MPHOSPH1: a potential therapeutic target for hepatocellular carcinoma

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Running title: MPHOSPH1 as a drug target in HCC therapy.
Abstract

MPHOSPH1 is a critical kinesin protein that functions in cytokinesis. Here we show that MPHOSPH1 is overexpressed in hepatocellular carcinoma (HCC) cells where it is essential for proliferation. Attenuating MPHOSPH1 expression with a tumor-selective shRNA-expressing adenovirus (Ad-shMPP1) was sufficient to arrest HCC cell proliferation, in a manner associated with an accumulation of multinucleated polyploid cells, induction of post-mitotic apoptosis and increased sensitivity to taxol cytotoxicity. Mechanistic investigations showed that attenuation of MPHOSPH1 stabilized p53, blocked STAT3 phosphorylation and prolonged mitotic arrest. In a mouse subcutaneous xenograft model of HCC, tumoral injection of Ad-shMPP1 inhibited MPHOSPH1 expression and tumor growth in a manner correlated with induction of apoptosis. Combining Ad-shMPP1 injection with taxol administration enhanced antitumor efficacy relative to taxol alone. Further, Ad-shMPP1 tail vein injection suppressed formation of orthotopic liver nodules and prevented hepatic dysfunction. Taken together, our results identify MPHOSPH1 as an oncogenic driver and candidate therapeutic target in HCC.

Key words: hepatocellular carcinoma, gene therapy, oncolytic vector, M-phase phosphoprotein 1

Precis: Results highlight a critical role for a mitotic kinesin as a critical oncogenic driver and candidate therapeutic target in liver cancer.
Introduction

Kinesin superfamily (KIF) proteins are a group of proteins with a highly conserved motor domain, some of which move toward the plus end of microtubules in an ATP dependent process reliant on their adenosine triphosphatase (ATPase) activity (1). KIF proteins participate in many essential cellular biological activities including mitosis, meiosis and the transport of macromolecules (2). Recently, there has been increasing evidence of aberrant expression of KIF proteins in a variety of cancers, suggesting the oncogenic potential of KIFs in addition to their normal cellular physiological functions (3, 4). Among these KIFs, M-phase phosphoprotein 1 (MPHOSPH1, also referred to as KIF20B) was reported to be a plus-end-directed kinesin protein, playing a critical role in completion of cytokinesis in late telophase of mitosis (5). Recently, an oncogenic role for MPHOSPH1 in bladder and colorectal cancer cells was reported (4, 6).

Hepatocellular carcinoma (HCC) accounts for 90% of human primary liver cancers (PLC), which is the fifth most common cancer and the third most common cause of cancer-related death, particularly in East Asia. Advanced HCC often has a poor prognosis and only few chemotherapeutic drugs, such as sorafenib, demonstrate efficacy in increasing overall survival in advanced or metastatic HCC (7). However, due to the rapid development of drug resistance, the effective course of chemotherapy for HCC often lasts for only a few months (8). Resistance to chemotherapeutic agents therefore remains a major challenge to HCC therapy. Recently, KIF proteins have been shown to play critical roles in the development of
resistance to anti-mitotic drugs, such as taxanes (7, 9). Combined chemotherapy targeting kinesins may thus represent a promising anticancer strategy, especially in drug-resistant solid tumors.

Another challenge in treating HCC is reduced hepatic function. Poor hepatic function is common in HCC, resulting from a variety of causes, such as hepatic ascites and malnutrition. In many cases, hepatic dysfunction results in a bad prognosis for HCC patients. Therefore, maintaining hepatic function is critical for HCC therapy. For this reason, improving liver function of HCC patient is usually initiated before carrying out further treatment, especially surgery. Consequently, due to the cytotoxicity of chemotherapy, an improved, combined strategy that prevents hepatic injury is urgently needed.

In the present study, we found that MPHOSPH1 expression is increased in HCC tissues compared with non-tumor tissues, and that knockdown of endogenous MPHOSPH1 using an adenoviral vector inhibited HCC cell proliferation by triggering mitotic arrest and apoptosis. We also demonstrated that combining taxol treatment with MPHOSPH1 interference resulted in synergistic repression of HCC cell viability. Additionally, we demonstrated that Ad-shMPP1 treatment not only suppressed HCC development, but also significantly reduced hepatic dysfunction caused by orthotopic HCC xenografts, suggesting that use of Ad-shMPP1 produced a dual effect; anti-HCC activity and protection of liver function in vivo.
Materials and Methods

Cell lines, animals and tissue specimens

The human liver cell lines L02 and QSG7701 and the human hepatocellular carcinoma cell lines Hep3B, HepG2 and BEL-7404 were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) or China Center for Type Culture Collection (CCTCC, Wuhan, China) within 6 months of the experiments carried out. All cell lines were authenticated by STR profiling and were confirmed to be mycoplasma-negative before use. Cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 10% FBS. The human samples including 4 non-malignant liver diseases (cirrhosis, choledocholithiasis and cavernous hemangioma) and 14 HCC and 6 adjacent tissues were obtained from Wuhan Union Hospital, Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). The Institutional Review Board of Wuhan Union Hospital approved acquisition of tissue specimens and collection of human samples was in accordance with the established guidelines.

Immunohistochemistry assays

Clinical human tissues or the HCC xenograft samples were fixed in 4% formaldehyde, dehydrated with an ethanol gradient and embedded in paraffin. Tissue sections were dewaxed and rehydrated according to a standard protocol. The sections were washed with PBS, treated with 3% H₂O₂, and blocked with a blocking solution. These steps were followed by overnight
incubation with primary antibody at the proper dilution for IHC or hematoxylin - eosin (H&E) staining assays as previously described (10). To avoid experimental bias, the clinical human samples were scored from 0 to 3, based on the IHC staining of MPHOSPH1, by two people using a double-blind procedure.

Recombinant adenovirus

Recombinant adenoviruses (rec-Ads) were generated by respective homologous recombination between the shuttle plasmids and the packaging plasmid pBHGE3 in HEK293 cells. Generation, identification, production, purification and titration of the recombinant adenovirus were performed as previously described (6).

RNA extraction and quantitative reverse transcriptase PCR

Total RNA was isolated from cells using Trizol (Invitrogen). A total of 2 μg of RNA was used to synthesize the first single-strand cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). The relative quantification of MPHOSPH1 cDNA and adenovirus gene E3 cDNA was performed using an ABI 7500 Fast System or a Bio-Rad CFX96™ Real-Time PCR Detection System with Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). GAPDH cDNA was used as an internal standard. The primers used are provided in Supplementary Materials.

Cytotoxicity assay
HCC cells and normal cells were seeded in 24-well plates at a density of 20,000 cells per well. 24 hours later, cells were infected with Ads at various MOIs (multiplicity of infection). Four days after infection, the medium was removed, and the cells were exposed to 2% crystal violet in 20% methanol for 20 min. The plates were then washed with water and photographed.

**Cell proliferation assays**

Hep3B and HepG2 cells were seeded in 96-well plates at a density of 2,000 cells per well. For the combined treatment (Ads and taxol), cells were infected with Ads at an MOI of 2. 24 hours after infection, taxol (Sigma-Aldrich, St. Louis, MO) at a final concentration of 10 μg/ml was added to each well. 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well at 48 hours later, and the plates were incubated at 37 ºC for 4 hours. The medium was removed and 150 μl of dimethyl sulfoxide (DMSO) was added to each well. Absorbance at 490 nm was measured using a Microplate Reader (Thermo, Asheville, NC). 6 replicate wells were counted for each assay.

**In vitro combination studies**

Synergism was determined using the software package Calcusyn (Biosoft, Cambridge, United Kingdom). The combination index (CI) was calculated by the Chou-Talalay equation (11). A combination index (CI) less than 0.9 was defined as synergism. The detailed description on the combination is provided in the Supplementary Materials.
Cell nuclei and cytoskeleton staining assay

Cells were seeded on glass cover slips in six-well plates. 24 hours later, cells were infected with Ads at an MOI of 2. Cells were then fixed with 0.5 ml of 4% paraformaldehyde for 10 minutes, washed twice with PBS, stained with Phalloidin Alexa Fluor 555 (Molecular Probes, Eugene, OR), washed twice with PBS, stained with DAPI (Sigma-Aldrich, St. Louis, MO) and washed twice with PBS. Cell staining was analyzed using a BX51 upright microscope (Olympus, Tokyo, Japan) or a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

DNA fragmentation assays

HepG2 cells were seeded in 10 cm culture dishes and infected with various Ads at MOI=5. 72 hours post infection, DNA was extracted and analyzed using agarose gel (12).

Flow cytometric analyses

Cells fixed with 70% ethanol were incubated for 2 hours with an RNase A (40 μg/ml; Promega)/propidium iodide solution (50 μg/ml; Sigma-Aldrich). Fluorescence-activated cell sorting (FACS) was performed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

SA-β-gal staining assay
β-Galactosidase staining was performed using a senescence-associated β-Galactosidase Staining Kit (Beyotime, Shanghai, China). Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Next, the cells were incubated overnight at 37°C in the dark with a solution containing 0.05 mg/ml X-gal.

**Western blot assays**

Cells were harvested and washed with PBS, and then lysed for analysis of proteins. Western blot analysis was performed as previously reported (6). Detailed antibody information is provided in the Supplementary Materials.

**Animal experiments**

Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals set forth by the Huazhong University of Science and Technology. Male BALB/c nude mice (4-week old) were purchased from the Beijing HFK Bioscience Co. Ltd. (Beijing, China). Details of the experiments are provided in the Supplementary Materials.

**Statistical analyses**

All data were expressed as the mean ± SD and were analyzed using independent sample t-tests and one-way analyses of variance (ANOVA) using SPSS Base 10.0. Results were considered statistically significant when $p < 0.05$. 
Results

Overexpression of MPHOSPH1 in HCC tissues

MPHOSPH1 was previously classified as an oncogene based on studies of bladder cancer as well as in colorectal and lung cancer cell lines (4, 6). To determine whether MPHOSPH1 is upregulated in HCC, IHC detection of MPHOSPH1 was performed in samples from 19 HCC patients (including 4 samples of normal adjacent tissues) and 10 non-malignant tissues (Table S1 & Fig. 1A). Two people scored each IHC assay of all samples on a 0-3 point scale based on the MPHOSPH1 staining ranges and color degrees as shown in Table S1 & Fig. 1B (Fig. 1B shows examples of MPHOSPH1 scoring 0-3). An IHC score of $\geq 2$ is considered overexpression of MPHOSPH1. Approximately 84% (16 of 19) of HCC tissues demonstrated overexpressed MPHOSPH1, while, in contrast, only 14% (3 of 14) of non-malignant liver tissues showed overexpression of MPHOSPH1 (Table S1). Further statistical analysis showed a significant difference in the MPHOSPH1 expression level between HCC tissues and non-HCC tissues (Fig. 1C). In addition, although the HCC samples exhibited a range of Edmondson-Steiner grading (an index reflecting the differentiation degrees of HCC cells), we found no statistically significant correlation between the differentiation state of the HCC cells and the MPHOSPH1 expression level (Table S1).

The MPHOSPH1 mRNA levels in three HCC cell lines were also compared by qRT-PCR. The HCC cell lines HepG2, Hep3B and BEL-7404 all showed elevated mRNA levels of
MPHOSPH1 compared to that of the normal liver cell line L02 (Fig. 1D), suggesting that both MPHOSPH1 protein and mRNA levels may be used as potential biomarkers for HCC.

**HCC-specific replication and MPHOSPH1 knockdown effects of the oncolytic recombinant adenovirus**

We previously constructed a recombinant adenoviral vector based on the oncolytic Ad in which the viral E1B55KD gene was deleted (6). This Ad-vector has a tumor-specific survivin promoter controlling the expression of the adenoviral early gene E1A (Fig. 2A), thus restricting replication to tumor cells (6). To verify its ability to replicate in HCC cells, we infected HCC and normal liver cells with the Ad-vector at an MOI=0.1. The ability to replicate was evaluated by assessing the adenoviral E3 gene mRNA level. Ad-vector replication in HepG2 cells was at least 100-fold higher than in the normal QSG7701 cells at 48 hours after infection (Fig. 2B) suggesting that it is a potent HCC-targeting oncolytic virus.

To construct small hairpin RNA (shRNA) expressing Ad, an MPHOSPH1-targeted shRNA (shMPP1) expression plasmid was used (6). We cloned the shMPP1 expression cassette into the oncolytic vector to generate Ad-shMPP1 (Fig. 2A). As expected, this tumor-specific oncolytic Ad-shMPP1 displayed potent interference ability against endogenous MPHOSPH1 expression in HCC cells. The relative mRNA level of MPHOSPH1 in HepG2 and Hep3B cells was significantly reduced following Ad-shMPP1 infection (95% and 60%...
downregulation at an MOI of 2 respectively; Fig. 2C).

**Ad-shMPP1 inhibits HCC cell growth and potentiates the efficacy of taxol**

To investigate the cytopathic effect (CPE) and the potential anti-HCC activity induced by Ad-shMPP1, different liver cancer cell lines and a normal liver cell line were infected with Ad-vector and Ad-shMPP1 at various MOIs. 96 hours later, CPEs were determined by crystal violet staining. As shown in Figure 3A, in normal liver L02 cells, infection with Ad-shMPP1 at an MOI of 5 did not cause a CPE, while in HepG2, Hep3B and BEL-7404 cells, treatment at the same MOI (in HepG2 cells, even at a lower MOI of 2, Fig. 3A) caused obvious CPE, demonstrating HCC cell-selective cytotoxicity and the relative safety of Ad-shMPP1.

Some KIF proteins seem to be involved in the development of drug resistance by HCC to some microtubule-targeted agents, such as taxol (13). Thus, to further clarify whether Ad-shMPP1 could enhance the anti-HCC effects of taxol, 24 hours before taxol treatment, we infected HepG2 and Hep3B cells with Ad-shMPP1 at an MOI of 2, and 48 hours later the cell viability was determined using an MTT assay. Ad-shMPP1 produced a significant cell growth arrest effect on HepG2 and Hep3B cells compared with Ad-vector control groups (Fig. 3B). More importantly, compared with the Ad or taxol mono-treatment group, the combined treatment amplified the anti-HCC effect ($p < 0.01$), indicating significantly enhanced cytotoxicity of taxol (Fig. 3B). To further investigate whether the combination of
Ad-shMPP1 and taxol act synergistically, the combination index (CI) value of Ad-shMPP1/taxol was calculated both in HepG2 and Hep3B cells using CalcuSyn software, yielding CI values of 0.538 and 0.705, respectively (Fig. 3C), suggesting that an increased anti-tumor effect of taxol was achieved by its combination with Ad-shMPP1 treatment.

**Ad-shMPP1 knockdown induces apoptosis, polyploidy and senescence in HCC cells**

To investigate whether apoptosis is promoted during the course of HCC cells growth arrest induced by MPHOSPH1 downregulation, we infected HepG2 cells with Ad-vector and Ad-shMPP1 at MOI=2, and performed Hoechst staining 48 hours after infection. Nuclear fragmentation and chromatin condensation, which are typical apoptotic features, were clearly observed in the Ad-shMPP1 infected cells (Fig. 4A). Apoptotic DNA fragmentation, a key feature of apoptosis, was also induced by Ad-shMPP1 infection in HepG2 cells (Fig. 4B).

Another obvious morphologic alteration following Ad-shMPP1 infection is the appearance of multinuclear cells due to the mitotic slippage from cell cycle arrest without the formation of two daughter cells by cytokinesis discussed previously (6). In the current study, 24 hours after infection with Ad-shMPP1 at a relatively low MOI (MOI=1), multinuclear cells were clearly observed by confocal microscopy (Fig. 4C). FACS analysis demonstrated accumulation of polyploid cells 24 hours after infection (>4N DNA content), which represents part of the multinucleate cells that were induced by Ad-shMPP1 (Fig. 4D). In agreement with our previous work, these results suggest that at an early stage of infection,
prolonged mitotic arrest is induced due to failure of cytokinesis caused by reducing MPHOSPH1.

Moreover, 48 hours after infection with Ad-shMPP1 at an even lower MOI (MOI=0.5), cellular senescence, rather than apoptosis, was accompanied by the growth arrest of HCC cells, as shown by altered cell shape and expression of pH-dependent β-galactosidase activity (Fig. 4E). Taken together, these results suggest that loss of MPHOSPH1 has a cytostatic effect on HCC cells probably through triggering mitotic arrest, and subsequent senescence or apoptosis depending on the MOI.

The p53 signal pathway inhibits proliferation of HCC cells after MPHOSPH1 knockdown

To study the mechanism underlying the MPHOSPH1 downregulation-induced HCC cell growth arrest, we analyzed the proteins involved in the process of apoptosis on western blots. The tumor suppressor p53, which is normal in HepG2 cells (14), was significantly elevated both in protein and mRNA levels when MPHOSPH1 was reduced (Fig. 5A & E). Phosphorylation of Signal Transducers and Activators of Transcription 3 (STAT3), down-stream of p53, was suppressed in HepG2 cells after infection with Ad-shMPP1, while the base expression level of STAT3 was unaltered in comparison with the control (Fig. 5A, B & C), suggesting that STAT3 signal transduction was blocked in the process, since STAT3 is activated by phosphorylation (15). Expression of the G1 phase cyclin-D1, a critical
oncoprotein in several cancers as the driver for entering the cell cycle (16), was significantly reduced by the downregulation of MPHOSPH1 (Fig. 5A), suggesting a potential mechanism for mitotic arrest. Intriguingly, although infection with an Ad vector upregulates cyclin-B1 (Fig. 5A), the expression level of this M-phase cyclin was independent of MPHOSPH1 expression (Fig. 5A). In addition, Mad2, a critical spindle assembly checkpoint (SAC) promoting protein (17), was increased following Ad-shMPP1 infection (Fig. 5A), which suggested that mitotic arrest was induced by SAC. However, the expression of the mitotic inhibitor p27, which is also downstream of p53 and represses cell cycle progression, was downregulated by MPHOSPH1 knockdown (Fig. 5A); this contradictory result indicates a complex molecular mechanism in the mitotic arrest process induced by reducing MPHOSPH1.

Additionally, p-STAT1, which is induced by IFN-α and is a part of the ISGF3 transcription factor complex (18), was significantly upregulated after Ad-vector and Ad-shMPP1 treatment (Fig. 5B & C), suggesting that an interferon effect is triggered by Ad infection, which may explain why the Ad-vector also induces some cytotoxicity (Fig. 3A & B) since the interferon effect is capable of enhancing the antitumor effect of the oncolytic Ad (19). Interestingly, Ad-shMPP1 slightly downregulated the total expression level of STAT1 compared with Ad-vector (Fig. 5B & C), which shows a dual-effect of Ad-shMPP1 on the cells. First, it reduces the total level of STAT1, presumably due to reducing MPHOSPH1 expression; and secondly, as an oncolytic Ad, it significantly induces the phosphorylation of
STAT1. This result indicates that infection with Ad-shMPP1 initiates a complicated process.

Other proteins involved in the combined treatment with Ad-shMPP1 and taxol were also analyzed by western blotting. KIF inhibitors have been reported to induce the anti-apoptotic heat-shock protein 70 (Hsp70) in myeloma cells (20), potentially revealing an underlying mechanism by which cancer cells resist the KIF-targeted drugs through the anti-apoptotic pathway. In the present study, we did not detect upregulation of Hsp70 following infection of HCC cells with Ad-shMPP1 (Fig. 5D), which suggests that Hsp70 may be not involved in the taxol resistance of HCC cells. In addition, cyclin-D1 was downregulated, not only in Ad-shMPP1 infected HepG2 cells, but also after the combined treatment with taxol to an even greater extent (Fig. 5D), suggesting a critical role for mitotic arrest in the affect of the Ad-shMPP1/taxol combined treatment. Additionally, the expression of CDKI (cyclin-dependent kinase inhibitor) p15, which is also an anti-mitotic protein, was not affected by MPHOSPH1 knockdown (Fig. 5D), suggesting that the CDKI pathway may not be involved.

In order to minimize the interference of the Ad system (Fig. 5A & B), we also used a plasmid containing the shMPP1 cassette (p-shMPP1) to downregulate MPHOSPH1 in HCC cells (6). MPHOSPH1 was significantly downregulated by p-shMPP1 transfection in HepG2 cells (Fig. 5F), and, consistent with the Ad results (Fig. 5A), MPHOSPH1 knockdown significantly induced p53 expression while reducing the expression of p27, p-STAT3 and
cyclin-D1 (Fig. 5F). The MTT assay also demonstrated that the downregulation of MPHOSPH1 by p-shMP1 significantly attenuated HCC cell proliferation (Fig. 5G), in agreement with the MTT results from the Ad infection (Fig. 3B).

**Knockdown of endogenous MPHOSPH1 inhibits HCC xenografts growth in vivo**

To evaluate the efficacy of an MPHOSPH1-targeted strategy against HCC, a subcutaneous HepG2 cell xenograft model was used. Intratumoral injections of $2 \times 10^9$ plaque-forming unit (pfu) Ads or taxol were carried out after the mean tumor volume reached 80-120 mm$^3$. As shown in Fig. 6A, at the end of a 6-week experiment, the Ad-shMP1 treated animals demonstrated significant suppression of tumor growth with an average tumor volume of about 250 mm$^3$, while the average tumor volumes of PBS or Ad-vector injected animals were 960 mm$^3$ and 770 mm$^3$ respectively, indicating approximately 74% suppression of the xenograft tumor growth by Ad-shMP1 ($p < 0.05$). The efficacy of combined treatment was also investigated. The combined treatment with Ad-shMP1 and taxol demonstrated enhanced anti-HCC activity, with the mean tumor volumes of 180 mm$^3$, while the groups of taxol and taxol/Ad-vector were 790 and 700 mm$^3$ respectively in contrast ($p < 0.05$, Fig. 6B), indicating the potent anti-HCC activity of the MPHOSPH1 knockdown in combination with taxol treatment.

To study whether MPHOSPH1 knockdown also triggers apoptosis in HCC cells *in vivo*, pathological examination was performed 7 days after the injection of Ads. IHC indicated
significant reduction of the endogenous MPHOSPH1 in HCC cells induced by Ad-shMMP1 (Fig. 6C), demonstrating its ability to downregulate MPHOSPH1 \textit{in vivo}. Furthermore, hematoxylin-eosin (H&E) staining showed large areas of nuclear fragmentation in HCC cells from Ad-shMPP1 treated xenografts, while few apoptotic cells were detected in the control groups (Fig. 6C). All these results suggest that Ad-shMPP1 may exert anti-HCC effects \textit{in vivo} by interfering with the expression of endogenous MPHOSPH1 and inducing apoptosis in HCC tissues.

Furthermore, an orthotopic xenograft model was also used to better evaluate the anti-HCC efficacy of Ad-shMPP1 \textit{in vivo} through i.v injection. At 7 days after intrahepatic implantation of HepG2 cells, a total of $2 \times 10^9$ pfu of Ads were injected through the tail vein. Three weeks after the injection, only 33.3% (2 of 6) of the Ad-shMPP1 treated mice developed liver tumor nodules, and in the other 4 mice receiving Ad-shMPP1 (66.6%, 4 of 6), no macroscopic or microscopic tumors were found in their livers (Table S2 & Fig. S1). In contrast, all animals (6 of 6) in the control groups (PBS and Ad-vector groups) developed visible liver tumor nodules (Table S2 & Fig. S1).

Ad-shMPP1 also prevented the hepatic damage normally caused by liver cancer. Compared with the control groups, the aspartate transaminase (AST) value, which is often elevated in HCC patients due to hepatic injury, was significantly lower after i.v injection of Ad-shMPP1 in the \textit{in situ} HCC xenograft implanted mice (Table S2 & Fig. 6D). Another hepatic marker,
albumin (ALB), which reflects the protein synthesis ability of liver and is commonly impaired by hepatic ascites or malnutrition, was significantly improved by Ad-shMPP1 treatment of HCC animals ($P < 0.05$ vs PBS group and $P < 0.07$ vs Ad-vector group, Table S2 & Fig. 6D). The alanine transaminase (ALT) and alkaline phosphatase (ALP) values were not statistically different between groups (Table S2), although a trend toward improvement was nevertheless observed for ALT ($p = 0.1107$, Table S2). To assess how long the Ad replicated after injection, qRT-PCR was used to evaluate the Ad content in animal’s liver by determining the relative mRNA levels of the adenoviral E3 gene. We found that the oncolytic Ad persists in liver tissues for at least 8 days after i.v. injection, and that by 12 days post-injection, the Ad were nearly completely eliminated (Fig. 6E).
**Discussion**

Recently, kinesins have attracted significant attention as potential new targets for cancer therapy in the clinic (13, 21). Some agents that target mitotic kinesin proteins, such as Eg5, have demonstrated potent anti-tumor effects in preclinical models (22). However, the results from current phase II clinical trials for these agents are disappointing. For instance, ispinesib (SB-715992, Cytokinetics and GlaxoSmithKline), the first Eg5 inhibitor to enter clinical trials, has been closed for further trials as a monotherapy due to its lack of clinical efficacy for a number of solid tumors (23-25). An explanation for the clinical failure of mitosis-specific suppressors is the much smaller proportion of mitotic cells in human tumors compared to the animal xenograft models (26). Due to the insufficient anti-tumor effect of monotherapy, several ongoing clinical studies have incorporated KIF inhibitors with chemotherapeutic agents including carboplatin, capecitabine, and docetaxel to explore potential synergistic effects (27). In addition, drug resistance continues to be a major challenge in HCC chemotherapy (8). Notably, some KIF proteins, such as KIFC3 and MCAK, play a critical role in the development of drug resistance in cancer cells (9, 28, 29). Therefore, a combined strategy that brings synergistic efficacy and reduces development of drug resistance is urgently required for combating malignant HCC.

In searching for novel HCC targets that potentially increase chemotherapy efficacy, we focused on the kinesin protein MPHOSPH1, first identified through its *in vivo* interaction with the mitotic peptidyl-prolylisomerase PIN1 (30). In the current study, we investigated the
efficacy of the combined treatment of targeting MPHOSPH1 in combination with taxol in HCC xenograft models, and demonstrated that reducing endogenous MPHOSPH1 significantly enhanced the cytotoxic effect of taxol against HCC, which suggests that MPHOSPH1 is a potential target for HCC therapy. Due to the intrinsic cytotoxicity of the Ad-vector, however, the observed combined effect (Fig. 3C) may simply indicate the synergistic interaction of Ad-shMPPI as a complex with taxol, which would not be interpreted as the interaction of taxol and the shRNA of MPHOSPH1.

We recently reported that depletion of MPHOSPH1 in colorectal cancer cells resulted in mitotic arrest due to failure of cytokinesis and subsequent apoptosis (6). Intriguingly, our previous study indicated that the observed multinucleate (polyploidy) cells eventually underwent apoptosis (6). In the present study, we also found significant mitotic arrest was induced by downregulation of endogenous MPHOSPH1 in HCC cells, leading to polyploidy (the multinucleated cells in Fig. 4C & D), which suggested the mitotic slippage was induced presumably by the prolonged mitotic arrest (6). Intriguingly, mitotic slippage that causes polyploid cells is considered to be a protective mechanism that cancer cells use against mitotic inhibition (31). However, the infected cells with polyploidy observed here eventual underwent apoptosis (Fig. 4A & B), suggesting that the mitotic slippage process triggered post-mitotic cell death instead.

Single nucleotide polymorphism (SNP) is a useful tool to study the potential pattern of gene
expression. By applying “SNPinfo” tool to predict the SNPs in the MPHOSPH1 promoter region (32), we found three SNPs with Minor Allele Frequency (MAF) > 0.05 (rs10881625, rs9325443 and rs9325444) to be located at the transcription factor binding sites (TFBS), which may regulate gene expression by impacting transcription factors binding. However, this is only a prediction and experimental verification is still needed.

Moreover, p53, which promotes post-mitotic apoptosis due to its accumulation following mitotic arrest (33), was significantly elevated in Ad-shMPP1 treated HepG2 cells (Fig. 5A). Expression of the oncogene cyclin-D1 as well as phosphorylation of STAT3, which were reported to be regulated by p53 (34, 35), were also markedly inhibited by MPHOSPH1 knockdown, suggesting that STAT3 activation was blocked through the p53 pathway. A probable mechanism was proposed previously by Blagosklonny (33) suggesting that during the mitotic arrest when the nuclear envelope is dissolved and chromosomes are condensed, transcription is absent, while the p53 mRNA is long-lived and accumulates (Fig. 5A & E). However, p53 cannot induce its targets since transcription is blocked. When the cell escapes from the mitotic arrest (mitotic slippage), transcription resumes, and the accumulated p53 suddenly induces its pro-apoptotic targets such as PUMA, Bax and CD95, which can trigger apoptosis (Fig. 4A & B) (6, 33). This hypothesis is consistent with the present study and our previous work (6).

Interestingly, in addition to HCC with wild type p53, we also demonstrated in Hep3B cells,
which are p53-null, sensitivity to MPHOSPH1 interference, although to a lesser extent (Fig. 3). In a recent study, Forys and colleagues reported that loss of p53 leads to an increase in ARF protein levels, which function to limit the proliferation and tumorigenicity of p53-null cells, suggesting a complementary effect of ARF when p53 is deficient (36). It will be of great future interest to further investigate whether ARF or other tumor suppressors are involved in the p53-null cells to better understand the alternative regulation mechanism of MPHOSPH1 knockdown.

A carcinogenic link to MPHOSPH1 was previously found in bladder cancer (4), and we recently reported its upregulation in colorectal cancer cells (6). Here, we demonstrated that infection with Ad-shMPP1 not only suppressed HCC development, but also protected hepatic function from HCC impairment (Fig. 6D). Significantly improved AST and ALB were observed following Ad-shMPP1 treatment. Since the estimation of the hepatic function reserve (HFR) is critical in the prognosis of PLC patients, improving poor hepatic function of patients is critical for facilitating proper treatment and reducing the risk of surgery. Another potential benefit of Ad-shMPP1 treatment is the interferon effect, which can be induced by Ad infection (Fig. 5B), and might further enhance the anti-tumor effects of the oncolytic Ad (19), although the interferon itself may also impair the replication of oncolytic Ad (37). In summary, the observed HCC inhibition and the improved hepatic function following treatment with Ad-shMPP1 may present an outstanding advance in clinical outcomes of HCC treatment.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

8. Kim HY, Park JW. Molecularly targeted therapies for hepatocellular carcinoma: sorafenib as a


Figure Legends

Figure 1. Overexpression of MPHOSPH1 in HCC patient samples and human HCC cell lines. A, immunohistochemistry was conducted on normal liver and HCC tissues for MPHOSPH1 protein expression. Magnification, ×20. B, intensity of MPHOSPH1 staining was scored on a scale of 0–3. C, MPHOSPH1 mRNA was measured by RT-PCR in 3 liver cancer cell lines and a normal liver cell line (** P<0.001).

Figure 2. HCC-specific replication and MPHOSPH1 knockdown effects of Ad-shMPP1
A, a schematic drawing of the Ad-shMPP1. Ψ, the encapsidation signal; ITR, the inverted terminal repeat. B, analysis of the replication ability of Ad-shMPP1 in the normal liver cell (QSG7701) and the HCC cell (HepG2) by RT-PCR. The adenovirus E3 region was amplified to estimate the viral DNA content 48 hrs after infection (MOI = 0.1). C, quantification of the MPHOSPH1 transcript level by RT-PCR in HepG2 and Hep3B cells 48 hours after infection with Ad-vector and Ad-shMPP1 at MOI=1. GAPDH was used as an internal control.

Figure 3. Anti-proliferation effects of Ad-shMPP1 in HepG2 cells. A, the cytopathic effects of Ads on HCC cells and normal liver cells at varied MOIs were determined by crystal violet staining 96 hours after infection. B, measurement of cell viability of HepG2 and Hep3B HCC cells 96 hours after infection with Ads or combined treatment with taxol using an MTT assay (** P < 0.01, *** P < 0.001). C, HepG2 and Hep3B cells were treated with Ad-shMPP1 or taxol or both in a series of concentrations and the combination index was calculated using CalcuSyn software. CI values less than 0.9 is considered synergism.

Figure 4. MPHOSPH1 knockdown induces polyploidy and apoptosis in HCC cells.
A, The nuclear morphology of HepG2 cells stained with Hoechst33258 dye were visualized 48 hours after infection. The arrows indicate apoptotic cells. Magnification, ×40. B, DNA fragmentation assays of HepG2 cells 48 hours after infection with Ads. DNA marker, DL2000. C, 24 hours after infection, the multinuclear HepG2 cells stained with DAPI and Phalloidin Alexa Fluor 555 were visualized by confocal microscopy. Scale bar, 10 μm. D, analysis of the polyploid HepG2 cells with PI staining by FACS 24 hours after infection. The numbers show the percentage of cells with >4N DNA content.

**Figure 5. Proteins involved in the inhibition of proliferation of HCC cells induced by MPHOSPH1 knockdown.** A, western blot analysis of proteins from HepG2 cells treated with Ads. B, western blot analysis of proteins from HepG2 cells treated with Ads at different MOIs. C, densitometric quantitative analysis of western results. D, western blot analysis of proteins from HepG2 cells with combined treatment of Ads and taxol. E, p53 mRNA was measured by RT-PCR in HepG2 cells treated with Ads. F, western blot analysis of proteins from HepG2 cells transfected with shMPP1 expression plasmid at different times. G, measurement of cell viability of HepG2 cells 48 or 96 hours after transfection with shMPP1 expression plasmid or control plasmid using an MTT assay (*P < 0.05, ***P < 0.001).

**Figure 6. In vivo knockdown of endogenous MPHOSPH1 inhibits HCC xenograft growth.** A and B, measurement of the tumour volume of different groups treated with Ads or taxol every 4 days (*P < 0.05). C, IHC and H&E staining analysis of tumor sections derived from tumors. Magnification, ×20. D, AST and ALB values of the orthotopic HCC exnografts implanted mice 21 days after injection of Ads or PBS (*P < 0.05, #0.05 < P < 0.07). E,
analysis of the relative content of rec-Ad in mouse livers by RT-PCR after i.v. injection. The adenovirus E3 region was amplified to estimate the viral DNA content using GAPDH as an internal control.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
MPHOSPH1: a potential therapeutic target for hepatocellular carcinoma

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