Final Report

Cellular adaptation in response to oxidative stress during cholangiocarcinogenesis caused by liver fluke infection

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รายงานวิจัย

เรื่อง

การปรับตัวของเซลล์เพื่อตอบสนองต่อภาวะ oxidative stress ในกระบวนการก่อมะเร็งท่อน้ำดีที่เกิดจากการติดเชื้อพยาธิใบไม้ตับ

(Cellular adaptation in response to oxidative stress during cholangiocarcinogenesis caused by liver fluke infection)

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ทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ 2554
ชุดโครงการพยาธิใบไม้ตับและมะเร็งท่อน้ำดี

คำนำ

โครงการวิจัยนี้เป็นส่วนหนึ่งของชุดโครงการ “กลไกการก่อมะเร็งท่อน้ำดีในระดับโมเลกุลเพื่อการป้องกันด้วยเคมีอย่างมีประสิทธิภาพ” ซึ่งได้รับทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ 2554 (โครงการต่อเนื่อง 2554-2555) ภายใต้การสนับสนุนครุภัณฑ์วิจัย วัสดุชีวภาพ (เนื้อเยื่อผู้ป่วยและเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี) และบุคคลากรของศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี มหาวิทยาลัยขอนแก่น

บทคัดย่อ

กลไกการปรับตัวในการปกป้องยีนจะเกิดในเซลล์ที่มีภาวะ genotoxic stress เนื่องจากการสร้างอนุมูลอิสระจำนวนมากผ่านทางกระบวนการอักเสบและการติดเชื้อ สถานการณ์ดังกล่าวเซลล์พยายามปรับตัวเพื่อให้อยู่ในสภาพปกติด้วยกลไกการปกป้องนี้ อย่างไรก็ตามมีหลักฐานบ่งบอกว่าการปรับตัวของเซลล์อาจมีผลเสียต่อเซลล์ เช่น ถ้าพบการแสดงออกและ/หรือการทำงานของเอนไซม์ต้านอนุมูลอิสระลดลง รวมทั้งมีความไม่สมดุลของระบบซ่อมแซมดีเอ็นเอจะทำให้เกิด microsatellite instability (MSI) ดังนั้นคณะผู้วิจัยได้ตั้งสมมติฐานว่า MSI ซึ่งเป็นปัจจัยหนึ่งที่ก่อให้เกิดโรคมะเร็งท่อน้ำดี (cholangiocarcinoma, CCA) เกิดจากการตอบสนองที่ไม่สมดุลของเอนไซม์ต้านอนุมูลอิสระและหรือเอนไซม์ซ่อมแซมดีเอ็นเอเนื่องจากภาวะ oxidative/nitrative stress ที่เกิดขึ้นจากการอับเสบอันเป็นผลมาจากการติดเชื้อพยาธิใบไม้ตับ เพื่อพิสูจน์สมมติฐานนี้คณะผู้วิจัยทำการศึกษารูปแบบการแสดงออกของเอนไซม์ต้านอนุมูลอิสระ ได้แก่ superoxide dismutase (SOD2) และ catalase (CAT) และเอนไซม์ซ่อมแซมดีเอ็นเอ ได้แก่ alkyladenine DNA glycosylase (AAG), apurinic endonuclease (APE) และ DNA polymerase beta (DNA pol β) และยังศึกษาการทำงานของเอนไซม์ต้านอนุมูลอิสระ ได้แก่ SOD2 และ CAT ในเนื้อเยื่อผู้ป่วย CCAและเนื้อเยื่อตับหนูแฮมสเตอร์ที่ถูกชักนำให้เป็น CCA ส่วน MSI จะศึกษาเฉพาะในเนื้อเยื่อ CCA ของผู้ป่วย ผลการศึกษา พบทั้งการแสดงออกของเอนไซม์ SOD2 และ CAT ลดลงและการทำงานลดลงอย่างมีนัยสำคัญเมื่อเทียบกับเนื้อเยื่อตับคนปกติ สำหรับการแสดงออกของเอนไซม์ซ่อมแซมดีเอ็นเอ เราพบการแสดงออกของเอนไซม์ AAG และ DNA pol β สูงขึ้น ขณะที่เอนไซม์ APE ลดลง นอกจากนี้เรายังพบ MSI-high คิดเป็น 69% ในขณะที่ MSI-low คิดเป็น 31% และไม่พบ microsatellite stability (MSS) ผู้ป่วยที่มี MSI-high มีความสัมพันธ์กับการพยากรณ์โรคที่ไม่ดีโดยมีระยะเวลาการรอดชีพที่สั้นลง จากผลการศึกษาแสดงให้เห็นว่าการลดลงของเอนไซม์ต้านอนุมูลอิสระและการปรับตัวที่ไม่สมดุลของเอนไซม์ซ่อมแซมดีเอ็นเอในเนื้อเยื่อผู้ป่วย CCA อาจก่อให้เกิด MSI และส่งเสริมกระบวนการก่อมะเร็งได้

ผลการศึกษาในเนื้อเยื่อตับหนูแฮมสเตอร์ที่ถูกชักนำให้เป็นมะเร็งท่อน้ำดีพบการติดสีเข้มของเอนไซม์ SOD2 และ CAT ในเนื้อเยื่อตับทุกช่วงเวลาของหนูแฮมสเตอร์ที่ถูกชักนำให้เป็นมะเร็งท่อน้ำดี ขณะที่การทำงานของเอ็นไซม์ทั้งสองลดลงอย่างมีนัยสำคัญในกลุ่มหนูที่เหนี่ยวนำให้เป็น CCA เมื่อเทียบกับกลุ่มหนูปกติ ผลการศึกษานี้สอดคล้องกับผลการศึกษาในเนื้อเยื่อผู้ป่วย CCA แสดงให้เห็นว่าแม้มีการแสดงออกของเอนไซม์ SOD2 และ CAT ที่สูงแต่ความสามารถในการทำงานนั้นเสียไป นอกจากนี้เรายังทำการศึกษารูปแบบการแสดงออกของเอนไซม์ BER (APE และ DNA pol β) ในกระบวนการก่อมะเร็งท่อน้ำดี ผลการศึกษาพบว่ามีการแสดงออกของเอนไซม์ APE และ DNA pol β สูงตั้งแต่ระยะแรกของการถูกชักนำให้เป็นมะเร็งท่อน้ำดีจนกระทั่งพัฒนาเป็นมะเร็ง จากผลการศึกษานี้ชี้ให้เห็นว่าการลดลงของเอนไซม์ต้านอนุมูลอิสระและการปรับตัวที่เพิ่มขึ้นของเอนไซม์ซ่อมแซมดีเอ็นเออาจนำไปสู่ความเสียหายของดีเอ็นเอและนำไปสู่การเป็นมะเร็งท่อน้ำดีในหนูแฮมสเตอร์

Abstract

An adaptive response of the genome-protection mechanism occurs in cells when exposed to genotoxic stress due to overproduction of free radicals via inflammation and infection. Such circumstances, cells attempt to keep them healthy by many genome protection machineries. However, there are increasing evidences that this adaptive response may have deleterious effects, for instance if reduction of antioxidant enzymes and/or imbalance DNA repair system occur, they will generate microsatellite instability (MSI), which has procarcinogenic implication. Therefore, we hypothesized that MSI caused by imbalanced responses of antioxidant enzymes and/or DNA repair enzymes due to the oxidative/nitrative stress arising from inflammatory response is involved in liver fluke-associated cholangiocarcinogenesis. To prove this, we proposed to identify the expression patterns of antioxidant enzymes i.e. superoxide dismutase 2 (SOD2) and catalase (CAT) and DNA repair enzymes i.e. alkyladenine DNA glycosylase (AAG), apurinic endonuclease (APE) and DNA polymerase beta (DNA pol β) and also determined activities of antioxidant enzymes i.e. SOD2 and CAT in human and hamster CCA tissues. MSI was investigated only in human CCA tissues. In human CCA, our data demonstrated that expression of SOD2 and CAT enzymes were mainly decreased. Both SOD2 and CAT activities in human CCA were parallelly shown that they were significantly decreased when compared with hepatic tissue of cadaveric donors. Among expression of DNA repairing enzymes, we found that the expression of AAG and DNA pol β enzymes was increased while APE was decreased. Moreover, we found MSI-high at 69% whereas MSI-low at 31% and none were classified as microsatellite stability (MSS). Patients with MSI-high were correlated with poor prognosis as indicated by shorter survival. Our data indicate that the reduction of antioxidant enzymes and adaptive imbalance of base excision repair (BER) enzymes in human CCA could cause MSI and may increase susceptibility to tumorigenesis.

In hamster CCA, our results showed that strong staining of SOD2 and CAT in liver tissue at every time point during cholangiocarcinogenesis of CCA-induced hamster, whereas, SOD2 and CAT activities were significantly decreased when tumor developed when compared with the controls. This was consistent with our data in human CCA. It could indicate that although expressions of SOD2 and CAT are high but activities are impaired. We further investigated expression patterns of BER enzymes (APE and DNA pol β) as duration time of carcinogenesis. Our results demonstrated that expressions of APE and DNA pol β were increased as a time dependent manner from acute phase of CCA-induced hamster until tumor had developed. Taken together, our results suggest that reduction of antioxidant enzymes and adaptive increase of BER enzymes may contribute to DNA translesion-mediated cholangiocarcinoma in liver fluke-associated CCA in hamster.

Acknowledgment

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สารบัญ

รายงานวิจัย

เรื่อง

การปรับตัวของเซลล์เพื่อตอบสนองต่อภาวะ oxidative stress ใน กระบวนการก่อมะเร็งท่อน้ำดีที่เกิดจากการติดเชื้อพยาธิใบไม้ตับ (Cellular adaptation in response to oxidative stress during cholangiocarcinogenesis caused by liver fluke infection)

ทุนอุดหนุนการวิจัย มหาวิทยาลัยขอนแก่น ประจำปีงบประมาณ 2554
ชุดโครงการ กลไกการก่อมะเร็งท่อน้ำดีในระดับโมเลกุลเพื่อการป้องกันด้วยเคมีอย่างมีประสิทธิภาพ

1. หน่วยงานที่รับผิดชอบงานวิจัยภาควิชาชีวเคมี คณะแพทยศาสตร์
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โทรศัพท์/โทรสาร 043-348-386

2. **คณะผู้วิจัย**
   1. **หัวหน้าโครงการวิจัย ผศ.ดร.วัชรินทร์ ลอยลม**
   2. **ผู้ร่วมวิจัย รศ.ดร.พวงรัตน์ ยงวณิชย์**
   3. **ผู้ร่วมวิจัย รศ.ดร.สมชาย ปิ่นละออ**
   4. **ผู้ร่วมวิจัย นางสาวศศิธร คาดสนิท**

3. **เป็นส่วนหนึ่งของชุดโครงการ พยาธิใบไม้ตับและมะเร็งท่อน้ำดี**
4. **ประเภทของงานวิจัย งานวิจัยพื้นฐาน**
5. **สาขาวิชาการที่ทำการวิจัย วิทยาศาสตร์การแพทย์**
6. **คำสำคัญของเรื่องที่ทำการวิจัย** *Opisthorchis viverrini*, liver fluke infection, oxidative stress, cellular adaptation, DNA damage, DNA repair proteins, apoptosis proteins, antioxidant enzymes, carcinogenesis

7. **ความสำคัญและที่มาของปัญหาการวิจัย (Background and rational)**

Cholangiocarcinoma (CCA) is the malignant tumor arising from bile duct epithelial cells which is the common cancer in Thailand and the major public health problem of northeast region. Many evidences from epidemiological and experimental studies support that liver fluke (*Opisthorchis viverrini*; Ov) infection is the etiology of CCA development. At present the mechanistic link between host-parasite interaction and CCA carcinogenesis is gradually being

uncovered. Our studies in hamster model reported that chronic inflammation induced by infection repeatedly with Ov has been postulated to be a risk factor for CCA. More frequent Ov infections can induce the accumulation of inducible nitric oxide synthase (iNOS) not only in inflammatory cells but also in the epithelium of bile ducts and subsequently cause nitrative and oxidative damages to nucleic acids. Furthermore, NO may be involved in carcinogenesis through heme oxygenase-1 (HO-1)-derived iron accumulation, which may participate in enhancement of oxidative DNA damage in epithelium of small bile ducts (Pinlaor et al., 2004a; Pinlaor et al., 2004b; Pinlaor et al., 2004c; Pinlaor et al., 2003). It can be concluded that repeated-infection with Ov could mediate oxidative and nitrative DNA damage which may play role in initiation and/or promotion steps of CCA carcinogenesis through chronic inflammation. It could also be demonstrated that inflammatory cell infiltration triggered by repeated infection occurred earlier than single infection and is associated with altered liver enzymes, and severity of periductal fibrosis. Furthermore, Thanan and colleague have reported that human infected with liver fluke had the accumulation of urinary 8- oxo- 7, 8- dihydro-2'- deoxyguanosine which was positively associated with plasma nitrate/ nitrite and AST level, indicating that Ov infection can induce oxidative and nitrative stress via reactive oxygen and reactive nitrogen species production during liver injury in a human model (Thanan et al., 2008). Moreover, DNA damage due to Ov infection mediated lipid peroxidation has been also reported in both hamster and human studies (Dechakhamphu et al., 2010a; Dechakhamphu et al., 2008). Many cellular proteins in CCA tissue is recently found to exist in carbonylated forms via oxidative damage and become dysfunction, leading to CCA progression (Thanan et al., 2012). Altogether these incidences suggested the association between Ov infection, chronic inflammation and CCA development.

An adaptive response of the genome-protection mechanism occurs in cells when exposed to genotoxic stress due to overproduction of free radicals via inflammatory process and infection. Such circumstances, cells attempt to keep them healthy by many genome-protection machineries. However, there are increasing evidences that this adaptive response may have deleterious effects, for instance if reduction of antioxidant enzymes and/or imbalance DNA repair system occur, they will generate microsatellite instability (MSI), which has procarcinogenic implication. Evidences to support these phenomena were studies by the groups of Guner and Hofseth (Guner et al., 1996; Hofseth et al., 2003). They reported that low level of antioxidant enzymes in cancerous tissues could be a relevant event in the carcinogenic process and the adaptive imbalance in base excision-repair enzymes generates MSI in chronic inflammation. Therefore, we hypothesized that MSI caused by imbalanced responses of antioxidant enzymes and/or DNA repair enzymes due to the oxidative/nitrative stress arising from inflammatory response is involved in liver fluke-associated cholangiocarcinogenesis. To prove this hypothesis, we proposed to identify the expression

patterns of antioxidant enzymes i.e. superoxide dismutase type 2 (SOD2) and catalase (CAT) and DNA repair enzymes i.e. alkyladenine DNA glycosylase (AAG), apurinic endonuclease (APE) and DNA polymerase beta (DNA pol β) and also activity of antioxidant enzymes i.e. SOD2 and CAT in human and hamster CCA tissues. MSI was investigated only in human CCA tissues. These determinations may help to explain pro-carcinogenic effect of cholangiocarcinoma caused by the liver fluke infection.

8. วัตถุประสงค์ของโครงการวิจัย (Objectives)

8.1 To identify the expression patterns of antioxidant enzymes and DNA repair enzymes in human CCA tissues.

8.2 To determine activities of antioxidant enzymes in human CCA tissues.

8.3 To examine microsatellite instability in human CCA tissues.

8.4 To study parameters as 8.1 & 8.2 in carcinogenic pathway of Ov-induced CCA in hamster.

9. ประโยชน์ที่คาดว่าจะได้รับ (Anticipated outcome)

Microsatellite instability caused by the host-parasite interaction via oxidative/nitrative stress and the imbalance of adaptive responses in cholangiocarcinogenesis will be revealed.

10. หน่วยงานที่จะนำเอาผลการวิจัยไปใช้ประโยชน์

1. คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น
2. กระทรวงสาธารณสุข

11. งานวิจัยที่เกี่ยวข้อง (Literature review)

11.1 Liver fluke infection and Cholangiocarcinoma

Infection and inflammation have been postulated to be risk factors of several cancers. Chronic inflammation of biliary cells induced by Ov infection causes production of nitric oxide (NO) and other oxygen radicals from inflammatory cells upon activation of inflammatory cytokines in infected and inflamed tissues (Ohshima et al., 1994a; Pinlaor et al., 2004b). Vastly increased NO and its derivative radicals contribute to carcinogenesis by causing damage to DNA and proteins. Endogenous NO formation is catalyzed by the inducible nitric oxide synthase (iNOS), which is mainly produced by inflammatory cells, especially activated macrophages induced by inflammatory cytokines (Mayer and Hemmens, 1997). It can be demonstrated that the activation of iNOS and excess NO production in response to inflammatory cytokines causes oxidative DNA damage and inactivation of DNA repair proteins in CCA cells (Jaiswal et al., 2000). Recently, Pinlaor and coworkers demonstrated that 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-nitroguanine are biomarkers for DNA damage in the liver of Ov-infected hamster and suggested that these oxidative and nitrative DNA damages and iNOS expression induced by Ov infection via inflammation may play a key role in several processes such as modulation or alteration of gene expression especially genes involved in carcinogen metabolism leading to form the ultimate carcinogens, which enhance the effect of DNA adducts (Pinlaor et al., 2004b). Additionally, Thanan and coworkers (Thanan et al., 2008) have reported that the highest 8-oxodG level was observed for CCA patients and a higher level was found in the urine and leukocytes of Ov-infected patients than in healthy subjects. In addition, DNA damage due to Ov infection mediated lipid peroxidation has been also reported in both hamster and human model (Dechakhamphu et al., 2010a; Dechakhamphu et al., 2008). Many cellular proteins in CCA tissue is recently found to exist in carbonylated forms via oxidative damage and become

dysfunction, leading to CCA progression (Thanan et al., 2012). Usually genotoxic events with DNA damage lead to either DNA mismatched repair mechanism or, if the damage is beyond repaired, or beyond cell death through apoptosis, then the mutated cells are permitted to survive and going on to transform to malignant cells.

Yongvanit et al (Yongvanit et al., 2012a) has intensively reviewed possible mechanisms that link Ov-induced chronic inflammation to the onset of cholangiocarcinogenesis. Liver fluke, particularly repeated infections, cause chronic inflammation *via* pro-inflammatory cytokines (i.e. IL-6) and transcription factor NF-**K**B that control oxidative stress response enzymes COX-2 and iNOS to generate ROS and RNS. The resulting persistent oxidative/nitrative stress disturbs homeostasis of many adaptive response systems such as oxidant/antioxidant ratio, DNA repair enzymes including many altered candidate genes involved in controlling cell proliferation, apoptosis and fibrogenesis. Overproduction of ROS and RNS leads to genotoxic DNA damage as evidenced by the high level of 8-oxodG, 8-nitroguanine and etheno-DNA adducts (**ε**dA and **ε**dC) detected in affected tissues. Excess ROS and RNS can also increase endogenous nitrosation reactions to yield carcinogenic *N*-nitrosamines such as NDMA which alkylate DNA bases. Taken together, all these events (figure 2.1) may act as driving force to cholangiocarcinogenesis.

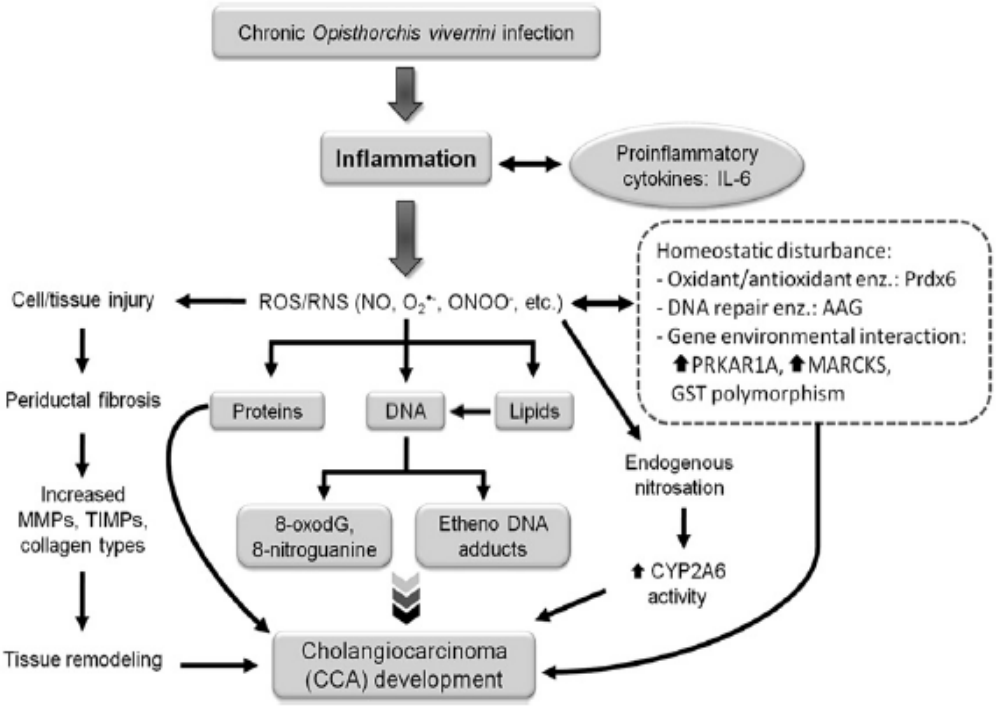


Figure 1 A possible mechanisms that link Ov-induced chronic inflammation to the onset of cholangiocarcinogenesis (Yongvanit et al., 2012a).

11.2. Oxidative stress and antioxidant system

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species (ROS) produced as a consequence of aerobic respiration and substrate

oxidation. Small amounts of reactive oxygen species (ROS), as hydroxyl radicals ($HO^{\cdot}$), superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli. Low levels of ROS are indispensable in many biochemical processes, including intracellular messaging in the cell differentiation and cell progression or the arrest of growth, apoptosis, immunity, and defense against micro-organisms. In contrast, high doses and/or inadequate removal of ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules.

A wide array of enzymatic and non-enzymatic antioxidant defenses exist, including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), beta-carotene, vitamin A, ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E). There is a interrelationship between both, the activities, and the intracellular levels of these metabolites, protecting themselves from oxygen toxicity (Mates and Sanchez-Jimenez, 1999b).

Antioxidant enzymes, such as SOD, CAT, GPx, thioredoxin reductase, and glutamate cysteine ligase (GCL) function co-operatively to destroy excess ROS, thereby rescuing cells from oxidative injuries. SOD inactivates superoxide anion by converting it to H_2O_2, which in turn, is detoxified by CAT or GPx. While CAT reacts with H_2O_2 to form water and molecular oxygen, GPx detoxifies H_2O_2 in the presence of reduced glutathione (GSH), producing H_2O and oxidized glutathione (GSSG) which is recycled to GSH by glutathione reductase (GR). GSH can be replenished through *de novo* synthesis which is catalyzed by GCL. When a cellular GSH level is low, H_2O_2 can be produced to extremely reactive hydroxyl radicals in the presence of transition metal ions via the Fenton reaction. If SOD is formed at a very high level, excessive amounts of H_2O_2 can be generated, provoking a hydrogen peroxide stress. Thus, it is desirable that SOD induction should be accompanied by timely induction of a gene coding for an H_2O_2- inactivating enzyme, such as CAT or GPx. Therefore, the response of cells to oxidative stress should be tightly regulated. The network of cellular defense systems is summarized in figure 2.2.

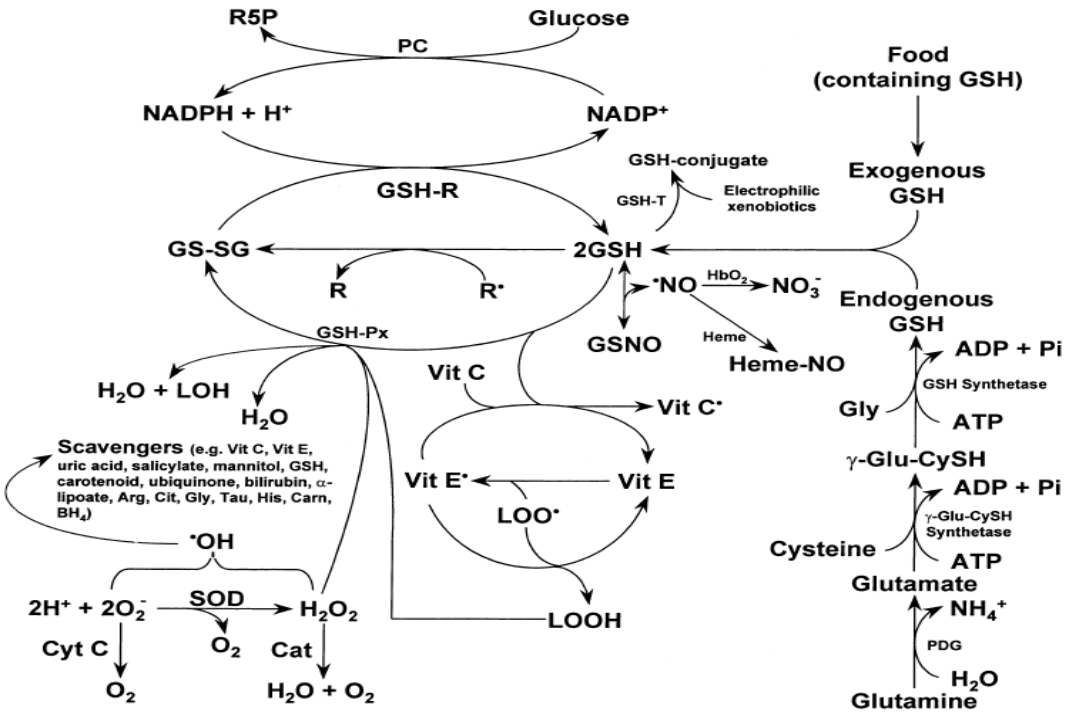


Figure 2 The roles of major antioxidants defense to free radicals species. ADP, adenosine diphosphate; Arg, arginine; BH4, (6R)-5,6,7,8,-tetrahydro-L-biopterin; Carn, carnosine; Cat, catalase; Cit, citrulline; Cyt C, cytochrome C; ETS, electron transport system; Glu, glutamate; Gly, glycine; γ-Glu-CySH, γ-glutamyl-cysteine; GS-SG, oxidized glutathione (glutathione disulfide); GSH, glutathione (reduced form); GSH-Px, glutathione peroxidases; GSH-R, glutathione reductase; GSH-T, glutathione S-transferase; GSNO, nitrosylated glutathione; HbO_2, oxyhemoglobin; Heme-NO, heme-nitric oxide; His, histidine; LOH, lipid alcohol; $LOO^{\bullet}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; $^{\bullet}NO$, nitric oxide; NO_3^-, nitrate; $O_2^{\bullet-}$, superoxide anion radical; $ONOO^-$, peroxynitrite; PC, pentose cycle; $R^{\bullet}$, radicals; R, non-radicals; R5P, ribulose 5-phosphate; SOD, superoxide dismutase; Tau, taurine; Vit C,vitamin C (ascorbic acid); Vit $C^{\bullet}$, vitamin C radical; Vit E, vitamin E (α-tocopherol); Vit $E^{\bullet}$, vitamin E radical (Fang et al., 2002).

11.2.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is the antioxidant enzyme that catalyses the dismutation of the highly reactive superoxide anion to and to the less reactive species H_2O_2.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

In human, there are three forms of SOD: SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 is an extracellular enzyme. The first is a dimer, while the others are tetramers. SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive centre. In this study, we selected SOD2 to be investigated because SOD2 can be

induced by a variety of stimulants and cytokines and its induction serves as an indicator of conditions where superoxide anion is being generated at high levels. On the other hand, SOD1 is constitutively expressed in cells with no direct response to oxidative stress, cytokines, or other stimulants.

SOD2 is a homotetramer (96 kDa) containing one manganese atom per subunit that cycle from Mn (III) to Mn (II) and back to Mn (III) during the two-step dismutation of superoxide. The respiratory chain in mitochondria is a major source of oxygen radicals. Inactivation of recombinant human mitochondrial SOD2 by peroxynitrite is caused by nitration of a specific tyrosine residue. The biological importance of Mn-SOD is demonstrated among others by the following observations: (a) inactivation of Mn-SOD genes in *Escherichia coli* increases mutation frequency when grown under aerobic conditions; (b) elimination of the gene in *Saccharomyces cerevisiae* increases its sensitivity to oxygen; (c) lack of expression in Mn-SOD knockout mice results in dilated cardiomyopathy and neonatal lethality; (d) expression of human Mn-SOD genes in transgenic mice protects against oxygen induced pulmonary injury and adriamycin-induced cardiac toxicity (Mates et al., 1999a).

Measurement of SOD2 activity in this study based on colorimetric assay as shown in figure 2.3. Superoxide ions (substrate) are generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase. The superoxide anion will convert tetrazolium salt to formazan dye (color product) but if have SOD activity, it will also convert superoxide ion to oxygen and water. Thus, color formazan dye is lower. SOD activity is determined from percent inhibition of the rate of formazan dye formation.

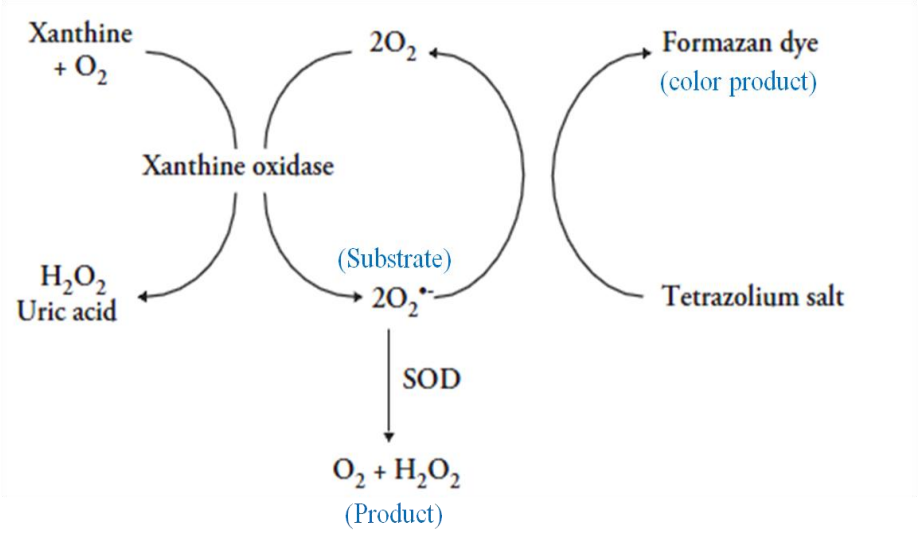


Figure 3 Measurement principle of SOD2 activity in this study

11.2.2 Catalase (CAT)

Catalase (CAT) is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa that contains a single ferriprotoporphyrin group per subunit, and has a molecular mass of about 240 kDa. CAT reacts with H_2O_2 to form water and molecular

oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity (Mates et al., 1999a).

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

CAT is one of the fastest known enzymes and its turnover number is 6 million, which means the number of substrate molecules with one molecule of the enzyme turns to products per minute. Therefore, it is an important neutralized antioxidant in cells exposed to hydrogen peroxide.

Measurement of CAT activity in this study based on colorimetric assay as shown in figure 2.4. The peroxidatic function of CAT is used determine enzyme activity. CAT converts hydrogen peroxide to water and formaldehyde. Then formaldehyde reacts with purpald, which acts as the chromogen to form purple color.

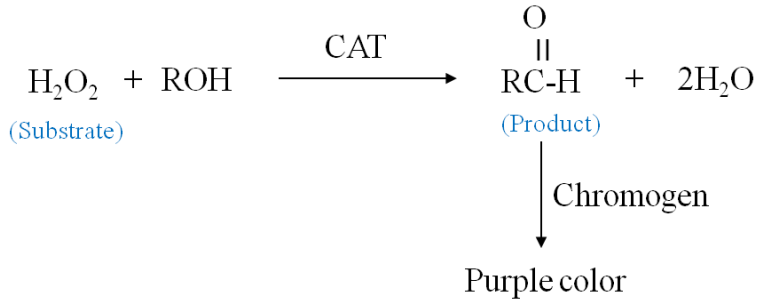


Figure 4 Measurement principle of CAT activity in this study

11.3 Base excision repair system (BER)

Persistent oxidative/ nitrative stress and excess lipid peroxidation are induced by chronic inflammatory processes that lead to massive DNA damage in target organs and represent an important step in carcinogenesis. The oxidation of lipids by reactive oxygen and nitrogen species results in byproducts such as trans-4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and crotonaldehyde. These intermediates can react with DNA bases to form exocyclic DNA adducts such as 1, N^6-etheno-2'-deoxyadenosine (**ε**dA) and 3, N^4-etheno-2'-deoxycytidine (**ε**dC) (el Ghissassi et al., 1995; Nair et al., 2007a; Singer and Hang, 1999). Etheno-DNA adducts are eliminated by base excision repair (BER), whereby DNA glycosylases hydrolyze the *N*-glycosidic bond between the damaged base and 2'-deoxyribose, leaving an abasic site in DNA. Since DNA repair must play an important role in eliminating LPO-derived adducts, insufficient and impaired repair by inflammatory mediators has been hypothesized as a risk factor for increasing mutation rates and genomic instability

(Feng et al., 2004; Wink et al., 1998), acting as driving force in chronic degenerative diseases including cancer. Inflammation-induced inactivation of the BER pathway contributes to neoplastic conversion of cells, there is an emerging paradoxical hypothesis that adaptive activation of the BER system may also induce microsatellite instability and hence represent one of the mechanisms underlying inflammation-driven carcinogenesis (Kundu and Surh, 2012). APE also acts as a redox chaperone and enhances DNA binding of various transcription factors, such as NF-**K**B and AP-1, through reduction of cysteine residues present in these transcription factors either directly or indirectly by recruiting glutathione and thioredoxin (Ando et al., 2008).

Base excision repair (BER) pathway plays a major role in counteracting the cytotoxic and mutagenic effects of a broad range of DNA lesions caused by exogenous as well as endogenous factors. The key enzymes in this process are DNA glycosylases, which remove different types of modified or remove different kinds of damage, and the specificity of the repair pathway is determined by the type of glycosylase involved. Once the base is eliminated, the apurinic/apyrimidinic (AP)-site is removed by an AP-endonuclease or an AP-lyase, which cleaves the DNA strand 5' or 3' to the AP-site, respectively. The remaining deoxyribose phosphate residue is excised by a phosphodiesterase; the resulting gap is filled by a DNA polymerase, and the strand is sealed by DNA ligase.

There are two pathways of BER: Short-patch BER pathway is initiated when the damaged base is recognized and excised by a DNA glycosylase, leaving an abasic site, then apurinic/apyrimidinic (AP) endonuclease 1 (APE1) incises the DNA backbone 5' to the abasic site, leaving a 3'-OH and a 5'-deoxyribosephosphate (dRP) group. DNA polymerase beta (Pol β) catalyzes the excision of the dRP group with its dRP lyase activity and fills in the single nucleotide gap using its DNA polymerase activity. The XRCC1-DNA ligase IIIa (LigIIIa) complex then seals the nick, completing repair. In some cases a bifunctional DNA glycosylase removes the damaged base and incises the AP site, then APE1 or another 3'-repair diesterase removes the 3'-terminal 4-hydroxypentanal phosphate to generate a 3'-OH group. Pol β fills in the gap and LigIIIa seals the nick. Long-patch BER pathway is thought to have evolved to repair sites that are refractory to the dRP lyase activity of Pol β. This process results in a repair patch that is 2–12 nucleotides in length and involves other additional proteins including proliferating cell nuclear antigen (PCNA), replication factor C (RFC), flap endonuclease 1 (FEN1), the replicative polymerases delta and epsilon (Pol δ and ε), and DNA ligase I (LIGI) (Figure 2.5) (Ide and Kotera, 2004; Sweasy et al., 2006).

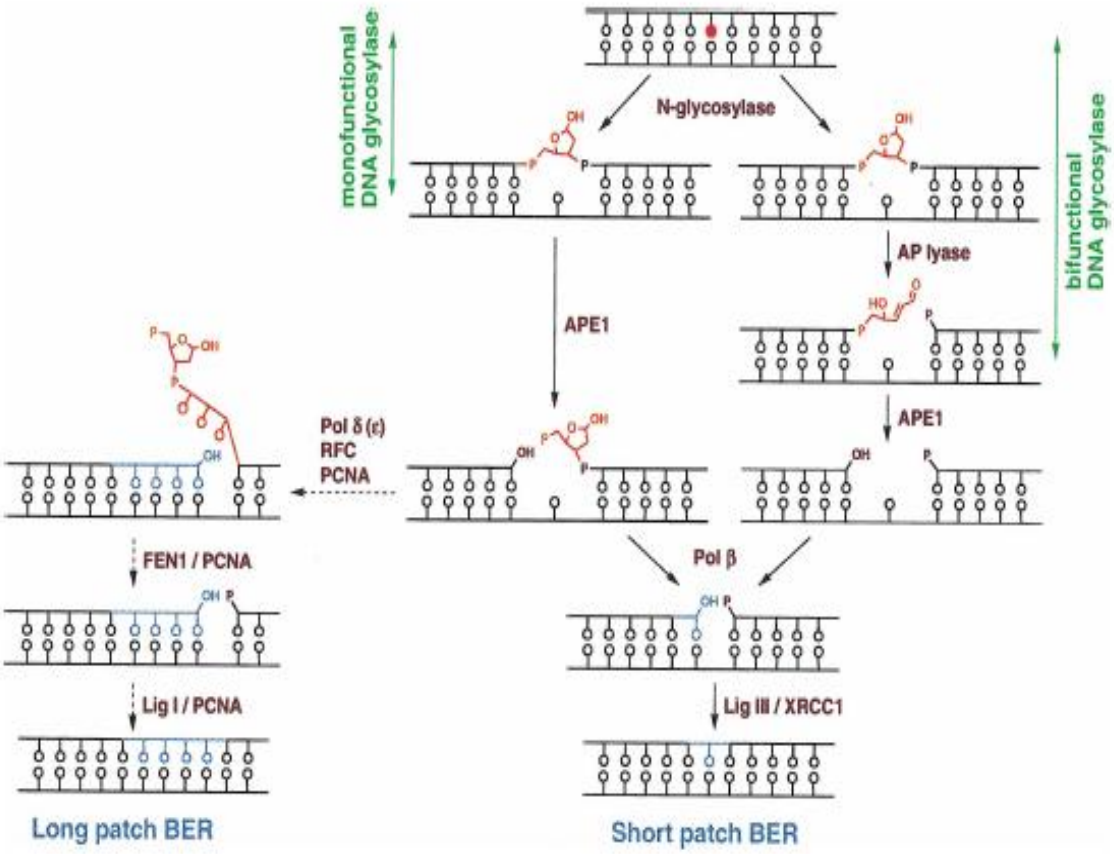


Figure 5 Pathway of long patch and short patch BER (Ide and Kotera, 2004).

11.3.1 Alkyladenine DNA glycosylase (AAG also called ANPG or MPG)

AAG is a 33-kDa monomeric protein that excises alkylation-damaged bases such as 3-methyladenine, 7-methylguanine, and 1, N^6-ethenoadenine (**ε**A) by hydrolysis of the *N*-glycosylic bond, forming a free DNA base and a basic sugar residue. Once the damaged base is removed, other enzymes in the pathway remove the remaining sugar residue and resynthesize DNA to fill in the gap. It shares this property with the bacterial enzyme AlkA, although they are not related in their amino acid sequences (Krokan et al., 1997). This glycosylase has been shown to have a broad substrate specificity (Lau et al., 1998). Substrate selection appears to be governed by combination of selectivity filters. The first selectivity filter occurs at the nucleotide flipping step, since AAG preferentially selects lesions that are presented in unstable base pairs. The catalytic mechanism constitutes a second selectivity filter. Once the lesions are bound, the use of general acid catalysis ensures that AAG excises only purine bases, even though smaller pyrimidines can fit into the active site (Biswas et al., 2002; O'Brien and Ellenberger, 2003). The third selectivity filter consists of unfavorable steric clashes with the exocyclic amino groups of guanine and adenine, so that purine lesions lacking these functional groups are preferentially recognized.

Finally, since alkylation of N3 and N7 of the purine ring leads to destabilization of the *N*-glycosidic bond, AAG is able to effectively excise *N*-alkyl lesions with a relatively modest rate enhancement.

11.3.2 Apurinic (apyrimidinic) endonuclease/redox-factor 1 (APE/Ref-1)

APE/Ref-1 is an ubiquitously expressed multifunctional protein of 36.5 kDa which is encoded by a ~3-kb gene localized on chromosome 14 q11.2–12 and consists of four introns and five exons (Harrison et al., 1992). The human APE cDNA is about 1.4 kb in length and encompasses a coding region of 954 nucleotides, encoding a protein of 318 amino acids (Seki et al., 1992). Deletion analysis showed that the C-terminal part of APE is important for the DNA binding and the AP-endonuclease activity of the protein (Barzilay et al., 1995; Izumi and Mitra, 1998). Site directed mutagenesis of Asp-219 leads to loss of both DNA binding and AP-endonuclease activity, indicating that this amino acid is crucial for the repair function of APE (Barzilay et al., 1995). Further amino acids which are functionally relevant for DNA repair are Asp-90, Glu-96 and Asp-308; their replacement by alanine causes loss of endonuclease function, yet without influencing DNA binding activity (Figure 2.6). A ~6 kDa N-terminal domain is of particular relevance for the redox function of APE/Ref-1 (Xanthoudakis et al., 1994). The amino acid which is essentially required for the Ref-1-mediated redox activation of transcription factors is Cys-65 being in a reduced state (Walker et al., 1993). In oxidized, redox deficient state, Cys-65 is hypothesized to form a disulfide bridge with Cys-93.

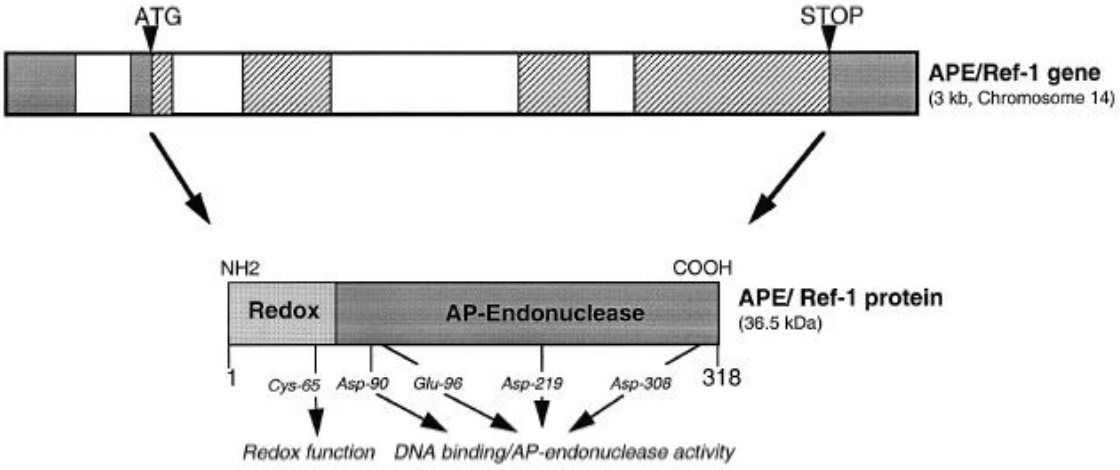


Figure 6 Organization of apurinic (apyrimidinic) endonuclease/redox-factor 1 (APE/Ref-1) gene and structure–function relationship of the APE/Ref-1 protein (Fritz, 2000).

APE/Ref-1 is a multifunctional protein which possesses several completely different physiological activities. Thus, besides exhibiting DNA repair activity, APE/Ref-1 also acts as a regulator of gene expression. Regarding its DNA repair activity, APE:Ref-1 functions as 5'AP-endonuclease in base excision repair. Moreover, by its 3'-diesterase activity, it removes phosphoglycolate residues from DNA damaged by ionizing radiation. Furthermore, APE/ Ref-1 stimulates the activity of transcription factors such as AP1 via a redox-based mechanism,

thereby increasing the expression of corresponding target genes. Interestingly, APE/Ref-1 also acts as a transcriptional repressor of its own gene and other genes such as that coding for the parathyroid hormone (Izumi et al., 1996). Depletion of the cellular amount of APE/Ref-1 protein by overexpression of APE/Ref-1 antisense mRNA, which gives rise to specific blockage of APE/Ref-1 mRNA translation, results in hypersensitivity to DNA damaging agents such as methyl methanesulfonate and hydrogen peroxide (Walker et al., 1994). The expression of the APE gene is inducible by oxidative stress. Overexpression of APE protein protects cells from the genotoxicity of oxidizing agents (Grosch et al., 1998). Thus, APE is obviously part of the cellular defense to particular types of genotoxic stress. However, it is rather unclear so far whether the protective effects of APE/Ref-1 are mainly due to its DNA repair functions and/or are based on its activity as transcriptional regulator. Summarized functions of APE/Ref-1 are depicted in figure 2.7.

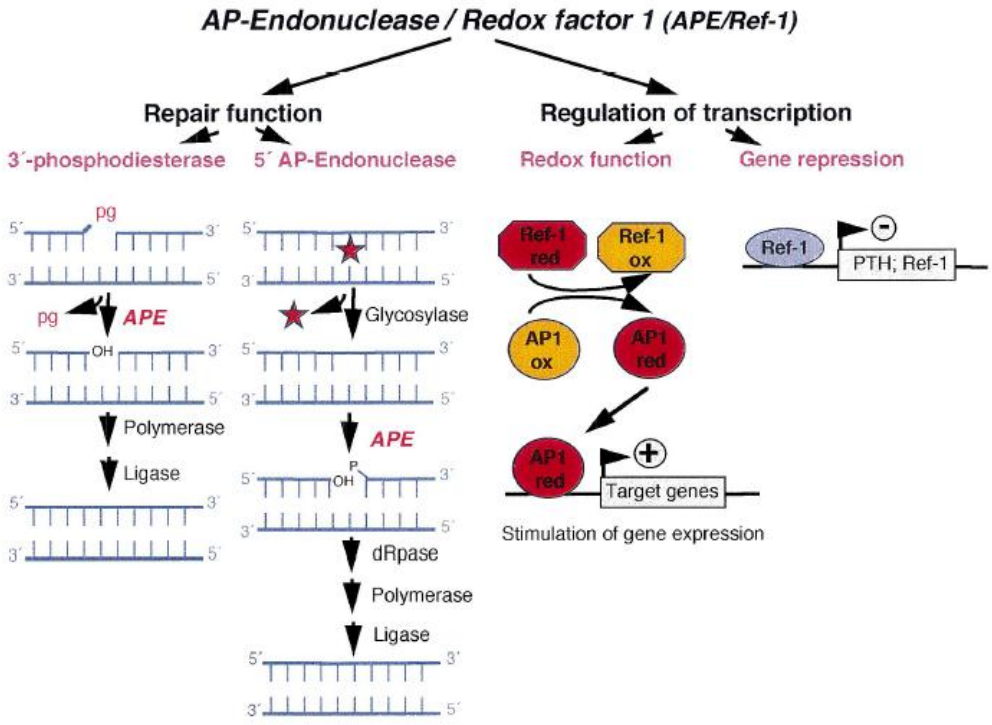


Figure 7 Apurinic (apyrimidinic) endonuclease/redox-factor 1 (APE/Ref-1) is a multifunctional protein. Human APE/Ref-1 acts both as a DNA repair protein (exhibiting 3'-phosphodiesterase and 5'AP-endonuclease activity) and as a positive or negative regulator of transcription (via redox-based activation of transcription factors such as AP1, p53, and NF-kB or by repression of genes, including its own gene). PTH, parathyroid hormone gene; dRpase, deoxyribophosphodiesterase; ★, damaged DNA base; ox, oxidized state; red, reduced state; +, stimulatory effect; -, repressive effect (Fritz, 2000).

11.3.3 DNA polymerase β (DNA pol β)

DNA pol β is a 39-kDa single chain polypeptide comprising 335 amino acid residue (Figure 2.8). The amino-terminus (8 kDa) is connected to the polymerase domain (31 kDa) by

a protease sensitive hinge region (Prasad et al., 1998). The 8-kDa domain has a lyase activity that removes the 5-deoxyribose phosphate generated after incision by an apurinic/apyrimidinic (AP) endonulcease during base excision repair (BER) (Matsumoto and Kim, 1995). The nucleotidyl transferase reaction is catalyzed by the 31-kDa polymerase domain. In addition to the significant movement of the amino-terminus domain upon binding gapped DNA, the C-terminus is observed to 'close' around the correct incoming dNTP and its complementary template base (Pelletier et al., 1994; Sawaya et al., 1997).

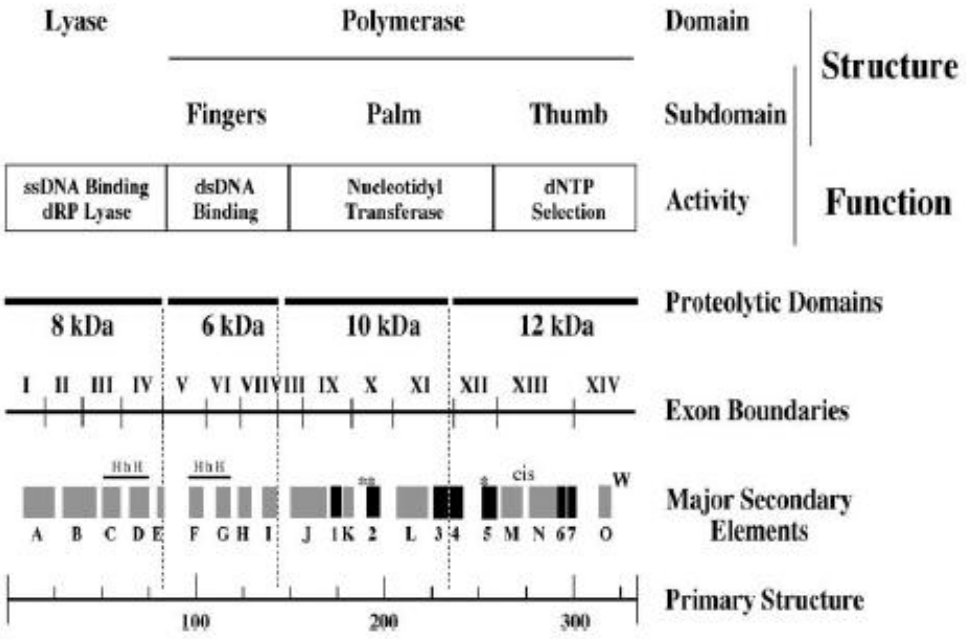


Figure 8 Genomic structure of DNA polymerase β, illustrating the major domains in the protein molecule (Idriss et al., 2002).

DNA pol β has been shown to be primarily involved in DNA repair. There are two biochemical pathways of BER in mammalian cells: short-patch (single nucleotide replacement) and long-patch BER (multi-nucleotide replacement) (Foiani et al., 1997). Experimental evidence indicates that DNA pol β is involved in short-patch BER. In addition, DNA pol δ or ε are probably involved in PCNA-dependent, long-patch BER with gaps of 2–13 nucleotides (Klungland and Lindahl, 1997; Stucki et al., 1998). In this pathway, the flap endonuclease 1 (Fen1) is needed to cleave a reaction intermediate generated by template strand displacement during gap filling. A variation of the long-patch BER pathway with gap lengths of 2–6 nucleotides has also been characterized in mammalian cells. This Fen1 and PCNA-dependent pathway can be reconstituted with DNA pol δ and DNA pol ε. However, lack of DNA pol β in DNA pol β deficient cells or in the presence of neutralizing antibody, causes a reduction in DNA repair activity. This evidence strongly suggests a role for DNA pol β in this pathway *in vivo* (Dianov et al., 1999). Another role for DNA pol β is found in meiosis

and double strand break repair. DNA pol β has been implicated in the meiotic events associated with synapsis and recombination (Plug et al., 1997). In addition, a 67-kDa homologue of mammalian DNA pol β, encoded by the non-essential *POL*4 gene of *S. cerevisiae*, has recently been implicated in double strand break repair through a non-homologous end joining mechanism (Wilson, 1998).

11.4 Microsatellite instability (MSI)

Microsatellite is repeated sequences of 1-6 base pairs of DNA and localized in non-coding and coding region of DNA. Although the length of these microsatellites is highly variable from person to person, each individual has microsatellites of a set length. These repeated sequences are common, and normal. The most common microsatellite in humans is a dinucleotide repeat of cytosine and adenine, abbreviated $(CA)_n$, which occurs in tens of thousands of locations in our germ line**.**

Microsatellite instability (MSI) is a situation in which a germ-line microsatellite allele has gained or lost repeated units and has thus undergone a somatic change in length. Because this type of alteration can be detected only if many cells are affected by the same change, it is an indicator of the clonal expansion that is typical of a neoplasm. MSI is a key factor in several cancers including colorectal, endometrial, ovarian and gastric cancers. For instance, MSI in hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome, where an inherited mutation in a mismatch-repair gene causes a microsatellite repeat replication error to go unfixed. The replication error results in a frameshift mutation that inactivates or alters major tumor suppressor genes - key genes in the regulation of the cell cycle and, ultimately, the prevention of cancer (de la Chapelle, 2003).

11.5 Genoxotic stress, adaptive imbalance, microsatellite instability (MSI) and cancer

When cells are exposed to infection, inflammatory process would be induced to release a variety of reactive oxygen and nitrogen species. Free radicals can inflict cellular damage, and then several defenses have evolved to protect cells from genotoxic stress. However, there are increasing evidences that this adaptive response may have deleterious effects, for instance if reduction of antioxidant and/or imbalanced DNA repair enzyme system occurs, it will generate microsatellite instability (MSI) and has procarcinogenic implication. There are a number of studies supporting this phenomena. Jaruga et al (Jaruga et al., 1994) reported that higher levels of DNA lesions were observed in cancerous tissues than in cancer-free surrounding tissues. Antioxidant enzyme levels were lower in cancerous tissues. These results indicate an association between decreased activities of antioxidant enzymes and increased levels of DNA lesions in cancerous tissues. Higher levels of DNA lesions suggest that free radical reactions may be increased in malignant tumor cells. Moreover, Guner et al (Guner et al., 1996) reported that low levels of SOD and CAT enzymes in lung cancerous tissues can lead to elevated levels of reactive oxygen metabolites, resulting in damage to the key subcellular structurcs such as DNA, cell membranes, and other vital cellular components. Glassner et al (Glassner et al., 1998) demonstrated that generation of a strong mutator phenotype in yeast by imbalanced base excision repair. The data presented a mechanism by which spontaneous mutation rates may be modulated. An imbalance between AP endonuclease and one particular DNA glycosylase produced a striking mutator phenotype in yeast. Glycosylase to AP

endonuclease ratio imbalances could arise by promoter mutations that affect DNA repair gene expression. Imbalances in base excision repair pathway enzymes may represent a hitherto unrecognized source of increased cancer risk. In 2003, Hofseth et al (Hofseth et al., 2003) reported the adaptive imbalance in base excision-repair enzymes and proposed the generation of microsatellite instability (MSI) in noncancerous colon of ulcerative colitis (UC) patients. Mechanistic studies in yeast and human cell model showed that overexpression of AAG and/or APE1 were associated with increased frameshift mutations and MSI. AAG and APE1 were significantly increased in inflamed colon epithelium, with MSI being positively correlated with their imbalanced enzymatic activities.

Recently, Kundu and Surh (2012) proposed that persistent local inflammation perturbs the homeostatic control of cell signaling pathways, which may predispose cells to premalignant and malignant conversion. Various types of inflammatory immune cells, host stromal cells, and cancerous cells within a tumor microenvironment also release a large excess of proinflammatory mediators, which accelerates tumor invasion and metastasis. The link between inflammation and cancer has two paradigms: one is inflammation driven carcinogenesis (extrinsic mechanism) and the other involves accelerated cancer progression by tumor-derived inflammatory triggers (intrinsic mechanism) (Figure 2.9)(Kundu and Surh, 2012).

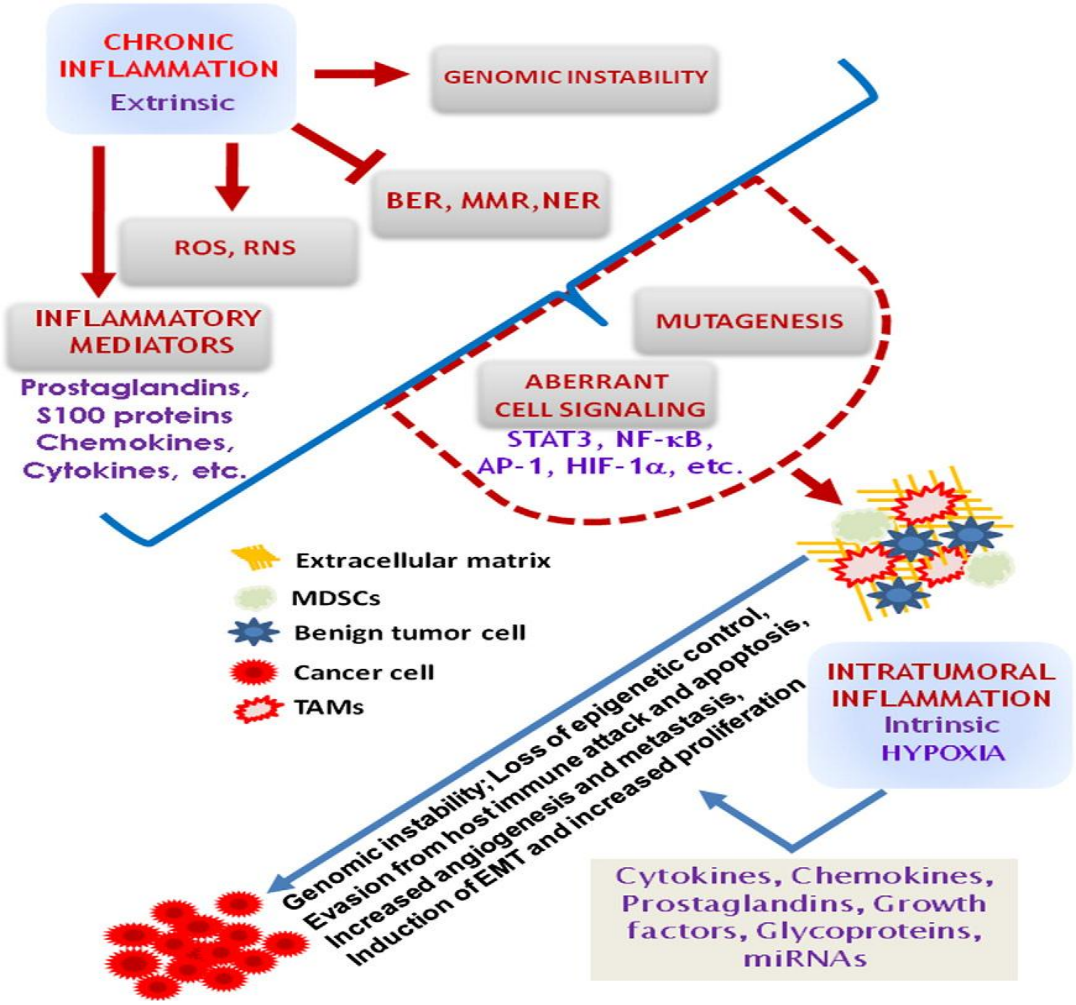


Figure 9 Two paradigms of the link between inflammation and cancer. Chronic inflammation (extrinsic) often accompanies generation of ROS, which can cause DNA damage and genomic instability, and simultaneously impairs the cellular DNA repair system, thereby initiating carcinogenesis. In addition, excessive generation and/or expression of a variety of proinflammatory mediators perturb cell signaling pathways and promotes the induction of growth-promoting genes. On the other hand, an intratumoral inflammatory state (intrinsic), created by a complex interaction between cytokines and growth factors released by tumor cells or surrounding stromal cells, further facilitates tumor growth by increasing angiogenesis and metastasis, inducing epithelial-to-mesenchymal transition and reducing the susceptibility of tumor cells to the host immune response (Kundu and Surh, 2012).

12. ระเบียบวิธีวิจัย (Research methodology)

12.1 Materials and Methods

12.1.1 White blood cells (WBCs) and cholangiocarcinoma tissues

WBCs and CCA tissues were collected from cholangiocarcinoma patients admitted at surgical wards of Srinagarind Hospital, Khon Kaen University. The protocol of collection and

study was approved by the Ethic Committee for Human Research, Khon Kaen University (HE 521209).

12.1.2 Hamster cholangiocarcinoma tissues

Hamster CCA tissues were taken from Dr. Somkid's project (Dechakhamphu et al., 2010a). The animal experiments were conducted according to the guidelines of the National Committee of Animal Ethics. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand (AEKKU 18/2550). In brief, hamsters were divided into 4 groups: (1) Untreated control (2) OV-infected, (3) *N*-nitrosodimethylamine (NDMA), and (4) OV plus NDMA. Fifty metacercariae of *O. viverrini* were infected to hamsters by intragastric intubations, while oral administration NDMA in drinking water (12.5 ppm) was provided *ad libitum* for 2 months. The animals were sacrificed at 21 days, 1, 2, 3, 4, and 6 months post infection. Liver tissue from each animal was fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin for histological and immunohistochemical examinations.

12.1.3 Immunohistochemical study of antioxidant and DNA repair enzymes

Immunohistochemical method was performed to determine the expression patterns of antioxidant enzymes and DNA repair enzymes in human and hamster CCA tissues. In brief section of human liver tissues were deparaffinized and rehydrated with stepwise decreasing concentration of ethanol. Antigen retrieval was performed by microwave treatment in 10 mM citrate buffer pH 6.0 at high power for 10 min then sections were immersed for 20 min in 3% (v/v) hydrogen peroxide in PBS for endogenous hydrogen peroxide activity blocking and non specific binding was blocked by skim milk in PBS for 30 min. Sections were incubated with the primary antibody in the moisture chamber 1 hr at room temperature and 4°C overnight. After that, sections were washed in PBS (three times) and incubated with peroxidase conjugated secondary antibody. After washing in PBS (three times), the color was developed with DAB (3, 3'diaminobenzidine tetrahydrochloride) substrate kit for 5 min, then counterstained with Mayer's haematoxylin. The sections were rehydrated with stepwise increasing concentration of ethanol, cleared with xylene and mounted with permount. The stained sections are reviewed under a microscope. Each primary and secondary antibody for IHC of human and hamster CCA are shown in table 3.1 and 3.2 respectively.

Table 1 Antibodies for IHC of human CCA tissue

Antibody	Dilution for IHC		Company of Primary antibody
	Primary antibody	Secondary antibody	
Rabbit anti-SOD2 polyclonal antibody	1:200	1:400	Millipore, USA
Rabbit anti-CAT polyclonal antibody	1:1000	1:2000	Abcam, USA
Goat anti-MPG polyclonal antibody	1:100	1:200	Abcam, USA
Mouse anti-APE1 monoclonal antibody	1:50	1:100	Santa Cruz Biotechnology, USA
Rabbit anti-DNA polymerase beta polyclonal antibody	1:50	1:100	Abcam, USA

Table 2 Antibodies for IHC of hamster CCA tissue

Antibody	Dilution for IHC		Company of Primary antibody
	Primary antibody	Secondary antibody	
Rabbit anti-SOD2 polyclonal antibody	1:200	1:400	Abnova, USA
Rabbit anti-CAT polyclonal antibody	1:500	1:1000	Abcam, USA
Mouse anti-APE1 monoclonal antibody	1:100	1:200	Santa Cruz Biotechnology, USA
Rabbit anti-DNA polymerase beta polyclonal antibody	1:50	1:100	Abcam, USA

12.1.4 Enzymatic reaction study of antioxidant enzymes

12.1.4.1 Protein extraction

Superoxide dismutase 2 (SOD2)

Frozen tissue of human and hamster CCA was washed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots. Then the tissue was homogenized in 1 ml of cold 20 mM HEPES buffer, pH 7.2, which was consisted of 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. The homogenate was centrifuged at 1,500×g for 5 min at 4°C. The supernatant was removed for assay of total SOD activity (cytosolic and mitochondrial). To separate the two compartment of enzymes, centrifuged the 1,500×g

supernatant at 10,000×g for 15 min at 4°C, the supernatant contained cytosolic SOD and the pellet contained mitochondrial SOD. Homogenize the mitochondrial pellet in HEPES buffer. It was used for determination of mitochondrial SOD (SOD2) activity.

Catalase (CAT)

Frozen tissue of human and hamster CCA was washed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots. Then the tissue was homogenized in 1 ml of 50 mM potassium phosphate (K_3PO_4), pH 7.0, containing 1 mM EDTA The homogenate was centrifuged at 10,000×g for 15 min at 4°C. The supernatant was used to assay CAT activity**.**

12.1.4.2 Assay of SOD2 and CAT activities

SOD2 and CAT proteins were extracted to perform enzymatic reactions. SOD and CAT assay kits were purchased from Cayman Chemical Company, USA. The principle of SOD assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Measure the absorbance at 440-460 nm on ELISA reader from TECAN. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical, while, CAT assay kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol, in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1, 2, 4-trizazole as the chromogen. Read the absorbance at 540 nm using ELISA reader from TECAN.

12.1.5 Study of microsatellite instability (MSI)

12.1.5.1 Hematoxylin staining

Paraffin sections of CCA tissue were deparaffinized in xylene for 5 min (two times) to remove the paraffin wax. After deparaffinization, the slide was hydrated by decreasing concentrations of ethanol as following steps; absolute alcohol for 3 min, 70% alcohol for 3 min and finally rinsed in the tap water. After hydration, the slide was stained with hematoxylin for 5 min and washed in the running tap water for 2 min, following by destaining in acid alcohol (1% acid in 70 % alcohol), washing in the running tap water. After that, the slide was dehydrated by 70% alcohol for 2 min and absolute alcohol for 2 min (two times). The appropriated result for nuclei was stained blue color.

12.1.5.2 DNA extraction

By using hematoxilin technique, tumorous and non-tumorous tissues staining were visualized. After separation of tumorous and non-tumorous tissues, they were extracted by QIAamp DNA formalin-fixed, paraffin-embadded (FFPE) tissue kit. Matched White blood cells were prepared for DNA by digesting in 1 ml of lysis buffer, 0.2 µl of 10 mg/ml RNase and 10 µl of 17 mg/ml proteinase K incubate 50 °C, 30 min. Protein was precipitated by adding 300

µl of NaI solution. Isopropanol was added to the supernatant in order to precipitate DNA. The DNA pellet was then resuspended in TE buffer pH 8.0.

12.1.5.3 Microsatellite instability (MSI) analysis

Tumor, non tumor of adjacent area and WBC DNA of the same patient were analyzed for MSI using five microsatellite markers (Yamamoto et al., 2005): BAT25, BAT26, D5S346, D2S123 and D17S250. Oligonucleotide forward primers were fluorescent-5'-labeled. PCR was performed in a 20 ml reaction mixture with 10 to 100 ng genomic DNA, 0.2 mM each dNTP, 1x high fidelity PCR buffer, 0.2 µM each primer and 1 U Taq DNA polymerase high fidelity (Invitrogen, USA). Each primer sequence is shown in table 3.3.

Table 3 Specific primers for microsatellite PCR (Yamamoto et al., 2005)

Primer name	Linked genes	Sequence
BAT25	c-kit	forward primer 5'-TCG CCT CCA AGA ATG TAA GT
		reverse primer 5'-TCT GCA TTT TAA CTA TGG CTC
BAT26	hMSH2	forward primer 5'-TGA CTA CTT TTG ACT TCA GCC
		reverse primer 5'-AAC CAT TCA ACA TTT TTA ACC C
D2S123	hMSH2	forward primer 5'-ACA TTG CTG GAA GTT CTG GC
		reverse primer 5'-CCT TTC TGA CTT GGA TAC CA
D5S346	APC	forward primer 5'-ACT CAC TCT AGT GAT AAA TCG G
		reverse primer 5'-GTT TCC ATT GTA GCA TCT TGA C
D17S250	p53	forward primer 5'-GCT GGC CAT ATA TAT ATT TAA ACC
		reverse primer 5'-GGA AGA ATC AAA TAG ACA AT

Each amplification condition was as follows: BAT25, BAT26 and D17S250, 5 min initial denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 s at 50°C and 1 min at 68°C, with a final extension of 10 min at 68°C; D2S123 and D5S346, 5 min initial denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 s at 55°C and 1 min at 68°C, with a final extension of 10 min at 72°C. Then MSI was analyzed in ABI Prism 3130 Genetic Analyzer (Applied Biosystems) using GeneScan Analysis v3.2.1 software (Applied Biosystems). MSI was defined as changes of size bands observed in the tumor tissue and non-tumor of adjacent area that was not seen in normal control WBC from the same patient. Tumors exhibiting MSI at two or more markers were defined as MSI-high (MSI-H). Tumors showing instability at only one marker was defined as MSI-low (MSI-L). Tumor in which no markers exhibited MSI was considered to be microsatellite stability (MSS).

12.2 Statistical analysis

Mann-Whitney U test was used to compare the differences cadaveric donor and human CCA. The Student's t-test was used to analyze statistical significance between untreated and Ov+NDMA groups. Clinicopathological characteristics and microsatellite instability (low and high) in human CCA tissue were compared using the Fisher's exact

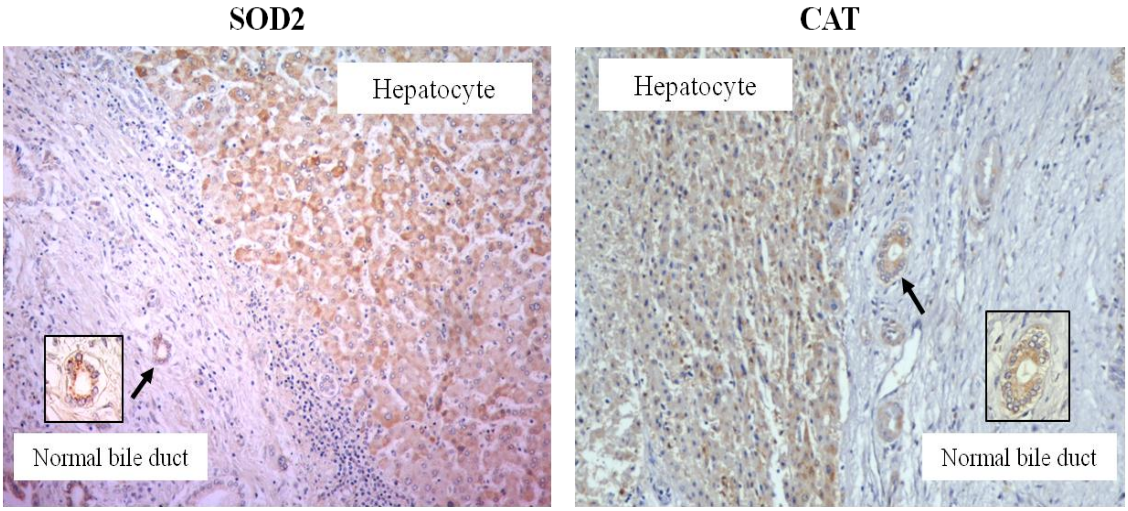
probability test. The Kaplan-Meier method was used to calculate the survival curves, and the Log-rank test was performed to compare differences in the survival rates of patients who were subjected to curative surgery. A *P*-value < 0.05 was considered as a statistically significance.

13. ผลการวิจัย (Results)

134.1 Protein expression of antioxidant enzymes in human CCA tissues

Immunohistochemical analysis was performed to detect SOD2 and CAT expressions in human CCA tissue. SOD2 and CAT expressions were evaluated by calculating a total immunostaining index as the product of a frequency and intensity score. The proportion score described the estimated fraction of positive stained tumor cells (0= 0%; 1= 1-25%; 2= 26-50%; 3= >50%). The intensity score represented the estimated staining intensity (0, negative staining; 1, weak; 2, moderate; 3, strong). The IHC index ranged from 0 to 9. Negative, low, moderate and high expressions of SOD2 and CAT were defined as IHC index = 0; 1 to 2; 3 to 4 and >4 respectively. Our data demonstrated that SOD2 and CAT were cytosolic stained in bile duct CCA tissues. Most of normal bile duct epithelia residing in an adjacent tissue of tumor sections were strongly positive staining with SOD2 and CAT. SOD2 and CAT expressions were also seen in hepatocytes (Figure 4.1A). Levels of SOD2 with low, moderate and high expression were 15 (55%), 8 (30%) and 4 (15%) respectively, while levels of CAT with negative, low, moderate and high expressions were 4 (15%), 11 (40%), 8 (30%) and 4 (15%) respectively (Figure 4.2). Examples of low, moderate and high expression for SOD2 and CAT in CCA tissue are shown in figure 4.1B.

A



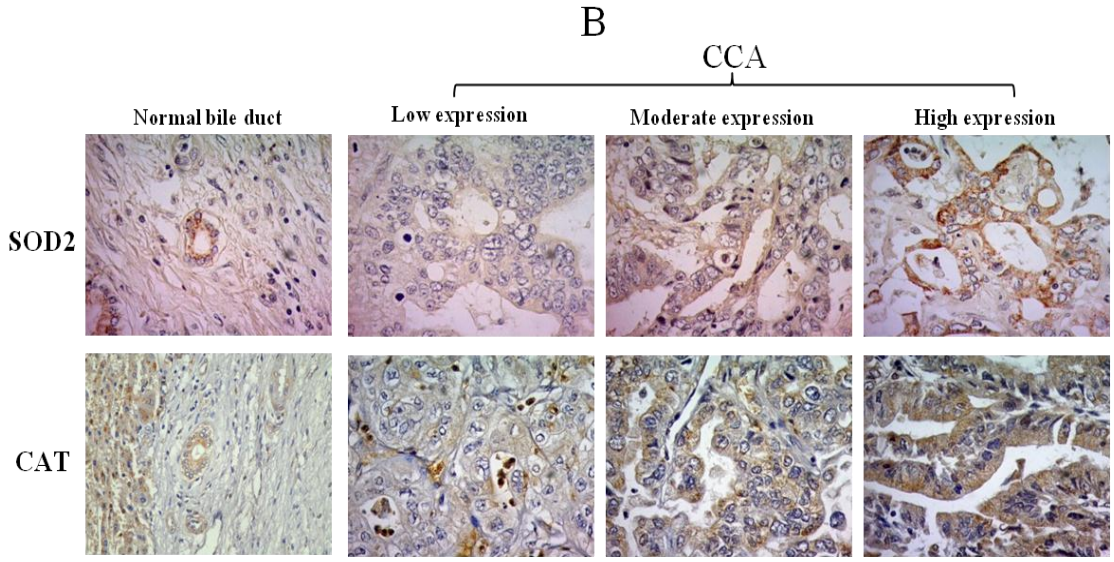


Figure 4.1 Immunohistochemical analysis of SOD2 and CAT expression in human CCA tissues. (A) Expression of SOD2 and CAT in normal bile duct and hepatocyte. The original magnification was 100x. (B) Normal bile duct, low, moderate and high expression of SOD2 and CAT. The original magnification was 400x.

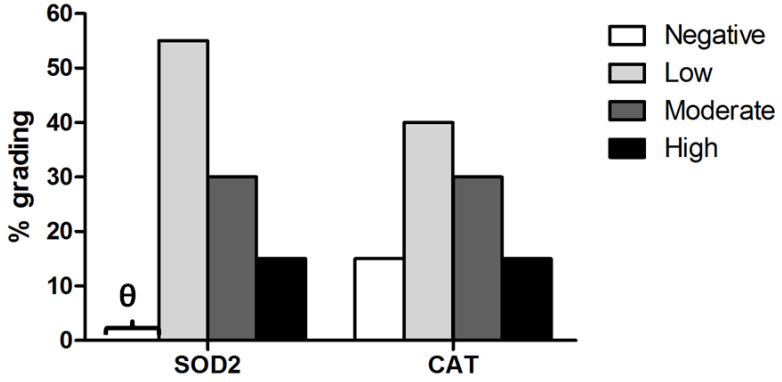


Figure 4.2 Grading of expressed SOD2 and CAT enzymes presented in percentage. θ, miss bar indicates that grading was negative expression.

4.2 Antioxidant enzymes activities in human CCA tissues

The activities of SOD2 and CAT were examined in human CCA tissues. These results showed that both SOD2 and CAT activities were significantly decreased in CCA when compared with cadaveric donor (P=0.04 and P=0.004 respectively) as shown in figure 4.3.

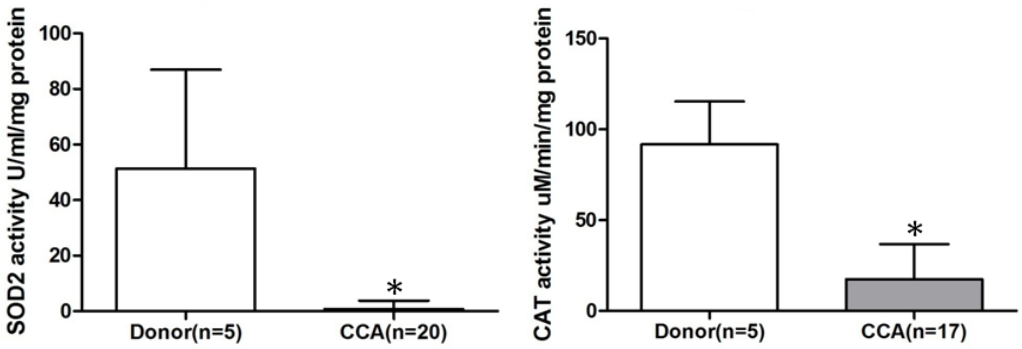


Figure 4.3 SOD2 and CAT activities (mean ± SD) in CCA and cadaveric donor. Mann-Whitney U test was used to analyze statistical significance between CCA and cadaveric donor. Differences were considered statistically significant at $P<0.05$. * Denotes statistical significance when compared between CCA and cadaveric donor.

4.3 Protein Expression of DNA repair enzymes in human CCA tissues

We examined the expression of AAG, APE and DNA pol β of human CCA tissues using immunohistochemistry. AAG, APE and DNA pol β expressions were evaluated by calculating a total immunostaining index as the product of a frequency and intensity score was describe in previous study. The result showed that AAG, APE and DNA pol β expressions were nuclear stained in bile duct CCA tissues. Mostly normal bile duct epithelia residing in an adjacent tissue of tumor sections were weakly stained with AAG, APE and DNA pol β. AAG and DNA pol β staining were seen in hepatocyte except APE staining (Figure 4.4A). Level of AAG with low, moderate and high expressions were 4 (15%), 8 (30%) and 15 (55%) respectively; levels of APE with negative, low, moderate and high expressions were 5 (19%), 15 (55%), 5 (19%) and 2 (7%) respectively and levels of DNA pol β with moderate and high expressions were 8 (30%) and 19 (70%) respectively as shown in figure 4.5. Example of low, moderate and high expressions for AAG, APE and DNA pol β in CCA patients are shown in figure 4.4B.

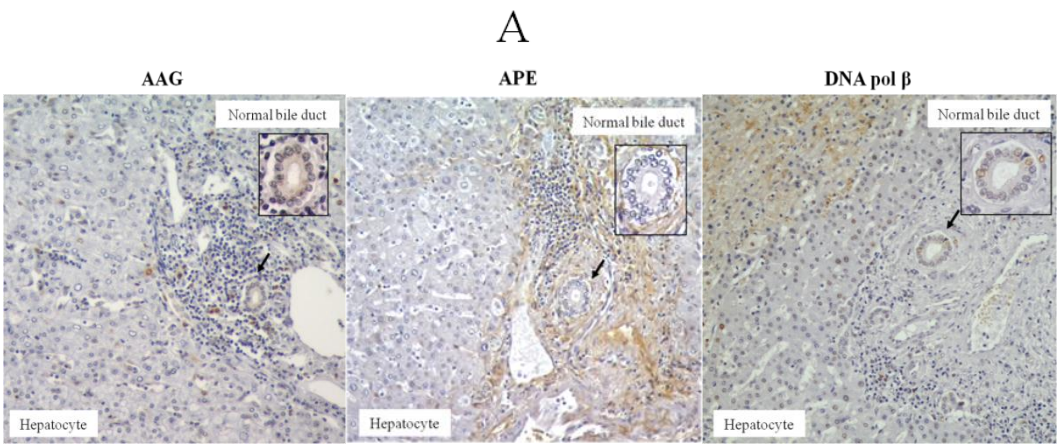


Figure 4.4 Immunohistochemical analysis of AAG, APE and DNA pol β expression in human CCA tissues. (A) Expression of AAG, APE and DNA pol β in normal bile duct and hepatocyte. The original magnification was 100x. (B) normal bile duct, low, moderate and high expression of AAG, APE and DNA pol β. The original magnification was 400x.

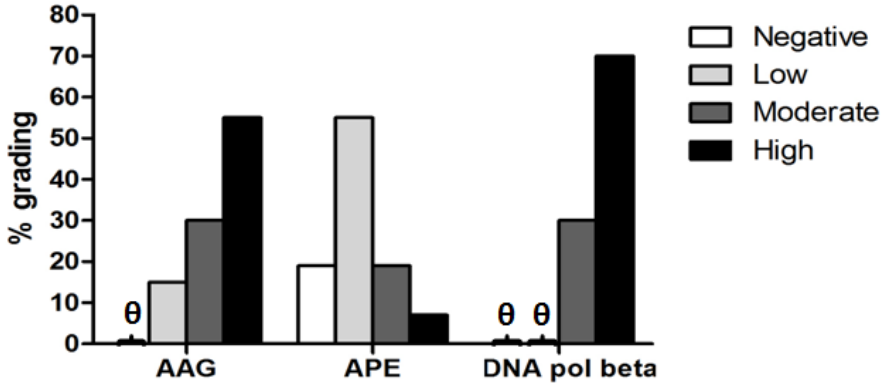


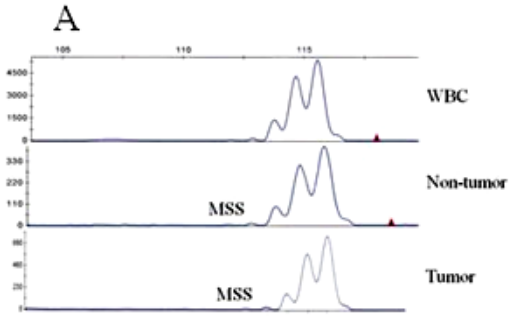
Figure 4.5 Grading of expressed AAG, APE and DNA pol β enzymes presented in percentage. θ, miss bar indicates that grading was negative expression.

4.4 Microsatellite instability analysis in human CCA tissues

We studied the microsatellite instability (MSI) in 13 CCA patients by using five microsatellite markers according to Yamamoto *et al.* (Yamamoto et al., 2005). MSI was defined as changes of size bands observed in the tumor tissue and non tumor of adjacent area that was not seen in normal control WBC from the same patient. A tumor was defined as MSI-high if more than one of the five markers showed instability. Tumors were declared as MSI-low if only one marker showed instability. Microsatellite stability (MSS) is classified if no microsatellite instability was found. The results are presented in table 4.1. Four of 13 CCA samples 4 (31%) were identified as MSI-low and 9 (69%) were identified as MSI-high, none of the cases were determined as microsatellite stability (MSS). Representative electropherogram for assessment of MSI is shown in figure 4.6.

Table 4.1 Microsatellite markers analysis and microsatellite instability status

Patient code (n=13)	Microsatellite markers					MSI diagnosis
	BAT25	BAT16	D2S123	D5S346	D17S250	
R23	+	-	+	-	+	MSI-high
R26	+	-	+	-	+	MSI-high
R49	+	-	-	-	-	MSI-low
R52	+	-	-	-	-	MSI-low
R81	-	-	-	+	+	MSI-high
R100	+	-	+	+	-	MSI-high
R101	+	-	-	+	-	MSI-high
R103	-	-	-	+	-	MSI-low
R104	-	-	+	+	+	MSI-high
R118	-	-	+	+	-	MSI-high
R123	+	-	+	+	-	MSI-high
R134	-	-	-	+	-	MSI-low
R149	+	-	+	-	-	MSI-high



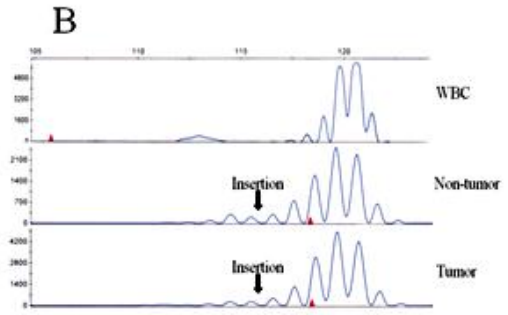


Figure 4.6 Representative electropherogram for assessment of MSI. Size in bp is shown on the x-axis at the top of the figure and the peak heights in fluorescence are shown on the y-axis on the left. The upper trace shows alleles of the WBC, middle trace shows alleles of the non-tumor tissue and the lower trace those of the corresponding tumorous tissue. (A) An example of a microsatellite stability (MSS) case. (B) An example of a MSI-insertion case. (C) An example of a MSI-deletion case. Arrow indicates abnormal allelic shifts.

4.5 Correlation of MSI with clinical-pathological features and survival of CCA patients

We compared the clinical and histological tumor characteristics of the patients with MSI-high tumors with those of patients with MSI-low tumors (Table 4.2). No differences were observed in age, sex, histological type and metastasis. In a comparison between the patients with MSI-low and patients with MSI-high, the 5-year survival rate of the MSI-low (n = 4) was 31% whereas the group with MSI-high (n = 9) was 69%, indicating the better survival rate of MSI-low as depicted in figure 4.7. However no significant difference between these two groups was found (P = 0.14 by Log rank test).

Table 4.2 Correlation between MSI in CCA tissues and clinico-pathological data

Variables	No.of patients	MSI		
		Low	High	*P* value
Age (Year)				
≤ 56	8	3(37.5%)	5(62.5%)	1.000
> 56	5	1(20%)	4(80%)	
Gender				
Female	3	0(0%)	3(100%)	0.497
Male	10	4(40%)	6(60%)	
Histological type				
Papillary	4	1(25%)	3(75%)	1.00
Non-papillary	8	3(37.5%)	5(62.5%)	
Metastasis stage				
Negative	4	0(0%)	4(100%)	0.208
Positive	8	4(40%)	4(50%)	

* Fisher's Exact probability was used in comparison of variables that had 2 categories.

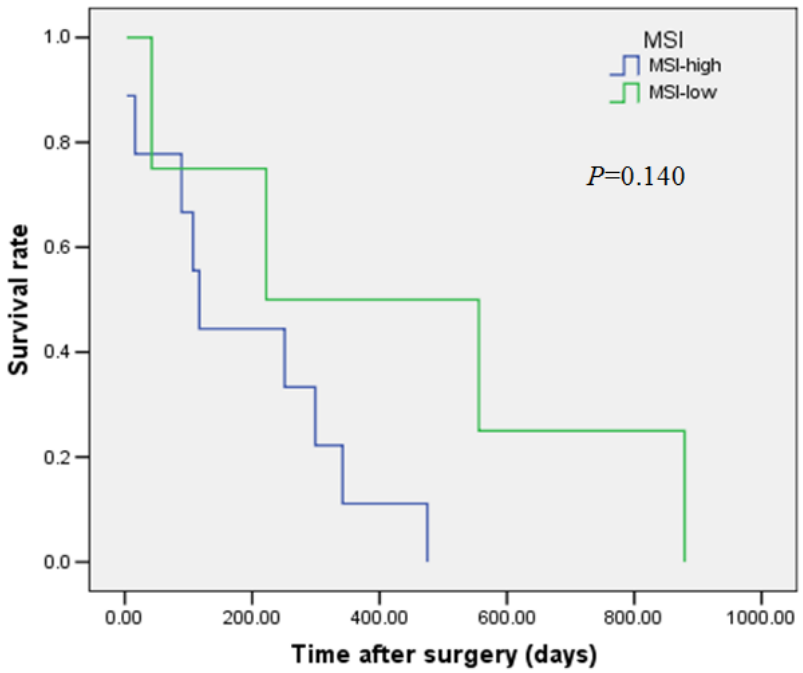


Figure 4.7 Five-year survival curve of MSI by the Kaplan-Meier method. Patients with MSI-low (—) had higher survival rates than those with MSI-high (—) CCA (*P* = 0.14, Log-Rank test).

4.6 Histopathological changes in hamster liver tissue

There was no pathological change observed in the untreated group, whereas the liver sections of NDMA administration group developed mild periductal inflammation and a low degree of bile ductule proliferation and cholangiofibrosis. In Ov-infected alone group, the inflammatory reaction in the acute phase (on day 21) of infection was predominantly eosinophilic infiltration of the portal areas, with some neutrophils and mononuclear cells. Inflammatory cells were seen around the adult worms, particularly eosinophils and mononuclear cells. The dilated bile ducts showing hyperplasia with atypical epithelia lining were found on day 21. In the chronic phase (in month 1-6), we observed. an increased mononuclear cells, decreased eosinophils and the thickness of fibrosis. In the Ov plus NDMA administration 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, tumor was observed at month 6.

4.7 Expression patterns of antioxidant enzymes in hamster liver tissues during CCA development

Immunohistochemical staining of SOD2 in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls are demonstrated in figure 4.8. Untreated control livers showed slightly staining of SOD2 in the bile duct at the beginning until month 6. Liver sections of NDMA administration group showed light staining at the beginning until month 3. At month 4 and 6, these groups were increased in intensity. Strong intensity of immunoreactivity was observed in the bile duct epithelia of Ov-infected hamsters either with or without NDMA administration at the beginning until month 6. Intensed SOD2 staining was strongly seen in hapatocyte of all groups at day 21 to month 6.

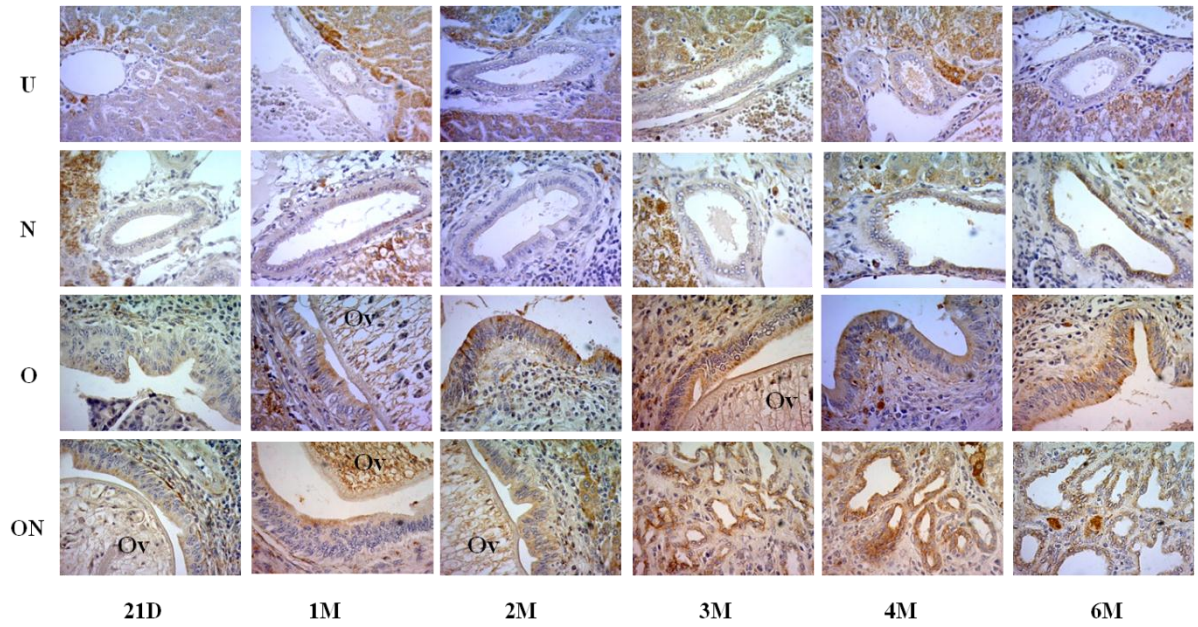


Figure 4.8 Immunohistochemical staining of SOD2 in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls. Abbrevation used: U, untreated control; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x.

Immunohistochemical staining of CAT in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls are shown in figure 4.9. Untreated control livers showed weakly staining of CAT in the bile duct at the beginning until month 6. Liver sections of NDMA administration group showed slightly staining at day 21 and month 1. The strong staining of CAT was observed at month 2 until month 6. Infection of hamster by Ov with or without NDMA administration showed strongly staining at the beginning until month 6. Intensed CAT staining was strongly seen in hepatocyte of all groups at day 21 to month 6.

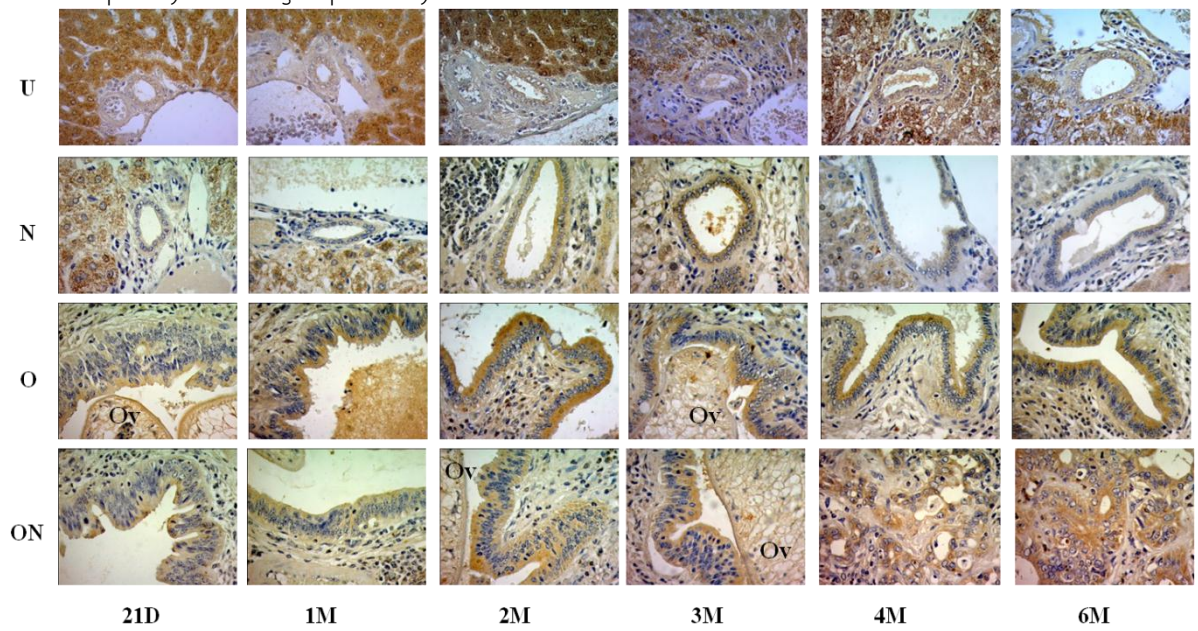


Figure 4.9 Immunohistochemical staining of CAT in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls. Abbreviation used: U, untreated control; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x.

4.8 Activities of antioxidant enzymes in hamster liver tissues during CCA development

SOD2 activities along carcinogenic pathway in CCA-inducd hamster is depicted in figure 4.10. Expression units of SOD2 activities of all experimental groups were normalized with its control of each time point. In the acute phase of Ov infection (on day 21), the SOD2 activity in Ov plus NDMA administration group was significantly higher than untreated control ($P<0.05$). At month 1 which is defined as chronic phase of Ov-infection, activity of SOD2 in Ov plus NDMA administration group had declined when compared with day 21. Nevertheless activity of SOD2 in Ov plus NDMA administration group was higher than untreated control but not statistically significant. At month 2, the SOD2 activity in the experimental group reached maximal level and was significantly higher than untreated control ($P<0.05$), whereas, SOD2 activity had decreased respectively at month 3 and 6. However, in increased SOD2 activities in Ov plus NDMA group showed statistical significance when compared to untreated control at month 2 and 3 ($P<0.05$). Notably, when tumor developed, the activity of SOD2 in CCA induced group was decreased to similar level of the untreated control.

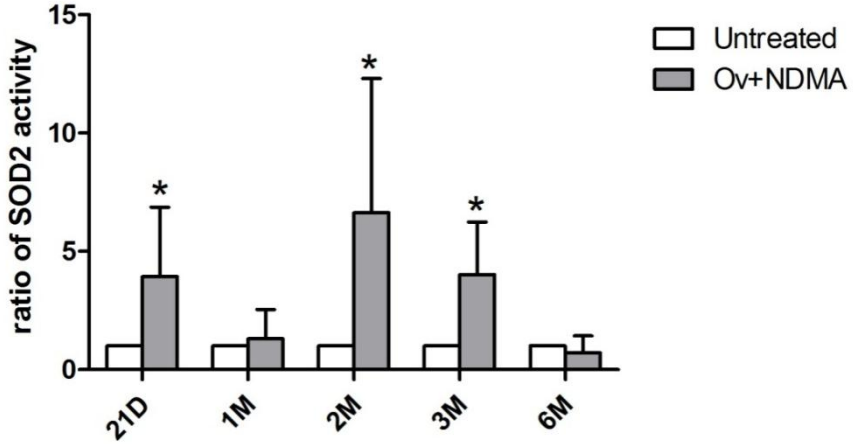


Figure 4.10 SOD2 activity (as ratio of control groups) during carcinogenesis in CCA-induced hamster. Student's t-test was used to analyze statistical significance between untreated and Ov+NDMA groups. Differences were considered statistically significant at $P<0.05$. * Denotes statistical significance when compared between untreated and Ov+NDMA groups.

CAT activities is shown in figure 4.11. Expression units of CAT activities of all experimental groups were normalized with its control of each time point. Overall activities of CAT in all experimental groups were decreased when compared to the controls but only at month 3 that statistical difference was seen, this may be due to less individual variation of this group. Similar to SOD2 activity, when tumor had developed, the activity of CAT in Ov plus NDMA group was mostly declined but no statistical significance was shown when compared to untreated control.

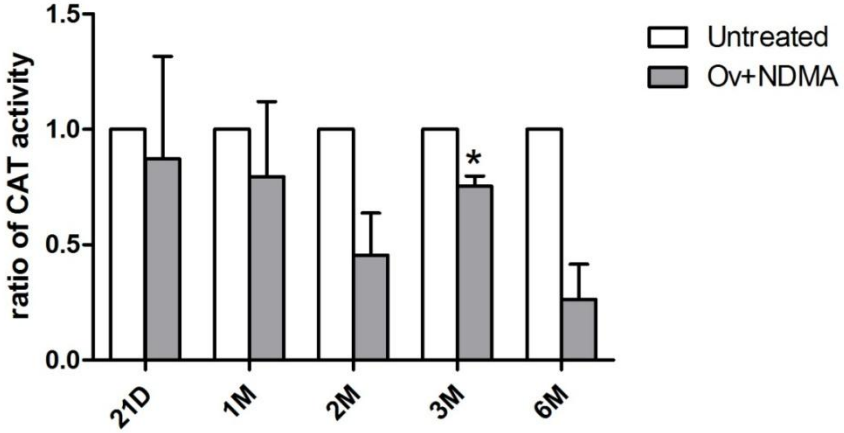


Figure 4.11 CAT activity (as ratio of control groups) during carcinogenesis in CCA-induced hamster. Student's t-test was used to analyze statistical significance between untreated and Ov+NDMA groups. Differences were considered statistically significant at $P<0.05$. * Denotes statistical significance when compared between untreated and Ov+NDMA groups.

4.9 Expression patterns of DNA repair enzymes in hamster liver tissues during CCA development

Immunohistochemical staining of APE in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls are demonstrated in figure 4.12. Untreated control liver showed weakly nuclear staining of APE in the bile duct at every time point of carcinogenesis. Liver section of NDMA administration group showed weak to negative staining of APE on day 21 until month 2. At months 3, 4 and 6 showed increased intensity of the staining over day 21,

month 1 and month 2. Liver sections of Ov-infected hamster without NDMA administration group showed weak to negative staining on day 21. Intensity of APE was increased in month 1 until month 6 of the experiment. Liver sections of Ov-infected hamster with NDMA administration group showed weak to negative staining on day 21 and month 2. Intensity of APE was increased in month 3 until month 6.

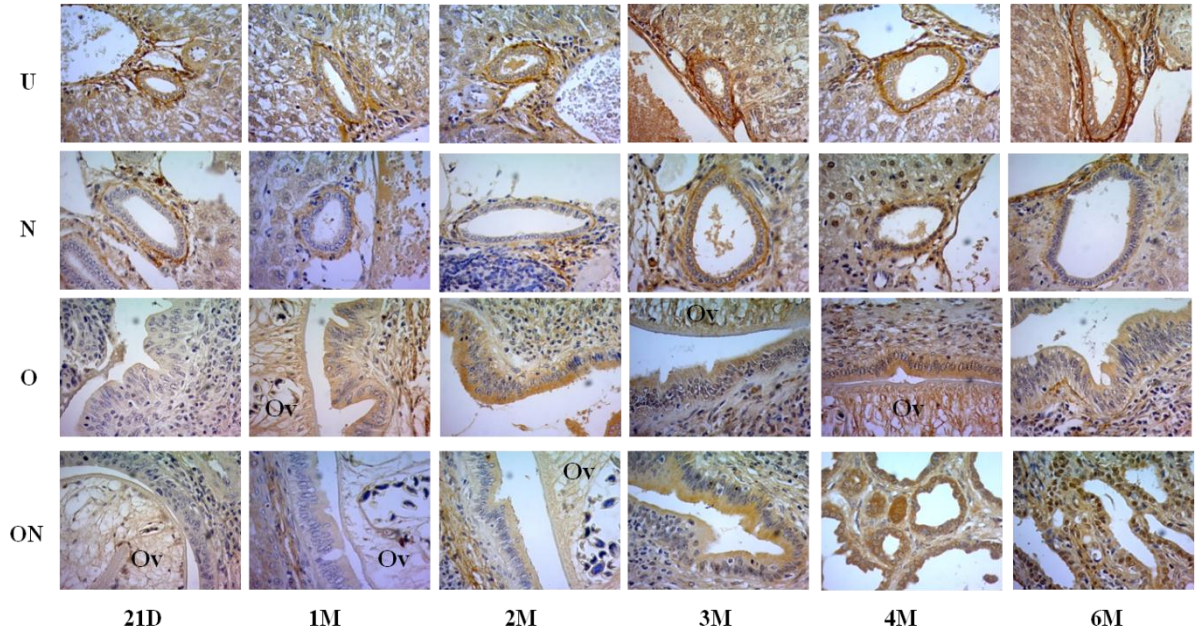


Figure 4.12 Immunohistochemical staining of APE in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls. Abbreviation used: U, untreated control; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x.

Immunohistochemical staining of DNA pol β in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls are demonstrated in figure 4.13. Untreated control liver showed weak nuclear staining of DNA pol β in the bile duct at the beginning until month 6. Liver section of NDMA administration group showed weak to negative nuclear staining of DNA pol β on day 21 until month 2, but the intensity was higher in month 3 until month 6. Liver sections of Ov-infected hamster without NDMA administration group showed weak to negative staining on day 21. Intensity of DNA pol β was increased in month 1 until month 6 of the experiment. Intensities of DNA pol β in Ov-infected hamster with NDMA administration were increased as time dependent manner from acute phase of Ov infection until tumor had developed.

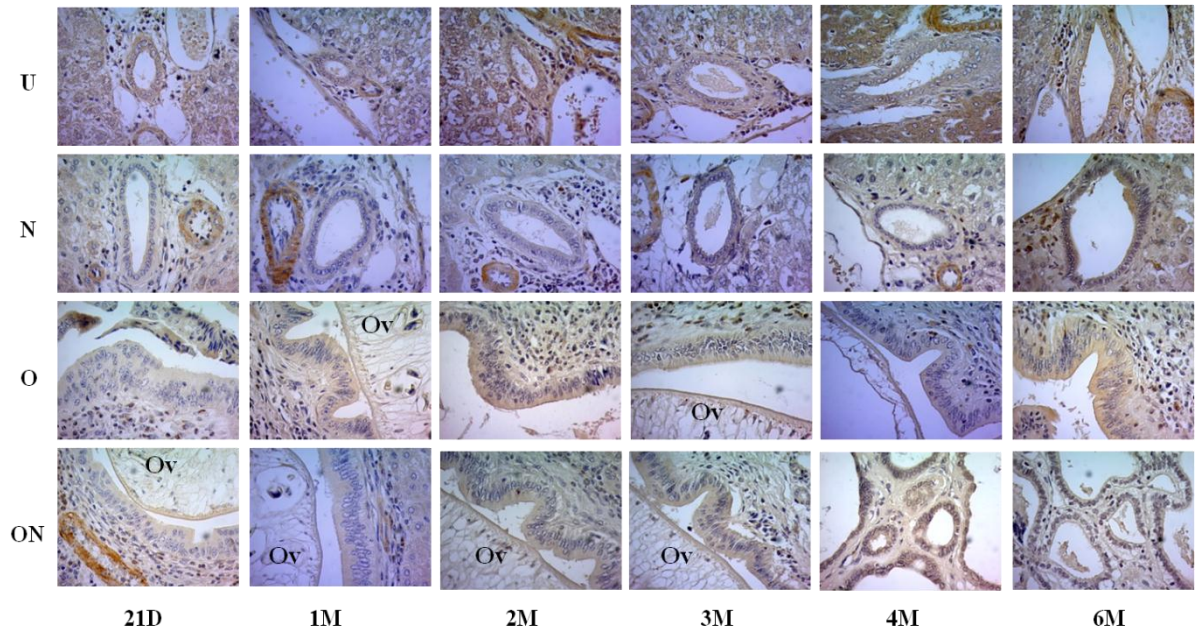


Figure 4.13 Immunohistochemical staining of DNA pol β in the liver sections of Ov-infected hamster with or without NDMA administration, NDMA administration alone and untreated controls. Abbreviation used: U, untreated control; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400.

14. สรุปและวิจารณ์ผลการทดลอง (Summary and Discussion)

5.1 Oxidative responses, DNA repairing and MSI in human CCA tissues

The impaired antioxidant system may favor accumulation of free radicals. It has been found that low levels of essential antioxidants are associated with an increased risk of cancer (Scibior et al., 2008). The results of the present study demonstrated that expression of SOD2 and CAT enzymes were mainly decreased in human CCA tissue. This result was consistent with the previous observations of prostatic (Baker et al., 1997) and bladder cancer (Jeon et al., 2007), both studies showed that the expression of SOD and CAT were significantly lower in cancer tissue than that in normal tissue. In addition, we found that activities of SOD2 and CAT in human CCA were significantly decreased when compared with cadaveric donor. There was a report that decreased activities of both SOD and CAT and increased levels of DNA lesions, probably caused by free radicals in human lung cancerous tissues (Jaruga et al., 1994). In a study performed on brocho alveolar lavage, in 11 lung cancer patients and 21 patients without lung cancer, both SOD and CAT activities were found lower in cancerous group (Tang, 1991). Parallel with these results, Guner et al (Guner et al., 1996) have also found lower total SOD and CAT activities in lung carcinoma tissue. Moreover, it was demonstrated that levels of lipid peroxidation products and nitric oxide products were significantly elevated, whereas SOD and CAT enzymes were significantly lowered in oral cavity cancer patients when compared to normal healthy subjects (Beevi et al., 2004). Induction of an oxidant-generating system and reduction of an antioxidant system are well-recognized causes of oxidative stress. Oxidant-generating enzymes such as inducible

nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) were found to be upregulated in animals with Ov-induced CCA, *in vitro* cell lines, and clinical samples of CCA (Itatsu et al., 2009; Pinlaor et al., 2006; Wu, 2005). Plasma levels of nitrate/nitrite, by-products of reactive nitrogen species, were significantly increased in Ov-infected subjects and Ov associated CCA patients in comparison with uninfected healthy subjects (Thanan et al., 2008). Moreover, inflammatory cytokines induced formation of NO and suppression of redox ratios of glutathione (GSH) and glutathione disulfide (GSSG) in cholangiocarcinoma cells (Buranrat et al., 2007; Prawan et al., 2009). Recently, our group (Thanan et al., 2012) demonstrated that Mn-SOD protein expression was significantly decreased in the cancer cells of CCA tissues in comparison to the individual normal bile duct cells located at tumor-adjacent areas. We could also show that decreased expression of CAT genes in the peripheral blood leukocytes of opisthorchiasis patients induced by Ov antigen (Yongvanit et al., 2012b). Additionally CAT were down regulated in an animal model of opisthorchaiasis-associated cholangiocarcinoma detected by a kinetic analysis using the cDNA microarray technique (Wu et al., 2011).

In agreement with other liver fluke, *Fasciola hepatica*, antioxidant potential was decreased in rat livers after post-infection (Kolodziejczyk et al., 2005). Such reduction of antioxidant systems may increase oxidative damage to all kinds of biomolecules: DNA, lipids, carbohydrates and proteins. The oxidation of lipids by excess amounts of ROS/RNS generates by-products such as trans-4-hydroxy-2- nonenal (HNE), malondialdehyde (MDA) and crotonaldehyde which can react with DNA bases to form a variety of exocyclic DNA adducts (el Ghissassi et al., 1995; Nair et al., 2007a; Singer and Hang, 1999). Reaction of DNA bases with the major LPO-product HNE yields *inter alia* the etheno-DNA adducts 1, N^6-etheno-2′-deoxyadenosine (**ε**dA) and 3, N^4-etheno-2′-deoxycytidine (**ε**dC). Recently, we found the increased formation of **ε**dA and **ε**dC in CCA tissues (Kadsanit et al., 2011). These etheno modified DNA bases are highly miscoding lesions, and are thought to contribute to the initiation of carcinogenesis through induction of specific point mutations in DNA. Therefore base excision repair is a key pathway used in the protection of free radical attack on DNA (Bohr, 2002). Our data revealed that increased expression of AAG and DNA pol **β** enzyme while APE expression was decreased.

Moreover, we found MSI-low (31%) and MSI-high (69%) in human CCA. Patients with MSI-high correlated with poor prognosis as indicated by shorter survival when compared with the group of MSI-low, although statistical significance was not obtained which may be due to the small sample size. However, Liengswangwong et al (Liengswangwong et al., 2003) reported that MSI-low or MSS in liver fluke infection-associated intrahepatic cholangiocarcinoma (ICC). Previously, it was demonstrated that generation of a strong mutator phenotype in yeast by imbalanced base excision repair (Glassner et al., 1998). The data presented a mechanism by which spontaneous mutation rates may be modulated. An imbalance between AP endonuclease and one particular DNA glycosylase produced a

striking mutator phenotype in yeast. Glycosylase to AP endonuclease ratio imbalances could arise by promoter mutations that affect DNA repair gene expression. Imbalances in base excision repair pathway enzymes may represent a hitherto unrecognized source of increased cancer risk. Furthermore, Imbalanced base excision repair increases spontaneous mutation and alkylation sensitivity in *Escherichia coli* (Posnick and Samson, 1999). Recent study in human colon adenoma showing dysregulation of BER, a striking correlation between AAG and iNOS expression was observed. Nitration *in vitro* in the AAG active site of tyrosine 162 impaired εA-excision activity, whereas nitrosation of cysteine 167 increased εA-excision. Thus posttranslational modification of AAG by reactive nitrogen species via NO overproduction appears to be one mechanism of BER dysregulation leading to malignancy (Jones et al., 2009). In ulcerative colitis, it was shown that the adaptive imbalanced in base excision repair enzymes generate MSI in chronic inflammation. Mechanistic studies using yeast and human cell models in which overexpression of AAG and/or APE1 was associated with frameshift mutations and MSI. These observations indicated that the adaptive imbalanced increase in BER enzymes may have DNA-damaging effects and contribute to carcinogenesis in chronic inflammation (Hofseth et al., 2003). Furthermore, there are evidences that showed overexpression of DNA pol β may associate MSI leading to malignancy. DNA pol β has high level of infidelity in the replicating DNA *in vitro*, due to the lack of associated proofreading activity and a poor ability to discriminate nucleotides. Probably because of these error-prone features, its expression was tightly regulated in *vivo*, being constant and low throughout the cell-cycle (Zmudzka et al., 1988). Overexpression of DNA pol β in mammalian cells has been demonstrated to significantly increased mutation rate (Canitrot et al., 1998), this could consequently induce chromosomal instability and enhance tumorigenicity of xenografts (Bergoglio et al., 2002). Besides, Yamada et al (Yamada and Farber, 2002) reported that induction of a low level of MSI by overexpression of DNA pol β. They have identified a mechanism for the induction of microsatellite mutations, using a selection-based cell culture system that is sensitive to relatively small increases in mutation rates. These results suggest that DNA pol β overexpression may affect increase genetic instability. Altogether, we demonstrated that reduction of antioxidant enzymes and adaptive imbalance of BER enzymes may cause MSI in human CCA. Moreover, it was shown that patients with MSI-low possessed longer survival than the ones with MSI high. This may increase susceptibility to tumorigenesis.

5.2 Oxidative responses and DNA repairing in hamster liver tissues during CCA development

Our earlier studies in humans revealed decreased expressions and activities of SOD2 and CAT in CCA patients. This finding suggests that reduction of SOD2 and CAT enzymes in epithelial bile duct may increase the risk of CCA development. To further elucidate the activities of antioxidant enzymes and its cellular localization during the genesis of CCA, the

hamster model of cholangiocarcinogenesis was investigated. The expression of antioxidant enzymes were analyzed by immunohistochemical with specific antibodies against SOD2 and CAT in the epithelial bile duct of liver fluke-associated CCA hamsters, while antioxidant activities were investigated by enzymatic reactions. Our results showed the strong staining of SOD2 and CAT at every time point during carcinogenesis in CCA–induced hamster. However, SOD2 and CAT activities were shown to decrease in CCA-induced hamsters when tumor developed. Both enzyme activities were consistent with our data in human CCA. This result indicates that in spite of high expressions of SOD2 and CAT in CCA-induced hamster when tumor was developed but impaired activities.

Dechakhamphu and coworkers have previously reported the accumulation of miscoding etheno-DNA adducts and highly expressed DNA repair during liver fluke-induced cholangiocarcinogenesis in hamsters (Dechakhamphu et al., 2010a). Of particular interest, it was observed that a repair protein (AAG) eliminating promutagenic etheno-DNA adduct (**ε**dA) could play a crucial role in maintaining the stability of genetic information, insufficiency of such protein may associate with susceptibility to carcinogenesis in OV-infected hamsters. They hypothesized that BER imbalances might predispose to cancer. Therefore, we investigated expression patterns of other BER enzymes by immunohistochemistry during carcinogenesis in CCA-induced hamster. Our results revealed that APE and DNA pol **β** revealed increased expression as a time dependent manner from acute phase of CCA-induced hamster until tumor developed. This result was consistent with the previous observation of Hofseth et al (Hofseth et al., 2003), who showed that ulcerative colitis (UC) patients have increased AAG and APE1 enzyme activities in epithelial areas of their colon undergoing active inflammation and overexpression of the AAG and APE1/ APN1 enzymes was associated with frameshift mutations in *S. cerevisiae* and MSI in human cells. More errors may create while the rate of repair is high, especially the high activity of DNA pol **β**, this is one of the explanation. These observations indicate that the adaptive imbalanced increase in BER enzymes may have DNA-damaging effects and contribute to carcinogenesis in chronic inflammation. Moreover, overexpression of DNA pol **β** was deleterious effects. Albertella et al (Albertella et al., 2005) reported that the major BER DNA polymerase, DNA pol **β**, was overexpressed at the mRNA and protein level in approximately one-third of all tumours sampled and DNA pol **β** was most frequently overexpressed in uterus, ovary, prostate and stomach cancer samples. Frequent mutation related with overexpression of DNA pol **β** in primary tumors and precancerous lesions of human stomach (Tan et al., 2005).

Taken together our results in hamster liver tissues during CCA suggest that reduction of antioxidant enzymes in CCA-induced hamsters when tumor had developed and adaptive increase in BER enzymes as a time dependent manner from acute phase of CCA-induced hamster until tumor develop may contribute to DNA translesion-mediated cholangiocarcinoma in liver fluke-infected hamsters.

15. เอกสารอ้างอิง

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16. ภาคผนวก

16.1 การนำเสนอผลงานทางวิชาการจำนวน 2 ครั้ง

16.1.1 Sasithorn Kadsanit *et al*. Imbalanced adaptive responses in liver fluke associated cholangiocarcinogenesis. The 3rd Biochemistry and Molecular Biology (BMB) Conference: From Basic to Translational Research for a Better Life. Chiang Mai University, Chiang Mai, Thailand 2011. ***(Poster presentation)*** April 6-8.

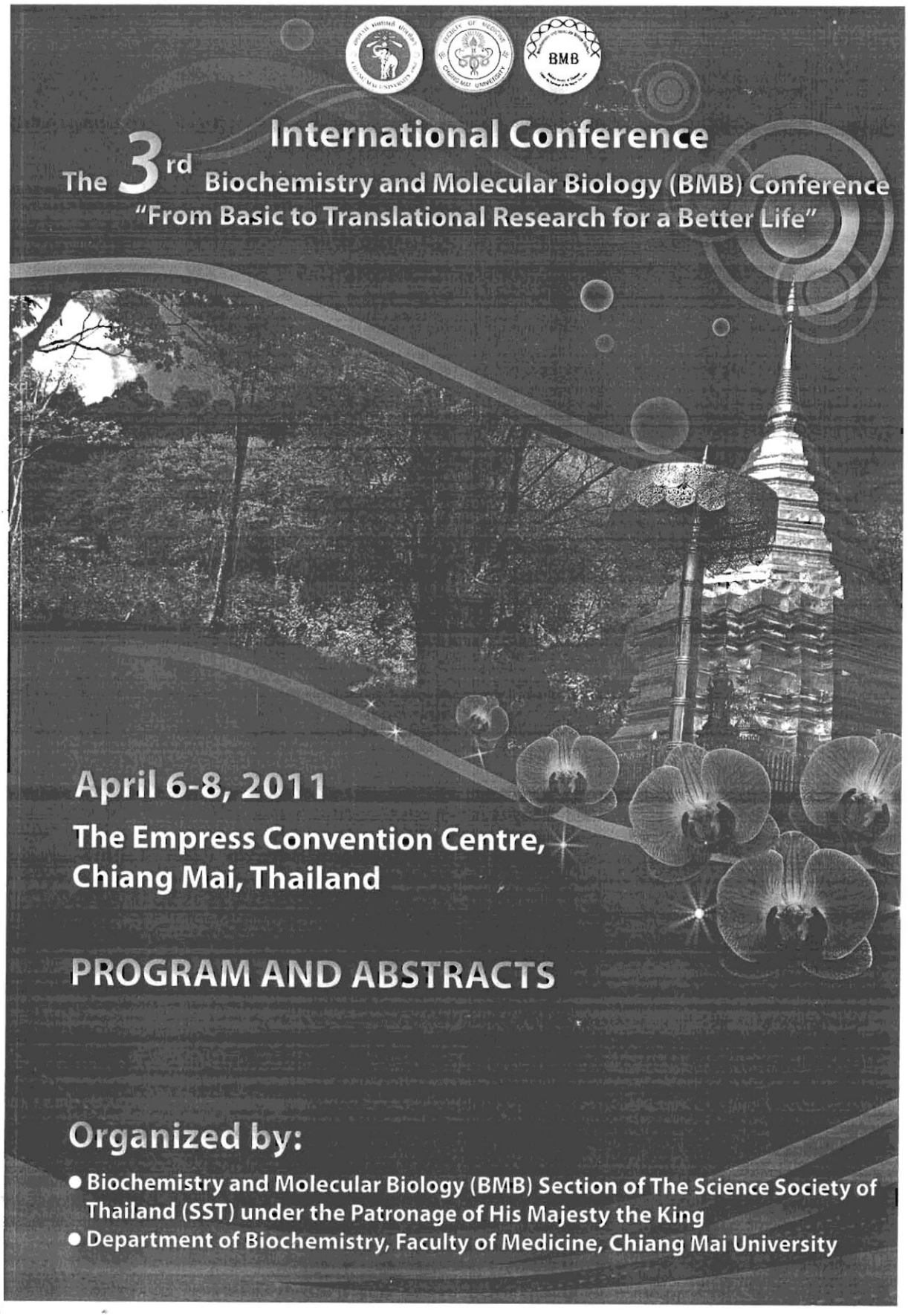
16.2.2 Sasithorn Kadsanit *et al*. Etheno DNA Adducts in Cholangiocarcinoma. Annual Meeting of Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand 2011. ***(Poster presentation)*** October 4-6.

16.2 ผลงานตีพิมพ์

16.2.1 ศศิธร คาดสนิท, ชฎามาศ พินิจสนุทร, วัชรินทร์ ลอยลม, พวงรัตน์ ยงวณิชย์, ผลเสียต่อเซลล์เมื่อมีการปรับตัวที่ไม่สมดุลต่อภาวะเครียดที่เกิดกับยีนภายหลังการติดเชื้อและการอักเสบ. Srinagarind Med J 2011: 26(2).

16.2.2 Sasithorn Kadsanit, Somkid Dechakhamphu, Watcharin Loilome, Anucha Puapairoj, Nisana Namwat and Puangrat Yongvanit. Etheno DNA Adducts In Cholangiocarcinoma. Srinagarind Med J 2011; 26, 199-201.

ภาคผนวก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P5-01
Imbalanced Adaptive Responses in Liver Fluke Associated Cholangiocarcinogenesis

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An adaptive response of the genome-protection mechanism occurs in cells when exposed to genotoxic stress due to overproduction of free radicals via inflammatory process and infection. Such circumstances, cells attempt to keep them healthy by many genome-protected machines. However, this adaptive response may have deleterious effects, i.e. if imbalance of the antioxidant or DNA repair enzyme system occurs, it will generate microsatellite instability (MSI) and has procarcinogenic implication. This study aims to determine the expression patterns of antioxidant enzymes: catalase and superoxide dismutase-2 (SOD-2) and DNA repair enzymes: alkyladenine DNA glycosylase (AAG), apurinic endonuclease (APE) and DNA polymerase beta (DNApol-β) in 29 cases of human cholangiocarcinoma (CCA) tissues by using immunohistochemistry. Our data demonstrated that SOD-2 level with low, moderate and high expression were 38%, 41% and 21% respectively, while catalase expression with low, moderate and high expression were 35%, 41% and 24% respectively. However, 16 from 29 cases or 55% showed an inverse expression of both enzymes in the same patient. Those particular cases were supposed to be an adaptive imbalance of SOD-2 and catalase. Among expression levels of DNA repairing enzymes, we found that low, moderate and high expressions of AAG were 48%, 31% and 21% respectively; APE expressions were 48%, 24% and 28% respectively, and DNApol-β expressions were 21%, 38% and 41% respectively. High expression of AAG and APE has been previously shown to cause MSI, which is planned to perform among these cases. This finding may help to explain pro-carcinogenic effect of CCA caused by liver fluke infection.

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Keywords: antioxidant enzymes, DNA repair enzymes, cholangiocarcinoma


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Etheno DNA Adducts in Cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is a bile duct cancer, which is still a serious health problem in the northeast of Thailand and increasing worldwide[1]. Liver fluke (*Opisthorchis viverrini*) infection is the main risk factor of CCA in this region, consequently causing chronic inflammation around bile ducts where liver flukes reside. Persistent cellular oxidative/nitrative stress and enhanced lipid peroxidation (LPO) leading to macromolecular damage and disruption of signaling pathways, which are implicated in the development of human malignancies associated with chronic inflammatory processes[2,3]. The oxidation of lipids by reactive oxygen and nitrogen species results in by products such as *trans*-4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and crotonaldehyde. These intermediates react with DNA bases and form exocyclic DNA adducts[4]. Reaction of DNA bases with the principal LPO end-product HNE yields *inter alia* the etheno-DNA adduct 1, N^6-etheno-2'-deoxyadenosine (εdA) and 3,N^4-etheno-2'-deoxycytidine (εdC). These etheno-modified DNA bases are highly miscoding lesions; they are thought to initiate the carcinogenic process through specific point mutations. The high frequency of mutations and the deregulation of cell homeostasis could play a pivotal role in the pathogenesis of human chronic diseases[5]. Our previous study reported that high εdA and εdC excretion were found in urine of opisthorchiasis[6]. Levels of etheno DNA adduct were also found in white blood cell of liver fluke infected patients, which were decreased to control level after 2 months of praziquantel treatment[6,7]. Therefore this study aimed to confirm whether εdA and εdC formations are persistently occurred until CCA development. CCA tissues obtained from patients were investigated by using immunohistochemical technique.

Materials and Methods

Tissue samples

CCA tissues were collected from cholangiocarcinoma patients admitted at surgical ward of Srinagarind Hospital, Khon Kaen University. The protocol of collection and study were approved by the Ethic Committee for Human Research, Khon Kaen University (HE 480316). Immonohistochemistry

LPO-derived DNA adduct formations were localized in paraffin-embedded section by immunohistochemistry as described by Yang et al[8] with major modifications[9]. In brief, paraffin tissue sections were deparaffinized in xylene for 7 min (two times) to remove the paraffin wax. After deparaffinization, slides were hydrated by decreasing concentrations of ethanol as following steps; absolute alcohol for 5 min (two times), 90% alcohol for 5 min (two times), 80% alcohol for 5 min (two times), 70% alcohol for 5 min (two times) and finally rinsed in the tap water. Antigen was retrieved in 100 mM sodium citrate buffer (pH6.0) in a microwave for 15 min. Endogenous peroxidase activity was quenched, and histone and non-histone proteins from DNA were removed from slides with liver sections by treatment with proteinase K (10 ug/ml, at RT for 10 min) and endogenous RNA were removed by RNase A treatment (100 ug/ml, at 37 °C, for 1 hour). Non-specific binding

บทฟื้นฟูวิชาการ • Review Article

ผลเสียต่อเซลล์เมื่อมีการปรับตัวที่ไม่สมดุลต่อภาวะเครียดที่เกิดกับยีน ภายหลังการติดเชื้อและการอักเสบ

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ภาควิชาชีวเคมี และศูนย์วิจัยพยาธิใบไม้ตับและมะเร็งท่อน้ำดี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

Adverse Effects Caused by Adaptive Imbalance to Genotoxic Stress After Infection and Inflammation

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มะเร็งเป็นผลที่เกิดตามมาจากความเสียหายต่อดีเอ็นเอหรือการกลายพันธุ์ของยีนซึ่งมีสาเหตุได้มากมาย เช่น การติดเชื้อ การอักเสบ การสัมผัสรังสีหรือสารก่อมะเร็ง เป็นต้น ดังนั้นเพื่อป้องกันไม่ให้เกิดความผิดปกติหรือการกลายพันธุ์ เซลล์จึงมีกลไกการปรับตัวเพื่อตอบสนองต่อภาวะเครียดของยีนโดยการกระตุ้นกลไกการซ่อมแซมที่เหมาะสม แต่หากการซ่อมแซมนั้นไม่สำเร็จ ก็จะมีการเหนี่ยวนำให้เกิดการตายของเซลล์ กลไกปกป้องเซลล์จากอันตรายนี้ประกอบด้วย กลไกการกำจัดสารแปลกปลอม การต้านอนุมูลอิสระ การหยุดวงจรชีวิตของเซลล์ ระบบการซ่อมแซมดีเอ็นเอ และการตายของเซลล์แบบอะพอพโทซิส อย่างไรก็ตามได้มีหลักฐานบ่งบอกว่าการปรับตัวที่ไม่สมดุลต่อภาวะเครียดที่เกิดกับยีนอาจมีผลเสียต่อเซลล์และยิ่งเร่งให้เกิดเป็นมะเร็งได้เร็วขึ้น กล่าวคือ เมื่อเซลล์อยู่ในภาวะเครียดที่ส่งผลถึงความผิดปกติของยีนแบบไม่ต่อเนื่อง จะมีการกระตุ้นการสร้างโปรตีนและเอนไซม์ในการปรับตัวของเซลล์แบบขึ้นๆ ลงๆ ไม่ต่อเนื่องด้วยเช่นกัน ทำให้เซลล์เสียหายมากกว่าการอยู่ในภาวะเครียดของยีนแบบต่อเนื่องเรื้อรัง ดังตัวอย่างจากการศึกษาทางระบาดวิทยาพบว่าปัจจัยเสี่ยงในการเกิดมะเร็งผิวหนังเพิ่มมากขึ้นเมื่อได้รับแสงแดดอย่างไม่สม่ำเสมอหลายครั้งต่อปี นอกจากนี้ข้อมูลผลการศึกษารูปแบบในการแสดงออกของยีนที่ตอบสนองต่อภาวะเครียดในหนูที่สัมผัสควันบุหรี่พบว่า มีการแสดงออกของกลุ่มยีนดังกล่าวเพิ่มขึ้น และจะกลับสู่ภาวะปกติเมื่อหยุดให้

Cancer is a consequence of genomic damage and mutation, which can arise from various causative agents including infection, inflammation and exposure to genotoxic agents, i.e. ultraviolet light, carcinogens. To protect the genome against the abnormality or mutation, cells have evolved genotoxic stress responses to activate an appropriate repair pathway, or, in the case of irreparable, apoptosis would be induced. The defense mechanism consists of a set of different pathways that are xenobiotic mechanism, antioxidant process, cell cycle arrest, DNA repair system, and apoptosis. Nevertheless, numbers of evidence show that these responses may have adverse effects arising from the imbalanced action of the above-mentioned pathways which, in turn, may accelerate cancer development. It has been hypothesized that if the stress is not consistent with intermittent lapses in genotoxic stress, there will be the down-regulation of these adaptive proteins and enzymes, possibly leading to a greater amount of cellular damage than with chronic exposure. This hypothesis is supported by the epidemiological finding of significantly increased risk of skin cancer with multiple sunburns. Another supported data is the cigarette smoke in experimental animals causing an adaptive increase in gene expression patterns which return to normal when the exposure is interrupted. These findings suggested that the imbalance of adaptive responses to

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