DJ-1 knockdown inhibits growth and xenograft-induced tumor generation of human hepatocellular carcinoma cells

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Abstract. The aim of this study was to identify potential downstream effectors of the oncogene DJ-1 in hepatocellular carcinoma (HCC) cell lines, and examine its role in the Akt signaling pathway and HCC oncogenesis. Expression of the DJ-1 protein was assessed by immunoblotting in several human HCC cell lines. Knockdown of DJ-1 was achieved by transfecting DJ-1-specific short hairpin RNAs into the HepG2 HCC cell line. The effect of DJ-1 downregulation on phosphatase and tensin homolog (PTEN) and phosphorylated Akt was evaluated. In addition, cell cycle, proliferation, adhesion and invasion were analyzed in the DJ-1 knockdown of HepG2 cells. The growth of HepG2-induced tumor was evaluated in a nude mouse model after DJ-1 silencing. Stable DJ-1 knockdown was achieved in HepG2 cells using a shRNA eukaryotic expression vector. Downregulation of DJ-1 increased PTEN expression but decreased phosphorylation of Akt in HepG2 cells. In addition, DJ-1 knockdown resulted in the decreased proliferation, adhesion and invasion of HepG2 cells *in vitro*, and inhibited the growth of HepG2-induced tumor *in vivo*. DJ-1 knockdown altered the malignant behavior of HepG2 cells, potentially through the Akt signaling pathway, suggesting a crucial role for DJ-1 in the oncogenesis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and causes approximately one million deaths each year (1-3). The incidence of HCC in the USA has increased in the past two decades, possibly due to increased incidence in cirrhosis and longstanding hepatitis C infection (1). HCC is considered a major health problem due to endemic hepatitis B and C and regional exposure to environmental pathogens (3). HCC is invasive and resistance to chemotherapeutic agents limits treatment options. At present, the only curative treatment is surgery (4). Surgical resection offers the best prognosis when the tumor is <5 cm in diameter and limited to one lobe of the liver, has not invaded the liver vasculature, and liver function is well preserved. However, long-term survival is rare, mainly due to the recurrence and metastasis of HCC (5). Identification of target genes associated with the progression of HCC and improved understanding of the early molecular events in tumorigenesis may lead to improvements in diagnostic efficiency and aid in the development of new therapeutic strategies for HCC.

Apoptosis is a genetically determined process of controlled cellular suicide (6). Deregulation of apoptosis is involved in the pathophysiology of liver disease including hepatocarcinogenesis (7). HCC cell resistance to apoptosis impairs the efficacy of HCC therapeutic regimens (8). The oncogene DJ-1 encodes a ubiquitously expressed 189-amino acid integral mitochondrial protein reported to confer resistance to apoptosis (9-13). DJ-1 has been reported to regulate cell death, and appears to play a role in tumorigenesis, invasion and metastasis in breast cancer, HCC, non-small cell lung carcinoma and prostate cancer (9-17). The oncogenic effect of DJ-1 is mainly attributed to its anti-apoptotic ability. A number of DJ-1 downstream effectors, such as PTEN, Akt, IKK and NF-κB, are involved in regulating the progression and invasion of tumors (16,18).

In the present study, we aimed to investigate the role of DJ-1 in HCC tumorigenesis. HepG2 cells provide an *in vitro* model system for the study of polarized human hepatocytes, exhibiting robust morphological and functional differentiation with apical and basolateral cell surface domains. Selective shRNA expression vector-mediated RNAi is an effective method for suppressing oncogene products *in vitro* (19). Therefore, shRNA can be therapeutically used for the inhibition of aberrant mRNA expression in cancer cells (20).

Using a shRNA expression vector, stable DJ-1 knockdown of HepG2 cells was obtained. We found that DJ-1 downregulation resulted in the growth inhibition of HepG2 cells *in vitro*

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and *in vivo*. Increased PTEN expression and decreased Akt phosphorylation were detected in DJ-1 knockdown HepG2 cells.

These findings suggested that DJ-1 knockdown altered the malignant behavior of HepG2 cells, potentially through Akt signaling, indicating a crucial role for DJ-1 in the oncogenesis of HCC.

Materials and methods

Cell culture. The human HepG2, SMMC-7721 and Huh-7 HCC cell lines were obtained from the China Center for Type Culture Collection (Wuhan University, Wuhan, China). Human metastatic MHCC-97L and MHCC-97H HCC cell lines were obtained from the Liver Cancer Institute of Zhongshan Hospital (Fudan University, Shanghai, China) (21). Cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) high-glucose medium containing 10% calf serum (both from Gibco-Life Technologies, Carlsbad, CA, USA) and 1% penicillin and streptomycin.

Construction of DJ-1 shRNA expression plasmid. A candidate DJ-1 cDNA sequence (GenBank NM_007262: AGTGTAGCC GTGATGTGGT) was selected for RNA interference (RNAi). The oligonucleotide sequence used for DJ-1 silencing was *Bam*HII + sense + loop + antisense + terminator + *Sal*I + *Hind*II, with shRNA-DJ-1 (forward), 5'-CACCAGTGTAGC CGTGATGTGGT̲T̲T̲C̲A̲A̲G̲A̲C̲G̲ACCACATCACGGCT ACACTTTTTTTG-3' and (reverse), 5'-AGCTCAAAAAAA GTGTAGCCGTGATGTGGT̲C̲G̲T̲C̲T̲T̲G̲A̲A̲ACCACATCA CGGCTACACT-3'. The negative control sequences were: shRNA-HK (forward), 5'-CACCTTTT̲T̲T̲C̲A̲A̲G̲A̲C̲G̲G̲ATG AACTTCAGGGTCAGCTTTTTTG-3' and (reverse), 5'-AGC TCAAAAAAGCTGACCCTGAAGTTCATC̲C̲G̲T̲C̲T̲T̲G̲A̲ ̲A̲AAAA-3'. The underlined sequences denote the loop. An oligonucleotide targeting GAPDH served as a positive control while an irrelevant oligonucleotide served as a negative control. Recombinant plasmid synthesis and purification was performed by Wuhan Genesil Biotechnology Co., Ltd. (Wuhan, China).

Stable transfection of HepG2 with recombinant human DJ-1 shRNA plasmid. HepG2 cells at 60-80% confluency were transfected with constructed plasmids using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection 450 µg/ml G418 was added to the cell culture medium. The cells were cultured in medium containing G418 for 8 weeks to select stable transfectants. Individual clones were isolated and expanded. The green fluorescence expressed by stable transfectant cells was observed by fluorescence microscopy.

RNA extraction and semi-quantitative PCR. Total RNA was isolated using the TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, 48-72 h after transfection. Total RNA (2 µg) was used for semi-quantitative reverse transcription PCR on a Bio-Rad S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) (Promega, Madison, WI, USA). The primers used were: DJ-1, 5'-AGCAGAGGAAATGGAGACG-3' (forward), and 5'-GCCAACAGAGCAGTAGGAC-3' (reverse); internal control β-actin 5'-TCTACAATGAGCTGCGTGTG-3' (forward), and 5'-ATCTCCTTCTGCATCCTGTC-3' (reverse). PCR products (5 µl) were analyzed by electrophoresis on 1.2% agarose gels.

Immunoblotting. Cells were collected, washed with phosphate-buffered saline (PBS) three times, and lysed in cell lysis buffer containing RIPA (ProMab, Changsha, China) and protease inhibitor (Promega). The cell lysates were submitted to centrifugation at 12,000 x g for 30 min and the supernatants were collected as total protein, and quantified by the bicinchoninic acid (BCA) method. Protein (50 µg) was used for electrophoresis followed by transfer on PVDF membranes, and probed with rabbit polyclonal antibodies against human DJ-1 (Proteintech, China), PTEN (Epitomics, Inc., Burlingame, CA, USA) or rabbit monoclonal antibodies against human β-actin (Abzoom Biolabs, Dallas, TX, USA). Following incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibodies, signals were detected with an enhanced chemiluminescence (ECL) chromogenic substrate. Visualization and quantification were carried out on a digital image analyzer.

Flow cytometric analysis. A single-cell suspension of HepG2 cells in the logarithmic phase was seeded in new culture flasks and incubated for 48 h followed by fixation with 75% ethanol at 4°C overnight. The washed cells were resuspended in PBS containing RNase A, and incubated at 37°C for 30 min prior to the addition of 50 µg/ml propidium iodide (PI) for 30 min in the dark. After washing, the cells were analyzed using a flow cytometer (Chemunex, Vernon Hills, IL, USA).

Cell growth assay. Cell proliferation was assessed by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells in the logarithmic phase were seeded at a density of $1x10^2$/µl in triplicate in 96-well plates (100 µl/well). After 24, 48, 79 and 96 h, 50 µl MTT (5 mg/ml) were added to each well and incubated at 37°C. After 4 h, the supernatants were carefully aspirated and the purple crystals dissolved with 150 µl DMSO. Absorbance was measured at 570 nm on a microtiter plate reader.

Adhesion and invasion assays. Cellular adhesion to plastic and laminin- or fibronectin-coated surfaces was evaluated in 96-well plates. The plates were coated with 20 µl DMEM containing 2% BSA at 37°C for 1 h and rinsed three times with PBS. The cells ($1x10^4$) were incubated in the plate for 1 h and non-adherent cells were removed by washing with PBS. Cell viability was assessed by MTT (0.5 µg) as described above.

The invasive activity of cells was determined using transwell chambers. Matrigel (20 µl) (BD Biosciences, Bedford, MA, USA) at 500 µg/ml in cold PBS was spread on the inner side of the filter. The cells in the logarithmic phase were harvested and suspended at $1x10^5$/ml in DMEM. Then, 100 µl of cell suspension was added to the upper compartment and incubated at 37°C for 24 h. Filters were subsequently fixed with 10% neutral formaldehyde for 30 min and stained with crystal violet for 5 min. The cells on the upper side of the filter were removed with a cotton swab and any cells that had migrated through the Matrigel to reach the reverse side were counted under a microscope.
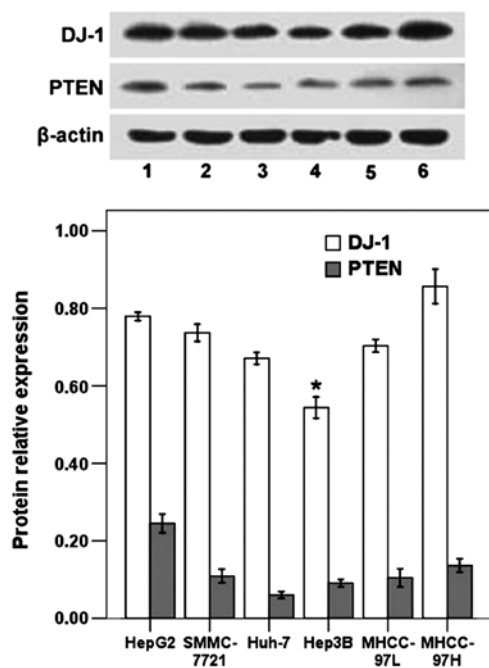
Figure 1. Expression levels of DJ-1 and PTEN in HCC cell lines. Extracts of HepG2, cell extracts from SMMC-7721, Huh-7, Hep3B, MHCC-97L and MHCC-97H were probed for DJ-1, PTEN and β-actin protein by immuno-blotting. Quantities of DJ-1 and PTEN proteins were normalized to β-actin. Values present the means ± standard deviation, to indicate a statistically significant result. *P<0.05 was considered to indicate a statistically significant result. PTEN, phosphatase and tensin homolog.
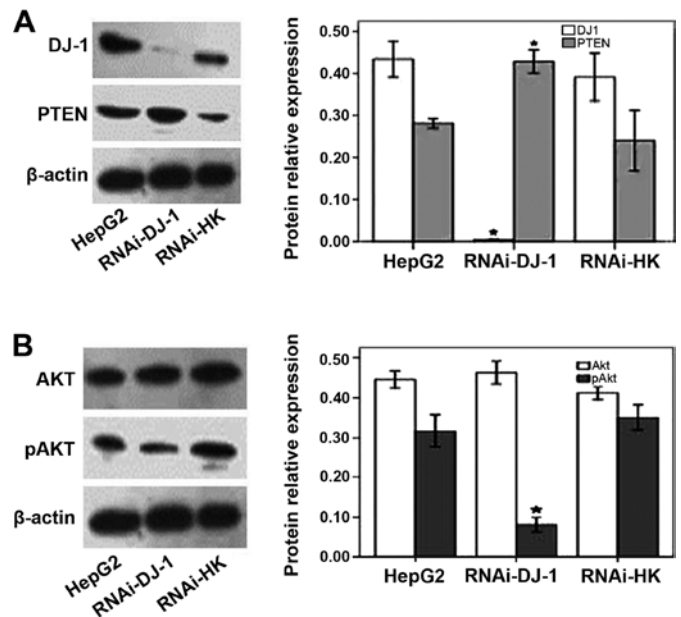

Figure 2. Effects of DJ-1 knockdown on PTEN and AKT in HepG2 cells. Cells were transfected with DJ-1 shRNA-expressing plasmids or control plasmid RNAi-HK for 8 weeks. The levels of the proteins in the control and shRNA cell lines were determined from whole cell extract using immuno-blot analysis. (A) DJ-1 and PTEN protein expression levels. (B) Total and phoshorylated Akt (pAkt) levels. Quantities of proteins were normalized to β-actin. Values present means the ± standard deviation, to indicate a statistically significant result. *P<0.05 was considered to indicate a statistically significant result. PTEN, phosphatase and tensin homolog.

Animal experiments. Six- to eight-week male BALB/c-nu mice were obtained from the Experimental Animal Center of the Huazhong University of Science and Technology (Wuhan, China). Animal experiments were conducted according to the guidelines of and approved by the Committee on Animals of the Huazhong University of Science and Technology. Animals were provided with food and water *ad libitum* and allowed one week adaptation. The mice were housed at 25±1°C under pathogen-free conditions with a 12-h light/dark cycle and 40-60% relative humidity. Single-cell suspensions (HepG2, RNAi-DJ-1 or RNAi-HK) were prepared and $5x10^6$ cells in 0.2 ml Hanks solution injected subcutaneously into mice. Tumor size was assessed every 4 days as $ab^2/2$, where a and b are the major and minor diameter, respectively. Four weeks after the first measurement, the mice were sacrificed.

Statistical analysis. The results were expressed as means ± standard deviation (SD). The SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform the analysis. One-way ANOVA was used for comparisons among different groups. P<0.05 was considered to indicate a statistically significant result.

Results

DJ-1 and PTEN expression in human HCC cell lines. The expression levels of DJ-1 and PTEN were assessed by immunoblot analysis in human HCC cell lines including HepG2, SMMC-7721, Huh-7, Hep3B, MHCC-97L and MHCC-97H. As shown in Fig. 1, DJ-1 expression varied significantly between cell lines. Significantly lower DJ-1 levels were

observed for Hep3B cells, which possess low metastatic potential, in comparison with the MHCC-97L cell line with low metastatic potential and the MHCC-97H cell line with high metastatic potential (P<0.05). The protein expression of DJ-1 in MHCC-97L cells was significantly lower than that in MHCC-97H cells (P<0.05; Fig. 1). The results suggested that DJ-1 expression may correlate with the metastatic potential of HCC cell lines. Concerning PTEN expression, no significant differences were obtained when these cell lines were compared (Fig. 1). HepG2 cells were selected to examine the high level of DJ-1 expression in this cell line. This cell line has been widely used for the *in vitro* and *in vivo* studies of treatment of HCC and the mechanism involved.

shRNA knockdown of DJ-1 expression. The DNA segment encoding a shRNA for DJ-1 was inserted into a pGenesil-1 plasmid expression vector containing the human U6 promoter, between the *Bam*HII and *Sal*II restriction sites. The shRNA expression was directly driven by the cytomegalovirus promoter. The correct inserts in the RNAi plasmids shRNA-DJ-1 and negative control shRNA-HK were confirmed with DNA sequencing (data not shown). The plasmids were transfected into HepG2 cells, with a transfection efficiency of ~70% after 48 h (data not shown). After 8 weeks of G418 selection stable sub-clones expressing green fluorescent protein were isolated and expanded for subsequent assays (data not shown).

RT-PCR analysis confirmed that the DJ-1 shRNA recombinant plasmid inhibited DJ-1 mRNA expression in HepG2 cells (data not shown). DJ-1 protein expression levels were reduced by 98.78% after silencing (Fig. 2A).
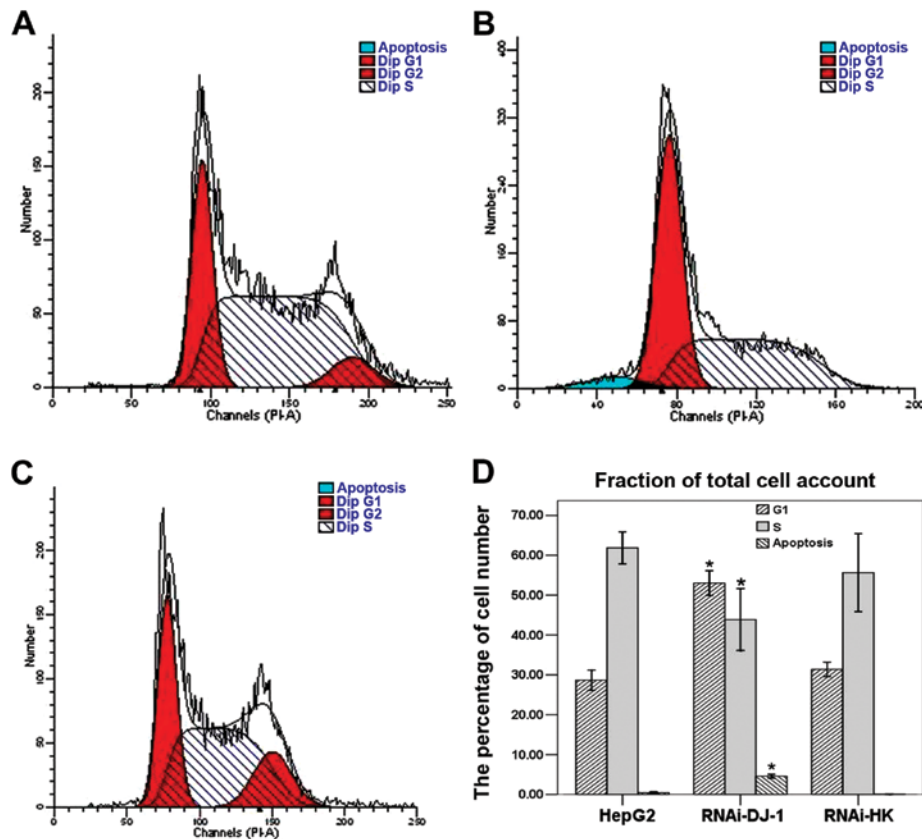
Figure 3. Impact of DJ-1 knockdown induced cell death. Cells were stained with propidium iodide staining and analyzed using flow cytometry. (A) The populations of cells in different cell cycle stages, as well as cells in apoptosis were estimated by measuring the area shown on the flow cytometry diagram. Compared to untreated cells, the (C) RNAi-HK-transfected cells showed similar cell population distribution. However, an increased number of cells with fewer DNA contents (indicating apoptosis) were observed in (B) RNAi-DJ-1 cells. The data were quantified as shown in (D). Results present the means ± standard deviation of three independent experiments, to indicate a statistically significant result. *$P<0.05$ was considered to indicate a statistically significant result. PTEN, phosphatase and tensin homolog.

DJ-1 shRNA inhibited Akt phosphorylation in HepG2 cells. In cells treated with RNAi-DJ-1, PTEN protein levels increased, compared to the untreated cells or cells transfected with the control plasmid RNAi-HK ($P<0.05$; Fig. 2A). Akt phosphorylation (pAkt) levels were significantly reduced in the RNAi-DJ-1-treated cells compared to the control cells ($p<0.05$; Fig. 2B). By contrast, total Akt levels were not significantly altered by RNAi-DJ-1 ($P>0.05$; Fig. 2B). These data indicated that the downregulation of DJ-1 resulted in an increase of PTEN protein levels as well as the inhibition of Akt phosphorylation.

DJ-1 shRNA suppressed the proliferation, adhesion and invasion of HepG2 cells. PI-stained cells were analyzed using flow cytometry. In RNAi-DJ-1-transfected cells, we found a significant decrease in cell populations with diploid DNA as well as an accumulation of apoptotic cells ($P<0.05$; Fig. 3). These findings suggested that DJ-1 knockdown arrested the cell cycle and induced apoptosis.

Using MTT assay (Fig. 4A), we detected that the proliferation of RNAi-DJ-1-transfected cells was significantly reduced in comparison with untreated cells and cells transfected with the control plasmid ($P>0.05$; Fig. 4A).

Cell adhesion and invasion rates were also significantly reduced in DJ-1 knockdown cells: binding to fibronectin and laminin was reduced by 56.07 and 60.73% in RNAi-DJ-1-

transfected cells, respectively ($P<0.05$; Fig. 4B). Compared to untreated cells, there were 53.34% fewer RNAi-DJ-1 cells that migrated through a transwell filter after 24 h ($P<0.05$; Fig. 4C).

RNAi-DJ-1 suppression of xenograft tumor formation in nude mice. Thirty nude mice received subcutaneous injections of normal HepG2 cells with or without transfected shRNA plasmid. As shown in Fig. 5, the xenograft of untreated HepG2 induced tumor growth in transplanted animals in a time-dependent manner. The RNAi-HK-transfected cells, when transplanted, induced similar level of tumor growth in SD mice. However, tumor growth in the RNAi-DJ-1 group was significantly reduced as compared to that of normal HepG2 cells and cells transfected with RNAi-HK ($P<0.05$). These data indicated that inhibition of DJ-1 by shRNA inhibited the *in vivo* growth of HepG2 cells in the xenograft transplant.

Discussion

Results of the present study have shown that DJ-1 was expressed in a variety of human HCC cell lines including HepG2, SMMC-7721, Huh-7, Hep3B, MHCC-97L and MHCC-97H, and that DJ-1 expression correlated positively with the metastatic potential of HCC cell lines (22). However, Zhang *et al* recently described DJ-1 downregulation in the early stages of HBV-infected HCC, suggesting an abnormal
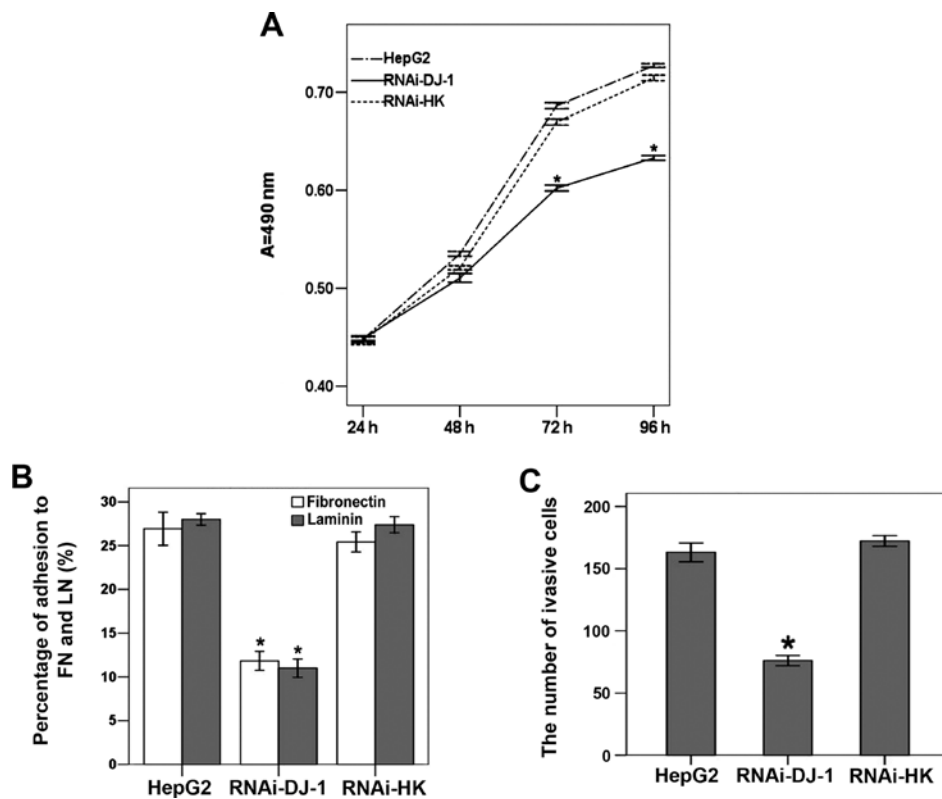
Figure 4. Impact of DJ-1 shRNA on HepG2 cell growth, adhesion and invasion. The abilities of cell growth (MTT assay), adhesion and invasion were tested in RNAi-DJ-1 cell lines as well as in control cells as described in Materials and methods. (A) Growth curves of transfectant HepG2 cells. (B) Transfectant HepG2 adhesion to fibronectin and laminin. (C) Invasion of transfectant HepG2 through transwell filters. Data represent means ± standard deviation obtained from three independent experiments, to indicate a statistically significant result. *$P<0.05$ was considered to indicate a statistically significant result. PTEN, phosphatase and tensin homolog.
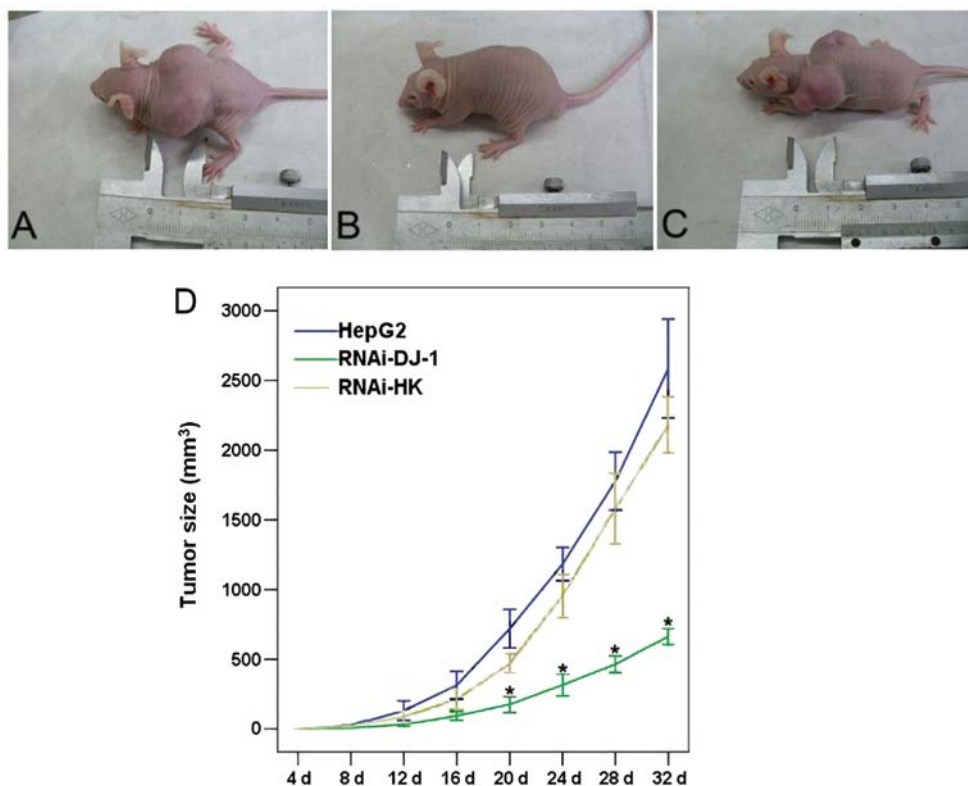

Figure 5. Effects of DJ-1 shRNA on tumor formation *in vivo*. HepG2 and HepG2 transfected with RNAi-DJ-1 and RNAi-HK were inoculated subcutaneously into nude mice. Typical images of tumor formation in nude mice of the (A) HepG2, (B) RNAi-DJ-1, (C) RNAi-HK groups. (D) Tumor growth was monitored and tumor volumes calculated. Results present the means ± standard deviation obtained from three independent experiments, to indicate a statistically significant result. *$P<0.05$ was considered to indicate a statistically significant result. PTEN, phosphatase and tensin homolog.

pathophysiological state of the mitochondria (17). Authors of that study suggested that mitochondrial disease occurs before tumorigenesis in HBV-infected HCC (17). The conflicting findings on DJ-1 levels may reflect the differences between tumorigenesis in HBV-infected primary cells and HCC cell lines (17). Nevertheless, the observations strongly suggest that DJ-1 is involved in HCC, although its precise role in tumorigenesis remains to be determined.

The effects of DJ-1 on cell proliferation and apoptosis have been reported in other studies. For examples, cells harboring DJ-1 are more resistant to UV-induced apoptosis than DJ-1 knockdown cells (9). Overexpression of DJ-1 in a prostatic benign hyperplasia cell line (BPH-1) or prostate carcinoma cells (PC-3) results in resistance to the apoptosis normally induced by cytotoxic agents (10). By contrast, knockdown of DJ-1 enhances apoptosis of prostate carcinoma cells (PC-3) when treated with the same toxic reagents (11,12) as well as TNF-related apoptosis-inducing ligand/Apo-2L (TRAIL)-mediated apoptosis (13). Overexpression of DJ-1 blocks neuronal apoptosis by inhibiting the transcriptional silencing activity of the pyrimidine tract-binding protein-associated splicing factor, or by preventing the translocation of Daxx from the nucleus to the cytoplasm, where Daxx activates the apoptotic signal-regulating kinase-1 death pathway (10). In the present study, we employed RNAi technology to construct a shRNA plasmid to silence DJ-1 mRNA and demonstrated DJ-1 expression was markedly reduced in HepG2 cells after knockdown. Notably, DJ-1 silencing resulted in decreased proliferation, adhesion and invasion of HepG2 cells *in vitro* and inhibited tumor formation in nude mice. These results are consistent with a previous study (11,12) suggesting DJ-1 in HepG2 cells plays a similar role in inhibiting apoptosis and promoting cell proliferation, adhesion and invasion.

DJ-1 was identified as a suppressor of the PTEN function through a genetic screening in *Drosophila* for gain-of-function mutants (16). PTEN negatively regulates the Akt signaling pathway through the dephosphorylation of PIP3 (23,24). Activation of Akt is involved in the regulation of cell proliferation, apoptosis and migration (25).

We found that DJ-1 knockdown in HepG2 cells resulted in the upregulation of PTEN and decreased Akt phosphorylation. These results suggest that the PI3P/Akt is the potential signaling pathway in which DJ-1 functions.

In summary, the downregulation of DJ-1 increased PTEN expression, and decreased phosphorylation of Akt in HepG2 cells. In addition, DJ-1 knockdown resulted in decreased proliferation, adhesion and invasion of HepG2 cells *in vitro*, and inhibited the growth of HepG2-induced tumor *in vivo*. These findings suggest that DJ-1 plays a crucial role in the oncogenesis of HCC, thereby providing a potential target for drug development for HCC.

Acknowledgements

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