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Original Article

Mistletoe alkali increases Glucose-6-phosphatase, Succinate dehydrogenase, and adenosine triphosphatase expression and decreases GGP and NF-κB expression in precancerous hepatic lesions in rats

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Abstract: Precancerous hepatic lesions are closely correlated with hepatocellular carcinoma (HCC). Specific Chinese herbs may be able to regulate cell metabolism in hepatocarcinogenesis. In this study, we sought to elucidate the effect of mistletoe alkali on some metabolism enzymes and nuclear factor-kappa B (NF-κB) in diethylnitrosamine (DEN)-mediated precancerous hepatic lesions in rats. A rat model of prehepatocarcinoma was established by DEN and 2-acetylaminofluorene (AAF), and the rats in the treatment group received mistletoe alkali by gavage (0.12 g/kg of body weight) for four weeks. The expression of metabolism enzymes, including gamma-glutamyltranspeptidase (GGT), glucose-6-phosphatase (G-6-Pase), succinate dehydrogenase (SDH), and adenosine triphosphatase (ATPase), was detected by immunohistochemistry analysis of precancerous lesions in the rat livers. Nuclear factor-kappa B (NF-κB) was also detected by immunohistochemistry and Western blotting. Our results showed that mistletoe alkali improved the activity of G-6-Pase, SDH, and ATPase ($P<0.05$), while it suppressed the expression of GGT ($P<0.05$) and inhibited the activation of NF-κB in the DEN-induced hepatic precancerous lesions. Mistletoe alkali can regulate the abnormal metabolism enzymes and suppress the activation of NF-kB in the occurrence and developmental process of prehepatocarcinoma. Thus mistletoe alkali is a potent therapeutic agent against HCC.

Keywords: Metabolism enzymes, mistletoe alkali, nuclear factor-kappa B, precancerous, Chinese herbs

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Precancerous hepatic lesions are closely correlated with HCC, and blocking or reversing the pathological processes of the precancerous hepatic lesions may prevent its occurrence [1].

Cancerous cells show enhanced glycolysis, even in the presence of ample oxygen, and glycolysis might be crucial in the evolution of malignancies [2]. Glucose-6-phosphatase (G-6-Pase) catalyzes the final reactions of glycolysis and gluconeogenesis and plays an important role in the regulation of glucose homeostasis. L-G6pc(-/-) mice experienced hyperlipidemia, lactic acidosis, and uremia at 1 month, hepatomegaly caused by glycogen accumulation and hepatic steatosis 6 months later, and finally, multiple hepatocellular adenomas 18 months later [3]. Succinate dehydrogenase (SDH) is a mitochondrial enzyme involved in two essential energy-producing metabolic cellular processes, namely Krebs cycle and cellular electron transport chain. SDH has been implicated as a tumor suppressor. SDH dysfunction alters the epigenetic and metabolic landscape in ovarian cancer, and loss-of-function mutations in germline SDH genes lead to hereditary paraganglioma/pheochromocytoma syndrome (HPGL/PCC) [4, 5].

Gamma-glutamyl transpeptidase (GGT) is expressed on the surface of hepatocytes, where it hydrolyzes serum glutathione and provides the cell with necessary amino acids for replenishing intracellular glutathione levels.

Elevated serum GGT activity can be found in liver diseases and different types of cancers including HCC [6]. According to 384 patients in an HCC study, patients with GGT >50 U/L showed lower survival rates than did patients with GGT ≤50 U/L [7]. Nuclear factor-kappa B (NF-κB), a ubiquitous transcription factor, is kept inactive in the cytoplasm by inhibitor of κB (IκB), the inhibitory subunit of NF-κB complex. Constitutive activation of NF-κB is the underlying mechanism behind tumorigenesis. NF-κB regulates the expression of genes involved in proliferation, survival, drug resistance, angiogenesis, and metastasis. NF-κB has been considered an important target for the treatment of HCC [8].

Mistletoe is an important herb that acts as a complementary and alternative medicine for cancer therapy [9]. At present, there are approximately 1500 species of mistletoes worldwide. It has been reported that mistletoe extract has anticancer activity and minimal side effect, especially in breast cancer; it also relieves hypertention [10-15]. Hu Qisan, a Chinese herb prescription, has notable therapeutic effectiveness on hepatocarcinogenesis. There have been reports of obvious increase in the activities of G-6-Pase, SDH, and ATPase, and a simultaneous decrease in the expression of GGT and AFP. Mistletoe is one of the important elements of Hu Qisan. Mistletoe alkali is a major active constituent of Chinese mistletoe (Viscum coloratum Kom nakai) extract. In rat models, mistletoe alkali has been reported to be able to inhibit Ca^{2+} mobilization from intracellular stores, inhibit peroxidation in the liver during oxidative stress, and decrease hepatic AFP activity in precancerous lesions. Hence it has been hypothesized that mistletoe alkali is the main active component of Hu Qisan [16, 17].

In this study, we investigated the effect of mistletoe alkali on GGT, G-6-Pase, SDH, and ATPase as well as the potential contribution of NF-κB in precancerous hepatic lesions to test whether mistletoe alkali is the main active component of Hu Qisan.

Materials and methods

Animals and treatment

Male Wistar rats weighing 135-149 g (6 weeks of age) were purchased from the Animal Department of Capital Medical University, Beijing. The animals received humane care and were fed a standard mouse chow diet ad libitum. The animal experiments were approved by the Laboratory Animal Welfare Ethics Committee of Beijing Institute of TCM. The rats were allowed free access to a pellet diet and water and divided into the following three groups: normal, model, and mistletoe alkali groups. The experiment was conducted based on the Solt-Farber model [18]. The two experimental groups were administered an intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg body weight), and the normal group received saline. After 2 weeks, the test entered a selective promoting stage, and the DEN-initiated groups received a diet containing 0.015% 2-acetylaminofluorene (2-AAF) feed for 6 weeks. Following standard surgical techniques, we removed the large lobes of the liver of the rats (partially hepatectomized; PH) at the end of the third week. The treatment group began mistletoe alkali (0.12 g/kg body weight) through the stomach 1 week after PH; this process continued throughout the experiment, and mistletoe alkali was administered for 4 weeks. The rats in the normal group were continued on the former routine. At the end of week 8, the rats were sacrificed under anesthesia with pentobarbital after 24 h of fasting. The livers were excised and weighed and the sliced samples were fixed in 10% phosphate-buffered formalin for immunohistochemistry analysis (**Figure 1**). The remaining liver sections were frozen in liquid nitrogen and stored at -80°C.

Preparation of mistletoe alkali

Chinese mistletoe (Viscum coloratum Komar. Nakai) was commercially obtained from Beijing Hospital of TCM (Beijing, China), mistletoe alkali was extracted with an acidic aqueous solution method by Beijing Institute of Traditional Chinese Medicine (Beijing, China). Mistletoe alkali is a red-brown powder insoluble in water and soluble in organic solvents. It was added with an appropriate amount of DMSO prior to dissolution, made up with saline solution to be used for the experiment [16].

Histochemical staining for GGT

The frozen liver sections were fixed in acetone (1 h, 4°C), homogenized in 50 mM Tris-HCl (pH 7.4, 10 min), and further incubated in a solution

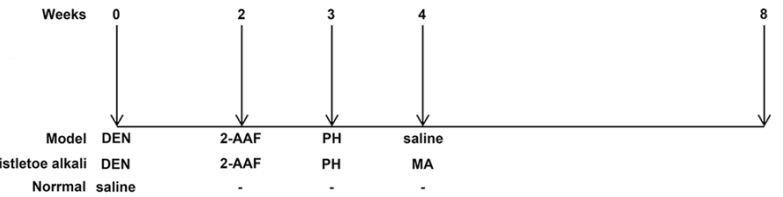

Figure 1. Flow diagram of experimental preneoplastic lesions in rat livers.

containing γ-glutamic-acid-4-methoxy-β-naphthylamide (Sigma, St. Louis, MO) as the GGT substrate, Fast Blue B (1.25 mg and 5 mg in 10 mL of incubation medium; Beijing Dingguo Biotechnology, Beijing, China.) and glycyl-glycine (5 mg in 10 mL of incubation medium, pH 7.4, Sigma) for 30 min at 37°C. The sections were then submerged in 100 mM cuprum sulphuricum (2 min), washed in normal sodium, and mounted in glycogelatin. In the control group, the GGT substrate was omitted in the incubation medium. The positive foci were quantified using a color video image processor (FW4000-Leica, Wetzlar, Germany).

histochemical detection of G-6-Pase

The G-6-Pase activity was determined by histochemical analysis. Sodium glucose-6-phosphate (G-6-P; Sigma) was used as the substrate. After incubation for 12 min at 37°C in 40 mL of 0.1 M acetate buffer (pH 6.5) containing 26 mg of G-6-P and 1 mL of 100 mM Pb $(NO3)_2$, the slides were rinsed in distilled water for 1 min, 0.5% ammonium sulfide for 2 min followed by rinsing with plain distilled water. After that, the specimens were mounted with glycogelatin. The G-6-Pase-positive foci were quantified using a color video image processor (FW4000-Leica). For each animal, 3-5 images were quantified.

SDH activity

The cryostat sections were immersed for 10 min at 37°C in an incubation medium containing 10 mL of 0.1 M phosphate buffer (pH 7.8), 80 mg of sodium succinate dibasic hexahydrate (Sigma), 1 mg of phenazine methosulfate, and 10 mg of NBT (Beijing Dingguo, Beijing, China.). All of the media were freshly prepared prior to incubation. After incubation, the tissue sections were rinsed in distilled water to stop the reaction, and they were mounted in glycogelatin. The SDH-positive foci were quantified using a color video image processor (FW4000-Leica).

Activity of ATPase

The cryostat sections were immersed for 30 min at 37°C in an incubation medium containing 5 mg of adenosine 5-triphosphate disodium salt (Sigma), 4 mL of 0.1 M tris-maleate buffer solution (pH 7.2), 0.6 mL of 2% Pb $(NO3)_2$, 1 mL of 2.5% $MgSO_4$, and 4.4 mL of distilled water in 10 mL of incubation medium. Then, the slides were rinsed in distilled water for 1 min, 0.5% ammonium sulfide for 2 min followed by rinsing with plain distilled water. After rinsing, the specimens were mounted with glycogelatin. For each enzyme, the control samples were incubated in substrate-free media. The ATPase-positive foci were quantified with a color video image processor (FW4000-Leica).

immunohistochemistry and Western Blot analyses of NF-κB p65

The paraffin-embedded liver tissues were dewaxed and rehydrated. After briefly washing in PBS, the slides were blocked with 5% normal goat serum for 1 h and then incubated with a rabbit polyclonal anti-NF-κB p65 antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). After an overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG (Alexa Fluor 594; 1:100) for 1 h, DAB chromogenic reaction for 5 min, and the nuclei were counterstained with haematoxylin. Negative controls using the corresponding IgG were included to check for nonspecific staining. For the preparation of whole tissue lysates, the tissues were lysed in a buffer (containing 20% glycerine, 1 mmol/L EDTA, 10 mmol/L chloratum Kalium, 0.42 mmol/L sodium chloride, 20 mmol/L Hepes, 1 mmol/L DTT, 1 mmol/L PMSF and 0.1 mmol/L sodium orthovanadate Sigma Chemical). Nuclei were separated by centrifugation and cytoplasmic extracts were obtained. Protein concentrations of protein lysates were determined using the Bicinchoninic Acid Kit for Protein Determination (Pierce, Rockford, IL). For the detection of nuclear-localized NF-κB family members, 100 μg of nuclear extracts were separated on a 10% SDS-PAGE gel (Sigma) and transferred onto nitrocellulose membrane. The blot was

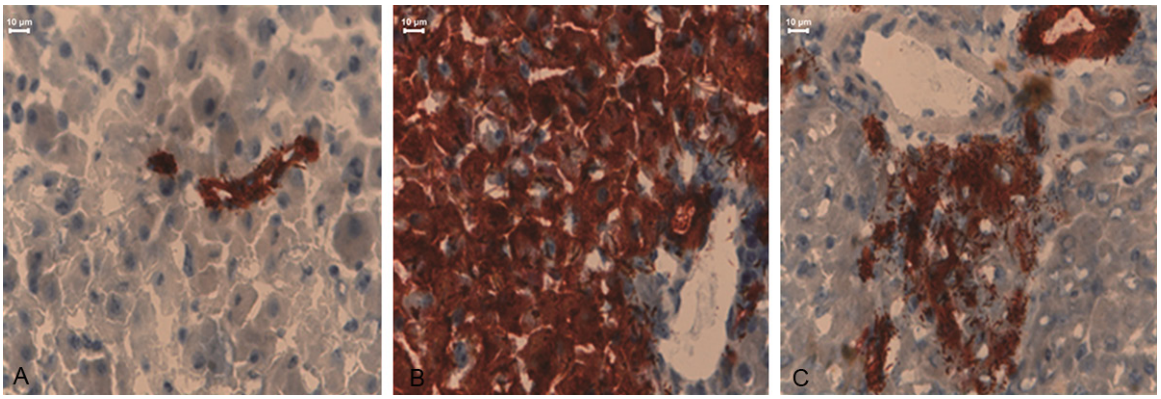

Figure 2. GGT in rat livers of the normal, model, and mistletoe alkali groups with histochemistry (×100). The red-stained areas are the GGT-positive foci of various sizes. A: A small amount of GGT-positive foci was observed in the livers of the normal group, predominantly distributed in the periportal regions; B: Increased GGT-positive foci in the livers of the model group; C: The area of the GGT-positive foci was significantly reduced in the mistletoe alkali group compared with the model group.

Table 1. Effect of mistletoe alkali on GGT, G-6-Pase, SDH, and ATPase in precancerous hepatic lesions in rats (mean ± SD, n=9)

		Mean grey		
Groups	GGT (mm^2)	G-6-Pase	SDH	ATPase
Normal	0.20±0.06*	1.17±0.12*	1.45±0.01*	0.41±0.02
Model	35.51±1.40	0.67±0.49	1.21±0.05	0.09±0.02*
Mistletoe alkali	10.81±1.34*	0.99±0.03*	1.34±0.01*	0.70±0.01*

*P<0.05 vs. model group.

incubated with antibodies against NF-κB p65 (1:400 Santa Cruz). Incubation of all primary antibodies was followed by incubation with an appropriate horse radish peroxidase-conjugated secondary antibody (1:2000 Santa Cruz). Bands were visualized by enhanced chemiluminescence staining. Western blotting was carried out in at least three independent experiments.

Statistical analysis

The data are presented as mean ± SD. The data analysis was performed using one-way ANOVA followed by the least significant difference (LSD) test. The differences were considered significant at P<0.05. Data was analyzed using SPSS15.0.

Results

H&E and GGT staining were used to demonstrate that DEN and liver resection promotes carcinogenesis in rats. We have previously reported that hepatic cells demonstrated early HCC transformation, and the normal hepatic cord structure and sinusoids were damaged in the model group according to H&E staining [19]. GGT is present in the bile ducts of normal adult rats. However, it is highly upregulated in carcinogen-induced HCC rats and is used as a marker of precancerous lesions [6]. The histochemical analysis showed that a few GGT-positive foci were observed in the liver sections of the normal group; however, in the model group, the abundance of GGT-positive foci of various sizes was significantly increased. The area of the GGT-positive foci was significantly reduced in the mistletoe alkali-treated group compared with the model groups (**Figure 2**; **Table 1**).

The expression of G-6-Pase, observed as a brown staining in the rat livers, was decreased in the model group. In the mistletoe alkali group, the G-6-Pase expression was increased significantly (P<0.001, compared with the model group) (**Figure 3**; **Table 1**). When a similar detection system was used to localize the SDH enzyme activity in the rat livers, the results showed significantly decreased SDH in the model group compared with the normal group (P<0.01). The SDH (images not shown) expression was significantly increased (P<0.01) by the addition of mistletoe alkali compared with the model group (**Table 1**). In addition, similar results were observed in the study of ATP-

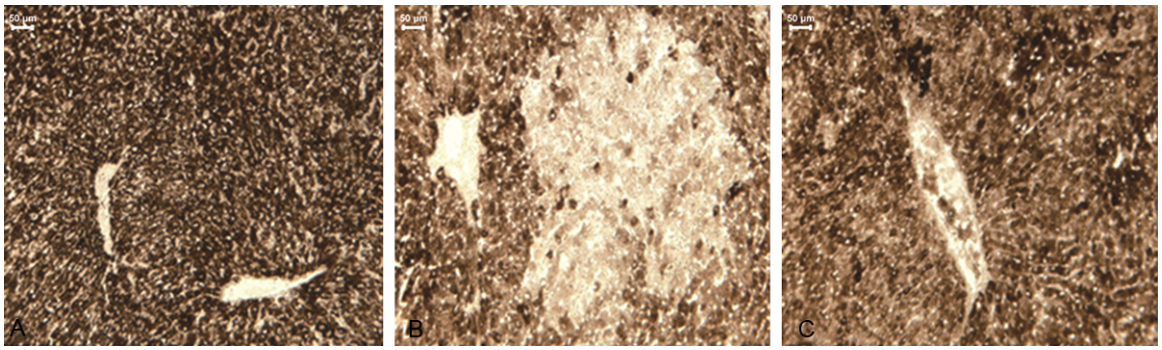

Figure 3. G-6-Pase in the rat livers of the normal, model, and mistletoe alkali groups with histochemistry (×100). The brown-stained areas show various levels of G-6-Pase activity. A: The G-6-Pase expression was increased in the livers of the normal group; B: The G-6-Pase expression was significantly decreased in the model group compared to the normal group; C: The G-6-Pase expression was significantly increased in the mistletoe alkali group compared with the model group.

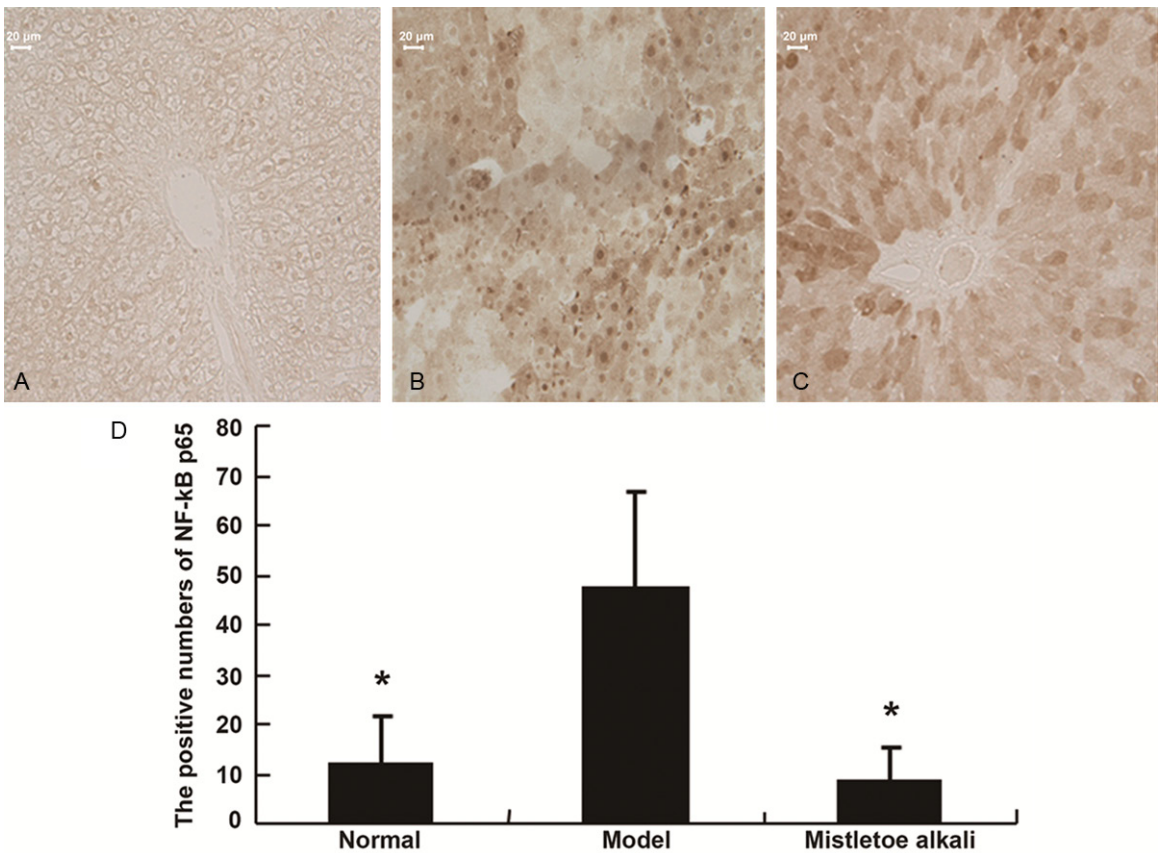

Figure 4. Effect of mistletoe alkali on the expression of NF-κB p65 in rat livers by immunohistochemistry. The brown-stained areas showed the NF-κB p65 proteins. A: NF-κB p65 proteins were predominantly expressed in the cellular cytoplasm in the normal group; B: NF-κB p65 expression was predominantly observed in the cellular nucleus in the model group; C: Expression of the NF-κB p65 proteins occurred predominantly in the cytoplasm. D: Quantification of the NF-κB p65-positive areas. The data shown are mean ± SD (n=9). *P<0.05, compared with the model group.

ase (images not shown). ATPase expression increased after the mistletoe alkali treatment compared with the model group (P<0.01) (**Table 1**).

To detect the expression of the NF-kB p65 proteins in hepatocarcinogenesis induced by DEN, immunohistochemistry and Western-blot analyses were performed (**Figures 4**, **5**). **Figure 4**

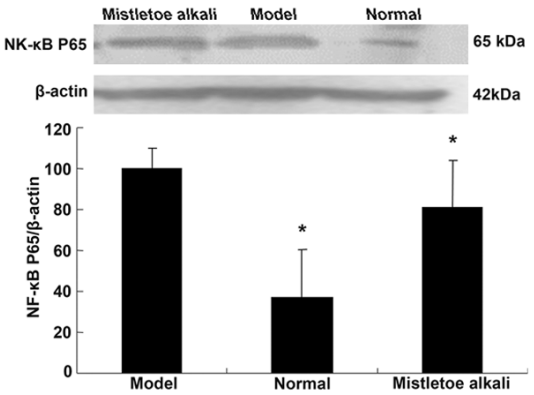

Figure 5. Effect of mistletoe alkali on the expression of NF-κB p65 in the nuclear region in rat livers by Western blotting. NF-κB p65 protein levels in the nucleus increased significantly in the model group compared with normal group, and decreased significantly following treatment with mistletoe alkali. Data are mean ± SD, *P<0.05 vs. model group.

shows that NF-κB p65 was predominantly observed in the cellular nucleus in the model group, whereas it was located mainly in the cytoplasm after the mistletoe alkali treatment. The result indicates that the transcription of NF-κB was downregulated after treatment with mistletoe alkali. **Figure 5** verifies the above results further, NF-κB p65 protein levels in the nuclear region increased significantly in model group compared with normal group, and decreased significantly following treatment with mistletoe alkali.

Discussion

Tumor cell metabolism has been considered a hallmark of cancer. GGT is a key enzyme involved in glutathione metabolism, and its expression is often significantly increased in malignancies in humans. The growth of rapidly dividing tumors can be limited by cysteine. In vitro transfection of GGT into mouse tumor cells provides cells with a growth advantage when cultured in media containing physiologic concentrations of cysteine. GGT-positive cells are able to use extracellular glutathione as a secondary source of cysteine, thereby overcoming the growth restriction imposed by serum cysteine levels. Carcinogen-induced rat hepatocellular carcinomas, mouse skin tumors, and hamster squamous cell carcinomas have highly induced levels of GGT activity. Synthesis of GGT prodrugs is used as a new approach in chemotherapeutic drugs to treat GGT-positive tumors [20]. Our results demonstrated a dramatic protective effect by mistletoe alkali on the formation of GGT-positive foci resulting from DEN exposure. Hence mistletoe alkali could be a new therapeutic agent against GGT-positive tumors.

Glycometabolism is the key point of energy metabolism [21]. G-6-Pase is a microsomal enzyme that catalyzes the conversion of G-6-P to glucose and inorganic phosphate. SDH is a marker enzyme in the TCA cycle, and succinate, phosphate, and ATP promote its activity. It is a regulatory enzyme, and its properties are altered when solubilized. Mitochondrial electron transport complexes play a critical role in generating cellular energy disorders that affect respiratory chain activity and could cause dysfunction in any organ system. SDH is formed and neutralized by coordinated interactions between the mitochondria and the nuclear genome [22]. ATPases are spread widely throughout all life forms [23]. Their function is associated with motor-like activities, and many of them undergo extensive conformational changes to transform chemical energy stored in the form of ATP into mechanical movement. In this study, mistletoe alkali elevated the activity of SDH, ATPase, and G-6-Pase. These results are consistent with the report on Hu Qisan [16]. Thus, we conclude that mistletoe alkali improved mitochondrial function and energy metabolism. It was newly reported that Hu Qisan may induce apoptosis by reducing the inhibitory effects of X-linked inhibitor of apoptosis protein (XIAP) on caspase-3. The components responsible for this effect are identified as mistletoe alkali and mistletoe polysaccharide [19]. These indicate that mistletoe alkali is the main active component of Hu Qisan.

The inhibitors suppressing NF-κB activation is of great therapeutic importance in the treatment of HCC in recent years [24]. Our results showed that mistletoe alkali could downregulate the transcription of NF-κB. Therefore, mistletoe alkali may be a potent anti-carcinogenic agent by inhibiting the activation of NF-κB.

We found that in precancerous lesions, glycometabolism enzymes such as G-6-Pase and SDH were decreased significantly. Mistletoe alkali could increase the levels of G-6-Pase, SDH, and ATPase, while decreasing the levels of GGT and inhibiting the activation of NF-κB.

The study limitations included non-rigorous animal grouping and unitary type of data and results. Further studies are warranted to fully elucidate these mechanisms.

In summary, mistletoe alkali can regulate abnormal cell metabolism in precancerous liver lesions through G-6-Pase, SDH, ATPase, and GGT, and it can inhibit activation of NF-κB. Thus, mistletoe alkali is a new potent therapeutic agent against HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

HCC, Hepatocellular carcinoma; G-6-Pase, Glucose-6-phosphatase; SDH, Succinate dehydrogenase; 2-AAF, 2-acetylaminofluorene; GGT, gamma-glutamyl transpeptidase; ATPase, adenosine triphosphatase.

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