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Mouse hepatic neoplasm formation induced by trace level and low frequency exposure of diethylnitrosamine through β-catenin signaling pathway

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Abstract

It has been reported that massive levels or/and high frequency exposure of diethylnitrosamine could induce hepatic neoplasm. However, it would be more interesting to figure out the hepatotoxic effects of diethylnitrosamine exposure at trace level and low frequency, which could be more common in our daily life. We found that both the mRNA and protein expression levels of β-catenin were aberrant in all liver tissues, accompanied by inflammation, steatosis, fibrosis and hepatic neoplasm after 10-week exposure of diethylnitrosamine (dissolved in sesame oil, 0.16 mmol/kg body weight) to mice. In addition, gradual increases in the mRNA expressions of several pivotal risk factors (TNF-α, COX-2, PPAR-γ, AP-2, Smad-2, TGF-β1, and C-myc), as well as their protein expression levels, were associated with the aberrant expression or/and nucleus localization of β-catenin. Taken together, our results show that long-term diethylnitrosamine exposure at trace amount and low frequency can also induce hepatotoxicity (including inflammation, steatosis and fibrosis) and consequently aberrant activation of β-catenin which in turn plays an important role in the initiation and promotion of liver tumor.

Key Words: diethylnitrosamine; trace level; low frequency; β-catenin; hepatotoxicity; hepatic neoplasm

1. Introduction

Humans are exposed to more and more environmental pollutants, which come from the emissions of various chemical compounds due to their production and extensive uses.[1] Therefore, public concerns over the past decade are significantly inspired for the effects of exposure to environmental pollutants on human health. Risk assessment of pollutants is commonly realized by evaluating the acute toxicity or/and chronic toxicity of the pollutants. However, many studies have shown that the chronic toxicity differs from the acute toxicity,[2-5] which may be caused by the difference of the chemical model of action.[6] The acute toxicity is often evaluated under the exposure conditions of the pollutants at high concentrations or/and high frequencies. Although there is a great amount of available information on acute toxicity of environmental pollutants, the information on chronic toxicity is still relatively scarce.[7,8] It is easily understood that during the daily life, humans are typically exposed to environmental pollutants over long periods of time,[9] which is quite different from the acute toxicity evaluation conditions.[10] Therefore, the chronic toxicity is a better selection for the risk assessment of environmental pollutants and food ingredients, which is conducted with the exposure way at trace levels and low frequencies. There are a great number of environmental pollutants and food ingredients which should be checked for their chronic toxicity. Among these chemical compounds, carcinogenic environmental or food contaminants, including N-nitroso compounds (NOCs), are a group of highly toxic pollutants, which significant attention must be paid to.

NOCs are a class of widespread potential environmental carcinogens. Among about three-hundred tested NOCs, more than 90% of them have been found to be carcinogenic toward a wide variety of animal species.[11] They are also potentially important in the etiology of human cancer. Human exposure to NOCs occurs readily via diet, cigarette smoking, occupational exposure, and endogenous nitrosation.[12] Hepatic neoplasm, especially hepatocellular carcinoma (HCC) is a primary malignancy of the liver, and is the third leading cause of cancer-related death in the

world.[13] The high incidence of HCC is related to high exposure to carcinogenic compounds, including NOCs.[14]

Diethylnitrosamine (DEN) is a representative member of the NOC family and has been detected frequently in foods.[15-17] DEN has been evaluated as one of the most important potential carcinogens.[18] Therefore, DEN was selected as a target carcinogen to investigate the chronic toxicity effects of NOCs and the underlying mechanisms. According to the researches on DEN-induced hepatocarcinogenesis, reactive oxygen species (ROS) is suspected to play an important role in DEN-induced hepatocarcinogenesis. It was reported that the exposure to DEN caused hepatocellular accumulation of ROS, leading to oxidative damage of DNA and other nucleophiles.[19,20] DEN induced DNA damage by alkylation and the resultant O^6-alkylguanine caused GC-AT transition mutations, being largely responsible for DEN-induced carcinogenesis.[21] The association of circadian disruption with DEN exposure was shown to accelerate mouse liver carcinogenesis.[22] However, these conclusions were obtained under the exposure conditions with mass dose as high as 1.96 mmol/kg body weight, or/and high frequency, usually once a day, which is not in conformity with the actual human exposure conditions. Few studies have elucidated the hepatotoxic effects of trace level and low frequency DEN exposure and the underlying mechanisms, especially the process of hepatic neoplasm formation.

The role of the canonical Wnt/β-catenin signaling pathway in liver biology has come to the forefront over the last several years.[23] In normal liver, as an essential downstream transcriptional activator of this signaling pathway, β-catenin maintains its dual characteristics and is clearly of essence in several physiological events such as development, regeneration, and growth.[24] In hepatic pathological conditions, β-catenin aberrant activation is evident in chronic inflammation, steatosis and fibrosis changes and in many different tumors of the liver, such as HCC and hepatoblastoma.[25] Therefore, the aberrant activation of β-catenin is likely an initiating or contributory factor from inflammation towards cirrhosis and carcinogenesis in the progression of hepatic neoplasm. Therefore, β-catenin is a well-recognized oncogene. Indeed, our preliminary experiments demonstrated that trace level and low frequency DEN

exposure resulted in aberrant activation of β-catenin, contributing to hepatocarcinogenesis. The major purpose of the present work was to study the hepatotoxic effects of trace level and low frequency DEN exposure and clearly uncover the role of β-catenin in the formation process of hepatic neoplasm induced by such an exposure. For this purpose, the effects of the specified DEN exposure were systematically investigated on the expression changes of β-catenin and other factors correlated with the inflammation, steatosis and fibrosis stages (such as TNF-α, COX-2, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc) in different liver lesions on the initiation and progression of liver tumors in the present study. It should be emphasized here that the DEN exposure dose and frequency were only 0.16 mmol/kg and 1 time/week, which were correspondingly much less than the amount (1.96 mmol/kg and 7 times/week) often used in the literature.

2. Materials and Methods

2.1. Animal

Sixty young male Kunming mice (18-20 g) were acclimatized for seven days under SPF conditions before the initiation of experiments. The animals were kept in a temperature controlled laboratory (22-25 ^{o}C) with a 12-hour light-dark cycle. In this study, the animals were randomly divided into two groups: control group and DEN group. After one week on a basal diet, thirty-six mice belonging to the DEN group underwent oral administration of DEN (0.16 mmol/kg body weight) in sesame oil once a week, consecutively for 10 weeks, whereas the other twenty-four mice received sesame oil (isotonic) as the control. Food and water intakes and body weights were measured daily until the mice were sacrificed at 10, 15, 20, 25, 30, and 35 weeks after the beginning of DEN oral administration. At the sacrifice, their livers were immediately collected for histology, mRNA profiling, and protein analysis. All animal procedures were performed in accordance with the Guide for Animal Experimentation, South-Central University for Nationalities and the Committee of Research Facilities for Laboratory Animal Sciences, South-Central University for Nationalities, China.

2.2. Sample collection and histological analysis

After autopsy, all liver tissues were examined grossly and weighted. The selected liver tissues were observed for gross changes, divided into pieces of about 0.1 g, snap-frozen directly in liquid nitrogen, and then stored at -80°C prior to RNA and protein isolation for RT-PCR. The remained liver tissues were preserved in 10% phosphate-buffered formalin and embedded in paraffin, followed by sectioning and hematoxylin and eosin (H&E) or Masson's trichrome staining by standard techniques. Histopathologic examinations of the liver sections were conducted by a pathologist and peer-reviewed using a Nikon 50i light microscope (Nikon Inc, Tokyo, Japan).

2.3. Immunohistochemical staining and multispectral imaging analysis of β-catenin expression

Briefly, four-micron sections of liver tissues were incubated overnight at 4°C with rabbit anti-β-catenin primary antibody (dilution of 1:300; Cayman chemical, Michigan, USA) overnight at 4°C. After washing, the sections were incubated with appropriate biotin-conjugated secondary antibody (dilution of 1:100; Santa Cruz Biotechnology, CA, USA) for 30 minutes at room temperature. The color development (brown) was performed using a DAB substrate kit (Nichirei, Tokyo, Japan), and the sections were counterstained with hematoxylin (blue). Then, multispectral imaging analysis of sections were performed by using a Nikon 50i light microscope (Nikon) with a Nuance Multispectral Imaging System (Cambridge Research and Instrumentation Inc., Woburn, MA) according to the method instructions.[26] Spectral optical density data were automatically acquired from 420–720 nm in 10 nm increments. Spectral unmixing was accomplished by Nuance software v1.42 and pure spectral libraries of individual chromogens. Nonspecific background staining was subtracted from each image individually. For the quantification in each experiment, three equal-sized fields of each photograph per group were randomly chosen.

2.4. Quantitative real-time RT-PCR

Total RNA was harvested from the liver tissues with RNAiso Plus (TaKaRa, Dalian,

China), followed by cDNA synthesis according to the manufacturer instructions with PrimeScriptII 1ststrand cDNA synthesis kit (TaKaRa). Quantitative real-time PCR was performed on a Thermal Cycler Dice TP800 system (TaKaRa Bio, Japan) using SYBR Premix Ex Taq II (Takara) with 40 cycles of 95 ºC for 5 s and 60 ºC for 30 s. *GAPDH* was used as an internal standard. The primer pairs used in the present study were showed in Table 1.

2.5. Immunohistochemical staining of factors associated with the inflammation, steatosis, fibrosis, and cancer stages in different liver lesions

Briefly, four-micron sections of liver tissues were incubated overnight at 4°C with associated primary antibodies (mouse anti COX-2 polyclonal antibody, dilution of 1:300, Cayman chemical, Michigan, USA; rabbit anti AP-2 polyclonal antibody, dilution of 1:400, Boster, Wuhan, China; rabbit anti PPAR-γ polyclonal antibody, dilution of 1:400, Boster; rabbit anti TGF-β1 polyclonal antibody, dilution of 1:400, Boster; Boster; rabbit anti Smad-2 polyclonal antibody, dilution of 1:200, Boster; Boster; rabbit anti HGF polyclonal antibody, dilution of 1:200, Boster; mouse anti GAPDH monoclonal antibody, dilution of 1:1000, Boster) overnight at 4^{o}C. After washing, the sections were incubated with appropriate biotin-conjugated secondary antibodies (dilution of 1:100; Santa Cruz Biotechnology, CA, USA) for 30 minutes at room temperature. The color development (brown) was performed using a DAB substrate kit (Nichirei), and the sections were counterstained with hematoxylin (blue). Then, imaging analysis of sections was performed by using a Nikon 50i light microscope imaging system (Nikon).

Western blot analysis

The cell cytoplasmic and nuclear proteins were extracted from cultured HepG2 cells using a Nucl-Cyto-Mem Preparation Kit (Applygen, Beijing, China). Primary antibodies were raised against with specific rabbit anti-β-catenin primary polyclonal antibody (1:1000 dilution; Cayman) and rabbit anti-GAPDH polyclonal antibody (1:1000 dilution; Boster). The horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution; rabbit polyclonal anti-immunoglobulin G; Cell

Signaling Technology, Beverly, MA) were used for chemiluminescence detection according to the manufacturer's instructions, respectively.

2.6. Statistical analysis

GraphPad Prism 5 was used for all statistical analyses. All data are presented as mean ± S.E.M. Differences were analyzed using one- or two-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test or Bonferroni post hoc test, with p values < 0.05 considered to be statistically significant.

3. Results

3.1. Histopathological changes in the liver tissues of the DEN-treated mouse

DEN and control male mice received a single-dose oral administration of DEN (0.16 mmol/kg) or sesame oil (isotonic) once a week, consecutively for 10 weeks, respectively. The changes in body weight of the mice were measured daily until the day of sacrifice. The specified DEN exposure in a mode of trace amount and low frequency significantly reduced the body weights of the tested mice and increased their liver weights in comparison with the control group (Fig. 1A and B). The changes in the body and liver weights of the mice suggest that the "trace level and low frequency" DEN exposure is a primary factor to induce liver injuries towards neoplasm in the progression of liver tumor as observed in the present work.

After the oral administration, each experimental mouse bearing specific liver disease(s) was analyzed and compared (Fig. 2A and B). Initially, from the first week to 35th week, liver inflammation frequently (61%; 22/36) occurred in the forms such as cellular swelling, necrosis and inflammatory infiltration. During the stage from the 10th to the 35th week, a gradual increase (17% to 83%) in liver fatty change of the DEN group occurred, such as macrovesicular fat within hepatocytes compared to the control. In addition, from the 20th to the 35th week, liver fibrosis occurred infrequently (20.8%; 5/24) and weakly with extensive collagen deposition and pseudolobular formation, whereas liver cancer (62.5%; 15/24) occurred frequently with an increased nuclear-to-cytoplasmic index, enlarged and hyperchromatic nuclei, and expansive

growth. From the 20th to the 35th week, increases in the number and size of cancerous nodules were obviously observed on the surface of the livers in DEN group, but not in the control group.

3.2. Expressions of important factors associated with the inflammation, steatosis, fibrosis, and cancer stages in liver tissues during the DEN-induced tumor formation

To further assess the histological phases of liver tissues observed above, we investigated mRNA and protein changes of several important factors associated with the inflammation (*COX-2* and *TNF-α*), steatosis (*PPAR-γ* and *AP-2*), fibrosis (*Smad-2* and *TGF-β1*), and tumor (*C-myc*) stages in different liver lesions. As shown in Fig. 3A, the trace level and low frequency DEN exposure caused gradual increases in mRNA levels of *COX-2* (approximately 230% to 380% of the control), *TNF-α* (approximately 280% to 590% of the control), *PPAR-γ* (approximately 160% to 380% of the control), and *AP-2* (approximately 170% to 400% of the control) in liver tissues on the 10th to the 35th week. Consistently, the trace level and low frequency DEN exposure induced delayed increases in mRNA levels of *Smad-2* (approximately 106% to 180% of the control) and *TGF-β1* (approximately 104% to 280% of the control). Similar to the expression pattern of *TNF-α* mRNA, *HGF* (approximately 180% to 430% of the control) and *C-myc* (approximately 100% to 590% of the control) mRNA levels were dramatically increased in mice treated with DEN in comparison to that in control group. Maximal mRNA levels of both *HGF* and *c-myc* were obviously observed at 25th or 30th week after the beginning of DEN oral administration, respectively.

Moreover, the immunohistochemistry experiments confirmed that the protein expression levels of *COX-2*, *AP-2*, *PPAR-γ*, *TGF-β1*, *Smad-2* and *HGF* were also up-regulated in accordance with their mRNA levels during the trace level and low frequency DEN exposure as shown in Fig. 3B.

3.3. Aberrant changes of β-catenin in different liver lesions during the DEN-induced tumor formation

To investigate whether aberrant activation of β-catenin initiates inflammation towards steatosis, fibrosis and neoplasm during the tumor formation induced by trace level and low frequency DEN exposure, the mRNA and protein expressions of β-catenin were firstly examined at the 10^{th}, 15^{th}, 20^{th}, 25^{th}, 30^{th}, and 35^{th} week after the beginning of DEN oral administration. As shown in Fig. 4, both mRNA level and protein expression of β-catenin (approximately 300-600% and 5000-17000% of the control, respectively) were dramatically increased in mice after the DEN exposure in comparison to those in control group at same time point. Maximal protein levels of both total and cytoplasm/membrane β-catenin (approximately 17000% and 45000% of the control, respectively) were obviously observed at the 25^{th} week after the beginning of DEN oral administration, while a maximal protein level of nuclear β-catenin (approximately 2800% of the control) was observed at the 30^{th} week. Namely, there was a differential distribution in the level of β-catenin expression between different stages during the development of neoplasm. All of these results suggest that cytoplasmic accumulation and nucleus translocation of β-catenin is associated with liver inflammation, steatosis, fibrosis, and neoplasm.

3.4. Different expressions of β-catenin and several factors associated with the inflammation, steatosis, fibrosis, and tumor stages in tumor and adjacent non-tumor liver tissues

Based on the results described above, the trace level and low frequency DEN exposure induced early high-expressions of β-catenin protein and its mRNA accompanied with the histopathological changes of liver tissues: such as inflammation, steatosis, fibrosis, and neoplasm. To more-deeply assess a possible role of β-catenin in the initiation and promotion of liver histopathological changes induced by the DEN exposure, we examined the expression of β-catenin of tumor and adjacent non-tumor liver tissues from DEN-treated mice by immunohistochemical staining and multispectral imaging analysis. In Fig. 5A, we found that the expression of β-catenin was much higher in the tumor region. We also observed a highly significant increase of *β-catenin* mRNA level in tumor liver tissues in comparison to adjacent non-tumor

liver tissues (Fig. 5B).

To investigate the possible molecular mechanism of tumor formation, we further examined mRNA changes of several important factors associated with the inflammation (*COX-2* and *TNF-α*), steatosis (*PPAR-γ* and *AP-2*), fibrosis (*Smad-2* and *TGF-β1*), and cancer (*HGF* and *C-myc*) stages in tumor and adjacent non-tumor liver tissues from the mice treated by trace level and low frequency DEN exposure. As shown in Fig. 5C, significant increases in mRNA levels of *COX-2*, *TNF-α*, *PPAR-γ*, *AP-2*, *Smad-2*, *TGF-β1*, and *C-myc* were observed in tumor liver tissues from DEN-treated mice compared to the adjacent non-tumor region. These results were in accordance with the change of *β-catenin* mRNA level, suggesting the involvement of *β-catenin* and these factors in the initiation and promotion of tumor. The only exception was *HGF*, which showed significant decrease in mRNA level. *HGF* is secreted by mesenchymal cells and acts as a multi-functional cytokine to play a central role in angiogenesis, tumorogenesis, and tissue regeneration.[27] Therefore, we further assessed amount of mesenchymal cells in liver tissues from DEN-treated mice and observed a significant decrease in tumor regions of neoplastic mice (Fig. 6). This explained in part the decrease of mRNA level of *HGF*.

3.5. Relationship between nuclear β-catenin accumulation and tumor formation

To further characterize the role of β-catenin accumulation in tumor formation, we investigated the protein distribution of β-catenin in the tumor region. It was found that the ratios of both MP/CP and nuclear β-catenin-positive cells in tumor liver tissues were significantly greater than those in adjacent non-tumor liver tissues (Fig. 7A). Moreover, there were significant increases in numbers of both HCC cell mitosis and microscopic nodule with nuclear β-catenin accumulation (Fig. 7B). Interestingly, by searching in GenBank, we found that the nearby upstream domains of *COX-2*, *TNF-α*, *PPAR-γ* and *AP-2*, *Smad-2*, *TGF-β1* and *C-myc* contain the β-catenin/Tcf-Lef consensus binding site sequence (5'-CTTTT/G-3' or 5'-CAAAG-3'; Fig. 7C). In accordance with this notion, except for *HGF*, the mRNA levels of other seven factors, *COX-2*, *TNF-α*, *PPAR-γ*, *AP-2*, *Smad-2*, *TGF-β1*, and *C-myc*, were greater in nuclear

β-catenin-positive liver tissues than those in nuclear β-catenin-negative liver tissues (Fig. 7D).

3.6. Activation of β-catenin and induction of several factors described above in Wnt 3a-treated HepG2 cells

To clarify the impact of β-catenin on the expressions of those genes, we mimicked the activation of β-catenin with an agonist of β-catenin transcriptional activity (Wnt 3a, 100 ng/ml) in cultured HepG2 cells. As shown in Fig. 8A, the mRNA level of β-catenin increased (up to 154% of the control) in a time-dependent manner after Wnt 3a treatment. Consistent with mRNA level, β-catenin protein level of either cytoplasm, nuclear or total fractions from cultured HepG2 cells was also increased by the Wnt 3a treatment in a time-dependent manner (up to 337%, 161% and 142% of the control, respectively; Fig. 8B and C).

Furthermore, mRNA levels of several factors including *COX-2*, *TNF-α*, *PPAR-γ* and *AP-2*, *Smad-2*, *TGF-β1* and *HGF*, *C-myc* in cultured HepG2 cells treated with Wnt 3a. As expected, exposure of HepG2 cells to Wnt 3a also induced significant increases (Fig. 8D) in mRNA levels of *COX-2* (up to 150% of the control at 0 h), *TNF-α* (up to 266% of the control at 0 h), *PPAR-γ* (up to 136% of the control at 0 h), and *AP-2* (up to 126% of the control at 0 h), *Smad-2* (up to 132% of the control at 0 h) and *TGF-β1* (up to 159% of the control at 0 h), *HGF* (up to 178% of the control at 0 h) and *C-myc* (up to 164% of the control at 0 h), respectively.

4. Discussion

The present study focused on the hepatotoxic effects of the trace level and low frequency DEN exposure and the role of β-catenin in the process of hepatic neoplasm formation. DEN, one of the most important potential carcinogens, has been detected frequently in foods. Because the liver is the first detoxification organ, and very sensitive to dietary pollutants,[28] it is prominent to investigate the hepatotoxic effects of DEN.

Previous studies have demonstrated that DEN exposure may induce hepatic

neoplasm.[19-22] In liver cancer researches with experimental animals, DEN is used either as a complete carcinogen or as an initiator in multistage models. When used as a tumor initiator, DEN is usually given at a single dose of 1.96 mmol/kg, which can induce pronounced liver necrosis.[29,30] The relevant mechanism is thought to be as follows: (1) DEN is bioactivated by metabolic enzymes CYP2E1 and CYP2A5 in the liver; (2) After DEN is bioactivated to an ethyldiazonium ion, it undergoes a reaction with DNA bases to form adducts; (3) Ethyl DNA adducts can interrupt base pairing, resulting in mutations and the consequent activation of proto-oncogenes and inhibition of tumor-suppressor genes, which often result in HCC.[31] However, the exposure concentration of DEN in the past studies was usually massive, which cannot simulate the actual human exposure conditions. Generally speaking, people will not be easily exposed to such a high concentration of DEN. It is necessary to clarify what will happen if the DEN exposure is carried out at trace level of DEN with low exposure frequency. Therefore, the aim of this study is to investigate the hepatotoxic effects of trace level and low frequency DEN exposure and the underlying molecular mechanisms.

DEN can be produced by the transformation of nitrite through many ways,[32,33] and DEN has been detected in foods frequently.[15-17] When humans are accustomed to eat pickles and bacon, their DEN exposure risk will be increased drastically because such pickled foods often contain high level nitrites.[34] It was recently reported that the nitrite content in overnight foods increased significantly. It is certain that DEN will be formed during the production and storage of nitrite preserved foods. The levels of DEN in nitrite preserved foods may be as high as in the order of thousands μg kg^{-1}.[35] The dosage of DEN in this paper is 0.16 mmol/kg body weight once a week, which can almost be close to the actual exposure level. The results from the present study shows that trace level and low frequency DEN exposure can also induce liver injuries or even tumor formation (Fig. 2A and B), which is particularly dangerous because people tend to ignore its hepatotoxic effects. Therefore, trace level and low frequency DEN exposure is worth more attentions.

As β-catenin is clearly of essence in several physiological events such as

development, regeneration, and growth,[24] a detailed characterization of expression changes of β-catenin during the DEN-induced tumor formation is very important in order to understand the mechanisms of carcinogenesis. The contribution of inflammation to carcinogenesis has received major attention in hepatocarcinogenesis because more than 90% of HCCs develop in the context of chronic liver damage and inflammation.[36] It has been shown that prolonged inflammation can increase the cancer risk by accumulating genetic and epigenetic damage.[37] We therefore investigated whether inflammation was a component of β-catenin–induced tumorigenesis after trace level and low frequency DEN exposure. The results from the present study showed that over-expression and nuclear translocation of β-catenin initiate inflammation towards steatosis, fibrosis and carcinogenesis during the trace level and low frequency DEN-induced hepatocarcinogenesis.

The histological changes in DEN-induced liver cancer in mice are similar to those seen in human hepatic neoplasm. Therefore, studying animal models of liver tumor provide reliable data on the primal biology of liver tumorigenesis.[38] In the present study, we have investigated the pathologic changes of livers in mice after trace level and low frequency DEN exposure included non-specific injuries, regeneration and repair, fibrosis, and cirrhosis, dysplastic nodules, early tumorous nodules, advanced tumorous nodules and metastasis foci, better reflecting the true process of human hepatic neoplasm (Fig. 3A and B).

Many pathways broadly categorized into Ras/MAPK, PIK3CA/AKT, and Wnt/β-catenin signaling, have been shown to be of signaling in HCC. β-catenin, the central orchestrator of Wnt signaling, is a known oncogene due to its implications in a variety of cancer, including 20%-40% of all HCCs. Recently, the activity of Wnt/β-catenin have also been identified in human liver diseases associated with a high incidence of HCC or cholangiocarcinoma (such as chronic viral and alcoholic hepatitis, sclerosing cholangitis).[23,39] Our data support such a role for Wnt/β-catenin signaling in the development of hepatic neoplasm after DEN exposure. In this study, we showed that mRNA and protein levels of β-catenin were significantly higher in livers with non-specific injuries (Fig. 4A and B), suggesting an association between

β-catenin and the chronic inflammation of liver. Similar results were also observed in livers with fatty change and fibrosis, suggesting β-catenin is correlated with steatosis and fibrosis as well. Meanwhile, mRNA and protein expression levels of several pivotal risk factors (including TNF-α, COX-2, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc) were also up-regulated. Moreover, the fact that the mRNA level of β-catenin was significantly higher in tumor areas than in non-tumor areas suggesting a role of β-catenin in the initiation and formation of liver tumor after trace level and low frequency DEN exposure (Fig. 5).

Abnormal expression of β-catenin were identified in numerous human neoplasm and correlated with tumor metastasis.[40,41] It has been shown that the accumulation of β-catenin in cytosol and translocation of activated β-catenin into nucleus could lead to hepatocarcinoma.[42] It has also been shown that COX-2 is down-regulated by APC and up-regulated by β-catenin in HuH7, hepatocellular carcinoma cell line.[43] We found that the translocation of β-catenin into the nucleus was also positively correlated with the develop stages of hepatic neoplasm after trace level and low frequency DEN exposure. Consequently, in non-specific injury and inflammation stages (10^{th} week to 15^{th} week) there were significantly higher levels of β-catenin in the cytoplasm and significantly less membrane-bound β-catenin than the control group. In these stages, β-catenin was mostly concentrated in the cytoplasm, although some was located in the nucleus. In tumor stage (25^{th} week to 35^{th} week), there were significantly higher levels of β-catenin in the nucleus than the inflammation, steatosis and fibrosis stage liver cells (Fig. 7A).

Moreover, we also observed a co-relation between nucleus located β-catenin and high expressions of several pivotal risk factors (including TNF-α, COX-2, PPAR-γ, AP-2, Smad-2, TGF-β1, and C-myc; Fig. 7D). Together with the fact that the β-catenin/Tcf-Lef consensus binding site sequence could be found in the upstream domains of these factors, we speculated that nucleus localization of β-catenin turned on the expression of these risk factors. In accordance with this speculation, Wnt 3a treatment in HepG2 cells successfully stimulated the expression and nucleus localization of β-catenin, and hence the expression of these risk factors (Fig. 8).

5. Conclusions

We have successfully demonstrated the hepatotoxic effects of trace level and low frequency DEN exposure and the underlying molecular mechanisms for the formation of tumors. Once the liver subjected to the chronic injury under the trace level and low frequency DEN exposure, the Wnt/β-catenin pathway is over activated and translocated into nucleus. These nucleus localized β-catenin will gradually initiate and promote the formation of tumor. For these reasons, we conclude that trace level and low frequency DEN exposure can induce liver injuries even tumor formation and β-catenin plays a key role during the DEN-induced hepatocarcinogenesis. Therefore, trace level and low frequency DEN exposure is worth more attention for its neglected influence on health.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

1 B. Hennig, L. Ormsbee, C.J. McClain, *et al.*, Nutrition can modulate the toxicity of environmental pollutants: implications in risk assessment and human health, *Environ. Health Perspect.*, 2012, **120**, 771-774.

2 Y.Y. Hou, Y. Wang, H.H. Wang and Y.Y. Xu, Induction of glutathione synthesis in human hepatocytes by acute and chronic arsenic exposure: Differential roles of mitogen-activated protein kinases, *Toxicology*, 2014, **325**, 96-106.

3 H. Hanafusa, Y. Morikawa, T. Uehara, *et al.*, Comparative gene and protein expression analyses of a panel of cytokines in acute and chronic drug-induced liver injury in rats, *Toxicology*, 2014, **324**, 43-54.

4 U. Blaschke, A. Paschke, I. Rensch and G. Schüürmann, Acute and chronic toxicity toward the bacteria vibrio fischeri of organic narcotics and epoxides: structural alerts for epoxide excess toxicity, *Chem. Res. Toxicol.*, 2010, **23**, 1936-1946.

5 V.W. Bao, G.C. Lui and K.M. Leung, Acute and chronic toxicities of zinc pyrithione alone and in combination with copper to the marine copepod Tigriopus japonicas, *Aquat Toxicol.*, 2014, **157**, 81-93.

6 J. Ahlers, C. Riedhammer, M. Vogliano, R.U. Ebert, R. Kühne and G. Schüürmann, Acute to chronic ratios in aquatic toxicity-variation across trophic levels and relationship with chemical structure, *Environ. Toxicol. Chem.*, 2006, **25**, 2937-2945.

7 B. Antizar-Ladislao, Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review, *Environ. Int.*, 2008, **34**, 292-308.

8 H.R. Foster, G.A. Burton, N. Basu and E.E. Werner, Chronic exposure to fluoxetine (Prozac) causes developmental delays in Rana pipiens larvae, *Environ. Toxicol. Chem.*, 2010, **29**, 2845-2850.

9 X.M. Zou, Z.F. Lin, Z.Q. Deng, D.Q. Yin and Y.L. Zhang, The joint effects of sulfonamides and their potentiator on Photobacterium phosphoreum: Differences between the acute and chronic mixture toxicity mechanisms, *Chemosphere*, 2012, **86**, 30-35.

10 R. Kovács, Z. Csenki, K. Bakos, *et al.*, Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (Danio rerio) two-generation study, *Water Res.*, 2015, **77**, 201-212.

11 S. González-Mancebo, J. Gaspar, E. Calle, *et al.*, Stereochemical effects in the metabolic activation of nitrosopiperidines: correlations with genotoxicity, *Mutat. Res.*, 2004, **558**, 45-51.

12 A.P. Keszei, R.A. Goldbohm, L.J. Schouten, P. Jakszyn and P.A. van den Brandt, Dietary N-nitroso compounds, endogenous nitrosation, and the risk of esophageal and gastric cancer subtypes in the Netherlands Cohort Study, *Am. J. Clin. Nutr.*, 2012, ajcn-043885.

13 X.T. Jin, M.L. Chen, L. Song, H.Q. Li and Z.Y. Li, The evaluation of p, p'-DDT exposure on cell adhesion of hepatocellular carcinoma, *Toxicology*, 2014, **322**, 99-108.

14 N.P. Santos, A. Colaco, R.M.G. da Costa, M.M. Oliveira, F. Peixoto and P.A. Oliveira, N-diethylnitrosamine mouse hepatotoxicity: time-related effects on histology and oxidative stress, *Exp. Toxicol. Pathol.*, 2014, **66**, 429-436.

15 F. Shahidi, R.B. Pegg and N.P. Sen, Absence of volatile N-nitrosamines in cooked nitrite-free cured muscle foods, *Meat Sci.*, 1994, **37**, 327-336.

16 S. Yurchenko and U. Mölder, Volatile N-nitrosamines in various fish products, *Food chem.*, 2006, **96**, 325-333.

17 S. Yurchenko and U. Mölder, The occurrence of volatile N-nitrosamines in Estonian meat products, *Food Chem.*, 2007, **100**, 1713-1721.

18 R. Peto, R. Gray, P. Brantom and P. Grasso, Nitrosamine carcinogenesis in 5120 rodents: chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age of starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters). *IARC Sci. Publ.*, 1983, **57**, 627-665.

19 K. Pradeep, C.V.R. Mohan, K. Gobianand and S. Karthikeyan, Silymarin modulates the oxidant–antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats, *Eur. J. Pharmacol.*, 2007, **560**, 110-116.

20 N.P. Santos, I.C. Pereira, M.J. Pires, *et al.*, Histology, bioenergetics and oxidative stress in mouse liver exposed to N-diethylnitrosamine, *In Vivo*, 2012, **26**, 921-929.

21 K. Inami, S. Ishimura, Y. Akaike, *et al.*, Oxidation products of N-nitrosodialkylamines generated by Fenton's reagent in the presence of copper are direct acting mutagens, *J. Health Sci.*, 2010, **56**, 576-580.

22 E. Filipski, P. Subramanian, J. Carriere, C. Guettier, H. Barbason and F. Levi, Circadian disruption accelerates liver carcinogenesis in mice, *Mutat. Res.*, 2009, **680**, 95-105.

23 J. Behari, The Wnt/β-catenin signaling pathway in liver biology and disease. *Expert Rev. Gastroenterol. Hepatol.*, 2010, **4**, 745-756.

24 M.D. Thompson and S.P. Monga, WNT/β-catenin signaling in liver health and disease. *Hepatology*, 2007, **45**, 1298-1305.

25 K. Taniguchi, L.R. Roberts, I.N. Aderca, *et al.*, Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. *Oncogene*, 2002, **21**, 4863-4871.

26 Y.S. Li, J.X. Wang, M.M. Jia, M. Liu, X.J. Li and H.B. Tang, Dragon's blood inhibits chronic inflammatory and neuropathic pain responses by blocking the synthesis and release of substance P in rats, *J. Pharmacol. Sci.*, 2012, **118**, 43-54.

27 M. Gos, J. Joanna Miłoszewska, M. Przybyszewska, Epithelial-mesenchymal transition in cancer progression, *Postepy Biochem.*, 2009, **55**, 121-128.

28 C. Gasnier, C. Dumont, N. Benachour, E. Clair, M.C. Chagnon and G.E. Séralini, Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines, *Toxicology*, 2009, **262**, 184-191.

29 D.J. Liao, A. Blanck, P. Eneroth, J.A. Gustafsson and I.P. Hällström, Diethylnitrosamine causes pituitary damage, disturbs hormone levels, and reduces sexual dimorphism of certain liver functions in the rat, *Environ. Health Perspect.*, 2001, **109**, 943-947.

30 D. Solt and E. Farber, New principle for the analysis of chemical carcinogenesis, *Nature*, 1976, **263**,701-703.

31 N. Matsunaga, Y. Kohno, K. Kakimoto, A. Hayashi, S. Koyanagi and S. Ohdo, Influence of CLOCK on cytotoxicity induced by diethylnitrosamine in mouse primary hepatocytes, *Toxicology*, 2011, **280**, 144-151.

32 A. Martelli, L. Robbiano, S. Grossi, F. Mattioli and G. Brambilla, Formation of DNA-damaging N-nitroso compounds from the interaction of calcium-channel blockers with nitrite, *Toxicology*, 2007,**238**, 211-215.

33 Y.A. Simonov, M.S. Fonari, G.G. Duca, *et al.*, Nitrosation of hydrochlorothiazide and the modes of binding of the N-nitroso derivative with two macrocycles possessing an 18-membered crown ether cavity, *Tetrahedron*, 2005, **61**, 6596-6601.

34 J.N. Sofos, F.F. Busta and C.E. Allen, Botulism control by nitrite and sorbate in

cured meats: a review, *J. Food Prot.*, 1979, **42**, 739-770.

35 S.S. Herrmann, K. Granby and L. Duedahl-Olesen, Formation and mitigation of N-nitrosamines in nitrite preserved cooked sausages, *Food Chem.*, 2015, **174**, 516-526.

36 S.I. Grivennikov, F.R. Greten and M. Karin, Immunity, inflammation, and cancer, *Cell*, 2010, **140**, 883-899.

37 M. Erreni, A. Mantovani and P. Allavena, Tumor-associated macrophages (TAM) and inflammation in colorectal cancer, *Cancer Microenviron.*, 2011, **4**, 141-154.

38 Y.F. Liu, B.S. Zha, H.L. Zhang, et al., Characteristic gene expression profiles in the progression from liver cirrhosis to carcinoma induced by diethylnitrosamine in a rat model, *J. Exp. Clin. Cancer Res.*, 2009, **28**, 1-16.

39 R. Dahmani, P.A. Just and C. Perret, The Wnt/β-catenin pathway as a therapeutic target in human hepatocellular carcinoma, *Clin. Res. Hepatol. Gastroenterol.*, 2011, **35**, 709-713.

40 A. Schmidt, A. Braeuning, P. Ruck, et al., Differential expression of glutamine synthetase and cytochrome P450 isoforms in human hepatoblastoma, *Toxicology*, 2011, **281**, 7-14.

41 M. Sharma, M. Johnson, M. Brocardo, C. Jamieson and B.R. Henderson, Wnt signaling proteins associate with the nuclear pore complex: implications for cancer, *Adv. Exp. Med. Biol.*, 2014, **773**, 353-372.

42 J. Cui, X.D. Zhou, Y.K. Liu, Z.Y. Tang and M.H. Zile, Abnormal beta-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China, *World J. Gastroenterol.*, 2001, **7**, 542-546.

43 Y. Araki, S. Okamura, S.P. Hussain, *et al.*, Regulation of cyclooxygenase-2 expression by the Wnt and ras pathways, *Cancer Res.*, 2003, **63**, 728-734.

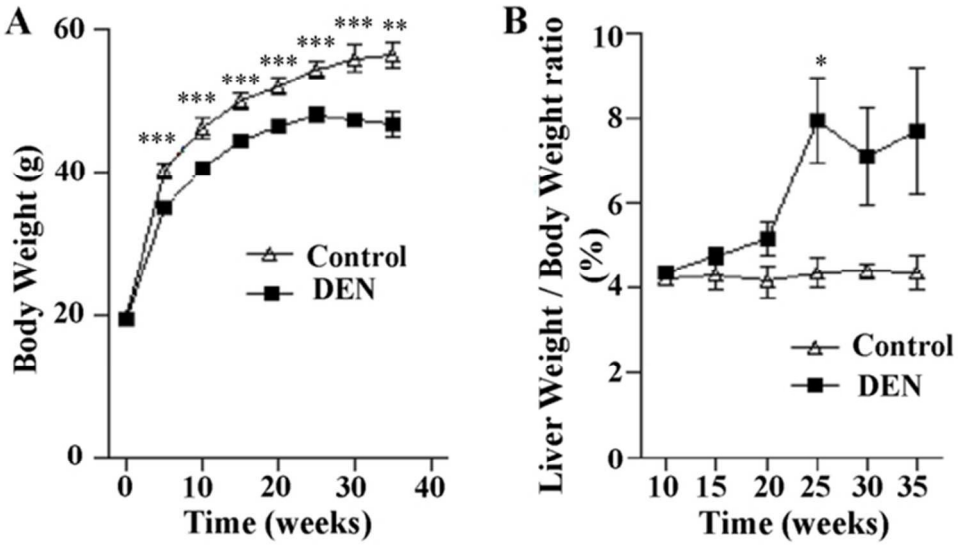

Fig. 1. Influence of trace level and low frequency DEN exposure on body weight and liver weight/body weight ratio of Kunming mice. (A) Body weight changes of Kunming mice during the whole experiment. (B) Liver weight/body weight ratio changes of Kunming mice from 10th to 35th week after the beginning of DEN exposure. *, ** and *** denote P < 0.05, 0.01 and 0.001 (two-way repeated-measures analysis of variance, followed by Bonferroni post hoc test), in comparison to respective controls, respectively.
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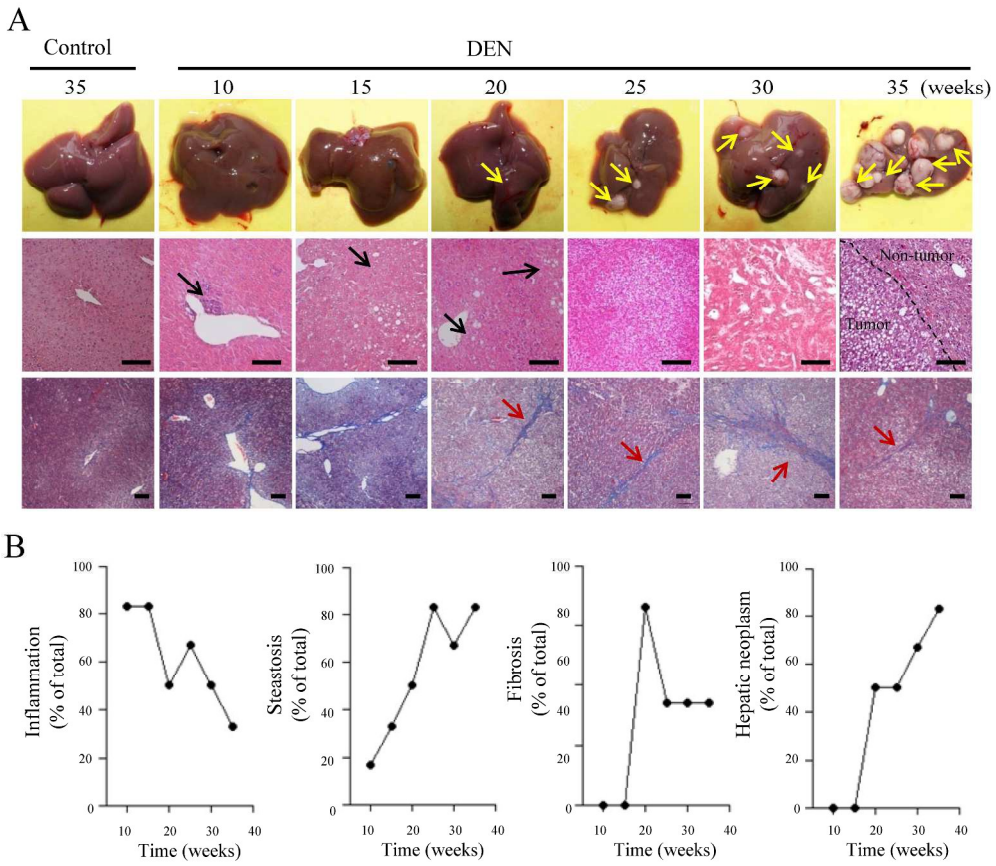

Fig. 2. The gross appearance and histological changes of livers from control and DEN-treated mice. (A) The liver tissues were harvested at 10, 15, 20, 25, 30, and 35 weeks after the beginning of trace level and low frequency DEN exposure (n = 6). Gross observation of livers from normal control (n = 4) and DEN-treated mice (yellow arrows stick to cancerous nodules). Hematoxylin and eosin (H&E) staining showing representative liver sections with inflammatory infiltration, fatty degeneration, necrotic damage, and microscopic tumor foci from DEN-treated mice (Middle panel). Masson staining showing representative liver sections from mice treated with DEN (red arrows stick to liver fibrosis; bottom panel). Scale bars: 50 μm. (B) Histological features (inflammation, steastosis, fibrosis, and tumor) comparison of liver sections from the DEN-treated mice.
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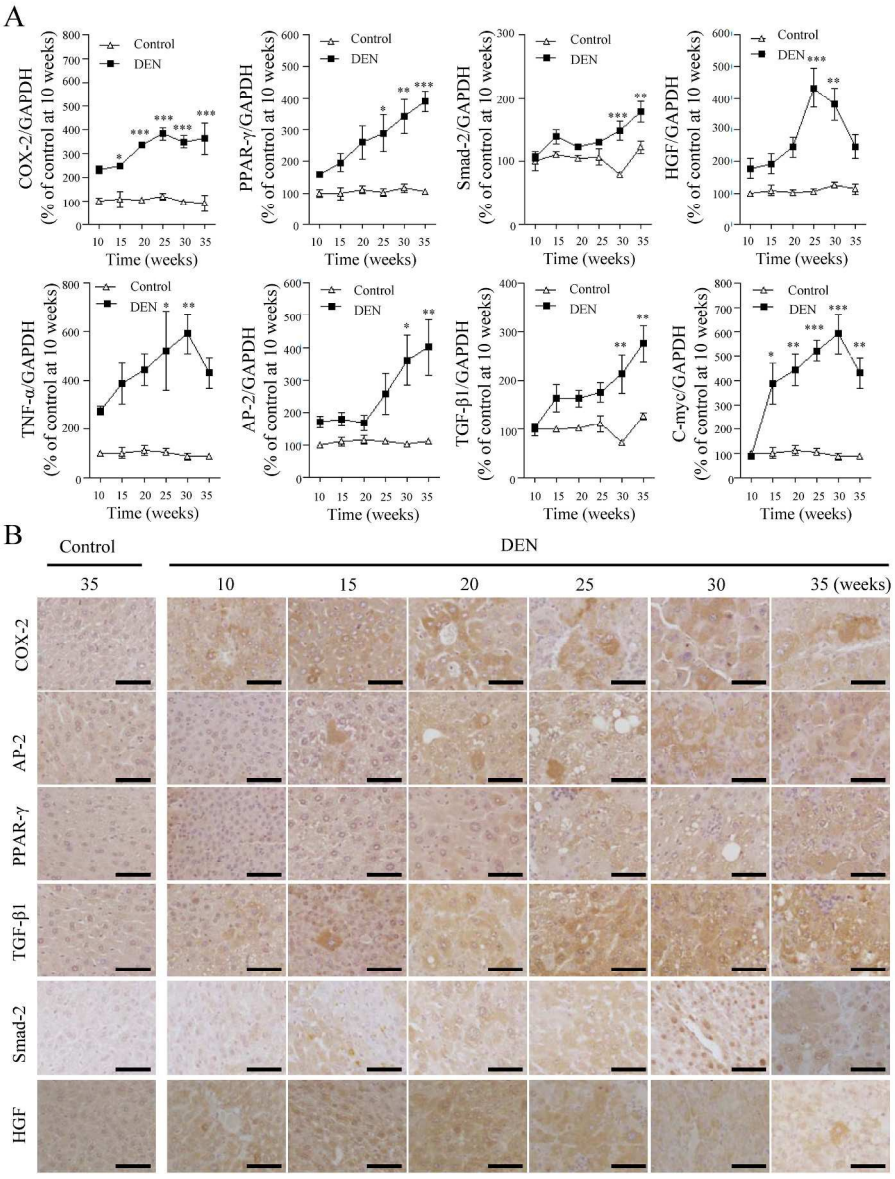

Fig. 3. Messenger RNA and protein changes of various factors associated with the inflammation, steatosis, fibrosis, and tumor stages in different liver lesions. (A) Messenger RNA expressions of COX-2, TNF-α, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc in liver tissues at 10, 15, 20, 25, 30, and 35 weeks after the beginning of trace level and low frequency DEN exposure. *, ** and *** denote $P < 0.05$, 0.01 and 0.001 (two-way repeated-measures analysis of variance, followed by Bonferroni post hoc test), in comparison to respective controls, respectively. (B) Protein expressions of COX-2, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc in liver tissues at 10, 15, 20, 25, 30, and 35 weeks after the beginning of trace level and low frequency DEN exposure. Scale bars: 50 μm.
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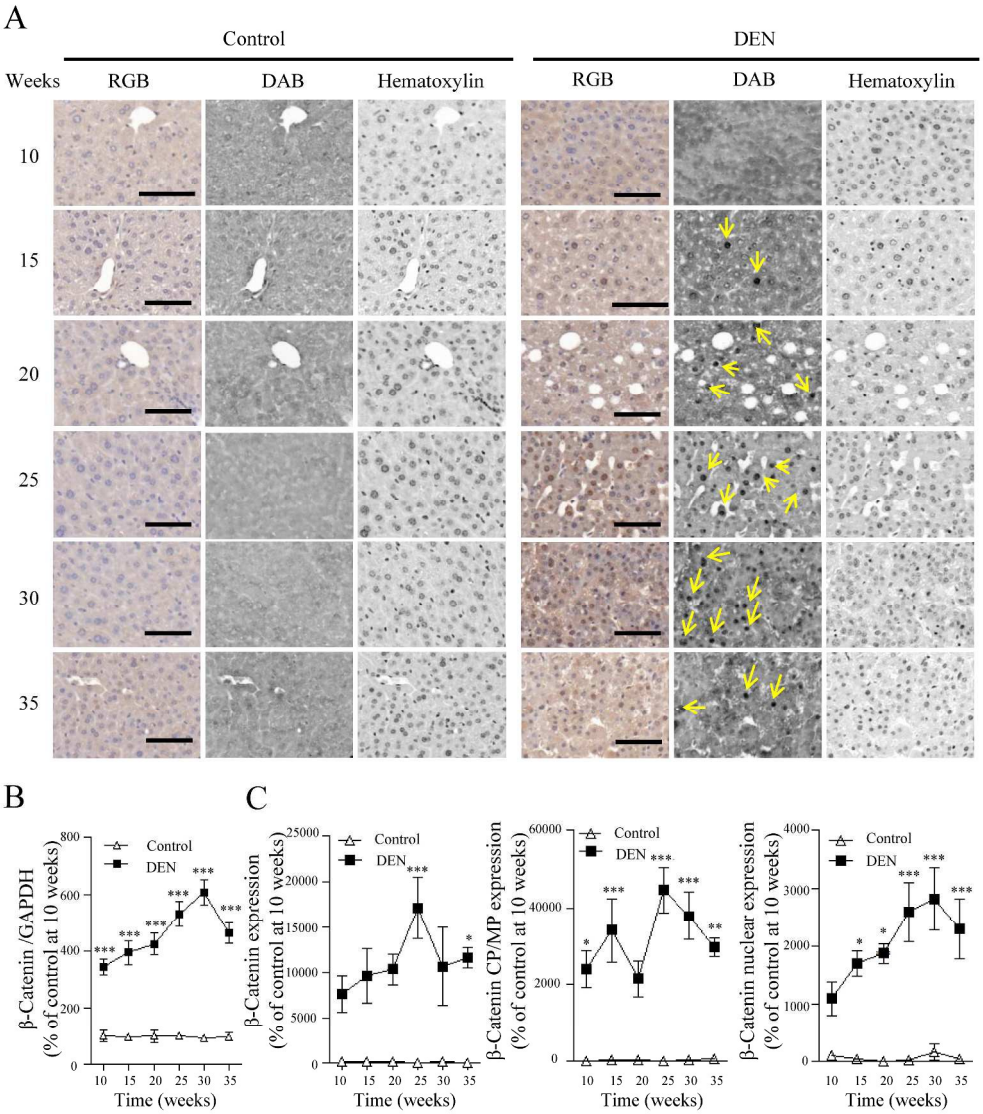

Fig. 4. Changes of β-catenin in different liver lesions of DEN-treated mice. (A) Representative multispectral images for immunohistochemical staining of β-catenin in liver tissues of control and DEN mice at 10, 15, 20, 25, 30, and 35 weeks after the beginning of trace level and low frequency DEN exposure. Brown is positive β-catenin stain and blue is hematoxylin counterstain. Left panel: RGB images of six time points; middle panel: unmixed DAB images of left panel; right panel: unmixed hematoxylin images of left panel. Scale bars: 50 μm. (B) Determination of spectral optical density of β-catenin protein expression in different liver lesions of control and DEN mice. MB, membrane; CP, cytoplasm. (C) β-Catenin mRNA expression in different liver lesions of control and DEN mice assayed by RT-PCR. *, ** and *** denote P < 0.05, 0.01 and 0.001 (two-way repeated-measures analysis of variance, followed by Bonferroni post hoc test), in comparison to respective controls, respectively.
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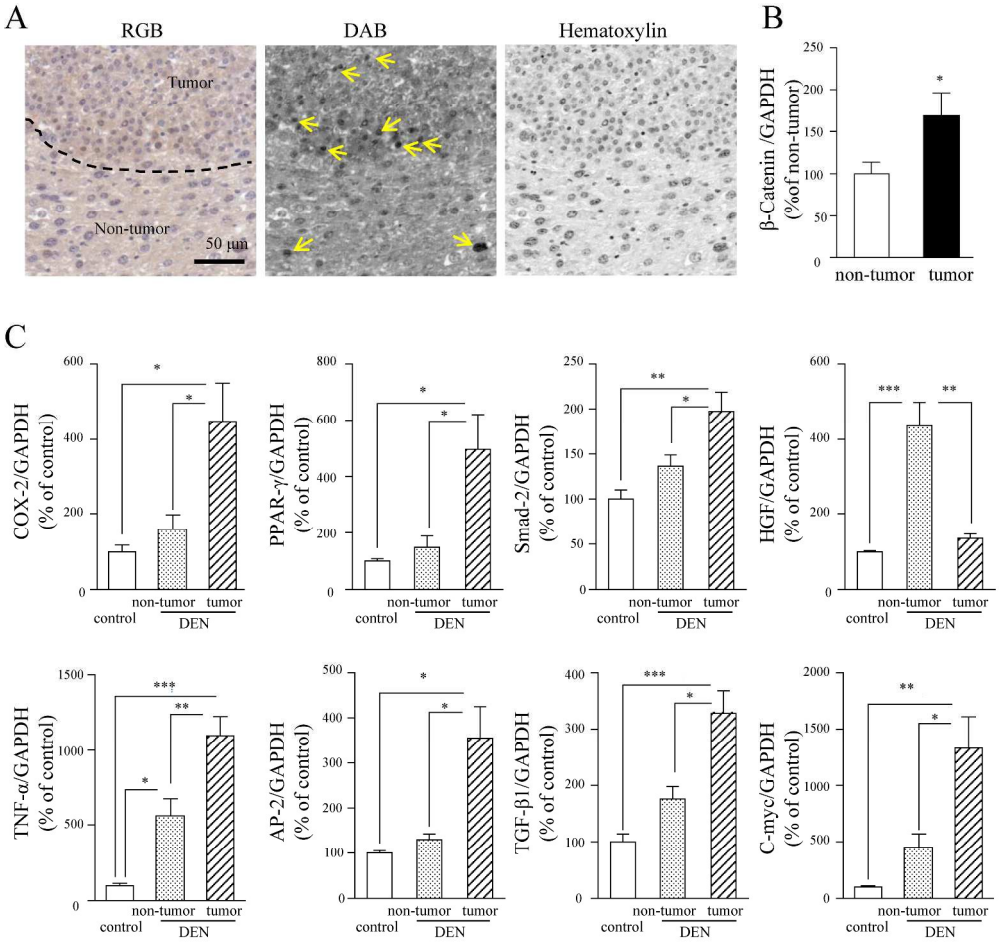

Fig. 5. Different expression of β-catenin and several factors associated with the inflammation, steatosis, fibrosis, and tumor stages in tumor and adjacent non-tumor liver tissues. (A) Representative multispectral images for immunohistochemical staining of β-catenin distrbution in tumor and non-tumor liver tissues from DEN-treated mice. The β-catenin proteins were stained to show brown color in membrane (MB), cytoplasm (CP) and nuclei. (B) β-Catenin mRNA expression in tumor and non-tumor liver tissues of (A). (C) Messenger RNA changes of COX-2, TNF-α, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc in tumor and non-tumor liver tissues from DEN-treated mice. *, ** and *** denote $P < 0.05$, 0.01 and 0.001 (two-way repeated-measures analysis of variance, followed by Bonferroni post hoc test), in comparison to respective controls, respectively.
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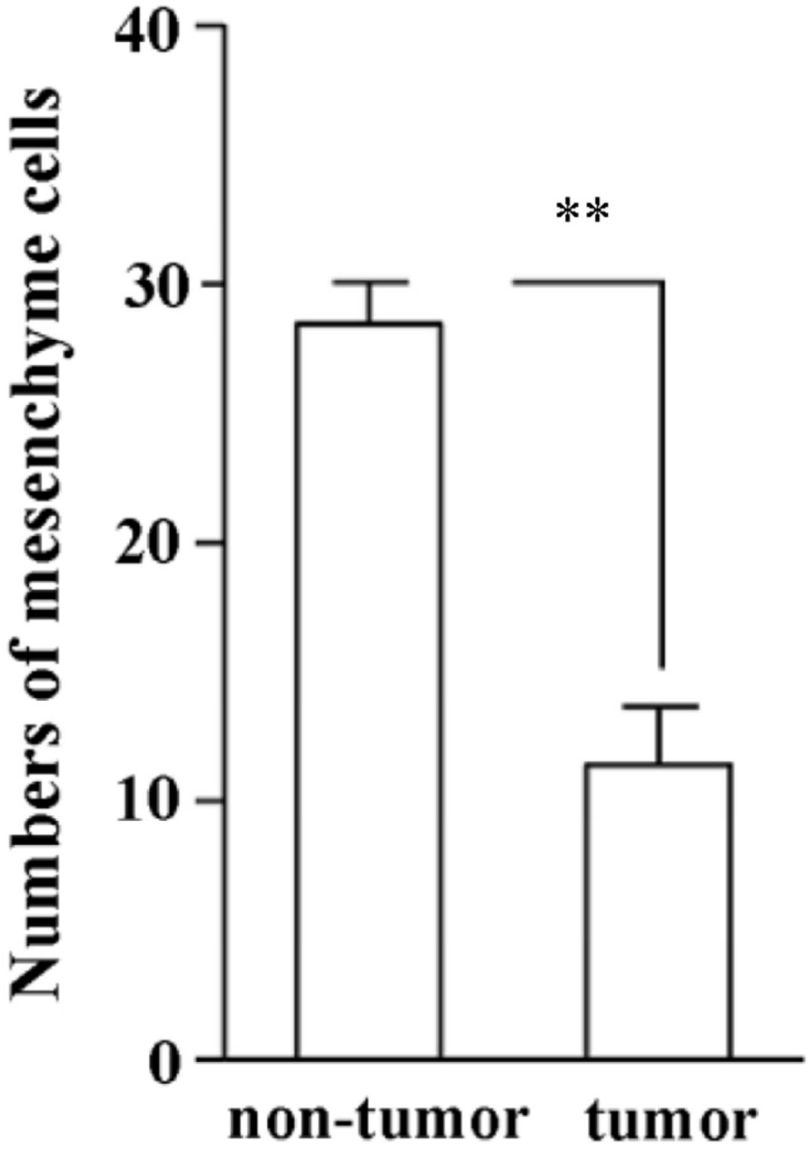

Fig. 6. Number of mesenchymal cells in liver tissues from DEN-treated mice. Determination of mesenchymal cell numbers in tumor and non-tumor region. ** denotes P < 0.01.
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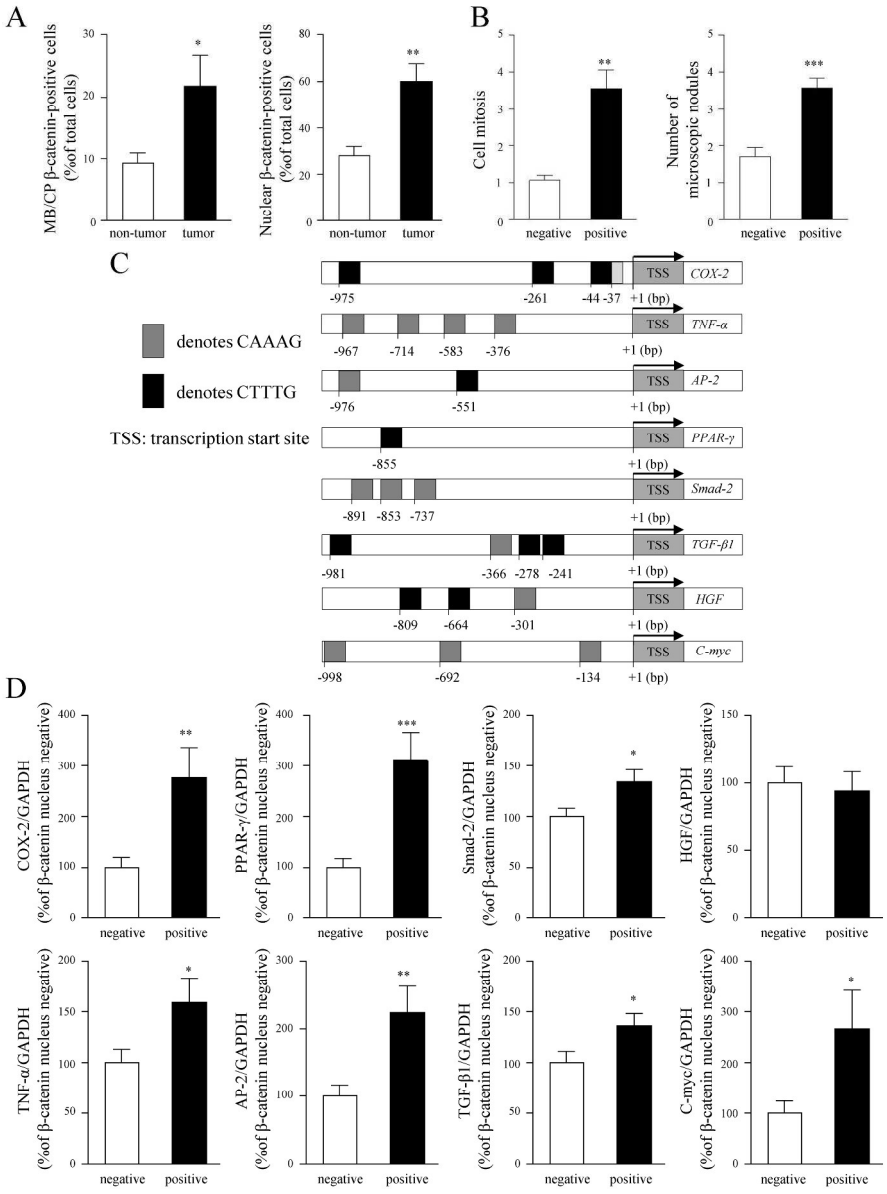

Fig. 7. Cell mitosis and high expression of several factors associated with the inflammation, steatosis, fibrosis, and tumor stages is connected to the protein distribution of β-catenin in mouse hepatic neoplasm induced by trace level and low frequency DEN exposure. (A) Determination of spectral optical density of β-catenin protein expression in tumor and non-tumor liver tissues. MB, membrane; CP, cytoplasm. (B) Determination of cell mitosis and microscopic nodule numbers in hepatic neoplasm. (C) β-Catenin/Tcf-Lef consensus binding site sequences (5′-CAAAG-3′, 5′-CTTTT-3′ and 5′-CTTTG-3′) located at the nearby upstream domain of mouse genes (including COX-2, TNF-α, AP-2, PPAR-γ, samd-2, TGF-β1, HGF, and c-myc) by searching GenBank. (D) Determination of mRNA expressions for COX-2, TNF-α, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc with β-catenin nucleus negative or positive in hepatic neoplasm. *, ** and *** denote $P < 0.05$, 0.01 and 0.001 (one-way analysis of variance, followed by Newman-Keuls post hoc test), in comparison to respective controls, respectively.
254x338mm (300 x 300 DPI)

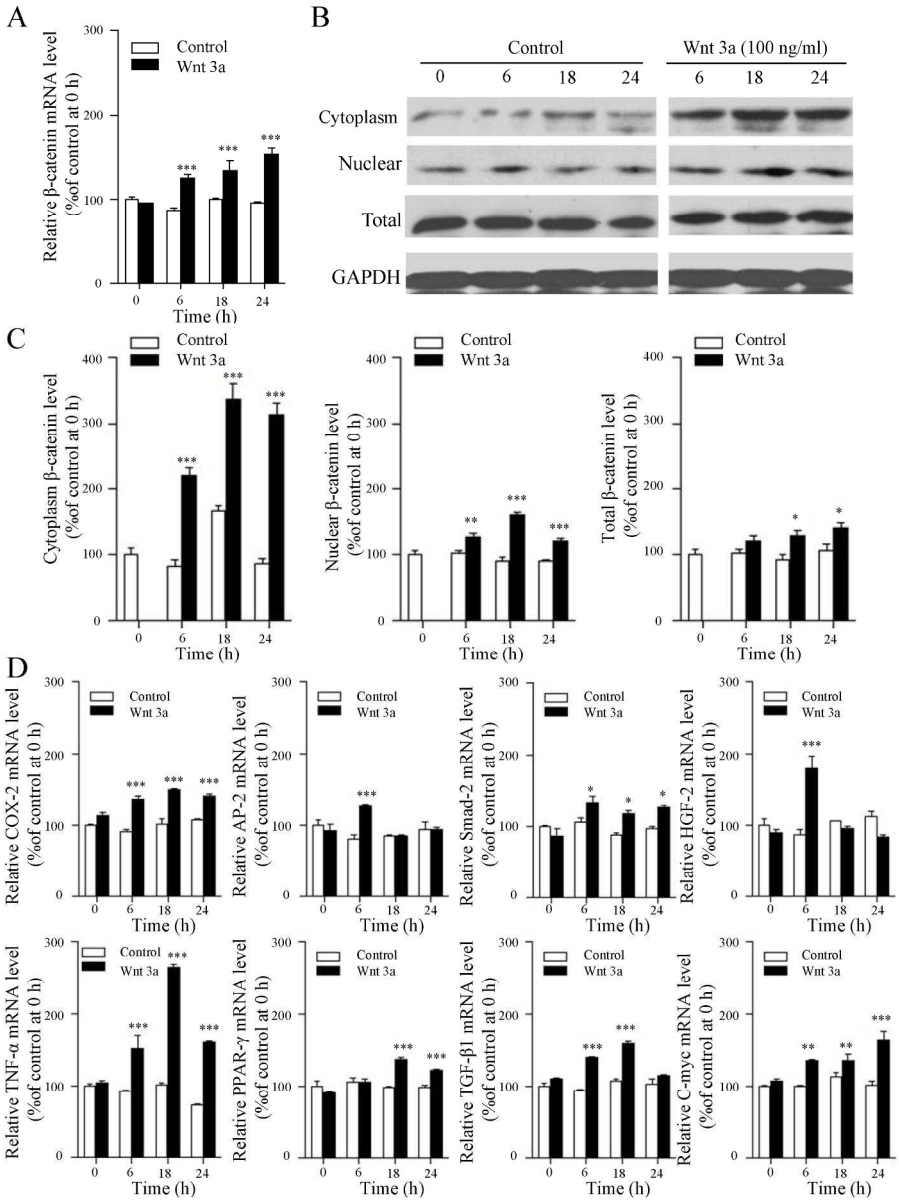

Fig. 8. Messenger RNA and protein changes of β-catenin and several factors associated with the inflammation, steatosis, fibrosis, and cancer stages in cultured HepG2 cells treated with Wnt 3a. Exposure of HepG2 cells to Wnt 3a (100 ng/ml) obviously increased mRNA expression (A) and protein level (B and C) of β-catenin in a time-dependent manner (different blots have been prepared under the same experimental conditions). (D) Exposure of HepG2 cells to Wnt 3a (100 ng/ml) also induced mRNA expression changes of COX-2, TNF-α, PPAR-γ, AP-2, Smad-2, TGF-β1, HGF, and C-myc within 24 h. *, ** and *** denote P < 0.05, 0.01 and 0.001 (n = 3; two-way repeated-measures analysis of variance, followed by Bonferroni post hoc test), in comparison to respective controls, respectively.
254x338mm (300 x 300 DPI)

Table 1 Summary of the primers designed and used in the present work.

Gene	direction	Primers for HUMAN (5'-3')	PCR Product (bp)	Primers for MOUSE (5'-3')	PCR Product (bp)
β-catenin	F	GAGTGCTGAAGGTGCTATCTGTCTG	116	CCTAGCTGGTGGACTGCAGAA	137
	R	GTTCTGAACAAGACGTTGACTTGGA		CACCACTGGCCAGAATGATGA	
TGF-β1	F	GCGACTCGCCAGAGTGGTTA	143	GTGTGGAGCAACATGTGGAACTCTA	174
	R	GTTGATGTCCACTTGCAGTGTGTTA		CGCTGAATCGAAAGCCCTGTA	
Smad-2	F	TGTTAACCGAAATGCCACGGTA	125	AACCCGAATGTGCACCATAAGAA	174
	R	GGCTCTGCACAAAGATTGCACTA		ATGCTTGAGCATCGCACTGAA	
C-myc	F	GCAGCTGCTTAGACGCTGGA	136	CCTAGTGCTGCATGAGGAGACAC	93
	R	CGCAGTAGAAATACGGCTGCAC		TCCACAGACACCACATCAATTTCTT	
COX-2	F	CCAGCACTTCACGCATCAG	119	CTGGAACATGGACTCACTCAGTTTG	109
	R	GCTGTCTAGCCAGAGTTTCACC		AGGCCTTTGCCACTGCTTGTA	
TNF-α	F	TGCTTGTTCCTCAGCCTCTT	132	ACCCTCACACTCAGATCATCTTCTGG	71
	R	CAGAGGGCTGATTAGAGAGAGGT		TGGTTTGCTACGACGT	
PPAR-γ	F	ATTCCATTCACAAGAACAGATCCAG	195	CGCTGATGCACTGCCTATGA	100
	R	TTTATCTCCACAGACACGACATTCA		AGAGGTCCACAGAGCTGATTCC	
AP-2	F	CAGGAAAGTCAAGAGCACCATAACC	198	CATGGCCAAGCCCAACAT	101
	R	GCGAACTTCAGTCCAGGTCAAC		CGCCCAGTTTGAAGGAAATC	
HGF	F	GGAAAACTGAATGGCTGACAAGA	197	CCATGAATTTGACCTCTATGA	262
	R	CCCCTCCCCAAATACTCCA		CTGAGGAATCTCACAGACTTC	
GAPDH	F	GCACCGTCAAGGCTGAGAAC	138	TGTGTCCGTCGTGGATCTGA	150
	R	TGGTGAAGACGCCAGTGGA		TTGCTGTTGAAGTCGCAGGAG