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Shanxian Granule Serum Suppresses Hepatic Oval Cell Malignant Transformation by Targeting the Wnt/β-Catenin Pathway

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Abstract: Aim In this study, we aimed to investigate how SXG serum attenuates the malignant phenotype in HOC. **Methods** Rat hepatic oval cells (HOC), WB-F344 was induced to undergo malignant transformation using N-methyl-N'-nitro-N-nitroso (MNNG). MNNG-treated WB-F344 cells were administered dose-dependent levels (low/medium/high) SXG-containing serum to evaluate its inhibitory effects. To examine pathway involvement, the Wnt agonist CP21R7 was co-administered with high-dose SXG serum. Cellular proliferation was quantified by MTT assay, while Wnt/β-catenin pathway components (wnt3a, β-catenin, C-Myc) were analyzed at mRNA levels using qPCR. **Results** Compared with the normal group, the Model showed a significantly enhanced cell proliferation capacity ($P<0.05$). Relative to the Model, the experimental group exhibited a significant decrease in cell proliferation ($P<0.05$). Moreover, SXG-containing serum significantly downregulated wnt3a β-catenin and C-Myc expression. which are important components of the Wnt/β-catenin signaling pathway and CP21R7 can antagonize the SXG effect ($P<0.05$). **Conclusion** SXG inhibits oncogenic transformation of WB-F344 cells by suppressing the Wnt/β-catenin pathway.

Keywords: Shanxian granule-containing Serum; Precancerous Lesions of Liver; Wnt/β-catenin Signaling Pathway; Hepatic Oval Cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer, characterized by its high malignancy and poor prognosis. Based on the most recent global cancer epidemiology statistics, HCC ranks as the sixth most prevalent malignant tumor worldwide, with the third highest fatality rate among all cancer types [1]. HCC development correlates strongly with risk factors such as viral hepatitis, alcoholic, non-alcoholic liver diseases, and cirrhosis. The surrounding tumor microenvironment, characterized by a dynamic interplay of extracellular components and stromal cells, is recognized as a critical regulator of hepatocarcinogenesis and malignant progression [2]. Studies have shown that HCC is unique due to its characterized chronic hepatic damage milieu [3]. The malignant progression from hepatic cirrhosis to hepatocellular carcinoma involves an extended precancerous phase characterized by dysplastic lesions. Precancerous lesions of the liver are intermediate stages between benign lesions and cancer, closely associated with HCC, and may progress to liver cancer if not promptly treated [4].

TCM theory suggests that SXG can inhibit abnormal liver cell proliferation and the development of liver cirrhosis [5]. However, it remains unclear whether SXG inhibit EMT and Cancerization process hepatic oval cells through Wnt signaling pathway, forestalling evolution from precancerous to malignant liver pathology. Therefore, this study harnessed serum pharmacology to investigate effects SXG-containing serum on hepatic oval cells and the molecular changes associated with the Wnt pathway.

2. Materials and Methods

Preparation of SXG-containing drug serum Male Sprague-Dawley rats were purchased from Sichuan Dashuo Laboratory Animals Co., LTD. (Sichuan, China) with license No. SCXK (CHUAN) 2020-030. The animal experimental protocols were approved by the Ethics Committee of Shaanxi University of Traditional Chinese Medicine (Approval number: SUCMDL-20190309001). Twenty male Sprague-Dawley rats (180±10g body weight) were acclimated to Prior to experimentation, animals underwent 5-day facility acclimatization and were maintained under IACUC-supervised care (Shaanxi University of TCM) implementing NIH Laboratory Animal Welfare standards. The rats relative humidity of 40-70%, a 12-hour light-dark cycle, fixed water supply system, and were fed three times a day. A 4.06 g/ml SXG stock solution was prepared in physiological saline (0.9% NaCl). Rats received twice-daily oral gavage at 1ml/100g body weight for 7 consecutive days. On the terminal day, animals underwent 12-hour fasting prior to dosing. One-hour post-administration, anesthesia was induced via intraperitoneal injection of 2% pentobarbital sodium. Terminal blood samples (2-5 ml/rat) were subsequently collected by transabdominal aortic puncture under deep anesthesia. Blood samples were allowed to clot at ambient temperature (0.5–1 h) prior to centrifugation (3000 rpm, 15 min). Post-aspiration, supernatant underwent thermal sterilization (56°C/30 min) and bacterial exclusion via 0.22-μm pore-size filtration. The filtered solution was then repackaged and stored at -80°C. Three serum dose groups were established: High-dose (SXG-H): Undiluted original

serum; Medium-dose (SXG-M): 50% serum concentration (diluted 1:1 with vehicle); Low-dose (SXG-L): 25% serum concentration (diluted 1:3 with vehicle).

Cell line maintenance and SXG-containing serum treatment Cells were cultured in high-glucose DMEM containing 10% FBS and incubated at 37°C with 5% carbon dioxide. The culture medium was changed every 2-3 days. The cells were divided into the following groups: Control, Model, SXG serum: High-dose (SXG-H), Medium-dose (SXG-M), Low-dose (SXG-L), SXG-H+Wnt activator (SXG-H+CP21R7). Except for the control group, all other groups were treated with 3 μg/ml MNNG for 24 hours to induce a malignant transformation model[6]. Subsequently, they were continuously stimulated with 7×10^{-7} mol/L H2O2 for 12 hours to induce EMT formation[7,8]. Cells in the Control and Model groups received DMEM. The SXG-H group received the original SXG-containing drug serum, SXG-M received a half-concentration (diluted with FBS), and SXG-L received a quarter-concentration (diluted with FBS). All treatments were applied at 20% of the culture medium volume. After 24 hours and 48 hours of SXG-containing serum treatment, the cells were harvested for subsequent experiments.

MTT tetrazolium assay for cell proliferation Cellular proliferation was assessed using MTT. After seeding WB-F344 cells at 2,000 cells/well in 96-well plates, cultures were exposed to SXG-containing serum for 24, 48, or 72 h before assay termination. To each well, 10 μl of MTT (Sigma) was added and incubated in a cell incubator for 4 hours. After the incubation, the medium was discarded, and 150 μl of DMSO was added to each well. The plate was shaken at a low speed on a shaker for 10 minutes to fully dissolve the crystals. The absorbance (OD value) of each well was measured at 490 nm using a microplate reader. The experimental results were statistically analyzed based on the OD values at various time points.

Quantitative reverse transcription PCR Total RNA was isolated from WB-F344 cells using RNAiso Plus following the manufacturer's protocol. To evaluate expression level of wnt3a, β-catenin, and C-Myc, Complementary DNA (cDNA) was generated from total RNA via PrimeScript reverse transcription system. The reverse transcription (RT) process involved incubation at 7°C for 15 minutes, followed by a brief heating step at 85°C for 5 seconds. For the qPCR reactions, we employed the StepOne Plus device from Applied Biosystems (Thermo Fisher Scientific, Inc.) and the SYBR Premix Ex Taq kit as per the manufacturer's instructions. The qPCR thermocycling conditions consisted of an initial denaturation step at 95°C for 10 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, and annealing/extension at 60°C for 20 seconds. To determine the relative mRNA expression levels, we utilized the $2^{-\Delta\Delta Cq}$ method [17], normalizing them to the internal reference gene GAPDH. All primers were synthesized by TsingKe Biological Technology and are listed in Table 1. Sequences of primers used in reverse transcription-quantitative PCR.

Table 1. normalizing them to the internal reference gene GAPDH

Gene	Sequence (5'→3')
GAPDH	F: ACAGCAACAGGGTGGTGGAC R: TTTGAGGGTGCAGCGAACTT
wnt3a	F:TGCTGGACAAAGCTACCAGG R:CGAGACACCATCCCACCAAA
β-catenin	F: CTCTAGTGCAGCTTCTGGGTT R: AGATGGCAGGCTCGGTAATG
C-Myc	F: CGAGCTGAAGCGTAGCTTTT R: CTCGCCGTTTCCTCAGTAAG
	F, forward; R, reverse

3. Statistical Analysis

All statistical analyses were conducted using SPSS 22.0 software. Data are reported as mean±standard deviation based on three independent repetitions. Intergroup differences were analyzed by Student's t-test (two groups) or one-way ANOVA (≥3 groups), with statistical significance defined as $p<0.05$.

4. Results

SXG serum suppressed proliferation in MNNG-stimulated WB-F344 cells. Proliferation was quantified via MTT at 24h, 48h, and 72h. Relative to controls, model group proliferation increased significantly ($P<0.05$). Conversely, SXG-L/M/H and SXG-H+CP21R7 groups exhibited dose-responsive inhibition vs. model ($P<0.01$), maximal in SXG-H (Fig. 1). Furthermore, as the duration of treatment and dosage increased, the cell proliferation activity gradually diminished. In comparison to the SXG-H group, the cell proliferation activity in the SXG-H+CP21R7 group exhibited a significant increase ($P<0.01$). These data demonstrate time- and dose-dependent inhibition of malignant WB-F344 cell proliferation by SXG-containing serum.

SXG-containing serum inhibited the activation of Wnt signaling pathway-related proteins in MNNG-stimulated WB-F334 cells. The quantitative real-time polymerase chain reaction analysis was performed to evaluate the expression of WB-F344 cells were treated for 24h and 48h with Control, Model, SXG-L, SXG-M, SXG-H, or SXG-H combined with the Wnt activator CP21R7; Wnt pathway-related proteins were analyzed. As shown in Fig.2, Wnt pathway genes (wnt3a, β-catenin, c-Myc) were markedly overexpressed in model cells relative to control($P<0.05$). However, the mRNA expression levels of wnt3a, β-catenin, and C-Myc were significantly decreased in the SXG-L, SXG-M, and SXG-H group compared to the Model ($P<0.01$). Additionally, compared to the SXG-H, the mRNA expression levels of wnt3a, β-catenin, and C-Myc were significantly increased in the SXG-H+CP21R7($P<0.01$).

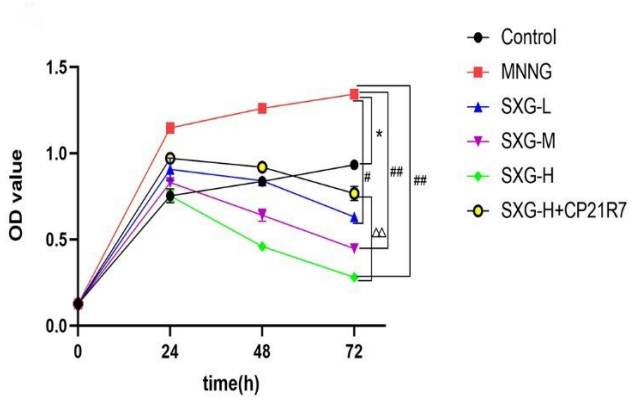

Fig 1. Anti-proliferative effects of SXG serum in MNNG-stimulated WB-F344 cells. Compared to the Control, *P<0.05; compared to the Model, #P<0.05, ##P<0.01; compared to SXG-H, △△P<0.01; MNNG, N-methyl-N '-nitro-N-nitroso; SXG-L, low dose containing serum ; SXG-M, middle dose drug-containing serum; SXG-H, high dose medicated serum; SXG-H+ CP21R7, Wnt activator.

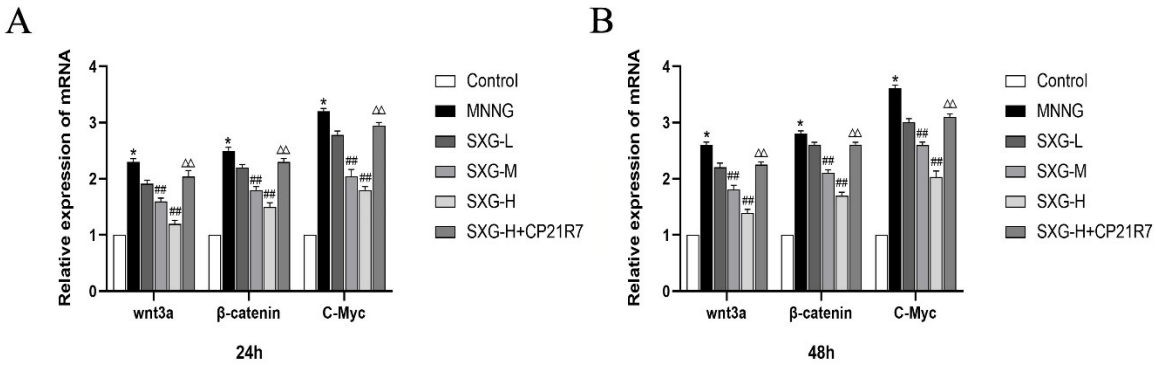

Fig 2. SXG serum regulates Wnt pathway transcripts at 24h and 48h. (A) Wnt3a/β-catenin/c-Myc mRNA expression after 24h SXG treatment. (B) Transcript levels of Wnt targets following 48h SXG exposure. Quantitative PCR data shown as mean±SD. Compared with the Control, *P<0.05, **P<0.01; compared with the Model, #P<0.05, ##P<0.01; compared with SXG-H, △△P<0.01.

5. Discussion

The development of liver cancer is a complex and multi-stage process that typically begins with hepatitis and progresses to liver fibrogenesis, advanced hepatic fibrosis, hepatic precancerous lesions, and ultimately, the development of liver cancer [9]. Precancerous lesions in liver cirrhosis refer to benign liver lesions that have the potential to transform into malignant liver tumors. Therapeutically efficient prevention and treatment of these precancerous lesions can inhibit their progression to liver cancer and reduce the occurrence of the disease[10]. These models are induced by the experimental carcinogen MNNG, which replicates some of the conditions observed during the transformation of hepatocytes into hepatocellular carcinoma cells [11]. In this study, a model of MNNG/H_2O_2-induced malignant transformation was constructed using the WB-F34 cell line. It was found that the SXG drug-containing serum could significantly inhibit cell proliferation.

Numerous studies have confirmed that abnormal activity in the Wnt/β-catenin signaling pathway is a significant factor in the occurrence and progression of liver cancer, as well as its metastasis [12]. The Wnt pathway orchestrates WB-F344 cell malignant transformation. Wnt3a-overexpressed in hepatoma cells - drives tumorigenesis/metastasis [13], while aberrant β-catenin expression mediates hepatic oncogenesis[14]. Upon Wnt-Frizzled binding, Dishevelled aggregates nucleate a phosphorylation cascade that de-represses β-catenin, enabling its nuclear translocation. This results in the inactivation of GSK-3, preventing the phosphorylation of cytoplasmic β-catenin by GSK-3β. As a result, β-catenin gradually accumulates and translocates into the nucleus, where it interacts with the nuclear transcription factor T lymphocyte factor/lymphoid enhancer factor (TCF/LEF). This interaction regulates the expression of target genes such as C-myc, ultimately contributing to the malignant transformation of hepatocytes[15,16], culminating in hepatocarcinogenesis.

The Chinese medicine compound granule SXG is carefully designed, and the combination of various herbs in the prescription has been found to have beneficial effects such as improving blood flow and clearing stagnation, improving qi and nourishing yin, softening and eliminating diseases, as well as strengthening timmune defenses and cancer-fighting mechanisms. It has shown positive therapeutic effects in clinical practice. This study aims to further explore the mechanism and potential role of SXG-containing serum in the treatment of these conditions. Through Wnt/β-catenin signaling, SXG suppresses malignant progression in WB-F344 cells.

Declarations

Animal research ethical approval

The animal experimental protocols in this study were approved by the Ethics Committee of Shaanxi University of Traditional Chinese Medicine (Approval number: SUCMDL-20190309001). All experimental procedures were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals provided by Shaanxi University of Traditional Chinese Medicine, China

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