

RESEARCH ARTICLE

Impaired Antioxidant Enzyme Activity and Increased DNA Repair Enzyme Expression in Hamster Liver Tissues Related to Cholangiocarcinoma Development

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Abstract

A possible mechanism of liver fluke (*Opisthorchis viverrini*; Ov)-associated cholangiocarcinoma (CCA) genesis may be imbalance in responses of antioxidant enzymes and/or DNA repair enzymes which are the consequence of oxidative/nitrative stress, arising from inflammatory processes. This study aimed to investigate changes in the expression patterns of antioxidant enzymes, including superoxide dismutase 2 (SOD2) and catalase (CAT), as well as their activities in Ov-associated hamster CCA tissues. Expression of DNA repair enzymes including apurinic endonuclease (APE) and DNA polymerase beta (DNA pol β) was also investigated. Our results showed that SOD2 and CAT levels were increased in CCA-induced liver hamster tissues at every time point during cholangiocarcinogenesis. However, once tumors were well established, activities of both enzymes were significantly decreased. Expression of APE and DNA pol β was increased in the acute phase of Ov infection and this persisted until tumors developed. These findings suggest that a reduction in antioxidant enzymes and an increase in DNA repair enzymes may contribute to DNA translesion-mediated CCA in liver fluke-associated cholangiocarcinogenesis in the hamster model.

Keywords: *Opisthorchis viverrini* - cholangiocarcinoma - antioxidant enzymes - DNA repair enzymes

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Introduction

Cholangiocarcinoma (CCA), a malignant tumor of bile duct epithelial cells, is one of the most common cancers and a significant public health problem in the northeast Thailand. Epidemiological and experimental evidences have shown that liver fluke (*Opisthorchis viverrini*; Ov) infection is the etiology of this CCA development. The mechanism that governs the pathogenic effects of inflammation and immunity in liver fluke-associated cholangiocarcinogenesis has been recently reviewed (Yongvanit et al., 2012). It has been postulated that chronic inflammation induced by repeated infection with Ov is related to CCA development (Pinlaor et al., 2004a). 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. This subsequently causes nitrative and oxidative damage to nucleic acids. In addition, nitric oxide (NO) may be involved in carcinogenesis through heme oxygenase-1 (HO-1)-derived iron accumulation,

which may participate in the enhancement of oxidative DNA damage in epithelium of small bile ducts (Pinlaor et al., 2004b). Moreover, inflammatory cell infiltration triggered by repeated infections occurred earlier than a single infection and was associated with altered liver enzymes and severity of periductal fibrosis (Pinlaor et al., 2004c). Notably, it has been reported that humans infected with Ov had the accumulation of urinary 8-oxo-7, 8-dihydro-2'-deoxyguanosine which was positively associated with plasma nitrate/nitrites and AST levels, indicating that Ov infection can induce oxidative and nitrative stress via reactive oxygen and reactive nitrogen species produced during liver injury in humans (Thanan et al., 2008). In addition, lipid peroxidation-mediated DNA adducts has been reported in both hamsters and humans (Dechakhamphu et al., 2008; 2010). 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

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the inflammatory process and infection. In such circumstances, cells attempt to keep healthy by many genome-protection machineries. However, there is increasing evidence that this adaptive response may have deleterious effects, for instance, if an imbalance of the antioxidant process and/or DNA repair enzyme systems occurs, it will generate microsatellite instability (MSI), which has procarcinogenic implications. An hypothesis was formulated by Hofseth and Policastro (Hofseth et al., 2003; Policastro et al., 2004) that the imbalance of antioxidant enzymes in tumor cells as well as the adaptive imbalance in the base excision-repair enzymes in chronic inflammatory condition could be a relevant event in the carcinogenic process. Therefore, we sought to determine whether Ov-associated cholangiocarcinogenesis is involved in an adaptive imbalance of antioxidant and DNA repairing enzymes that occurs due to the oxidative/nitrative stress arising from inflammatory responses. This work is therefore aimed to identify the expression patterns and activities of antioxidant enzymes including catalase and superoxide dismutase-2 (SOD-2) as well as DNA repairing enzymes including apurinic endonuclease (APE) and DNA polymerase beta (DNA pol- β) in liver fluke infection induced in hamster CCA tissues.

Materials and Methods

Hamster cholangiocarcinoma tissues

Hamster CCA induction was prepared as previously described (Dechakhamphu et al., 2010) 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 (Ethical Clearance No. 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 into hamsters by intragastric intubations, while oral administration of NDMA in the drinking water (12.5 ppm) was provided *ad libitum* for 2 months. Five of animals from each group 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.

Immunohistochemical staining of antioxidant and DNA repair enzymes

An immunohistochemical method was performed to determine the expression patterns of antioxidant enzymes and DNA repair enzymes in human and hamster CCA tissues. In brief, sections of liver tissues were deparaffinized and rehydrated with stepwise decreasing concentrations of ethanol. Antigen retrieval was performed by microwave treating in 10 mM citrate buffer pH 6.0 at high power for 10 min then the sections were immersed for 20 min in 3% (v/v) hydrogen peroxide in PBS for endogenous hydrogen peroxide activity blocking and nonspecific binding was blocked by skim milk in PBS for 30 min. Sections were incubated with the primary antibody

for designated proteins which are Rabbit anti-SOD2 polyclonal antibody (1:200, Millipore, Billerica, MA), Rabbit anti-CAT polyclonal antibody (1:1000, Abcam, Cambridge, MA), Mouse anti-APE1 monoclonal antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and Rabbit anti-DNA polymerase beta polyclonal antibody (1:50, Abcam, Cambridge, MA) in the moisture chamber 1 h 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 concentrations of ethanol, cleared with xylene and mounted with permount. The stained sections were reviewed under a microscope.

Determination of SOD2 and CAT activities

For the SOD activity assay, frozen tissues of hamster CCA were washed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots. Then the tissues were 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 \times g for 5 min at 4°C. The supernatant was removed and retained for total SOD activity assays (cytosolic and mitochondrial activities). To separate the two compartments of enzymes were centrifuged at 10,000 \times g for 15 min at 4°C, and the supernatant contained cytosolic SOD while the pellet contained mitochondrial SOD. The mitochondrial pellet was homogenized in HEPES buffer and was further used for determination of mitochondrial SOD2 activity. For the CAT activity assay, frozen tissues of hamster CCA were washed with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots. Then the tissues were homogenized in 1 ml of 50 mM potassium phosphate (K_3PO_4), pH 7.0, containing 1 mM EDTA. The homogenates were then centrifuged at 10,000 \times g for 15 min at 4°C. The supernatants were used to assay for CAT activity. SOD and CAT assay kits were purchased from Cayman Chemical (Ann Arbor, MI). The principle of the SOD assay kit is to utilize a tetrazolium salt for detecting superoxide radicals, which are generated by xanthine oxidase and hypoxanthine. The absorbance was measured at 440-460 nm on an ELISA reader (TECAN, Männedorf, Switzerland). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The CAT assay kit utilizes the peroxidative function of the 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 . Formaldehyde produced is measured spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole as a chromogen. The absorbance was recorded at 540 nm using an ELISA reader (TECAN, Männedorf, Switzerland).

Statistical analysis

The student's *t*-test was used to analyze the statistical

significance between untreated and treated groups. A P -value <0.05 was considered as a statistically significant.

Results

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

The 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 1. Untreated control livers showed weak positive staining of SOD2 in the bile duct from the beginning until month 6. Liver sections of the NDMA administration group showed light staining from the beginning until month 3. At months 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 from the beginning until month 6. Intense SOD2 staining was seen on hepatocytes in all groups at day 21 to month 6.

Likewise, the untreated control group showed weak staining of CAT in the bile duct from the beginning until

month 6. Liver sections of the NDMA administration group showed slight staining at day 21 and month 1, while the strong staining of CAT was observed from month 2-6. Infection of hamsters by Ov with or without NDMA administration showed strong staining from the beginning until month 6. Intense CAT staining was seen in hepatocytes in all groups at day 21 to month 6 (Figure 2).

Activities of antioxidant enzymes in hamster liver tissues during CCA development

SOD2 activities along with the carcinogenic pathway in CCA-induced hamsters is depicted in Figure 3A. Expression units of SOD2 activities of all experimental groups were normalized with the control group at each time point. In the acute phase of Ov infection (on day 21), the SOD2 activity in the Ov plus NDMA administration group was significantly higher than the untreated controls ($P < 0.05$). At month 1 which is defined as chronic phase of Ov-infection, the activity of SOD2 in the Ov plus NDMA administration group had declined when compared with day 21. Nevertheless activity of SOD2 in the Ov plus NDMA administration group was higher

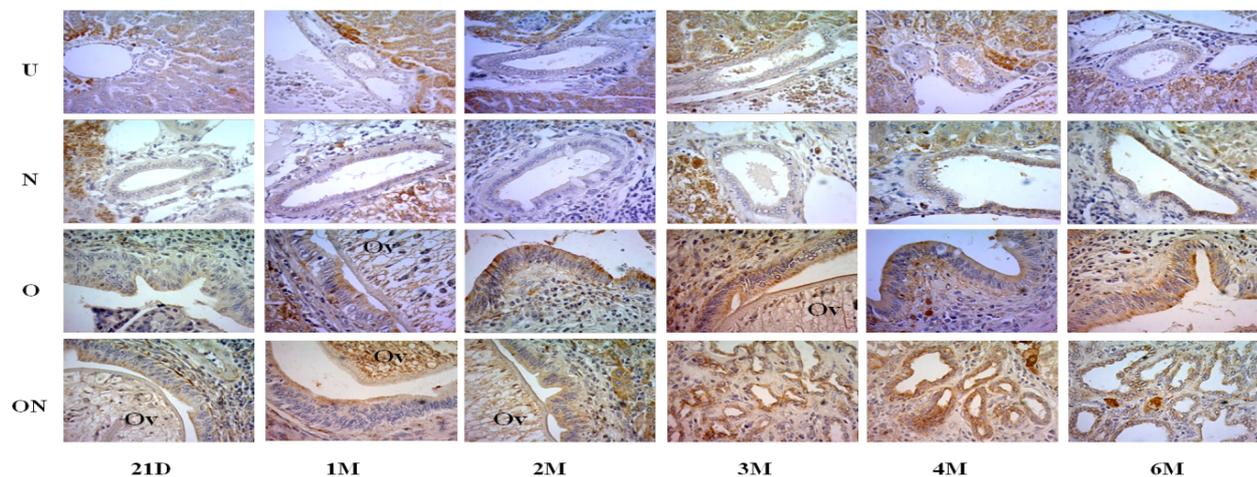


Figure 1. Immunohistochemical Staining of SOD2 in the Liver Sections of Ov-Infected Hamsters with or without NDMA Administration, NDMA Administration Alone and Untreated Controls. U: untreated controls; N: NDMA administration; O: Ov infection; ON: Ov infection with NDMA administration. The original magnification was 400x

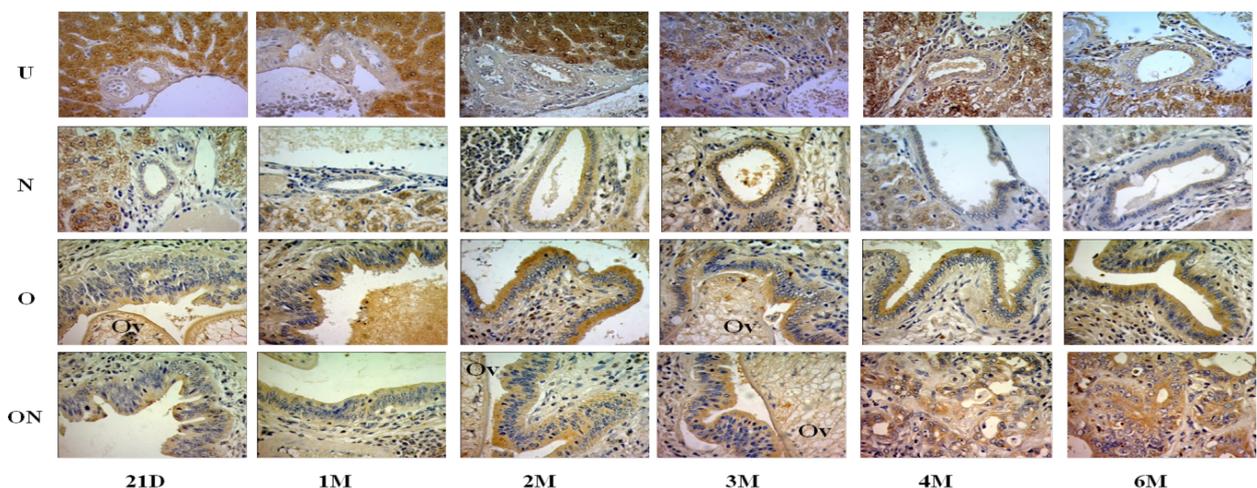


Figure 2. CAT Staining in Liver Sections of Ov-Infected Hamsters with or without NDMA Administration, NDMA Administration Alone and Untreated Controls. U, untreated controls; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x

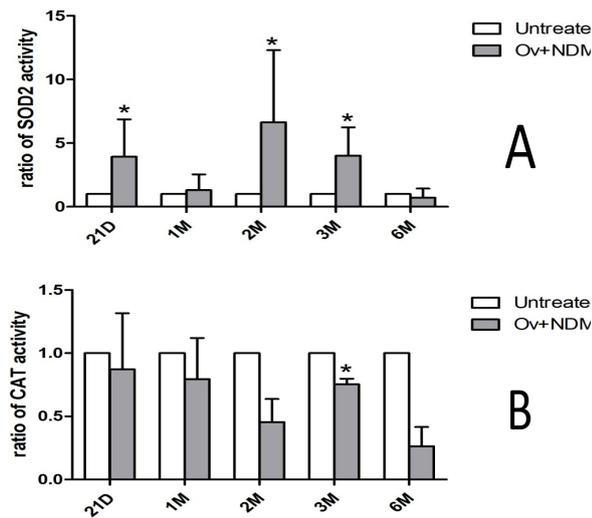


Figure 3. (A) SOD2 and (B) CAT Activity During Carcinogenesis in CCA-Induced Hamsters. The Student's *t*-test was used to analyze the statistical significance between untreated and Ov+NDMA groups. Differences were considered statistically significant at $P < 0.05$. *Denotes statistical significance when compared between untreated and Ov+NDMA groups

than the untreated control group but the activity was not statistically significant. At month 2, the SOD2 activity in the experimental group reached the maximal level and was significantly higher than the untreated control ($P < 0.05$), whereas, SOD2 activity had decreased at months 3 and 6. However, the Ov plus NDMA group showed a statistically significant increase in SOD2 activities when compared to the untreated control group at months 2 and 3 ($P < 0.05$). Notably, when tumor developed, the activity of SOD2 in the CCA induced group was decreased.

Overall activities of CAT in all experimental groups were decreased when compared to the controls but only at month 3 was a statistical difference seen. This may be due to the less individual variation of this group (Figure 3B). Similar to SOD2 activity, when tumors had developed, the activity of CAT in Ov-infection plus NDMA group was mostly declined but no statistical significance was shown when compared to untreated controls.

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

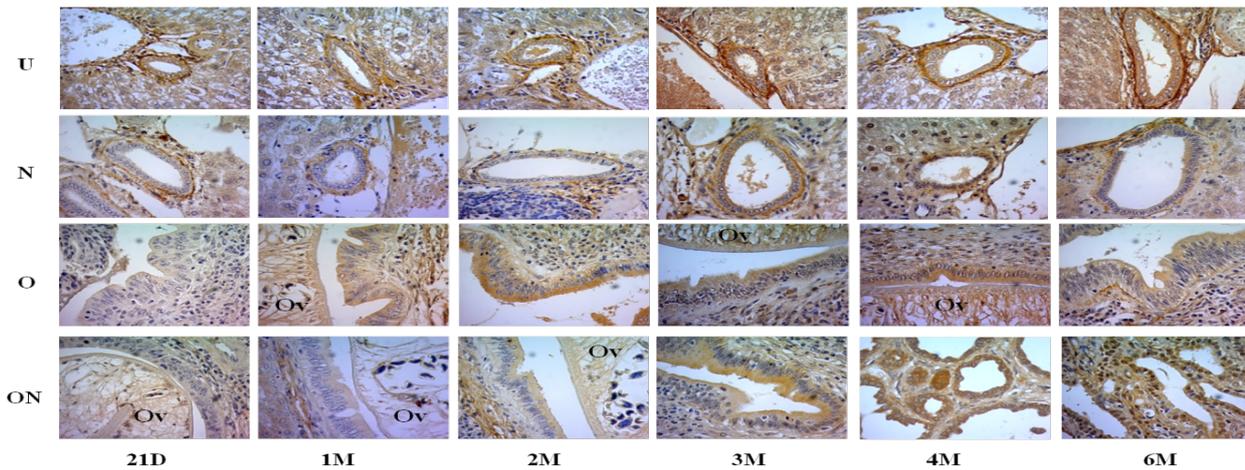


Figure 4. Expression of APE in the Liver Sections of Ov-Infected Hamsters with or without NDMA Administration, NDMA Administration Alone and Untreated Controls. U, untreated controls; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x

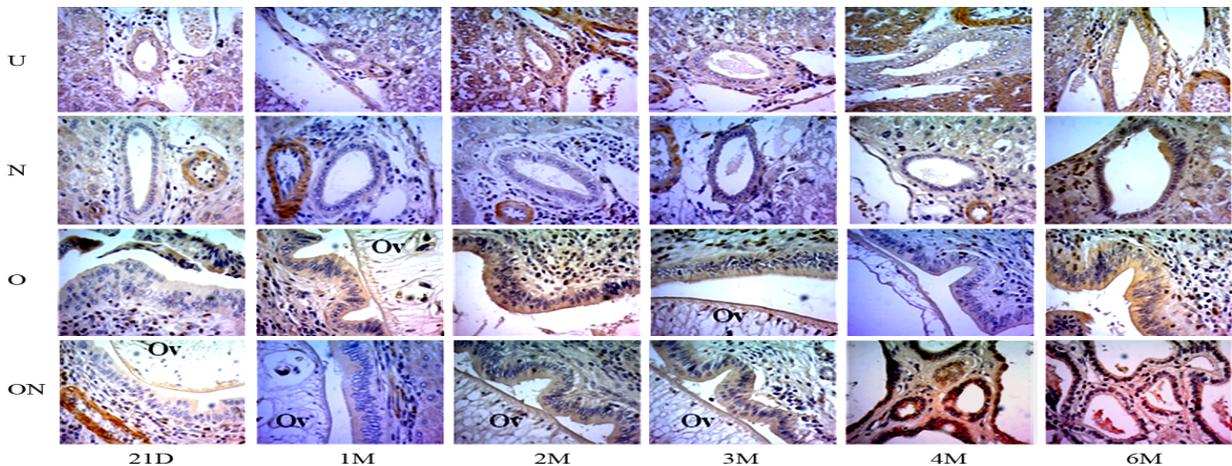


Figure 5. Expression of DNA pol β in the Liver Sections of Ov-Infected Hamsters with or without NDMA Administration, NDMA Administration Alone and Untreated Controls. U, untreated controls; N, NDMA administration; O, Ov infection; ON, Ov infection with NDMA administration. The original magnification was 400x

controls are shown in Figure 4. The untreated control livers showed weak nuclear staining of APE in the bile duct at every time point of carcinogenesis. Liver sections of the NDMA administration group showed weak to negative staining of APE on day 21 until month 2. At months 3, 4 and 6 the intensity of the staining increased over day 21, month 1 and month 2. Liver sections of Ov-infected hamsters in the without NDMA administration group showed weak to negative staining on day 21. The intensity of APE was increased in month 1 and continued through month 6 of the experiment. In Ov-infected hamsters with the NDMA administration group showed weak to negative staining for day 21 and month 2. Intensity of APE was increased in month 3 through month 6.

In addition, expression of DNA pol β in the hamster liver sections showed the similar pattern as in APE. The untreated control livers showed weak nuclear staining of DNA pol β in the bile duct at the beginning through month 6. Liver sections of the NDMA administration group showed weak to negative nuclear staining of DNA pol β on day 21 through month 4, but the intensity was higher in month 6. Liver sections of Ov-infected hamsters in the without NDMA administration group showed weak to negative staining on day 21. Intensity of DNA pol β was increased in month 1 through month 6 of the experiment. Intensities of DNA pol β in Ov-infected hamsters with NDMA administration were increased in a time dependent manner from the acute phase of Ov infection until tumor had developed.

Discussion

In the present study, the expression of antioxidant enzymes was analyzed by the immunohistochemical staining method with specific antibodies against SOD2 and CAT in the epithelial bile ducts 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 hamsters. SOD activities seemed to be increased at day 21, months 2 and 3, although decreased activity was observed at month 6 when the tumor has fully developed. CAT activities were shown to decrease in every time point of CCA-induced hamsters. These results indicate that in spite of high expressions of SOD2 and CAT in CCA-induced hamsters, when tumors were developed activities of these enzymes were impaired.

Dechakhamphu and co-workers 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., 2010). Of particular interest, it was observed that a repair protein (AAG) eliminating promutagenic etheno-DNA adducts (ϵ dA) could play a crucial role in maintaining the stability of genetic information; insufficiency of such a protein may be associated with susceptibility to carcinogenesis in OV-infected hamsters. They hypothesized that base excision repair (BER) imbalances might predispose to cancer. Therefore, we investigated expression patterns of other BER enzymes by immunohistochemistry during

carcinogenesis in CCA-induced hamsters. Our results revealed that APE and DNA pol β were increasingly expressed in a time dependent manner from the acute phase of CCA-induced hamsters until the tumors 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 the epithelial areas of their colon undergoing active inflammation and the overexpression of the AAG and APE1/ APN1 enzymes overexpressed with frameshift mutations in the *S. cerevisiae* and MSI in human cells. More errors may occur when the rate of repair is high, especially the high activity of DNA pol β . 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 has deleterious effects. Albertella et al. (Albertella et al., 2005) reported that the major BER DNA polymerase, DNA pol β , was overexpressed at both mRNA and protein levels in approximately one-third of all tumors sampled and DNA pol β was most frequently overexpressed in uterus, ovary, prostate and stomach cancer samples. Frequent mutation is 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 a reduction of antioxidant enzymes in CCA-induced hamsters when tumors had developed and adaptive increase in BER enzymes in a time dependent manner from the acute phase of CCA-induced hamsters by tumor development may contribute to DNA translation-mediated cholangiocarcinoma in the liver fluke-infected hamster model. This can help to explain the role of adaptive imbalances in response to chronic Ov infection that led to cholangiocarcinogenesis in human.

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