The effect of hericium polysaccharides combined with aspirin on the expression of CD133 and ABCG2 in lung cancer

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Background: The study was designed to investigate the effects of aspirin and hericium polysaccharides (HP) on the expression of CD133 and ABCG2 in lung cancer growth.

Methods: In vitro, A549 cells were treated with aspirin alone, HP alone, and a combination of both substances. Cell proliferation and the expression of CD133 and ABCG2 were examined using the CCK-8 assay, colony formation assay, and western blot. In vivo, eight C57BL/6J male mice were randomly selected as a blank group. The remaining 42 were inoculated with A549 cells to establish tumor-bearing model. The tumor-bearing mice were giving aspirin alone, HP alone, and a combination of both substances and sacrificed after 30 days of drug treatment. After tumor volumes were calculated, and tumor tissues were weighed, Western blot and quantitative fluorescence PCR were used to detect the expression of CD133 and ABCG2 in tumor tissues from different groups.

Results: Aspirin alone, HP alone and combined aspirin and HP inhibited cell proliferation significantly. Western blot results showed that Aspirin alone, HP alone and combined HP and aspirin inhibited the expression levels of CD133 significantly, but HP alone did not inhibit the expression levels of ABCG2. Combined HP and aspirin are synergistic. In vivo, HP alone, aspirin alone, and combined aspirin and HP inhibited tumor volume and tumor weights significantly. According to results from the Western blot and PCR analyses, HP alone, aspirin alone, and combined HP and aspirin inhibited the expression levels of CD133 significantly, but HP alone did not inhibit the expression levels of ABCG2. Combined aspirin and HP applications are synergistic.

Conclusions: The combined effect of aspirin and HP inhibited tumor growth significantly by obstructing CD133 and ABCG2 expression in lung cancer.

Keywords: Aspirin; hericium polysaccharides (HP); CD133; ABCG2; lung cancer

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Introduction

Lung cancer, a relatively common malignant tumor, is increasing its number of victims year after year, but its pathogenesis remains unclear. Many studies have suggested that lung tumors are caused by the presence of cancer stem cells (CSCs) in tumor tissues, and the number of these CSCs is extremely small, but their role in tumorigenesis is very significant. Given that CD133 and ABCG2 can be used as markers (1) for CSCs, an in-depth study on the characteristics of CD133 and ABCG2 and their role in lung tumors could provide new therapeutic targets for the prevention of lung tumors.

CD133, also known as Prominin-1, is distributed
mainly in the cell membrane and is one of the well-studied CSC markers. Several studies have shown that CD133 is closely related to the formation of many tumors. For example, the expression of CD133 increases sequentially in cirrhotic tissues, adjacent tissues, and liver cancer tissues and is expressed in multiple tumor tissues, such as prostate cancer, bladder cancer, and glioma (2-4). Also, the level of CD133 in lung cancer is reportedly also significantly higher than that in normal tissues (5).

ABCG2, also known as breast cancer resistance protein (BCRP), is a gene that belongs to the G subfamily of the ABC family and is a transmembrane transporter composed of 663 amino acids with an ATP-dependent drug efflux function (6,7). This gene can unselectively transport harmful substances out of the cells and plays a physiological protective role in normal organisms, but it exhibits multi-drug resistance (MDR) to tumor tissues. Existing studies have shown that both highly malignant tumor tissues and CSCs have strong drug resistance and high expression of ABCG2. According to the CSC theory, the MDR of tumors is derived from CSCs; for its resistance mechanism, in addition to stem cells’ better “self-renewal” ability and stationary phase characteristics, the most important factor is the high expression of the ABC transporter (8). Experiments have shown that there are multiple ABC transporters on the surface of CSCs, including ABCB1 (MDR1), ABCC1 (MRP1), and ABCG2 (BCRP), and per in vitro experiments, the efficacy of anti-tumor drugs (9) can be enhanced using ABC protein inhibitors.

Aspirin is a non-steroidal anti-inflammatory drug used widely in the treatment of cardiovascular diseases, cervical cancer, and pulmonary arterial hypertension (10,11). Its role in preventing tumors may be related to, among others, the non-selective inhibition of cyclooxygenase (COX-1 and COX-2), inhibition of tumor cell proliferation, promotion of tumor cell apoptosis, and inhibition of tumor angiogenesis. *Hericium erinaceus* is a type of edible fungus used for medical purposes and has as its main active substance hericium polysaccharide (HP). It has been reported that it has efficacies including anti-tumor, anti-bacteria, immune regulation, anti-oxidation, and lowering blood lipids (12,13). Our unpublished study also showed that HP inhibits PD-1 and enhances the activity of natural killer (NK) cells and cytotoxic T (CTL) cells.

In this experiment, we evaluated the inhibitory effect of HP combined with aspirin on lung CSCs in vitro and in vivo.

**Methods**

**Cell proliferation assay**

Cells were cultured in DMEM containing 10% fetal bovine serum with 5% CO₂ at 37 °C. The A549 cells were inoculated into a 96-well plate at a cell density of 5,000 cells per well. The cells were then partitioned into: (I) the control group; (II) the HP low-dose group (HPL, 25 µg/mL); (III) the HP high-dose group (HPH, 100 µg/mL); (IV) the aspirin low-dose group (AL, 8 µg/mL); (V) the aspirin high-dose group (AH, 16 µg/mL); (VI) the HPL (25 µg/mL) + AL group (8 µg/mL); and (VII) the HPH(100 µg/mL) + AH group (16 µg/mL). HP was purchased from Nuoyuan Biotech Shanghai, China (the purity >98%), and aspirin (molecular weight: 180) was acquired from Sigma USA. Three parallel wells were set for each group and were cultured for 12, 24, 36, 48, 60, and 72 h. At the end of each culture, 10 µL of CCK8 solution was added to each well and cultured for another 4 h. The culture solution was carefully removed, and the absorbance value was measured at 490 nm using a microplate reader (14).

**Colony formation assay**

Cells were seeded at a density of 1×10³ cells/well in a six-well plate and after incubation for 24 hours, were treated with aspirin alone, HP alone, or a combination of both drugs for 7 days. At the end of the 7 days, the cells were stained with 0.1% crystal violet, and the number of colonies was counted.

**In vivo model**

Forty-eight C57BL/6J male mice (6-8 weeks old) weighing 20±2 g were purchased from the Children Hospital Affiliated to Zhengzhou University and fed adaptively for one week and numbered with picric acid in a dry and clean animal room under 25 °C and 8 h/d light. The LL/2-luc-M38s lung cancer cell line (ATCC, USA) was cultured and adjusted to 10⁶ cells/mL. Six mice were randomly selected for the control group, receiving standard feeding, and the remaining mice were inoculated with 0.2 mL of the Lewis lung cancer cell line subcutaneously in the dorsal area of the right axilla of the mice. At 24 h after the inoculation, the animals were weighed and randomly divided into 7 groups, with 6 mice in each group: (I) the model group;
(II) the HPL group (mg/kg/d, i.p.); (III) the HPH group (100 mg/kg/d, i.p.); (IV) the AL group (8 mg/kg/d by gavage); (V) the AH group (16 mg/kg/d by gavage); (VI) the HPL (25 mg/kg/d) + AL group (8 mg/kg/d); (VII) the HPH (100 mg/kg/d) + AH group (16 mg/kg/d). The body weights of the mice were measured at the same time every 5 d. The size of the tumor was measured with a vernier caliper every 5 d after tumor formation, and the tumor volume was calculated according to the formula $V = \frac{a^2b}{2}$; $a$ represents the longest diameter of the tumor volume, $b$ is the shortest diameter of the tumor volume; the length is measured in mm. The mice were weighed and sacrificed by cervical dislocation on the 30th day after the administration of our curative substances.

**Western blot**

The mice were left to fast for 24 h before sacrifice. After anesthetization by intraperitoneal injection with 10% chloral hydrate (0.35 mL/100 g), the mice were then placed supinely and fixed on an anatomical plate and the lungs were removed and weighed. Total protein was extracted from cells or lung cancer tissues using RIPA, and 75 µg of protein was applied to SDS-PAGE and then transferred to the PVDF membrane. Additionally, TBST (containing 5% skim milk) was used to block the non-specific binding for 2 h, and 1× TBST was washed 4 times (each wash lasted 5 min). Anti-CD133 (1:200), ABCG2 (1:250), and GAPDH (1:1,000) were added to incubate overnight at 4 ℃. After washing the membrane the next day, the corresponding secondary antibody was added to incubate for 2 h at room temperature, followed by 1× TBST washing (3 times). Results were acquired using ECL luminescence in a dark room. All antibodies were procured from Santa Cruz Biotech, Dallas, Texas, USA. Grayscale analysis of bands was performed using the Image J software. Semi-quantitative analysis was achieved using GAPDH as an internal control, and the value was determined by the ratio of the target band to the gray value of the corresponding GAPDH.

**Real-time fluorescent quantitative PCR**

Total RNA was extracted from tumor tissues or cells using Trizol Reagent, and 2 µg of the RNA was used for reverse transcription. The resulting cDNA was amplified using a fluorescent quantitative PCR kit manufactured by Thermo Fisher Scientific, pre-denatured at 95 ℃ for 10 min and cycled (40 times) at 95 ℃, 15 s and 60 ℃, 60 s. Results were analyzed with $2^{-\Delta\Delta CT}$. The relevant primer sequences were as follows: CD133, 5‘-TGTTGAAAGATGGCTTCA-3’ and 5‘-GAGGGTCAATTCCACAGCA-3’; ABCG2, 5‘-TTACATCAGGGTTAAAAAGCACAGG-3’ and 5‘-CCTGTGCTTTTTTAACCTGTGAATA-3’; β-actin, TCCTTTCTGGGTATGGAATCCT and GCTCAGTAACAGTCCGCCTA.

**Statistical analysis**

The results were processed with ONE-WAY ANOVA of the statistical software SPSS16.0 for windows and were expressed as mean ± standard deviation ($\bar{x}$±s). P<0.05 showed that differences were statistically significant.

**Results**

**Aspirin alone, HP alone, and the two drugs combined inhibited proliferation, colony formation, and the expression of CD133 and ABCG2 in A549 cells**

Cell proliferation was assayed using the CCK-8 assay and colony formation assay. CCK-8 assay results showed that HPH reduced the proliferation of A549 cells significantly after 48 h of culture (P<0.05, Figure 1A). Both AL and AH also reduced the proliferation of A549 cells significantly after 48 h (Figure 1B). Furthermore, both HPL + AL and HPH + AH inhibited the proliferation of A549 cells, and the proliferation rates were significantly lower than those in the control group after 36 h of culture (Figure 1A). Cells in HPH + AH showed the lowest proliferation rate. Colony formation assay findings also suggested that HP alone, aspirin alone, and combined aspirin and HP markedly suppressed cell proliferation (Figure 1B), while combined aspirin and HP exhibited stronger inhibition when compared with aspirin alone or HP alone (Figure 1B).

The expression levels of CD133 and ABCG2 were evaluated by the Western blot method after treatment of A549 cells with aspirin alone, HP alone, and the two drugs combined for 24 h. The results showed that the expression levels of CD133 decreased in the HPH, AL, AH, HPL + AL, and HPH + AH groups when compared with the control group. The expression of ABCG2 also decreased in the AL, AH, HPL + AL, and HPH + AH groups, and the expression levels of CD133 and ABCG2 was statistically lower in combined aspirin and HP when compared with
aspirin alone or HP alone (Figure 1C).

**Aspirin alone, HP alone, and the two substances combined inhibited the growth of lung tumors**

None tumor-bearing mice died during the procedure. Activities of tumor-bearing mice decreased significantly, and their mental states were poor in the model group, but the administration of aspirin alone or HP alone or the two combined increased the activities significantly and improved the mental states of mice. As seen in Figure 2A, most of the mice developed tumors around the 20th day, and the mice in the model group began to have a significant increase in body weight on the 20th day, with their body weights higher than those in other groups. Figure 2B shows that the tumor volume increased rapidly in the model group on the 20th day, which was significantly inhibited by HP alone, aspirin alone, or combined aspirin and HP. HPH showed significantly better inhibitory effects than HPL, but AL and AH showed no differences. Combined aspirin and HP displayed significantly better inhibitory effects than HP alone or aspirin alone (Figure 2B). Judging by the presentation in Figure 2C, the tumor weights of the mice in the model group were significantly higher than those of the mice in the other groups, which were inhibited significantly by HP alone, aspirin alone, and combined aspirin and HP. The combined application of aspirin and HP is synergistic.
The growth of lung tumors was inhibited by aspirin and HP alone or in combination (n=6). (A) The body weights of mice were recorded every 5 days (*P<0.05 and **P<0.01 vs. control). (B) Tumor volumes were examined in each group every 5 day (*P<0.05 and **P<0.01 vs. model; †P<0.05 vs. HP alone or aspirin alone). (C) The tumor weights of the mice in the model group were significantly higher than those of the mice in the other groups, which were significantly inhibited by HP alone, aspirin alone or combined aspirin and HP (*P<0.05 and **P<0.01 vs. control vs. model; †P<0.05 vs. HP alone or aspirin alone). HP, hericium polysaccharides; HPL, HP low-close group; HPH, HP high-close group; AL, aspirin low-close group; AH, aspirin high-close group.

**Figure 2**

**Aspirin alone, HP alone, and the two substances combined inhibited the expression levels of CD133 and ABCG2**

Western blot and real-time qPCR results showed that CD133 was upregulated in the model group but decreased significantly in the HP alone, aspirin alone, or combined HP and aspirin groups. The expression levels of CD133 in the combination group were lower than those in the HP alone or aspirin alone groups, and the difference was statistically significant, but there was no significant difference between the two doses (Figure 3A).

Western blot and real-time qPCR results showed ABCG2 was upregulated in the model group but decreased significantly in the HP alone or combined HP and aspirin groups. HP alone did not inhibit the expression of ABCG2. The expression levels of ABCG2 in the combination group were lower than those in the HP alone or aspirin alone groups (Figure 3B).

**Discussion**

The formation of lung tumors is the result of a combination of factors such as genes, environment, and body conditions (15,16). CD133 and ABCG2, as two of the markers of lung CSCs, are located mainly in the cell membrane, but some are situated in the cytoplasm. Many studies have shown that the biological functions of CD133 and ABCG2 are closely related to the formation of many tumors, but their role in the occurrence and development of tumors is still unclear. The results of this experiment demonstrate that there are self-renewing cell populations that express CD133 and ABCG2 in lung cancer. CD133 and ABCG2 are expressed primarily in the cell membrane of lung cancer cells, and a few of them are found in the cytoplasm, with the positive expressions of CD133 and ABCG2 related to the invasion and metastasis of tumor tissues.

At present, most studies believe that CSCs play an
 extremely crucial role in the formation, development, infiltration, and metastasis of tumors. Aspirin, a non-steroidal anti-inflammatory drug, has been revealed to reduce the incidence of colorectal tumors significantly in some studies (17). *In vitro* analyses have demonstrated that aspirin alone or in combination with 5-FU can inhibit the survival of colon cancer cells, the progression of the cell cycle, the expression of cyclooxygenase-2, and other processes (18). In accordance with the preceding claims, the results of our experiment showed that the growth of lung cancer and the expression levels of CD133 and ABCG2 in the aspirin groups were significantly lower than those in the control group in *vitro* and in *vivo*, indicating that aspirin has a certain inhibitory effect on the growth of lung cancer. However, the difference between the high-dose group and the low-dose group was not statistically significant, which could be due to the relatively small samples used.

HP is a traditional Chinese medicine ingredient that has an anti-cancer effect. HP inhibits gastric cancer growth via cell cycle arrest and apoptosis and enhances doxorubicin-induced apoptosis in human hepatocellular carcinoma cells (12,19). A previous study in our lab showed that HP could inhibit lung cancer growth by inhibiting PD-1 and enhancing the activity of NK and CTL cells. Our experimental results in the present study suggest that HP had significant inhibitory effects on the proliferation and colony formation of A549 cells and lung cancer growth in *vivo*. The ingredient also inhibited the expression of CD133, but not ABCG2. The effect of the combined use of HP and Aspirin showed better efficacies in inhibiting cancer cell proliferation and CD133 and ABCG2 expression than those of the single substance-treated groups, suggesting that the combined use had a more beneficial anti-cancer effect. However, further studies are needed to define specific
combined dosage.

At present, no obvious side effects have been encountered using high doses of HP. The most common side effect of Aspirin is gastrointestinal dyspepsia. Although high-doses of Aspirin can cause a gastric ulcer, gastric bleeding, and other symptoms, no significant adverse reactions were registered in mice during our study using the combination of HP and Aspirin.

Conclusions

The results of this experiment confirm that Aspirin and HP play inhibitory roles in the expression of CD133 and ABCG2 and that the combined use of the two drugs has a synergistic effect. In-depth studies on non-steroidal, anti-inflammatory drugs and HP’s prevention and treatment of lung cancer may provide the theoretical and experimental bases for finding new therapeutic targets for lung cancer.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.06.01). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Animal Ethic Committee of People’s Hospital of Guizhou Province (GZPH-2016-116).

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