

## Chemoprevention of HBV-related hepatocellular carcinoma by the combined product of resveratrol and silymarin in transgenic mice

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### ABSTRACT

**Background:** Patients with chronic hepatitis B virus (HBV) infection are at a high risk to develop hepatocellular carcinoma (HCC). Recently, metabolic syndrome has been found to carry a risk for HCC development. Considering the limitation of chemotherapeutic drugs for HCCs, the development of chemopreventive agents for high risk chronic HBV carriers is urgently demanded. In this study, we used combined silymarin and resveratrol extract which have been shown to exhibit biologic effects on activating peroxisome proliferator activated receptors (PPAR) and inhibiting mTOR signaling in a transgenic mice model harboring HBV viral oncoproteins.

**Methods:** The transgenic mice model harboring HBx and pre-S2 mutant constructs which develop HCC was adopted. First, we *in vitro* tested the ideal combination dosages of the silymarin and resveratrol product, and then we fed the natural product to the transgenic mice. The chemopreventive effects on preventing the development of HCC were evaluated.

**Results:** MTT assay showed an enhanced effect of the combined silymarin and resveratrol product on the reduction of cell proliferation in two hepatoma cell lines, Huh-7 and Hep G2. *In vitro* reporter assay and Western blot analyses revealed that the combined product could activate PPAR/PGC-1 $\alpha$  signaling and inhibit mTOR expression. *In vivo*, the combined products could significantly ameliorate fatty liver and reduce HCCs in transgenic mice harboring HBV oncoproteins.

**Conclusions:** The combined silymarin and resveratrol product exhibits a synergistic effect on the reduction of HCC development in transgenic mice model and may represent a potential agent for the prevention of HCC in high risk chronic HBV carriers.

**Key words:** HBV, HCC, Transgenic mice, Chemoprevention

## **INTRODUCTION:**

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and is the second most frequent cause of cancer death in men [1]. The major risk factors for HCC include chronic infection with hepatitis C virus (HCV) or hepatitis B virus (HBV), and excessive alcohol consumption [2]. Chronic hepatitis B virus (HBV) infection and HBV-related hepatocellular carcinoma (HCC) remain one of the major health problems in Taiwan, mainland China, and other parts of the world [3]. Among the mechanisms of HBV hepatocarcinogenesis, HBx has been recognized as a viral oncoprotein and many HBV-related signal pathways have been reported to be potential oncopathways, including the recently reported mTOR (mammalian target of rapamycin) signaling [4-6]. Besides HBx, we proposed that the emergence of a second HBV oncoprotein, the pre-S2 deletion mutant in Type II ground glass hepatocytes (GGH) at the advanced stage of chronic HBV infection [7, 8]. The pre-S2 deletion mutant can induce endoplasmic reticulum (ER) stress and initiate ER stress-dependent and -independent signals, leading to NF- $\kappa$ B activation, COX-2 induction, oxidative DNA damage, and genomic instability [9-11]. The pre-S2 deletion mutants can also activate the VEGF-A and mTOR signal pathway [12, 13]. Therefore, GGHs are now recognized as a pre-neoplastic lesion of HCC and pre-S2 deletion mutant as a new viral oncoprotein.

Although anti-viral drugs have been shown effective in controlling the HBV infection, the progression from chronic HBV infection to HCC still remains a nightmare for the majority of chronic HBV carriers. Target drugs have been developed for therapeutic trial of HCC, but the efficacy is still limited. Furthermore, no chemopreventive agents or natural products have been tried to prevent or revert the disease progression from chronic HBV infection to HCC. The reasons for this difficulty to apply chemoprevention for HBV-related HCC is due to the lack of knowledge for the molecular mechanism of HBV tumorigenesis or the disease progression from their precursor lesions to HCC, and also the lack of suitable chemopreventive products. Recently, patients with metabolic syndrome have been found to carry a high risk to develop HCC [14, 15]. For chemoprevention of cancer development or recurrence, natural products with active ingredients and without side effects are most ideal considering the long-term usage.

Besides HBV or HCV infection, nonalcoholic fatty liver disease, inflammation, and metabolic syndrome have been recognized to be the risk factors for HCC development. Furthermore, small-sized HCC, either HBV- or HCV-related, frequently showed fatty change, suggesting that lipid metabolism is disturbed at the advanced stage of chronic liver disease and HCC [16, 17]. Recently, mTOR signaling has been found to play a regulator role in the metabolic syndrome and HBV tumorigenesis [18-20]. It can regulate the lipid biosynthesis through the SREBP-1 to activate peroxisome proliferator activated receptors (PPAR)- $\gamma$  and lipid-related genes which may contribute to tumorigenesis [18, 21, 22].

Among the natural products, silymarin is the ideal candidate product for chemoprevention of HCCs because of their popular and long-term usage for chronic liver diseases in human community (23). Silymarin, a compound from milk thistle (*Silybum marianum*), has been widely consumed as a dietary supplement and an empirical product widely used for liver diseases. The active component-silibinin has been evaluated clinically in the treatment of hepatitis and liver damage because of its anti-inflammation and anti-oxidant effects [23, 24]. Recent evidence also suggests that silymarin and silibinin may be useful in the chemoprevention of malignancies in a variety of sites including liver cancer [25, 26]. The other ideal candidate for HCC chemoprevention is the resveratrol from grape skin extract which has been verified to be effective to prevent cancer development at various stages of carcinogenesis including initiation, promotion, tumor invasion, and metastasis [25, 27-29]. Resveratrol is a strong anti-oxidant and anti-inflammatory agent [30, 31], and also a most promising product for metabolic syndrome and longevity through mTOR inhibition and upregulation of PPARs and PGC-1 $\alpha$  [31-33]. Therefore, the combination of silymarin and resveratrol will be a potential candidate for chemoprevention of HCCs in high risk chronic HBV carriers.

In this study, the *in vitro* and *in vivo* effects of silymarin and resveratrol, either alone or in combination, were evaluated in inhibiting the proliferation of HCC cell lines by MTT assay. Their activities in regulating PPARs, PGC-1 and mTOR activities were tested by the reporter assay or Western blot analyses. The ideal combination of these two products were then fed to the transgenic mice harboring the double HBV oncoproteins (HBX and pre-S2 mutants) for chemoprevention of HCC development.

## **METHODS:**

### **Cell proliferation assay**

A total  $5 \times 10^3$  cells/well was seeded in the 96 well plate. After treatment of silymarin (Sigma, St. Louis, MO), resveratrol (Calbiochem, Darmstadt, Germany), or both for 24 hour and 48 hour, Cell Counting Kit-8 solution (Sigma, St. Louis, MO) was added to each well and then incubated the plate for three hours at 37°C Then the plate was measured the absorbance at 450 nm.

### **Luciferase reporter assay**

PPAR- $\gamma$ -LBD and pRL-TK plasmids were used in PPAR- $\gamma$  activity analysis. After transfection with lipofectamine 2000 (Invitrogen, Carlsbad, CA92008), luciferase-expressed cells were assayed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Renilla luciferase activities were measured for normalization.

### **Western blot analyses**

Cells were harvested after treated with silymarin, resveratrol, or both for 48 hours and then lysed in RIPA buffer (Upstate Biotechnology, NY) containing proteinase inhibitor cocktail and phosphatase inhibitor (Roche Applied Science, Indianapolis, IN). Total protein lysate was then harvested via centrifugation at 13,000 rpm for 20 minutes, resolved on SDS-PAGE and transferred to PVDF membranes. After incubated with primary antibodies and following

secondary antibodies, the membranes were visualized by ECL (PerkinElmer Life Science, Boston, MA). Antibodies used in this study were anti-PPAR- $\gamma$  (Abcam, MA, USA), anti-PGC1 $\alpha$  (Abcam, Cambridge, UK), anti-SIRT1 (Abcam, Cambridge, UK), anti-p-mTOR (Abcam, Cambridge, UK) and anti- $\beta$ -actin (Chemicon, Temecula, CA).

### **Transgenic animal model harboring double HBx and pre-S2 mutant constructs**

Transgenic mice harboring HBx and pre-S2 mutant were established using the liver-specific transgenic vector pAlb-In-pA-HS4 as described previously [26]. The albumin-HBx or pre-S2 mutant (nt 4-57 deletion) [9] transgenic mice were generated by pronucleus microinjection of C57BL/6 fertilized eggs. The transgenic mice harboring both HBx and pre-S2 mutants were obtained by hybridizing the HBx-transgenic mice and pre-S2 mutant-transgenic mice. The HBx and pre-S2 mutant transgenic mice are bred and screened for the HCC chemoprevention study. All of the mice used in this study were male and housed in a specific pathogen free facility. All the animal experiments have been performed under the approval of the Institutional Animal Care and Use Committees of the National Cheng Kung University Medical School and National Health Research Institutes.

### **Preparation of natural products for chemoprevention**

The natural product-silymarin was purchased from Indena (Indena, Milan, Italy). This product is an extract from Milk Thistle and contained 35% silybin in a phytosome complex as assayed by HPLC. The natural product-Vineatrol<sup>®</sup> 20-6 was purchased from Breko (Breko GmbH, Bremen, German). This product is an extract from grape vine and contained resveratrol of 20% as revealed by HPLC assay. Silymarin (0.875 mg Silymarin/g chow), resveratrol (0.875 mg Vineatrol<sup>®</sup>/g chow), or both were added and mixed with normal chow diet.

### **Animal study for HCC chemoprevention**

Four groups of transgenic mice were fed with normal chow diets or chow diets containing silymarin, resveratrol or both at the age of 8 months for six months (at the age of 14 months) and then sacrificed for evaluation. The residual chow diets were weighed weekly for controlling amount of mice uptake. These mice were weighed each week and measured ALT monthly. The tumor development (including tumor size and tumor number) in each group was evaluated and analyzed by biostatistics.

### **Histopathology**

Mice were sacrificed after being fed natural products for 6 months. Liver tissues were then collected, fixed with 10% neutral formalin, and embedded in paraffin. These paraffin-embedded liver tissues were sectioned at 4  $\mu$ m thickness and further subjected to hematoxylin-eosin (H&E) staining for conventional histopathological observation. Fat accumulation in liver was examined by Oil-Red O staining of cryostat frozen sections as described previously [34].

### **Statistical analysis**

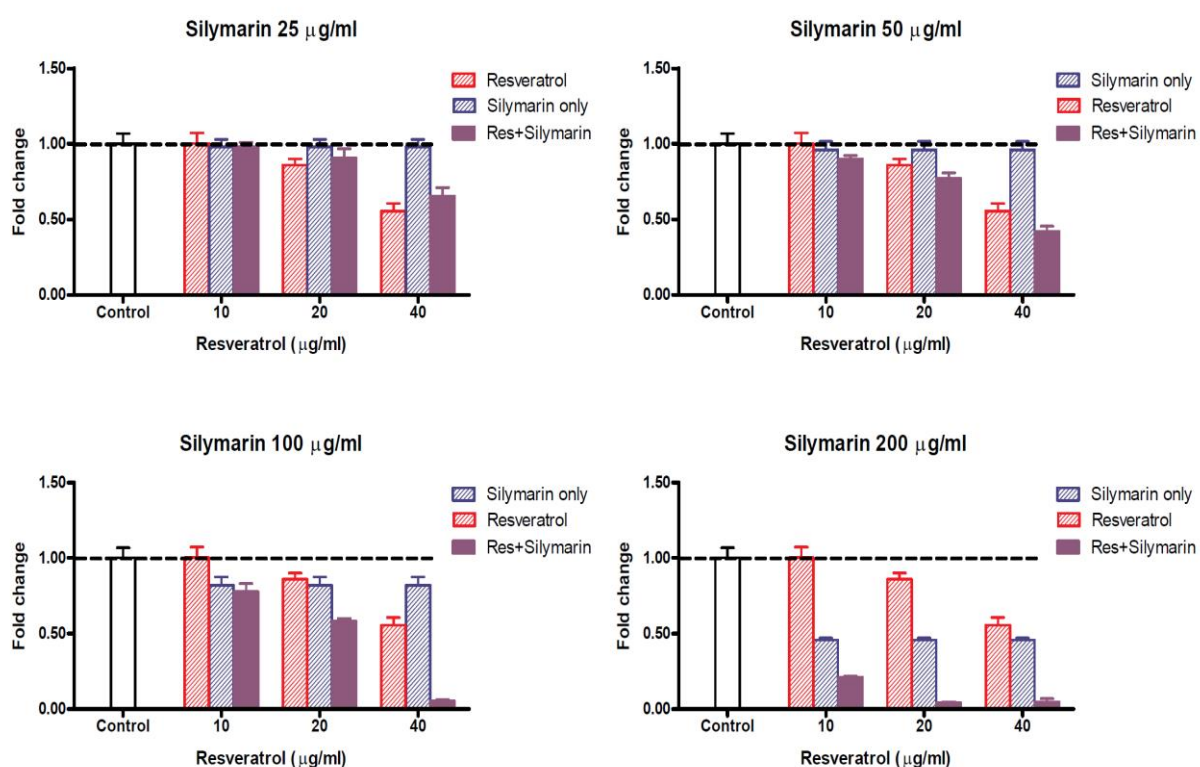
Statistical analyses were carried out using GraphPad Prism 4. Data was analyzed by

Kruskal-Wallis test and Dunn's post test. P value <0.05 was considered significant.

## RESULTS and DISCUSSIONS:

### *In Vitro* Study: enhanced activity of combined silymarin and resveratrol product on the inhibition of tumor cell proliferation

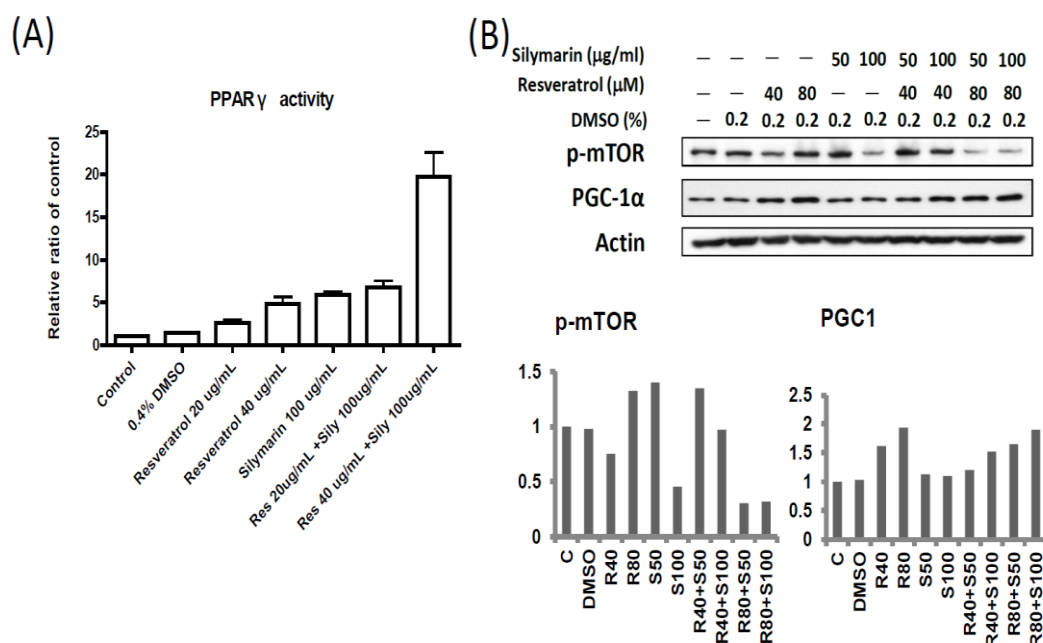
We first tested the effect of silymarin and resveratrol on two hepatoma cell lines, Huh-7 and Hep-G2. Different doses of silymarin (0, 25, 50, 100 and 200  $\mu\text{g/ml}$ ) and resveratrol (0, 10, 20, and 40  $\mu\text{g/ml}$ ) were studied by MTT assay on both cell lines. The results showed a reduction of cell proliferation on both Huh 7 and Hep G2 cell lines. The best combination dosage for the anti-proliferation effect on both cell lines were determined at around the ratio of 1:2.5-1:5 for resveratrol and silymarin, respectively in combination (Figure 1).



**Figure 1.** Anti-proliferation effect of resveratrol combined with silymarin on Huh-7 hepatoma cell line

We then determined the effects of the combined product on the regulation of PPAR $\gamma$ , PGC-1 $\alpha$ , and p-mTOR. Resveratrol has been reported that can activate PGC-1 $\alpha$  which acts as a co-activator of PPAR- $\gamma$ . We chose 100  $\mu\text{g/ml}$  silymarin combined with 40 or 80  $\mu\text{M}$  resveratrol based on the above-described MTT results to test their effects on the regulation of PPAR- $\gamma$ , PGC-1 $\alpha$  and p-mTOR. The PPAR- $\gamma$  activities were determined by the luciferase reporter assay, while Western blot analyses were used to evaluate the expression of PGC-1 $\alpha$  and p-mTOR. Our results showed that resveratrol combined with silymarin had synergistic effects on increasing the PPAR- $\gamma$  activities in both Huh-7 and Hep-G2 cell lines (Figure 2A).

Western blot analyses showed that the combined silymarin (100 µg/ml) and resveratrol (80 µM) exhibited the significant enhanced activities to inhibit mTOR and increased PGC-1α ( Figure 2B ).



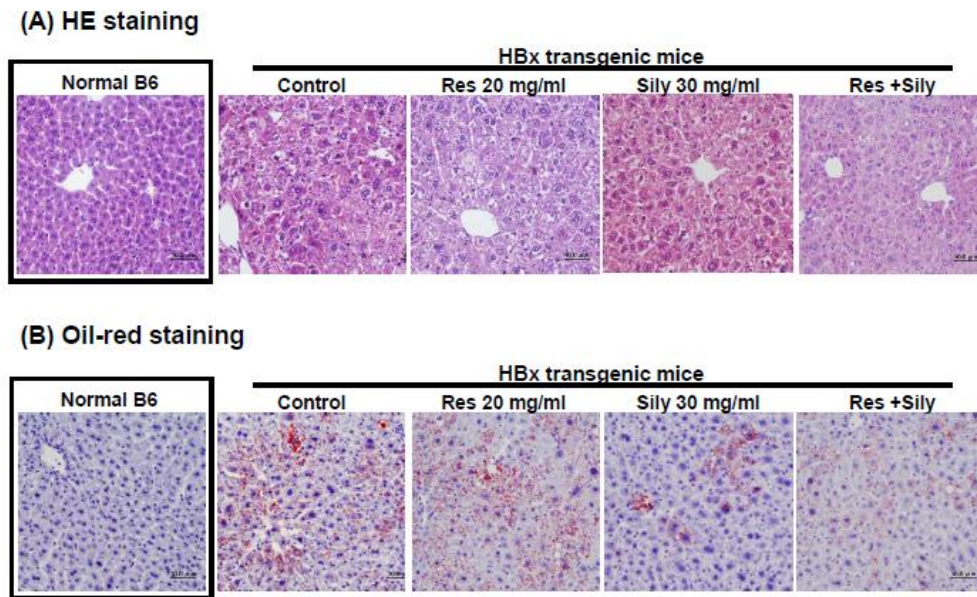
**Figure 2.** Synergistic effect of resveratrol combined with silymarin on PPAR- $\gamma$  activity, p-mTOR inhibition, and enhanced PGC-1 $\alpha$

Considering the absorbability and catabolism *in vivo*, we then performed a pilot *in vivo* study for testing the effects of the different dosages of the combined product on mice.

**In Vivo dosage test**

In this study, we used 4-week-old of HBx transgenic male mice (C57BL/6 background) for testing the effect of combined product on the early stage of liver pathogenesis. The HBx transgenic male mice and their wild type male littermates were randomly assigned into different groups. Resveratrol (20 mg/kg) combined with various doses of silymarin (15, 30, and 60 mg/kg) were fed to mice daily through p.o administration using feeding needles for two weeks (14 days) and then mice were sacrificed for analysis. From H&E and oil-red staining, the liver of HBx-transgenic mice without any treatment showed minor fatty change at the age of 6 weeks, but it improved after treatment of resveratrol (20 mg/kg) combined with Silymarin (15 mg/kg, 30 mg/kg and 60 mg/kg). The HBx transgenic livers recovered to almost normal morphology in the group of 20 mg/kg resveratrol combined either 30 mg/kg silymarin or 60 mg/kg silymarin group. In the group of 20 kg/ml resveratrol combined with silymarin 30 mg/kg, it showed significant improvement of fatty change as compared to the group of either resveratrol or silymarin alone (Figure 3A and 3B).





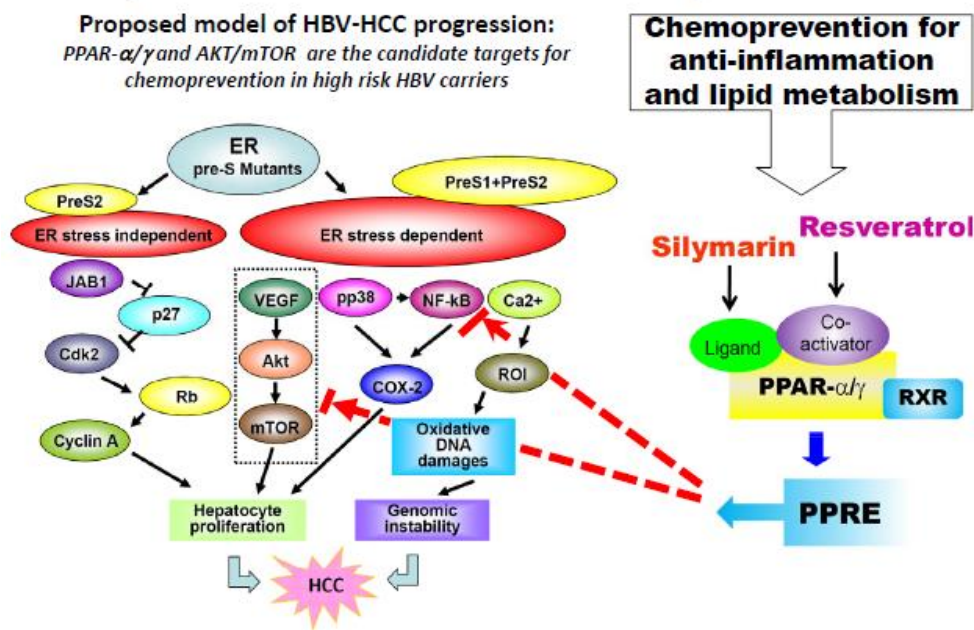
**Figure 3.** Beneficial effects of resveratrol combined with silymarin on liver damage and fatty changes.

### Toxicity studies

Serum ALT observed in both wild-type and HBx transgenic mice are in a normal range (20-50 U/L) at the age of 4-6 weeks. No obvious difference in the serum ALT was detected among control and treatment groups. Taken together the liver pathology and serum biochemistry, the findings indicate that resveratrol (20 mg/kg), silymarin (15, 30 and 60 mg/kg) or the combined product showed no toxic effect on liver at this age of mice. Therefore, the combination of these two natural products (20 mg/kg resveratrol and 30 mg/kg silymarin) will be a safe agent for chemoprevention on HBV-related HCC animal model.

### Chemoprevention design based on the molecular mechanism of HBV tumorigenesis by using the combined products on transgenic mice harboring both HBx and pre-S2 mutant

Recently, we demonstrated that GGHs co-expressing both HBx and pre-S2 mutant exhibited an enhanced tumorigenesis pertinent to human HBV/HCC development. A transgenic animal model harboring the double-construct HBx and pre-S2 mutant has been successfully established in our laboratory and was adopted in this study for chemoprevention study. The study design is based on the molecular mechanism of HBV tumorigenesis we previously proposed [34] (Figure 4).

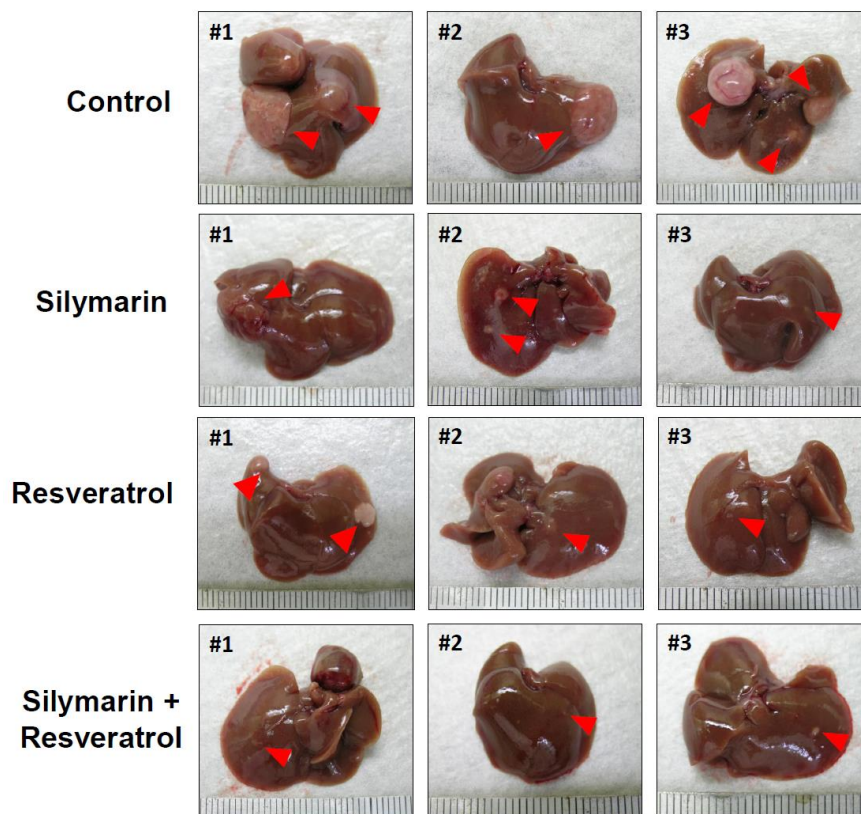


**Figure 4.** Design of chemoprevention based on the molecular mechanism of HBV tumorigenesis.

#### **Tumor development was reduced in transgenic mice harboring double HBx plus pre-S2 mutants fed with combined silymarin and resveratrol product**

The natural product for chemoprevention is prepared by combining extracts of silymarin and resveratrol, based on the concentration and dosages used in *in vitro* and *in vivo* dosage studies. The component of the extract for resveratrol is 20% purity, while that of silymarin is 35% purity based on the HPLC profile analyses provided by the manufacturers. These products were prepared as chow diets to feed the transgenic mice (4 g chow/mouse/day), including control group (normal chow diets), silymarin group, resveratrol group and combined silymarin plus resveratrol group. In the transgenic mice harboring double HBx and pre-S2 constructs, the results showed that tumor development was significantly reduced in the group fed with resveratrol or silymarin alone, or in the combined product group (Figure 5). However, the combined product showed remarkable for the tumors larger than 5 mm diameter, from 50% in the control group to 12.5% in the combined product group, as compared to that in the group of resveratrol (45 %) or silymarin (47%) alone. The data indicate that the combined resveratrol and silymarin product exhibited a synergistic effect on reducing HCC development in this transgenic mice model which is pertinent to human HBV-related HCC. In this study, to ensure whether other ingredients in these extracts have additional effects, these chow diets (containing silymarin, resveratrol or both) were also fed to wild-type mice (for control and toxicity observation). Both ALT in serum and liver pathology showed no abnormal change after feeding these diets.





**Figure 5.** Tumor development in transgenic mice was significantly reduced in the group of feeding combined silymarin and resveratrol product.

### CONCLUSION:

In this study, our *in vitro* studies revealed that resveratrol could activate the PGC-1 $\alpha$  and PPAR signaling and reduce cell proliferation in the two hepatoma cell lines by MTT assay. We also verified that the combined product can exhibit inhibition on mTOR signaling which represents an important mechanism in HBV tumorigenesis. Our results in the transgenic mice harboring double HBx and pre-S2 mutant construct showed that the combined silymarin and resveratrol product at a defined ratio of dosages may represent an ideal candidate agent for chemoprevention of HCCs because of their popular and long-term usage in human community. Silymarin and resveratrol have been reported to be effective at various stages of carcinogenesis including initiation, promotion, tumor invasion, and metastasis [25-27, 29]. Clinical trial will be designed along this line to conduct on high risk chronic hepatitis B patients to prevent HCC development or to prevent the recurrence of their HCC after surgery.

### Competing Interests:

The authors have no financial interests or conflicts of interest.

### Authors' Contributions:

Prof. Ih-Jen Su is the principal investigator for this study and wrote the manuscript. Wen-Chuan Hsieh contributed the study design and manuscript writing. Ching-Wen Yang organized the *in vitro* study and performed the MTT assay. Yi-Sheng Haung performed the

PPAR activity assay and Western Blotting. Ting-Wei Chao took care of animal and Prof. Tin-Fen Tsai established the transgenic mice. Wen-Chuan Hsieh and Ching-Wen Yang contributed equally to these studies.

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