


# Polysaccharides Produced by the Mushroom *Trametes robiniophila* Murr Boosts the Sensitivity of Hepatoma Cells to Oxaliplatin via the miR-224-5p/ABCBI/P-gp Axis

Integrative Cancer Therapies  
Volume 21: 1–15  
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DOI: 10.1177/15347354221090221  
journals.sagepub.com/home/ict  


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## Abstract

**Aim:** To investigate the mechanisms employed by PS-T (polysaccharides of *Trametes*, PS-T), the main active ingredient of Huaier granules, to improve the susceptibility of hepatoma cells to oxaliplatin (OXA). **Methods:** Cell proliferation in response to PS-T was determined both in vitro and in vivo. The effects of PS-T on miRNAs were analyzed with the use of a microarray. MiRNAs were screened under specific conditions ( $P < .05$ ,  $\log_{2}\text{FoldChange} > \text{ABS} [1.5]$ ) and further silenced or overexpressed by liposome transfection. Levels of ABCBI mRNA and P-gp were detected by qRT-PCR and western blot analysis, respectively. A dual fluorescence assay was performed to determine whether miRNA directly targets ABCBI. **Results:** PS-T enhanced the inhibitory effect of OXA in human hepatoma cells and xenografts. Among 5 up-regulated miRNAs, overexpression of only miR-224-5p inhibited the expression of ABCBI mRNA and P-gp, while silencing of miR-224-5p had an opposite effect. Moreover, miR-224-5p can directly target the 3'-UTR of ABCBI. **Conclusion:** PS-T increases the sensitivity of human hepatoma cells to OXA via the miR-224-5p/ABCBI/P-gp axis.

## Keywords

chemotherapy resistance, *Trametes robiniophila* Murr, hepatocellular carcinoma, miR-224-5p, microRNA array

Submitted November 11, 2021; revised February 19, 2022; accepted March 8, 2022

## Introduction

Chemotherapy resistance is a major burden to the efficacy of therapeutic drugs against hepatocellular carcinoma (HCC). The main mechanisms underlying the resistance of human hepatoma cells to oxaliplatin (OXA) involve members of the ATP-binding cassette (ABC) transporter family, abnormal enzymatic activity of glutathione S-transferase or topoisomerase II, DNA repair, and cell autophagy.<sup>1,2</sup> Multidrug resistance 1 gene (MDR1), also known as permeability glycoprotein (P-gp), is a member of the ABC transporter family that has been positively correlated with MDR1 expression and the degree of chemotherapy resistance.<sup>3</sup> P-gp expression is significantly higher in liver cancer tissue than normal liver tissue,<sup>4</sup> thus inhibition or down-regulation of P-gp could strengthen the sensitivity of hepatoma cells to chemotherapy drugs.<sup>5</sup>

OXA,<sup>6</sup> a third generation platinum drug, is extensively applied for the treatment of HCC. Various microRNAs

(miRNAs), including miR-199a-5p,<sup>7</sup> miR-340,<sup>8</sup> miR-363,<sup>9</sup> and miR-182,<sup>10</sup> are reportedly involved in regulating the susceptibility of hepatoma cells to cisplatin, a first generation platinum drug. Although many studies have investigated the relationship between miRNAs and resistance of

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colorectal cancer to OXA,<sup>11,12</sup> there are relatively few reports on the regulatory relationships of miRNAs and resistance of hepatoma cells to OXA.

The mushroom *Trametes robiniophila* Murr (Huaier) is used as a traditional Chinese herbal medicine approved by the State Food and Drug Administration of China and widely employed for the treatment of multiple cancers.<sup>13</sup> Previous studies have shown that Huaier granules or extract can down-regulate mRNA expression of MDR1 and, subsequently, P-gp levels.<sup>14,15</sup> Moreover, an in-depth study reported that Huaier granules can be used to modulate miRNAs to achieve silencing of target genes.<sup>16</sup> MiRNAs are short endogenous noncoding RNAs, consisting of approximately 22 nt, that can bind to the 3' untranslated region (3'-UTR) of a target gene and promote its degradation or inhibit translation, thereby regulating protein expression at the post-transcriptional level.<sup>17,18</sup> Hence, the aim of the present study was to determine whether Huaier-induced activation of miRNAs targeting MDR1 can regulate P-gp expression to enhance the sensitivity of hepatoma cells to chemotherapy drugs.

## Methods and Materials

### Cell Culture

Human hepatoma HepG2 and Bel-7404 cells and human embryonic kidney (HEK)-293T cells were purchased from Jiangsu KeyGEN BioTECH Co., Ltd. (Nanjing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Inc., South Logan, UT, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin under an atmosphere of 5% CO<sub>2</sub>/95% air at 37°C.

### Drugs

PS-T, the main active ingredient of Huaier granules, was obtained from Fanying Life Sciences, Ltd. (Shanghai, China) and 2.0 g were dissolved in 40 ml of serum-free DMEM, mixed well with an oscillator, and placed on a decolorizing shaker for 72 hours to promote drug dissolution. Then, the drug was filtered through a MILLEX® GP non-pyrogenic filter (pore diameter, 0.22 µm; Sigma-Aldrich Corporation, St. Louis, MO, USA), aliquoted, and stored at -20°C for future use. OXA was purchased from Hangzhou Sanofi-Aventis Minsheng Pharmaceuticals Co., Ltd. (Hangzhou, China) and prepared in accordance with the manufacturer's instructions. OXA lyophilized powder was dissolved in 5% glucose solution and stored at -20°C for following-up use.

### CCK-8 Assay

The influence of drugs on cell viability was determined with the Cell Counting Kit-8 assay (CCK-8; MedChemExpress,

Monmouth Junction, NJ, USA). Briefly, hepatoma cells in the exponential growth phase were digested with 0.25% trypsin-ethylenediaminetetraacetic acid (NCM BioTECH, China) and seeded in the wells of 96-well plates at a density of 3000 cells/well. After adherence to the wells, the HepG2 and Bel-7404 cells were treated with the prepared drugs for 24, 48, and 72 hours. Subsequently, the DMEM was replaced with 100 µl of serum-free DMEM with the addition of 10 µl of CCK-8 solution and incubated under an atmosphere of 5% CO<sub>2</sub>/95% air for 3 hours at 37°C. Afterward, the absorbance of each well was calculated at a wavelength of 450 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

### EdU Assay

The measurement of cell viability was performed using the Cell Light EdU Apollo 567 In Vitro Imaging Kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) in accordance with the manufacturer's instructions. Initially, Bel-7404 and HepG2 cells ( $4.0 \times 10^3$ /well) were spread evenly on the bottom of the wells of 96-well microplates. Approximately 24 hours later, the cells were treated with prepared drugs for 48 hours. After removing the DMEM, the cells were labeled with 5-ethynyl-2'-deoxyuridine (EdU) diluted to 1:1000 in serum-free DMEM for 3 hours in a thermostatic incubator (5% CO<sub>2</sub>, 37°C). After discarding the DMEM containing EdU, the cells were fixed with 4% formaldehyde solution in phosphate-buffered saline (PBS; HyClone Laboratories, Inc.) for 30 minutes and then permeabilized with 0.5% Triton X-100 solution in PBS for 10 minutes. Finally, the cells were stained with Apollo® 567 reagent (Guangzhou RiboBio Co., Ltd.) for 30 minutes in the dark, while the DNA was stained with Hoechst 33342 in deionized water (1:100) for 30 minutes in the dark. Fluorescence images of cells were captured with a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

### Clone Forming Assay

Bel-7404 cells, Bel-7404 cells treated with an miR-224-5p inhibitor or a negative control (NC), HepG2 cells, and HepG2 cells treated with an miR-224-5p inhibitor or NC in suspension were carefully plated in 6-well plates at a density of  $0.80 \times 10^3$ /well (Bel-7404 cells) or  $2.0 \times 10^3$ /well (HepG2 cells) and treated with drugs at various concentrations for 24 hours. Specifically, Bel-7404 cells were treated with an equivalent volume of serum-free DMEM (control group), 0.27 mM PS-T, 10.08 µM OXA, or 0.27 mM PS-T + 10.08 µM OXA, while HepG2 cells were treated with an equivalent volume of serum-free DMEM (control group), 0.20 mM PS-T, 5.04 µM OXA, or 2.0 mM PS-T + 5.04 µM OXA. The cells were cultured in DMEM for 14 days with fresh medium every 3 days. After removal of the DMEM, the cells were washed twice with PBS, fixed

with 4% paraformaldehyde solution (Biosharp Life Sciences, Hefei, China) for 15 min, stained with crystal violet (Beijing Leagene Biotech Co., Ltd., Beijing, China) for 10 minutes, washed with running water, and air dried. Photographs of the cells were captured using an automatic chemiluminescence/fluorescence image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China) and analyzed with ImageJ software (version 4.0; <https://imagej.nih.gov/ij/>).

### Microarray Analysis

Six samples for miRNA microarray (GeneChip® miRNA Array 4.0; Affymetrix, Santa Clara, CA, USA) analysis were divided into 2 groups: an experimental group treated with 0.20 mM PS-T or a control group treated with an equal volume of DMEM. HepG2 cells plated in a 6-well plate overnight were treated with 0.20 mM PS-T dissolved in DMEM for 72 hours. Total RNA was extracted using the RNeasy Pure Cell/Bacteria Kit (Qiagen Biotech Co., Ltd., Beijing, China) and the quality and quantity were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). MiRNA microarray analysis of PS-T was conducted by Jiangsu KeyGEN BioTECH Co., Ltd. All procedures, including the addition of a Poly(A) tail, RNA labeling, hybridization, washing, staining, and scanning as well as data analysis were conducted in accordance with manufacturer's instructions (FlashTag™ Biotin HSR, RNA Labeling Kit for Affymetrix® GeneChip® miRNA Arrays).

### Prediction and Screening of miRNAs Targeting ABCB1 Genes

The TargetScan Human 7.2<sup>19</sup> and miRBase 20.0<sup>20</sup> databases were searched to identify potential miRNAs targeting the 3'-UTR of ABCB1. Thereafter, the clinical significance and differential expression of the screened miRNAs in tumor and normal tissues of HCC patients were determined using The Cancer Genome Atlas (TCGA, <https://portal.gdc.cancer.gov/>) database.<sup>21</sup>

### Cell Transfection

HepG2 and Bel-7404 cells ( $3.0 \times 10^4$ /well) were seeded evenly in the wells of 6-well microplates. After adherence, the cells were transfected with miRNA mimics, inhibitors, or a NC for 48 or 72 hours with Lipofectamine 3000 at a concentration of 50 nM diluted in Opti-MEM medium (Gibco, Carlsbad, CA, USA). Total RNA was collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and the protein content was collected for western blot analysis.

### Dual-Luciferase Reporter Assay

The pmirGLO-UTR reporter plasmid, containing the 3'-UTR of wild-type or mutant ABCB1, was obtained from Jiangsu KeyGEN BioTECH Co., Ltd. HEK-293T cells were co-transfected with a pmirGLO plasmid and a miR-224-5p or NC mimic using Lipofectamine 3000 reagent (Invitrogen Corporation, Carlsbad, CA, USA) for 24 hours. Then, luciferase activity was detected using the Dual-Glo® Luciferase Assay System (E2920; Promega Corporation, Madison, WI, USA) with the *Renilla* luciferase gene as the NC.

### Western Blot Analysis

To detect P-gp expression, total membrane protein was extracted by the sequential stain remover method. The protein concentration was quantified with the bicinchoninic acid assay method (BCA, Jiangsu KeyGEN BioTECH Co., Ltd.). Equal amounts of proteins were separated by 6% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel for glyceraldehyde 3-phosphate dehydrogenase) and then transferred onto polyvinylidene fluoride membranes (250 mA, 3 hours), which were blocked for 1 hour, and then incubated overnight with primary rabbit monoclonal antibodies against MDR1/ABCB1 or glyceraldehyde 3-phosphate dehydrogenase (dilution, 1:1000; Cell Signaling, USA) at 4°C, followed by horseradish-conjugated secondary goat anti-rabbit antibodies against immunoglobulin G (dilution, 1:10000; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 1 hour at room temperature. Chemiluminescence of the antibodies was conducted with High-sig ECL Western Blotting Substrate (Tanon Science & Technology Co., Ltd.) and signals were captured using a gel imaging system (Tanon Science & Technology Co., Ltd.).

### RT-qPCR Analysis

Total RNA was extracted using the RNeasy Pure Cell/Bacteria Kit (Jiangsu KeyGEN BioTECH Co., Ltd.) and the quantity and quality were determined using a K5600 spectrophotometer (Beijing Kaiuo Technology Development Co., Ltd. Beijing, China). Complementary DNA was synthesized using 5X All-In-One MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada) and then amplified by qRT-PCR using EvaGreen 2X qPCR MasterMix-Low Rox (Applied Biological Materials Inc.) with a 7500 Real-Time PCR System (ABI 7500, USA) and gene-specific primers for ABCB1 (5'-TTG CTG CTT ACA TTC AGG TTT CA-3'/5'-AGC CTA TCT CCT GTC GCA TTA-3'),  $\beta$ -actin (5'-CCT TCC TGG GCA TGG AGT C-3'/5'-TGA TCT TCA TTG TGC TGG GTG-3'), miR-224-5p reverse

primer (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAA AC-3'), miR-224-5p-F (5'-TCA AGT CAC TAG TGG TTC CGT TTA G-3'), miR-224-5p-R (5'-ATC CAG TGC AGG GTC CGA GG-3'), and U6 (5'-CTC GCT TCG GCA GCA CA-3'/5'-AAC GCT TCA CGA ATT TGC GT-3'). Relative expression of the target genes was normalized to  $\beta$ -actin using the  $2^{-\Delta\Delta Ct}$  method.

### Animal Study

The animal study was conducted in accordance with the ethical guidelines for animal care and approved by the Jiangsu KeyGen Ethics Committee (No. IACUC-001-22). BALB/c nude mice (age, 4–5 weeks; weight,  $20 \pm 0.6$  g) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). After 5 days of adaptive feeding in a pathogen-free environment, the mice were subcutaneously injected with 0.1 ml of HepG2 and Bel-7404 cell suspensions at a concentration of  $1.0 \times 10^7$ /ml. After the tumor volume reached 90–100 mm<sup>3</sup>, the mice were randomly assigned to 1 of 8 groups ( $n=6$ /group) and treated with various drugs for 21 days. Mice in the control group were gavaged with an equivalent volume of normal saline once per day, while mice in the PS-T group were gavaged with 2.6 g/kg body weight (BW) of PS-T once per day, those in the OXA group were intraperitoneally injected with 10 mg/kg BW once per week, and those in the PS-T + OXA group were gavaged with 2.6 g/kg BW of PS-T once per day and intraperitoneally injected with 10 mg/kg BW once per week. The tumor volume, calculated as  $\frac{1}{2} \times a$  (length)  $\times b^2$  (width), was recorded during drug administration, and xenograft weight was calculated after the mice were sacrificed.

### Statistical Analysis

The data are presented as the mean  $\pm$  standard deviation. All data analyses were conducted using IBM SPSS Statistics for Windows, version 23.0. (IBM Corporation, Armonk, NY, USA) or GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between 2 groups were conducted using the Student's *t*-test and among 3 or more groups with 1-way analysis of variance. A probability (*P*) value of  $<.05$  was considered statistically difference.

## Results

### *PS-T Enhanced the Ability of OXA to Inhibit Cell Proliferation and Down-Regulated P-gp Expression in Bel-7404 and HepG2 Cells*

The viability of cells treated with PS-T was evaluated with the CCK-8 kit. PS-T significantly inhibited the viability of

human HepG2 and Bel-7404 cells in a dose-dependent manner at 48 and 72 hours (Figures 1A and B). Since the anti-tumor activity of PS-T itself will affect the sensitization of cells to OXA, low-dose PS-T (HepG2: IC<sub>10</sub> 0.20 mM; Bel-7404: IC<sub>10</sub> 0.27 mM) was chosen for the following studies. For subsequent CCK-8 assays, human HepG2 and Bel-7404 cells were treated with various doses of different drugs. The results showed that PS-T combined with OXA had a greater inhibitory effect than OXA alone at 48 and 72 hours (Figures 1C and D). For the EdU study, human Bel-7404 and HepG2 cells were treated with the same doses of drugs as for the CCK-8 assay. The ratio of EdU-positive to Hoechst 33342-positive Bel-7404 or HepG2 cells in the combined treatment group was significantly lower than that in the OXA group (Figure 1E–H). The cell clone formation assay (Figure 1I–K) showed that the number of clones in the combined treatment group was significantly lower than in the OXA group, indicating greater inhibition of cell proliferation in the PS-T + OXA group than the OXA group. Additionally, the results of western blot analysis revealed that P-gp expression was down-regulated in human Bel-7404 and HepG2 cells after treatment with PS-T (Figure 1L–N).

### *PS-T Promoted the Ability of OXA to Inhibit the Growth of Xenografts and down-Regulate P-gp Expression in Nude Mice Inoculated With Bel-7404 and HepG2 Cells*

An *in vivo* experiment was conducted to verify the ability of PS-T to increase the sensitivity of human Bel-7404 and HepG2 cells to OXA. The xenograft model of BALB/c nude mice was successfully established and further underwent 3-week drug administration (Figure 2J). The xenograft volume of the nude mice inoculated with human Bel-7404 and HepG2 cells was significantly lower in the PS-T + OXA group than the OXA group after 10 or 13 days of drug administration (Figures 2A, B, D and E). Also, the xenograft weight was lower in the combined group than the OXA group (Figure 2C and F). Besides, PS-T lowered the expression of P-gp in the xenografts (Figure 2G–I).

### *Five miRNAs Were Eligible for the Following Experiments After Series of Screening*

To explore the regulatory relationship between PS-T and miRNAs, a miRNA microarray (Affymetrix GeneChip 4.0, MiRBase 20.0 version) was utilized to comprehensively detect differences in the miRNA expression profiles of human HepG2 cells before and after treatment with PS-T. After treatment of human HepG2 cells with 0.20 mM PS-T for 48 hours, 1257 miRNAs were up-regulated, 1259 were down-regulated, and 62 remained unchanged (Figure 3A).

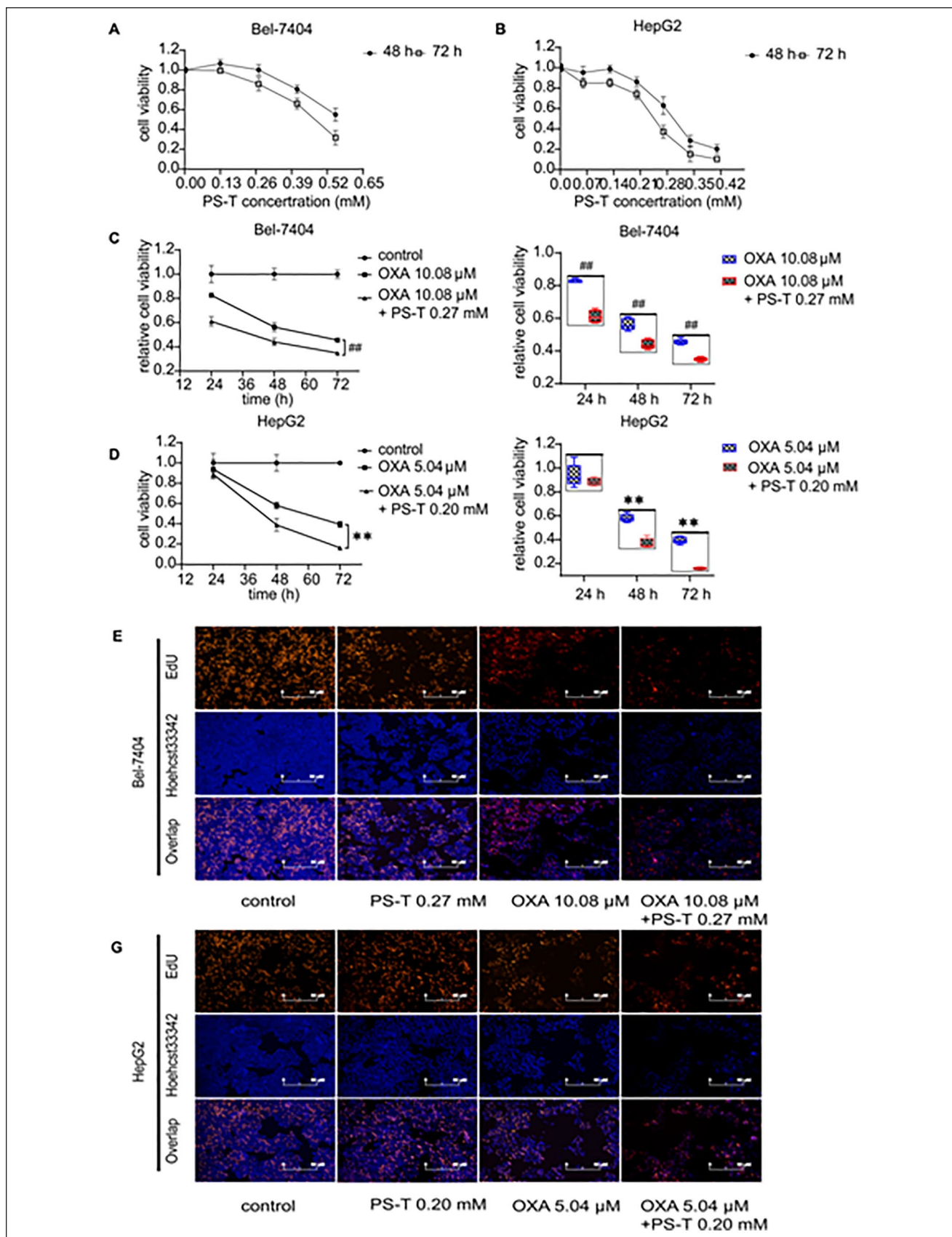
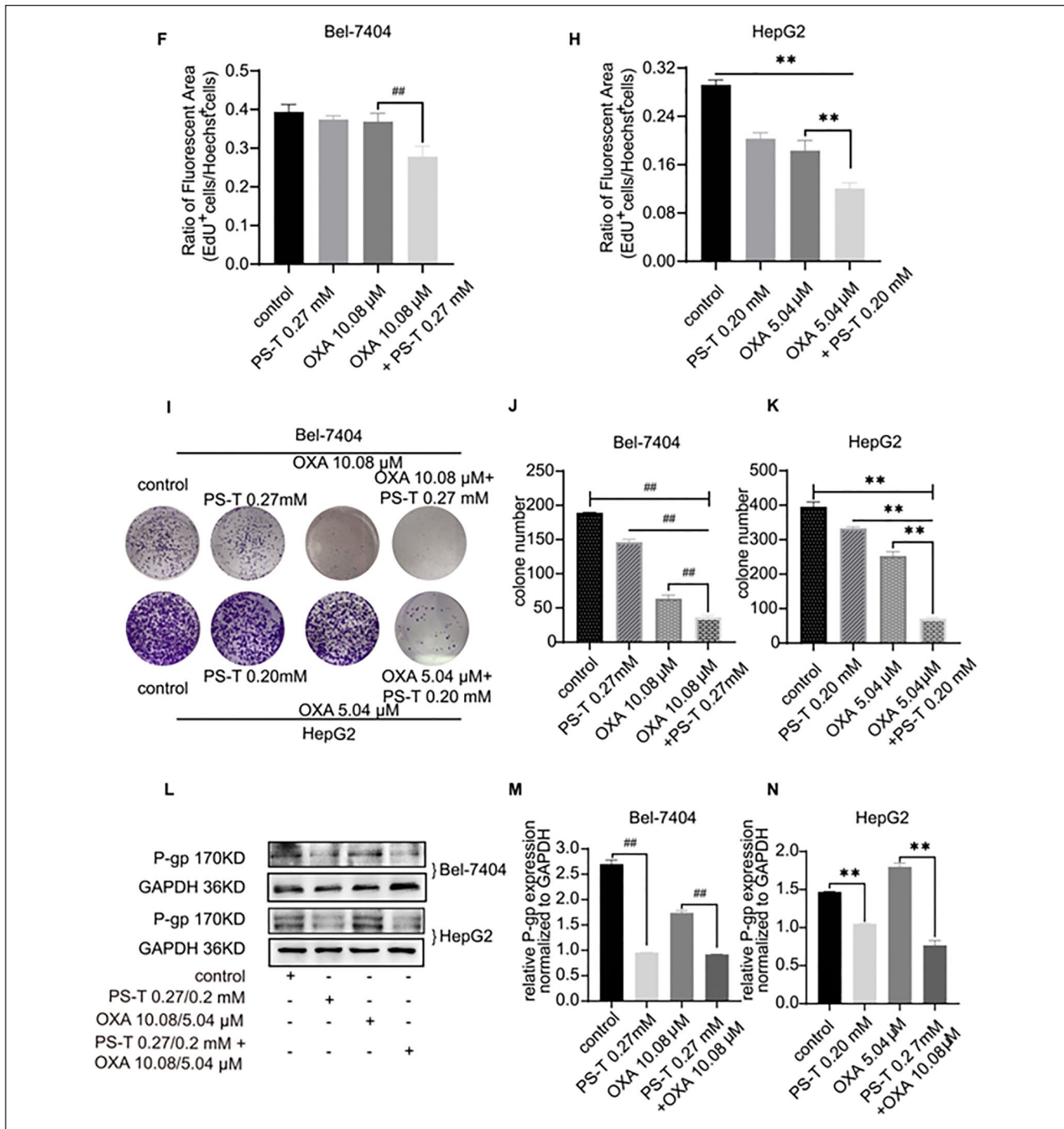


Figure I. (continued)



**Figure 1.** (A–N) PS-T enhanced the anti-tumor effects (cell proliferation) of OXA and down-regulated P-gp level in human Bel-7404 and HepG2 hepatoma cells. (A and B) The viability of human hepatoma cells treated with PS-T was measured by CCK-8. (C and D) The viability of cells treated with PS-T, OXA, or PS-T plus OXA was estimated by CCK-8. (E–H) The estimation of cell proliferation was measured by EdU assay. (I) The representative images of clones indicated more powerful inhibition of cell proliferation explored to PS-T, OXA, or OXA plus PS-T. (J and K) Numbers of clones of human BEL-7404 and HepG2 cells were counted using Image J software and analyzed by Graphpad Prism 8.0. (L) PS-T down-regulated P-gp expression in human Bel-7404 and HepG2 cells. (M and N) Relative P-gp expression of cells, normalized to GAPDH, treated with PS-T in Bel-7404 and HepG2 cells. Data were shown as mean  $\pm$  SD. Each experiment was repeated in triplicate. <sup>##</sup> $P < .05$ , <sup>\*\*</sup> $P < 0.05$  represents statistically difference.

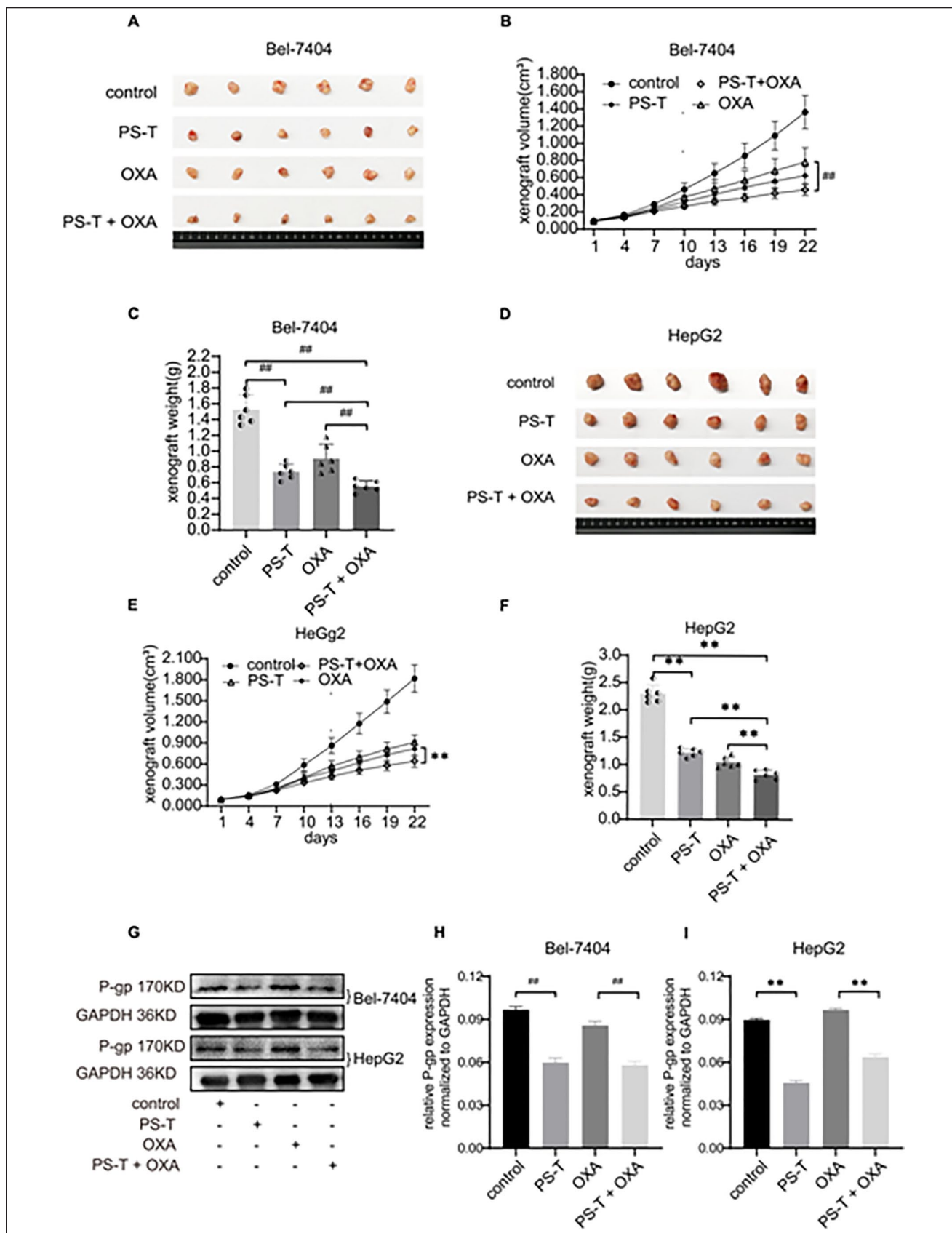
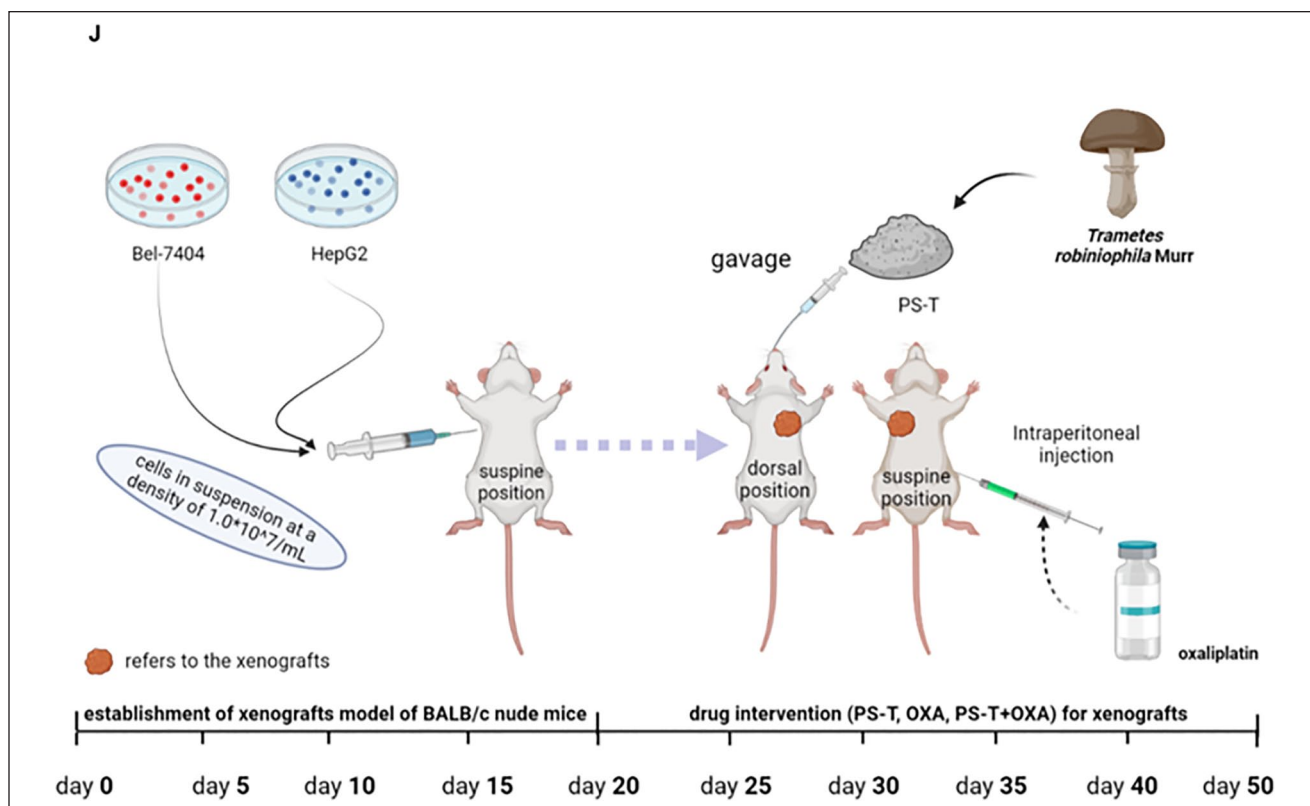


Figure 2. (continued)



**Figure 2.** (A–J) PS-T promoted effects of OXA in inhibiting the growth of xenografts and down-regulated P-gp expression in nude mice inoculated with human Bel-7404 and HepG2 hepatoma cells. (A–C) The volume and weight of xenograft of BALB/c nude mice inoculated with human Bel-7404 cells in 4 groups, respectively. (D–F) The volume and weight of xenografts of nude mice inoculated with human HepG2 cells in 4 groups, respectively. (G) PS-T down-regulated P-gp expression in nude mice inoculated with human Bel-7404 or HepG2 cells. (H and I) Relative P-gp expression in xenograft tissues in HepG2 and Bel-7404 cells. (J) The establishment of xenograft model of BALB/c nude mice and drug intervention. The picture was made by BioRender (<https://biorender.com/>). Data were showed as mean  $\pm$  SD.  $##P < .05$ ,  $**P < .05$ .

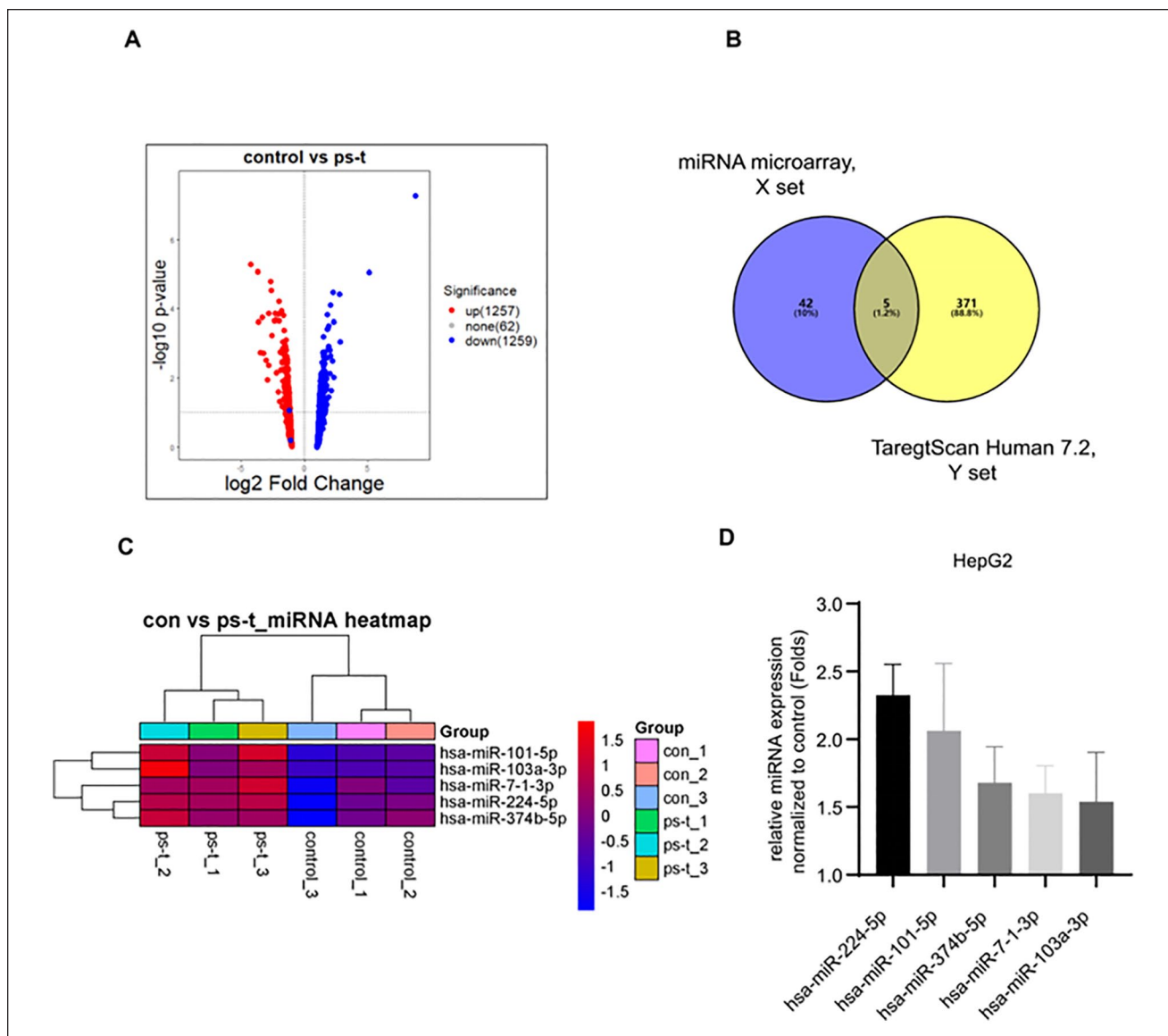
After preliminary screening ( $\log_{\text{FoldChange}} > \text{ABS}(1.5)$  and  $P < .05$ ), 47 miRNAs were obtained and defined as the *X* data set. TargetScanHuman 7.2 identified 376 miRNAs that potentially targeted the ABCB1 gene, which were defined as the *Y* set. As shown in Figures 3B to D, after exclusion (intersection of the *X* and *Y* data set), 5 remaining miRNAs were screened for following experiments, and their expression levels (compared with control group) were analyzed and presented by ranking from high levels to low levels.

#### *MiR-224-5p* Directly Regulated ABCB1 mRNA and Further Down-Regulated P-gp Expression in HepG2 and Bel-7404 Cells

Many studies have confirmed that P-gp is encoded by the ABCB1 gene. The results of the present study confirmed that PS-T can down-regulate P-gp both in vivo and in vitro. Five up-regulated miRNAs were screened based on PS-T. To further explore the specific regulatory relationship between miRNAs and ABCB1, liposome transfection was

performed. First, human Bel-7404 and HepG2 cells were transfected with mimics or inhibitors of 5 miRNAs (miR-224-5p, miR-103a-3p, miR-101-5p, miR-713-3p, and miR-374b-5p) with 50 nm Lipofectamine 3000 reagent. Then, the expression levels of the 5 miRNAs of cells transfected with miRNAs mimics were detected for cells with by stem-loop qPCR. The results showed that all were significantly increased, indicating successful transfection (Figures 4A and B). Because miRNA inhibitors interact with target genes differently than miRNA mimics, the NC miRNA inhibitors were labeled with FAM. Fluorescence signal detection at 48 hours showed that the hepatoma cells were successfully transfected with the NC inhibitors (Figure 4C). After 48 hours of transfection, total RNA was extracted to detect ABCB1 mRNA. As shown in Figures 4D and E, ABCB1 mRNA was slightly up-regulated in cells transfected with the miR-224-5p inhibitor and mildly down-regulated in those transfected with the miR-224-5p mimic. Meanwhile, at 72 hours post-transfection, P-gp expression was significantly up-regulated in cells transfected with the





**Figure 3.** (A–D) The screening procedure of miRNAs potentially targeting ABCB1 gene. (A) (Volcano map): Expressions of miRNAs regulated by PS-T via microarray (Affymetrix Gene Chip 4.0): 1257 were up-regulated miRNAs, 1259 were down-regulated miRNAs, and 62 were unchanged miRNAs. (B) (Venn diagram): The results of the intersection of the X and Y data set: 5 miRNAs possibly targeting ABCB1 gene were remained (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). (C) (Heat map) The differential expressions of 5 screened miRNAs: 5 were up-regulated and 0 was down-regulated. (D) (Bar plot): The differential expressions of 5 screened miRNAs (arranging from high level to low level). (A) and (C) were conducted by using R package version 4.0.

miR-224-5p inhibitor and significantly down-regulated in those transfected with the miR-224-5p mimic, indicating a negative regulatory relationship between miR-224-5p and P-gp (Figure 4H–L). Subsequently, a double luciferase gene reporting experiment was conducted to determine whether there is a direct targeted relationship between miR-224-5p and ABCB1. The luciferase activity of wild-type miR-224-5p was significantly inhibited following co-transfection with the plasmid containing the 3'-UTR region of the target gene and the miR-224-5p mimic, while that of the

mutant miR-224-5p remained mostly unchanged, suggesting that miR-224-5p directly targeted or regulated the 3'-UTR of ABCB1 (Figure 4F and G).

#### *PS-T Increased the Sensitivity of Hepatoma Cells to OXA via Regulation of the miR-224-5p/ABCB1/ P-gp Axis*

The results of qRT-PCR analysis showed that miR-224-5p was highly expressed in human HepG2 cells treated with

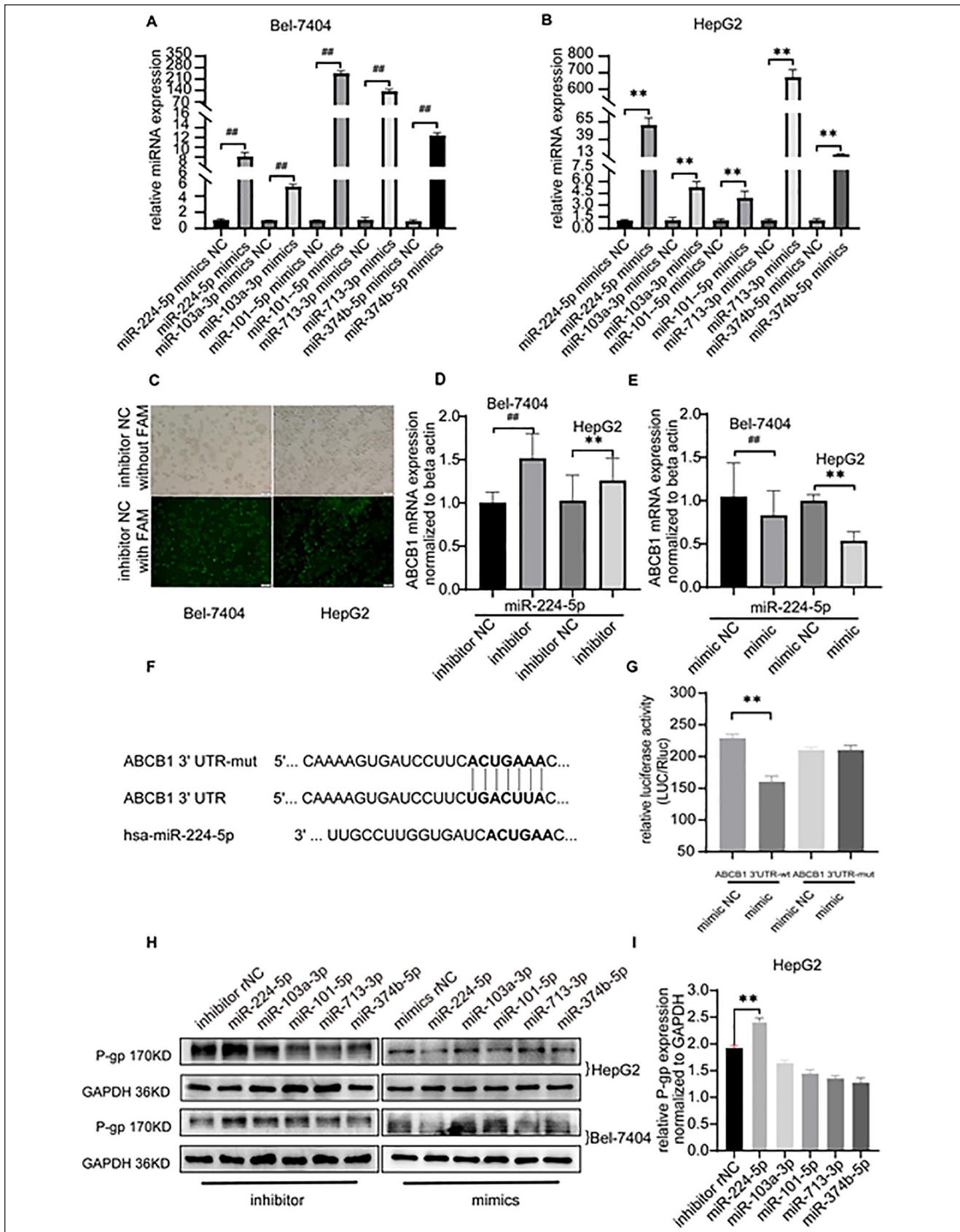
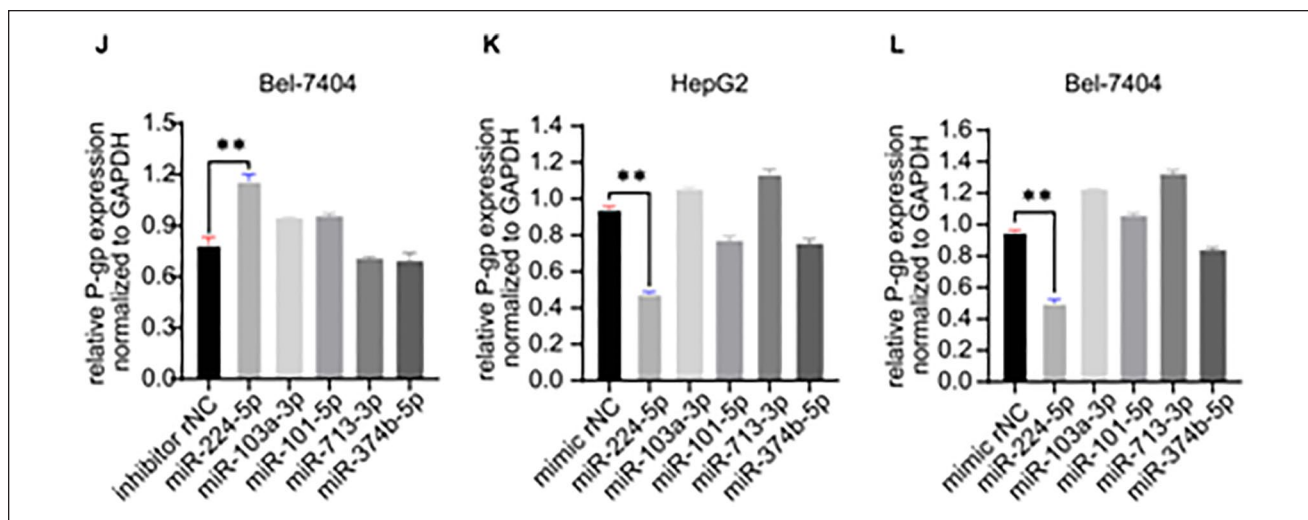


Figure 4. (continued)



**Figure 4.** (A–L) MiR-224-5p directly regulated the ABCB1 mRNA and further downregulated P-gp expression in human HepG2 and Bel-7404 cells. (A and B) Relative expression of 5 miRNAs in cells transfected with mimics or mimics NC. (C) Fluorescent images of human HepG2 and Bel-7404 cells transfected with FAM-labeled inhibitors or inhibitors NC. (D) Real time qPCR showed that relative ABCB1 mRNA levels in human HepG2 and Bel-7404 cells with miR-224-5p inhibitor was slightly up-regulated relative to cells with miR-224-5p inhibitor NC. (E) Relative low level of ABCB1 mRNA was observed in human HepG2 and Bel-7404 cells with miR-224-5p mimic compared with cells with miR-224-5p mimic NC. (F) The sequence of miR-224-5p and its potential binding site of ABCB1 3'UTR. (G) Dual luciferase reporter assay showed that miR-224-5p can directly target ABCB1 3'UTR. *Renilla luciferase* was used as internal reference. (H) Western blot analysis indicated that P-gp expression was overexpressed in human HepG2 and Bel-7404 cells only transfected with miR-224-5p inhibitor and was inhibited in cells only transfected with miR-224-5p mimic. GAPDH was used for internal control. (I–L) Relative P-gp expression of 5 miRNAs in HepG2 and Bel-7404 cells transfected with mimics or inhibitors. Data were showed as Mean  $\pm$  SD. Each experiment was repeated 3 times. ## $P < .05$ , \*\* $P < .01$ .

PS-T, consistent with the results of the miRNA microarray (Figure 5J). P-gp was highly expressed in HepG2 cells transfected with the miR-224-5p inhibitor and down-regulated after intervention with PS-T (Figure 5A–C). In addition, the clone formation assay was performed again to evaluate the effect of OXA on the proliferation of Bel-7404 and HepG2 cells following transfection with the miR-224-5p inhibitor. The results showed that the number of clones was significantly increased in the O + M group as compared with cells without the miR-224-5p inhibitor (the O group). As compared with the O + M group, the number of clones in the combined treatment group (O + M + P) was decreased (Figure 5D–F). The ability of OXA to inhibit cell proliferation was weakened because of decreasing concentrations driven by high expression of P-gp in cell transfected with the miR-224-5p inhibitor. Since PS-T can inhibit P-gp expression by up-regulating miR-224-5p, the ability of OXA to inhibit cell proliferation was somewhat recovered after treatment with PS-T.

Survival analysis showed that high rather than low expression of miR-224-5p was correlated with improved overall survival (OS) and disease-free survival (DFS) (Figures 5G and H; <http://kmplot.com/analysis/index.php>). Also, there were significant differences in miR-224-5p expression between tumor and adjacent tissues according to TCGA database (Figure 5I).

In a word, as is shown in Figure 5K, PS-T strengthens the sensitivity of human hepatoma cells to OXA via the miR-224-5p/ABCB1/P-gp axis.

## Discussion

In recent years,<sup>22</sup> especially following the approval of sorafenib by the Food and Drug Administration, the advent of targeted therapy and immunotherapy has greatly improved the survival of patients with HCC. However, the diversity of therapeutic methods and the efficacy of targeted drugs as well as immune-checkpoint inhibitors for patients with HCC failed to achieve longer survival expectations. Furthermore, drug-induced resistance has even worsened patient survival. Hence, further studies are urgently needed to address resistance to chemotherapeutics.<sup>23</sup>

A growing number of studies have demonstrated that P-gp is involved in resistance to chemotherapeutic agents by pumping anti-tumor drugs out of the cell and decreasing the accumulated concentration in tumor cells.<sup>24</sup> Based on this mechanism, several generations of MDR/P-gp inhibitors, such as VX-710 (Bircodar),<sup>25</sup> cyclosporin A,<sup>26</sup> zosuquidar (LY 335979),<sup>27</sup> and tariquidar (XR9576),<sup>28</sup> have been developed to overcome chemotherapy resistance. Unfortunately, patients with HCC<sup>5</sup> seem to benefit less from these drugs due to unavoidable side effects, drug

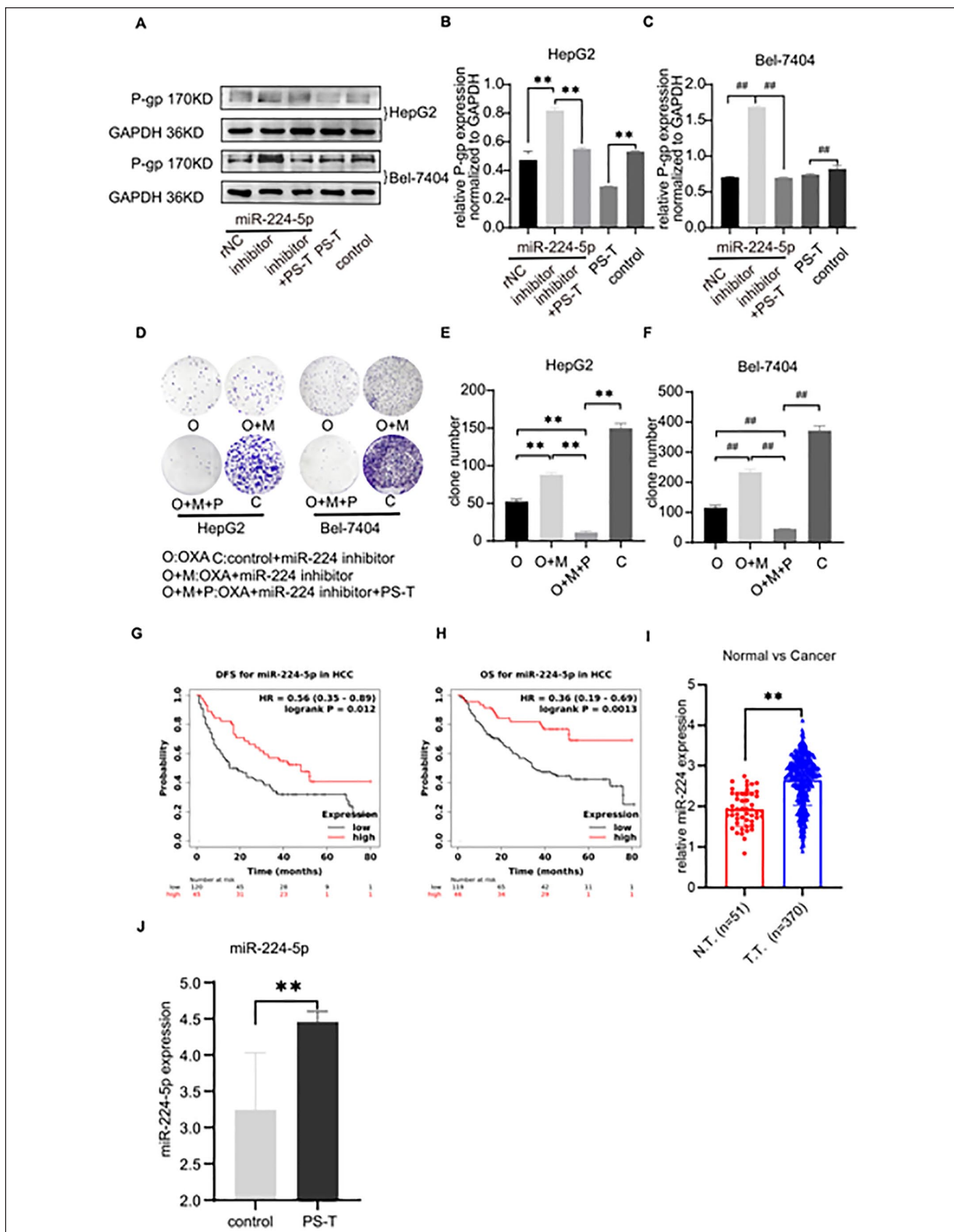
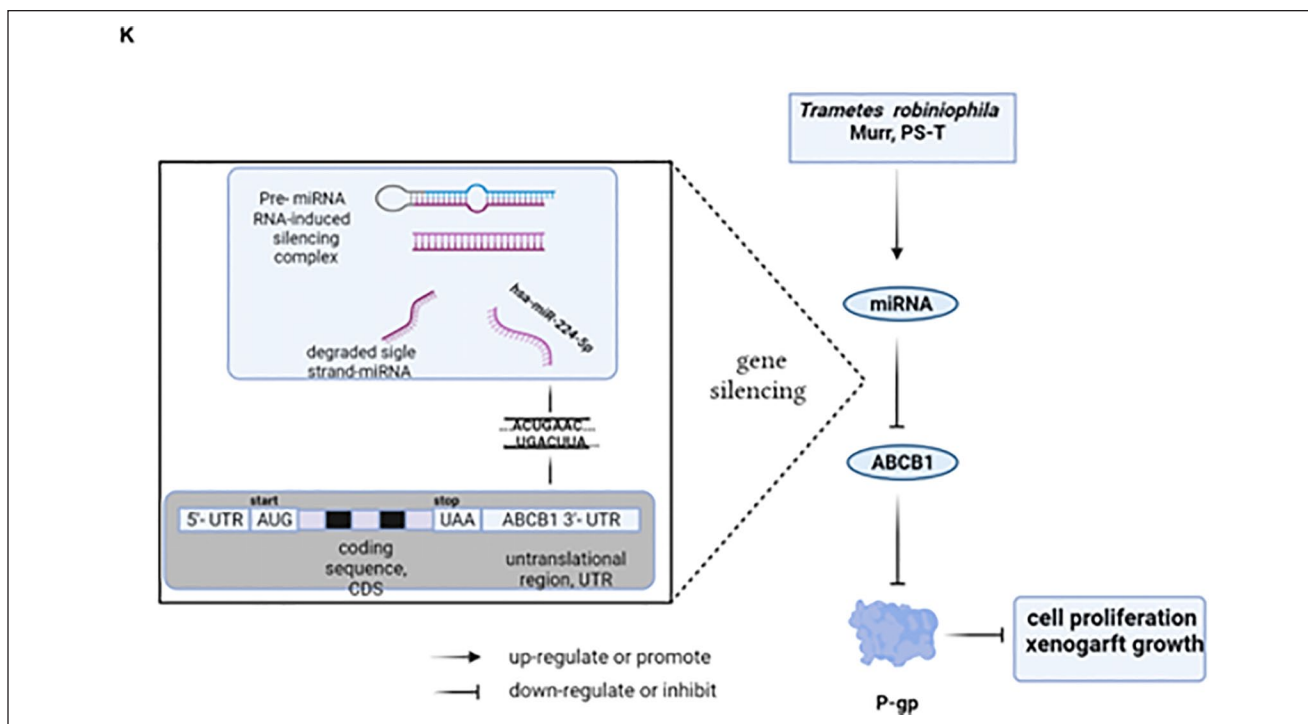


Figure 5. (continued)



**Figure 5.** (A–K) PS-T increased the sensitivity of human hepatoma cells to OXA by regulating miR-224-5p/ABCB1/P-gp axis. (A) Western blot analysis showed that PS-T could down-regulate P-gp expression in human HepG2 or Bel-7404 cells with miR-224-5p inhibitor, respectively. GAPDH was considered as internal control. (B and C) Relative P-gp expression in human HepG2 and Bel-7404 cells with miR-224-5p inhibitor or inhibitor NC. (D) The photographs of clones of human HepG2 and Bel-7404 cells transfected with miR-224-5p inhibitor. The results showed the ability of inhibiting cell proliferation of OXA was recovered after the treatment of PS-T. (E and F) Clone numbers of human Bel-7404 and HepG2 cells were counting by Image J software. (G and H) Survival analysis showed that HCC patients with high expression of miR-224-5p had a better overall survival (HR=0.36 [0.19-0.69], logrank  $P=.0013$ ) and disease-free survival (HR=0.56 [0.35-0.89], logrank  $P=.012$ ). (I) MiR-224-5p was highly expressed in tumor tissue compared with normal tissue in patients with HCC in TCGA database (N.T. means normal tissues; T.T. means tumor tissues). (J) Real time qPCR indicated that PS-T can upregulate miR-224-5p expression compared with control group. Beta-actin was regarded as negative control. (K) The overview diagram of mechanism: PS-T strengthens the sensitivity of human hepatoma cells to OXA via the miR-224-5p/ABCB1/P-gp axis. The picture was made by BioRender (<https://biorender.com/>). ## $P<.05$ , \*\* $P<.05$ .

toxicity, and minor limitations of application.<sup>24</sup> Commonly used chemotherapy regimens for HCC include cisplatin, doxorubicin (ADM), 5-fluorouracil, and OXA. Numerous studies have reported that miR-491-3b,<sup>29</sup> miR-122,<sup>30</sup> miR-223,<sup>31</sup> and miR-503<sup>32</sup> can confer less resistance of hepatoma cells to ADM by binding to the 3'-UTR of targeted ABCB1 gene. Other miRNAs, such as 520b,<sup>33</sup> miR-26b,<sup>34</sup> and miR-145,<sup>35</sup> also suppress ADM resistance by regulating related targeted genes. Since one miRNA probably targets multiple genes, the mechanism of chemotherapy resistance differs with not only various chemotherapeutic drugs but also among cancer types.<sup>36</sup> Therefore, it is extremely difficult to solve chemotherapy resistance in HCC.

The intensity of the regulation between miRNA and mRNA seems to be mild.<sup>37</sup> The first miRNA, discovered in *Caenorhabditis elegans*, was suppressed in translation, but its target mRNA level was only slightly down-regulated.<sup>38</sup> The results of microarray analysis conducted by Lim et al.<sup>39</sup>

demonstrated that some miRNAs can down-regulate a large number of target mRNAs, but only slightly by less than 2-fold. Interestingly, a similar phenomenon was noted in the present study. The levels of most miRNAs in the miRNA microarray were mildly altered after intervention with PS-T, while expression of 5 miRNAs was up-regulated, but by less than 2-fold. Moreover, there was a mild down-regulation in the relationship between miR-224-5 and ABCB1 mRNA, even though miR-224-5p can directly target ABCB1. There has been no relevant research to explain the cause and mechanism of this “mild regulation” phenomenon.

According to the current literature, this is the first study to systematically explore the regulatory effect of PS-T on the chemosensitivity of hepatoma cells to OXA at the miRNA level. The results preliminarily confirmed that miR-224-5p can down-regulate P-gp by modulating ABCB1 mRNA levels. The results of the dual-luciferase reporting assay indicated that the regulation between miR-224-5p and ABCB1 was directly targeted. Zhao et al.<sup>29</sup> found that

miR-491-3p can inhibit the expression of ABCB1 at both the post-transcriptional and transcriptional levels. On the one hand, miR-491-3p inhibited ABCB1 mRNA expression at the post-transcriptional level by directly targeting the 3'-UTR region of ABCB1. On the other hand, miR-491-3p down-regulated ABCB1 expression by indirectly regulating the upstream promoter SP3 of the ABCB1 gene, which suggests that miR-224-5p also possibly regulates a certain ABCB1 transcription factor or promoter at the transcriptional level. Subsequent studies are needed to identify the transcription factor or promoter directly involved in the regulation between miR-224-5p and ABCB1.

There were some limitations to this study. For some unavoidable reasons, we only explored the anti-tumor effect of PS-T combined with OXA in nude mice and discussed the regulatory relationship between miR-224-5p, ABCB1, and P-gp at the cellular level. However, animal experiments are needed to elucidate the detailed mechanisms. Also, the scope of this study was limited to the regulatory relationship between 5 miRNAs and ABCB1. Similar to the above-mentioned mild regulatory relationship between miRNAs and ABCB1, the other miRNAs may also have direct or indirect regulatory effects on ABCB1.

In conclusion, the results of this study confirmed that PS-T enhanced the sensitivity of HCC cells to OXA via a mechanism possibly related to the miR-224-5p/ABCB1/P-gp axis. Meanwhile, miR-224-5p may be a potential prognostic biomarker for patients with chemotherapy-resistant HCC.

### Acknowledgments

Many thanks to Director Yang Aizhen of Clinical Laboratory and Professor Wang Feng of Oncology Department of Qinhua Medical Area of Eastern Theater Command General Hospital (Nanjing city, Jiangsu Province, China) for their help and support for this paper. Thanks to Qidong Gaitianli pharmaceutical Co. Ltd (Jiangsu province, China) for providing the latest research data of Huaier granules. The authors would like to thank Enago (www.enago.com) for the English language review.

### Author Contributions

Y.D.G. designed and conducted the study, and also completed the initial version of this manuscript. Y.D.G., X.Z., W.M.L., and H.Y.D. collected and analyzed the data. S.K.Q. supervised the procedure of this project and revised the manuscript. All the authors approved the final manuscript submitted.

### Availability of data and materials

The datasets deriving from this study are available from the corresponding author on reasonable request.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

### Ethics Approval and Consent to Participate

The animal study was conducted in accordance with the ethical guidelines for animal care and approved by the Jiangsu KeyGen Ethics Committee (No. IACUC-001-22).

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### References

1. Mansoori B, Mohammadi A, Davudian S, Shirjang S, Baradaran B. The different mechanisms of Cancer Drug Resistance: A Brief Review. *Adv Pharm Bull.* 2017;7:339-348.
2. Wei L, Wang X, Lv L, et al. The emerging role of microRNAs and long noncoding RNAs in drug resistance of hepatocellular carcinoma. *Mol Cancer.* 2019;18:147. Published 2019 Oct 25.
3. Amawi H, Sim HM, Tiwari AK, Ambudkar SV, Shukla S. ABC transporter-mediated multidrug-resistant cancer. *Adv Exp Med Biol.* 2019;1141:549-580.
4. Waghray D, Zhang Q. Inhibit or evade multidrug resistance P-Glycoprotein in cancer treatment. *J Med Chem.* 2018;61:5108-5121.
5. Mollazadeh S, Sahebkar A, Hadizadeh F, Behravan J, Arabzadeh S. Structural and functional aspects of P-glycoprotein and its inhibitors. *Life Sci.* 2018;214:118-123.
6. Ashrafzadeh M, Zarrabi A, Hushmandi K, et al. MicroRNAs in cancer therapy: their involvement in oxaliplatin sensitivity/resistance of cancer cells with a focus on colorectal cancer. *Life Sci.* 2020;256:117973.
7. Xu N, Zhang J, Shen C, et al. Cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell. *Biochem Biophys Res Commun.* 2012;423:826-831.
8. Shi L, Chen ZG, Wu LL, et al. miR-340 reverses cisplatin resistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway. *Asian Pac J Cancer Prev.* 2014;15:10439-10444.
9. Ou Y, Zhai D, Wu N, Li X. Downregulation of miR-363 increases drug resistance in cisplatin-treated HepG2 by dysregulating Mcl-1. *Gene.* 2015;572:116-122.
10. Qin J, Luo M, Qian H, Chen W. Upregulated miR-182 increases drug resistance in cisplatin-treated HCC cell by regulating TP53INP1. *Gene.* 2014;538:342-347.
11. Moradi Marjaneh R, Khazaei M, Ferns GA, Avan A, Aghae-Bakhtiari SH. MicroRNAs as potential therapeutic targets to predict responses to oxaliplatin in colorectal cancer: from basic evidence to therapeutic implication. *IUBMB Life.* 2019;71:1428-1441.
12. Xie T, Huang M, Wang Y, Wang L, Chen C, Chu X. MicroRNAs as regulators, biomarkers and therapeutic targets in the drug resistance of colorectal cancer. *Cell Physiol Biochem.* 2016;40:62-76.

13. Chen Q, Shu C, Laurence AD, et al. Effect of Huaier granule on recurrence after curative resection of HCC: a multicentre, randomised clinical trial. *Gut*. 2018;67:2006-2016.
14. Zhang LI. *Effects and Mechanisms of Huaier Extract on Doxorubicin Resistance Cancer*. Master's thesis. Shangdong University; 2018.
15. Danwei R. *Huaier Reverses the Resistances of Human Acute T-lymphocytic Leukemia CEM-C1 Cells to Glucocorticoids*. Master's thesis. Southwest Medical University; 2020.
16. Tanaka M, Tanaka T, Teng F, et al. Drastic genomic alterations in a human transcriptome and transcription factor control identified in the course of cancer recovery by Huaier administration. In preparation.
17. Tétreault N, De Guire V. miRNAs: their discovery, biogenesis and mechanism of action. *Clin Biochem*. 2013;46:842-845.
18. Ali Syeda Z, Langden SSS, Munkhzul C, Lee M, Song SJ. Regulatory mechanism of MicroRNA expression in cancer. *Int J Mol Sci*. 2020;21:1723. Published 2020 Mar 3.
19. Garcia DM, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. *Nat Struct Mol Biol*. 2011;18:1139-1146. Published 2011 Sep 11.
20. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. *Nucleic Acids Res*. 2019;47:D155-D162.
21. Nagy Á, Lániczky A, Menyhart O, Györfy B. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. *Sci Rep*. 2018;8:9227.
22. McGlynn KA, London WT. The global epidemiology of hepatocellular carcinoma: present and future. *Clin Liver Dis*. 2011;15:223-243.
23. Villanueva A. Hepatocellular carcinoma. *New Engl J Med*. 2019;380:1450-1462.
24. Dewanjee S, Dua TK, Bhattacharjee N, et al. Natural products as alternative choices for P-glycoprotein (P-gp) inhibition. *Molecules*. 2017;22:871. Published 2017 May 25.
25. Minderman H, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. *Clin Cancer Res*. 2004;10:1826-1834.
26. Qadir M, O'Loughlin KL, Fricke SM, et al. Cyclosporin A is a broad-spectrum multidrug resistance modulator. *Clin Cancer Res*. 2005;11:2320-2326.
27. Tang R, Faussat AM, Perrot JY, et al. Zosuquidar restores drug sensitivity in P-glycoprotein expressing acute myeloid leukemia (AML). *BMC Cancer*. 2008;8:51.
28. Pusztai L, Wagner P, Ibrahim N, et al. Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer*. 2005;104:682-691.
29. Zhao Y, Qi X, Chen J, et al. The miR-491-3p/Sp3/ABCB1 axis attenuates multidrug resistance of hepatocellular carcinoma. *Cancer Lett*. 2017;408:102-111.
30. Yahya SMM, Fathy SA, El-Khayat ZA, et al. Possible role of microRNA-122 in modulating multidrug resistance of hepatocellular carcinoma. *Indian J Clin Biochem*. 2018;33:21-30.
31. Yang T, Zheng ZM, Li XN, et al. MiR-223 modulates multidrug resistance via downregulation of ABCB1 in hepatocellular carcinoma cells. *Exp Biol Med*. 2013;238:1024-1032.
32. Wang D, Zhang N, Ye Y, Qian J, Zhu Y, Wang C. Role and mechanisms of microRNA-503 in drug resistance reversal in HepG2/ADM human hepatocellular carcinoma cells. *Mol Med Rep*. 2014;10:3268-3274.
33. Gao AM, Zhang XY, Hu JN, Ke ZP. Apigenin sensitizes hepatocellular carcinoma cells to doxorubicin through regulating miR-520b/ATG7 axis. *Chem Biol Interact*. 2018;280:45-50.
34. Zhao N, Wang R, Zhou L, Zhu Y, Gong J, Zhuang SM. MicroRNA-26b suppresses the NF- $\kappa$ B signaling and enhances the chemosensitivity of hepatocellular carcinoma cells by targeting TAK1 and TAB3. *Mol Cancer*. 2014;13:35. Published 2014 Feb 24.
35. Ju BL, Chen YB, Zhang WY, Yu CH, Zhu DQ, Jin J. miR-145 regulates chemoresistance in hepatocellular carcinoma via epithelial mesenchymal transition. *Cell Mol Biol (Noisy-le-grand)*. 2015;61:12-16.
36. Si W, Shen J, Zheng H, Fan W. The role and mechanisms of action of microRNAs in cancer drug resistance. *Clin Epigenetics*. 2019;11:25. Published 2019 Feb 11.
37. Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature*. 2008;455:58-63.
38. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 2008;9:102-114.
39. Lim LP, Lau NC, Garrett-Engle P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*. 2005;433:769-773.