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RESEARCH

Tumor-Related Gene Changes in Immunosuppressive Syrian Hamster Cholangiocarcinoma

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Abstract The results of a previous study demonstrated that prednisolone enhanced cholangiocarcinogenesis. Therefore, to clarify molecular changes during immunosuppressive cholangiocarcinogenesis, Syrian hamsters were divided into 8 groups: uninfected controls; immunosuppressed Syrian hamsters using prednisolone (P); normal Syrian hamsters administered *N*-nitrosodimethylamine (ND); immunosuppressed Syrian hamsters administered *N*-nitrosodimethylamine (ND*is*); normal Syrian hamsters infected with *Opisthorchis viverrini* (OV); immunosuppressed Syrian hamsters infected with *O. viverrini* (OV*is*); normal Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA); and immunosuppressed Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA*is*). Syrian hamster livers were used for analysis of tumor-related gene expression and immunohistochemistry through cytokeratin 19 (CK19) and proliferating cell nuclear antigen (PCNA) staining. The tumor-related gene expression results show that CCA*is* groups at all time points exhibited upregulation of COX-2, IL-6, SOD1, CAT and iNOS and downregulation of p53, which correlated with the predominant expression of CK19 and PCNA in liver tissue. These results suggest that prednisolone enhances cholangiocarcinoma development, which was confirmed by molecular changes.

Keywords Immunosuppressive · Cholangiocarcinogenesis · Tumor-related gene · Immunohistochemistry

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Introduction

Cholangiocarcinoma (CCA) in northeast Thailand is caused by a combination of factors such as *Opisthorchis viverrini* infection, nitrosamines, and host immune response, leading to mutagenesis via a multistep process involving several groups of genes. However, it is well known that many inflammatory tumor-related gene expressions are involved in cholangiocarcinogenesis. According to our previous report, prednisolone was shown to inhibit inflammatory cell response but enhanced early CCA as determined by gross histopathological changes and cyclooxygenase-2 (COX-2) immunohistostaining in an animal CCA model [1], but without molecular results. Therefore, to clarify the inflammatory tumor-related gene expression changes in immunosuppressed hamsters, Syrian hamsters were infected with liver flukes and administered *N*-nitrosodimethylamine (NDMA) or prednisolone. Livers were subsequently examined for immunohistochemistry via cytokeratin19 (CK19), proliferating cell nuclear antigen (PCNA) staining, and tumor-related gene expression (COX-2, iNOS, SOD1, CAT, IL-6 and p53) using real-time RT-PCR.

Materials and Methods

Animals

Syrian hamsters, 6 to 8 weeks old, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into 8 groups: i) uninfected controls; ii) immunosuppressed Syrian hamsters using prednisolone (P); iii) normal Syrian hamsters administered *N*-nitrosodimethylamine (ND); iv) immunosuppressed Syrian hamsters administered *N*-nitrosodimethylamine (ND*is*); v) normal Syrian hamsters infected with *Opisthorchis viverrini* (OV); vi) immunosuppressed Syrian hamsters infected with *O. viverrini* (OV*is*); vii) normal Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA) and viii) immunosuppressed Syrian hamsters infected with *O. viverrini* and administered *N*-nitrosodimethylamine (CCA*is*). Five Syrian hamsters per group were sacrificed at 1, 2, 3 and 6 month (s) post-infection; photographs were taken for comparison of the gross anatomy of the livers. The protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU43/2553).

Parasite Preparation and Animal Infection

Naturally infected freshwater cyprinoid fish were captured from a water reservoir in an endemic area of opisthorchiasis in Khon Kaen province, northeast Thailand. Fish were processed followed as the previous reports [2–4]. In brief, Fish were minced and digested with pepsin-HCl at 37 °C for 1 h then filtered through sieves (1,000, 425 and 106 μm, respectively) and sedimented. *O. viverrini* metacercariae were isolated and identified under a stereomicroscope. Each Syrian hamster was infected with 50 *O. viverrini* metacercariae via intragastric intubation.

Prednisolone Preparation and Immunosuppressed Syrian Hamsters Using Prednisolone

For suppression of host immunity, 5 mg tablets of prednisolone (Inpac Pharma, Thailand) were dissolved with absolute ethanol and then mixed into drinking water [5]. This was orally administered to the assigned groups every day for 2 months (5 mg/kg/day).

Preparation of *N*-nitrosodimethylamine

Diluted *N*-nitrosodimethylamine (NDMA), 12.5 ppm in drinking water, was administered to hamsters beginning at 7 days post-infection and every day thereafter for 2 months in order to induce cholangiocarcinogenesis [4, 6].

Immunohistochemistry

To determine whether prednisolone had an effect on suppressing inflammation in Syrian hamsters infected with *O. viverrini*, deparaffinized liver tissue sections of ND, ND*is*, OV, OV*is*, CCA and CCA*is* groups were processed for immunostaining.

Immunohistochemical Staining for Proliferating Cell Nuclear Antigen (PNCA) and Cytokeratin 19 (CK19)

The 1st antibodies were anti-PCNA, monoclonal antibody (Novocastra, UK) and CK19 polyclonal antibody (Abcam, UK). Biotin conjugated goat anti-mouse IgG (Invitrogen, USA) and EnVision System-HRP labeled polymer anti-rabbit antibody (Dako, USA) were used as the 2nd antibodies respectively. Amino-9-ethylcarbazole (AEC) was used for developing the color. All sections were counterstained with hematoxylin followed by mounting on slides using glycerol mounting medium. A positive immune reaction was visualized with red color as observed under a microscope.

Gene Expression Study

Total RNA Isolation

Liver was extracted for RNA followed as the previous reports [3, 6]. In brief, 300 mg of the right lobe of each liver was homogenized in a glass homogenizer with Trizol® solution (Invitrogen). The homogenized liver was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected in a new tube and added chloroform then precipitated RNA with isopropanol, washed with 75 % ethanol and dissolved with RNase-free water. Then removed DNA by DNase. RNA integrity was checked by visualizing the rRNA bands (28 s and 18 s) by agarose gel electrophoresis. Finally, the concentration and purity of total RNA in solution was assessed by spectrophotometry at wavelengths of 260 nm and 280 nm, respectively.

Complementary DNA Synthesis

Three μg RNA template was placed into a new tube, followed by adding 1 μl of oligo $(dT)^{15}$ primer and DEPC-treated water to 12 μl. This was mixed gently and spun down, then incubated at 65 °C for 5 min. After chilling on ice, the following components were added: 4 μl of 5X reaction buffer, 1 μl of 20 units ribonuclease inhibitor, 2 μl of 10 mM deoxyribonucleotide triphosphate mix, and 1 μl of 200 units M-MLV reverse transcriptase (Invitrogen). The solution was mixed gently and incubated at 42 °C for 1 h. The reaction was stopped by incubation at 70 °C for 10 min

and then chilled on ice. cDNA template was diluted with 180 μl deionized water.

For checking cDNA quality, polymerase chain reaction (PCR) technique was used. G3PDH primers were used as housekeeping genes. One μl of cDNA template was added to the reaction mixture as follows: 1 μl of 10X *Taq* buffer, 1 μl of 5 mM deoxyribonucleotide triphosphate mix, 1 μl of 25 mM $MgCl_2$, 1 μl of 5 μM G3PDH forward primer, 1 μl of 5 μM G3PDH reverse primer, 0.04 μl of *Taq* DNA polymerase (RBC Bioscience, Taiwan), and 4 μl of deionized water to give a final volume of 10 μl. The mixture was gently vortexed and briefly spun down. PCR analysis was performed using a thermocycler (GeneAmp® PCR system 9700; Invitrogen). PCR conditions were 1 cycle at 95 °C for 5 min, 25 cycles of denaturing (94 °C for 30 s), annealing (58 °C for 60 s), and extension (72 °C for 60 s) step followed by 1 cycle at 72 °C for 7 min. Finally, G3PDH primer specificity was checked by visualizing the positive band (228 bp) by agarose gel electrophoresis.

Relative Quantification by Real-Time RT-PCR

Real-time RT-PCR using the SYBR® Green method was performed to analyze the relative quantification of mRNA expression. In brief, a total of 20 μl of master mix was composed of 3 μl of cDNA, 2 μl of 10X TrueStart™ *Taq* buffer (Fermentas, USA), 2 μl of 5 μM forward and reverse primers, 1 μl of 5 mM deoxyribonucleotide triphosphate mix, 2.4 μl of 10 mM $MgCl_2$, 2 μl of SYBR® Green, 0.2 μl of TrueStart™ Hot Start *Taq* DNA polymerase (Fermentas) and 7.4 μl of deionized water. Real-time RT-PCR analysis was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA). The real-time RT-PCR conditions are shown in Table 1. Relative changes in gene expression were analyzed by 2^{-ddCt}. All values were in reference to the housekeeping gene (G3PDH) level, normalized with the untreated control group, and then reported as relative mean fold change.

Primers for Real-Time RT-PCR

Many pairs of primers were designed for examination of the expression of tumor-related genes in cholangiocarcinoma-related inflammation. The primer pairs for this experiment were G3PDH (housekeeping gene), COX-2, SOD1, iNOS, CAT, p53 and IL-6. All primers were designed based on the published sequences [2, 6, 7] as summarized in Table 2.

Statistical Analysis

The real time RT-PCR data was calculated as mean ± SEM. The statistic was analyzed using One-way ANOVA (SPSS version 19.0. USA). The results were considered statistically significant when P-value is less than 0.05 ($P<0.05$).

Results

Proliferating Cell Nuclear Antigen

PCNA was stained at the bile duct epithelial nucleus of all groups, but to different degrees. The ND group had a light expression at the nuclei of epithelial bile ducts at all time points (Fig. 1 A, G, M, S). The ND*is* group had no positive staining at all time points (Fig. 1 B, H, N, T). For the OV group, PCNA was predominantly expressed at the nuclei of the bile duct epithelium surrounding the parasite area at 1 month, and gradually decreased at 2, 3 and 6 months, respectively (Fig. 1 C,I,O,U). The OV*is* group had a similar staining pattern to the OV group, but lower expression (Fig. 1 D, J, P, V). Both CCA and CCA*is* groups showed very strong staining in all areas of bile duct proliferation and cancerous areas. The highest expression was found in the CCA*is* group at 2 months (Fig. 1 L), whereas the highest expression in the CCA group was observed at 6 months (Fig. 1 W)

Cytokeratin 19

CK19 staining was evident in the bile duct epithelial cytoplasm of all groups, but to different degrees. The ND group showed expression in the cytoplasm of bile duct epithelial cells at all time points (Fig. 2 A, G, M, S). The ND*is* group had positive staining at all time points, but showed a slight increase with time (Fig. 2 B, H, N, T). For the OV group, CK19 was expressed in the cytoplasm of hepatic bile duct epithelial cells surrounding the parasite area at the same level at 1, 2, 3 and 6 months (Fig. 2 C,I,O,U). The OV*is* group had a similar staining pattern to the OV group (Fig. 2 D, J, P, V). Both CCA and CCA*is* groups showed very

Table 1 Summary of real time RT-PCR conditions

Gene	Real time RT-PCR condition	Cycles
G3PDH	95 °C for 15s, 60 °C for 60s, 72 °C for 60s	35
p53, SOD1, CAT	95 °C for 15s, 62 °C for 60s, 72 °C for 60s	40
IL-6, COX-2, iNOS	95 °C for 15s, 64 °C for 60s, 72 °C for 60s	40

Table 2 Summary of real time RT-PCR primer pairs

Gene	Product length (bp)	Sequence Upper line : forward primer Bottom line : reverse primer	GenBank Accession number/reference
G3PDH	228	5′-GGCATTGTGGAAGGGCTCAT-3′ 5′-GACACATTGGGGGTAGGAACAC-3′	[2]
p53	232	5′-AAGGCGATAGTTTGGCTCCT-3′ 5′-CTGGGGTCTTCCAGTGTGAT-3′	[2]
IL-6	193	5′-CCAGATCTACCTGGAGTTTG-3′ 5′-CCACTCCTTTTGTGACTCC-3′	AB028635
SOD1	165	5′-CGGATGAAGAGAGGCATGTT-3′ 5′-CACCTTTGCCCAAGTCATCT-3′	[6]
CAT	179	5′-TTGACAGAGAGCGGATTCCT-3′ 5′-AGCTGAGCCTGACTCTCCAG-3′	[6]
COX-2	238	5′-CTGTATCCCGCCCTGCTGGTG-3′ 5′-ACTTGCGTTGATGGTGGCTGTCTT-3′	DQ485276
iNOS	139	5′-TCAACCTACTTCCTGGACATC-3′ 5′-TTTCTGAACTTCCAATCGTT-3′	[7]

strong staining in all areas of bile duct proliferation and cancerous areas. The highest expression was found in the CCA*is* group at 2 months (Fig. 2 L), whereas the highest in the CCA group was observed at 6 months (Fig. 2 W), which correlated with the increased bile duct proliferation and CCA area.

Tumor-Related Genes

Different expression levels of COX-2, SOD1, iNOS, IL-6, CAT and p53 were observed in all groups at all time points (Figs. 3, 4, 5, 6, 7, 8). COX-2, iNOS and IL-6 expression in CCA*is* groups were high at all time points when compared with other groups ($P<0.05$). The highest level was observed at 3 months. SOD1 expression was similar to COX-2, iNOS, and IL-6 expression, but the highest expression level was observed at 1 month. There was no difference in CAT expression level in the groups of P, ND, ND*is*, OV, CCA and CCA*is*, but in the OV*is* group CAT expression level was significantly increased at 3 and 6 months ($P<0.05$). p53 was lower expression in CCA*is* than similar to but high expression in ND group at all time points with significant difference ($P<0.05$).

Discussion

The present study confirms that immunosuppression by prednisolone induced early cholangiocarcinogenesis in *O. viverrini*-infected Syrian hamsters by increasing the expression of COX-2, IL-6, SOD1 and iNOS and by reducing p53; moreover, immunohistostaining for CK19 and PCNA was predominantly expressed.

It is well established that chronic inflammation is a key factor in carcinogenesis by causing DNA damage and mutation [8]. Many cancers are linked to chronic infection or inflammation, such as hepatocellular carcinoma induced by hepatitis B and C [9, 10], and chronic *O. viverrini* infection which is correlated with a high rate of CCA in the northern part of Thailand. This parasite has been reported to be one of the risks for cholangiocarcinogenesis by the International Agency for Research on Cancer [11]. Several researchers have hypothesized that reducing the rates of chronic infection or inflammation may reduce the risk of cholangiocarcinoma development. This hypothesis is true, but reducing the host immune response may effect on the unbalancing host immune response which may increase the susceptibility to many diseases, including cancers [12–15], as in our previous result which showed that prednisolone inhibited inflammatory cell response but induced early cholangiocarcinoma development. Molecular changes in OV-associated CCA in hamsters have been reported in many genes related to oncogenes, cell proliferation, differentiation and transformation, cell cycle regulation, apoptosis, metabolism, and some unknown functions, which were greatly up- or down regulated during CCA development [2–4, 16–19].

COX-2 is one of the key enzymes implicated in the modulation of inflammation, and acts by catalyzing the rate-limiting step that leads to the formation of prostaglandins (PGs) from arachidonic acid [20]. To date, most studies have focused on the expression of COX-2 and its possible role in oncogenesis and cancer growth [21]. According to a previous report by Juasook et al. [1], immunosuppressive prednisolone enhanced early cholangiocarcinogenesis in *O. viverrini*-infected Syrian hamsters as indicated by gross appearance, histopathology and COX-2 staining; this is in agreement with the present study showing the upregulation of COX-2 expression (Fig. 3). Several studies have shown that COX-2 is induced in human tumors, colon [22], pancreas [23], skin [24], lung [25], and hepatocellular carcinoma [26–29].

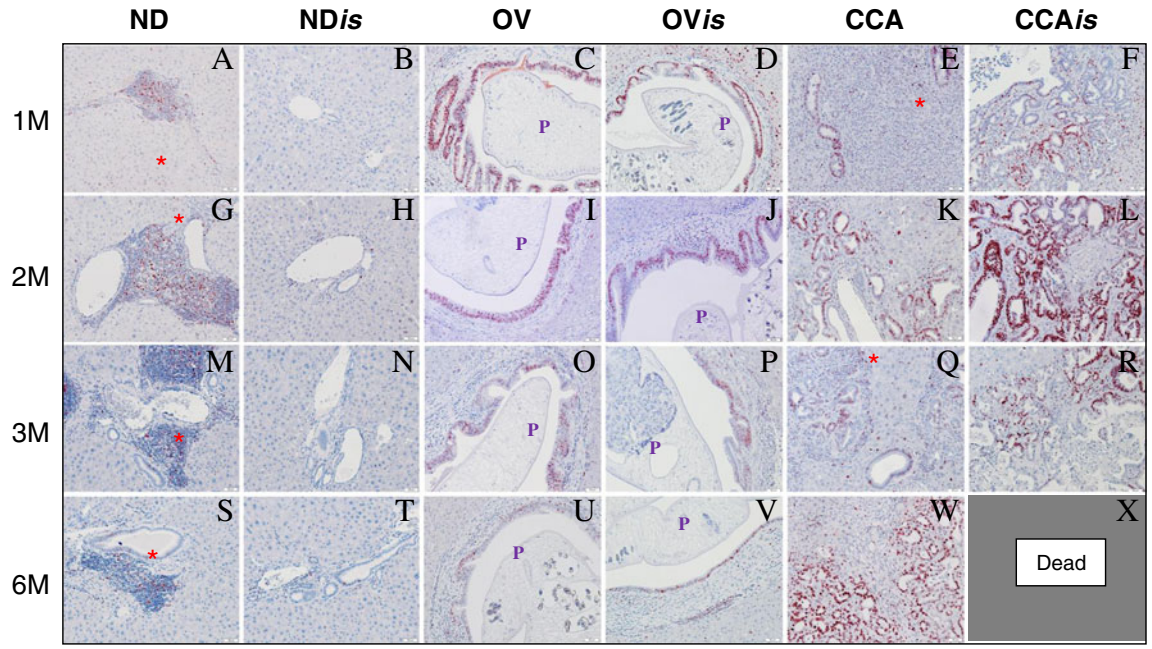

Fig. 1 The immunohistochemical staining for proliferating cell nuclear antigen (PCNA) of the Syrian hamster in each group at 1, 2, 3 and 6 month (s) post infection. *ND*; carcinogen administered group (A,G,M,S), ND*is*; carcinogen administered in immunosuppressive group (B,H,N,T), OV; *O. viverrini* infected group (C,I,O,U), OV*is*; *O. viverrini* infected in immunosuppressive group (D,J,P,V), CCA; cholangiocarcinoma group (E,K,Q,W), CCA*is*; cholangiocarcinoma in immunosuppressive group (F,L,R,X), p; parasite, *; inflammatory cells

Superoxide dismutase 1 (SOD1) is an enzyme that catalyzes the conversion of the superoxide ion (O^{2-}) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) to maintain low levels of reactive oxygen species (ROS); thus, it plays a key role in defending cells against oxygen radical toxicity [30]. The overexpression of SOD1 was observed in CCA*is* (Fig. 4), which agreed with several previous studies on many diseases such as central nervous system tumor,

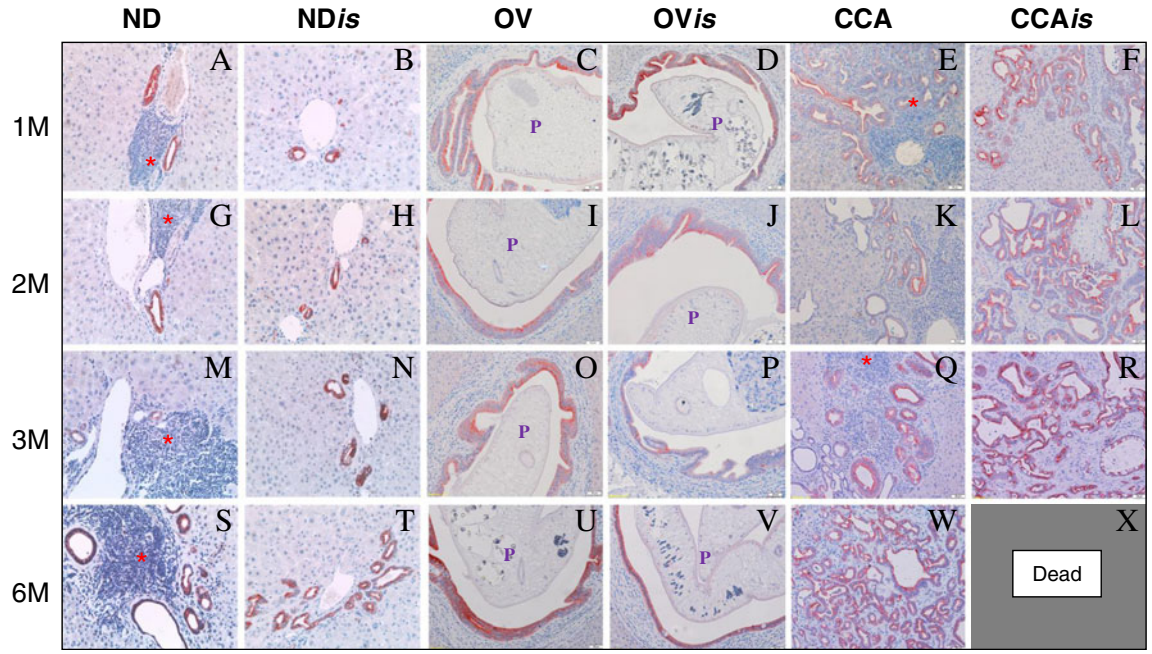

Fig. 2 The immunohistochemical staining for cytokeretin 19 (CK19) of the Syrian hamster in each group at 1, 2, 3 and 6 month (s) post infection. *ND*; carcinogen administered group(A,G,M,S), ND*is*; carcinogen administered in immunosuppressive group (B,H,N,T), OV; *O. viverrini* infected group (C,I,O,U), OV*is*; *O. viverrini* infected in immunosuppressive group (D,J,P,V), CCA; cholangiocarcinoma group (E,K,Q,W), CCA*is*; cholangiocarcinoma in immunosuppressive group (F,L,R,X), p; parasite

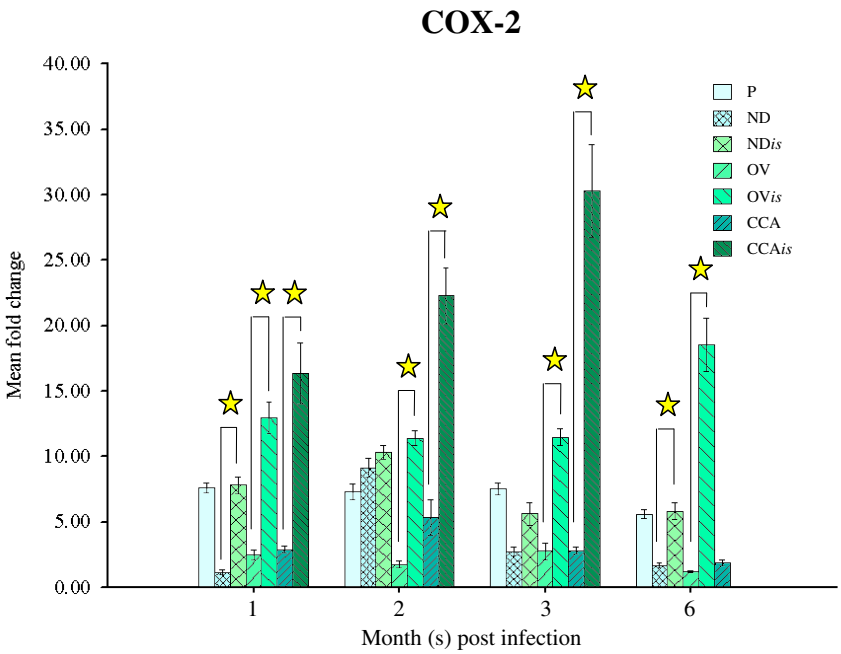

Fig. 3 The real time RT-PCR result of COX-2 expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

gastric cancer, colorectal adenocarcinoma [31–33], and cholangiocarcinoma [6].

Inducible nitric oxide synthase (iNOS) activity has been reported in a wide array of cells and tissues, and is not present in resting cells. To date, upregulation of iNOS has been reported to be associated with several malignant human tumors including breast [34], brain [35], lung [36], prostate [37], colorectal [38] and pancreatic carcinomas [39], melanoma [40] as well as hepatocellular carcinoma [41]. Moreover, increased expression of 8-nitroguanine formation and iNOS has been reported in the bile duct epithelium of hamsters repeatedly infected with *O. viverrini* [17]. Another mechanism by which NO may promote tumor growth is by activating COX-2, which modulates the production of prostaglandins, promotes angiogenesis and inhibits apoptosis [42]. Furthermore, it has been reported that overexpression of iNOS leads to DNA damage, mutation, increased cell proliferation, oxidative stress, resistance to apoptosis, increased tumor vascularity, and metastatic potential [43]. The above

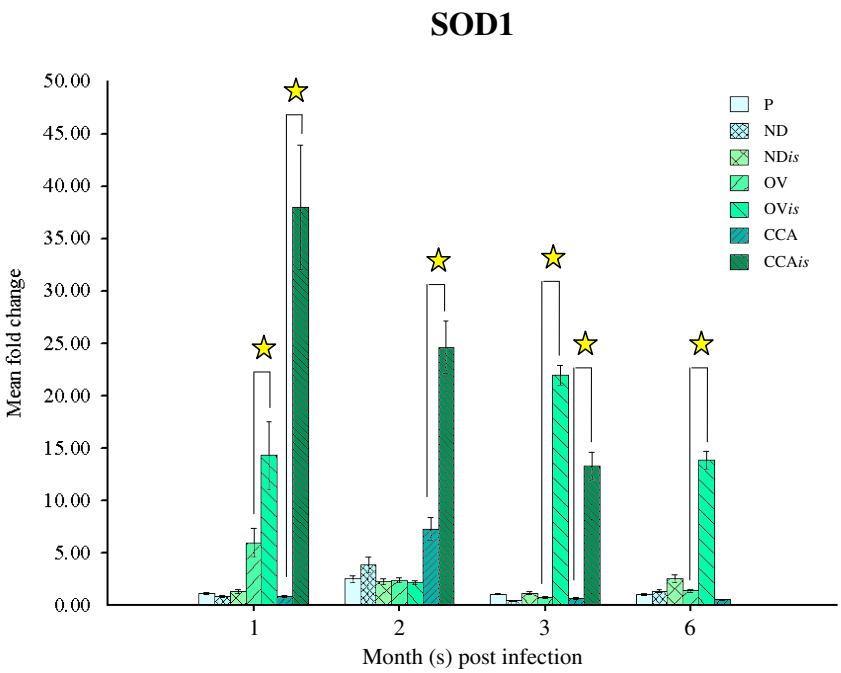

Fig. 4 The real time RT-PCR result of SOD1 expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

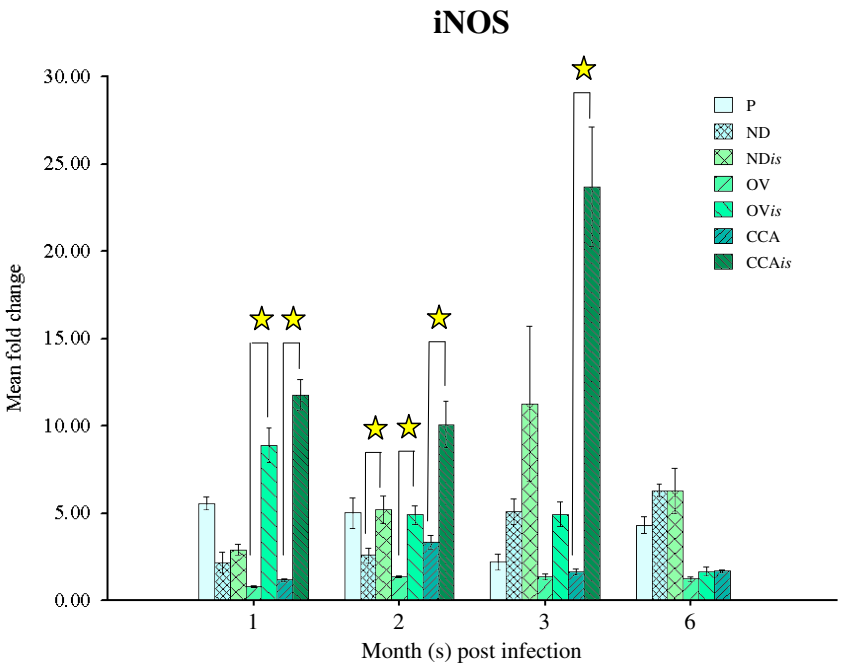

Fig. 5 The real time RT-PCR result of iNOS expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

results agreed with our present results, as shown in Fig. 5.

Catalase (CAT) plays a major role in cellular antioxidant defense by decomposing hydrogen peroxide, thereby preventing the generation of hydroxyl radicals. The OV*is* group at 3 and 6 months post-infection showed significantly increased CAT levels compared with the OV group including cholangiocarcinoma (Fig. 7); this was in accordance with others studies of the antioxidant property of CAT . The prevention of oxidation is an essential process in all cells, as decreased antioxidant protection may lead to cytotoxicity, mutagenicity and/or carcinogenicity such as breast cancer and hepatocellular carcinoma [44–47]. The increased level of CAT in this group was correlated with more improved pathology compared with the untreated group (Fig. 7).

The tumor suppressor gene p53 plays an important role in DNA repair and regulation of apoptosis, helping to conserve genetic stability; this supports our present result showing very low expression of p53 in the CCA*is* group (Fig. 8). Abnormal regulation of the p53 pathway is a characteristic hallmark of many types of human cancer, including the

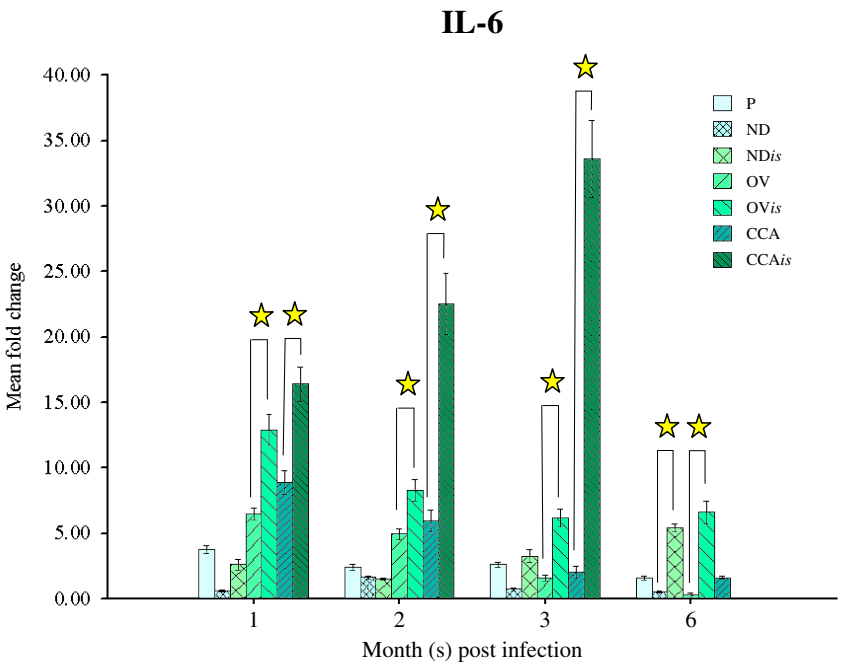

Fig. 6 The real time RT-PCR result of IL-6 expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

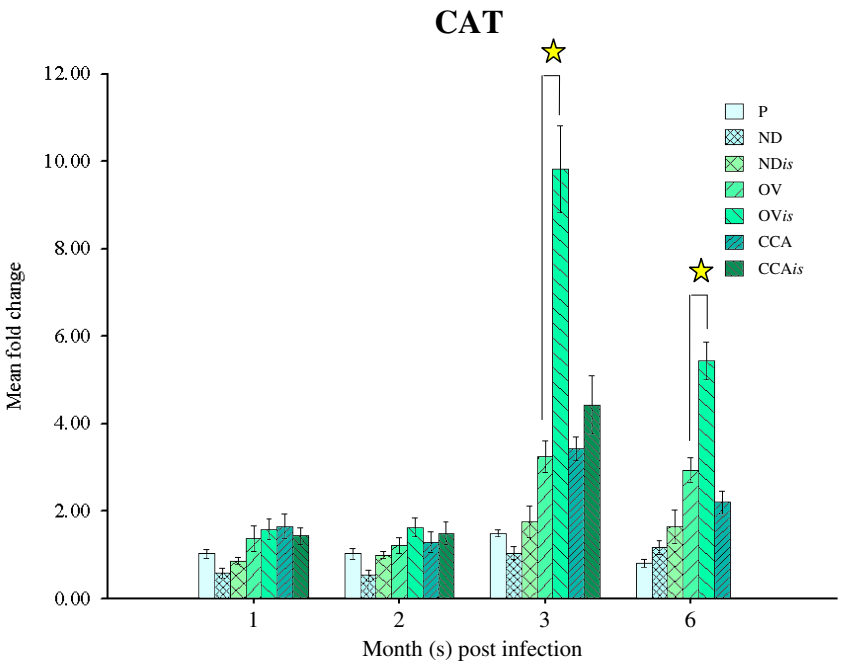

Fig. 7 The real time RT-PCR result of CAT expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

breast [48], brain, reticuloendothelial tissues, hematopoietic tissues, colon, lung, esophagus, liver [49], and primary intrahepatic cholangiocarcinoma [50].

IL-6 plays a role as an anti-inflammatory cytokine, which is produced by various types of lymphoid and non-lymphoid cells such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several tumor cells [51]. In the normal homeostatic state, IL-6 level is very low; but in response to the appropriate stimulus (e.g. inflammation). Colon carcinoma patients have been reported to have increased levels of IL-6, and that the levels are correlated with tumor size [52]. In addition, IL-6 has been shown to promote the growth of colon cancer epithelial cells in vitro, suggesting that IL-6 may drive cancer growth [53]. Several of these reports support our result that IL-6 is the most important in CCA development (Fig. 6), such as evidence for the involvement of IL-6 in the pathophysiology of CCA. IL-6, a known biliary mitogen, is a key cytokine involved in the hepatic response to inflammation; it is also an important mediator of hepatic proliferation [54]. Recent

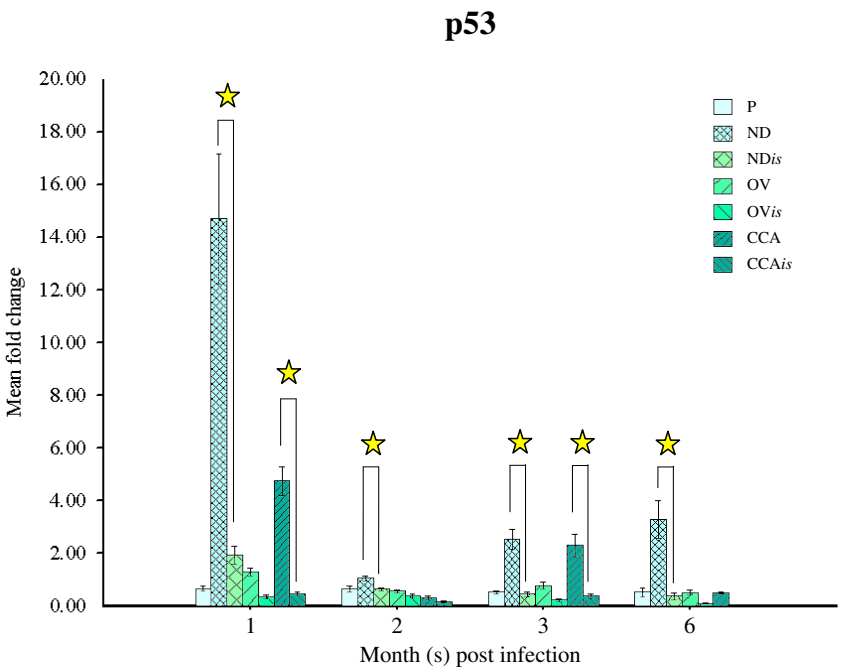

Fig. 8 The real time RT-PCR result of p53 expression level ralative to G3PDH in each group at 1, 2, 3 and 6 month (s) post infection. P; immunosuppressive group, ND; carcinogen administered group, ND*is*; carcinogen administered in immunosuppressive group, OV; *O. viverrini* infected group, OV*is*; *O. viverrini* infected in immunosuppressive group, CCA; cholangiocarcinoma group, CCA*is*; cholangiocarcinoma in immunosuppressive group,; significantly different compare with counter group ($P<0.05$)

studies have shown the ability of CCA cells to proliferate in response to IL-6 [55, 56]. Moreover, CCA is associated with elevated serum levels of IL-6. Indeed, biliary levels of IL-6 are elevated in cholangitis patients [57]. Thus, IL-6 is a potent biliary epithelial cell mitogen and may play a role in the growth regulation of normal and malignant cholangiocytes [54, 58, 59]. Recently, increased plasma IL-6 level was reported in a CCA patients [60].

Based on these results, we suggest that when host immunity is suppressed, this is followed by molecular changes leading to CCA development. Finally, we are more confident that prednisolone enhances carcinogenesis through molecular changes which result from the cancer microenvironment, and that chronic inflammation plays an important role in carcinogenesis. Moreover, host immune responses are crucially associated with pathogenesis. We can conclude that host immunity is strongly related to pathogenesis in CCA associated with *O. viverrini* infection. Pathology of the hepatic bile duct in cases of *O. viverrini* infection showed improvement when host immune responses were restricted; conversely, CCA development was enhanced by suppression of host immunity during *O. viverrini* infection. CCA development in an immunosuppressed Syrian hamster model implies that cancer risk patients should avoid immunosuppressive therapy, and that balancing of the immune response should be a primary concern in treatment of inflammation-related cancer.

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References

1. Juasook A, Boonmars T, Wu Z, Loilome W, Veteewuthacharn K, Namwat N, Sudsarn P, Wonkchalee O, Sriraj P, Aukkanimart R (2013) Immunosuppressive prednisolone enhances early cholangiocarcinoma in Syrian hamsters with liver fluke infection and administration of N-nitrosodimethylamine. Pathol Oncol Res 19(1):55–62
2. Boonmars T, Srirach P, Kaewsamut B, Srisawangwong T, Pinlaor S, Pinlaor P, Yongvanit P, Sithithaworn P (2008) Apoptosis-related gene expression in hamster opisthorchiasis post praziquantel treatment. Parasitol Res 102(3):447–455
3. Boonmars T, Wu Z, Boonjaruspinyo S, Pinlaor S, Nagano I, Takahashi Y, Kaewsamut B, Yongvanit P (2009) Alterations of gene expression of RB pathway in *Opisthorchis viverrini* infection-induced cholangiocarcinoma. Parasitol Res 105(5):1273–1281
4. Boonmars T, Wu Z, Boonjaruspinyo S, Puapairoj A, Kaewsamut B, Nagano I, Pinlaor S, Yongvanit P, Wonkchalee O, Juasook A, Sudsarn P, Srisawangwong T (2010) Involvement of c-Ski oncoprotein in carcinogenesis of cholangiocacinoma induced by *Opisthorchis viverrini* and N-nitrosodimethylamine. Pathol Oncol Res 17(2):219–227
5. Trune DR, Kempton JB, Gross ND (2006) Mineralocorticoid receptor mediates glucocorticoid treatment effects in the autoimmune mouse ear. Hear Res 212(1–2):22–32
6. Boonjaraspinyo S, Boonmars T, Aromdee C, Puapairoj A, Wu Z (2011) Indirect effect of a turmeric diet: enhanced bile duct proliferation in Syrian hamsters with a combination of partial obstruction by *Opisthorchis viverrini* infection and inflammation by N-nitrosodimethylamine administration. Parasitol Res 108(1):7–14
7. Laothong U, Pinlaor P, Hiraku Y, Boonsiri P, Prakobwong S, Khoontawad J, Pinlaor S (2010) Protective effect of melatonin against *Opisthorchis viverrini*-induced oxidative and nitrosative DNA damage and liver injury in hamsters. J Pineal Res 49(3):271–282
8. Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140(6):883–899
9. Barth H, Robinet E, Liang TJ, Baumert TF (2008) Mouse models for the study of HCV infection and virus-host interactions. J Hepatol 49(1):134–142
10. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, Osterreicher CH, Takahashi H, Karin M (2010) Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 140(2):197–208
11. IARC (2011) A review of human carcinogens part B: biological agents (*Opisthorchis viverrini* and *Clonorchis sinensis*). IARC Monogr Eval Carcinog Risks Hum 100:347–376
12. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E (1999) Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 59(13):3128–3133
13. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr, Rabkin CS (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 404(6776):398–402
14. de Martel C, Llosa AE, Farr SM, Friedman GD, Vogelman JH, Orentreich N, Corley DA, Parsonnet J (2005) *Helicobacter pylori* infection and the risk of development of esophageal adenocarcinoma. J Infect Dis 191(5):761–767
15. Zamarron BF, Chen W (2011) Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci 7(5):651–658
16. Pinlaor S, Hiraku Y, Ma N, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S (2004) Mechanism of NO-mediated oxidative and nitrative DNA damage in hamsters infected with *Opisthorchis viverrini*: a model of inflammation-mediated carcinogenesis. Nitric Oxide 11(2):175–183
17. Pinlaor S, Ma N, Hiraku Y, Yongvanit P, Semba R, Oikawa S, Murata M, Sripa B, Sithithaworn P, Kawanishi S (2004) Repeated infection with *Opisthorchis viverrini* induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis 25(8):1535–1542
18. Wu Z, Boonmars T, Boonjaraspinyo S, Nagano I, Pinlaor S, Puapairoj A, Yongvanit P, Takahashi Y (2011) Candidate genes involving in tumorigenesis of cholangiocarcinoma induced by *Opisthorchis viverrini* infection. Parasitol Res 109(3):657–673
19. Loilome W, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, Hanai S, Miwa M (2006) Altered gene expression in *Opisthorchis viverrini*-associated cholangiocarcinoma in hamster model. Mol Carcinog 45(5):279–287
20. Howe LR, Subbaramaiah K, Brown AM, Dannenberg AJ (2001) Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. Endocr Relat Cancer 8(2):97–114
21. Lakatos PL, Lakatos L (2008) Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. World J Gastroenterol 14(25):3937–3947

22. Dolcet X, Llobet D, Pallares J, Matias-Guiu X (2005) NF-kB in development and progression of human cancer. Virchows Arch 446(5):475–482
23. Sakamoto K, Maeda S, Hikiba Y, Nakagawa H, Hayakawa Y, Shibata W, Yanai A, Ogura K, Omata M (2009) Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. Clin Cancer Res 15(7):2248–2258
24. Fernandez-Majada V, Aguilera C, Villanueva A, Vilardell F, Robert-Moreno A, Aytes A, Real FX, Capella G, Mayo MW, Espinosa L, Bigas A (2007) Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. Proc Natl Acad Sci U S A 104(1):276–281
25. Leong KG, Karsan A (2006) Recent insights into the role of Notch signaling in tumorigenesis. Blood 107(6):2223–2233
26. Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M (1999) Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. Hepatology 29(3):688–696
27. Martin-Sanz P, Mayoral R, Casado M, Bosca L (2010) COX-2 in liver, from regeneration to hepatocarcinogenesis: what we have learned from animal models? World J Gastroenterol 16(12):1430–1435
28. Kundu JK, Surh YJ (2008) Inflammation: gearing the journey to cancer. Mutat Res 659(1–2):15–30
29. Burstein E, Fearon ER (2008) Colitis and cancer: a tale of inflammatory cells and their cytokines. J Clin Invest 118(2):464–467
30. McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244(22):6049–6055
31. Cobbs CS, Levi DS, Aldape K, Israel MA (1996) Manganese superoxide dismutase expression in human central nervous system tumors. Cancer Res 56(14):3192–3195
32. Izutani R, Asano S, Imano M, Kuroda D, Kato M, Ohyanagi H (1998) Expression of manganese superoxide dismutase in esophageal and gastric cancers. J Gastroenterol 33(6):816–822
33. Toh Y, Kuninaka S, Oshiro T, Ikeda Y, Nakashima H, Baba H, Kohnoe S, Okamura T, Mori M, Sugimachi K (2000) Overexpression of manganese superoxide dismutase mRNA may correlate with aggressiveness in gastric and colorectal adenocarcinomas. Int J Oncol 17(1):107–112
34. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S (1995) Nitric oxide synthase activity in human breast cancer. Br J Cancer 72(1):41–44
35. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA (1995) Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res 55(4):727–730
36. Masri FA, Comhair SA, Koeck T, Xu W, Janocha A, Ghosh S, Dweik RA, Golish J, Kinter M, Stuehr DJ, Erzurum SC, Aulak KS (2005) Abnormalities in nitric oxide and its derivatives in lung cancer. Am J Respir Crit Care Med 172(5):597–605
37. Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K (1998) Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer 82(10):1897–1903
38. Lagares-Garcia JA, Moore RA, Collier B, Heggere M, Diaz F, Qian F (2001) Nitric oxide synthase as a marker in colorectal carcinoma. Am Surg 67(7):709–713
39. Hajri A, Metzger E, Vallat F, Coffy S, Flatter E, Evrard S, Marescaux J, Aprahamian M (1998) Role of nitric oxide in pancreatic tumour growth: in vivo and in vitro studies. Br J Cancer 78(7):841–849
40. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA (2000) Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res 6(12):4768–4775
41. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Muroni MR, Simile MM, Frau M, Tomasi ML, De Miglio MR, Seddaiu MA, Daino L, Sanna V, Feo F, Pascale RM (2008) Aberrant iNOS signaling is under genetic control in rodent liver cancer and potentially prognostic for the human disease. Carcinogenesis 29(8):1639–1647
42. Cahlin C, Gelin J, Delbro D, Lonnroth C, Doi C, Lundholm K (2000) Effect of cyclooxygenase and nitric oxide synthase inhibitors on tumor growth in mouse tumor models with and without cancer cachexia related to prostanoids. Cancer Res 60(6):1742–1749
43. van der Woude CJ, Kleibeuker JH, Jansen PL, Moshage H (2004) Chronic inflammation, apoptosis and (pre-)malignant lesions in the gastro-intestinal tract. Apoptosis 9(2):123–130
44. Mates JM (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153(1–3):83–104
45. Marra M, Sordelli IM, Lombardi A, Lamberti M, Tarantino L, Giudice A, Stiuso P, Abbruzzese A, Sperlongano R, Accardo M, Agresti M, Caraglia M, Sperlongano P (2011) Molecular targets and oxidative stress biomarkers in hepatocellular carcinoma: an overview. J Transl Med. doi:10.1186/1479-5876-9-171
46. Min JY, Lim SO, Jung G (2010) Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the catalase promoter. FEBS Lett 584(11):2427–2432
47. Sen S, Kawahara B, Chaudhuri G (2012) Maintenance of higher H_2O_2 levels, and its mechanism of action to induce growth in breast cancer cells: important roles of bioactive catalase and PP2A. Free Radic Biol Med 53(8):1541–1551
48. Alawadi S, Ghabreau L, Alsaleh M, Abdulaziz Z, Rafeek M, Akil N, Alkhalaf M (2011) P53 gene polymorphisms and breast cancer risk in Arab women. Med Oncol 28(3):709–715
49. Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. Science 253(5015):49–53
50. O'Dell MR, Huang JL, Whitney-Miller CL, Deshpande V, Rothberg P, Grose V, Rossi RM, Zhu AX, Land H, Bardeesy N, Hezel AF (2012) Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. Cancer Res 72(6):1557–1567
51. Kishimoto T, Akira S, Narazaki M, Taga T (1995) Interleukin-6 family of cytokines and gp130. Blood 86(4):1243–1254
52. Chung YC, Chang YF (2003) Serum interleukin-6 levels reflect the disease status of colorectal cancer. J Surg Oncol 83(4):222–226
53. Schneider MR, Hoeflich A, Fischer JR, Wolf E, Sordat B, Lahm H (2000) Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. Cancer Lett 151(1):31–38
54. Park J, Tadlock L, Gores GJ, Patel T (1999) Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. Hepatology 30(5):1128–1133
55. Meng F, Yamagiwa Y, Ueno Y, Patel T (2006) Over-expression of interleukin-6 enhances cell survival and transformed cell growth in human malignant cholangiocytes. J Hepatol 44(6):1055–1065
56. Frampton G, Invernizzi P, Bernuzzi F, Pae HY, Quinn M, Horvat D, Galindo C, Huang L, McMillin M, Cooper B, Rimassa L, DeMorrow S (2012) Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 61(2):268–277
57. Alvaro D (2009) Serum and bile biomarkers for cholangiocarcinoma. Curr Opin Gastroenterol 25(3):279–284
58. Okada K, Shimizu Y, Nambu S, Higuchi K, Watanabe A (1994) Interleukin-6 functions as an autocrine growth factor in a cholangiocarcinoma cell line. J Gastroenterol Hepatol 9(5):462–467
59. Sugawara H, Yasoshima M, Katayanagi K, Kono N, Watanabe Y, Harada K, Nakanuma Y (1998) Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. Histopathology 33(2):145–153
60. Sripa B, Thinkhamrop B, Mairiang E, Laha T, Kaewkes S, Sithithaworn P, Periago MV, Bhudhisawasdi V, Yonglitthipagon P, Mulvenna J, Brindley PJ, Loukas A, Bethony JM (2012) Elevated plasma IL-6 associates with increased risk of advanced fibrosis and cholangiocarcinoma in individuals infected by *Opisthorchis viverrini*. PLoS Negl Trop Dis 6(5):e1654