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*Original Article*

# Diosgenin increased DDX3 expression in hepatocellular carcinoma

Hong Yu[1*], Yuanni Liu[1*], Chuanzhen Niu[1], Yu Cheng[2]

*[1]Department of Liver Disease, Yantai Infectious Diseases Hospital, Yantai, Shandong, China; [2]Department of Medical Oncology, Yantai Yuhuangding Hospital Affiliated with Qingdao University, Yantai, Shandong, China. [*]Equal contributors.*

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**Abstract:** Liver cancer, one of the most common malignant tumors occurred worldwide, has emerged as a main health trouble and accounts for leading cancer-related death. Diosgenin is provided as an important material in the pharmaceutical industry, and is used to manage various medical troubles such as cancer because of its multiple bioactivities. DEAD box polypeptide 3 (DDX3) is involved in cancer biogenesis and modulates cancer progression. However, the role of DDX3 in human hepatocellular carcinoma (HCC) has not been fully understood. In the present study, we investigated the anti-tumor effects of diosgenin on HCC cells and whether DDX3 is involved in its antitumor activity. We observed that diosgenin dramatically inhibited cell proliferation, triggered apoptotic cell death, induced G2/M phase arrest, suppressed cell migration and invasion abilities. Moreover, the expression of DDX3 was measured and the results showed that DDX3 was significantly up-regulated upon diosgenin exposure. All together, our data indicated that diosgenin shows a cytotoxic effect on HCC cells and has potential therapeutic values for HCC patients.

**Keywords:** DDX3, hepatocellular carcinoma, diosgenin, proliferation, invasion

## Introductions

Natural plant products have been revealed to inhibit the growth of cultured human cancer cell lines, which are potential innovative chemopreventive or therapeutic agents [1]. Diosgenin is the product of hydrolysis of saponins, extracted from the tubers of wild yam (*Dioscorea villosa*). Diosgenin has attracted global interests of many researchers for the pharmaceutical manufacturing industry [2-4]. As a matter of fact, most of steroidal drugs, representative of sex hormones and corticosteroids, are semisynthetic products predominantly from diosgenin [5]. Diosgenin itself is a typical traditional medicine which is used to manage various medical troubles, because of its multiple bioactivities, such as antitumor [6], anti-inflammatory [7], and antidiabetes [8]. The anticancer properties of diosgenin have been widely studied in recent years. Diosgenin was reported to induce G2/M arrest, apoptotic cell death, reactive oxygen species (ROS) generation, caspase-3 activation, and to down-regulate the antiapoptotic Bcl-2 and Bcl-xL proteins, and to up-regulate proapoptotic Bax, leading to inhibiting growth of human chronic myeloid leukemia cells [9]. Diosgenin was also described to inhibit migration and invasion of human prostate cancer cells via decreasing matrix metalloproteinases (MMPs) expression, and inhibiting ERK, JNK and PI3K/Akt signaling as well as NF-kappaB activity [10]. Diosgenin has antiproliferative activity, such as inhibition of cell proliferation and induction of apoptosis in squamous cell carcinoma [6]. Mao et al. found that diosgenin is a potent candidate for suppressing the proliferation and invasion abilities of gastric cancer cells [11]. Furthermore, they revealed that the anti-invasion property of diosgenin might be associated with E-cadherin, integrin-alpha 5 and integrin beta 6. In human hepatocellular carcinoma HepG2 cells, diosgenin was reported to induce apoptosis via Bcl-2 protein family-mediated mitochondria/caspase-3-dependent pathway [12]. ROS was also strong-

ly produced by diosgenin treatment and might promote apoptotic cell death via activation of ASK1 in HepG2 cells. Diosgenin triggers G2/M cell cycle arrest and apoptosis in human HCC cells by suppressing p-Akt, up-regulating p21 and p27, but not altering p53 level [13].

DEAD box helicases, named after the conserved amino acid sequences DEAD (Asp-Glu-Ala-Asp), are vitally involved in various aspects of RNA metabolism, and knockdown of these helicases is always embryonically lethal [14]. DDX3 (DEAD box polypeptide 3) is a member of the DEAD box family, has been identified to associate with many kinds of RNA metabolism, such as RNA transcription, splicing and mRNA transport, and protein translation initiation [15, 16]. DDX3 has attracted increasing attentions as it is involved in cancer biogenesis and modulates cancer progression [17]. Interestingly, contrary function of DDX3 was described both as tumor promoter and tumor suppressor [18]. In certain tumor types, such as breast cancer, overexpression of DDX3 has been reported in pathological samples and is associated with lower survival [19]. However, DDX3 was identified to inhibit growth and activate the expression of p21, so as to be a candidate tumor suppressor [20]. In this study, we detected the expression of DDX3 in liver cancer cells and measured whether DDX3 was affected after diosgenin treatment. We found that diosgenin treatment markedly inhibited cell growth and significantly promoted the expression of DDX3 in HepG2 and SMMC-7721 cells. Moreover, the expression of p21 and E-cadherin was activated. The level of Cyclin D1, Notch-1, β-catenin was suppressed after diosgenin treatment. Our results suggested that DDX3 functioned as a tumor suppressor in HCC and might benefit the inhibitory property of diosgenin.

## Methods and materials

### *Cell culture*

Human HCC cell lines HepG2 and SMMC-7721 were purchased from the Chinese Academy of Science (Shanghai, China) and maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco, Grand Island, NY, USA), supplemented with 10% FBS (fetal bovine serum) and 100 U/ml PS (penicillin/streptomycin, HyClone™). The cells were then incubated at 37°C in a humidified atmosphere with 5% $CO_2$, and 95% humidity.

### *Reagents*

Diosgenin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diosgenin was dissolved in DMSO and stored at -20°C. Series working concentrations of diosgenin were freshly diluted in DMED prior to each experiment. Fluorescein (FITC)-Annexin V Apoptosis Detection kit, PI (Propidium iodide) and BCA Protein assay kit were bought from Beyotime Biotechnology (Shanghai, China). Calcein-AM was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies for DDX3, Cyclin D1, Notch-1, E-cadherin, β-catenin, and p21 were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal anti-Tubulin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies were bought from Thermo Fisher Scientific.

### *Cell viability detection*

Freshly prepared HepG2 and SMMC-7721 cells ($5\times10^3$ cells/well) were seeded in 96-well plates. After incubated overnight, the cells were treated with different concentrations (0-100 μM) of diosgenin. The experiment was carried out in triplicate. And after exposed to diosgenin for 24 h, 48 h and 72 h, removed the media. Cell viability was detected by MTT assays following the manufacturer's instruments. For brief, added 10 μl MTT solutions (0.5 mg/ml) to each well, and incubated the 96-well plate for about 4 h. Removed the liquid supernatant carefully and added 100 μl DMSO and mixed thoroughly using the pipette. After incubated at 37°C for 10 minutes, read the absorbance at 540 nm with a Multimode Reader of SpectraMax M5 (Moleucular Devices, Sunnyvale, CA, USA).

### *Cell apoptosis analysis*

Freshly prepared HepG2 and SMMC-7721 cells were inoculated to 6-well plates at a density of $1\times10^5$ cells/well. The cells were exposed to 0, 50 and 75 μM of diosgenin for 48 h. Cells were collected by trypsinisation and centrifugation, and subsequently resuspended in 500 μl of

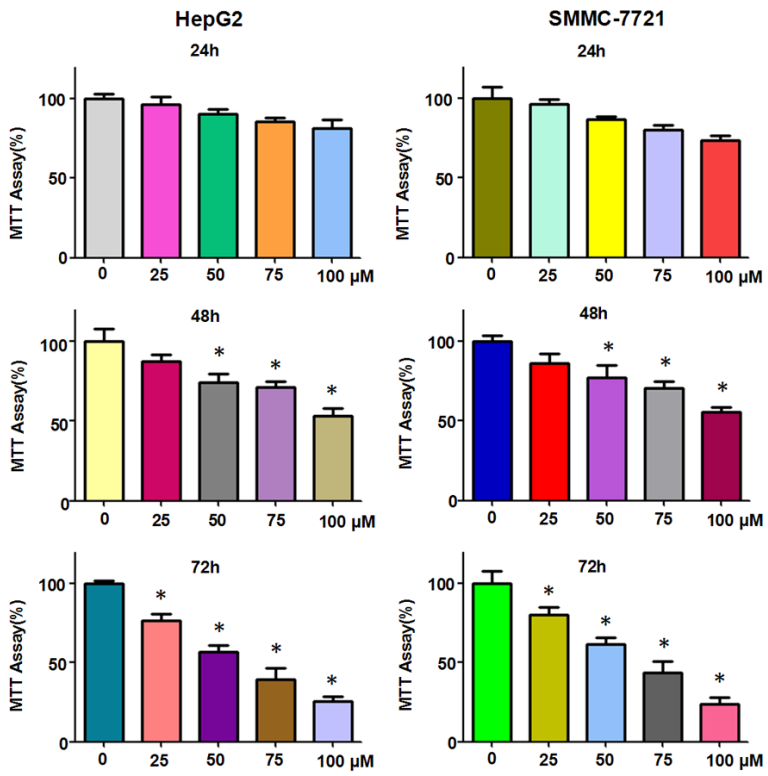


**Figure 1.** Diosgenin inhibited human hepatocellular carcinoma (HCC) cells growth. HepG2 and SMMC-7721 cells ($5\times10^3$ cells/well) were seeded in 96-well plates and treated with 0-100 μM of diosgenin. After exposed to diosgenin for 24 h, 48 h and 72 h, MTT assay was carried out to detect the cell viabilities. *P<0.05, compared to the control groups treated with DMSO.

binding buffer containing 5 μl annexin V-FITC and 5 μl PI. Flow cytometric analysis was carried out to measure the presence of apoptotic cells using a FACScalibur flow cytometer (BD, USA). At least 20,000 events from each specimen were measured to pledge sufficient data.

*Cell cycle stage detection*

HepG2 and SMMC-7721 cells were cultured in 6-well plates at a density of $1\times10^5$ cells/well overnight. Diosgenin treatment was performed as above. Cells were harvested with trypsin and washed twice with PBS. The collected cells were then fixed with 70% ice-cold ethanol and maintained at 4°C overnight. After washed twice with PBS, cells were suspended with 500 μl cold PBS containing 100 μg/ml RNase and 50 mg/ml PI at 37°C for 30 min in the dark. Cell cycle stage was measured using flow cytometer.

*Wound healing migration assay*

HepG2 and SMMC-7721 cells were incubated in 6-well plates till grew to around 90% confluence. Rectangular lesions were made on the cell monolayer by using sterile tips. Cells were washed with PBS and debris and detached cells were carefully sucked. After cultured for appropriate periods of time, those cells that migrated into the wound area were photographed using an inverted microscope. The lesion sizes were calculated by measuring the borders of the wound and comparing their initial lesions.

*Transwell cell invasion assay*

24-well Transwell chambers (8 μm pore size, Corning, NY, USA) with Matrigel coating were subjected to cell invasion assay for HepG2 and SMMC-7721 cells. Freshly cultured or diosgenin treated HCC cells were detached with trypsin and diluted in FBS free DMEM at appropriated concentration. Aliquot 200 μl cells and inoculated into the upper chamber of the insert in the 24-well plate. In the lower chamber, 500 μl complete DMEM was added. After cultured for certain time point, the cells were allowed to invade the lower chamber containing FBS. The non-invasive cells remaining on the upper surface of the membrane were removed. The invasive cells attached to the lower surface of the filter membrane were dyed with Calcein-AM for 10 min and then counted under a light microscope.

*Quantitative real-time reverse transcription-PCR (qPCR)*

Total RNA of HCC cells was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). For the quantification of DDX3 mRNAs, 2 μg total RNA was reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit. qPCR was performed using Power SYBR Green PCR Master Mix on an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), the relative expression of DDX3 was calculated. GAPDH was used as an endogenous reference.

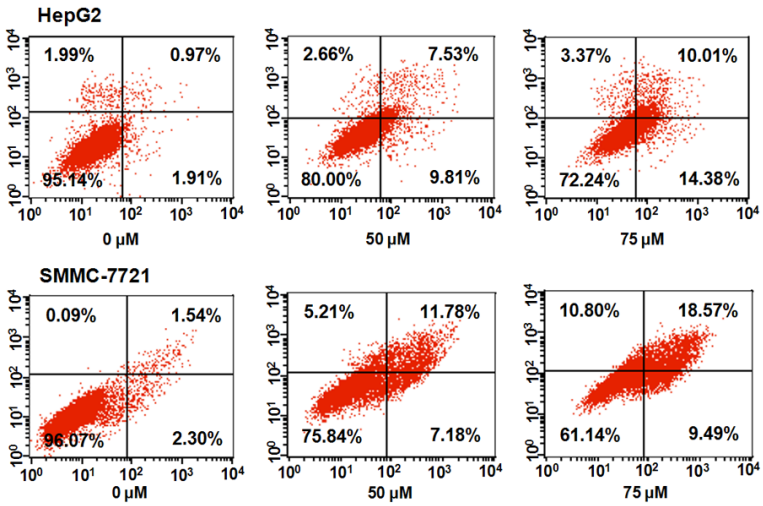


**Figure 2.** Diosgenin induced apoptotic cell death in HCC cells. HepG2 and SMMC-7721 cells were inoculated to 6-well plates at a density of $1\times10^5$ cells/well and were exposed to 0, 50 and 75 μM of diosgenin for 48 h. Annexin V-FITC/PI staining and flow cytometry assay were performed to detect the apoptotic cell death.

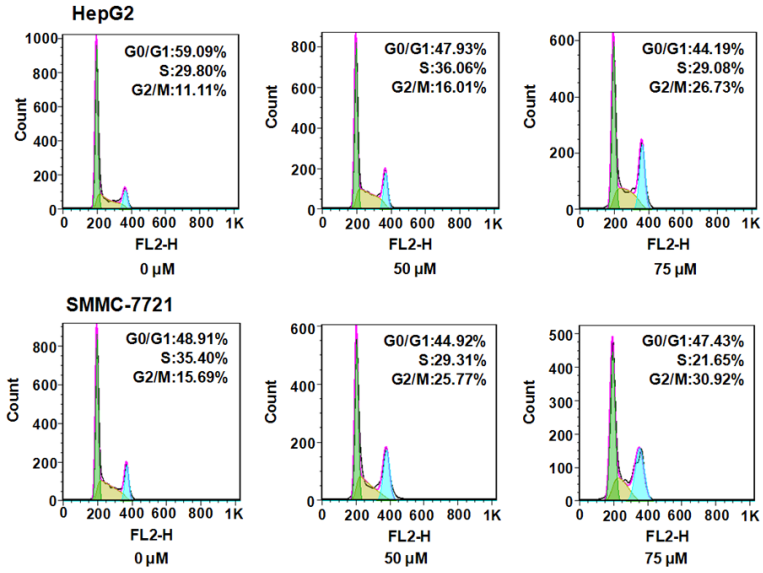


**Figure 3.** Diosgenin induced cell cycle arrest in HCC cells. Diosgenin-induced HCC cell cycle arrest was measured by PI staining and flow cytometry. Cells were harvested with trypsin and washed twice with PBS. The collected cells were fixed with 70% ice-cold ethanol and maintained at 4°C. After washed twice with PBS, cells were suspended with PBS containing 100 μg/ml RNase and 50 mg/ml PI at 37°C for 30 min in the dark. Cell cycle stage was measured using flow cytometer.

*Western blotting*

HepG2 and SMMC-7721 cells ($1\times10^6$ cells/well) were inoculated in 6-well plates. Following diosgenin treatment for the indicated times, cells were harvested by gentle scraping, and lysed in cell lysis buffer. BCA Protein Assay was performed to determine protein concentrations. Equal quantities of denatured protein samples were was subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane. The member was blocked with 5% defatted milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, subsequently incubated with appropriate primary antibodies at 4°C overnight. The member was washed with TBST and incubated with suitable secondary antibody at room temperature for 1.5 h. Lectrochemiluminescence (ECL) assay was used to detected specific protein bands.

*Statistical analysis*

The data were expressed as mean ± SD of triplicates determinants after analysis with Graph Pad Prism 4.0 (Graph Pad Software, La Jolla, CA). Statistical significance was assessed using a two-tailed *Student's t* test. Differences were considered significant when $P<0.05$.

## Results

*Diosgenin inhibited the proliferation of HCC cells*

In order to identify whether diosgenin affects the viability of HCC cells, HepG2 and SMMC-7721 cells were exposed to 0-100 μM diosgenin for 24 h, 48 h and 72 h, respectively. Cytotoxicity of diosgenin was detected by MTT assays. Our data showed that diosgenin suppressed HepG2 and SMMC-7721 cell prolifera-

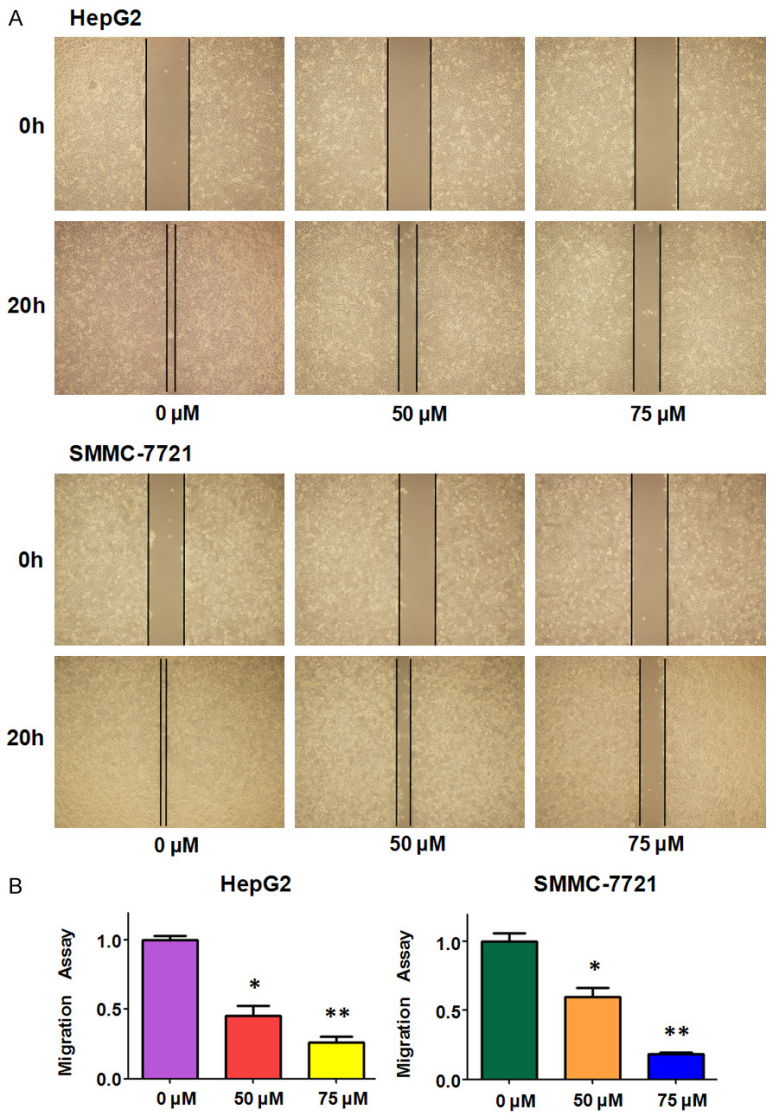


**Figure 4.** Inhibitory effects of diosgenin on HCC cell migration. (A) HepG2 and SMMC-7721 cells were incubated in 6-well plates till grew to around 90% confluence. Rectangular lesions were made on the cell monolayer by using sterile tips. Cells were washed with PBS and debris and detached cells were carefully sucked. After 20 h, cells that migrated into the wound area were photographed using an inverted microscope. (B) Quantitative results are illustrated for (A). *P<0.05, **P<0.01 vs control.

tion in a time- and dose-dependent manner (**Figure 1**). The results demonstrated that diosgenin exhibits an anti-proliferation activity in these HCC cell lines.

*Diosgenin triggered apoptosis of HCC cells*

The effect of diosgenin on cell death was assessed after a 48 h of challenge of HepG2 and SMMC-7721 cells with increasing concentration of diosgenin. Flow cytometry analysis was carried out after double staining with Annexin V-FITC and PI. We found that diosgenin triggered apoptotic cell death of HCC cells in a dose-dependent manner (**Figure 2**). 50 μM and 75 μM diosgenin expose induced apoptotic cell death to 17.34% and 24.39%, compared with control groups of HepG2. Similar results were found in SMMC-7721 cells. These findings proved that diosgenin caused cell growth suppression could be due to increased apoptotic cell death.

*Diosgenin induced G2/M cell cycle arrest of HCC cells*

We further conducted flow cytometric analysis to detect the mechanisms of diosgenin-promoted growth inhibition. After labeling with PI, a concentration-dependent addition of G2/M phase cell population was observed in diosgenin challenged HepG2 and SMMC-7721 HCC cells (**Figure 3**). The results indicated that diosgenin caused a G2/M phase cell cycle arrest in a dose-dependent manner, which could contribute to diosgenin-induced HCC cell growth inhibition.

*Diosgenin inhibited HCC cell migration*

As the migratory ability is considered an important property of cancer cells in the progress of cell death, wound healing assay was carried out in HepG2 and SMMC-7721 cells to evaluate the effect of diosgenin on HCC cell motility. We observed that cell migration was significantly suppressed in both diosgenin challenged HCC cells in a concentration dependent manner (**Figure 4**).

*Diosgenin inhibited HCC cell invasion*

Tranwell assay was further performed to examine whether diosgenin affect the invasive ability of HCC cells. Our results revealed that invasion

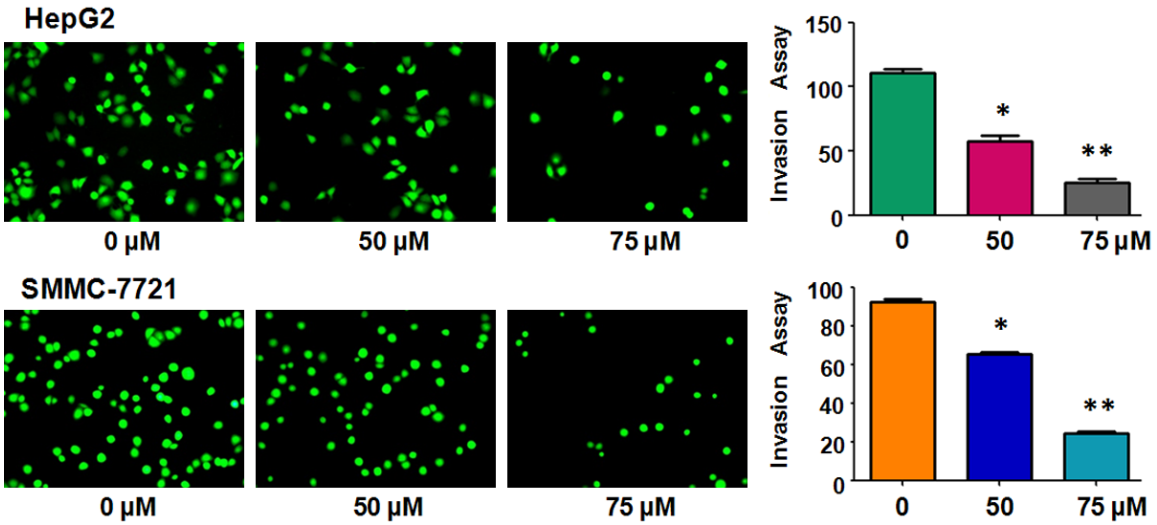


**Figure 5.** Diosgenin suppressed HCC cell invasion. Left panel: The inhibitory effect of diosgenin on HCC cell invasion was detected by Transwell chambers assay. 24-well Transwell chambers with Matrigel coating were subjected to cell invasion assay for HepG2 and SMMC-7721 cells. The cells were inoculated into the upper chamber of the insert in the 24-well plate. In the lower chamber, 500 μl complete DMEM was added. After 20 hours, the cells were allowed to invade the lower chamber containing FBS. The non-invasive cells remaining on the upper surface of the membrane were removed. The invasive cells attached to the lower surface of the filter membrane were dyed with Calcein-AM for 10 min and then counted under a light microscope. Right panel: Quantitative results of left panel. *P<0.05, **P<0.01 vs control.

was markedly impeded in diosgenin-treated HepG2 and SMMC-7721 cells in a dose-dependent manner (**Figure 5**). The number of invasive cells that transferred through the matrigel-coated transwell chamber membrane were significantly reduced in diosgenin challenged cells.

*Diosgenin up-regulated DDX3 expression in HCC cells*

DDX3 has been demonstrated to be involved in cancer biogenesis and modulates cancer progression. We determined here whether DDX3 plays a role in diosgenin-induced HCC cells death. After treated with 50 μM and 75 μM diosgenin, the mRNA level of DDX3 was measured by q-PCR. The results revealed that a drastic increase of DDX3 mRNA in both diosgenin-treated HepG2 and SMMC-7721 cells in a concentration determined manner (**Figure 6A**). The protein level of DDX3 was further measured by Western blotting assay. We observed that the expression of DDX3 protein was significantly up-regulated upon diosgenin expose (**Figure 6B**). Since an accumulation of cells in the sub G2/M phase was observed after exposed in diosgenin, the expression levels of cell cycle related proteins were determined subsequently. As shown in **Figure 6B**, cell cycle positive protein Cyclin D1 was significantly reduced respond to diosgenin treatment in both HCC cell lines, whereas the expression of cell cycle inhibitor p21 was dramatically induced (**Figure 6B**). We also measured the downstream targets of DDX3 including Notch-1, E-cadherin, β-catenin in HCC cells after diosgenin treatment. We found that diosgenin increased the E-cadherin expression, but decreased Notch-1 and β-catenin expression (**Figure 6B**). Taken together, these results indicated that diosgenin shows a cytotoxic effect on HCC cells possibly through the up-regulation of DDX3, suppression of Cyclin D1, Notch-1, β-catenin, and activation of p21 and E-cadherin.

## Discussion

In the present study, we evaluated the cytotoxic properties of diosgenin in two human liver cancer cell lines. Our findings demonstrated that cell proliferation was significantly suppressed by diosgenin treatment in both HepG2 and SMMC-7721 cells in a dose- and time-dependent manner (**Figure 1**). Then we observed that diosgenin triggered apoptotic cell death in both HCC cells after exposed to diosgenin and FITC-labeled annexin v/PI staining and flow cytometry assay (**Figure 2**). Furthermore, a concentra-

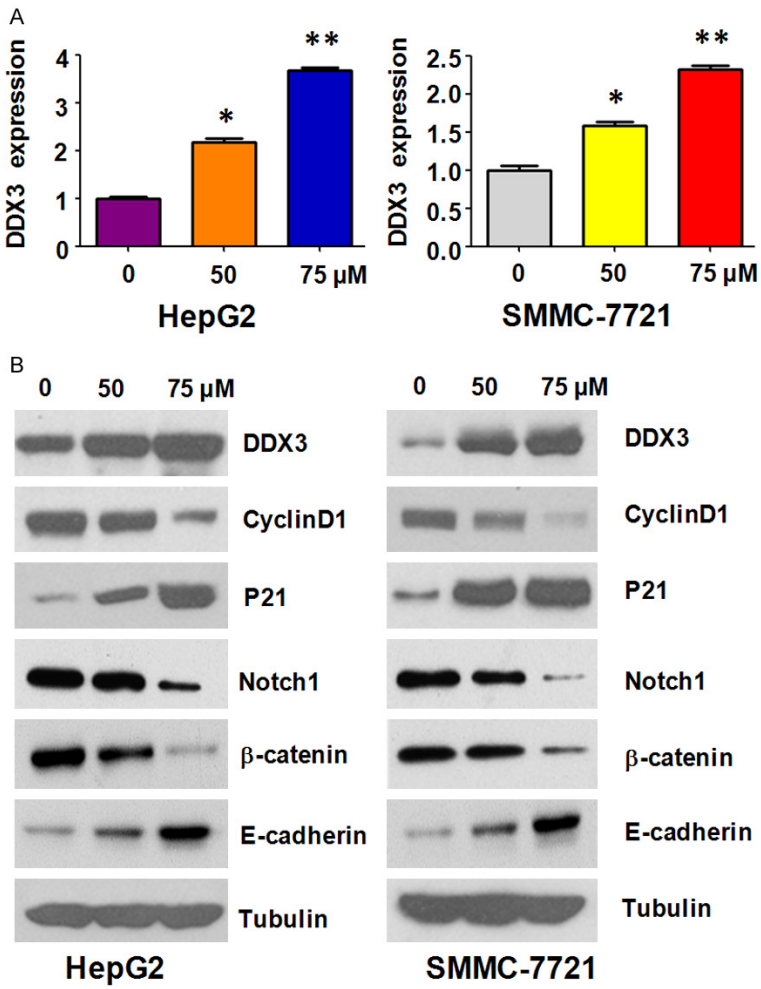


**Figure 6.** Diosgenin up-regulated DDX3 expression. A. mRNA level of DDX3 was measured by q-PCR in diosgenin treated HepG2 and SMMC-7721 cells using power SYBR Green PCR Master Mix on an ABI Prism 7500 Fast Real-Time PCR system. The relative expression of DDX3 was calculated. GAPDH was used as an endogenous reference. B. The expression of DDX3, Cyclin D1, p21, Notch-1, E-cadherin, and β-catenin was detected by Western blotting analysis in diosgenin treated HepG2 and SMMC-7721 cells. *P<0.05, **P<0.01 vs control.

tion-dependent accumulation of G2/M phase cell population was observed in diosgenin-treated HepG2 and SMMC-7721 HCC cells (**Figure 3**). We also found that the migratory and invasive abilities of HepG2 and SMMC-7721 HCC cells were strongly inhibited by diosgenin treatment (**Figures 4** and **5**); indicating diosgenin exhibited a strong migration inhibitory activity against both HCC cells. Mechanistically, the expression level of DDX3 mRNA and protein was significantly up-regulated upon diosgenin expose of HepG2 and SMMC-7721 HCC cells (**Figure 6**), suggesting DDX3 was a target of diosgenin. Subsequently, we observed that the expression of Cyclin D1, Notch-1, β-catenin was strongly decreased respond to diosgenin treatment in both HCC cells, whereas E-cadherin and p21 [21] were dramatically increased. All together, our data indicated that diosgenin shows a cytotoxic effect on HCC cells possibly through the up-regulation of DDX3.

Although multiple current therapeutic approaches are available, the restricted efficacy and intolerable toxicities of these treatments in most cases led to poor prognosis of patients with HCC. Lots of natural products and their extractions might have relative minimal toxicity and safety, which are used traditionally to manage various cancers [22-24]. Diosgenin is a phytosteroid saponin derived from many species of *Dioscorea* [25]. This bioactive phytochemical is widely used as raw materials for various drugs [26], such as steroidal drugs. In addition, diosgenin is used for the treatment of various medical troubles such as tumors [27]. It was observed to exhibit anti-carcinogenic properties. Diosgenin inhibited cell viability, promoted apoptosis, suppressed cell migration and induced cell cycle arrest in oral squamous cell cancer cell lines [28]. Diosgenin inhibits colon tumor growth *in vivo* and has no toxicity at the test condition, and suppresses tumor angiogenesis via modulating VEGFR2 and AKT/MAPK signaling pathways [29]. Nie *et al*. found that diosgenin reduced cell proliferation, activated apoptosis and autophagy via the suppression of the PI3K/Akt/mTOR signaling pathway in a human prostate cancer cell line [30]. Interestingly, diosgenin functionalized iron oxide nanoparticles have enhanced inhibitory effects on cell proliferation, migration and triggered apoptosis in breast cancer cells [31]. Kim *et al*. found that dios-

genin induced apoptosis in HepG2 cells via the generation of reactive oxygen species and mitochondrial pathway [12]. It was also reported that diosgenin dramatically reduced the growth of Bel-7402, SMMC-7721 and HepG2 HCC cells in a dose-dependent manner, caused G2/M cell cycle arrest, induced p53-independent upregulation of p21 and p57 [13]. Diosgenin activated caspase-3, -8 and -9 (increasing the cleaved caspase-3, -8 and -9 levels), but did not affect the expression of Bcl-2 and Bax, suggesting diosgenin-induced apoptotic death of HCC cells might be regulated through the death receptor pathway, but not the mitochondrial pathway [13]. In our study, diosgenin-mediated decrease of Cyclin D1, increase of p21 and G2/M cell cycle arrest could be associated with the apoptotic progression of HCC cells, which could led to cell growth inhibition. Diosgenin was also described to arrest cell cycle at G1 phase in C3a hepatoma cells [32], which suggested that the effect and mechanism of diosgenin on cell cycle might be in a cell type determined manner.

Previous data have revealed that DDX3 works as a host factor coopted by various viruses, including hepatitis B virus (HBV) and hepatitis C virus (HCV) [33, 34]. Ectopic expression of DDX3 restricts HBV genome replication and infection through two distinct mechanisms: by suppressing viral transcription of cccDNA (covalently closed circular) template and by promoting IRF signaling [34, 35]. DDX3 facilitates HCV RNA replication, although the underlying molecular mechanism is not clear yet [33, 36]. In recent years, DDX3 has attracted more and more attention on account of its important roles in regulating of hepatocarcinogenesis. Chang and colleagues examined the expression of DDX3 in surgically excised human HCC specimens and found that DDX3 was significantly downregulated in HCCs from hepatitis B virus (HBV)-positive patients, but not from HCV-positive ones [37]. They further investigated that inhibition of DDX3 expression up-regulated Cyclin D1 and down-regulated p21, resulting in an enhanced cell cycle progression and an accelerated cell growth [37]. Decreased expression of DDX3 was also found not only conversely associated with tumor grade, but also presented poor prognosis of patients with HCC [38]. Deletion of DDX3 in HepG2 cell line promoted stemness gene signature and led to tumorigenesis [38]. More recently, DDX3 was reported to be up-regulated the expression of in QGY7703 and SMMC7721 HCC cell lines after rottlerin treatment, accompanied by decreased expression of Cyclin D1 and increased expression of p21 [39]. In line with these previous studies, our results indicated that diosgenin treatment promoted DDX3 expression, reduced Cyclin D1 level, and induced p21 expression, leading to cell cycle arrest, apoptotic cell death and growth inhibition. These data suggested that DDX3 could act as a tumor suppressor in hepatocellular carcinoma.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Yu Cheng, Department of Medical Oncology, Yantai Yuhuangding Hospital, No. 20 Yuhuangding East Road, Yantai, Shandong, China. E-mail: yuchengyantai@126.com

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