time points compared to control (data not shown). The increase in both Triol and 3K4 levels in hamster livers were observed in the Ov infected-group.

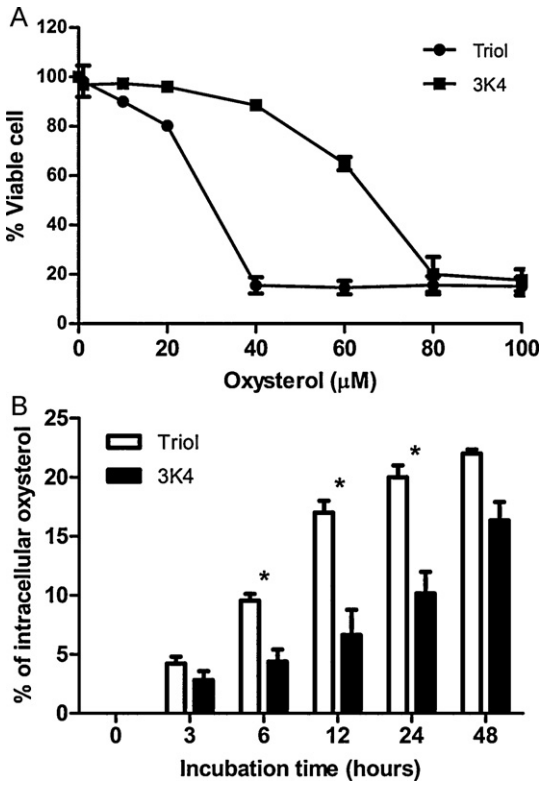

Fig. 2. (A) Cytotoxic effects of Triol and 3K4 on the induction of MMNK-1 cell death. MMNK-1 cells were cultured with Triol and 3K4 at the designated concentrations ranging from 1 to 100 μM in 0.5% ethanol. After 48 h, the cellular proteins of viable cells were measured using the sulforhodamine B assay. Values are expressed as the mean ± standard deviation of percentage of control cells incubated with 0.5% ethanol for three individual experiments. (B) Oxysterol uptake was analyzed by GC/MS at 0, 3, 6, 12, 24, 48 h after treatment with 20 μM Triol and 20 μM 3K4. The histograms are expressed as a percentage of the total amount of oxysterol added to the incubation medium. Significantly higher levels of intracellular Triol compared to 3K4 were found at 6, 12 and 24 h of incubation. Standard deviations were calculated from three individual experiments. *Significantly ($P < 0.05$) higher level of Triol compared to 3K4.

Notably, significantly increasing levels of 3K4 in the Ov infected-group were found at week 3 ($P < 0.05$ versus control). In the NDMA group, the levels of Triol and 3K4 were low and no significant differences were seen compared to the control group at all time points. Levels of Triol in the Ov plus NDMA group were statistically significantly higher than those in the control group ($P < 0.05$) from week 1 until week 24 after treatment. Moreover, levels of 3K4 tended to be higher at week 1 and 2, although these differences did not reach statistical significance. Increasing levels of 3K4 were found at week 3, 4, 12 and 24 ($P < 0.05$ versus control). Increased levels of oxysterols in Ov plus NDMA-treated hamsters were observed at an early stage of carcinogenesis (week 4) until tumor was developed (week 24). We therefore selected Triol and 3K4 for further studies aimed at identifying molecular mechanisms involved in the development of CCA using *in vitro* methods.

3.2. Effects of Triol and 3K4 on MMNK-1 cholangiocytes

The effects of Triol and 3K4 on MMNK-1 cell survival were studied. Oxysterols were added to cells at concentrations ranging from 1 to 100 μM for 48 h. Fig. 2A shows the effects of Triol and 3K4 on the induction of cell death, which occurred in a dose-dependent manner. The concentrations at which Triol and 3K4 inhibited cell growth to 50% of control (IC_{50}) were 27.5 ± 3.0 and 63.3 ± 5.8 μM, respectively. Triol had a greater inhibitory effect on cell growth than did 3K4. We therefore used higher concentrations of 3K4 than Triol in subsequent experiments.

3.3. Time course of oxysterol uptake

Intracellular oxysterol uptake by MMNK-1 cells (Fig. 2B) was determined by GC/MS. Low level uptake of Triol and 3K4 was found at 3 h after treatment (4.2% and 2.8% of levels in media, respectively). Mean Triol uptake was 9.5%, 13.7%, 19% and 22%, whereas mean 3K4 uptake was 5%, 6.6%, 10.1% and 16.3% of the total amount added at 6, 12, 24, 48 h, respectively. Notably, higher Triol uptake was seen when compared to 3K4 ($P < 0.05$).

3.4. Triol and 3K4 induce MMNK-1 cell apoptosis

We next investigated the mechanism of oxysterol-induced MMNK-1 cell death using flow cytometry. Cells were treated with 10, 20, 30 and 40 μM Triol, or 10, 20, 40, 80 μM 3K4 for 48 h. Oxysterol-treated cells were stained with annexin V and PI and then analyzed by flow cytometry. Fig. 3 shows the effects of Triol and 3K4 on the induction of MNNK-1 cell apoptosis. Increasing numbers of apoptotic cells (as determined by annexin V^+/PI^- and annexin V^+/PI^+) were observed with increasing concentrations of Triol and 3K4. Significant numbers of apoptotic cells were found following treatment with 30 and 40 μM Triol compared to the control ($P < 0.05$). However, only 80 μM 3K4 induced significant apoptosis ($P < 0.05$).

We further clarified the effect of oxysterols on the induction of apoptosis using a DNA fragmentation technique involving agarose gel electrophoresis. Cells were treated with Triol and 3K4 at the designated concentrations for 48 h then DNA was extracted. Oligonucleosomal cleavage was moderately and maximally elevated following treatment with 30 and 40 μM Triol, respectively (Fig. 4A). In contrast, treatment with 40 μM 3K4 slightly increased DNA fragmentation. A marked increase in DNA laddering was found when cells were treated with 80 μM 3K4 (Fig. 4B).

3.5. Activation of apoptosis-related proteins by Triol and 3K4

To investigate the molecular pathways of apoptosis induced by Triol and 3K4, we identified the induction of apoptosis-related proteins in MMNK-1 cells using western blot analysis. Cells were treated with 30 μM Triol or 80 μM 3K4 and proteins were extracted at 0, 3, 6, 12, 18 and 24 h. Fig. 5 shows the effects of Triol or 3K4 on cytochrome c release, activation of Bax and inhibition of Bcl-2. There was gradual release of cytochrome c in Triol- and 3K4-treated cells (Fig. 5A and B, respectively). Increasing levels of Bax were found in both Triol and 3K4-treated cells. Conversely, decreasing levels of Bcl-2 were found in Triol- and 3K4-treated cells. Thus, both Triol- and 3K4-induced apoptosis involve mitochondrial-dependent mechanisms.

3.6. Triol and 3K4 induce DNA damage in MMNK-1 cells

We investigated the effects of Triol and 3K4 on the induction of DNA damage using immunocytochemistry. DNA adducts were detected at 48 h after incubation. We used specific monoclonal antibodies against etheno-DNA base adducts called EMA-1 and EM-C-1 raised against εdA and εdC, respectively. A specific antibody against the free radical-derived DNA adduct, 8-oxodG, was also used.

Fig. 6 shows staining for εdA, εdC and 8-oxodG induced by Triol. The stains for these DNA adducts were located in the nuclei of MMNK cells by hematoxylin staining. No staining for εdA and εdC was observed in control groups. Significant increases in εdA, εdC and 8-oxodG levels were found when cells were treated with low concentrations of Triol (10 μM) ($P < 0.05$) compared to control

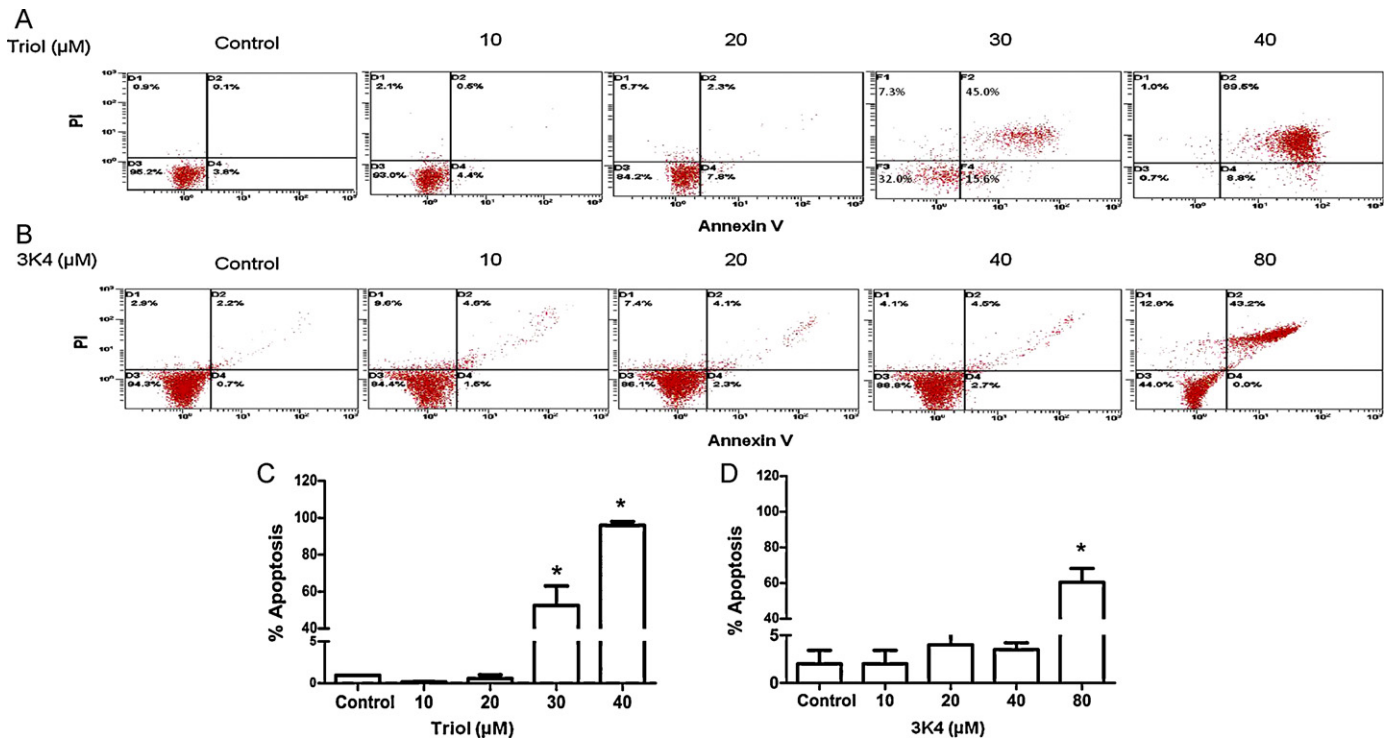

Fig. 3. MMNK-1 cells were treated with 10–40 μM Triol (A) and 10–80 μM 3K4 (B) for 48 h. After incubation, harvested cells were stained with annexin V and propidium iodide (PI). Stained cells were analyzed for apoptosis by flow cytometry. Live cells stained annexin V^-/PI^- (left bottom panel) and apoptotic cells stained annexin V^+/PI^- or annexin V^+/PI^+ (right bottom and right top panel). These cells were distinguished by fluorescent intensity of annexin V (abscissa) and PI (ordinate) in logarithmic scales. Bar graphs show mean ± SD of % apoptotic cells induced by Triol (C) and 3K4 (D) compared to controls which were 0.5% ethanol-treated cells. $^*P < 0.05$.

groups (Fig. 8A). Beyond these concentrations of Triol (10–30 μM), DNA adduct formation gradually increased.

Staining for εdA, εdC and 8-oxodG was also found in 3K4-treated cells (Fig. 7). Small numbers of positive cells were found in control groups. We found 20 μM and 30 μM 3K4 significantly increased the levels of εdA and εdC ($P < 0.05$). The lowest concentration of 3K4 (10 μM) significantly increased the level of 8-oxodG ($P < 0.05$) as shown in Fig. 8B. Thus, both Triol and 3K4 increased the formation of εdA, εdC and 8-oxodG in MMNK-1 cells.

4. Discussion

The discovery of increased expression of oxysterol-binding proteins in hamsters with Ov-induced CCA [22] led us to hypothesize that oxysterols participate in the processes that initiate and promote the development of malignancy in the biliary epithelium. We first analyzed oxysterols in the livers of hamsters induced to develop Ov-associated CCA. Five oxysterols were identified: 7KD, 3K4, 3K7, 3KD and Triol; these oxysterols were found in both normal liver and tumor tissues. Notably, only Triol and 3K4 showed

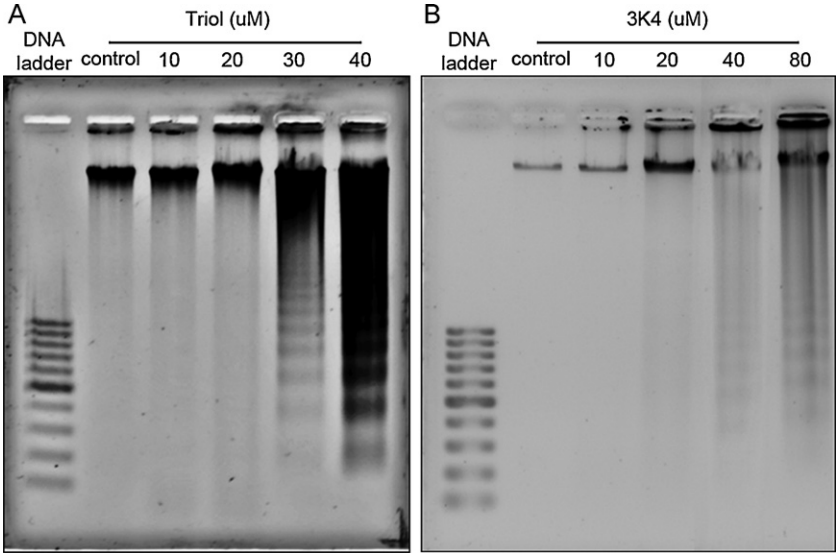

Fig. 4. DNA fragmentation in Triol (A)- and 3K4 (B)-induced MMNK-1 cell apoptosis analyzed by 1% agarose gel electrophoresis. Cells were treated with 10–40 μM Triol and 10–80 μM 3K4 for 48 h. First lanes were 100 bp DNA ladder. 0.5% ethanol-treated cells were used as control.

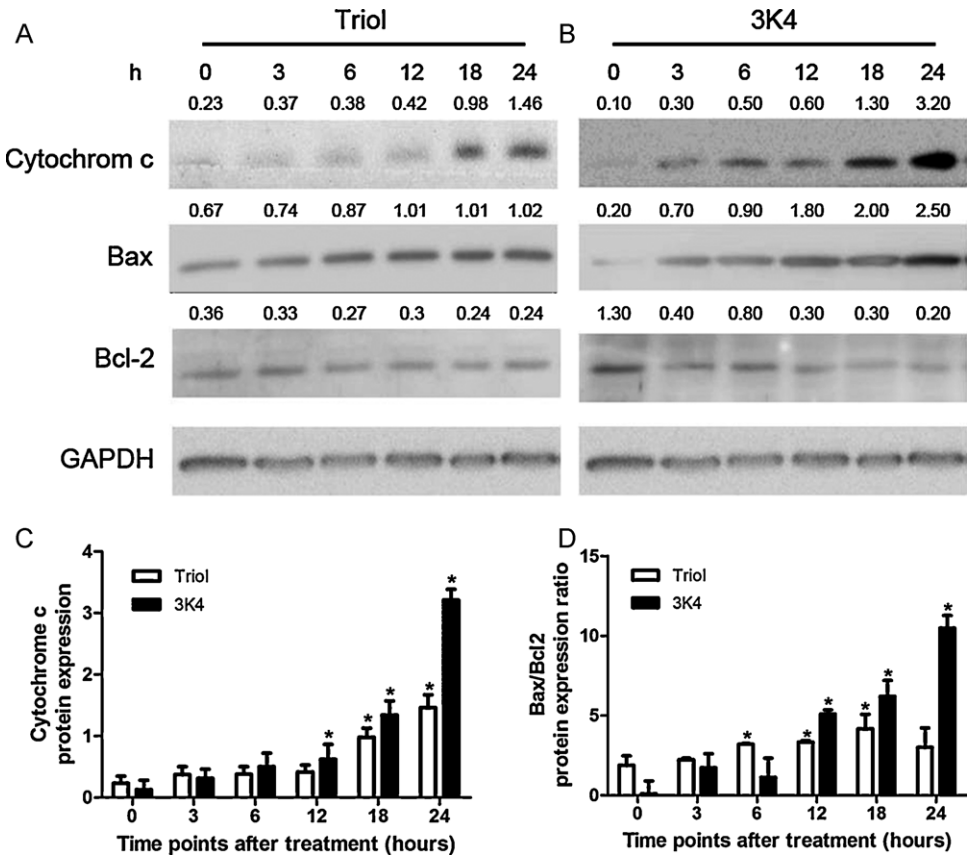

Fig. 5. Effects of Triol and 3K4 on the activation of apoptosis-related proteins. MMNK-1 cells were treated with 30 μM Triol (A) or with 80 μM 3K4 (B) for 3, 6, 12, 18 and 24 h. Protein extracts were analyzed by western blot with specific antibodies against cytochrome c, Bax, and Bcl-2. Bar graphs show relative densitometric values of western blot of Triol and 3K4 on induction of cytochrome c protein expression (C). The ratios of Bax/Bcl2 protein expression (D) were analyzed at 0, 3, 6, 12, 18 and 24 h after treatment. Values were normalized to GAPDH and expressed as mean ± SD from three individual experiments. *$P < 0.05$ compared to the result at 0 h.

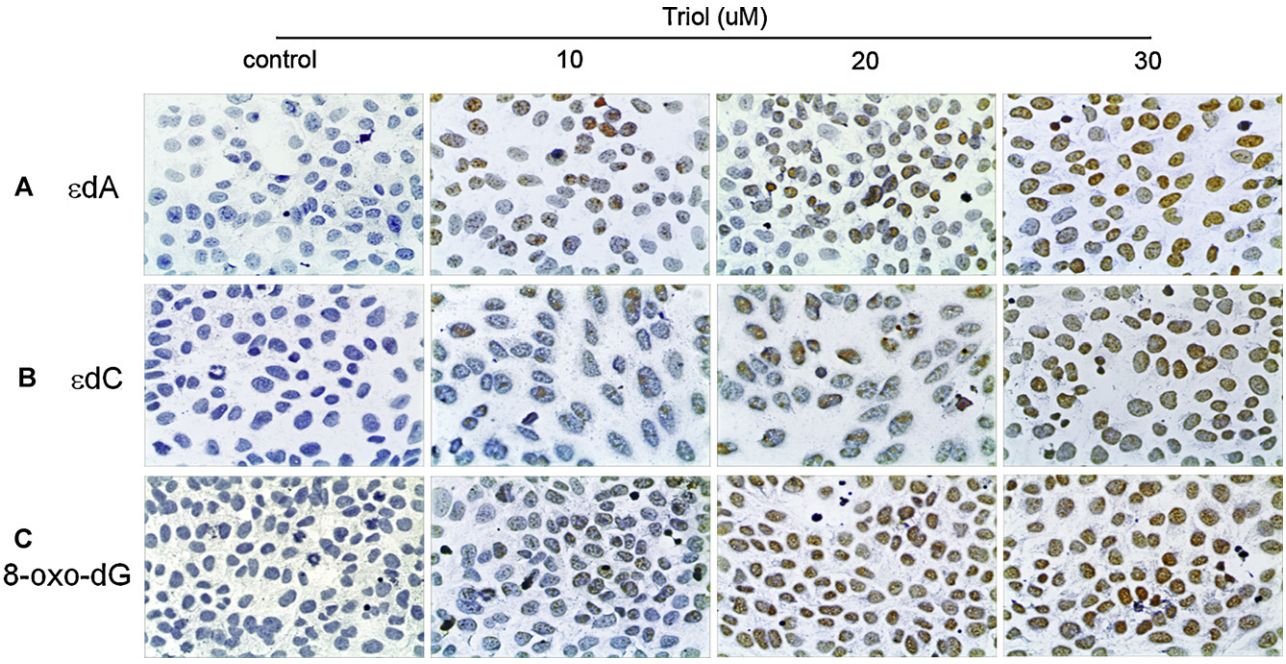

Fig. 6. DNA adducts formation in Triol-treated MMNK-1 cells. Cells were treated with 10, 20 and 30 μM Triol for 48 h and immunocytochemical staining was used to detect εdA (A), εdC (B) and 8-oxodG (C). The production of DNA adducts increased after incubation with increasing concentrations of Triol as compared to 0.5% ethanol-treated cells (control). Magnification 400×.

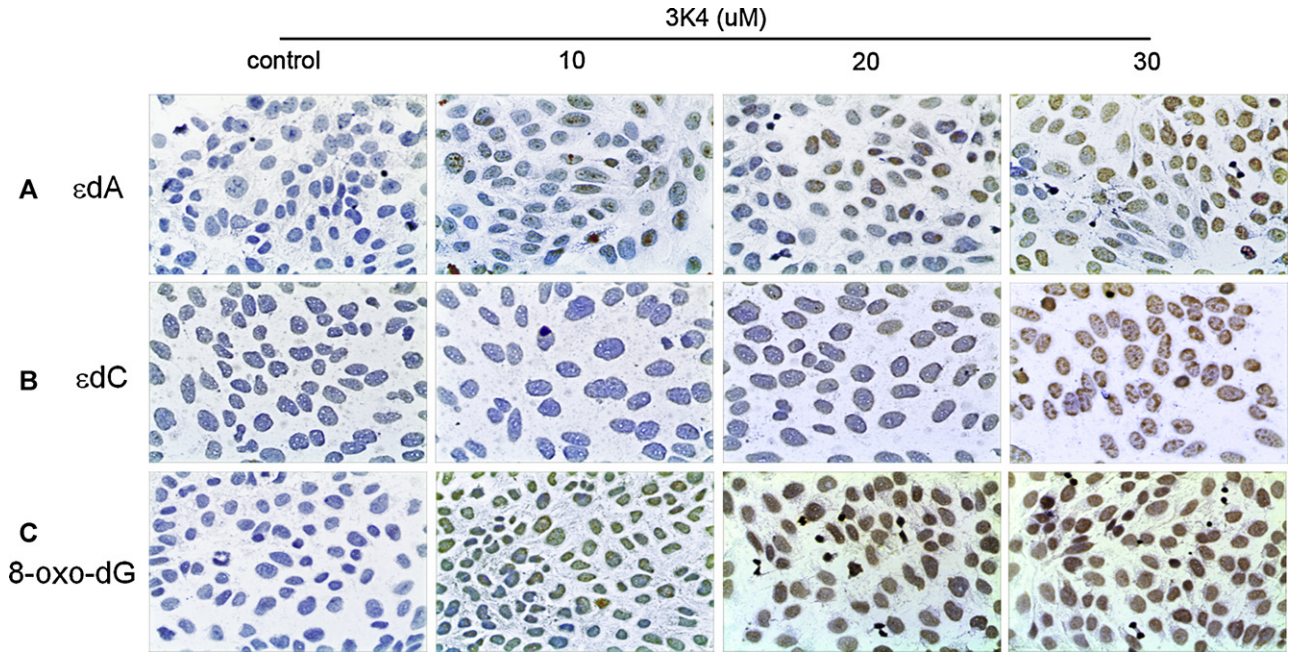

Fig. 7. DNA adducts formation in 3K4-treated MMNK-1 cells. Cells were treated with 10, 20 and 30 μM 3K4 for 48 h and immunocytochemical staining was used to detect εdA (A), εdC (B) and 8-oxodG (C). The production of DNA adducts increased after incubation with increasing concentrations of Triol as compared to 0.5% ethanol-treated cells (control). Magnification 400×.

significantly increased levels in tumor tissue compared to normal liver. A previous study reported that the same two oxysterols were present in hepatic bile of patients with biliary tract disease and pigment gallstones, with their levels correlating with serum C-reactive protein levels [26]. 7KD and 3KD were also found in higher levels in human gallstones [25]. We propose that Triol and 3K4 are involved in inflammation-associated diseases in the biliary tract, including Ov-induced CCA. The histopathological changes in livers of treated hamsters are similar to those described in previous reports [28,33]. There were no significant histological changes in untreated hamsters at any time point examined, whereas mild degrees of inflammation were observed in the livers of hamsters treated solely with NDMA. The histological changes in both untreated and NDMA-treated groups corresponded to oxysterols levels in our experiment. There were no significant differences in oxysterol levels between untreated and NDMA-treated groups. Nevertheless, the inflammatory response corresponded to the accumulation of inflammatory cells in Ov-treated hamsters. The highest numbers of inflammatory cells were observed at week 3 and markedly decreased from week 8 until week 24 [29,34]. There was no significant difference in Triol levels between Ov-treated hamsters and the control group. Notably, significantly increased levels of 3K4 were seen at week 3 compared to the control group. In Ov plus NDMA-treated hamsters, abundant inflammatory cells were detected at week 3. In the chronic phase, decreased inflammatory cells were observed [28]. However, accumulation of mononuclear cells and lymphoid follicles were also reported. The most prominent histological changes at these time points were periductal fibrosis, ductal dilation and granulomatous inflammation [33]. Along with rising levels of oxysterols in the Ov plus NDMA group, production of Triol and 3K4 showed an upward trend in the acute phase (week 1–week 3 after treatment). However, high levels of both Triol and 3K4 were found in the chronic phase when decreased inflammation was reported. Thus, we postulated that oxysterols may be involved in the inflammatory process but the level of oxysterols may not correlate linearly with the degree of inflammation.

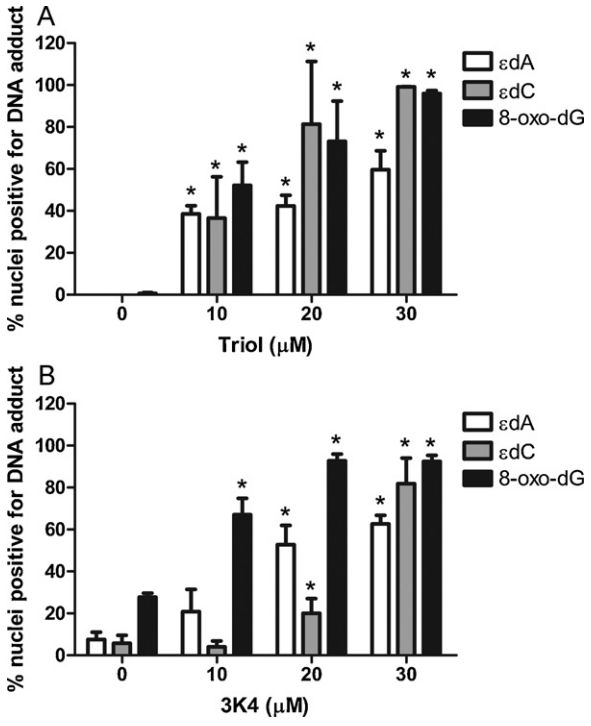

Fig. 8. Bar graph comparisons of % nuclei positive for DNA adducts induced by Triol (A) and 3K4 (B) in MMNK-1 cells. *$P < 0.05$ versus controls.

We studied the role of Triol and 3K4 on induction of cholangiocarcinogenesis using the immortalized human MMNK-1 cholangiocyte line. Both Triol and 3K4 induced cell death in a dose-dependent manner, although the doses at which cell death was induced differed. The difference in IC_{50} between Triol and 3K4 may be explained by their structures. We also determined the time course of oxysterol incorporation into MMNK-1 cells. As shown in Fig. 2B, we found higher accumulation of Triol than 3K4 in MMNK-1 cells. We propose that the cytotoxic effect of oxysterols may relate to different rates of cellular uptake as determined by oxysterol

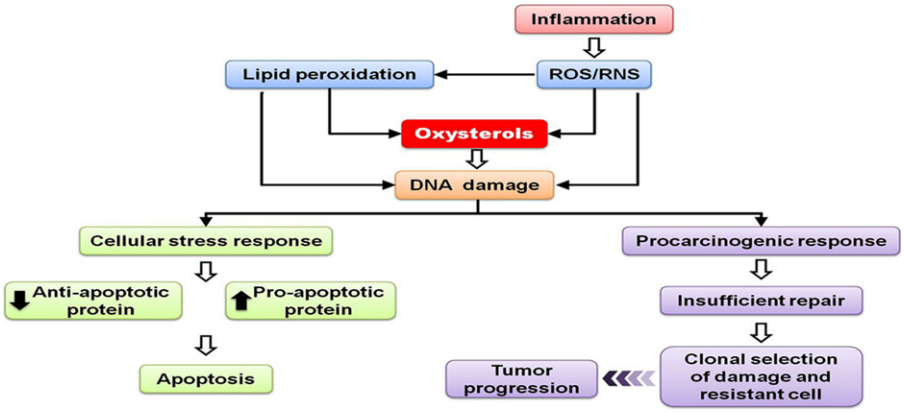

Fig. 9. Proposed scheme showing the role of oxysterols in cholangiocarcinogenesis. Oxidative stress triggered by liver fluke infection induces the release of ROS/RNS. These free radicals cause oxysterol production through induction of cholesterol oxidation and lipid peroxidation. Oxysterols, free radicals and lipid peroxidation products all cause DNA damage. Damaged cells are eliminated through apoptosis as a normal cellular defense. Ineffective or aberrant DNA repair contributes to increased mutation rate and genomic instability. Ongoing exposure to DNA damaging agents selects for apoptosis resistant cells that clonally expand to become malignant.

structure. Insertion of Triol (which is more polarized than 3K4) into cell membranes could more readily cause defects in membrane structure and packing, resulting in increased membrane permeability and membrane dysfunction [35–37]. Moreover, differences in oxysterol metabolism may alter cytoplasmic oxysterol levels that could in turn influence its cytotoxicity.

Concentrations of oxysterols used in our studies (1–100 μM) were of the same order of magnitude used in previous studies [38,39]. There is little information regarding oxysterol concentrations in various organ systems *in vivo*. Triol in human hepatic bile ranged from 18 to 387 nM [38], and 3K4 in gallbladder bile ranged from 218 to 1074 parts per million [25]. Concentrations of oxysterols in our study were higher. We justify the use of higher concentrations because under pathological conditions such as inflammation, the production of oxysterols is markedly increased through non-enzymatic reactions involving ROS/RNS [4]. This is supported by data from Haigh et al. who showed that infection and inflammation can lead to oxysterol production, including species known to be carcinogenic [26]. Secondly, cholangiocytes are exposed to bile containing oxysterols over a long time course in the setting of chronic inflammation induced by Ov infection. Such long term, low dose experiments would not be feasible in a laboratory setting, hence the need to accelerate and amplify the effects of oxysterols with higher doses.

Several studies have described the mechanisms of oxysterol-induced apoptosis in different cell systems and models, including neurons, gallbladder epithelial cells, colon adenocarcinoma cells, monocytes and hepatoma cells [15,40–42]. No previously reported study used human cholangiocytes. We also elucidated the mechanisms by which oxysterols induced cell death in MMNK-1 cells. Both Triol and 3K4-treated cells exhibited increasing levels of apoptosis with increasing concentrations of oxysterols. The apoptosis-inducing effects of Triol and 3K4 were confirmed by DNA fragmentation analysis. Both Triol and 3K4 induced formation of DNA ladders. Activation of Bax, a pro-apoptotic protein was found in Triol and 3K4-treated cells. We also found decreasing levels of the anti-apoptotic protein, Bcl-2, along with enhanced cytochrome c release. These results indicate that Triol and 3K4 triggered apoptosis via mitochondria-dependent mechanisms: activated Bax interacting with Bcl-2 resulting in Bcl-2 inactivation, followed by the disruption of mitochondrial membrane permeability resulting in cytochrome c release, activation of caspase proteins, and degradation of DNA.

DNA damage is theorized to be the mechanism by which chronic inflammation induced by Ov infection leads to cancer. Overproduction of ROS and RNS generates DNA adducts. For example, 8-oxodG and 8-nitroguanine were found in bile duct epithelia of Ov-infected hamsters [43]. Higher 8-oxodG levels were reported in Ov-infected patients compared to healthy subjects [44]. Ov-infected patients were found to have the lipid peroxidation (LPO)-derived etheno-DNA adducts εdA and εdC in white blood cell DNA and urine [45,46]. Accumulation of these etheno-DNA adducts correlated with cancer development in hamster bile ducts [28]. Based on this body of evidence, we decided to investigate whether Triol and 3K4 can induce DNA damage. We found that Triol triggers a significant increase in the production of εdA, εdC and 8-oxodG even at low concentrations (e.g. 10 μM). εdA, εdC and 8-oxodG were also induced by 3K4 but at higher concentrations (e.g. 20 μM) than Triol.

Effects of oxysterols on oxidative DNA damage have been described in human monocytic leukemia cells. 7-ketocholesterol and 7β-hydroxycholesterol, found at high levels in atherosclerotic lesions, induced 8-oxodG formation in U937 cells [47]. Oxysterols induced lipid peroxidation through increased malondialdehyde and 4-hydroxynonenal levels [47]. Triol also induced chromosomal aberrations and ROS in Chinese hamster ovary cells [18]. Based on these previously published data and our results, we propose that oxysterols are not only directly involved in the production of free radical-derived DNA adducts through the induction of ROS/RNS [15,18,20,48], but can also induce the formation of LPO-derived DNA adducts [47,48].

Thus, the proposed mechanisms whereby oxysterols induce apoptosis involve oxidation and inflammation [49,50]. Pro-apoptotic effects of oxysterols involve overproduction of free radicals [21]. Oxysterols increase intracellular ROS levels by inducing NADPH oxidase [15,51]. Oxysterol-induced ROS decrease transmembrane mitochondrial potential and intracellular levels of antioxidants such as glutathione [49]. Reduction of glutathione with corresponding overproduction of ROS leads to caspase activation [21]. Oxysterols that activate nuclear factor kappa B through ROS-mediated mechanisms enhance pro-inflammatory cytokines such as IL-1β and IL-8 [20,21,52], leading to the recruitment of immunocompetent cells [53].

Persistent production of pro-oxidative molecules such as ROS/RNS induced by oxysterols [47,49] perturb cellular stress responses. ROS/RNS and nitric oxide can impair DNA repair and inhibit apoptosis [54,55]. Excess nitric oxide may play an important role in cholangiocarcinogenesis [23]. Apoptosis induction by oxysterols may stimulate some cells to become resistant to apoptosis and thereby enhance the carcinogenic process. This could occur

when DNA repair systems or apoptosis are not sufficient to counteract it. The imbalance of DNA repair mechanisms was proposed to be an important mechanism in DNA damage-enhanced carcinogenesis [28,56]. Thus, oxysterol-induced impairments in the DNA repair system needs further investigation. Low concentrations (10 μM of Triol and 3K4) of oxysterols induce high levels of DNA-adducts but low cell death (Figs. 2A and 8), supporting the concept that certain cells may become resistant to apoptosis. Chronic exposure of biliary epithelial cells to pathogenic oxysterols may allow selective growth of apoptosis-resistant cells. This hypothesis has been previously described in bile acid-induced gastrointestinal cancer [57]. Long-term exposure to bile acids allowed selected cells to become resistant to apoptosis *in vitro* and *in vivo* [58,59].

Other concurrent causes may enhance cholangiocarcinogenesis. Increased production of proinflammatory cytokines such as IL-1, IL-6 and TNF alpha; and oxidative stress response enzymes such as COX-2 and free radical molecules, may disturb the balance of oxidant/anti-oxidant molecules and DNA repair mechanisms leading to enhanced survival [60]. Moreover, fibrosis caused by dysregulation of tissue repair in chronic inflammation may affect normal cell function leading to enhanced survival [61,62].

In conclusion, we identified the oxysterols Triol and 3K4 as potential mediators of cholangiocarcinogenesis. Triol and 3K4 induced cell apoptosis via mitochondrial-dependent mechanisms. Triol and 3K4 induced DNA damage in MMNK-1 cells. We propose that chronic liver fluke infection increases the production of ROS/RNS in the setting of chronic inflammation in the biliary system, leading to the formation of oxysterols via oxidation of biliary cholesterol. Oxysterols and free radicals can induce DNA damage. Damaged biliary epithelial cells are eliminated through induction of apoptosis. Ineffective DNA repair and ongoing exposure to DNA damaging agents select for resistant cells that clonally expand to become malignant (Fig. 9).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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RESEARCH ARTICLE

Identification of Biliary Bile Acids in Patients with Benign Biliary Diseases, Hepatocellular Carcinoma and Cholangiocarcinoma

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Abstract

Bile acids are implicated as aetiological factors in many types of gastrointestinal tract cancer including cholangiocarcinoma (CCA). Alterations in bile acid concentrations may affect the pathogenesis of these different types of cancer. Our aim was to determine the bile acid profile in gallbladder bile from patients who underwent liver resection. Thirty-seven patients with cholangiocarcinoma, 5 with hepatocellular carcinoma, and 7 with benign biliary diseases were studied. High pressure liquid chromatography was used to analyze conjugated and unconjugated bile acids. CCA patients with low (≤2 mg/dl) and high (>2 mg/dl) levels of total serum bilirubin had significantly higher total bile acid and conjugated bile acid concentrations than the benign biliary disease group. Markedly elevated levels of cholic and chenodeoxycholic acid were found in CCA cases with high levels of total serum bilirubin. Concentrations of total bile acids and primary bile acid were correlated with serum cholesterol, bilirubin and ALP in CCA. Notably, correlation of the carcinoembryonic antigen, a tumor marker, was found with level of total bile acids and chenodeoxycholic acid. These findings suggest a different pattern of bile acid concentration in cancer patients compared to patients with benign biliary diseases. Thus, accumulation of certain bile acids may be involved in carcinogenesis.

Keywords: Bile acids - cholangiocarcinoma - hepatocellular carcinoma

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Introduction

Bile acids are sterol-derived compounds synthesized and secreted by hepatocytes into bile canaliculi (Plaa et al., 1982). Bile acids are actively secreted by the liver into bile and discharged into the intestinal lumen upon ingestion of a meal. Bile acids confer detergent-like properties that are important for their physiological functions in absorption of dietary lipids and fat-soluble vitamins from the small intestine (Chiang, 2009). Bile acids that are synthesized from cholesterol in hepatocytes are termed primary bile acids. The most abundant primary C24 bile acids are chenodeoxycholic acid (CDCA) and cholic acid (CA). Bile acids that are formed by bacterial modification by removal, oxidation, or epimerization of the nuclear hydroxyl groups of primary bile acids are termed secondary bile acids. The most common C24 7-deoxy bile acids are lithocholic acid (LCA), formed by bacterial 7-dehydroxylation of CDCA, and deoxycholic acid (DCA), formed by bacterial 7-dehydroxylation of CA.

Bile acids are also implicated as causative agents in cancers of the gastrointestinal tract, including cancers of the esophagus, stomach, small intestine, biliary tract, pancreas and colon (Kuwahara et al., 1989; Reveille et al., 1990; Ross et al., 1991; Bayerdorffer et al., 1995; Kauer and Stein, 2002; Tucker et al., 2004). As bile fluid contains major pool of bile acids, the interaction of bile acids with cholangiocytes leads to alterations of cholangiocyte secretion, proliferation, apoptosis and differentiation (Alpini et al., 1999; Alpini et al., 2001; 2002a). Deleterious effects of bile acid exposure that are thought to be related to carcinogenesis include induction of reactive oxygen and reactive nitrogen species; induction of DNA damage, mutations, apoptosis in the short term, and selection for apoptosis resistance in the long term (Craven et al., 1987; Booth et al., 1997; Crowley-Weber et al., 2002). Bile acids may promote carcinogenesis by stimulating a variety of kinase signaling pathways; for example proliferation of cholangiocytes induced by bile acids occurs by a phosphatidylinositol 3-kinase-dependent pathway (Alpini et al., 2002b). Transactivation of the epidermal growth factor receptor by bile acids has been shown to stimulate cholangiocyte proliferation (Yoon et al., 2002a).

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Although studies have shown that bile acids can be carcinogenic, there are few reports evaluating bile acid composition in biliary tract cancer, especially for liver fluke-associated CCA. Increased levels serum bile acids such as CA and CDCA have been reported in human opisthorchiasis (Migasena et al., 1983). N-nitrosotaurocholic and N-nitrosoglycocholic acid are mutagenic in the Ames test (Puju et al., 1982). DCA is also a potent promoter of diethylnitrosamine-initiated hepatocarcinoma (Cameron et al., 1982). Most previous studies have reported bile acid levels in serum but not in bile or tumor tissue. The objective of this study is to compare bile acid concentrations and composition between subjects with cholangiocarcinoma, hepatocellular carcinoma and benign biliary diseases. This information may be useful to elucidate the contribution of certain bile acids in cancer development in future studies.

Materials and Methods

Patients and bile collection

Bile samples were collected from patients who were undergone liver resection at Srinagarind Hospital, Khon Kaen, Thailand. Forty-nine bile samples were successfully collected. There were 37 patients with cholangiocarcinoma, 5 patients with hepatocellular carcinoma, and 7 patients with benign biliary diseases, including cholangitis and cystadenoma. The diagnosis was based on clinical findings, ultrasonography and histological data. The study protocol has been approved by the Khon Kaen University Ethics Committee for Human Research (HE 531163). Informed consents were obtained from all subjects. Ten to fifteen ml of bile was collected via gallbladder puncture. Samples were supplemented with 100 ppm of butylated hydroxytoluene, flushed with nitrogen, and stored at -70°C until analyses. All biochemical analyses that were used in this study were obtained from the Clinical Chemistry Laboratory Unit of Srinagarind Hospital.

Bile acid analysis

Samples were centrifuged at 3500 rpm for 10 min. The bile samples (100 μl each) were transferred to a glass tube containing 500 μg of internal standard (5β-cholanic acid). Samples were dried under nitrogen at 50°C and re-dissolved in 1 ml of distilled water (DW). Samples were then subjected to solid-phase extraction using a Bond-Elute C18 cartridge (Varian Assoc, Walnut creek, CA). The C18 cartridge was preconditioned prior to loading with successive elutions by 2 ml of chloroform – methanol 2:1 (v/v), 5 ml of methanol and 5 ml of (DW). After loading each sample, the column was washed with 2 ml of DW and 2 ml of n-hexane. The column was left for 10 minutes to remove excess solvents. Bile acids were recovered from the cartridge by elution with 5 ml of methanol. The eluents were then evaporated to dryness under nitrogen at ~50°C and the residue was dissolved in 1 ml of methanol.

High pressure liquid chromatography (HPLC) analysis for conjugated and free bile acids was carried out with a three pump Rainin Dynamax system (Rainin Inst., Woburn, MA). The sample was applied to a 4.6x250 mm Spherisorb ODS2, 5 μM (Phase Separations Ltd., Clwyd, U.K) and eluted with gradient from 65% methanol/37% 0.03 M ammonium acetate, pH 4.5 to 90% methanol/10% ammonium acetate buffer, followed by 100% methanol. Detection was by an evaporative light-scattering detector (ELSD Mk III, Altech, Deefield, IL), which had been calibrated with 14 standard bile acids. The bile acid standards were tauroursodeoxycholic acid (TUDC), glycoursocholic acid (GUDC), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC), glycolithocholic acid (GLC), free ursodeoxycholic acid (UDCA), free lithocholic acid (LCA) (Calbiochem, Gibbstown, NJ), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDCA), free cholic acid (CA), free chenodeoxycholic acid (CDCA) and free deoxycholic acid (DCA) (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

Results are presented as mean±standard deviation. The data were analyzed by using Kruskal-Wallis one-way analysis. P-value <0.05 was considered statistically significant.

Results

Demography of patients and laboratory characteristics

Forty-nine total cases were analyzed: 37 with CCA; 5 with hepatocellular carcinoma; and 7 with benign biliary disease, including cholangitis and biliary cystadenoma. The male to female ratio was 2.5:1 and the average age was 58 years (range 35-96 years). Of the 37 patients with CCA, seven had a level of total serum bilirubin >2 mg/dl (HB). In addition, 30 of CCA patients had levels of total serum bilirubin ≤2 mg/dl (LB). Significant differences in total and direct serum bilirubin between the CCA with the LB group and the benign biliary disease group were not observed. Levels of cholesterol, total bilirubin, direct bilirubin, albumin and globulin in the hepatocellular carcinoma group were not different compared to other groups. Globulin levels in all patients were slightly higher than the normal range. There was no difference in cholesterol levels between patients (Table 1).

Liver function tests were also performed. Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels were significantly higher in the CCA with HB group compared to the benign biliary disease and the CCA with LB groups. Levels of ALT and ALP in the hepatocellular carcinoma group were significantly lower compared to the levels in the CCA with HB group (Figure 1).

Bile acid profile in human gallbladder bile

Comparison of total bile acids, total conjugated bile acids (glycine and taurine conjugated) as well as total unconjugated bile acids in the CCA, hepatocellular carcinoma and benign biliary diseases groups are shown in Figure 2.

Total concentrations of bile acids in the benign biliary diseases, hepatocellular carcinoma, CCA with LB and

Table 1. Biochemical Parameters in Serum of Patients with Benign Biliary Diseases, Hepatocellular Carcinoma and CCA

Parameters (normal range)		Benign biliary disease: BD (n=7)		Hepatocellular carcinoma: HCC (n=5)		CCA (n=37)			
						Total serum bilirubin ≤2 mg/dl (LB) (n=30)		Total serum bilirubin >2 mg/dl(HB) (n=7)	
Cholesterol	(127-262 mg/dl)	172±25	(132-199)	149±55	(110-228)	183±50	(124-280)	231.0±68.0	(130.0-327.0)
Total bilirubin	(0.25-1.5 mg/dl)	1.5±2*	(0.3-5.3)	2.4±3.2	(0.4-7.2)	0.4±1.9*	(0.2-1.0)	7.8±8.7	(2.1-26.6)
Direct bilirubin	(0-0.5 mg/dl)	0.8±1.4*	(0.1-3.7)	1.5±2.6	(0.1-5.4)	0.13±0.08*	(0.1-0.3)	5.8±8.0	(1.0-23.7)
Albumin	(3.8-5.4 g/dl)	3.5±0.7	(2.7-4.7)	3.8±0.8	(2.7-4.6)	4.1±0.6	(2.4-5.0)	3.7±0.6	(2.9- 4.8)
Globulin	(2.6-3.4 g/dl)	4.2±0.9	(3.4-5.9)	4.5±1.1	(3.1-5.5)	4.2±0.8	(2.7-6.0)	4.0±0.6	(3.3- 4.9)

*indicates significant differences compared to the CCA with total serum bilirubin >2 mg/dl (HB) along the same horizontal line, Each value represents mean±SD with range in parenthesis

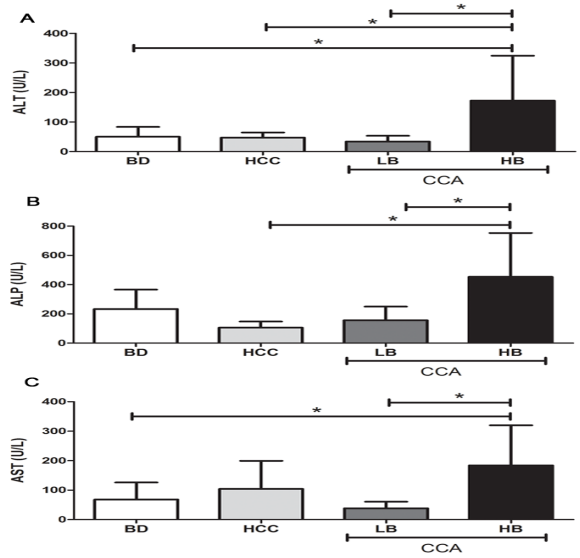

Figure 1. Bar Graphs Represent Serum Biochemical Parameters. Mean serum ALT (A), ALP (B) and AST (C) in patients with benign biliary disease (BD), hepatocellular carcinoma (HCC) and CCA patients with total serum bilirubin ≤2 mg/dl (LB) and >2 mg/dl (HB). P-values were obtained by One-Way ANOVA test. * indicates significantly different (P<0.05)

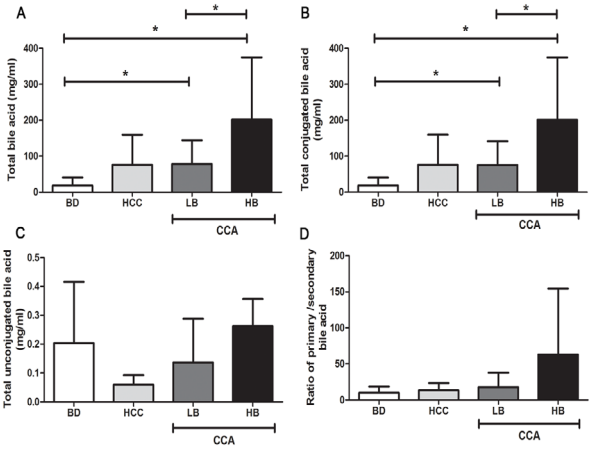

Figure 2. Mean Levels of Total Bile Acid (A), Total Conjugated Bile Acids (B) Total Unconjugated Bile Acids (C) and Ratios of Primary to Secondary Bile Acids in Gallbladder Bile from Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤2 mg/dl (LB) and >2 mg/dl (HB). *Indicates significantly different (P<0.05)

HB groups were 18.7±21.7 mg/ml, 76.1±83.4 mg/ml, 78.2±65.5 mg/ml and 201.8±173.2, respectively. The CCA groups with LB and HB had significantly higher total bile acid concentrations than the benign biliary disease group (P<0.05). Moreover, total bile acid concentrations in the CCA with HB group was increased compared to the CCA with LB group (P<0.05).

Levels of total conjugated bile acids in the benign biliary disease, hepatocellular carcinoma, CCA with LB and HB groups were 18.4±21.7 mg/ml, 76.0±83.3 mg/ml, 75.5±66.0 mg/ml and 201.5±173.1 mg/ml, respectively. Total conjugated bile acid concentrations in the CCA with LB and HB groups were significantly increased compared to the benign biliary disease group (P<0.05). Total unconjugated bile acid concentrations in the benign biliary disease, hepatocellular carcinoma, CCA without bile duct obstruction and CCA with LB and HB groups were 0.20±0.2 mg/ml, 0.06±0.03 mg/ml, 0.14±0.15 mg/ml and 0.3±0.1 mg/ml, respectively. No significant differences were observed between all groups.

The ratios of primary (CA, TCA, GCA, CDCA, TCDC and GCDC) to secondary (DC, TDC, GDC, UDCA, TUDC, GUDC, LCA, TLC and GLC) bile acids were also analyzed. Ratios of primary/secondary bile acids in the benign biliary diseases, hepatocellular carcinoma, CCA with LB and HB groups were 9.9±8.2 mg/ml, 13.2±10.0 mg/ml, 17.7±20 mg/ml and 62.98±91.16 mg/ml, respectively. The increasing primary:secondary ratio in CCA with HB was noted but this was not significantly different.

Levels of the primary bile acids CA and CDCA are shown in figure 3. Markedly elevated levels of CA were found in CCA patients with HB. Levels of CA in patient with benign biliary disease, hepatocellular carcinoma, CCA with LB and HB were 8.8±9.2 mg/ml, 11.7±5.3 mg/ml, 35.2±31.5 mg/ml and 122.3±144.0 mg/ml, respectively. Significant differences in CA concentration were observed in patients with CCA with LB and HB compared to patients with benign biliary disease (P<0.05). Levels of CA in the CCA with HB group were higher than in the hepatocellular carcinoma group, but this difference was not significant. Moreover, the level of CDCA was also significantly increased in patients with CCA with LB and HB compared to patients with benign biliary disease (P<0.05).

The concentrations of secondary bile acids including DCA, UDCA and LCA were analyzed and results are

Table 2. Bivariate Correlation between Bile Acid Levels and Biochemical Tests

		Cholesterol	Total bilirubin	Direct bilirubin	ALP	CEA
Total bile acid	Pearson Correlation	0.460*	0.514*	0.536*	0.510*	0.353*
	P value	0.004	0.001	0.001	0.001	0.050
Total primary bile acid	Pearson Correlation	0.456*	0.531*	0.554*	0.543*	0.369*
	P value	0.005	0.001	0	0.001	0.041
Total secondary bile acid	Pearson Correlation					
	P value	ns	ns	ns	ns	ns
Total CA	Pearson Correlation	0.402*	0.456*	0.477*	0.643*	
	P value	0.014	0.005	0.003	0.000	ns
Total CDCA	Pearson Correlation	0.404*	0.490*	0.509*		0.430*
	P value	0.013	0.002	0.001	ns	0.016

*indicates the significant correlation, ns indicates no significant correlation

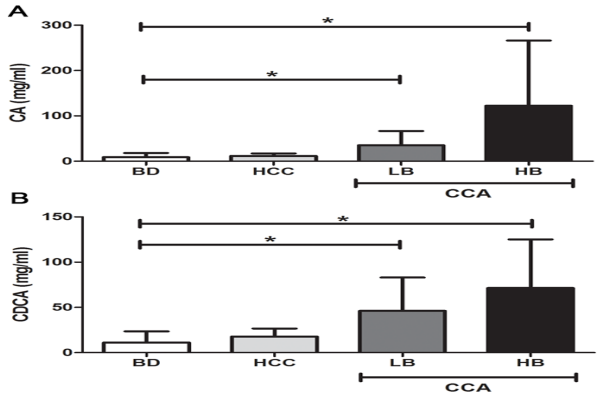

Figure 3. Levels of the Primary Bile Acids A; Cholic Acid (CA) and B; Chenodeoxycholic Acid (CDCA) in Gallbladder Bile from Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤2 mg/dl (LB) and >2 mg/dl (HB). Data are expressed as mean±SD. *indicates significantly different (P<0.05).

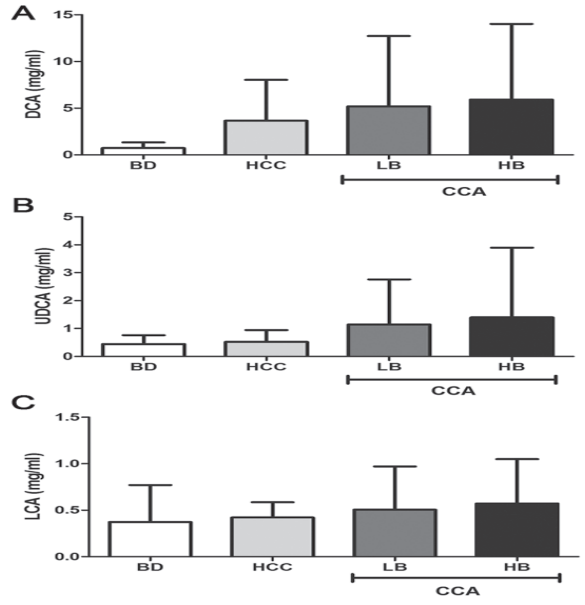

Figure 4. Levels of the Secondary Bile Acids, A) Deoxycholic Acid (DCA), B) Ursodeoxycholic Acid (UDCA) and C) Lithocholic Acid (LCA) in Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤2 mg/dl (LB) and >2 mg/dl (HB). Data are expressed as mean±SD.

shown in Figure 4. The level of DCA tended to be increased in patient with hepatocellular carcinoma and CCA with LB and HB compared to patients with benign biliary diseases, but this was not significantly different. Significant differences in UDCA and LCA were not observed in any group.

Correlation of bile acids and biochemical characteristics

To elucidate the effect of bile duct obstruction, the correlations between bile acid levels and liver function tests were analyzed in the CCA group by using bivariate analysis (Table 2). Levels of total bile acids and total primary bile acids were correlated with cholesterol, total bilirubin, direct bilirubin and ALP levels in CCA patients (P<0.01). No significant differences were found in levels of total secondary bile acids and these liver biochemical tests. The primary bile acids CA and CDCA correlated with cholesterol, total and direct bilirubin and ALP levels (P<0.05). In contrast, levels of the secondary bile acids including DCA, UDCA and LCA were not significantly correlated with cholesterol, total and direct bilirubin and ALP levels (data not shown). The correlation between bile acid and tumor markers was also investigated. Interestingly, the levels of total bile acid, total primary bile acid and chenodeoxycholic acid were significantly correlated (P<0.05) with the serum level of carcinoembryonic antigen (CEA). However, a significant correlation was not observed between levels of carbohydrate antigen (CA) 19-9 and alpha-fetoprotein (AFP) (data not shown).

Discussion

Many studies have also found that there is an excess risk of forming malignant tumors in some organs exposed to high concentration of bile acids, such as in the gastrointestinal tract (Bernstein et al., 2009). Alteration of bile acid composition increased the incidence of cholangiocarcinoma and colorectal adenocarcinoma (Kinami et al., 1993; Debruyne et al., 2002). In this study, we determined the bile acid profiles in bile from patients with CCA and hepatocellular carcinoma, and compared these profiles to those found in the bile of patients with benign biliary diseases. Higher levels of total bile acids were found in CCA and hepatocellular carcinoma patients. Markedly increased levels of total bile acids were observed in samples from patients with CCA with

HB. This finding suggests that bile duct obstruction may alter bile acid levels. Higher levels of primary bile acids resulting in an increased primary:secondary bile acid ratio in samples from patients with CCA with HB were also found. A positive correlation between total bile acids or total primary bile acids and biliary tract obstruction was also found, with total bilirubin and direct bilirubin representing markers of bile obstruction. CA and CDCA were also increased in the CCA group compared to the benign biliary disease group.

A previous study showed that bile duct obstruction increases bile acid synthesis. Bile duct ligation in rats increased serum, liver and urine bile acid levels (Kinugasa et al., 1981). The mechanism by which bile duct obstruction induces bile acid synthesis remain unclear but accelerated hepatic cholesterol synthesis and increased serum cholesterol levels have been proposed (Cooper and Ockner, 1974). A correlation between total bile acids and primary bile acids and cholesterol concentrations was also found in this study. Therefore, increased cholesterol synthesis is consistent with increased bile acid production. Park et al. (2006) also reported increased CA in bile from patients with biliary tract cancer compared to patients' bile with biliary tract stones and normal bile. In contrast, they found decreased total and secondary bile acids in biliary tract cancers. Low levels of secondary bile acids may be caused by primary bile acids that cannot reach the regions in bowel where secondary bile acids are produced. Production of secondary bile acids also depends on bacterial enzymes, so infection could be a contributing factor. Recently, *Helicobacter pylori* was found in 66% of bile samples in patients with CCA (Boonyanugomol et al., 2012). This finding supports higher production of secondary bile acids in CCA compared to benign biliary diseases. Although we used benign biliary disease as a control, significantly different results compared to the cancer groups were obtained. Sharif et al. (2010) reported different patterns of bile acids in bile from patients with CCA compared to bile from patients with benign biliary disease. They also suggested bile acids as potential disease biomarkers for distinguishing malignant from benign biliary disease. Although the differences were not significantly different, we also found increasing levels of total bile acids, total conjugated bile acids and primary bile acids in bile from patients with CCA with HB compared to bile from patients with hepatocellular carcinoma. Lower levels of ALT, AST and ALP were found in the bile from patients with hepatocellular carcinoma compared to the bile from patients with CCA and high levels of serum bilirubin. This suggested that liver function affected the bile acid concentration.

Alterations in bile acid concentrations in CCA were also previously reported in serum. Increased levels of CA and CDCA was found in human opisthorchiasis which is a CCA-associated liver fluke (Migasena et al., 1983). Elevations of total bile acids and total conjugated bile acids were reported in the serum of patients in the CCA group compared with normal controls (Changbumrung et al., 1990). Increased bile acids in serum may be caused by biliary obstruction (Hong et al., 1973). Chronic exposure to bile acids may play an important role in cholangiocarcinogenesis (Jansen, 2007). Concurrent with the present study, the positive correlation of total bile acids and CDCA with levels of serum CEA was observed in CCA patients. This result indicates the possible association of bile acid and cancer development. Previous studies suggested a role for bile acids in cholangiocarcinogenesis (Sirica, 2005). DCA, a potent promoter in the CCA hamster model, was shown to induce COX-2 in cultured immortalized human cholangiocytes via an EGFR-dependent mechanism and to block protein degradation of myeloid cell leukemia protein 1, a potent anti-apoptosis protein (Yoon et al., 2002a; 2002b). The underlying mechanism of bile acids on cholangiocarcinogenesis may involve their receptors. Adding the antagonist of farnesoid x receptor (FXR), a nuclear receptor for bile acid enhanced cholangiocarcinogenesis induced by free bile acids (Dai et al., 2011). Moreover, in bile duct obstruction, increased biliary bile acid concentration initiated enhanced cholangiocyte proliferation in an animal model (Alpini et al., 2002b).

In conclusion, total bile acids, conjugated bile acids, unconjugated bile acids and individual bile acids were analyzed in this study. Elevated levels of total bile acids, conjugated bile acids and primary bile acids were found in CCA. Bile duct obstruction may contribute to an alteration of bile acid concentration. Levels of CA, CDCA and DCA were increased in cancer groups. Moreover, levels of total bile acids and CDCA were also related to the level of CEA. These findings suggest a different bile acid concentration profile in the cancer groups compared to benign biliary disease groups. Alteration of bile acid concentration may be a reflection of the disease process. Since cholangiocytes are directly exposed to carcinogens in bile, profiling of bile acids in this study provides useful information about which bile acids may contribute to pathogenesis. Further studies are needed to determine the mechanisms underlying the correlation between bile acid composition and carcinogenesis.

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Anti-apoptotic phenotypes of cholestan-3β,5α,6β-triol-resistant human cholangiocytes: Characteristics contributing to the genesis of cholangiocarcinoma

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ABSTRACT

The oxysterols cholestan-3β,5α,6β-triol (Triol) and 3-keto-cholest-4-ene (3K4) are increased in *Opisthorchis viverrini*-associated hamster cholangiocarcinoma and induce DNA damage and apoptosis via a mitochondria-dependent mechanism in MMNK-1 human cholangiocytes. Based on these observations, we hypothesized that chronic exposure of cholangiocytes to these pathogenic oxysterols may allow a growth advantage to a subset of these cells through selection for resistance to apoptosis, thereby contributing to cholangiocarcinogenesis. To test this hypothesis, we cultured MMNK-1 cells long-term in the presence of Triol. Alteration in survival and apoptotic factors of Triol-exposed cells were examined. Cells cultured long-term in the presence of Triol were resistant to H_2O_2-induced apoptosis, and demonstrated an increase in the phosphorylation of p38-α, CREB, ERK1/2 and c-Jun. Elevations in the ratio of Bcl-2/Bax and in the protein levels of anti-apoptotic factors including cIAP2, clusterin, and survivin were detected. These results show that long-term exposure of MNNK-1 cells to low doses of Triol selects for kinase-signaling molecules which regulate resistance to apoptosis and thereby enhance cell survival. Clonal expansion of such apoptosis-resistant cells may contribute to the genesis of cholangiocarcinoma.
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1. Introduction

Oxysterols are oxygenated derivatives of cholesterol derived from enzymatic pathways mediated by CYP450 enzymes or from non-enzymatic mechanisms via reactive oxygen and/or nitrogen species (ROS/RNS) [1,2]. Numerous studies have demonstrated pathological effects of oxysterols such as 7-ketocholesterol, 24-hydroxycholesterol and Triol in a variety of diseases including cardiovascular diseases, neurological diseases and cancer [1–3]. Oxysterols also appear to play critical roles in multiple stages of carcinogenesis [3], such as in tumor initiation via enhancement of oxidative stress. Oxysterols increase intracellular ROS levels by inducing NADPH oxidase [4,5]. Persistent production of pro-oxidative molecules including ROS/RNS induced by oxysterols may perturb cellular stress responses [6,7] that can impair the DNA repair system and inhibit apoptosis [8,9]. Apoptosis induction by oxysterols may select a subset of cells that are resistant to apoptosis and thereby enhance carcinogenesis [10,11]. Infection with the liver fluke *Opisthorchis viverrini* (Ov) induces the generation of ROS/RNS in bile duct epithelia [12,13]. We have previously reported a possible role for oxysterols in the genesis of Ov-associated cholangiocarcinoma (CCA), a malignant tumor of bile duct epithelia [14]. Two oxysterols, cholestan-3β,5α,6β-triol (Triol) and 3-keto-cholest-4-ene (3K4), were increased in Ov-induced hamster CCA. Triol and 3K4 induced DNA damage and apoptosis in the human cholangiocyte MMNK-1 cell line [14]. Moreover, the expression of cytosolic oxysterol-binding proteins was increased in Ov-induced hamster CCA [15].

Mechanisms that explain the role of oxysterols in the initiation and progression of CCA remain undefined. Certain oxysterols can stabilize cyclooxygenase-2 (COX-2) mRNA via a p38

Abbreviations: CCA, cholangiocarcinoma; Triol, cholestan-3β,5α,6β-triol; 3K4, 3-keto-cholest-4-ene; p38-α, p38 mitogen-activated protein kinase α; CREB, cAMP response element-binding; ERK 1/2, extracellular signal-regulated kinase 1/2; cIAP2, cellular inhibitor of apoptosis 2.

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MAPK-dependent mechanism resulting in COX-2 protein accumulation in a CCA cell line [16]. Previous experiments, however, have examined the effects of high doses of oxysterols over short time courses, a situation that is diametrically opposed to that encountered in clinical Ov-induced CCA development where long term exposure to the liver fluke is present, and low concentrations of oxysterols are present in the biliary tract [14,17]. Indeed, culturing cholangiocytes with high concentration of oxysterols is expected to enhance their cytotoxicity. Interestingly, Gregorio-King and colleagues demonstrated that long-term culture of hematopoietic HL60 cells in the presence of low doses of the oxysterol 25-hydroxycholesterol (25-OHC) led to increased survival but it did not show resistance to apoptosis [18]. On the other hand, repeated exposure to oxysterols induced resistance to apoptosis in rat colon crypt cells and in a colon cell line [19,20]. Such resistance, was stable after at least 4 weeks growth in the absence of the oxysterol [20], and thus must have been due to somatically inherited changes in the cells, such as epigenetic or mutational alterations. Based on these observations, we hypothesized that chronic exposure of cholangiocytes to low doses of oxysterols may allow a growth advantage to a subset of these cells through selection for resistance to apoptosis, thereby contributing to cholangiocarcinogenesis. To test this hypothesis, we cultured MMNK-1 cells long-term in the presence of low doses of Triol, and investigated the molecular signaling pathways that have been associated with apoptosis resistance.

2. Materials and methods

2.1. Cell culture

The immortalized human cholangiocyte MMNK-1 cell line, transduced with SV40T and hTERT, was generated and supplied by Professor Naoya Kobayashi (Okayama University, Japan). Cells were cultured in Hams F12 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

2.2. Establishment of a cholangiocyte cell line chronically exposed to low doses of Triol

MMNK-1 cells were cultured by step-wise exposure to cholestan-3β,5α,6β-triol (Triol) (Steraloids Inc., USA). In brief, MMNK-1 cells were cultured repeatedly in the presence of 15 μM Triol. When the viability of Triol-treated cells was similar to that observed in untreated control cells, the concentration of Triol in the culture media was increased to 18 μM and 20 μM consecutively. Cell viability was determined using sulforhodamine B as described below.

2.3. Cell viability assay

MMNK-1 cells (2×10^3/100 μl) were seeded into 96-well plates and incubated overnight at 37 °C and 5% CO_2. Cells were treated with Triol at designated concentrations for 48 h. Triol was dissolved in 100% ethanol and added to culture media at different concentrations; the final concentration of ethanol was 0.5%. The number of viable cells was determined using sulforhodamine B (SRB, Sigma–Aldrich, MO, USA) as described previously [21]. Briefly, cells were fixed with 10% cold trichloroacetic acid for 1 h at 4 °C and stained with 0.4% SRB in 1% acetic acid for 30 min. Excess dye was washed with 1% acetic acid and stained cells were solubilized with 200 μl of 10 mM unbuffered Tris-base. The absorbance was measured with a microplate reader (Sunrise, TECAN Trading, Switzerland) at 540 nm. The results were expressed as a percentage of cell viability relative to control (cells treated with 0.5% ethanol).

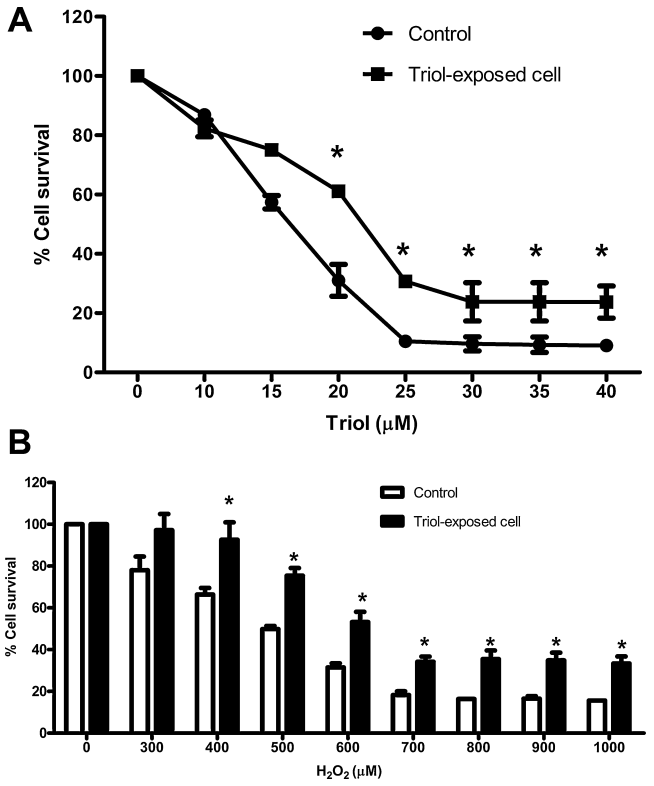

Fig. 1. Triol-exposed MMNK-1 cells are resistant to Triol and H_2O_2 treatment. (A) Both control and Triol-exposed cells were treated with Triol at designated concentrations for 48 h. After repeated exposure to Triol, cells were more resistant to Triol than control. (B) Control and Triol-exposed cells were treated with H_2O_2 at designated concentrations for 24 h. Increased numbers of viable cells were observed in Triol-exposed cells compared to control cells. Results are expressed as percent of control. Two individual experiments were performed. * indicates $P < 0.05$.

The concentration that inhibited cell growth to 50% of control (IC_{50}) was calculated by plotting the percentage of cell growth inhibition against oxysterol concentration.

2.4. Determination of apoptosis resistance

To determine resistance to apoptosis in MMNK-1 cells cultured in the presence of Triol, cells were treated with 300–1000 μM of hydrogen peroxide (H_2O_2), an apoptosis inducer [22], for 24 h. The number of viable cells was determined using sulforhodamine B as described above. Parental MMNK-1 cells were used as controls.

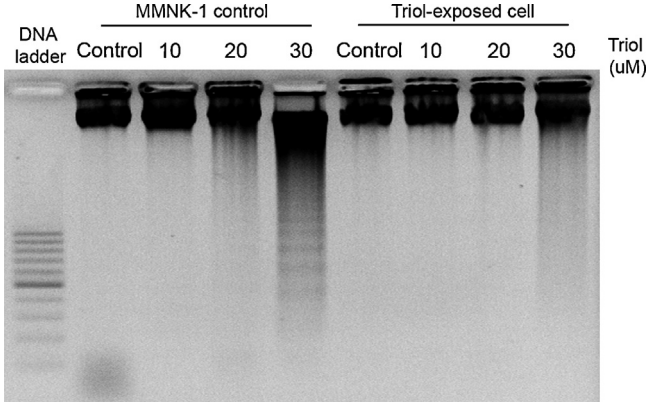

Fig. 2. Triol-exposed cells are resistant to apoptosis induction by Triol. Cells were treated with 10–30 μM Triol for 48 h. First lane was 100 bp DNA ladder. 0.5% ethanol-treated cells were used as control.

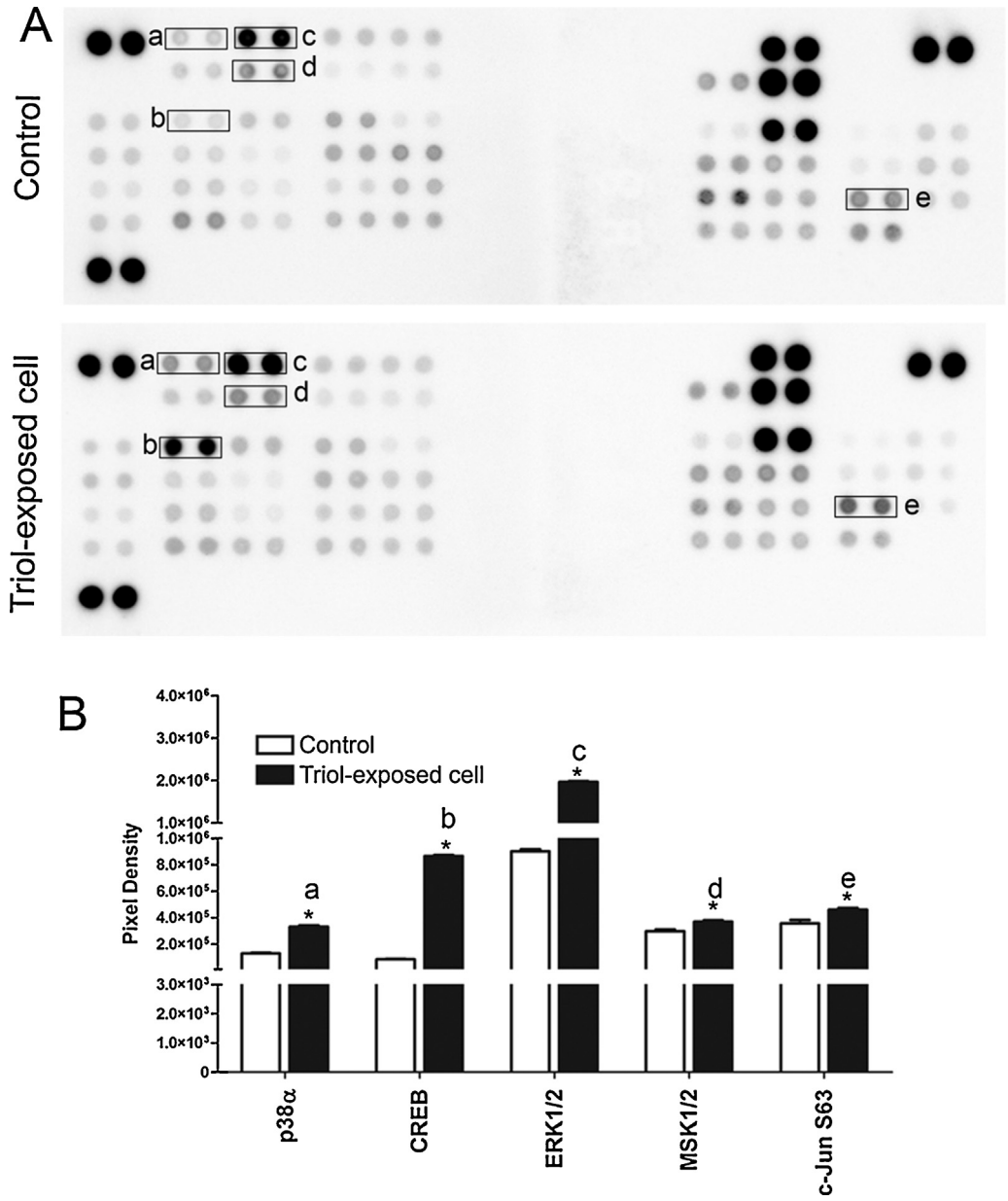

Fig. 3. Activation of kinase signaling cascade in Triol-exposed cells compares to MMNK-1 control cells. (A) Protein kinase phosphorylation in control and Triol-exposed cells were performed by using the phospho-kinase array. (B) Bar graph comparison of phosphorylated kinase protein expression in MMNK-1 control and Triol-exposed cells. Spot pixel densities were analyzed using ImageQuant™ and expressed as mean ± SD from two individual experiments. Results indicated the activation of kinase signaling cascade in Triol-exposed cells. * indicates $P < 0.05$.

DNA ladder formation was analyzed in Triol-exposed cells compared to MMNK-1 control cells. Cells were exposed to 10–30 μM Triol for 48 h, and then adherent cells were trypsinized and cell pellets were collected. Briefly, 10^6 cells were lysed twice with 1 ml lysis buffer (1% (w/v) Triton X-100, 0.32 M saccharose, 5 mM $MgCl_2$, 10 mM Tris–HCl) and centrifuged for 20 s at 10,000 × *g*. Cell pellets were collected and treated with 200 μl of enzyme reaction solution (1% (w/v) SDS, 5 mM EDTA-Na_2, 10 mM Tris–HCl) and 8 μl of 1 mg/ml of RNaseA for 20 min at 37 °C. Subsequently, 10 μl of 10 mg/ml Proteinase K was added to the sample and incubated for 1 h at 37 °C. Then, 300 μl of 7.6 M NaI solution (7.6 M NaI, 20 mM EDTA-Na_2, 40 mM Tris–HCl) and 500 μl of isopropanol were added to the sample. After centrifugation, the pellet was mixed with 40% isopropanol, washed with 70% ethanol and centrifuged. The pellet was dried and resuspended with 100 μl of TE buffer (10 mM Tris–HCl and 1 mM EDTA) and loaded onto 1% agarose gel in 1× TBE buffer (89 mM Tris Base, 89 mM boric acid, and 2 mM EDTA). Electrophoresis was done at 50 V for 1 h and the gel then stained with ethidium bromide for 15 min. Visualization was by ImageQuant 400 (GE Healthcare Bio-Science, UK).

2.5. Proteomic profiles of phospho-kinase and apoptosis proteins

Analysis of phosphorylation profiles of kinase proteins and expression profiles of apoptosis-related proteins were performed using Proteome Profiler Human Phospho-Kinase and Apoptosis Array Kits (R&D System, USA) according the manufacturer's instructions using protein extracts from MMNK-1 cells cultured in the presence of Triol in comparison to MNNK-1 cells cultured in the absence of Triol. Briefly, Triol-exposed MMNK-1 and control MMNK-1 cells were cultured in serum free media without oxysterol for 24 h. Cell lysates (800 μg per membrane)

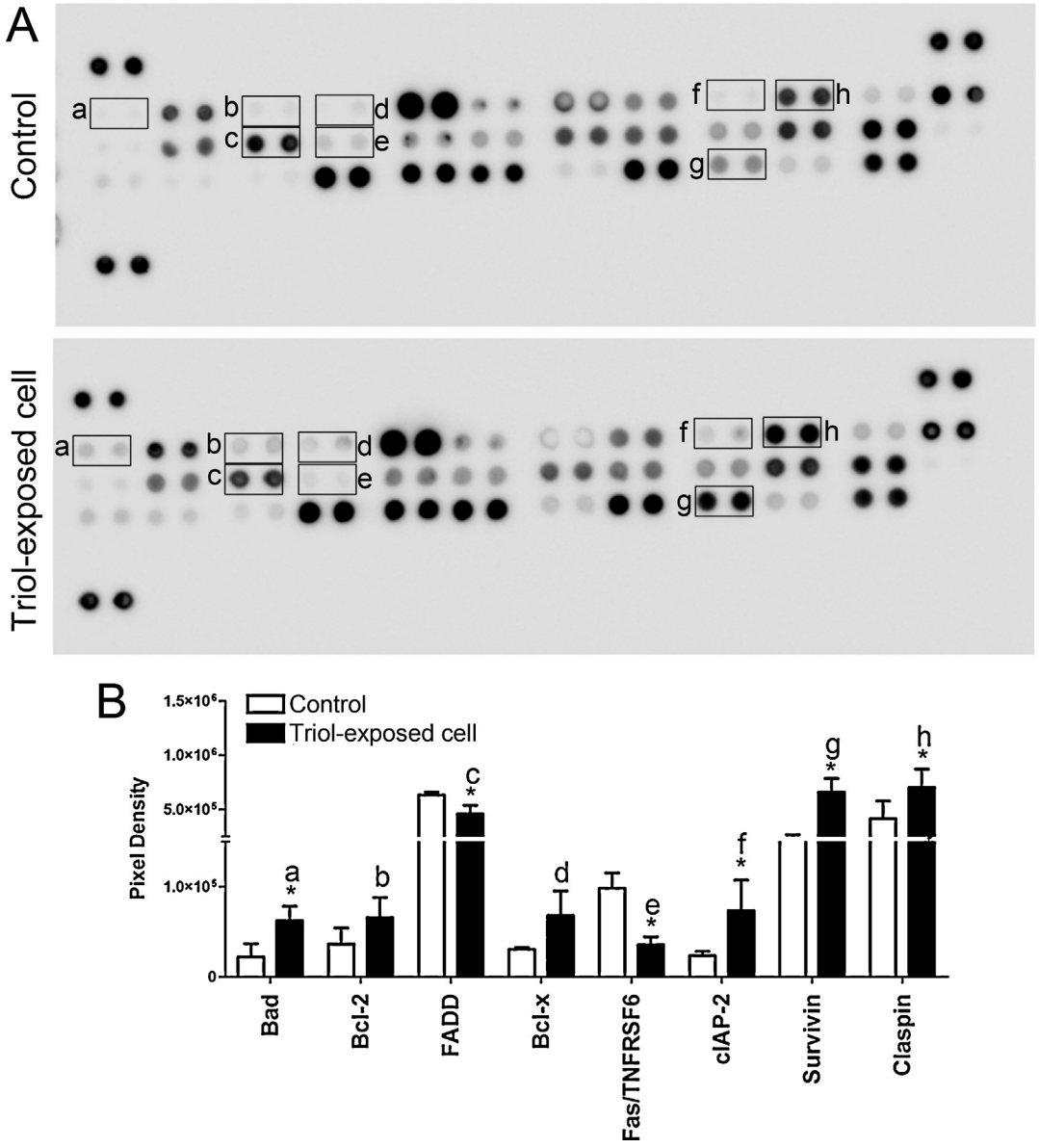

Fig. 4. Activation of apoptosis-related proteins in Triol-exposed cells compares to MMNK-1 control cells. (A) Profiling of apoptosis-related proteins expression in control and Triol-exposed cells was done by using the apoptosis array. (B) The quantification of mean spot pixel densities were analyzed using ImageQuant™ from two individual experiments. * indicates $P < 0.05$.

were incubated overnight with nitrocellulose membrane containing antibodies against 46 kinase phosphorylation sites and 35 apoptotic proteins printed in duplication. Then membranes were incubated with Detection Antibody Cocktail and subsequently with streptavidin-horseradish peroxidase. Signals were detected with ECL Plus Western blotting Detection Reagents (GE Healthcare UK Ltd., UK). The array images were analyzed and quantified using ImageQuant™ Imager (GE Healthcare UK Ltd., UK).

2.6. Western blot analysis

Cell lysate (30 μg) of Triol-exposed MMNK-1 and unexposed control cells were separated by 10% SDS–polyacrylamide electrophoresis and blotted to PVDF membrane (GE Healthcare Bio-Science, UK). Blots were probed with 1:100 of goat anti-Bax antibody (BD Biosciences, USA), 1:100 of rabbit anti-Bcl-2 (C21) antibody (Santa Cruz, Biochemical, USA), 1:1000 of rabbit anti-phospho-p44/42 MAPK (ERK1/2) antibody (Cell Signaling, USA), 1:1000 of rabbit anti-p44/42 MAPK (ERK1/2) antibody (Cell Signaling, USA), 1:1000 of rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling, USA), 1:1000 of rabbit anti-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling, USA) or 1:10,000 of the mouse anti-β-actin antibody (Sigma Aldrich, USA). Then, membranes were incubated in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biochemical, Santa Cruz, CA) at room temperature for 1 h. After that, detection was performed using the chemiluminescence for western blotting system (ECL, GE Healthcare Bio-Science, UK). Signals were analyzed by using Imagequant 400 and quantified by using ImageQuant™ Imager (GE Healthcare UK Ltd., UK).

2.7. Statistics

The results are presented as a mean ± SD for at least two individual experiments. Data were compared using Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

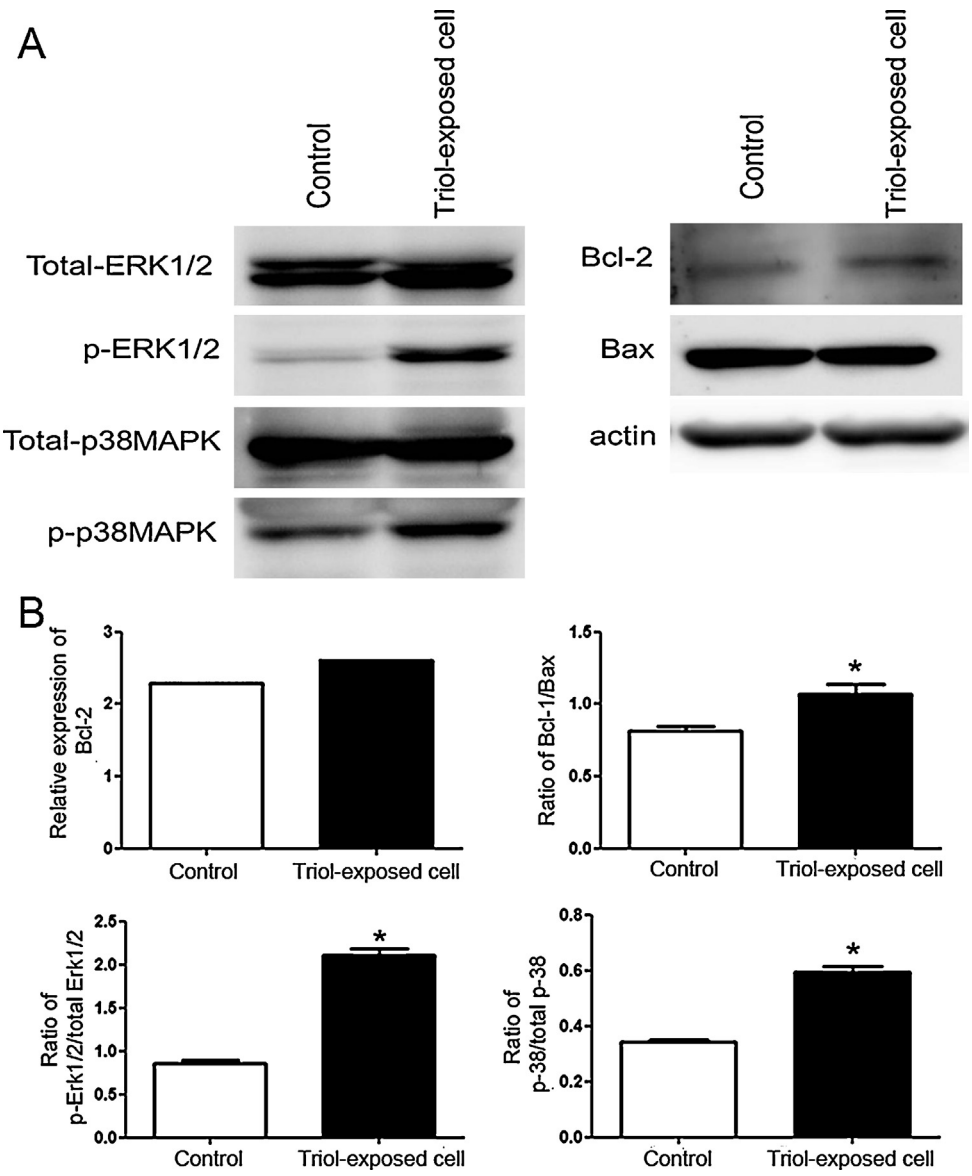

Fig. 5. Activation of survival signaling cascade in Triol-exposed cells. (A) Western blot analysis confirmed the protein expression status of kinase and apoptosis-related proteins in MMNK-1 control and Triol-exposed cells. (B) Bar graphs show relative densitometric values of western blot of Bcl-2, the ratio of Bcl-2/Bax, the ratio of phosphorylated ERK/total ERK and the ratio of phosphorylated p38α/total p38α, respectively. * indicates $P < 0.05$.

All analyses were performed using IBM SPSS 19 (Chicago, IL, USA) software.

3. Results

3.1. Triol-exposed MMNK-1 cells are resistant to H_2O_2-induced apoptosis

Triol-tolerant cholangiocytes were established by repeated exposure of MMNK-1 cells to Triol. Typically, only 20–30% of MMNK-1 cells survived within 5 days after exposure to 15 μM Triol. The surviving cells were cultured and repeatedly exposed to Triol until 80–90% viable cells were attained. The dose of Triol was then increased consecutively to 18 and 20 μM. The total time required to establish a Triol tolerance in MMNK-1 cell lines was approximately 12 months. After the 12-month exposure, Triol-exposed MMNK-1 cells showed a significant increase in IC_{50} (22.7 ± 1.1) when compared to the parental cells (16.7 ± 0.5 μM) as shown in Fig. 1A. To investigate whether Triol-exposed cells were resistant to apoptosis, cells were treated with 300–1000 μM H_2O_2 for 24 h. Triol-exposed MMNK-1 cells showed significantly higher cell viability than did the parental cells that were not exposed to Triol (Fig. 1B). Moreover, a marked decrease DNA laddering was found in Triol-exposed cells upon treatment with 30 μM of Triol (Fig. 2)

3.2. Profiling of kinase phosphorylation and apoptosis proteins in Triol-exposed cells

Patterns of activated kinases and apoptotic-associated proteins were investigated in Triol-exposed cells in comparison to the parental cells not exposed to Triol. Triol-exposed cells demonstrated increased phosphorylation of several kinases, including p38 mitogen-activated protein kinase α (p38-α), cAMP response element-binding (CREB), extracellular signal-regulated kinase (ERK1/2), mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2) and c-Jun (S63) when compared to the parental cells not exposed to Triol (Fig. 3A and B).

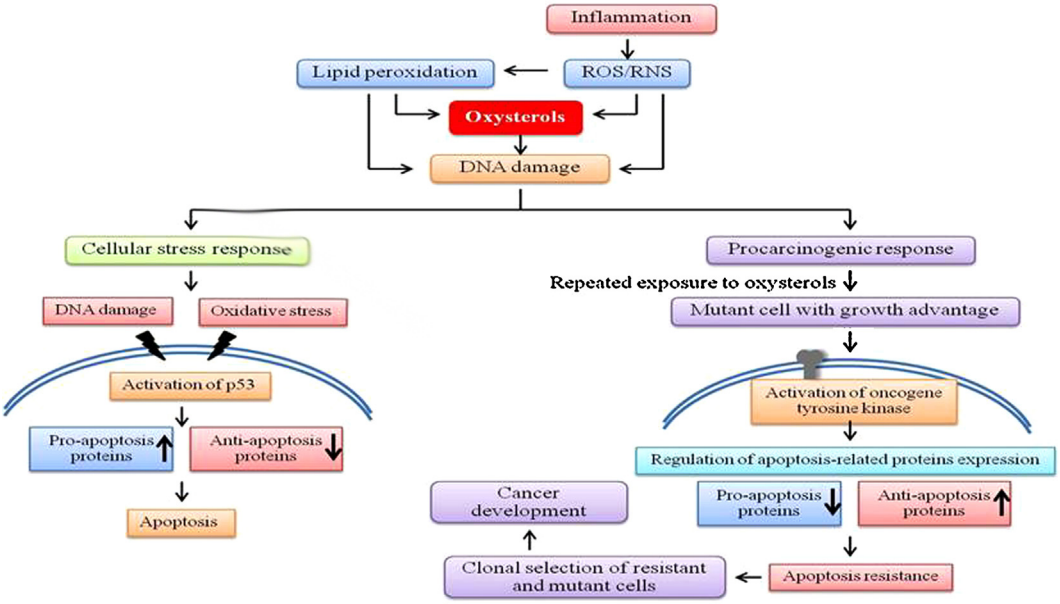

Fig. 6. Proposed scheme showing the mechanisms of action of oxysterols in cholangiocarcinogenesis.

The results also revealed a significant increase in anti-apoptotic proteins including Bad, cIAP-2, Claspin and Survivin in Triol-exposed cells compared to the parental cells (Fig. 4A and B). A marked reduction in pro-apoptotic proteins including Fas-associated protein with death domain (FADD) and Fas receptor (Fas/TNFRSF6) was detected in Triol-exposed cells when compared to the parental cells (Fig. 4A and B).

3.3. *Western blot analysis confirmed the phosphorylation status of kinase proteins and expression of apoptosis-related proteins*

To confirm the phosphorylation status of kinase proteins and expression of apoptosis-related proteins in Triol-exposed cells, we examined the protein levels of key-targeted proteins that included total-ERK1/2, phospho-ERK1/2, total-p38MAPK, phospho-p38MAPK, Bcl-2 and Bax. A significant induction in the ratio of phosphorylated to total forms of ERK1/2 and p38MAPK and Bcl-2 to Bax was observed for Triol-exposed cells when compared to the parental cells that were not exposed to Triol (Fig. 5A and B).

4. Discussion

Oxysterols have been proposed to contribute to multiple stages of carcinogenesis [3]. We reported increased levels of the pathogenic oxysterols Triol and 3K4 in Ov-induced hamster CCA tumor tissue. In addition, the cytotoxic effects of Triol and 3K4 were studied in human MMNK-1 cholangiocytes. Both Triol and 3K4 induced DNA damage and apoptosis via a mitochondria-dependent mechanism [14]. Studies have described the mechanisms of oxysterol-induced cytotoxicity in different cell systems [4,23]. Interestingly, those experiments were performed using single and/or high doses of oxysterols. High concentrations of oxysterols are cytotoxic to many cell types. Moreover, our previous study showed that low concentrations of oxysterols induce DNA damage without necessarily inducing cell death [14]. As previously suggested in bile-acid-induced gastrointestinal cancer [20], cells with an apoptosis-resistant phenotype are selected after long-term repeated exposure to pulses of bile acids, resulting in the survival of cells with unrepaired DNA damage, and a consequent increase in genomic instability leading to cancer progression. Long-term exposure to bile acids allowed cells to become resistant to apoptosis *in vitro* and *in vivo* [19,20]. Our study demonstrates that chronic exposure of cholangiocytes to low doses of the pathogenic oxysterol Triol leads cell to survival and resistance to apoptosis. Interestingly, we could not maintain 3K4-tolerant cells in a similar experiment. This may be due to greater cytotoxicity of Triol than 3K4 caused by higher polarity [14]. Another possible explanation is that a more extended treatment period may be required for establishing 3K4-tolerant cells. We proved that Triol-exposed cells were more resistant to Triol and H_2O_2 than the parental cells by using SRB assay. Moreover, a decrease in DNA ladder formation after treatment with Triol confirms that Triol-exposed cells are more resistant to apoptosis. This suggests that Triol-induced cytotoxicity may operate via similar mechanisms with apoptosis induction-related oxidative stress [24,25]. Nevertheless, cell death of 70% in Triol-exposed cells after treatment with 30 μM Triol using SRB with undetectable DNA laddering could happen due to a discrepancy between the two methods. Apoptotic and necrotic cells were excluded by collection of only adherent cells for assays.

Previous studies reported that oxysterols exert their survival effect through the activation of signal transduction pathways, especially kinase-dependent pathways [16]. An oxysterol-activated MAPK cascade exists in various cell types including in a human cholangiocarcinoma (KMBC) cell line [16,26]. We found that the Triol-exposed cholangiocytes exhibited pro-survival and anti-apoptotic properties in association with activation of signaling molecules in the MAPK pathway. The increase in activation of extracellular signal activated kinases including p38α MAPK and ERK1/2 were found in Triol-exposed cells. Activation of the MAPK cascade results in stimulation of a wide range of cellular responses including metabolism, survival, apoptosis and differentiation [27]. Moreover, activation of the MAPK cascade has profound effects on the regulation of the cell cycle and apoptosis [28]. An increase in phosphorylation of the transcription factors CREB and c-Jun, which are downstream molecules in the MAPK pathway, was also found in Triol-exposed cells. The activation of these downstream transcription factors may regulate the expression of survival-related proteins [28]. Induction of CREB stimulates a survival pathway in alveolar epithelial cells [29]. MAP kinase that activates c-Jun may underlie the common stimulation of this transcription factor by growth factors and oncogenes [30].

Oxysterols can induce apoptosis through both death receptor-dependent and mitochondrial pathways [14,31]. We found the alteration of both pathways in Triol-exposed cells. We demonstrated that cholangiocytes exposed to Triol exert anti-apoptotic properties by increasing the ratio of Bcl-2/Bax, enhanced expression of cIAP-2 and survivin. This finding contrasts to two other reports [18,32]. These contrasting findings may be due to the different types of oxysterols used and varied mechanisms of oxysterol-induced cytotoxicity in different cell types. Moreover, decreased expression of the death receptor Fas was found in our model. Even though previous reports have implicated Fas receptor mediated apoptosis by certain oxysterols, the mechanisms involved remain obscure [31,33]. Hence, the suppression of Fas ligand induced by Triol treatment should be investigated further. Notably, the activation of pro-apoptotic related proteins, Bad and Claspin, was observed in our study but did not trigger apoptosis.

In conclusion, we established a human cholangiocyte MMNK-1 cell line that was selected for resistance to cytotoxicity by Triol through long term, low dose exposure. The clonally selected cells expressed activated tyrosine kinases and their effector proteins allowed these cells to evade apoptosis. These findings support a model in which exposure of cholangiocytes to pathogenic oxysterols at a low dose and for a long duration (as occurs with Ov infection in humans) contributes to cancer development. Reduced DNA repair and ongoing exposure to DNA damaging agents cause an accumulation of DNA damage. Higher level of DNA damage leads to either increased mutation due to error-prone translational processes [34] or increased epimutations due to epigenetic alterations at the sites of DNA damage when repair is error-prone [35–37]. Such mutations and/or epimutations enable the selection of resistant cells. These resistant cells constitutively activate down-stream effectors of receptor tyrosine kinase signaling that may result in regulation of gene expression of apoptosis-related proteins, which are involved in the stimulation of cell survival and evasion of apoptosis. Clonal expansion of apoptosis-resistant cells may enhance the malignant transformation of biliary cells leading to the genesis of CCA (as depicted in Fig. 6). Pathogenic oxysterols found in the biliary tract, such as Triol, could play a key role in driving this process in CCA. Long term chronic inflammation due to Ov infection would allow the generation of reactive oxygen and nitrogen species, DNA damage, selection for apoptosis resistance, and increased mutation leading to carcinogenesis.

Acknowledgements

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RESEARCH ARTICLE

Association of CYP39A1, RUNX2 and Oxidized Alpha-1 Antitrypsin Expression in Relation to Cholangiocarcinoma Progression

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Abstract

Cytochrome P450 (CYP) enzymes are a large family of constitutive and inducible mono-oxygenase enzymes that play a central role in the oxidative metabolism of both xenobiotic and endogenous compounds. Several CYPs are involved in metabolism of oxysterols, which are cholesterol oxidation products whose expression may be dysregulated in inflammation-related diseases including cancer. This study focused on CYP39A1, which can metabolize 24-hydroxycholesterol (24-OH) that plays important roles in the inflammatory response and oxidative stress. We aimed to investigate the expression status of CYP39A1 and its transcription factor (RUNX2) in relation to clinical significance in cholangiocarcinoma (CCAs) and to determine whether 24-OH could induce oxidative stress in CCA cell lines. Immunohistochemistry showed that 70% and 30% of CCA patients had low and high expression of CYP39A1, respectively. Low expression of CYP39A1 demonstrated a significant correlation with metastasis. Our results also revealed that the expression of RUNX2 had a positive correlation with CYP39A1. Low expression of both CYP39A1 (70%) and RUNX2 (37%) was significantly related with poor prognosis of CCA patients. Interestingly, oxidized alpha-1 antitrypsin (ox-A1AT), an oxidative stress marker, was significantly increased in CCA tissues in which CYP39A1 and RUNX2 were down regulated. Additionally, immunocytochemistry showed that 24-OH could induce ox-A1AT in CCA cell lines. In conclusion, our study revealed putative roles of the CYP39A1 enzyme in prognostic determination of CCAs.

Keywords: CYP39A1 - RUNX2 - oxidized alpha-1 antitrypsin - cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is a tumor characteristic of bile duct epithelial cells which is related with a poor prognosis as well as responding poorly to current therapies (de Groen et al., 1999; Vatanasapt et al., 2002). The incidence of CCA burden greatly increases worldwide such as in the United Kingdom, United States, Western Australia and Thailand (Khan et al., 2008). Diverse exposure to risk factors for CCA result in varying geographic incidences with parasitic infections, such as liver fluke, Opisthorchis viverrini (O. viverrini), chronic viral hepatitis and cirrhosis including hepatitis C virus (HCV), hepatitis B virus (HBV), liver cirrhosis, and hepatolithiasis (Shin et al., 2010). A major mechanism linking between inflammation and CCA is related to the generation of free radicals such as superoxide anion ($O2^{-}\bullet$), nitric oxide (NO) and other reactive oxygen (ROS) and nitrogen species (RNS) which cause DNA damage and the alteration of gene expressions in both a hamster animal model and humans (Jaiswal et al., 2000; Pinlaor et al., 2003; Thanan et al., 2008; Yongvanit et al., 2012b). Regarding to the molecular mechanism underlining CCA progression, there are some studies have demonstrated aberrant expression of potential genes involving in CCA (Kunlabut et al., 2012; Namwat et al., 2012; Thongchot et al., 2014). In addition, several studies show that oxidative DNA damage plays a role in not only DNA mutation but also genetic instability and epigenetic changes which could be involved in all steps of inflammation-related carcinogenesis (Murata et al., 2012).

The cytochrome P450 enzymes (CYP), a family of mono-oxygenase enzymes that metabolize endogenous substances including vitamins, steroids, fatty acids and prostaglandins also play a crucial role in metabolizing xenobiotics to be in either detoxified forms or to be

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harmful reactive intermediates, including carcinogens (Simpson, 1997). Certain CYP enzymes, for instance CYP2A6 which is a major liver enzyme that biotransforms N-nitrosodimethyl amine (NDMA) into a carcinogen was previously shown to have increased activity in subjects infected with *O. viverrini* (Satarug et al., 1996). Increased CYP2A6 expression and activity were also found in chronic inflammation-related disease including hepatocellular, bronchogenic, esophageal carcinomas and CCA (Crawford et al., 1998; Raunio et al., 1998; Godoy et al., 2002; Yongvanit et al., 2012a). Decreased activity of CYP enzymes has been found to be associated with oxidative stress, for example, CYP2E1 in CCA (Yongvanit et al., 2012a) and HCC (Ho et al., 2004). CYP39A1, a steroid 7α-hydroxylation enzyme, is capable of metabolizing 24-hydroxycholesterol (24-OH) which is a cholesterol oxidation product (Li-Hawkins et al., 2000). In cancer, it has been reported that CYP39A1 was hypermethylated in ovarian tumor (Huang et al., 2009b).

On the other hand, 24-hydroxycholesterol (24-OH) plays important roles in the inflammatory response and oxidative stress. An experimental study reported an adverse effect of 24-OH on primary human neural cells via the significant induction of inflammatory gene expressions such as HSP70, COX-2, cPLA2 and beta-APP genes when compared to cholesterol stimulation (Alexandrov et al., 2005). Additionally, 24-OH significantly induces ROS production determined in primary porcine retinal pigment epithelial cells. (Joffre et al., 2007).

It is accepted that inflammation and oxidative stress play significant roles in inflammatory diseases including liver fluke-associated CCA (Yongvanit et al., 2012b). We recently determined oxidized alpha-1 antitrypsin (ox-A1AT) expression in human serum of CCA patients. Our results identified ox-A1AT as a potential risk indicator for opisthorchiasis-associated CCA which could be used as an oxidative stress biomarker in *O. viverrini*-associated CCA (Jamnongkan et al., 2013). In the current study, we hypothesize that there is an alteration of CYP39A1 expression in CCA, which might enhance the tumor progression. The gene expression pattern of CYP39A1 was investigated in order to address clinico-pathological associations. The underlying mechanism(s) of alteration of CYP39A1 expression governed by its transcription activator RUNX2 was investigated. In addition, ox-A1AT was also investigated in CCA to show the correlation of oxidative stress in alteration of CYP39A1 expression. We also confirmed whether 24-OH could induce ox-A1AT, an oxidative stress marker in CCA cell line.

Materials and Methods

Human CCA specimens

The 30 paraffin-embedded tissues collected from primary tumors of CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand during 2004-2011. Informed consent was obtained from each patient prior to surgery and the Ethics Committee for Human Research at Khon Kaen University approved the research protocols (HE521209 and HE531163).

Immunohistochemical analysis

The expression of CYP39A1, RUNX2 andox-A1AT in paraffin-embedded tissues was determined by immunohistochemical staining. The sections were deparaffinized and rehydrated by submerging respectively in xylene and ethanol with stepwise decreasing concentrations. For antigen retrieval, the sections were boiled in Tris-EDTA buffer (pH 9.0) and left to cool down for 20 min. Tissue sections were treated with 0.3% H2O2 in phosphate-buffered saline (PBS) to block the endogenous peroxidase activity, subsequently treated with 10% skim milk for non-specific binding blocking. The sections were incubated with primary antibodies against human CYP39A1 (1:200, rabbit polyclonal antibody; Sigma chemical, USA), RUNX2 (1:200, mouse monoclonal antibody; Abcam, USA) and oxA1AT (1:100, mouse monoclonal antibody; Ikagaku Co. Ltd, Kyoto, Japan) at 4ºC for overnight incubation. Subsequently, sections were incubated with peroxidase-conjugated Envision™ secondary antibody (DAKO,Glostrup, Denmark) at room temperature for 1h. Peroxidase activity was observed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate. The sections were counterstained with hematoxylin, dehydrated with stepwise increasing concentrations of ethanol, cleared with xylene and mounted with permount solution. Protein staining was evaluated by calculating a total immunostaining index (IHC index) as the product of a frequency and intensity score by two independent observers blinded to the protein type under analysis. The proportion score described the estimated fraction of positive stained tumor cells (0= none; 1= 1-25%; 2= 26-50%; 3= 51-75%; 4≥76%). The intensity score represented the estimated staining intensity (0=negative staining; 1= weak; 2= moderate; 3= strong) (Thanan et al., 2012a). These scores were calculated by multiplying the frequency score and intensity score. The mean of IHC index was defined as the cut-off value of low and high expression.

Cell lines and cell culture

M214 CCA cell line was cultured in Ham F'12 (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin and incubated at 37°C in a humidified incubator maintained with an atmosphere of 5% CO2. Subculture was done when the cell reached the confluent stage and the media were changed once every two to three day.

Immunocytochemical analysis

Immunocytochemical analysis was used to determine whether 24-OH could induce oxidative stress condition in CCA cell line. Cells were plated on slide chamber and treated with 24-OH (Enzo Life Sciences, Inc.USA) at concentrations of 6.25 and 12.5 mM. After incubation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% (v/v) Triton X-100. Fixed cells were then incubated with antibodies specific for ox-A1AT followed by secondary antibodies conjugated with Alexa

Table 1. Immunihistochemical Analysis and Clinicopathological Data of CCA Patients

Variable	No. of patients	CYP39A1 expression (%) Low 21 (70%)	CYP39A1 expression (%) High 9 (30%)	P value	RUNX2 expression (%) Low 11 (37%)	RUNX2 expression (%) High 19 (63%)	P value	Ox-A1AT expression (%) Low 23 (77%)	Ox-A1AT expression (%) High 7 (23%)	P value
Age (Year)										
< 56	15	10 (67%)	5 (33%)	1	6 (40%)	9 (60%)	1	13 (87%)	2 (13%)	0.39
> 56	15	11 (73%)	4 (29%)		5 (33%)	10 (67%)		10 (67%)	5 (33%)	
Gender										
Female	12	10 (83%)	2 (17%)	0.249	8 (67%)	4 (33%)	0.009*	8 (67%)	4 (33%)	0.392
Male	18	11 (61%)	7 (39%)		3 (17%)	15 (83%)		15 (83%)	3 (17%)	
Histological grading										
Non-papillary	13	9 (69%)	4 (31%)	1	4 (31%)	9 (69%)	0.708	10 (77%)	3 (23%)	1
Papillary	17	12 (71%)	5 (29%)		7 (41%)	10 (59%)		13 (76%)	4 (24%)	
Metastasis stage										
Non-metastasis	16	8 (50%)	8 (50%)	0.017*	4 (25%)	12 (75%)	0.257	14 (87%)	2 (13%)	0.204
Metastasis	14	13 (93%)	1 (7%)		7 (50%)	7 (50%)		9 (64%)	5 (36%)	

*P<0.05 was considered statistically significant

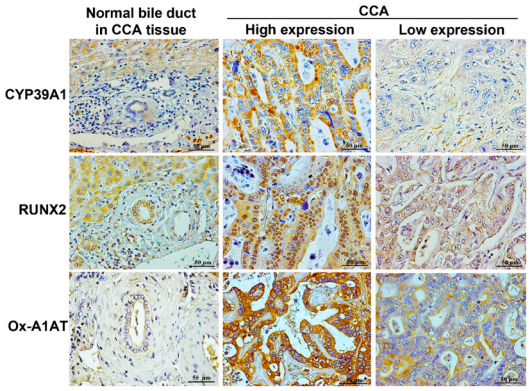

Figure 1. Immunohistochemical analysis of CYP39A1, RUNX2 and ox-A1AT in normal bile duct, which shows negative staining, whereas in CCA tissue shows positive immunoactivity that could be grouped into high and low expression. Original magnification is ×400 for normal bile duct and CCA tissues. Scale bars=50μm

Table 2. Correlation between Expression of CYP39A1 and its Regulator, RUNX2 and an Oxidative Protein Marker, ox-A1AT in Tumor Tissues of CCA Patients Demonstrated by Immunohistochemical Staining

Factors	CYP39A1	RUNX2	Ox-A1AT
CYP39A1	1	0.498	-0.361
Pearson correlation Sig. (2-tailed)	-	0.005	0.05
N	30	30	30
RUNX2			
Pearson correlation	0.498	1	-0.562
Sig. (2-tailed)	0.005*	-	0.001*
N	30	30	30
ox-A1AT			
Pearson correlation	-0.361	-0.562	1
Sig. (2-tailed)	0.050*	0.001*	-
N	30	30	30

*P<0.05 was considered statistically significant

fluor 555 for 1 hr. Preparations were mounted on slides with DAPI for nuclear staining and examined using a fluorescence microscope with a 40x objective lens.

Statistics

Statistical analysis was performed using SPSS software version 17 (IBM Corporation, NY, USA). The association of protein expression in CCA tissues and patients' clinico-pathological parameters assessed by Fisher's exact test was analyzed. The survival analysis was performed using Kaplan-Meier with Log-rank test. Correlations between CYP39A1, RUNX2 and ox- A1AT level in all groups were determined by Pearson's correlation analysis. A P-value less than 0.05 was considered statistically significant.

Results

BExpressions of CYP39A1, RUNX2 and ox-A1AT in human CCA tissues in relation to clinicopathological features

As shown in Table 1, results of immunohistochemical staining revealed high expressions of CYP39A1, RUNX2 and ox-A1AT were 30% (9/30), 63% (19/30) and 23% (7/30) of cases, respectively. In Figure 1, a strong expression of those proteins was detected in tumor cells and hepatocytes. RUNX2 was detected only in nuclei of CCA cells. The low expression of CYP39A1, RUNX2 and ox-A1AT was 70% (21/30), 37% (19/30) and 77% (23/30) of cases, respectively. Normal bile duct epithelia residing in non-tumor adjacent tissues showed weak staining in CYP39A1, RUNX2 and ox-A1AT for most cases. Among liver sections of 30 patients with intrahepatic CCA examined, 18 (60%) cases were male and 12 (40%) cases were female. The age of patients ranged from years 37-73 old (median age=56.5 years). In this study, the CCA histological types were classified as the papillary type of 57% (17/30) cases and tubular type of 43% (13/30) cases. CCA metastatic stage were classified as the metastasis of 47% (14/30) cases and non-metastasis of 53% (16/30) cases, Fisher's exact test showed a significant negative correlation between high CYP39A1 expression with tumor metastasis (p=0.017). Age, sex and histological grade did not show any association with CYP39A1 and ox-A1AT (Table 1). Notably, high expression of RUNX2 was significantly correlated with male gender (p=0.009). In Table 2, CYP39A1 expression was positively correlated with RUNX2 expression (r=0.498, p=0.005) but negatively correlated with ox-A1AT (r=-0.361, p=0.050) and RUNX2 expression was negatively correlated with ox-A1AT expression (r=-0.562, p=0.001).

Expressions of CYP39A1, RUNX2 and ox-A1AT and survival in CCA patients

The Kaplan-Meier method with log rank test showed

Table 3. Multivariate analysis by the COX proportional hazard regression model

Variable	Adjusted Hazard ratio	95% confidence interval	P value	Adjusted Hazard ratio	95% confidence interval	P value	Adjusted Hazard ratio	95% confidence interval	P value
CYP39A1				RUNX2			ox-A1AT		
Low	1	0.108-0.933	0.037	1	0.071-0.732	0.013	1	1.081-10.479	0.036
High	0.318			0.228			3.365		
Age (Year)									
< 56	1	0.368-2.518	0.938	1	0.467-3.064	0.71	1	0.280-2.156	0.628
> 56	0.963			1.196			0.777		
Gender									
Female	1	0.391-2.285	0.9	1	0.159-1.562	0.232	1	0.332-2.091	0.697
Male	0.945			0.499			0.833		
Histological grading									
Non-papillary	1	0.889-5.916	0.086	1	0.866-6.587	0.092	1	0.705-5.129	0.204
Papillary	2.293			2.389			1.902		
Metastasis stage									
Non-metastasis	1	0.424-2.842	0.848	1	0.760-3.998	0.189	1	0.782-3.976	0.171
Metastasis	1.097			1.744			1.764		

*P<0.05 was considered statistically significant

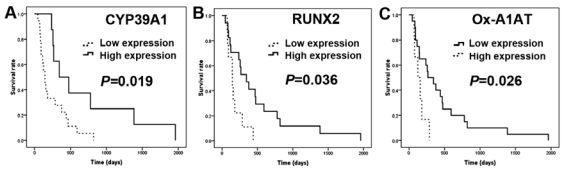

Figure 2. Comparison of survival in CCA tissue of patients, whose tumors showed high and low expression of CYP39A1 (log-rank test p=0.019) A); RUNX2 (log-rank test p= 0.036) B); and ox-A1AT (log-rank test p=0.026) C). Patients with low expression of the three proteins show the higher survival days than those with high expressions

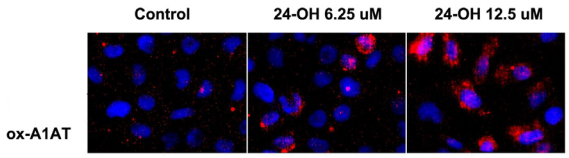

Figure 3. The immunofluoresence assay was performed to investigate the level of ox-A1AT switching in 24-OH-induced M214 cell lines. M214 cell lines were treated with 0 µM as control A), 6.25µM B) and 12.5µM C) respectively. The production of ox-A1AT increased after induction with increasing concentration of stimuli when compared to untreated

that CCA patients who had low expression of CYP39A1 and RUNX2 had significantly shorter survival (p=0.019 and p=0.036, respectively) when compared with patients who had high expression of those proteins (Figure 2). In contrast, low expression of ox-A1AT in CCA patients had significantly longer survival (p=0.026). Multivariate analysis was performed using the Cox proportional hazards model to investigate the independent value of each factor to predict overall survival. In Table 3, the result showed that CYP39A1 (hazard ratio, 0.318; 95% confidence interval, 0.108-0.933; p=0.037) and RUNX2 (hazard ratio, 0.228; 95% confidence interval, 0.071-0.732; p=0.013) remained significantly independently protective with the prognostic model whereas ox-A1AT (hazard ratio, 3.365; 95% confidence interval, 1.081-10.479; p=0.036) were independent prognostic risk factors

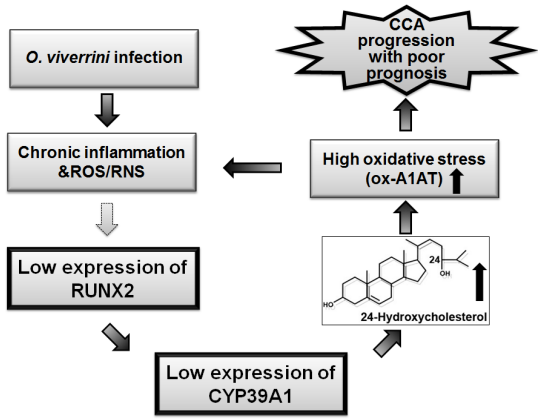

Figure 4. Proposed mechanism linking the altered expressions of RUNX2 and CYP39A1, that affects 24-OH level and oxidative stress in relation to CCA progression

for overall survival.

24-OH-induced ox-A1AT expression in CCA cell line

After 24 h incubation, 24-OH-induced M214 CCA cells significantly increased ox-A1AT, oxidative stress marker expression as dose dependent manner determined by immunocytochemistry analysis (Figure 3). This evidence suggests that 24-OH is involved in inflammatory-associated CCA.

Discussion

The cytochrome enzyme CYP39A1 (oxysterol 7α-hydroxylase) preferentially catalyzes the 7α-hydroxylation of 24-OH (Li-Hawkins et al., 2000) and the presence of CYP39A1 has been reported only in the liver (Bjorkhem et al., 1998). The major brain cholesterol metabolite 24-OH (Lutjohann et al., 1996) which is eliminated in the liver is catalyzed by CYP39A1 and CYP7A1 (Bjorkhem et al., 2006). It has been reported to be involved in the pathogenesis of Alzheimer's disease, which has an accumulation of oxidative brain damage (Bjorkhem et al., 2006). In cancer, CYP39A1 has been found to be hypermethylated in ovarian cancers as well as being related to poor prognosis (Huang et al., 2009). To

date the presence of CYP39A1 or its metabolite 24-OH has not been elucidated in CCA. In this study, we revealed that CYP39A1 showed low expression in most of CCA patients. Interestingly, CCA patients with high CYP39A1 expression in their tumor tissues had longer survival than patients with low expression. In addition, high expression of CYP39A1 was well-correlated with non-tumor metastasis status. These data suggest that CYP39A1 might be a promising protective-prognostic factor and altered expression of CYP39A1 might play roles in CCA progression, possibly leading to the accumulation of 24-OH. The molecular mechanism by which CYP39A1 expression is regulated and its role in CCA genesis and progression as well as the level of 24-OH in CCA requires further comprehensive investigation.

We demonstrated that CYP39A1 showed a positive correlation with RUNX2. In addition, patients with high RUNX2 expression had longer survival than those with low expression, suggesting a role in the prognostic indication for CCA. In contrast to ox-A1AT, patients with high ox-A1AT expression had shorter survival than those with low expression. It has been known that RUNX2 controls genes involved in sterol/steroid metabolism, including Cyp11a1, Cyp39a1, Cyp51, Lss, and Dhcr7, discovered in murine osteoprogenitor cells (Teplyuk et al., 2009). In addition, the tumor suppression property of RUNX2 has been reported in breast cancer cell lines in blocking the effect of estradiol, resulting in inhibiting tumor growth (Chimge et al., 2012). Notably, we found that high expression of RUNX2 in CCA was significantly related with male than female gender. Previously, estrogen level in male CCA patients' sera was shown to increase and correlated with clinical presentations (Hunsawong et al., 2012).

The oxidative damage occurs frequently in CCA. CCA patients who had high oxidative stress via protein damage formation significantly correlated with poor prognosis while low oxidative stress could be related with good prognosis (Thanan et al., 2012b). An oxidative damage marker, ox-A1AT, has been found to be highly expressed in CCA tissues (Jamnongkan et al., 2013). Our result demonstrated that ox-A1AT was highly expressed in CCA tissues and correlated with shorter patient survival. In addition, low CYP39A1 and RUNX2 expressions were positively correlated with high expression of ox-A1AT in CCA tissues. This implies that patients who present with low CYP39A1 and RUNX2 expression can have poorer prognosis than those who have high expressions due to the oxidative damage in CCA tissues which may enhance tumor progression.

In conclusion, significant roles of CYP39A1 and RUNX2 were confirmed in CCA clinical samples, suggesting that low expressions of those proteins were significantly related with high oxidative stress and correlated with poor prognosis and they could serve as a prognostic indicator of CCA. The cause and effect of 24-OH accumulation driven by the aberrant expression of these investigated proteins is depicted in Figure 4. The underlying mechanism by which CYP39A1 and RUNX2 are involved in the genesis of CCA requires further investigated.

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Differential profile of hepatobiliary oxysterols in subjects with and without cholangiocarcinoma

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Abbreviations: CCA, Cholangiocarcinoma; 7KD, 7-keto-cholesta-3,5-diene; 7β-OH, 7β-hydroxycholesterol; Epoxy, 5,6-epoxycholesterol; 3K4, 3-keto-cholest-4-ene; Triol, Cholestane-3β-5α-6β-triol; 7-keto, 7-ketocholesterol; 24-OH, 24-hydroxycholesterol.

Abstract

Oxysterols are reported to have inflammatory, cytotoxic and carcinogenic effects. We hypothesized that oxysterols in the hepatobiliary system play a role in the development of cholangiocarcinoma (CCA). The aim of this study was to characterize and quantify oxysterols in livers and gallbladder bile from patients with CCA and compare to patients without CCA using gas chromatography/mass spectrometry. Seven oxysterols were found in livers and gallbladder bile: 7-keto-cholesta-3,5-diene , 7β-hydroxycholesterol (7β-OH), 5,6-epoxycholesterol (Epoxy), 3-keto-cholest-4-ene (3K4), cholestane-3β-5α-6β-triol (Triol), 7-ketocholesterol (7-keto), 24-hydroxycholesterol (24-OH). Increased levels of 7β-OH, 3K4 and Epoxy were found in CCA tissue compared to patients with hepatocellular carcinoma and to cadaveric donors without liver disease. Significantly higher levels of 7β-OH, Epoxy and 7-keto were found both in CCA tissue and bile from CCA patients compared to livers and bile from patients with benign biliary diseases. Notably, CCA patients with high levels of 24-OH in their livers and in gallbladder bile had significantly reduced survival. Expression of CYP46A1, the enzyme responsible for synthesis of 24-OH, was significantly higher in CCA tissue. These findings support the hypothesis that specific hepatobiliary oxysterols are involved in the development of CCA.

Introduction

Oxysterols are derived from either enzymatic or non-enzymatic oxidation of cholesterol (1, 2). The chemical structures of oxysterols vary depending upon the number and position of oxygenated functional groups, and include hydroxy-, keto-, hydroxyperoxy-, and epoxy forms. Enzymatic pathways of oxysterol production mainly involve cytochrome P450 family enzymes. Examples include 27-hydroxylase (CYP27A1), the key enzyme in the alternative bile acid synthesis pathway which leads to the production of 27-hydroxycholesterol (3); and 7α-hydroxylase (CYP7A), the rate limiting enzyme in the classical bile acid synthesis pathway that leads to the production of 7α-hydroxycholesterol. Other oxysterols are produced by non-enzymatic oxidation (or auto-oxidation), a process that involves reactive oxygen species (ROS) and reactive nitrogen species (RNS). Examples of ROS that participate in non-enzymatic oxidation of cholesterol include hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) (4, 5). 7α-hydroperoxycholesterol is an example of a product of non-enzymatic oxidation of cholesterol, which can be further oxidized by non-enzymatic and enzymatic means.

Numerous *in vivo* and *in vitro* studies have described biological effects of oxysterols (6). Oxysterols act as intermediates in cholesterol catabolism (e.g. in bile acid synthesis) and are therefore involved in the elimination of excess cholesterol from the body. As oxysterols possess more hydrophilic moieties compared to the parent cholesterol molecule, they can pass more easily through cell membranes. Cholesterol removal from the brain is achieved via oxidation to 24-hydroxycholesterol (24-OH) by 24-hydroxylase (CYP46A1), with the 24-OH able to traverse the blood-brain barrier and be further metabolized by the P450 family enzymes CYP39A1 and CYP7A1 in the liver (7). Altered expression and/or function of these enzymes may affect oxysterol levels.

Many species of oxysterols have been identified in human tissues and plasma (8). High concentrations are found in atheromatous plaques (9), suggesting a role in the initiation of inflammation and progression of atherosclerosis in humans. Specific oxysterols have been found in various cancers, such as lung and colon (10, 11). Certain oxysterols were identified in gallstones and gallbladder bile (12). A previous study showed that hepatic bile from patients with biliary tract infection contained significantly higher levels of oxysterols than did such bile from patients without biliary tract infection (13). Various oxysterols have been described in human bile, some of which were proposed to be involved in the pathogenesis of biliary tract diseases and gallstones. Certain biliary oxysterols were reported to be involved in carcinogenesis in cholangiocytes (14). However, little is known about the types and concentrations of oxysterols in bile and hepatic tissue of patients with CCA.

Materials and methods

Chemicals

7-keto-cholesta-3,5-diene (7KD), 7β-hydroxycholesterol (7β-OH), 5,6-epoxycholesterol (Epoxy), 3-keto-cholest-4-ene (3K4), cholestane-3β-5α-6β-triol (Triol), 7-ketocholesterol (7-keto), 24-hydroxycholesterol (24-OH), and β-sitosterol were purchased from Steraloids (Newport, RI). Erythrodiol was purchased from Sigma-Aldrich.

Patients and sample collection

Liver tissue and gallbladder bile samples were collected from patients who underwent liver resection at Srinagarind Hospital, Khon Kaen, Thailand. Fifty-two tissue samples were collected, including seven samples from cadaveric donors, seven samples from patients with hepatocellular carcinoma (HCC), and 38 samples from CCA patients. Liver tissues were collected during surgery then immediately snap frozen in liquid nitrogen and stored at -80°C until use. For gallbladder bile collection, 10-15 ml of bile was collected via gallbladder puncture. Samples were supplemented with 100 ppm of butylated

hydroxytoluene and flushed with nitrogen to prevent autooxidation, then stored at -80°C until use. There were 48 bile samples collected, including 10 samples from patients with benign biliary diseases (bile duct dysplasia, cholangitis, cystadenoma, and bile duct stones). The diagnosis was based on clinical information, ultrasonography, and histological findings. Blood collection and analysis of biochemical tests were performed before the operation. Informed consent was obtained from each subject. The Khon Kaen University Ethics Committee for human research approved the research protocol (HE 531163).

Oxysterol analysis

Oxysterol concentrations in human livers and gallbladder were analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously (12) with modifications. In brief, the lipid fraction of liver tissue was extracted with chloroform/methanol (C/M) 2:1 containing the internal standard at room temperature. β-sitosterol and erythrodiol (Sigma-Aldrich, USA) were used as internal standards for oxysterol analysis in tissue and bile, respectively. The solvent was removed, dried and the residue was saponified at room temperature overnight with 2 N methanolic KOH. Nonsaponifiable lipids were extracted twice with diethyl ether; the combined extracts were then washed with distilled water. The ether was evaporated and the residue converted to the TMS derivative with BSTFA/pyridine Sigma. The solvent was evaporated and the sample was taken up in 200 µl of hexane. One µl of sample was injected into a Shimadzu QP2010 plus equipped with a 60m DB-5MS column.. Oven temperature was 215°C for 2 minutes, then 6°C/min to 290°C and 5°C/min to 320°C with 6 minutes at final temperature. Sterol derivatives were eluted at 21.5-26.5 min, and spectra were identified by comparison with authentic standards (Steraloids, Rhode Island, USA) and with the NIST library of spectra and the Wiley Registry of Mass Spectra, 7th edition. Standard curves were constructed and the contents in each sample were quantified.

Isolation of total RNA and Taqman® real-time polymerase chain reaction

Total RNA was isolated from seven cadaveric donors and 20 CCA patients. Total RNA was extracted using AllPrep® DNA/RNA/Protein Kit (Qiagen, CA). Real time RT-PCR was performed using TaqMan® Gene Expression assay kit and the ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA). The first-strand cDNA was synthesized from 2 ug of total RNA using SuperScript™ III (Invitrogen, NY). TaqMan® PCR primers and probes for cytochrome P450 family 46 subfamily A, polypeptide 1 (CYP46) (Hs01042347_m1) and β-actin were used in this experiment (Applied Biosystems, Foster City, CA). The TaqMan® Probes were labeled with quencher and reporter dyes. The reporter dye of targeted TaqMan® Probes in this experiment was labeled with FAM dye whereas β-actin probe was labeled with VIC dye. Briefly, single stranded cDNA was diluted with DEPC-treated water then subjected to mix with TaqMan® Master Mix and further mixed with TaqMan® Probe for specific targeted genes. The PCR reaction was performed at 2 steps PCR reaction including (i) step 1 at 50 °C for 2 min and 95 °C for 10 min for beginning of the hot start DNA polymerase, and (ii) step 2 at 95 °C for 15 sec for denaturing DNA to single strand and unfolding secondary form to primary structure of DNA then the temperature was decreased to 60 °C for 1 min for annealing of primer and DNA as well as DNA polymerase chain reaction; this stage was performed for 40 cycles.

Immunohistochemistry detection of CYP46A1

CYP46A1 protein was detected on the paraffin-embedded sections using standard immunohistochemistry protocols. In brief, the antigen were retrieved by using pressure boiler in 10 mM sodium citrate buffer pH6.0 and endogenous peroxidase was blocked with 0.3% H_20_2 (v/v) in 95 % ethanol. After non-specific blocking, slides were incubated with a 1:200 dilution of rabbit polyclonal anti-CYP46A1 antibody (Abnova, Taiwan) overnight in a humidified chamber at 4 °C. Sections were incubated with peroxidase-conjugated Envision

secondary antibodies (Dako, Glostrup, Denmark) and peroxidase activity visualized with diaminobenzidine solution. Hematoxylin was employed for counterstaining. The staining frequency of proteins was semiquantitatively scored based on the percentages of positive cells, as follows: 0 %=negative, 1–25 %=1, 26–50 %=2, and >50 %=3. The intensity of protein staining was scored as weak=1, moderate=2, and strong=3. The immunohistochemical scores were calculated by multiplying the frequency score with the intensity score.

Statistics

Biochemical tests, oxysterols and RNA expression were expressed as mean ± SD and statistically analyzed using one-way ANOVA. Bivariate Pearson correlation was used to study the correlation between the level of oxysterols and biochemical characteristic tests. The survival analysis was performed using Kaplan-Meier with Log-rank test. P-value $\leq$ 0.05 was considered statistically significant.

Results

Case demographics and laboratory characteristics

Among 52 samples of liver tissue, seven cases were identified as normal liver tissues from cadaveric donors, seven cases as HCC and 38 cases as CCA. In addition, 48 samples of gallbladder bile were collected. There were 10 cases of benign biliary disease, five cases of HCC and 33 cases of CCA. The male to female ratio was 2.3:1 and the average age was 57.1 years (range 35-77 years) (Table 1).

Levels of cholesterol, total bilirubin, direct bilirubin, albumin, globulin and white blood cell, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were not statistically different in all groups (Table 2).

Oxysterol profiles in liver tissue and gallbladder bile

Table 3 shows the levels of each oxysterol identified in normal liver tissues from cadaveric donors and tumor tissues from HCC and CCA patients. Seven types of oxysterols, including 7KD, 7β-OH, 3K4, Epoxy, Triol, 7-keto and 24-OH, were found in both normal liver tissues and tumor tissues. There was a significant increase in the levels of 7β-OH, 3K4 and Epoxy in CCA compared to normal liver tissue. The levels of 7β-OH in normal liver, HCC and CCA were 4.1 ± 5.6, 32.9 ± 22.5 and 80.9 ± 99.3 μg/g of tissue, respectively. The levels of 3K4 were 29.6 ± 47.4, 266.7 ± 40.5 and 394.4 ± 415.1 μg/g of tissue, respectively. The levels of Epoxy were 257.9 ± 255.2, 567.7 ± 105.5 and 739.1 ± 398.5 μg/g of tissue, respectively. In addition, higher levels of 7KD, Triol, 7-keto and 24-OH were found in the CCA groups compared to normal liver and HCC, although these differences were not statistically significant.

In the CCA group, levels of oxysterols in normal adjacent tissues were also determined. Significant differences in the levels of 7KD, 7β-OH, Epoxy, Triol and 7-keto in normal adjacent tissues versus tumor tissue were not observed (data not shown). Interestingly, significantly increased levels of 24-OH and 3K4 were found in CCA tumor tissue compared to normal adjacent tissue (Figure 1).

Level of oxysterols in gallbladder bile was analyzed in patients with benign biliary diseases, HCC and CCA (Table 4). Seven oxysterols were also identified in gallbladder bile. Significantly higher levels of 7β-OH, Epoxy and 7-keto were observed in bile from CCA patients compared to bile from patients with benign biliary diseases (8.6 ± 1.6 vs 2.2 ± 1.0; 155.0 ± 28.9 vs 30.4 ± 10.3; 49.8 ± 8.3 vs 19.8 ± 7.3 ug/ml, respectively). Notably, Epoxy levels in bile from HCC patients was significantly increased compared to levels in bile from patients with benign biliary diseases (245.7 ± 58.3 vs 30.4 ± 10.3 ug/ml).

Correlation of 24-OH level and the expression of its metabolizing enzyme (CYP46A1) in CCA

A comparison of all oxysterol levels with clinico-pathological data and cumulative survival was performed. The sole significant correlation was found with respect to the levels of 24-OH. The correlation between 24-OH levels, clinico-pathological findings and cumulative survival was analyzed. Histological review showed 21 cases of papillary type (58%). A significant correlation between 24-OH levels and histological grading was observed (P=0.05). Twelve of 21 patients with a low level of 24-OH were diagnosed with the papillary type (Table 5).

Cumulative survival was compared between CCA patients with low and high levels of 24-OH. Log-rank analysis indicated that CCA patients with high levels of 24-OH had significantly lower survival than patients with low levels of 24-OH in both tissue and gallbladder bile, as depicted in figure 2.

A multivariate progression analysis based on the Cox proportional hazard model was performed to analyze the independent value of each parameter predicting overall survival (Table 6). Factors that may affect survival were included in the model. In this analysis, only 24-OH level in tumor tissue (P=0.045) proved to be an independent prognostic survival factor in CCA. CCA patients who had high levels of 24-OH had a 3-fold higher risk of death compared to patients who had low 24-OH levels.

We also determined the expression of CYP46A1, the enzyme that is responsible for synthesis of 24-OH. We observed increased expression of CYP46A1 mRNA and protein in CCA tumor tissue compared to normal tissue from cadaveric donors. Among the liver sections of 34 patients with CCA, 27 cases showed high CYP46A1 staining (Figure 3).

Discussion

There is increasing interest in the biological functions of oxidative products of cholesterol, but it is surprising that so little is known about hepatobiliary oxysterols in the setting of cancers of the hepatobiliary system. We have profiled oxysterols in liver tissue and gallbladder bile from patients with CCA. Seven oxysterols--7KD, 7β-OH, 3K4, Epoxy, Triol, 7-keto, and 24-OH--were identified in normal human liver and hepatobiliary cancer tissue. Similar oxysterol species were found in gallbladder bile. Several other sterols were also present in tissue and bile. These include plant and fungal sterols (stigmasterol, lanosterol, stigmast-5-ene-3-ol and ergost-5-ene-3-ol).

Among oxysterols identified in our study, several were previously found in high concentrations in the hepatobiliary system. Oxysterols were found in human hepatic bile, with 7β-OH present at significantly higher concentrations in infected compared to uninfected bile. The levels of 7β-OH, Triol, 3K4 and 7-keto were also positively correlated with C-reactive protein levels (13). Haigh et al. (2006) demonstrated that human leukocytes, activated by the presence of bacterial lipopolysaccharide, oxidized cholesterol to a variety of oxysterols, including 3K4, 7KD, 7β-OH and 7-keto. GC/MS identified 3K4 and 3keto-4,6 diene in bile and stone samples. Markedly higher levels of these oxysterols were found in pigment gallstones, where bacterial DNA is most abundant. Moreover, we previously reported increasing levels of Triol and 3K4 in Ov-associated hamster CCA, and higher levels of these oxysterols have been hypothesized to be involved with inflammation (15). These observations suggest that biliary oxysterols are associated with the presence of bacteria and may play a role in the pathogenesis of gallstones and biliary tract cancers.

One concern is that oxysterols may be artifacts due to autooxidation that occurs during collection and extraction procedures, or are generated during the gas chromatography analysis. 7KD and 7-keto can be generated by autooxidation and thermal dehydration in the

injection port (6). Thus, we used solvents containing the antioxidant BHT and procedures were carried out under nitrogen. Pure standards processed through the same steps showed no autooxidation. Some samples in our study were processed in an identical manner but did not yield oxysterols. These observations suggest that oxysterol species found in this study were not artifacts but endogenous oxidized products of cholesterol formed *in vivo*.

Among the seven oxysterols reported in this study, we found only one, 24-OH, which is synthesized through an enzymatic pathway. 24-OH, synthesized in the brain in order to remove cholesterol, is metabolized by the liver into bile acids. This mechanism involves oxidation of cholesterol by CYP46A1, the product then passes through the blood-brain barrier (16). Although several studies describe the effects of 24-OH accumulation in the pathogenesis of inflammatory brain diseases, little is known about its role in the hepatobiliary system. We found high levels of 24-OH in CCA tumor tissue and bile compared to cadaveric donors and HCC, although these differences were not statistically significant. This may be due to high variation between individuals, as four cases in the CCA group did not have measurable 24-OH. However, significantly increased 24-OH levels were found in tumor tissue compared to normal adjacent tissue ($P<0.05$). In addition, the significant finding in this study is the correlation of 24-OH level and five-year survival rate in CCA patients. CCA patients with low levels of 24-OH showed significantly longer survival than patient with high levels of 24-OH. Moreover, we proposed that alteration of CYP46A1 activity may affect oxysterol level. Increased CYP46A1 expression in CCA patients may contribute to accumulation of 24-OH. Thus, the potential role of 24-OH in cholangiocarcinogenesis should be investigated further.

In conclusion, we profiled oxysterols in human CCA tissue and gallbladder bile and compared these results to liver tissues and bile obtained from normal liver, bile from patients with benign biliary diseases, and liver tissue from patients with HCC. Differences in

oxysterol levels in the cancer groups versus benign or normal samples were found. Some oxysterols found in our model have been previously proposed to be carcinogenic. Moreover, we provide the first report of 24-OH levels in the hepatobiliary system along with mRNA expression data on its metabolizing enzyme, CYP46A1. We propose that alteration of CYP46A1 expression and high 24-OH levels are involved in the pathological processes that lead to biliary tract cancer. Changes in hepatic and biliary oxysterol levels may be involved in cholangiocarcinogenesis. The mechanistic basis for the role of specific hepatobiliary oxysterols in the development and progression of cholangiocarcinoma remains to be elucidated.

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Table 1. Case demographics

Category	No. of Samples
Age (years) 57.1 ± 8.4 (range 35-77)	
Sex (Male:Female ratio) 2.3:1	
Liver tissue	52
- Cadaveric donor	7
- Hepatocellular carcinoma	7
- CCA	38
Gallbladder bile	48
- Benign biliary diseases	10
- Hepatocellular carcinoma	5
- CCA	33

Table 2. Biochemical characteristics

Biochemical parameters (Normal range)	**Groups (Mean ± SD)**		
	Benign biliary diseases	HCC	CCA
Cholesterol (127-262 mg/dl)	175±24.3	149.7±67.8	181.1±53.5
Albumin (3.8-5.4 g/dl)	3.5±0.8	3.4±1.1	3.9±0.6
Globulin (2.6-3.4 g/dl)	4.1±0.9	4.1±1	4.2±0.9
Total bilirubin (0.25-1.5 mg/dl)	1.5±1.5	2.7±3.9	2.8±6.8
Direct bilirubin (0-0.5 mg/dl)	0.8±1.2	1.9±3	2.1±5.8
ALT (4-36 U/L)	47.9±28.8	39.5±14.9	55.7±60.1
AST (12-32 U/L)	65.3±56.6	51±37	73.9±108.6
ALP (42-121 U/L)	227.6±147	88.3±30	204.9±139.4
WBC (4.6-10.6/µl)	8.9±3.2	14.5±9.3	10.4±3.3

Table 3. Level of oxysterols in liver tissue

Oxysterol	Level of oxysterols (ug/g of tissue)		
	Cadaveric donor (n=7)	**HCC (n=7)**	**CCA (n=38)**
7KD	10.7 ± 18.8	2.7 ± 4.7	64.2 ± 107.9
7β-OH	4.1 ± 5.6	32.9 ± 22.5	80.9 ± 99.3 [a]
3K4	29.6 ± 47.4	266.7 ± 40.5	394.4 ± 415.1[a]
Epoxy	257.9 ± 255.2	567.7 ± 105.5	739.1 ± 398.5 [a]
Triol	0.7 ± 1.8	N.D.	19.6 ± 34.2
7-keto	139.1 ± 142.9	115.9 ± 58.3	221.5 ± 138.5
24-OH	0.15 ± 0.3	N.D.	99.13 ± 164.3

[a] $p < 0.05$ one way ANOVA analysis; CCA vs Cadaveric donor
N.D.; Not detected

Table 4. Level of oxysterols in gallbladder bile

Oxysterol	Level of oxysterol (ug/ml)		
	Benign biliary disease (n=10)	**HCC (n=4)**	**CCA (n=33)**
7KD	N.D.	N.D.	0.1 ± 0.8
7β-OH	2.2 ± 1.0	3.0 ± 2.4	8.6 ± 1.6^{a}
3K4	12.7 ± 11.2	N.D.	18.0 ± 8.7
Epoxy	30.4 ± 10.3	245.7 ± 58.3^{b}	155.0 ± 28.9^{a}
Triol	0.05 ± 0.04	$2.6\text{x}10^{-7} \pm 1.8\text{x}10^{-7}$	0.08 ± 0.04
7-keto	19.8 ± 7.3	15.0 ± 2.3	49.8 ± 8.3^{a}
24-OH	2.3 ± 2.3	0.46 ± 0.46	5.8 ± 2.4

[a] $p < 0.05$ one way ANOVA analysis; CCA vs Cadaveric donor
[b] $p < 0.05$ one way ANOVA analysis; HCC vs Cadaveric donor
N.D.; Not detected

Table 5. Correlation between 24-OH levels in CCA tissues and clinico-pathological data

Variable	No. of patients	24-OH		
		Low expression	High expression	*P* value
Age (y)				
≤ 56	24	13	11	
> 56	12	3	9	0.095
Gender				
Male	19	8	11	
Female	17	8	9	0.515
Histological grading				
Papillary type	21	12	9	
Non Papillary type	15	4	11	0.05*
Metastasis stage				
No	19	7	12	
Yes	17	9	8	0.263

Fisher's exact probability test was used for the comparisons

* $P \leq 0.05$

Table 6. Multivariate analysis by Cox Proportional Hazards Regression Model

Variable	Hazard ratio	95% CI	*P*
24-OH			
Low	1		
High	3.292	1.024-10.577	0.045
Age, years			
<56	1		
≥56	0.227	0.435-3.325	0.722
Sex			
Male	1		
Female	0.558	0.216-1.439	0.227
Histopathology			
Papillary type	1		
Non-papillary type	1.833	0.586-5.731	0.298
Metastasis			
No	1		
Yes	1.216	0.471-3.143	0.686

95% CI indicates 95% confidence interval

Figure legends

Figure 1. Levels of 24-OH (A) and 3K4 (B) in liver tissue of CCA patients. Significantly increased levels of 24-OH and 3K4 were observed in tumor tissue of CCA patients compared to matched-pair adjacent tissue ($P < 0.05$).

Figure 2. Survival curves of CCA patients with levels of 24-OH. Kaplan-Meier log-rank test indicated that CCA patients with high levels of 24-OH had significantly lower survival than patients with low levels of 24-OH in both tissue (A) and gallbladder bile (B). $P < 0.001$ and $P < 0.05$, respectively.

Figure 3. mRNA and protein expression of CYP46 in CCA. A. qRT-PCR was performed to detect CYP46 expression in CCA tissue compared to normal liver tissue. Increased CYP46 mRNA expression in CCA tissue was found ($P < 0.05$). B. Protein expression of CYP46 was confirmed by immunohistochemical staining in CCA tissues (n = 34). a. CYP46 staining in normal bile duct of cadaveric donor (arrow indicates normal bile duct). b. Twenty-seven out of 34 cases (79%) showed high CYP46 staining scores. c. Seven out of 34 cases (21%) showed low CYP46 expression. Magnification was 400x.

Figure 1

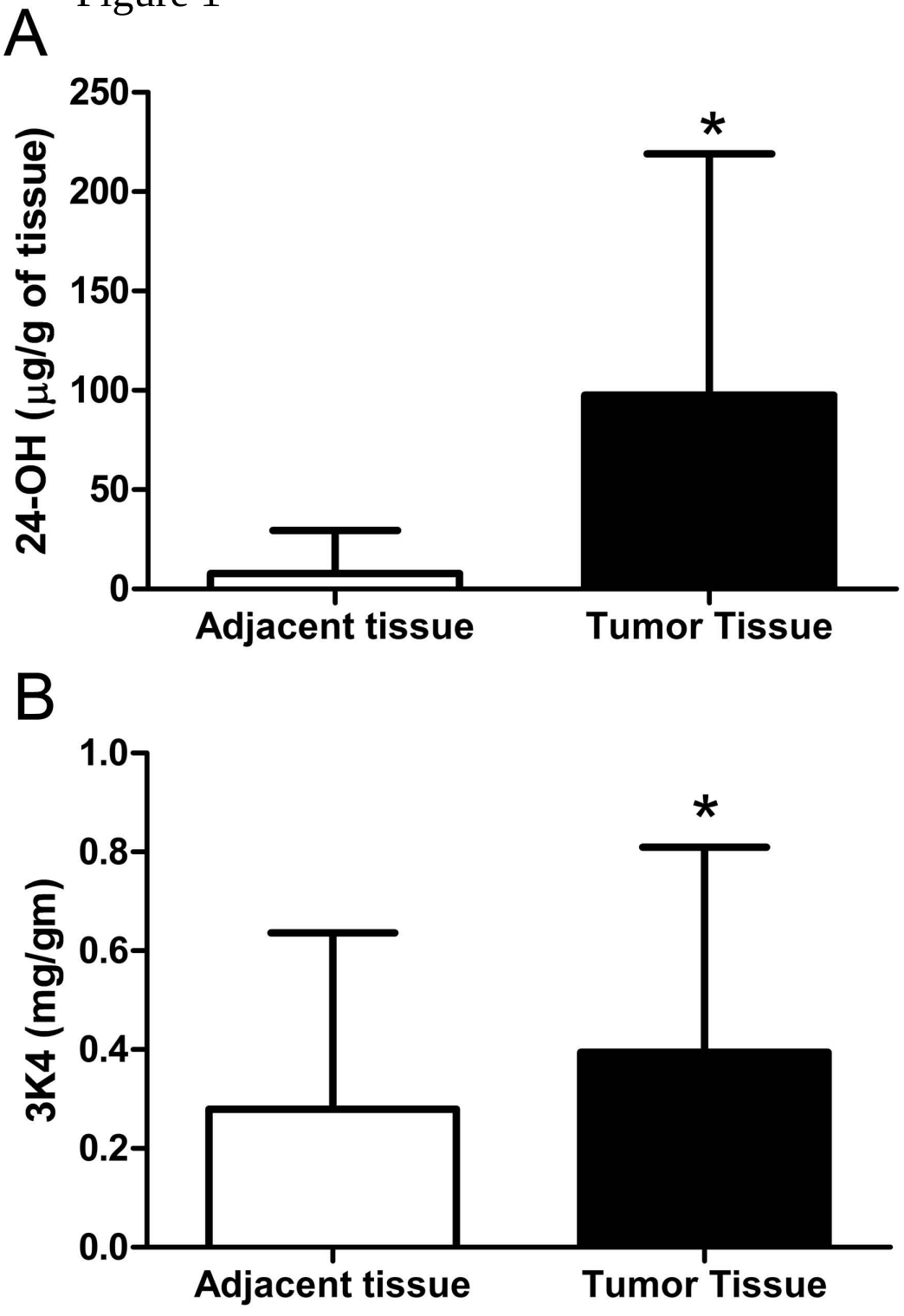
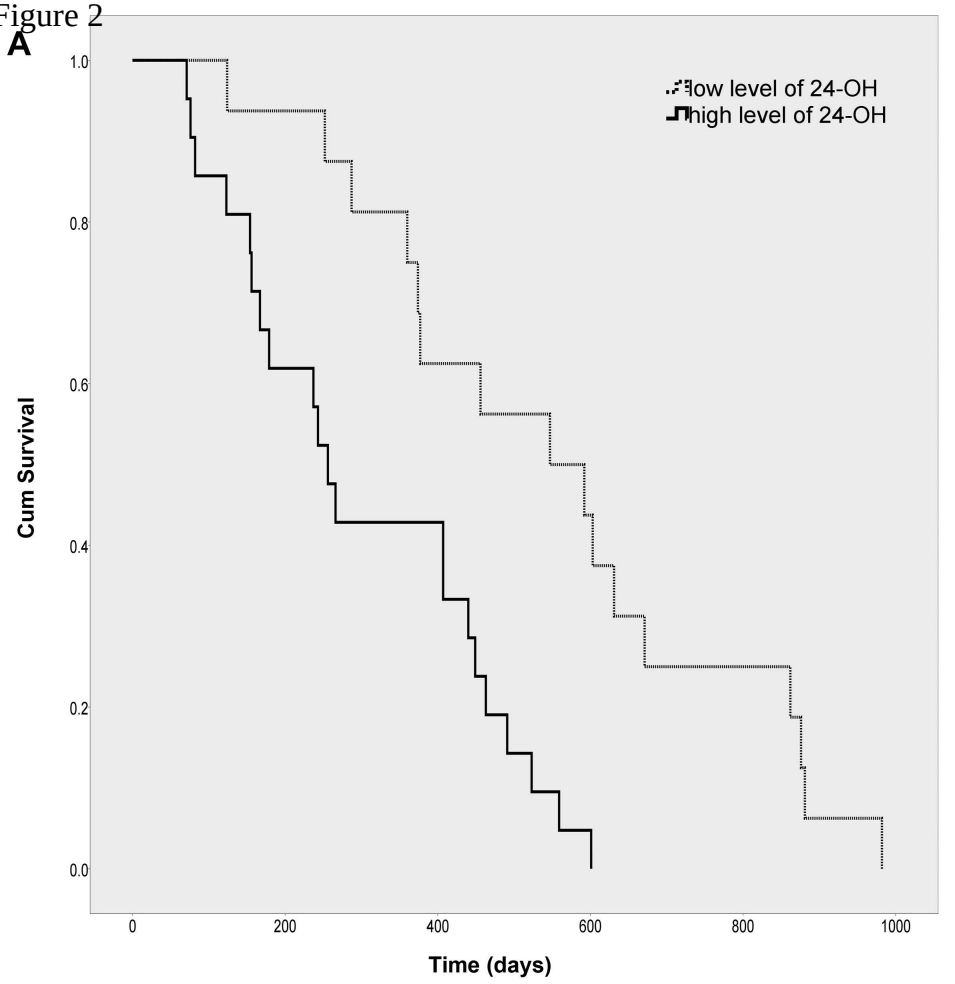

A
250
200
150
100
50
0
24-OH (μg/g of tissue)
*
Adjacent tissue
Tumor Tissue
B
1.0
0.8
0.6
0.4
0.2
0.0
3K4 (mg/gm)
*
Adjacent tissue
Tumor Tissue

Figure 2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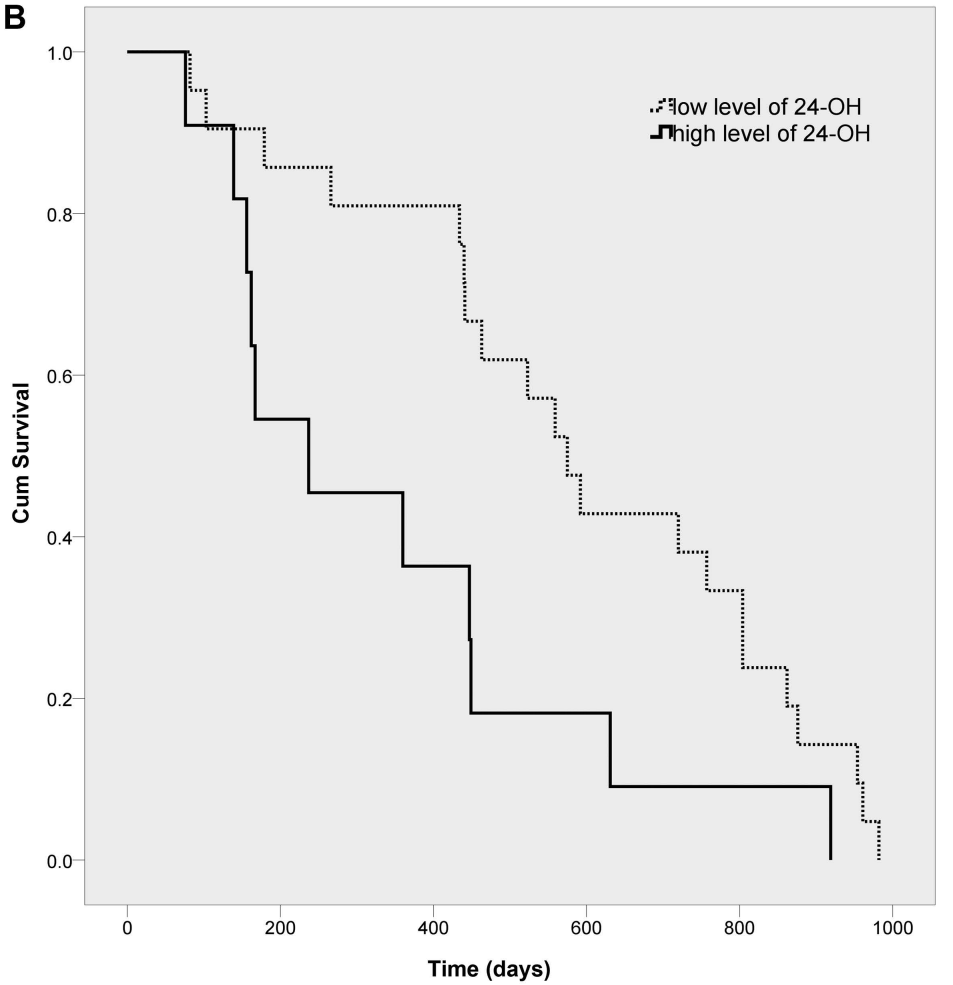
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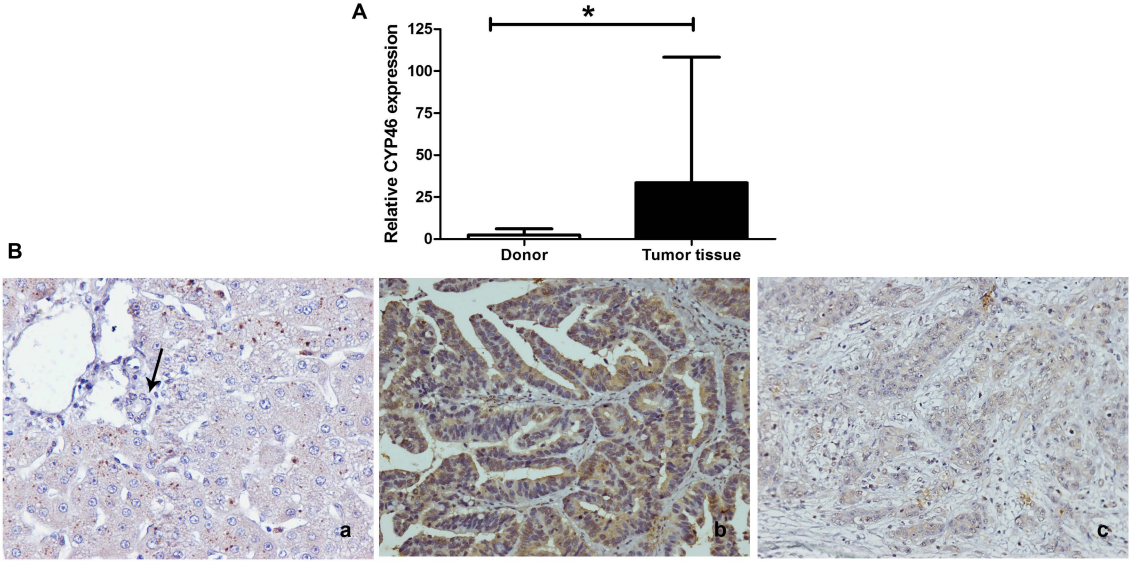
1000
Time (days)

Figure 3


A
*
Relative CYP46 expression
125
100
75
50
25
0
Donor
Tumor tissue
B
a
b
c

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Oral-01

Effect of carcinogenic oxysterols on induction of apoptosis is related to DNA damage in immortal cholangiocyte

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Oxysterols are oxygenated derivatives of cholesterol, which are produced by non-enzymatic reaction through reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Oxysterols, which may be produced upon the liver fluke (*Opithorchis viverrini*; Ov) infection are also suspected to play roles in genesis of cholangiocarcinoma (CCA). Our previous data showed that the level of particular oxysterols including cholestan-3β, 5α, 6β-triol (Triol) and cholest-4-ene-3-one (3K4), which are categorized as carcinogenic forms, were significantly higher in liver tissues of Ov-induced hamster CCA. We purpose to examine the effect of oxysterols including Triol and 3K4 on the induction of DNA damage and the alteration of cellular phenotype in human transformed cholangiocyte (MMNK-1). The immunocytochemical detection of etheno-DNA adduct in oxysterols-induced MMNK-1 cell was performed to determine the level of DNA adduct that caused by oxysterols. This study showed the increased level of carcinogenic ethano-DNA adducts (1-N^6-ethenodeoxyadenosine or εdA) that is formed by the reaction of lipid peroxidation. Besides that, the effect of oxysterols on cell proliferation was analyzed by using flow cytometer and western blot. It was shown that these oxysterols induced cells death via apoptosis pathway as a dose-dependent manner. These evidences suggest that cell death induced by Triol and 3K4, which is generated during carcinogenesis, may have the procarcinogenic impact, since oxysterol could also cause DNA damage via oxidative stress and if mutated clonal expansion occurs, it may play role in the changing of cellular phenotype from normal to malignancy. The proposed mechanistic involvement is under investigation.

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โรงพยาบาลสรรพสิทธิประสงค์ อุบลราชธานี

มะเร็งท่อน้ำดี : ปัญหาที่ยังมีทางออกของชาวอีสาน

พวงรัตน์ ยงวณิชย์ และวัชรินทร์ ลอยลม
ภาควิชาชีวเคมี และศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี
คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

ในรอบเวลา 30 ปีที่ผ่านมานี้ ได้ปรากฏหลักฐานที่พิสูจน์อย่างชัดเจนถึงบทบาทการก่อมะเร็งในมนุษย์ที่มีสาเหตุมาจากการติดเชื้อโรคชนิดต่างๆ โดย Parkin (Parkin, 2006) ได้รายงานถึงสถิติปี 2002 ของอุบัติการณ์ของโรคมะเร็งทั่วโลกที่เกิดจากการติดเชื้อ ว่าอยู่ที่ 1.9 ล้านราย หรือคิดเป็นร้อยละ 17.8 ของโรคมะเร็งจากสาเหตุทั้งหมด สาเหตุใหญ่ๆ นั้นมาจากเชื้อแบคทีเรีย Helicobactor pyroli ที่เป็นสาเหตุของมะเร็งกระเพาะอาหาร เชื้อไวรัส human papilloma ทำให้เกิดมะเร็งปากมดลูก และในกลุ่มมะเร็งตับคือ เชื้อไวรัสตับอักเสบชนิดบีและซี รวมถึงตัวปรสิตหลายชนิด นอกจากนี้ยังพบว่าโรคมะเร็งที่เกิดจากการติดเชื้อเป็นปัญหาของประเทศกำลังพัฒนามากกว่าประเทศพัฒนา สำหรับประเทศไทยโดยเฉพาะอย่างยิ่งในภาคตะวันออกเฉียงเหนือที่โรคมะเร็งท่อน้ำดีอันเกิดมาจากการติดเชื้อพยาธิใบไม้ตับ (Opisthorchis viverrini, OV) ก็ยังมีอุบัติการณ์สูงสุดเมื่อเทียบกับโรคมะเร็งชนิดอื่นๆ ยิ่งไปกว่านี้อุบัติการณ์ของมะเร็งตับของไทยนั้นมีตัวเลขที่สูงกว่าประเทศเพื่อนบ้านในแถบอาเซียตะวันออกเฉียงใต้เช่นฟิลิปปินส์และสิงคโปร์ ที่ไม่มีรายงานการติดเชื้อพยาธิใบไม้ตับ แต่มีการติดเชื้อไวรัสตับอักเสบใกล้เคียงกัน อยู่ถึงร้อยละ 50-100 (Ferlay, 2002)

การติดเชื้อพยาธิใบไม้ตับแบบเรื้อรังและซ้ำซากก่อให้เกิดการทำลายดีเอ็นเอ : หลักฐานจากสัตว์ทดลองและการศึกษาในผู้ป่วย

การศึกษาของคณะวิจัยเรา สามารถแสดงให้เห็นว่าการติดเชื้อพยาธิใบไม้ตับแบบเรื้อรังในหนูแฮมสเตอร์ก่อให้เกิดภาวะการอักเสบแบบเรื้อรังในท่อน้ำดี และส่งผลให้มีการสร้างสารประกอบที่เป็นอนุมูลอิสระชนิดออกซิเจน (reactive oxygen species ; ROS) และไนโตรเจน (reactive nitrogen species ; RNS) จำนวนมาก ซึ่งสารเหล่านี้สามารถทำลายดีเอ็นเอ ซึ่งอาจจะก่อให้เกิดการเปลี่ยนแปลงของยีน และมีโอกาสเสี่ยงนำไปสู่การเกิดมะเร็งท่อน้ำดีต่อไปได้ โดยผลการศึกษาครั้งนี้สามารถแสดงให้เห็นอย่างชัดเจนว่ามีการทำลายดีเอ็นเอในเซลล์เยื่อบุผิวท่อ

น้ำดีหลังจากสัตว์ทดลองได้รับเชื้อพยาธิใบไม้ตับ โดยการตรวจดูระดับของเบสบนดีเอ็นเอที่ถูกเปลี่ยนแปลงไป คือ 8-oxodG และ 8-nitroguanine ซึ่งเป็นตัวบ่งชี้การทำลายดีเอ็นเอจากอนุมูลอิสระของออกซิเจนและไนโตรเจนตามลำดับ (Pinlaor, 2003) เราได้ทำการทดลองต่อไปโดยให้หนูแฮมสเตอร์ติดเชื้อพยาธิซ้ำเป็นครั้งที่ 2 และครั้งที่ 3 จะเกิดการทำลายดีเอ็นเอใหม่ทุกครั้ง และความรุนแรงของการทำลายเกิดได้รวดเร็วขึ้นตามจำนวนครั้งของการติดเชื้อซ้ำ (Pinlaor, 2004a) นอกจากนี้ยังพบว่าในระยะเรื้อรังของทั้งการติดพยาธิครั้งเดียวและการติดพยาธิซ้ำ จำนวนเซลล์อักเสบจะลดลงแต่เนื้อเยื่อเกี่ยวพันจะเพิ่มขึ้น การติดพยาธิ OV ซ้ำสามครั้ง สองครั้งและครั้งเดียว จะเหนี่ยวนำจำนวนเซลล์อักเสบให้เคลื่อนที่มารวดเร็วขึ้นเป็นลำดับในระยะเริ่มต้น และเซลล์อักเสบนี้จะสัมพันธ์กับการเปลี่ยนแปลงของเอนไซม์ในตับและความหนาตัวของเนื้อเยื่อ (Pinlaor, 2004b) การทดลองนี้เป็นหลักฐานชิ้นสำคัญที่แสดงให้เห็นถึงความเสี่ยงในการเกิดมะเร็งที่เพิ่มมากขึ้นหากคนยังนิยมรับประทานปลาดิบ หรือปลาร้า ปลาส้มที่มีระยะติดเชื้อของ OV รวมทั้งการซื้อยาถ่ายพยาธิมารักษาตัวเอง แต่ยังไม่สามารถละเลิกจากนิสัยการกินดิบได้ การทำลายเบสบนดีเอ็นเอดังกล่าวได้รับการพิสูจน์ต่อมาว่าเกิดขึ้นในคนที่ติดเชื้อ OV เช่นเดียวกัน (Thanan, 2008)

การศึกษายีนที่มีบทบาทในกระบวนการก่อมะเร็งท่อน้ำดีเพื่อใช้เป็นเป้าหมายในป้องกัน การรักษา และการวินิจฉัยเราได้ศึกษาแบบแผนการแสดงออกของยีนในเนื้อเยื่อมะเร็งท่อน้ำดีของสัตว์ทดลองที่ถูกชักนำให้เป็นมะเร็งโดยการได้รับเชื้อ OV ร่วมกับการให้สารก่อมะเร็งชนิด N-nitrosodimethylamine (NDMA) เปรียบเทียบกับข้อมูลที่ได้จากกลุ่มควบคุม ทำให้เราสามารถค้นพบการแสดงออกของกลุ่มของยีนที่น่าจะมีบทบาทสำคัญในกระบวนการก่อมะเร็งท่อน้ำดี (Loilome, 2006) ซึ่งข้อมูลการแสดงออกของกลุ่มของยีนที่เปลี่ยนแปลงนี้พบได้ทั้งในกลุ่มที่ควบคุมการแบ่งตัว กลุ่มการตายและกลุ่มการแพร่ลุกลามของเซลล์ ยีนหลายตัว เช่น MARCKS ได้ทำการศึกษาต่อไปในเนื้อเยื่อมะเร็งของคนไข้ที่สัมพันธ์กับข้อมูลทางคลินิก (Techasen, 2010) เป็นต้น ความรู้เรื่องยีนที่เกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดีมีประโยชน์เป็นอย่างยิ่งในการใช้เป็นเป้าหมายของการตรวจวินิจฉัย การใช้สารยับยั้งเพื่อป้องกัน และการรักษา

ทางออกของปัญหา

การวิจัยที่เข้มแข็งทางวิทยาศาสตร์การแพทย์พื้นฐานถึงกลไกการเกิดมะเร็งท่อน้ำดีที่มีสาเหตุมาจากการติดเชื้อ OV น่าที่จะเป็นรากฐานที่สำคัญต่อการเฝ้าระวังกลุ่มเสี่ยงด้วยการพัฒนาตัวชี้วัดทางชีวภาพเพื่อการวินิจฉัยโรคได้แต่เริ่มต้น การค้นหาสารยับยั้งกระบวนการก่อมะเร็ง และการใช้ยาที่ออกฤทธิ์จำเพาะต่อโมเลกุลของมะเร็งที่เรียกว่าการรักษาแบบมุ่งเป้าหมาย (targeted therapy) (Loilome, in press) ก็ยังเป็นทางออกให้ศัลยแพทย์สามารถให้การรักษาได้อย่างมีประสิทธิภาพ ผู้ป่วยมีโอกาสหายจากโรคหรือมีอัตราการรอดชีพที่ยืนยาวขึ้น ทั้งนี้ต้องเกิดจากความร่วมมืออย่างจริงจังของนักวิจัย แพทย์ และผู้มีส่วนเกี่ยวข้องทุกฝ่าย ปัญหานี้ย่อมมีทางออกในอนาคตอันไม่ไกลนัก

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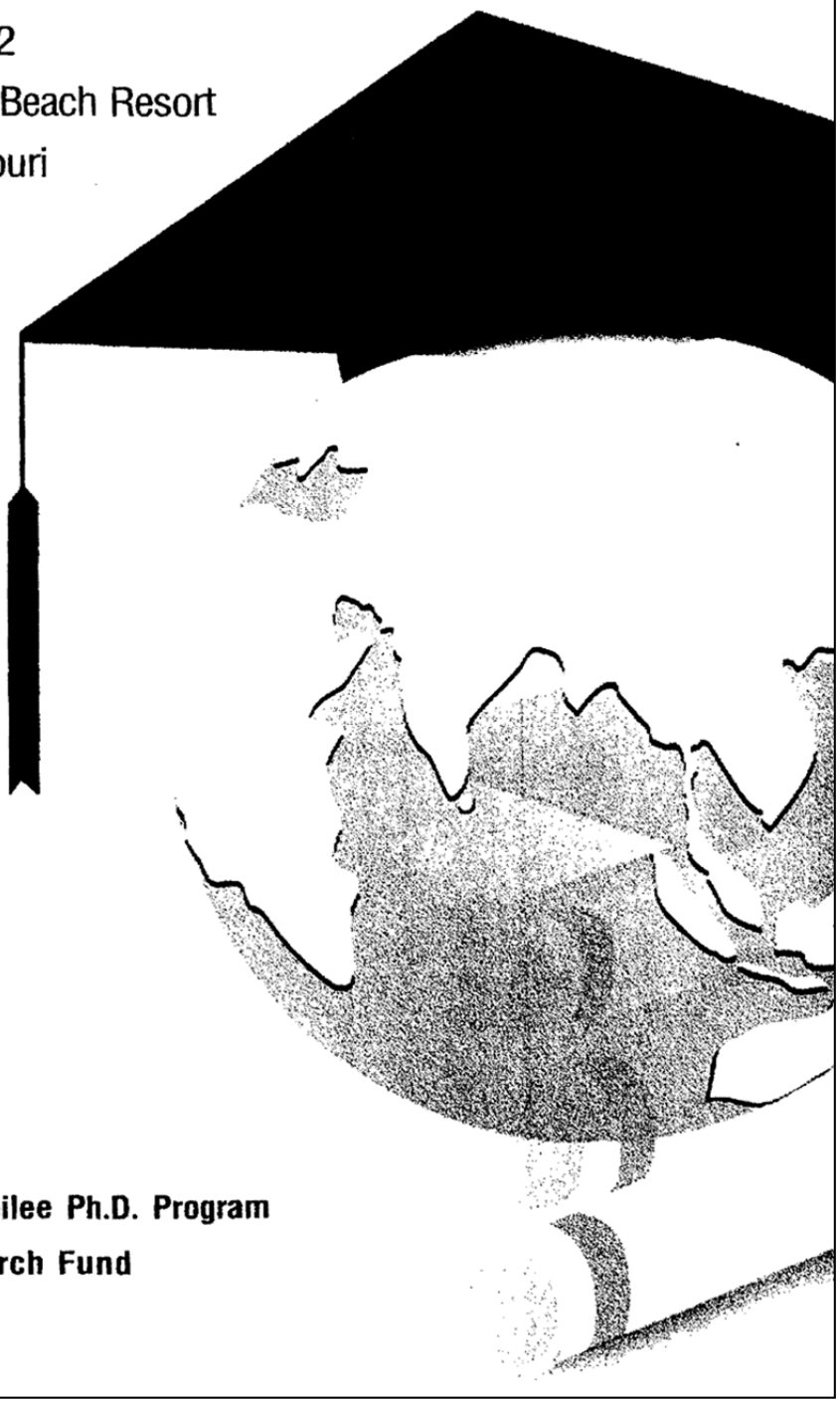

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Cholesterol Oxidation Products: A Possible Role in Cholangiocarcinogenesis

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Introduction and Objective

Cholesterol oxidation products or oxysterols are the oxygenated derivatives of cholesterol. They can be produced by enzymatic reaction which is involved with cytochrome P450 enzymes in bile acid synthesis. Moreover, oxysterols can be generated by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) in non-enzymatic reaction. They are the intermediates in bile acid synthesis as well as important signaling agents. However, some certain species are cytotoxic and carcinogenic. Oxysterols have been identified in bile in the setting of chronic inflammation. The increased expressions of oxysterol-binding proteins were also reported in cholangiocarcinoma. Then, we aim to identify the oxysterols in human cholangiocarcinoma and to explain the possible role of oxysterol in cholangiocarcinogenesis.

Methods

We identified oxysterols in liver tissue and bile in human and hamster cholangiocarcinoma using GC/MS. The possible effects of selected oxysterols on cholangiocyte cell line were studied using flow cytometry and western blot. Oxysterols-induced DNA damage was analyzed using immunocytochemistry.

Results

We found oxysterols in liver from hamsters infected with *Opisthorchis viverrini* (Ov). Level of some certain oxysterols, 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased in Ov and *N*-nitrosodimethylamine treated hamsters. The high levels of triol and 3K4 were also seen in both acute and chronic phases of inflammation along the cholangiocarcinogenesis process. Moreover, levels of both forms in human cholangiocarcinoma liver were higher than cadaveric donor and hepatoma tissue. We also found the rising level of these pathogenic oxysterols in human cholangiocarcinoma bile. Interestingly, triol and 3K4 could also induce the production of carcinogenic ethano-DNA adducts in human cholangiocyte cell line.

Conclusion

We proposed that chronic liver fluke infection increases the production of ROS/RNS in the setting of chronic inflammation in the biliary system, leading to the formation of oxysterols via oxidation of biliary cholesterol. Oxysterols and free radicals can induce DNA damage. Damaged biliary epithelial cells are eliminated through induction of apoptosis. Ineffective DNA repair and ongoing exposure to DNA damaging agents select for resistant cells that clonally expand to become malignant

Keywords: Oxysterols, Cholangiocarcinoma, DNA damage

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A possible role of oxidized cholesterol in cholangiocarcinogenesis

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Cholesterol oxidation products or oxysterols are the oxygenated derivatives of cholesterol. They can be produced by enzymatic reaction which is involved with cytochrome P450 enzymes in bile acid synthesis. Moreover, oxysterols can be generated by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) in non-enzymatic reaction. They are the intermediates in bile acid synthesis as well as important signaling agents. However, some certain species are cytotoxic and carcinogenic. Oxysterols have been identified in bile in the setting of chronic inflammation. The increased expressions of oxysterol-binding proteins were also reported in cholangiocarcinoma. Then, we aim to identify the oxysterols in human cholangiocarcinoma and to explain the possible role of oxysterol in cholangiocarcinogenesis. We identified oxysterols in liver tissue and bile in human and hamster cholangiocarcinoma using GC/MS. The possible effects of selected oxysterols on cholangiocyte cell line were studied using flow cytometry and western blot. Oxysterols-induced DNA damage was analyzed using immunocytochemistry. We found oxysterols in liver from hamsters infected with *Opisthorchis viverrini* (Ov). Level of some certain oxysterols, 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased in Ov and *N*-nitrosodimethylamine treated hamsters. The high levels of triol and 3K4 were also seen in both acute and chronic phases of inflammation along the cholangiocarcinogenesis process. Moreover, levels of both forms in human cholangiocarcinoma liver were higher than cadaveric donor and hepatoma tissue. We also found the rising level of these pathogenic oxysterols in human cholangiocarcinoma bile. Interestingly, triol and 3K4 could also induce the production of carcinogenic ethano-DNA adducts in human cholangiocyte cell line. We proposed that chronic liver fluke infection increases the production of ROS/RNS in the setting of chronic inflammation in the biliary system, leading to the formation of oxysterols via oxidation of biliary cholesterol. Oxysterols and free radicals can induce DNA damage. Damaged biliary epithelial cells are eliminated through induction of apoptosis. Ineffective DNA repair and ongoing exposure to DNA damaging agents select for resistant cells that clonally expand to become malignant.

Keywords: Oxysterols, Cholangiocarcinoma, Cholesterol oxidation products

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Molecular Mechanisms of Oxysterols in Liver Fluke-induced Cholangiocarcinogenesis

Yongvanit, P.[1,3*], Jusakul, A.[1,3], Loilome, W.[1,3], Namwat, N.[1,3], Techasean, A.[1,3], Haigh, W. G.[4], Pinlaor, S.[2,3], Kuver, R.[4]

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Abstract

Oxysterols are the oxygenated derivatives of cholesterol. They can be produced either by enzymatic reaction which is involved with cytochrome P450 enzymes or be generated by reactive oxygen/nitrogen radicals. Some certain species are cytotoxic and carcinogenic. We aim to identify the oxysterols in human cholangiocarcinoma (CCA) and to explain its possible role in cholangiocarcinogenesis. We identified oxysterols in liver tissue of *Opisthorchis viverrini* (Ov)-induced hamster cholangiocarcinoma using GC/MS. Level of oxysterols, 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased in CCA hamsters. The high levels of triol and 3K4 were seen in both acute and chronic phases of inflammation along the cholangiocarcinogenesis. It was shown that Triol- and 3K4-treated human cholangiocyte cells caused apoptosis via mitochondria-dependent mechanism. They could also induce the production of carcinogenic etheno-DNA adducts. Whether oxysterols induced cell death involve in the development of cholangiocarcinogenesis, we established the oxysterol-resistant cells. The resistant cell revealed resisted character to apoptosis induced by H_2O_2. Using protein arrays, we found activation of down-stream effectors in receptor tyrosine kinase signaling resulting in regulation of expression of apoptosis-related proteins. Those mechanisms involve in the stimulation of cellular survival and apoptosis evasion mechanism.

Keywords: oxysterols, cholangiocarcinoma

Outputs

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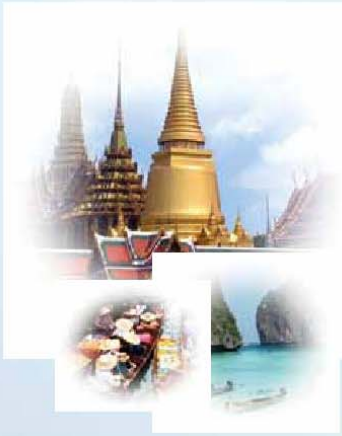

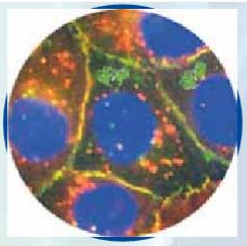

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Roles of Lipid Oxidation in Liver Fluke-induced Cholangiocarcinoma

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Introduction: Chronic inflammation induced by liver fluke (*Opisthorchis viverrini*, Ov) infection is the major risk factor for cholangiocarcinoma (CCA) in Northeastern Thailand. Increased levels of proinflammatory cytokines and nuclear factor kappa B that control cyclooxygenase-2 and inducible nitric oxide activities, disturb the homeostasis of oxidants / anti-oxidants and DNA repair enzymes, all of which appear to be involved in Ov-associated inflammatory processes and CCA. Consequently oxidative and nitrative stress-related cellular damage occurs due to the over production of reactive oxygen and nitrogen species (ROS/RNS), which do not only oxidize DNA and proteins but also lipids via lipid peroxidation (LPO) and cholesterol oxidation in inflamed target cells.

Objective: To prove the hypothesis that lipid peroxidation (LPO) and cholesterol oxidation triggered by Ov-infection plays a role in carcinogenesis of CCA through the formation of miscoding DNA adducts which can be prevented by some protective factors.

Materials & Methods: Our study was performed in both animal and human models: (i) etheno (ε) DNA adducts, 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytosine (εdC) were analyzed in the liver of Ov- and *N*-nitrosodimethylamine (NDMA)-induced CCA in hamster (ii) excretion and the accumulation of etheno adducts were analyzed in urine and white blood cells (WBCs) respectively in Ov infected patients (before and after treatment by anti-parasitic drug praziquantel) and healthy volunteers (iii) oxysterols, which are oxidized cholesterols were identified both in hamster and human CCA including their pathogenic effects in cell line study and (iv) activities of antioxidant enzymes, expression of DNA repairing enzymes and microsatellite instability (MSI) were performed in human CCA.

Results: Increased etheno DNA adducts (εdA and εdC) formations were associated with histopathological changes and CCA appearance time-dependently in hamster model. Plasma and urinary εdA- and εdC-levels of Ov-infected patients were related to indicators of inflammatory conditions such as malondialdehyde- (MDA), nitrate/nitrite-levels in urine including 8-isoprostane level and alkaline phosphatase (ALP) activity in plasma. Interestingly, mean of α-tocopherol levels in plasma were 2 times lower in Ov-infected patients than in healthy controls. Two months after a single dose of the anti-parasitic drug praziquantel, εdA- and εdC-levels in WBCs of Ov-infected subjects were decreased into normal levels; plasma 8-isoprostane, MDA, nitrate/nitrite and ALP were concomitantly lowered. WBC εdA- and εdC-levels were positively related with plasma 8-isoprostane-, MDA-, nitrate/nitrite-levels, and with plasma ALP activity. Plasma α-tocopherol-levels were inversely related with plasma 8-isoprostane-, nitrate/nitrite-levels and with plasma ALP activity.

Moreover cholesterol is oxidized via ROS/RNS or LPO to be oxysterol. We identified oxysterols in liver tissue of Ov-induced CCA in hamster and found that 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased. The high levels of triol and 3K4 were seen in both acute and chronic phases of inflammation along the cholangiocarcinogenesis. These two compounds are classified as carcinogenic oxysterols and could be detected in human CCA tissue and bile. It was additionally shown that triol- and 3K4-treated human cholangiocyte cells caused apoptosis via mitochondria-dependent mechanism. They could also induce the production of carcinogenic etheno-DNA adducts.

We also analyzed the adaptive responses of genome-protection mechanism and found that anti-oxidant enzyme activities, superoxide dismutase 2 and catalase were significantly increased. On the other hand the expressions of DNA repairing enzymes, alkyladenine DNA glycosylase and DNA polymerase beta were increased whereas apurinic endonuclease expression was decreased which reflects the imbalance of DNA repairing system. Among the same investigated specimen, we found MSI-high at 69 % while MSI-low at 31% in human CCA tissue.

Discussion: We conclude that chronic Ov infection through oxidative/nitrative stress induces lipid oxidation of both unsaturated fatty acids and cholesterol. Concurence with the imbalanced adaptive responses of genome protection mechanism; decreased activities of antioxidant enzymes while DNA repairing enzymes were aberrantly expressed leading to an accumulation of lipid peroxidation–derived DNA damage and MSI. This phenomenon is lowered by the plasma α-tocopherol and by antiparasitic drug therapy. Figure depicts the possible mechanism that link Ov-induced chronic inflammation to the onset of cholangiocarcinogenesis.

Conclusion: Our findings strongly suggest that LPO-derived DNA damage triggered by Ov infection plays a potential role in the genesis of CCA. εdA and εdC can be used as promising biomarkers for early detection of subjects at a high risk for CCA development as well as for the assessment the efficacy of preventive and therapeutic interventions.

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Keywords: lipid peroxidation, oxysterols, etheno DNA adducts, cholangiocarcinoma, *Opisthorchis viverrini*

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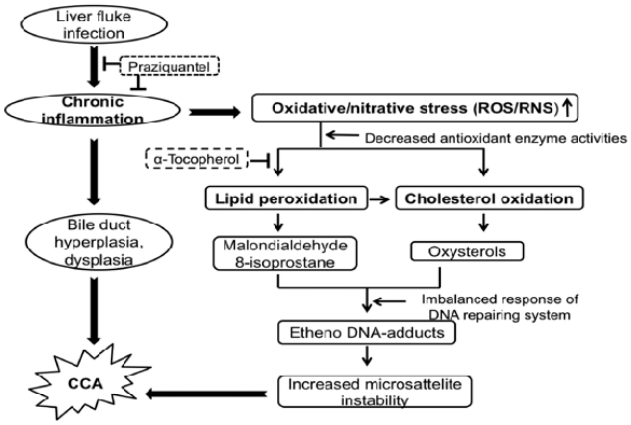

Figure: A possible link of liver fluke-induced inflammation through lipid peroxidation and cholesterol oxidation in relation to CCA development. (ROS, reactive oxygen species; RNS, reactive nitrogen species; CCA, cholangiocarcinoma)

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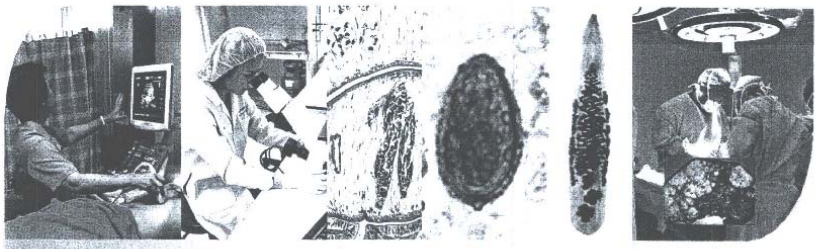

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Faculty of Medicine, Mahasarakham University

FORMATION OF CARCINOGENIC OXYSTEROLS AND THEIR ROLES IN LIVER FLUKE-INDUCED CHOLANGIOCARCINOGENESIS

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Introduction: Oxysterols are the oxygenated derivatives of cholesterol. They can be produced either by enzymatic reaction which is involved with cytochrome P450 enzymes or generated by reactive oxygen/nitrogen radicals. Some species are cytotoxic and carcinogenic. We hypothesized that biliary oxysterols resulting from liver fluke infection participate in cholangiocarcinogenesis.
Methods: We identified oxysterols in liver tissue of *Opisthorchis viverrini* (Ov)-induced hamster cholangiocarcinoma using GC/MS. The candidate carcinogenic oxysterol on induction of cholangiocarcinogenesis using an in vitro human cholangiocyte culture model was investigated.
Results: Levels of oxysterols, 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased in CCA hamsters. The high levels of triol and 3K4 were seen in both acute and chronic phases of inflammation along with the increasing cholangiocarcinogenesis. It was shown that triol- and 3K4-treated human cholangiocyte cells caused apoptosis via a mitochondria-dependent mechanism. They could also induce the production of carcinogenic etheno-DNA adducts. We established oxysterol-resistant cells to examine whether oxysterols induced cell death involved in the development of cholangiocarcinogenesis. The resistant cells were shown to exihbit resistant characteristics to apoptosis induced by H2O2. Using protein arrays, we found activation of down-stream effectors in receptor tyrosine kinase signaling resulting in regulation of expression of apoptosis-related proteins.
Conclusions: The data suggest that Triol and 3K4 may be carcinogenic since these oxysterols can cause DNA damage via oxidative stress. Clonal expansion of such mutated cells may play a role in driving the change in cellular phenotype from normal to malignant.
Keywords: oxysterol, cholestan-3β,5α,6β-triol, 4-cholesten-3-one, opisthorchiasis, cholangiocarcinoma, DNA damage
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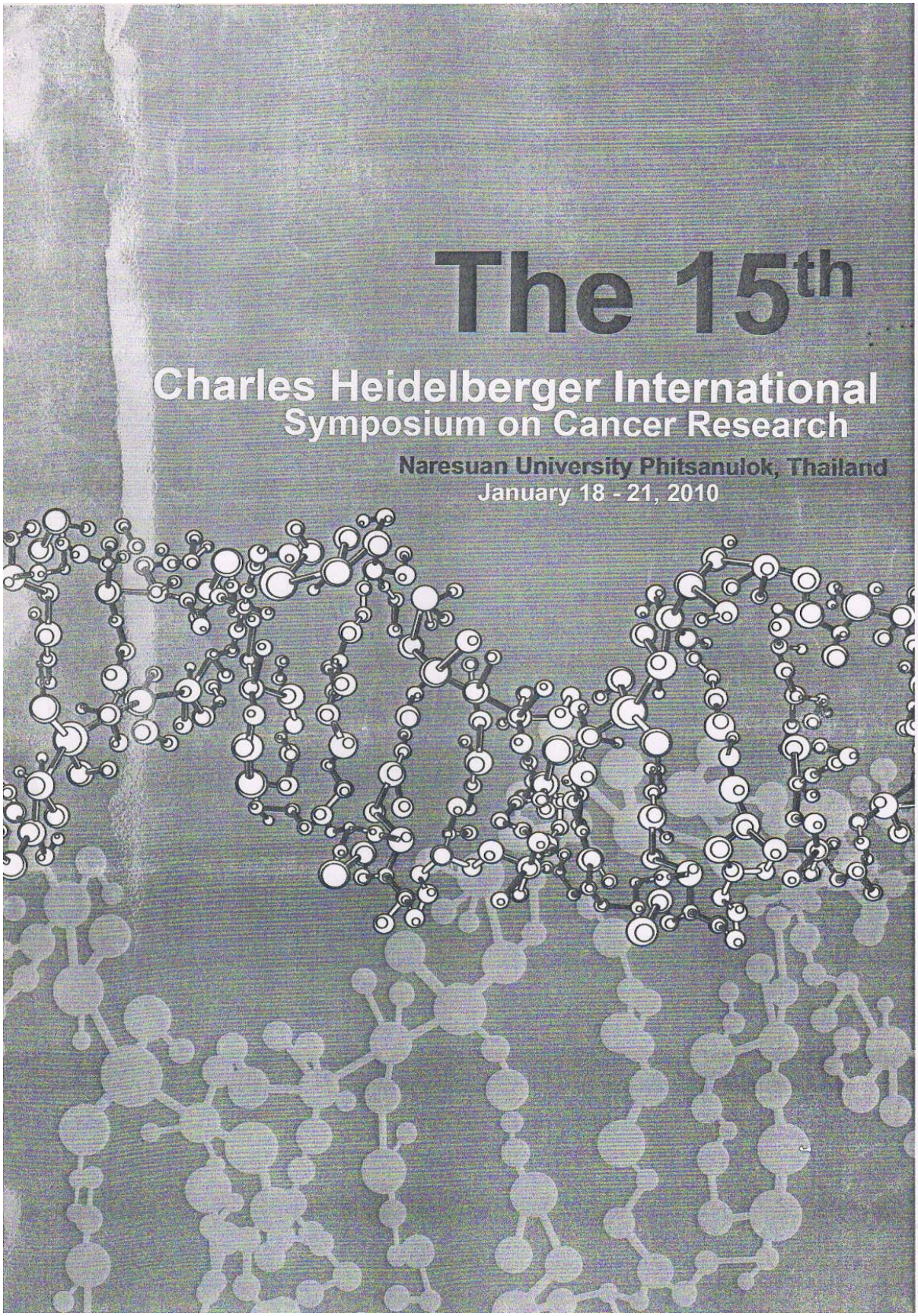
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Effect of carcinogenic oxysterols on induction of apoptosis is related to DNA damage in human cholangiocarcinoma cell lines

Apinya Jusakul[1,2], Watcharin Loilome[1,2], Nisana Namwat[1,2], Somkid Dechakhamphu[1,2] and Puangrat Yongvanit[1,2]

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Background: Oxysterols are oxygenated derivatives of cholesterol which are produced by enzymatic reaction mainly by cytochrome P450 family. They could be generated by non-enzymatic reaction through reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Oxysterols, which may be produced upon the liver fluke (*Opithorchis viverrini*; Ov) infection are also suspected to play roles in genesis of cholangiocarcinoma (CCA). Our previous data showed that the level of particular oxysterols including cholestan-3β, 5α, 6β-triol (Triol) and cholest-4-ene-3-one (3K4), which are categorized as carcinogenic forms, were significantly higher in liver tissues of Ov-induced hamster CCA. We hypothesize that these oxysterols distribute some parts to trigger cholangiocarcinogenesis.
Objective: The purpose of this study are to examine the effect of oxysterols including Triol and 3K4 on cellular phenotypes and to investigate their effects on the induction of DNA adduct of human cholangiocarcinoma cell line (M139) and transformed cholangiocyte (MMNK-1).
Methods: We have explored the effect of oxysterols on cell proliferation of M139 and transformed MMNK-1 by using sulforhodamine B assay. Cellular apoptosis and necrosis were determined by using annexin-v and propidium iodide staining then analyzed by a flow cytometer. The etheno-DNA adduct was detected by immunocytochemistry.
Results: It was shown that Triol and 3K4 induced cells death in both CCA cell lines and MMNK-1 cell as a dose-dependent manner, whereas the ability to stimulate cell proliferation could not be observed. We also found that Triol and 3K4 can induce cell death via apoptosis pathway in both M139 and MMNK-1 cells. In addition oxysterols caused the increasing of DNA adduct (εdA) was also observed.
Conclusion: These evidences preliminary suggest us that cell death induced by Triol and 3K4 which is generated during carcinogenesis may have the procarcinogenic impact, since oxysterol could also cause DNA damage via oxidative stress and if mutated clonal expansion occurs, it may play role in the changing of cellular phenotype from normal to malignancy. The proposed mechanistic involvement is under investigation.

Key Words: Oxysterol, Cholangiocarcinoma, Triol, 3K4

This work is supported by Thailand Research Fund through Window II (BRG 5280020) and Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0177/2549) to AJ and PY

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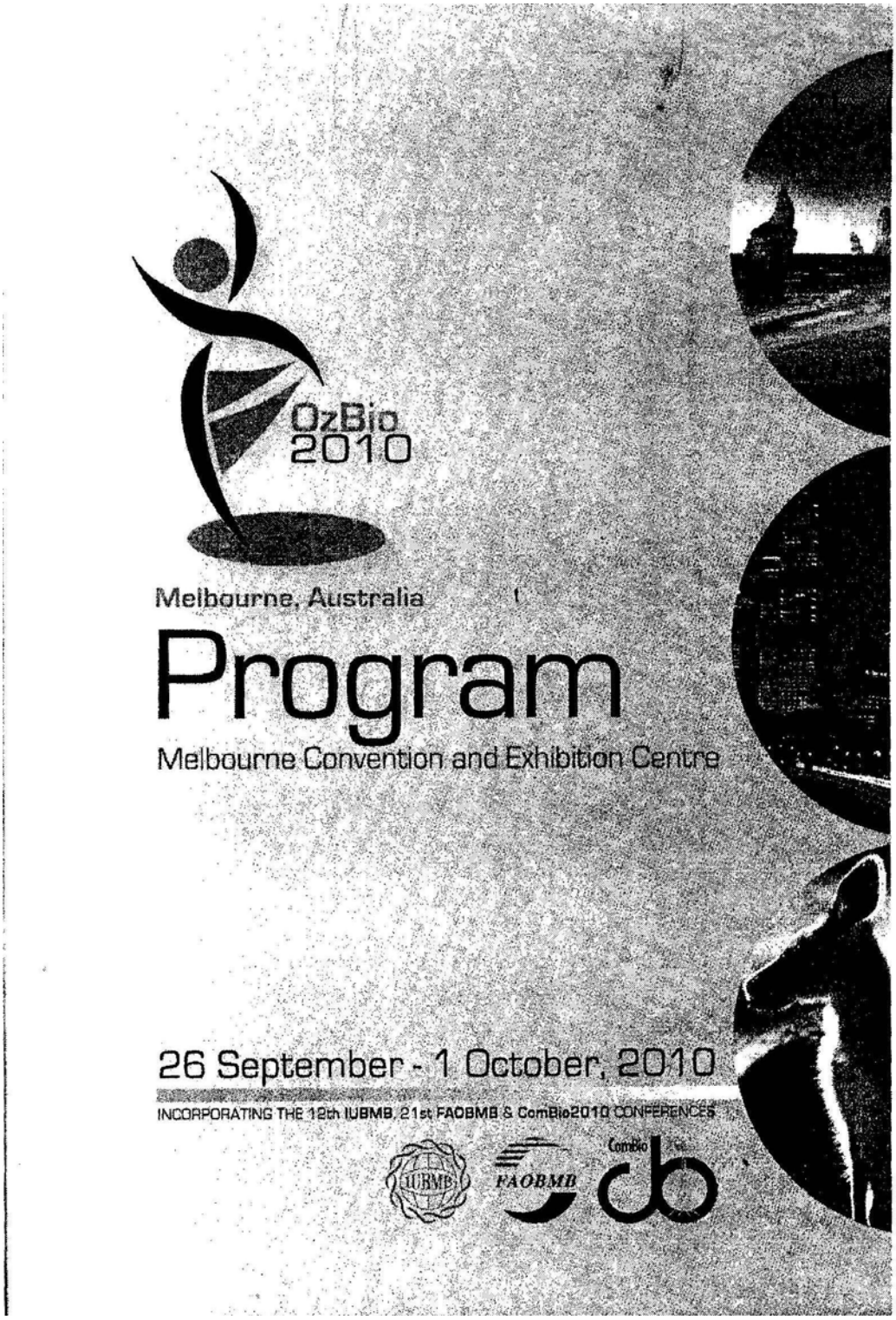
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INDUCTION OF ETHENO-DNA ADDUCT BY CARCINOGENIC OXYSTEROLS IN IMMORTALIZED CHOLANGIOCYTE

Yongvanit P.[1,2], Jusakul A.[1,2], Loilome W.[1,2], Namwat N.[1,2], Dechakhamphu S.[1,2] and Kobayashi N.[3]
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Oxysterols are oxygenated derivatives of cholesterol which are produced by non-enzymatic reaction through reactive oxygen species (ROS) and/ or reactive nitrogen species (RNS). Oxysterols, which may be produced upon the liver fluke (*Opithorchis viverrini*; Ov) infection, leading to inflammatory process and consequently overproduction of ROS, are also suspected to play roles in genesis of cholangiocarcinoma (CCA). Our previous data showed that the level of particular oxysterols including cholestan-3β, 5α, 6β-triol (Triol) and cholest-4-ene-3-one (3K4), which are categorized as carcinogenic forms, were significantly higher in liver tissues of Ov-induced hamster CCA. We proposed to examine the effect of oxysterols including Triol and 3K4 on the induction of DNA damage and the alteration of cellular phenotype in human immortalized cholangiocyte (MMNK-1). The immunocytochemical detection of etheno-DNA adduct in oxysterols-induced MMNK-1 cell was performed to determine the level of DNA adduct that caused by oxysterols. This study showed the increased level of carcinogenic etheno-DNA adducts (1-N^6-ethenodeoxyadenosine or εdA) that is formed by ROS *via* the reaction of lipid peroxidation. The effect of oxysterols on cell apoptosis induction was also analyzed by using flow cytometer. It was shown that these oxysterols induced cells death via apoptotic pathway as a dose-dependent manner. These evidences suggest that cell death induced by Triol and 3K4 which is generated during carcinogenesis may have the procarcinogenic impact, since oxysterol could also cause DNA damage via oxidative stress and if mutated clonal expansion occurs, it may play role in the changing of cellular phenotype from normal to malignancy. The proposed mechanistic involvement is under investigation.

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Pathogenic Oxysterols in Hepatic Tissue and Bile of Cholangiocarcinoma Patients

Apinya Jusakul[1,5,6], W. Geoffrey Haigh[6], Christopher Savard[6], Watcharin Loilome[1,5], Nisana Namwat[1,5], Narong Khuntikeo[3,5], Chawalit Pairojkul[4,5], Somchai Pinlaor[2,5], Rahul Kuver[6,7] and Puangrat Yongvanit[1,5]

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Oxysterols are oxidation products of cholesterol, which are generated by either cytochrome P450 (CYP) enzymes or reactive oxygen and nitrogen species. They are intermediates in bile acid synthesis as well asimportant signaling agents. However, some certain species are cytotoxic and carcinogenic. Oxysterols have been identified in bile in the setting of chronic inflammation. Interestingly, some species; 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol), which are carcinogenic, were indentified in hamster liver tissue of liver fluke-induced cholangiocarcinoma (CCA). Our previous study reported that liver fluke-induced oxysterols can generate DNA adducts in cholangiocytes. To explain carcinogenesis in human CCA, we aimed to identify and quantitate the oxysterols in liver tissue and bile from CCA patients by using gas chromatography and mass spectrometry. Focusing on the different profiles among CCA, hepatoma and cadaveric donor, we found the increased level of inflammation-related oxysterols; triol, 3K4 and 24-hydroxycholesterol (24-OH) in CCA liver tissue and bile. Moreover, higher levels of triol and 3K4 were also found in the hepatic bile compared to gallbladder bile from CCA patients, suggesting that there is the increasing production of free radical-induced pathogenic oxysterols in CCA. Furthermore, we found that low expression of CYP39A1 and CYP7A1 enzymes may involve in the increasing level of 24-OH in CCA patients. Thus, we proposed that pathogenic oxysterols areproduced during cholangiocarcinogenesis and the accumulation of pathogenic oxysterols may involve in the development of CCA.

Keywords: Oxysterols, Cholangiocarcinoma, Cholesterol oxidation products

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Roles of Lipid Oxidation in Liver Fluke-induced Cholangiocarcinoma

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Introduction: Chronic inflammation induced by liver fluke (*Opisthorchis viverrini*, Ov) infection is the major risk factor for cholangiocarcinoma (CCA) in Northeastern Thailand. Increased levels of proinflammatory cytokines and nuclear factor kappa B that control cyclooxygenase-2 and inducible nitric oxide activities, disturb the homeostasis of oxidants / anti-oxidants and DNA repair enzymes, all of which appear to be involved in Ov-associated inflammatory processes and CCA. Consequently oxidative and nitrative stress-related cellular damage occurs due to the over production of reactive oxygen and nitrogen species (ROS/RNS), which do not only oxidize DNA and proteins but also lipids via lipid peroxidation (LPO) and cholesterol oxidation in inflamed target cells.

Objective: To prove the hypothesis that lipid peroxidation (LPO) and cholesterol oxidation triggered by Ov-infection plays a role in carcinogenesis of CCA through the formation of miscoding DNA adducts which can be prevented by some protective factors.

Materials & Methods: Our study was performed in both animal and human models: (i) etheno (ε) DNA adducts, 1,N^6-ethenodeoxyadenosine (εdA) and 3,N^4-ethenodeoxycytosine (εdC) were analyzed in the liver of Ov- and *N*-nitrosodimethylamine (NDMA)-induced CCA in hamster (ii) excretion and the accumulation of etheno adducts were analyzed in urine and white blood cells (WBCs) respectively in Ov infected patients (before and after treatment by anti-parasitic drug praziquantel) and healthy volunteers (iii) oxysterols, which are oxidized cholesterols were identified both in hamster and human CCA including their pathogenic effects in cell line study and (iv) activities of antioxidant enzymes, expression of DNA repairing enzymes and microsatellite instability (MSI) were performed in human CCA.

Results: Increased etheno DNA adducts (εdA and εdC) formations were associated with histopathological changes and CCA appearance time-dependently in hamster model. Plasma and urinary εdA- and εdC-levels of Ov-infected patients were related to indicators of inflammatory conditions such as malondialdehyde- (MDA), nitrate/nitrite-levels in urine including 8-isoprostane level and alkaline phosphatase (ALP) activity in plasma. Interestingly, mean of α-tocopherol levels in plasma were 2 times lower in Ov-infected patients than in healthy controls. Two months after a single dose of the anti-parasitic drug praziquantel, εdA- and εdC-levels in WBCs of Ov-infected subjects were decreased into normal levels; plasma 8-isoprostane, MDA, nitrate/nitrite and ALP were concomitantly lowered. WBC εdA- and εdC-levels were positively related with plasma 8-isoprostane-, MDA-, nitrate/nitrite-levels, and with plasma ALP activity. Plasma α-tocopherol-levels were inversely related with plasma 8-isoprostane-, nitrate/nitrite-levels and with plasma ALP activity.

Moreover cholesterol is oxidized via ROS/RNS or LPO to be oxysterol. We identified oxysterols in liver tissue of Ov-induced CCA in hamster and found that 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol) were increased. The high levels of triol and 3K4 were seen in both acute and chronic phases of inflammation along the cholangiocarcinogenesis. These two compounds are classified as carcinogenic oxysterols and could be detected in human CCA tissue and bile. It was additionally shown that triol- and 3K4-treated human cholangiocyte cells caused apoptosis via mitochondria-dependent mechanism. They could also induce the production of carcinogenic etheno-DNA adducts.

We also analyzed the adaptive responses of genome-protection mechanism and found that anti-oxidant enzyme activities, superoxide dismutase 2 and catalase were significantly increased. On the other hand the expressions of DNA repairing enzymes, alkyladenine DNA glycosylase and DNA polymerase beta were increased whereas apurinic endonuclease expression was decreased which reflects the imbalance of DNA repairing system. Among the same investigated specimen, we found MSI-high at 69 % while MSI-low at 31% in human CCA tissue.

Discussion: We conclude that chronic Ov infection through oxidative/nitrative stress induces lipid oxidation of both unsaturated fatty acids and cholesterol. Concurence with the imbalanced adaptive responses of genome protection mechanism; decreased activities of antioxidant enzymes while DNA repairing enzymes were aberrantly expressed leading to an accumulation of lipid peroxidation–derived DNA damage and MSI. This phenomenon is lowered by the plasma α-tocopherol and by antiparasitic drug therapy. Figure depicts the possible mechanism that link Ov-induced chronic inflammation to the onset of cholangiocarcinogenesis.

Conclusion: Our findings strongly suggest that LPO-derived DNA damage triggered by Ov infection plays a potential role in the genesis of CCA. εdA and εdC can be used as promising biomarkers for early detection of subjects at a high risk for CCA development as well as for the assessment the efficacy of preventive and therapeutic interventions.

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Keywords: lipid peroxidation, oxysterols, etheno DNA adducts, cholangiocarcinoma, *Opisthorchis viverrini*

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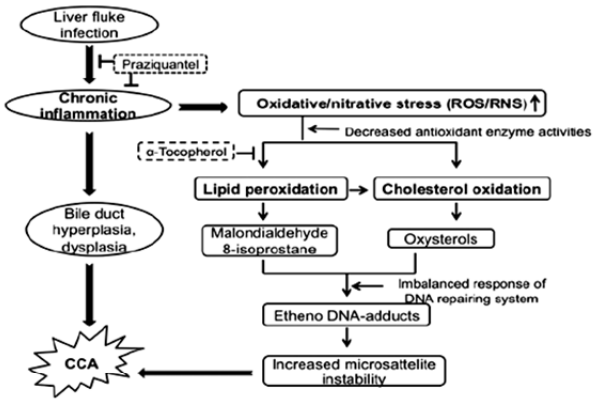

Figure: A possible link of liver fluke-induced inflammation through lipid peroxidation and cholesterol oxidation in relation to CCA development. (ROS, reactive oxygen species; RNS, reactive nitrogen species; CCA, cholangiocarcinoma)

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Identification of pathogenic cholesterol oxidation products in hepatic tissue and bile of cholangiocarcinoma patients

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Oxysterols are oxidation products of cholesterol, which are generated by either cytochrome P450 (CYP) enzymes or reactive oxygen and nitrogen species. They are intermediates in bile acid synthesis as well asimportant signaling agents. However, some certain species are cytotoxic and carcinogenic. Oxysterols have been identified in bile in the setting of chronic inflammation. Interestingly, some species; 3-keto-cholest-4-ene (3K4) and cholestane-3β-5α-6β-triol (triol), which are carcinogenic, were indentified in hamster liver tissue of liver fluke-induced cholangiocarcinoma (CCA). Our previous study reported that liver fluke-induced oxysterols can generate DNA adducts in cholangiocytes. To explain carcinogenesis in human CCA, we aimed to identify and quantitate the oxysterols in liver tissue and bile from CCA patients by using gas chromatography and mass spectrometry. Focusing on the different profiles among CCA, hepatoma and cadaveric donor, we found the increased level of inflammation-related oxysterols; triol, 3K4 and 24-hydroxycholesterol (24-OH) in CCA liver tissue and bile. Moreover, higher levels of triol and 3K4 were also found in the hepatic bile compared to gallbladder bile from CCA patients, suggesting that there is the increasing production of free radical-induced pathogenic oxysterols in CCA. Furthermore, we found the low expression of CYP39A1 and CYP7A1 enzymes, which may involve in the increasing level of 24-OH in CCA patients. Thus, we proposed that pathogenic oxysterols are produced during cholangiocarcinogenesis and the accumulation of those oxysterols may involve in the development of CCA.

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CP-20

Altered Expression and DNA Methylation of 24-Hydroxycholesterol Degrading Enzymes and Their Transcriptional Activators in Cholangiocarcinoma

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24-hydroxycholesterol (24-OH) is an oxidation product of cholesterol, which was found to be significantly increased in cholangiocarcinoma (CCA) tissue when compared to normal and hepatocellular carcinoma tissues. We aimed to investigate the expression of its degrading enzymes, CYP39A1, CYP7A1 and their transcriptional activators, RUNX2 and LXR-alpha in CCA tissue respectively. Immunohistochemical analysis and methylation-specific PCR were employed. It was found that these proteins were overexpressed in human CCA when compared to normal bile duct. Interestingly, patients with high expression of CYP39A1, CYP7A1, and RUNX2 had significantly longer survival rate than those with low expression. In addition, CYP39A1 was significantly correlated with RUNX2 expression. Methylation of CYP39A1 and RUNX2 promoter regions were also detected. There were three methylation patterns of RUNX2 promoter site including methylation only (M; 6.25 %), unmethylation only (U; 6.25 %) and both patterns (M + U; 87.75 %). CCA patients who have both methylation pattern of RUNX2 had a trend to correlate with its protein expression. All cases of CCA pateints showed only unmethylated pattern of promoter region of CYP39A1 gene and was not correlated with protein expression level. Moreover we found that H_2O_2 induced hypomethylation of RUNX2 (M + U pattern) in immortal cholangiocyte cell line. These results suggest that RUNX2 is over-expressed in CCA, which may be regulated by hypomethylation of its promoter region and lead to increase CYP39A1 expression. We hypothesized that free radicals generated by 24-OH can induce hypomethylation for RUNX2 and may play role in cholangiocarcinogenesis. This is on-going investigation.

Keywords: 24-hydroxycholesterol, cholangiocarcinoma, CYP39A1, RUNX2