# Anticancer Effects of Residual Powder from Barley-*Shochu* Distillation Remnants against the Orthotopic Xenograft Mouse Models of Hepatocellular Carcinoma *in Vivo*

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**Barely-*Shochu* is a traditional Japanese liquor distilled from fermented barley with *Saccharomyces cerevisiae*. Barely-*Shochu* distillation remnants (SDR) are by-products in the manufacturing process of barley-*Shochu*. We have already reported on valuable powder from *Shochu* distillation remnants (PSDR) including antioxidative compounds such as polyphenols. In this study, we investigated the therapeutic effects of barely-PSDR against orthotopic xenograft mouse models of hepatocellular carcinoma (HCC) *in vivo*. We constructed a mouse model of HCC by orthotopical inoculation of HepG2 cells into the liver of SCID mice. Barely-PSDR (2250 mg/kg) was orally treated once each day for 21 d after the inoculation of HepG2 cells. The livers were removed from anaesthetized mice after the treatment with barely-PSDR and fixed in formalin. The liver sections were analyzed by hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) methods. Remarkably high reduction of tumorigenesis was obtained in the mouse models of HCC after the oral administration of barely-PSDR *in vivo*. Induction of apoptosis in the liver section on the mouse models treated with barely-PSDR was observed. Furthermore, prolonged survival was obtained. Thus, therapeutic effects of barely-PSDR without side effects on the orthotopic xenograft mouse models were revealed for the first time.**

**Key words** barely-*Shochu* distillation remnant; apoptosis; human hepatocellular carcinoma; polyphenol

Human hepatocellular carcinoma (HCC) is one of the most frequent tumors in the world and the third most frequent oncological cause of death.[1,2] The mortality rate and prognosis for HCC's increasing incidence have not yet been greatly improved. Surgical resection is frequently limited due to metastasis, cirrhosis, and other pathological changes in the liver parenchyma, and the relapse rate is high in postoperative patients of HCC.[3,4] Furthermore, it is well known that drug-resistant genes including multi-drug resistance gene 1 (*MDR1*) are frequently overexpressed in HCC. Therefore, HCC is chemorefractory in chemotherapy. In fact, several anticancer agents such as doxorubicin have a response rate of up to 20%.[5] Therefore, the development of novel therapeutic and/or preventive agents without side effects is desirable for the treatment against HCC.

Barely-*Shochu* is a traditional Japanese liquor distilled from fermented barley with *Saccharomyces cerevisiae*. In the barley-*Shochu* manufacturing process, a large amount of *Shochu* distillation remnants (SDR) is generated. SDR used to be dumped into the sea as wastewater. However, the disposal method is now forbidden to prevent pollution of the sea. Therefore, the development of new and effective applications of SDR would serve an important function from the viewpoint of environmental preservation. On the other hand, it is important that the SDR do include various useful organic compounds derived from the fermentation of grains.[6] Therefore, there were some reports on biological and bioactive effects of SDR such as growth-stimulating effects on *Bifidobacterium* by sweet potato-SDR,[7] antioxidative cytoprotection activity of vinegars produced from rice-, barley, and sweet potato-SDR.[8]

We succeeded in developing membrane-targeted chemotherapy with hybrid liposomes composed of phospholipid (L-$\alpha$-dimyristoylphosphatidylcholine) and nonionic surfactants (polyoxyethylenelauryl ether) *in vitro*, *in vivo*, and in clinical applications.[9] On the basis of these studies, we have tried to develop a new processing method and medical applications for the effective utilization of SDR. We have already reported on inhibitory effects of freeze-dried supernatants of barley-, rice-, and sweet potato-SDR on the growth of human tumor cells *in vitro*.[10] Furthermore, we have obtained valuable powder from *Shochu* distillation remnants (PSDR) by treatment with ethanol. In addition, we found immunostimulatory effects of barley-PSDR on serum interferon (IFN)-$\gamma$ production and natural killer (NK) activity without toxicity to normal rats *in vivo*.[11] However, anticancer effects of barley-PSDR *in vivo* have not been elucidated. The aim of the present study is to clarify the anticancer effects of the oral administration of barley-PSDR on orthotopic xenograft mouse models of HCC *in vivo*.

## MATERIALS AND METHODS

**Preparation of Barely-PSDR** Barley-PSDR was prepared as described previously.[11]

**Cell Culture** A human hepatocellular carcinoma (HepG2) cell line was obtained from RIKEN Cell Bank (Ibaraki, Japan). HepG2 cells were grown in minimum essential medium (Invitrogen, CA, U.S.A.). The media was supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, UT, U.S.A.) and antibiotics (100 units/mL penicillin and 100 $\mu$g/mL streptomycin). The cells were cultured at 37°C in a humidified atmosphere containing 5% $CO_2$.

**Assessment of Antitumor Activity *in Vivo*** The mice were handled in accordance with the guidelines for animal experimentation set out in Japanese law. The animal studies were approved by the Committee on Animal Research of the Sojo University. Female SCID mice (C.B-17/Icr-scid) were obtained from CLEA Japan (Tokyo, Japan). HepG2 cells ($5.0\times10^6$ cells) suspended into matrigel (BD Co., U.S.A.) were

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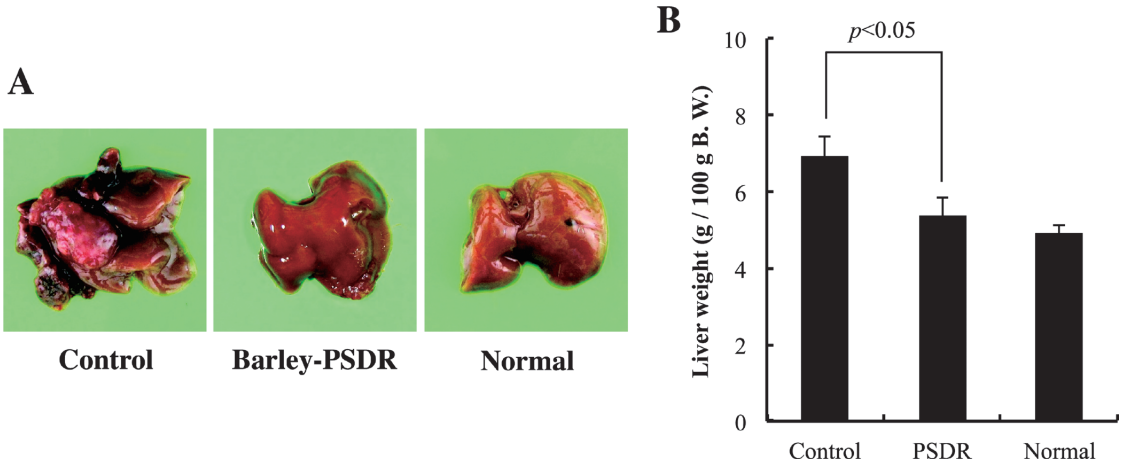


Fig. 1. Liver (A) and Relative Liver Weight (B) of Mice Orally Treated with Barley-PSDR after the Orthotopic Transplantation of HepG2 Cells
Data represented are the mean±S.D. Number of mice was three in each group.

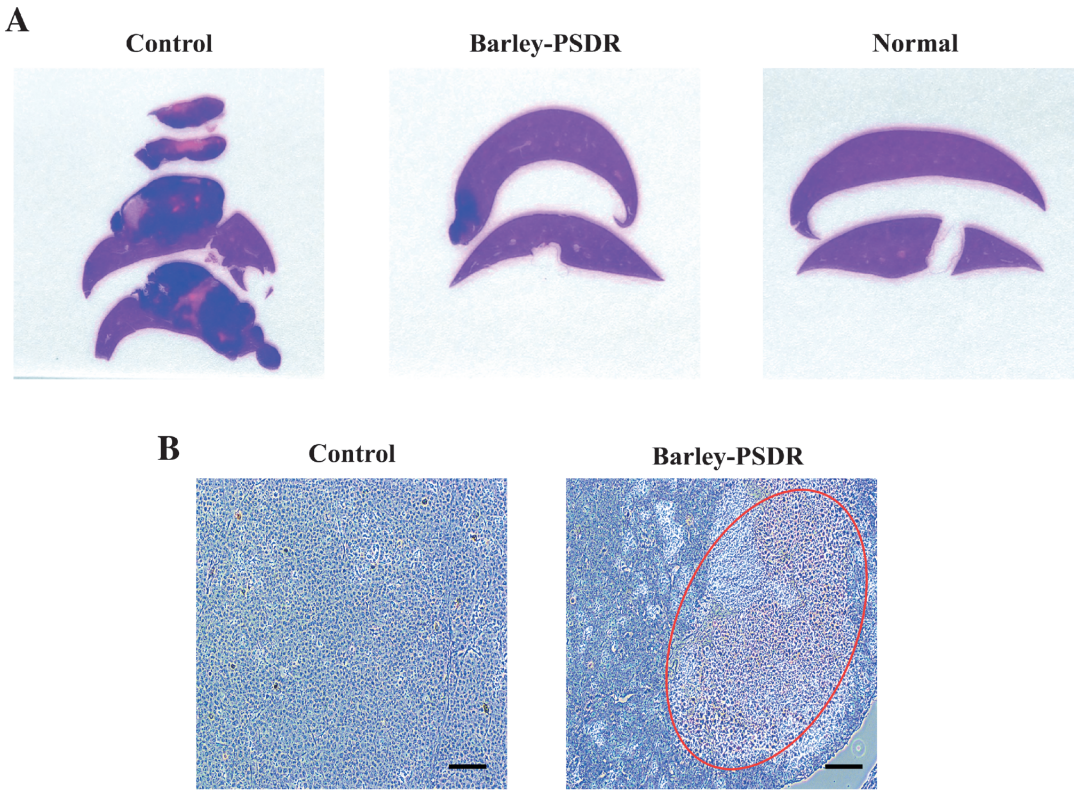


Fig. 2. Section of Liver in Xenograft Mice Models Using HE Staining (A) and TUNEL Method (B) after the Oral Administration of Barley-PSDR
Dose for barely-PSDR; 2250 mg/kg. (B) Apoptotic cells (red circle) were observed using TUNEL method. Scale bar: 100 $\mu$m, magnification ×100.

orthotopically inoculated to the liver of the mice. Barley-PSDR (2250 mg/kg) was orally treated once each day for 21 d after the inoculation of HepG2 cells. The liver was removed from anaesthetized mice after the treatment with barely-PSDR and fixed in a 10% formalin solution. Then, the liver was embedded in paraffin and sectioned at 5 $\mu$m of thickness. The liver sections were stained with hematoxylin and eosin (HE) and observed by an optical microscope (Nikon TS-100, Tokyo, Japan). The number of mice was three in each group.

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Method** Detection of apoptotic cells was performed on the basis of the TUNEL method using an *in situ* apoptosis detection kit (ApopTag Plus Peroxidase, Intergen, U.S.A.) according to the manufacturer's directions. A final observation was performed on the liver sections removed from the mice subjected to 21 d of treatment with barely-PSDR using an optical microscope (Nicon TS-100; Tokyo, Japan).

**Assessment of Survival Rate *in Vivo*** For assessment of survival rate, female SCID mice (C.B-17/Icr-scid) were obtained from CLEA Japan (Tokyo, Japan). SCID mice were randomly grouped ($n$=5) on the basis of body weight on the day of tumor cells inoculation using the stratified randomization method. HepG2 cells ($5.0\times10^6$ cells) were orthotopically

inoculated to the liver of SCID mice. Barley-PSDR was orally treated once each day for 21 d after the inoculation of HepG2 cells. The median lifespan was calculated using the following equation, median lifespan=(median survival days after the treatment)/(median survival days of control group)×100.

**Statistical Analysis** Results are presented as mean±S.D. Data were statistically analyzed using Student's *t*-test. A $p$ value less than 0.05 was considered to represent a statistically significant difference.

## RESULTS AND DISCUSSION

**Therapeutic Effects of Barley-PSDR on the Orthotopic Xenograft Mouse Models** We examined inhibitory effects of the oral administration of barley-PSDR on the growth of tumor in orthotopic xenograft mouse models of HCC. In this study, the dose (2250 mg/kg) was determined from the maximum solubility (150 mg/mL) of barley-PSDR in phosphate buffered saline (PBS)(−) and maximum sample volume (15 mL/kg/d) of oral administration. And we have already reported the safety of SDR in this dose using normal rats.[10] Therefore, this does (2250 mg/kg) is the safety maximum dose of barley-PSDR *in vivo* experiment. The results are shown in Fig. 1. The liver of the group treated with barely-PSDR was almost the same as that of the normal group, although enlargement and tumor-nodes of HCC in the liver of the untreated control group were confirmed (Fig. 1A). Furthermore, the relative liver weight of the group treated with barely-PSDR was close to that of the normal group, although that of the control group obviously increased. There was a significant difference ($p<0.05$) in the relative liver weight between the control group and the group treated with barley-PSDR (Fig. 1B). It is noteworthy that remarkable reduction of tumorigenesis was obtained after the oral administration of barley-PSDR on the orthotopic xenograft mouse models of HCC *in vivo*.

**Histological Bioanalysis** We histologically evaluated the inhibitory effects of barley-PSDR using the liver tissues of the orthotopic xenograft mouse models of HepG2 cells *in vivo*. First, we observed the liver tissues of mouse models with a microscope by HE staining method. The cross-sections of liver tissues stained with HE are shown in Fig. 2A. In the control group, a great portion of the section was occupied by the tumor. In contrast, a small fraction of the tumor was observed in the section of the group treated with barely-PSDR.

**Induction of Apoptosis by Barley-PSDR** We examined the mechanism of the inhibitory effects of barley-PSDR on the orthotopic xenograft mouse models of HCC *in vivo*. In our previous study, barley-PSDR were effective for inhibiting the growth of HCC cells through the induction of apoptosis *in vitro*.[11] Therefore, we assessed induction of apoptosis by barley-PSDR on the orthotopic xenograft mouse models of HCC *in vivo*. As a biochemical hallmark of apoptotic cell death, fragmentation of nuclear DNA was examined by TUNEL staining of paraffin-embedded liver tissue. The results are shown in Fig. 2B. A significant number of apoptotic cells appeared brown in the liver tissues of the group treated with barley-PSDR, while apoptotic cells were not observed in the control group. These results indicate that barley-PSDR have remarkable inhibitory effects on the growth of HepG2 cells along with apoptosis.

**Prolonged Survival Effects of Barley-PSDR on the Orthotopic Xenograft Mice Models *in Vivo*** We further examined the prolonged survival effects of barley-PSDR on the orthotopic xenograft mouse models of HCC *in vivo*. The results are shown in Fig. 3A. The median survival time of the control group and the group treated with barely-PSDR were 58.0 and 81.0 d, respectively. It is of interest that significantly prolonged survival rate of 168% ($p<0.01$) was obtained in the group treated with barely-PSDR. We have already reported the dose-dependent antitumor effects of barely-PSDR *in vitro*.[11] It is suggested that barely-PSDR should inhibit dose-dependently the growth of tumor *in vivo* as well as *in vitro*. Dose-dependent therapeutic effects and anti-tumor mechanism of barely-PSDR for carcinoma model mice are investigating in detail at present. These results demonstrate for the first time that barley-PSDR could strongly inhibit the growth of HepG2 cells *in vivo*.

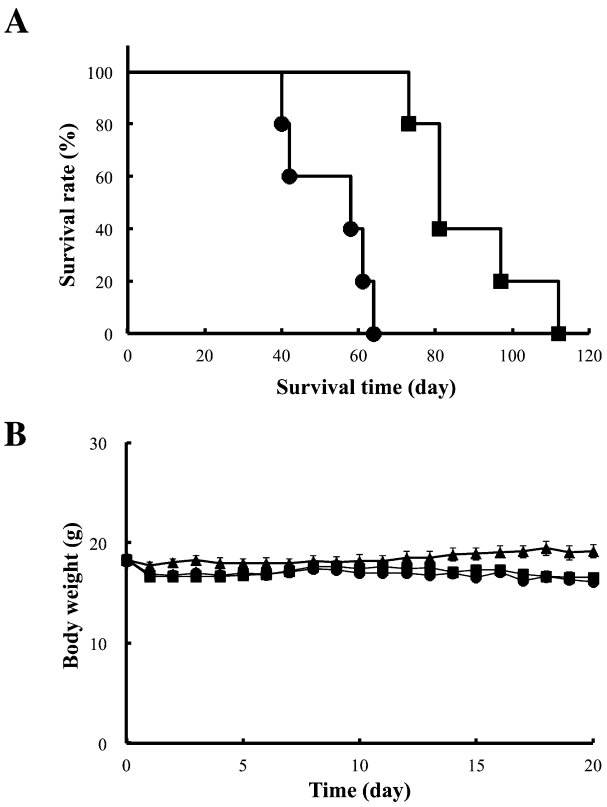


Fig. 3. Survival Curves (A) and Body Weight Changes (B) of Mice Orally Treated with Barley-PSDR after the Orthotopic Transplantation of HepG2 Cells

●: Control, ■: Barley-PSDR (2250 mg/kg), ▲: Normal. Five mice were employed in each experiment.

Furthermore, no weight loss was observed in the orthotopic xenograft mouse models of HCC during the oral treatment period of barley-PSDR for 21 d (Fig. 3B). There was no significant difference between the normal group and the group treated with barely-PSDR on the basis of gross pathology (Fig. 1) and histological analysis (Fig. 2). In addition, we have already reported on the safety of barley-SDR *in vivo*.[10] These results indicate that barley-PSDR should have no severe side effects *in vivo*.

What components of barley-PSDR are related to anticancer effects? It is well known that polyphenolic compounds contained in natural materials such as fruit and tea show

anticancer and cancer prevention effects *in vitro*, *in vivo*, and in the *meta*-analysis of several epidemiological studies.[12,13] Recently, we noted the antioxidative effects of barely-PSDR and identified some polyphenolic compounds from barley-PSDR by using HPLC.[14] Interestingly, it has already been reported that polyphenolic compounds such as ferulic acid, caffeic acid, catechin, and protocatechuic acid have anticancer effects along with apoptosis.[15–17] The polyphenolic compounds in barely-PSDR would be important for inhibiting the growth of tumors along with apoptosis.

In conclusion, our study demonstrates for the first time the remarkable therapeutic effects of barley-PSDR on the orthotopic xenograft mouse models of HCC *in vivo*. The noteworthy aspects are as follows. (a) Remarkably high therapeutic effects of barley-PSDR were obtained in the orthotopic xenograft mouse models. (b) Induction of apoptosis was observed in mice after the treatment with barely-PSDR using the TUNEL method. (c) Prolonged survival was observed in mice after treatment with barely-PSDR. The findings of this present study demonstrate that barley-PSDR could have the possibility of therapeutic and/or preventive agents of HCC.

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