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**Original Article**

# Resveratrol Downregulates Cyp2e1 and Attenuates Chemically Induced Hepatocarcinogenesis in SD Rats

Xiongfei Wu[1#], Chenggang Li[1#], Guozhen Xing[1], Xinming Qi[1*], and Jin Ren[1*]

[1] Center for Drug Safety Evaluation and Research, State Key Laboratory of New Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haike Road, Shanghai, 201203, China

**Abstract:** Cyp2e1 plays an important role in chemically induced hepatocarcinogenesis. Resveratrol (REV) is known to prevent diethylnitrosamine (DEN)-induced hepatocarcinogenesis, but its effects on this process induced by DEN and 2-acetylaminofluorene (2-AAF) and the role of Cyp2e1 remain unclear. In this study, glutathione S-transferase placental form (GST-P)-positive foci were used as a marker of hepatocarcinogenesis. REV or diallyl disulfide (DADS, an inhibitor of Cyp2e1) significantly reduced both the area and number of GST-P-positive foci induced by DEN and 2-AAF. Treatment with REV or DADS also markedly decreased the expression of Cyp2e1 in the rat liver. By immunohistochemical staining of serial liver sections, we found that the expression of Cyp2e1 in GST-P-positive foci showed three distinct patterns: decreased in GST-P foci, increased in GST-P foci when compared with surrounding liver tissue and mixed type. The number of GST-P foci with increased Cyp2e1 expression was greater than the number of GST-P foci with decreased Cyp2e1. Protein levels of GST-P and Cyp2e1 were also higher in foci compared with surrounding liver tissue. REV or DADS significantly reduced the expression of GST-P and Cyp2e1 in both foci and surrounding liver tissue. Taken together, these results suggested that REV has a significant inhibitory effect on chemically induced hepatocarcinogenesis, which may be attributed to downregulation of Cyp2e1. (DOI: 10.1293/tox.2013-0020; J Toxicol Pathol 2013; 26: 385–392)

**Key words:** resveratrol, hepatocarcinogenesis, laser microdissection, GST-P, Cyp2e1

## Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, the fifth most common malignant tumor type and the second leading cause of cancer-related death in the world[1,2]. Hepatitis B and C viruses, obesity, environmental pollutants, aflatoxin infection and nitrosamine consumption are the strongest risk factors for HCC development[3]. The overall five-year survival rate of HCC is estimated at only 20%, mainly because HCC is frequently diagnosed at an advanced stage. The recurrence rate can be as high as 50%. No effective therapy can be offered to patients with unresectable HCC[4]. Considering the limited treatment and negative prognosis of liver cancer, chemoprevention has been considered the best strategy to lower the morbidity and mortality rates associated with liver cancer[5,6].

The medium-term liver bioassay consists of initiation with diethylnitrosamine (DEN) followed by promotion with 2-acetylaminofluorene (2-AAF) and a partial hepatectomy. This protocol requires only 8 weeks for the formation of preneoplastic liver lesions, as identified by glutathione S-transferase placental form-positive hepatic foci[7,8]. DEN and 2-AAF are metabolically activated by Cyp2e1 and Cyp1a respectively, suggesting important roles for Cyp2e1 and Cyp1a in hepatocarcinogenesis induced by DEN or 2-AAF[9–12].

Resveratrol (REV, Fig. 1) is a phytochemical found in several dietary sources that prevents DEN-induced hepatocarcinogenesis by several mechanisms, acting as an antioxidant and anti-inflammatory agent and altering pro-inflammatory cytokines in rats livers[13–17]. REV downregulates the expression of Cyp1a due to its antagonist activity on the aryl hydrocarbon receptor[18–20]. Some previous studies have also shown that REV inhibited the activity of Cyp450 isoforms, including Cyp2e1[21,22].

Considering the important role of Cyp2e1 and Cyp1a in chemically induced hepatocarcinogenesis and the negative effects of REV on Cyp450 isoforms, this study investigated the effects of REV on hepatocarcinogenesis induced by DEN and 2-AAF and examined the expression of Cyp2e1, Cyp1a1/2 and GST-P in the livers of Sprague Dawley (SD) rats.

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*Corresponding authors: X Qi (e-mail: xmqi@cdser.simm.ac.cn), J Ren (e-mail: jren@cdser.simm.ac.cn)
[#] These authors contributed equally to this study.

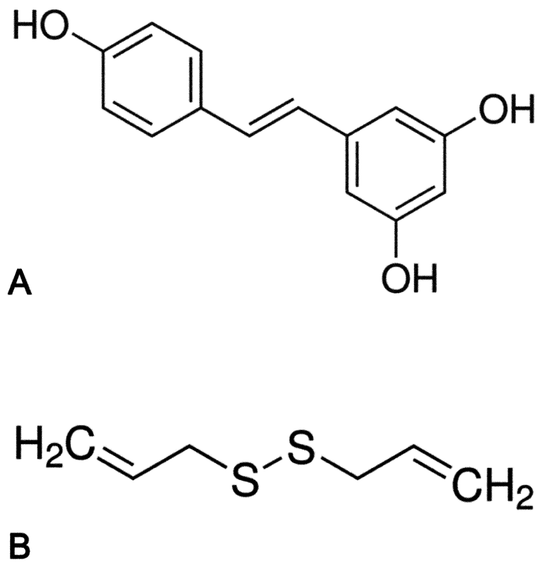

**Fig. 1.** Structure of *trans*-resveratrol (A) and diallyl disulfide (B).

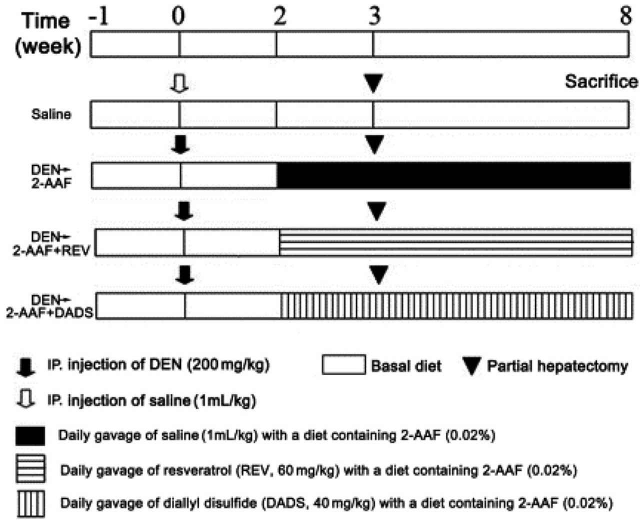


**Fig. 2.** Schematic representation of the experimental design.

## Material and Methods

*Chemicals*

DEN, 2-AAF and diallyl disulfide (DADS) (98%) were purchased from Sigma (St. Louis, MO, USA). Resveratrol (98%) was purchased from Shanxi Huike Botanical Development Co., Ltd (Xian, Shanxi, China). Rabbit anti-rat Cyp2e1, rabbit anti-rat Cyp1a1, rabbit anti-rat Cyp1a2, mouse anti-rat Cyp2b1, rabbit anti-rat cyp3a1 and rabbit anti-rat β-actin were purchased from Millipore (Billerica, MA, USA); rabbit anti-rat GST-P was purchased from Enzo (Waterloo, Australia). Other chemicals were commercially available and purchased as reagent grade from Sinopharm (Shanghai, China).

*Animal subjects*

Male SD rats (150–160 g body weight) were supplied by the Shanghai Sippr-BK Laboratory Animal Center (Shanghai, China), and housed in plastic cages in a temperature- and humidity-controlled SPF animal facility center with a 12-h light-dark cycle. All animal experiments were approved by the Shanghai Animal Care and Use Committee (Certificate No. SCXK [Shanghai] 2002-0010).

Twenty-four rats were divided into 4 groups: Saline, DEN→2-AAF, DEN→2-AAF+REV, and DEN→2-AAF+DADS (Fig. 2). REV (60 mg/kg) and DADS (40 mg/kg) were given by daily gavage from weeks 2 to 8. All groups received a partial hepatectomy (two-thirds of the total liver) in the third week. At week 8, all rats were sacrificed, and liver tissues were fixed in formalin, partially embedded with OCT and frozen in liquid nitrogen for cryosectioning.

*Immunohistochemical staining of GST-P and Cyp2e1*

Frozen serial sections of liver were prepared. One section was immunohistochemically stained with rabbit anti-rat GST-P antibody. Another section was stained with rabbit anti-rat Cyp2e1 antibody. Immunohistochemical assays were performed by the avidin-biotin complex method. Semiquantitative analysis of GST-P-positive foci was performed using a Leica QFAB image processing system (Leica Imaging Systems Ltd., Cambridge, England).

*Laser microdissection*

Sixteen-micron-thick frozen serial sections were prepared on Leica polyethylene terephthalate (PET) foil stretched on a metal frame. Rapid H&E staining was performed. Sections were dried at room temperature for 10 minutes and covered with a glass slide for subsequent dissection with a Leica AS LMD system (Leica Microsystems Ltd.). Target cells were dissected by laser beam (VSL-337ND-S nitrogen laser, Laser Science Inc.) and collected with a microcentrifuge tube (Supplementary Fig. 1: on-line only).

*Western blot*

Liver tissues and dissected samples were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Shanghai, China). Membranes were immunoblotted with rabbit anti-rat GST-P (1:800), rabbit anti-rat Cyp2e1 (1:800), rabbit anti-rat Cyp1a1 (1:1000), rabbit anti-rat Cyp1a2 (1:1000), mouse anti-rat Cyp2b1 (1:1000) and rabbit anti-rat β-actin (1:2000) and visualized using an ECL chemiluminescent detection system (GE Healthcare, Shanghai, China).

*Statistical methods*

Data were expressed as mean ± SD. After analysis of homogeneity, homogeneous data were analyzed by one-way analysis of variance followed by the least significant difference test as a post-hoc test. Heterogeneous data were analyzed using *t*-tests. $P<0.05$ was considered statistically significant.

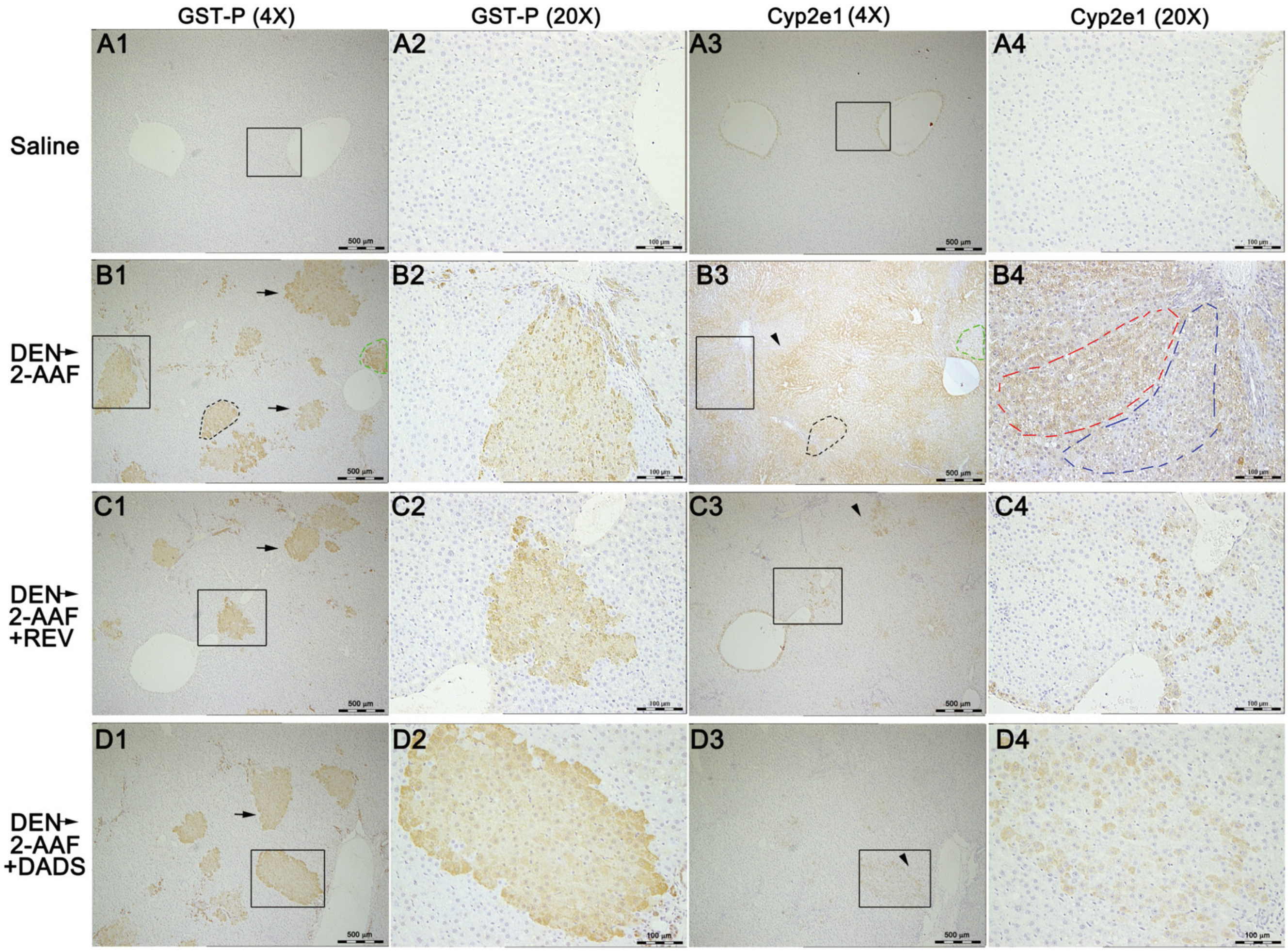


**Fig. 3.** Attenuation of DEN- and 2-AAF-induced hepatocarcinogenesis by REV or DADS. Liver serial sections were used to examine the expression of GST-P and Cyp2e1 by immunohistochemical staining. (A1–D1) Liver sections were stained with GST-P antibody (4×; scale bar, 500 µm); (A2–D2) A high magnification view of the GST-P-positive foci enclosed by a black rectangle in A1–D1 (20×; scale bar, 100 µm); (A3–D3) One serial section of liver stained with Cyp2e1 antibody (4×; scale bar, 500 µm); (A4–D4) a high magnification view of Cyp2e1 enclosed by a black rectangle in A3–D3 (20×; scale bar, 100 µm); (A1–A4) normal untreated liver showing the absence of GST-P foci and low Cyp2e1 expression; (B1–B4) liver sections from the DEN+2-AAF group showing numerous GST-P-positive foci (B1, B2) and extensive staining of Cyp2e1 (B3, B4); (C1–C4) Liver sections from the REV group showing fewer GST-P positive foci (C1, C2) and reduced Cyp2e1 staining compared with the DEN+2-AAF group (C3, C4); (D1–D4) liver sections from the DADS group showing fewer GST-P-positive foci (D1, D2) and reduced Cyp2e1 staining compared with the DEN+2-AAF group (D3, D4). Arrows indicate GST-P-positive foci; arrow heads indicate Cyp2e1 staining.

## Results

### *Effects of REV and DADS on DEN and 2-AAF-induced hepatocarcinogenesis*

The effects of REV and DADS on body and liver weight are shown in Table 1. DEN+2-AAF caused a significant decrease in body weight, and REV or DADS treatment did not result in recovery of body weight. There were no significant alterations in either absolute or relative liver weights (absolute liver weight/body weight) between Saline, DEN→2-AAF and DEN→2-AAF+REV groups. The absolute liver weight of the DEN→2-AAF+DADS group was higher when compared with that of the ‘DEN→2-AAF’ group.

Immunohistochemical staining of GST-P and Cyp2e1 was performed on serial liver sections. Numerous GST-P-positive foci were found in the liver of the ‘DEN→2-AAF’ group (Fig. 3B1). Treatment with REV or DADS significantly reduced the area and number of GST-P foci (Fig. 3C1 and D1, Fig. 4A-B). Expression of Cyp2e1 was markedly increased in the ‘DEN→2-AAF’ group (Fig. 3B3-4, Fig. 4C), and was decreased by REV or DADS in both GST-P foci and surrounding liver tissue (Fig. 3C3-4 and D3-4 and Fig. 4C).

### *Comparison of GST-P and Cyp2e1 expression between GST-P foci and surrounding liver tissue*

In this study, GST-P and Cyp2e1 displayed different staining patterns in livers of the ‘DEN→2-AAF’ group.

**Table 1.** Body and Liver Weights

| Group | Body weight/g | Liver weight | |
|---|---|---|---|
| | | Absolute/g | Relative/% |
| Saline | 458 ± 28 | 12.1 ± 1.1 | 2.66 ± 0.12 |
| DEN→ 2-AAF | 393 ± 14* | 11.9 ± 0.6 | 3.04 ± 0.18 |
| DEN→2-AAF+REV | 377 ± 25* | 11.2 ± 0.7 | 2.99 ± 0.29 |
| DEN→2-AAF+DADS | 410 ± 12 | 13.8 ± 0.5# | 3.08 ± 0.16 |

The results are Mean ± SD. n=6. * P<0.05 *vs*. Saline group; # P<0.05 *vs*. DEN→2-AAF group.

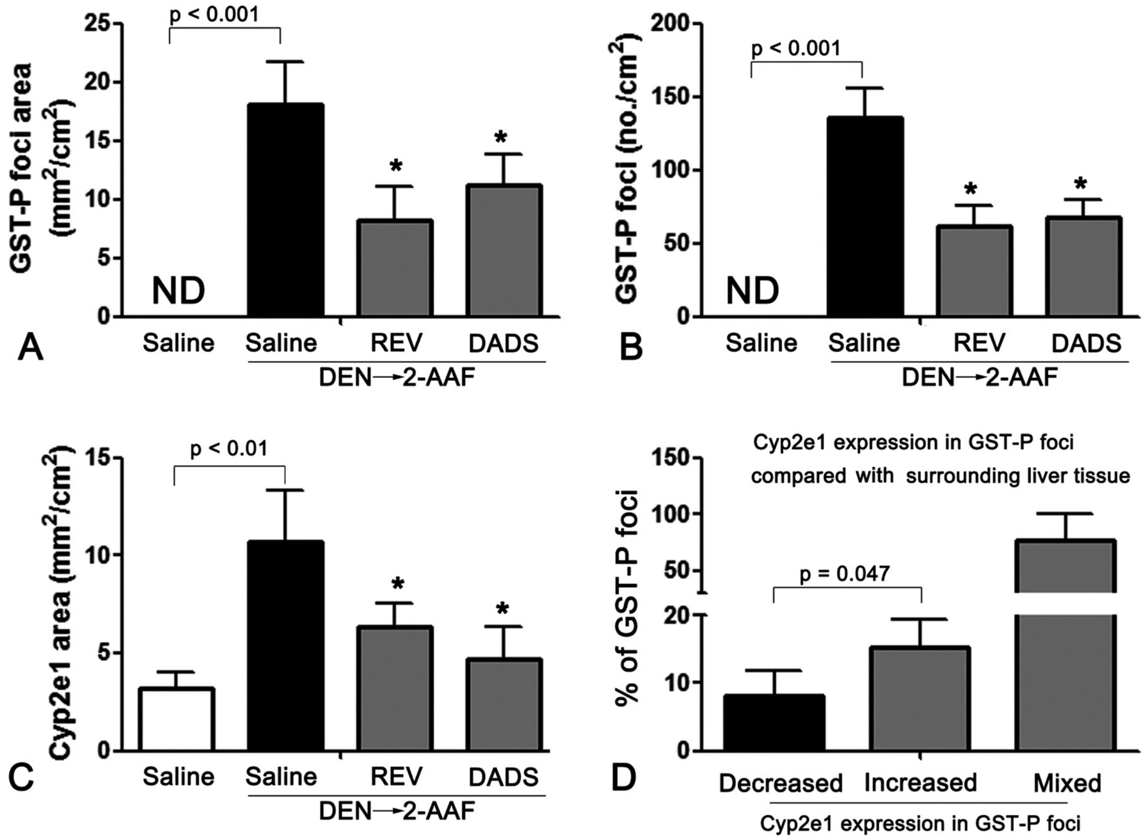


**Fig. 4.** Semiquantitative analysis of the expression of GST-P foci and Cyp2e1 in the liver serial sections. GST-P foci with a diameter greater than 0.2 mm were selected for analysis of area and number of foci. (A) Area of GST-P foci in liver sections; (B) number of GST-P foci in liver sections; (C) area of Cyp2e1 staining in liver sections; (D) percentage of GST-P foci with differential Cyp2e1 expression in the liver of the DEN→2-AAF group. Cyp2e1 expression was compared between GST-P-positive foci and surrounding liver tissue. Each bar represents mean ± SD. (n=6). * P<0.05 when compared with the DEN→2-AAF group.

GST-P staining showed a focal distribution with sharp demarcation from surrounding liver tissue (Fig. 3B1-2); Cyp2e1 showed strong diffuse staining around the centrilobular vein (Fig. 3B3-4). Using serial liver sections, we found that Cyp2e1 expression in GST-P-positive foci and surrounding liver tissue showed three distinct patterns: (1) decreased: in which the expression of Cyp2e1 was decreased in GST-P foci when compared with surrounding liver tissue (the region enclosed by the green dotted line in Fig. 3B1 and B3); (2) increased: in which the expression of Cyp2e1 in GST-P foci was higher when compared with surrounding liver tissue (the region enclosed by the black dotted line in Fig. 3B1 and B3); (3) mixed type: in which in one GST-P focus (the region enclosed by the rectangle in Fig. 3B1-4), Cyp2e1 staining in the region enclosed by the blue dotted line was lower when compared with the region enclosed by the red dotted line (Fig. 3B4). We further analyzed the percentage of GST-P foci with the three patterns of Cyp2e1 expression in the DEN→2-AAF group. As shown in Fig. 4D, most GST-P foci showed mixed-type Cyp2e1 expression. Approximately 10% of GST-P foci showed decreased Cyp2e1 expression, while 15% of GST-P foci had higher Cyp2e1 expression when compared with the surrounding liver tissue.

GST-P-positive foci and the surrounding liver tissue

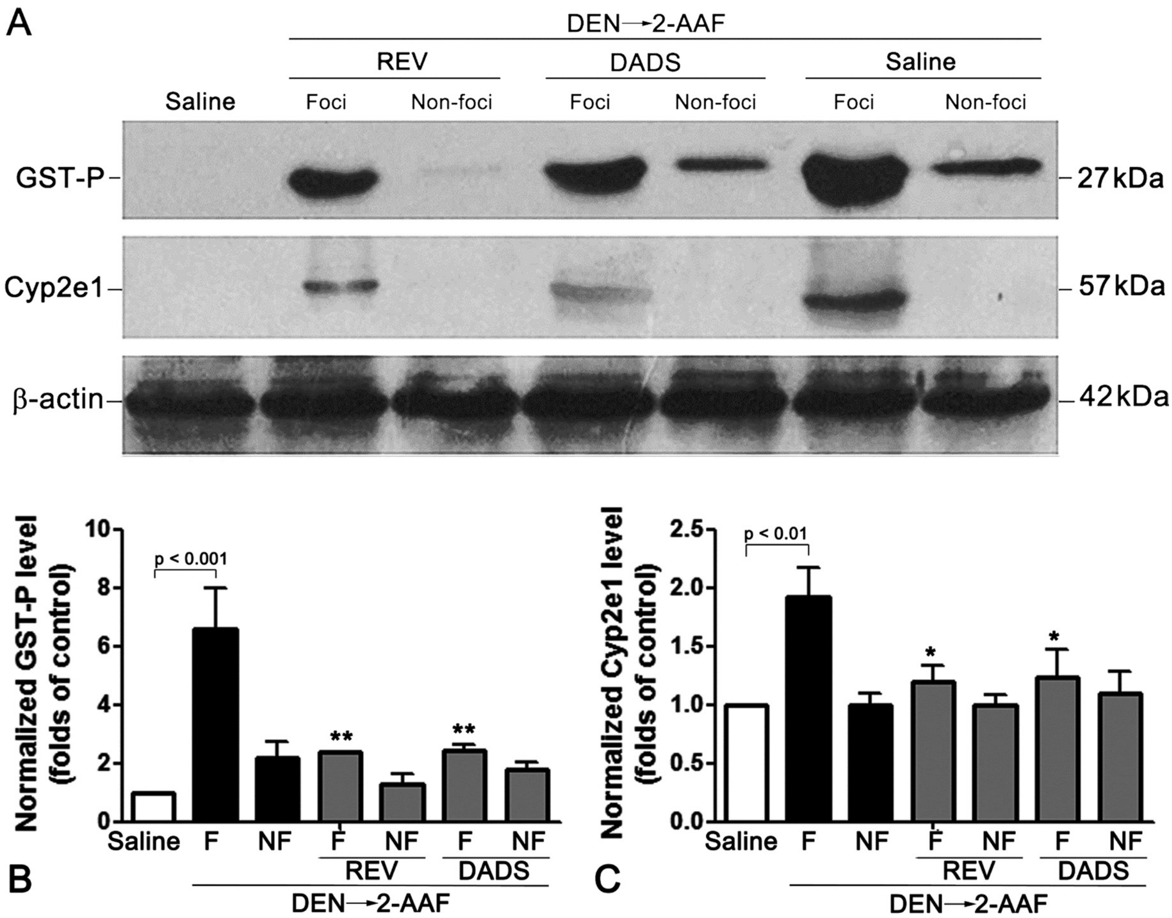


**Fig. 5.** Comparison of GST-P and Cyp2e1 expression between GST-P foci and the surrounding liver tissue. To ensure all types of GST-P foci will be collected, most of the GST-P-positive foci and surrounding GST-P/Cyp2e1-negative liver tissue in one section are dissected. (A) Western blot analysis of levels of GST-P and Cyp2e1 protein in foci and surrounding tissues (non-foci). (B, C) Semiquantitative analysis of GST-P (B) and Cyp2e1 (C) protein levels in foci and surrounding tissues (non-foci, negative staining for GST-P and Cyp2e1). The levels of GST-P and Cyp2e1 protein were normalized to β-actin. Each bar represents the mean±SD. (n=6). * $P<0.05$; ** $P<0.01$ compared with the level of protein in foci of the DEN→2-AAF group. F, foci; NF, non-foci.

were dissected and collected to detect the levels of GST-P and Cyp2e1 proteins. To ensure all types of GST-P foci will be collected, most of the GST-P-positive foci and surrounding GST-P/Cyp2e1-negative liver tissue in one section are dissected. As shown in Fig. 5, the levels of GST-P and Cyp2e1 proteins were higher in foci (F) when compared with surrounding liver tissues (non-foci, NF) (Fig. 5A and B). REV or DADS treatment decreased the GST-P protein in both foci and NF tissues (Fig. 5A and B). The level of Cyp2e1 protein in foci was also reduced by REV or DADS treatment (Fig. 5A and C).

### *Effects of REV on the expression of Cyp1a1, Cyp1a2, Cyp2b1 and Cyp3a1*

Protein levels of Cyp1a1, Cyp1a2 and Cyp2b1 were increased in the 'DEN→2-AAF' group, and reduced by REV treatment. Cyp3a1 expression was unchanged in the DEN→2-AAF and DEN→2-AAF+REV groups when compared with the Saline group (Fig. 6).

## Discussion

Previous studies have shown that resveratrol prevents DEN-induced hepatocarcinogenesis by several mechanisms, including suppression of NF-κB and COX-2[13–15]. In the present study, resveratrol downregulated the expression of Cyp2e1 and markedly attenuated hepatocarcinogenesis induced by DEN+2-AAF treatment in rats.

The role of Cyp2e1 in the initiation of hepatocarcinogenesis induced by DEN has been well characterized[9]. Recent studies have also shown the critical role of Cyp2e1 overexpression in the progression of hepatocarcinogenesis[12,23,24]. Furthermore, in some previous studies, Cyp2e1 was induced in the liver in DEN→2-AAF groups[25,26], suggesting overexpressed Cyp2e1 has a potential role in DEN+2-AAF-induced hepatocarcinogenesis. Here, we found that treatment with REV or DADS (an inhibitor of Cyp2e1) two weeks after DEN-initiation efficiently decreased the level of Cyp2e1 protein and reduced the number and area of GST-P

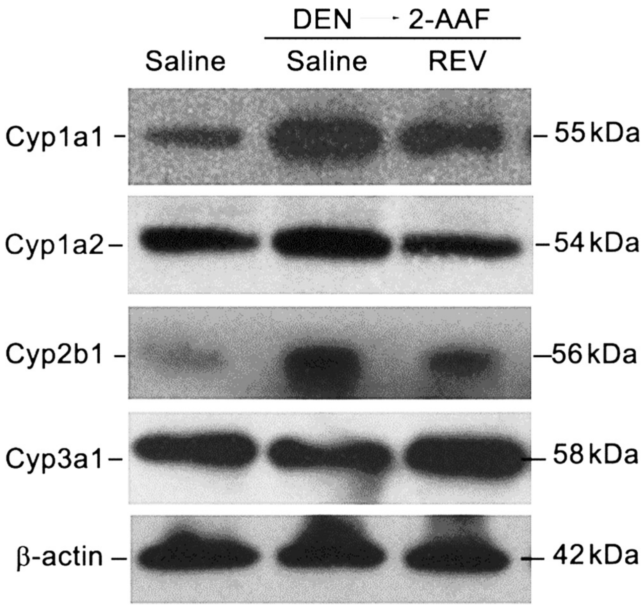


**Fig. 6.** The effects of resveratrol on the expression of Cyp1a1/2, Cyp2b1 and Cyp3a1 in the whole rat liver. The levels of Cyp1a1/2 and Cyp2b1 protein were increased in the liver of the 'DEN→2-AAF' group; both were decreased by resveratrol (60 mg/kg); Neither DEN +2-AAF nor resveratrol changed the level of Cyp3a1 protein.

foci (Figs. 3, 4). The inhibitory effects of REV are unlikely due to inhibition of metabolic activation of DEN.

Several studies have reported that Cyp2e1 expression was decreased in some GST-P-positive foci[25,27,28], while Cyp2e1 expression or activity in the whole liver was increased[25,27]. Additionally, the percentage of GST-P foci with decreased Cyp2e1 was not shown in these studies. Here, we tried to evaluate Cyp2e1 expression in GST-P foci using serial sections and immunochemical staining. It is difficult to obtain the real percentage of GST-P foci with differential Cyp2e1 expression. Three factors will affect the accuracy of the percentage: (1) different staining pattern of GST-P and Cyp2e1; (2) distortion and squeezing of liver tissue in the process of cutting serial section; and considering the diameter of hepatocytes (20–30 μm) and the thickness of the sections (16 μm), differences in the hepatocytes in the serial sections. Finally, we found that Cyp2e1 expression in GST-P foci showed three different patterns (Figs. 3, 4D). The percentage of GST-P foci with increased Cyp2e1 expression was greater than the percentage of those with decreased Cyp2e1 expression (Fig. 4D). Combining the protein level of Cyp2e1 in GST-P foci and surrounding liver tissue (Fig. 5), our results suggested that GST-P foci with decreased Cyp2e1 expression was present. However, the overall protein level of Cyp2e1 in GST-P foci and the expression of Cyp2e1 in the entire liver are increased in the DEN→2-AAF group.

In this study, REV or DADS treatment decreased the Cyp2e1 level in the rat whole liver including GST-P foci and surrounding liver tissue (Fig. 3B3-D3, Fig. 5). Noticeably, after REV or DADS treatment, the Cyp2e1 protein level in most of the surrounding liver tissue was close to that in the Saline group (Fig. 3A3-D3, Fig. 5). Due to the low level of Cyp2e1 protein in surrounding liver tissue, REV or DADS treatment changed the staining pattern of Cyp2e1 in GST-P foci. In the DEN→2-AAF+REV group, half of the GST-P foci showed a similar low Cyp2e1 expression when compared with surrounding liver tissue, and GST-P foci with decreased Cyp2e1 expression were not found. Approximately 10% of GST-P foci showed a bit higher Cyp2e1 expression when compared with the surrounding liver tissue. In the remaining GST-P foci (~40%), Cyp2e1 expression showed a mixed type. The staining pattern of Cyp2e1 in the DEN→2-AAF+DADS group was similar to that of REV-treated group. REV and DADS treatment decreased Cyp2e1 expression and showed similar effects on the staining pattern of Cyp2e1 in GST-P foci. Considering the significance of Cyp2e1 in hepatocarcinogenesis, more work is needed to clarify the role of the changed staining pattern of Cyp2e1 in the protective effects of REV or DADS.

Oxidative stress plays an important role in hepatocarcinogenesis[29,30]. Multiple studies have shown the important role of CYP2E1 overexpression in generating oxidative stress[12,31,32]. Cyp2e1 induction was also found in DEN+2-AAF-induced hepatocarcinogenesis[25,26], indicating the potential role of Cyp2e1 in this process. Here, we found that treatment of REV or DADS two weeks after DEN-initiation efficiently decreased the level of Cyp2e1 protein and reduced the number and area of GST-P foci (Figs. 3, 4). Some reports have shown that resveratrol significantly prevents DEN-induced hepatocarcinogenesis by acting as an antioxidant and anti-inflammatory agent in rats. Our results suggested that the antioxidant effects of REV may be due to the downregulation of Cyp2e1. More work is needed to clarify the mechanisms of decreased Cyp2e1 expression by REV.

2-AAF is metabolically activated primarily by Cyp1a, suggesting that its carcinogenic potential may be inhibited or enhanced by simultaneous exposure to either an inhibitor or inducer of Cyp1a. As our first research target, we investigated the role of Cyp1a in DEN+2-AAF-induced hepatocarcinogenesis and the protective effect of REV. Alpha-naphthoflavone (a strong inhibitor of Cyp1a with *Ki* values of 2.3 and 1.6 μM for Cyp1a1 and Cyp1a2, respectively; weak inducer of Cyp1a) and β-naphthoflavone (strong inducer of Cyp1a1 and Cyp1a2, no inhibitory effects on Cyp1a)[33–35] were used as controls for REV. However, coadministration of α-naphthoflavone or β-naphthoflavone with 2-AAF did not show any effects on the number or area of GST-P foci in the liver in the DEN→2-AAF group (unpublished data). These results were consistent with previous reports[36–38]. Additionally, REV did not completely suppress the expression of Cyp1a1/2 (Fig. 6). Considering the low dose of 2-AAF (0.02%), the remaining Cyp1a may be sufficient for metabolic activation of 2-AAF. Taken together, we hypothesize that Cyp1a may not be involved in DEN+2-AAF-induced hepatocarcinogenesis as well as the protective effect of REV under our experimental conditions.

In conclusion, our results show that resveratrol exerts a suppressive effect on hepatocarcinogenesis induced by DEN+2-AAF and has a potential chemopreventive effect on hepatocellular carcinoma. Inhibition of Cyp2e1 activity and expression by resveratrol may contribute to this process. Our study provides new insight into the mechanisms of resveratrol preventing chemically induced hepatocarcinogenesis and will be helpful in the clinical use of resveratrol.

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