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MARVELD1 inhibited cell proliferation and enhance chemosensitivity via increasing expression of p53 and p16 in hepatocellular carcinoma

Youtao Yu,[1,2,4] Yubao Zhang,[2,4] Jianran Hu,[1] Hao Zhang,[1] Shan Wang,[1] Fang Han,[1] Lei Yue,[1] Youpeng Qu,[1] Yao Zhang,[1] Hongjian Liang,[1] Huan Nie[1] and Yu Li[1,3]

[1]School of Life Science and Biotechnology, Harbin Institute of Technology, Harbin, Heilongjiang; [2]The Affiliated Tumor Hospital, Harbin Medical University, Harbin, Heilongjiang, China

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We have previously found that expression of *MARVELD1* was remarkably downregulated in multiple tumor tissues, but unclear in hepatocellular carcinoma (HCC) and its function has not been explored yet. In the present study, to uncover the underlying mechanism of *MARVELD1* in the pathogenesis and development of HCC, we investigated the expression pattern of *MARVELD1* and its effect on tumor proliferation in HCC. The results indicated the frequent downregulation of *MARVELD1* in clinic samples and cell lines of HCC resulted from promoter methylation, as well as genetic deletion. Furthermore, treatment of *MARVELD1* unexpressing Hep3B2.1-7 and PLC/PRF/5 cells with the demethylating agent 5-aza-2′ deoxycytidine restored its expression. Overexpression of *MARVELD1* suppressed the proliferation of HCC cells *in vitro* and *in vivo*, whereas downregulation of endogenous *MARVELD1* by shRNAs significantly enhanced these characters. *MARVELD1* overexpression could enhance chemosensitivity of HCC cells to epirubicin and 10-hydroxycamptothecin. Corresponding to these results, the expression of p-ERK1/2 and cyclin D1 were decreased, whereas p16 and p53 were increased in *MARVELD1*-transfected cells. We also demonstrated that knockdown of *MARVELD1* resulted in upregulation of p-ERK1/2 and cyclin D1, and downregulation of p16 and p53. Moreover, the effect of the decreased cell growth rate was significantly reversed when *MARVELD1*-overexpressing cells were trasfected with p53 or p16 siRNA. Our findings suggest that *MARVELD1* is a tumor suppressor by negatively regulating proliferation, tumor growth and chemosensitivity of HCC cells via increasing p53 and p16 *in vitro* and *in vivo*. *MARVELD1* may be a potential target for HCC therapy. (*Cancer Sci* 2012; 103: 716–722)

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide, and its incidence is still rising.[1] Currently there are about 600 000 cases of HCC each year, and nearly 78% of them are from Asian countries.[2] Substantial evidence from epidemiological studies indicates that HCC is strongly associated with alcohol abuse, chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV), and liver cirrhosis.[3–5] More than one million people die of liver cancer worldwide every year.[6] Few patients are diagnosed in the early stage, and <20% of HCCs can be resected completely.[7,8] Resistance to many of chemotherapy agents is a major obstacle to successful HCC treatment.[9–11] Therefore, a better understanding of the molecular mechanisms underlying HCC progression is urgently needed for leading to a more effective treatment.

Genetic and epigenetic aberrations, leading to the activation of oncogenes and inactivation of tumor suppressor genes, are thought to play major roles in the pathogenesis of HCC. Recently, several MARVEL (proteins of the myelin and lymphocytes [MAL] and related proteins for vesicle trafficking and membrane link) domain-containing proteins have attracted increasing interest because they exhibit tumor suppressor activities and are frequently decreased via promoter methylation in breast, cervical, prostate, hepatocellular, esophageal and gastric carcinoma or cell lines.[12–15] *MARVELD1* is a member of MARVEL domain-containing proteins, located on human chromosome 10q24, a locus associate with multiple cancers. Our previous study shows that *MARVELD1* is frequently downregulated in multiple cancers.[16] In addition, *MARVELD1* could be induced by multiple chemotherapeutic agents (data not shown), but its function is not investigated yet.

To investigate the expression and role of *MARVELD1* in HCC, the expression pattern of *MARVELD1* was firstly detected in primary HCC and matched adjacent normal/benign liver tissues. Then, we determined promoter methylation and DNA copy number of *MARVELD1* in clinic samples and cell lines of HCC. Subsequently, we examined tumor cell proliferation, anchorage-independent growth, tumorigenicity in xenograft mouse model and chemosensitivity following upregulation or downregulation of *MARVELD1*. We finally found that expression of p53 and p16, inhibitors of the cell proliferation, could be increased by *MARVELD1*.

Materials and Methods

Clinical samples. Thirty-three pairs of matched HCCs and adjacent normal tissues were obtained from the Affiliated Tumor Hospital, Harbin Medical University. We also obtained 115 formalin fixed and paraffin embedded HCC tissues, in which 23 were provided by the same hospital and 92 were from a tissue microarray (Shanghai Outdo Biotech, Shanghai, China). This tissue array comprised 27 hepatitis, 37 hepatocirrhosis, two fatty liver and 26 normal liver samples. All samples were collected between December 2008 and July 2010, and written informed consent was obtained from each donor. The study was approved by the Medical Ethics and Human Clinical Trial Committee at the Affiliated Tumor Hospital, Harbin Medical University.

Cell lines. Human HCC cell lines HepG2, Hep3B2.1-7, and PLC/PRF/5 were purchased from the American Type Culture Collection (ATCC). BEL7402 and SMMC7721 were from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS). All cell lines were cultured in DMEM supplemented with 10% FBS (Gibco, Grand Island, NY, USA) at 37°C in 5% CO_2 incubator.

Immunohistochemical staining. The immunohistochemical staining was performed as described previously. Immunohisto-

[3]To whom correspondence should be addressed. E-mail: liyugene@hit.edu.cn
[4]These authors made equal contributions to this work.

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chemical staining was determined by the staining intensity and the percentage of immunoreactive cells as reported previously.[17] The criteria of intensity are as follows: 0 (negative), absent or staining <5% of cells; grade 1, mild to moderate staining of 5–50% of cells; grade 2, moderate to intense staining of more than 50% of the cells.

Cell transfection and generation of stable *MARVELD1* knockdown cells. *MARVELD1* ORF region were cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA, USA). HepG2 cells were transfected with *MARVELD1* construct using Lipofectamine 2000. Two siRNAs targeting *MARVELD1* correspond to the sequences 5′-CCTCAAGGATTACCCGCTCTT-3′, and 5′-ATTGGAACCAGGCTTCTGGTT-3′. HeLa cells were transfected with siRNA constructs and cultured for 3 weeks in 800 μg/mL G418 (Merck, Darmstadt, Germany). *MARVELD1* overexpressing HepG2 cells were transfected with p53 siRNA pool or p16 siRNA pool (GenePharma, Shanghai, China) using Lipofectamine 2000.

Quantitative real-time PCR. Quantitative real-time PCR (qPCR for DNA and qRT-PCR for cDNA) was performed using the Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). Primers for qPCR are described in Table S1. Normal human peripheral blood lymphocytes (PBL) DNA acted as control. PCR conditions were: 95°C 10 s; 95°C 5 s, 60°C 34 s for 31 cycles at GAPDH or 38 cycles at *MARVELD1*. Reaction specificity was controlled by post-amplification melting curve analyses and gel electrophoresis of products. Relative gene expression levels were calculated by the formula $2^{-\Delta C_t}$, where ΔC_t (critical threshold) = C_t of *MARVELD1*−C_t of *GAPDH*.

Bisulfite DNA sequencing. Bisulfite modification of DNA was performed with Applied Biosystems methylSEQr Bisulfite Conversion kit. Bisulfite-treated DNA was amplified using nest PCR with primers described in Table S1. After the PCR products were cloned into the pMD18-T vector (Takara, Dalian, China), not <10 colonies in each case were randomly sequenced.

5-aza-2′-deoxycytidine treatment. Hep3B2.1-7, PLC/PRF/5 and HepG2 cells were separately treated with 2.0 and 5.0 μM 5-aza-2′-deoxycytidine (5-aza-dCyd; Sigma, St. Louis, MO, USA) for 24, 48 and 72 h. Control dishes received DMEM complete culture medium. Cells were harvested and used for RNA analysis and demethylation analysis of promoter region at the end of the experiments.

Western blotting. Cells were directly lysed in 2× Laemmli buffer. The same amount of cell lysates were electrophoresed in 12% SDS-polyacrylamide gels and blotted onto PVDF membranes, which were subsequently probed with the indicated primary antibodies and incubated with secondary antibodies conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). ECL western blotting analysis system (Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK) was used to detect the substrates.

Cell proliferation and soft-agar colony formation assay. Cells were seeded in 12-well plates at 3×10^4 cells/well. The number of the cells was counted 2 days after seeding, and every day from the fifth day, each in fourfold. The cells were continuously counted for 10 days. Meanwhile, cells (1×10^5) were seeded into 6-well plates with a bottom layer of 0.8% low-melting-temperature agar in DMEM and a top layer of 0.4% agar in DMEM. Colonies were scored after 10 days of growth.

***In vivo* tumor xenograft study.** HepG2/*MARVELD1* and HepG2/vector cells were inoculated subcutaneously into right backside with 4×10^6 cells. Tumor size was measured weekly using calipers until 7 weeks and their volumes were calculated using the following standard formula: width^2 × length × 0.52.

Epirubicin and 10-hydroxycamptothecin treatment. Mice were randomized into six groups (six mice per group, three groups for *MARVELD1* experiment and other three groups for vector control) and injected subcutaneously with 4×10^6 cells (HepG2/*MARVELD1* cells of stably expressing *MARVELD1* and HepG2/vector cells) into the right flank of each animal. Mice subsequently were treated with epirubicin (ADM), 10-hydroxycamptothecin (HCPT), or ADM and HCPT combination by abdomen after xenograft of mice was approximately 0.3 cm^3. Physiological saline was used as a blank control. The concentrations of ADM and HCPT were 0.5 and 1.5 mg/kg body weight in 0.5 mL, respectively, and the same dosage combined ADM and HCPT were also used in 0.5 mL. Tumor size was monitored two times weekly, and tumor tissues were fixed in 10% formaldehyde for routine paraffin sections and H&E staining.

Statistical analysis. Two-tailed Student's *t*-test and chi-square tests were applied where appropriate. Results were considered to be statistically significant at $P \leq 0.05$.

Results

***MARVELD1* was frequently downregulated in HCC.** To investigate *MARVELD1*'s role in HCC, the expression of *MARVELD1* mRNA was first examined in tumors and histologically normal adjacent tissues from HCC patients ($n = 33$, primary tumors) by quantitative real-time RT-PCR (qRT-PCR). As expected, pairwise comparison between individual HCC and paired normal tissues revealed that *MAEVELD1* expression in tumors was reduced by more than 50% in 23 of 33 (69.7%) tumors (Fig. 1a). Mean *MARVELD1* mRNA levels observed in HCCs were 50% lower than those observed in normal surrounding tissues.

To further confirm qRT-PCR results, immunohistochemistry was performed in 115 formalin fixed, paraffin embedded HCC tissues in which normal/benign liver tissues were found in 74 cases. Representative immunoreactivity results are shown in Figure 1b. The positive staining was localized in cytoplasm and nuclei, occasionally in the plasma membrane. *MARVELD1* proteins were significantly decreased compared with that of normal/benign liver tissues. Further quantitative analysis revealed that positive *MARVELD1* staining (staining score of grade 1 and 2) was found in 89 of 100 cases of normal/benign liver tissues (89%) (Fig. 1c), whereas only 34 of 115 cases (29.6%) in HCC specimens. Moreover, the expression of *MARVELD1* in benign liver tissues was not downregulated compared with that of HCC specimens, including hepatitis, hepatocirrhosis and fatty liver tissues. No correlation of *MARVELD1* expression with sex, age or clinical stage was observed (Table S2). Collectively, these findings indicated that downregulation of *MARVELD1* was a common and tumor-specific event in the pathogenesis of HCC.

Meanwhile, we analyzed *MARVELD1* expression in five HCC cell lines by qRT-PCR to select suitable cell lines for function analysis. We found that *MARVELD1* expression was lower observably in Hep G2, Hep 3B2.1-7 and PLC/PRF/5 cells (Fig. 1d).

DNA copy number of *MARVELD1* altered in HCC. To determine loss of the *MARVELD1* expression whether to related deletions of this gene, we screened *MARVELD1* gene region of genomic DNA of low expression *MARVELD1* in HCC clinical samples and HCC cells by quantitative PCR, using specific primer for *MARVELD1* of the genomic DNA (Fig. S1a). Normal human PBL DNA was used as control criterion. We identified that the number of *MARVELD1* DNA copies significantly decreased in five tumor tissues of 16 pairs of HCC samples than their normal adjacent tissues and normal human PBL DNA, and *MARVELD1* DNA of tumor tissue in case 22 was lowest (Fig. S1b). Furthermore, we examined *MARVELD1* DNA content in five HCC cell lines. The results from cell lines showed that *MARVELD1* DNA copies of HepG2 cells were 10-fold lower, and

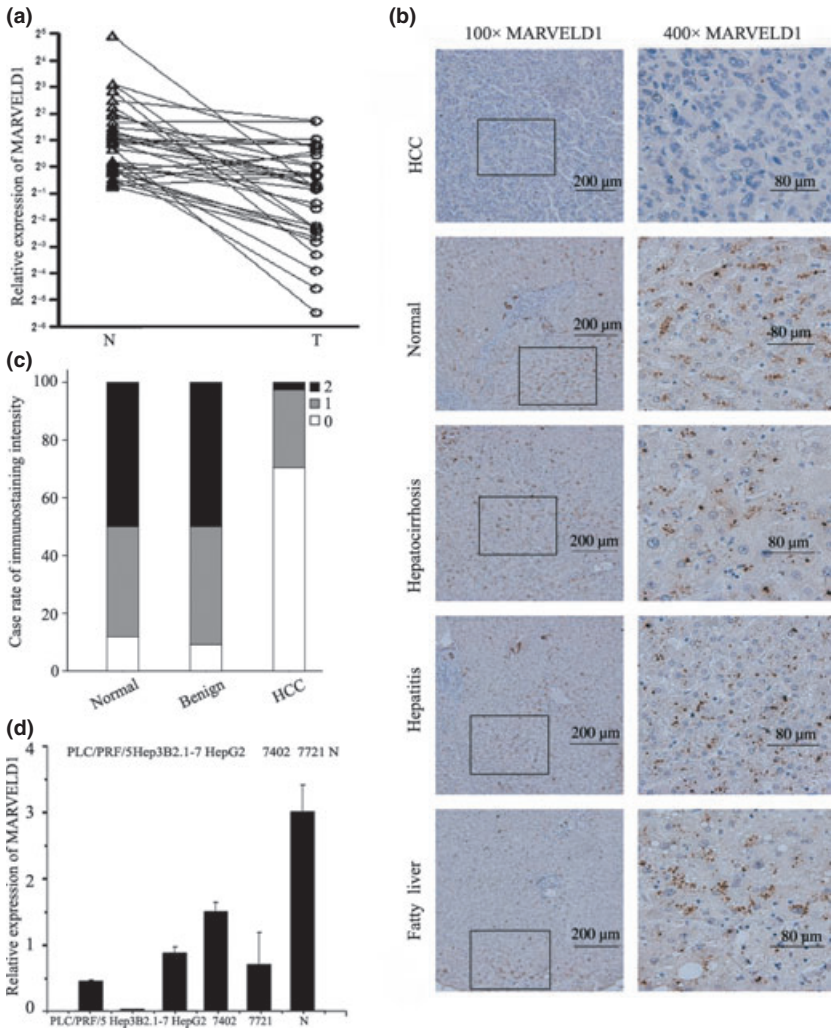

Fig. 1. The downregulated expression of *MARVELD1* in hepatocellular carcinoma (HCC). (a) Relative *MARVELD1* expression was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) from 33 pairs of frozen HCC (T) and adjacent normal tissues (N). Statistical analysis was performed comparing HCCs with normal surrounding tissues, $P < 0.01$. (b) Immunostaining of *MARVELD1* in HCC, normal benign liver, hepatitis and hepatocirrhosis tissues. (c) Statistics of *MARVELD1* levels in HCC, normal and benign liver disease tissues. The highest expression was scored as 2, and the lowest scored as 0. (d) Real-time PCR analysis of *MARVELD1* mRNA analysis was performed in five HCC cell lines, with normal liver tissue as control (N). Copies of *MARVELD1* were normalized to 1 000 000 copies of *GAPDH*.

MARVELD1 DNA copies of PLC/PRF/5 was half of the normal human PBL DNA (Fig. S1c). The results suggested that intragenic deletions represented an important genetic mechanism for inactivating *MARVELD1* in HCC cells and that this event was not an artifact that arose during establishment of the cell lines. In addition, *MARVELD1* DNA copies in five cases: cases 8, 9, 14, 15 and 18, were superabundant in the 16 pair samples (Fig. S1b), suggesting that other mechanisms of inactivating of *MARVELD1* should be detected.

***MARVELD1* promoter was methylated in HCC.** Based on the above results, we set out to investigate the promoter region (−1 to approximately −447 bp) methylation status of five pairs of samples (cases 3, 4, 8, 9, 12) with low expression of *MARVELD1* in HCC tissue. Bisulfite-sequencing showed that the *MARVELD1* promoter region was clearly methylated in those tumors and methylation rate of 29 CpG sites[16] was 80.9% in the promoter region of tumor samples (32.18% in normal adjacent tissues). We also found that all of 29 CpG sites were full methylated in a lot of clones from tumor tissues. Moreover, the third and fourth of five SP1 binding sites on upstream of transcript start sites were 100% methylated in tumor samples, and hypermethylation close-by the third SP1 binding sites. Considering *MARVELD1* DNA content in the analyzed five samples, two tumors (cases 8 and 9) have more *MARVELD1* DNA than PBL, two tumors (cases 3 and 4) were similar *MARVELD1* DNA levels to PBL, and one tumor (case 12) had quite lower *MARVELD1* DNA copies than its normal adjacent tissue and PBL. However, the hypermethylation was detected in all of the five tumor cases (Fig. 2a) and also in PLC/PRF/5 (Fig. 2b) and Hep3B2.1-7 cells lacking MARVELD1expression. The results suggested that hypermethylation of the promoter region was a considerable common mechanism for downregulating *MARVELD1* level in HCC. These observations led us to conjecture that, in addition to homozygous deletion, hypermethylation of the CpG sites might be a frequent cause of silence of *MARVELD1* in HCCs.

5-Aza-dCyd induced restoration of *MARVELD1* expression by demethylation in HCC cell lines. To explore whether demethylation treatment could restore expression of *MARVELD1* mRNA in HCC cells, we treated cells with 2.0 and 5.0 μM 5-Aza-dCyd. Induction of *MARVELD1* mRNA occurred after treatment with 5-Aza-dCyd in PLC/PRF/5 and Hep3B2.1-7

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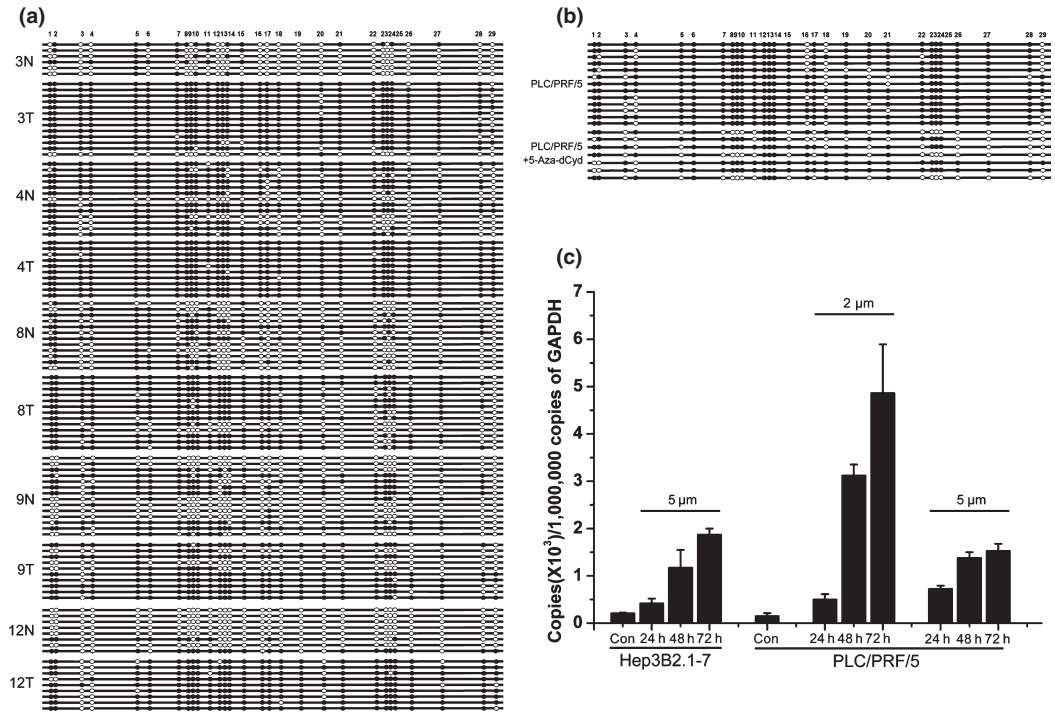

Fig. 2. Frequent methylation of *MARVELD1* promoter in hepatocellular carcinoma (HCC) and 5-Aza-dCyd induced restoration of *MARVELD1* expression by demethylation. (a) Methylation status of *MARVELD1* promoter region by bisulfite sequencing in five paired HCCs. N, normal adjacent tissue; T, tumor. (b) PLC/PRF/5 cell was treated with 2.0 μM 5-aza-dCyd for 24 h and methylation status of *MARVELD1* promoter region was detected by bisulfite sequencing. (c) The restoration of *MARVELD1* at mRNA levels in PLC/PRF/5 and Hep3B2.1-7 cells was analyzed by real-time PCR.

cells but without its DNA deletion, and *MARVELD1* expression was rush hour at 24 h in PLC/PRF/5 cells treated with 2.0 μM 5-Aza-dCyd (Fig. 2c). In contrast, 5-Aza-dCyd treatment of Hep G2 cells, which do harbor a homozygous deletion of *MARVELD1*, failed to enhance the expression of *MARVELD1* (data not shown). With the performance of bisulfite-sequencing, the demethylation of *MARVELD1* promoter region was observed distinctly in PLC/PRF/5 (Fig. 2b) and Hep3B2.1-7 cells. The results indicated that restoration of *MARVELD1* expression in HCC cells can be induced by 5-Aza-dCyd.

***MARVELD1* inhibited cell proliferation.** With respect to the potential tumor-suppressor activity of *MARVELD1*, we analyzed the role of *MARVELD1* on cell proliferation *in vitro*. After exogenous *MARVELD1* recombinants were transfected into HepG2 cells with this gene deletion and NIH3T3 cells without expression of *MARVELD1*, stable cell clones were established. As shown in Figure 3a, Fig. S2 and Fig. S3, the proliferation of HepG2 cells and NIH3T3 cells with overexpressing *MARVELD1* was clearly suppressed. Furthermore, the soft agar colony formation assay showed that the number of colonies produced by *MARVELD1*-transfected HepG2 cells decreased remarkably compared with control (Fig. 3b). On the other hand, downregulation of *MARVELD1* greatly enhanced the proliferation (Fig. S 4a) and anchorage-independent growth of HeLa cells (Fig. S4b). The results indicated that anchorage-independent growth ability of HepG2 was also influenced (Fig. 3b). Corresponding to the above results, we found an upward trend in p16 and p53 protein expression and a downtrend in cyclin D1 and p-ERK1/2 protein expression in *MARVELD1*-transfected HepG2 cells (Fig. 3c). Besides, we also observed a downtrend in p16 and p53 protein expression and an upward trend in cyclin D and p-ERK1/2 protein expression in *MARVELD1*-knockdown HeLa cells (Fig. S4c). To further elucidate the mechanisms underlying the function of *MARVELD1* in HCC, the *MARVELD1*-overexpressing Hep G2 cells were transfected with p53 or p16 specific siRNA by using Lipofectamine 2000 Transfection Reagent. Interestingly, the effect of the decreased cell growth rate was significantly reversed when *MARVELD1*-overexpressing cells were transfected with p53 or p16 siRNA (Fig. S5).

Overexpression of *MARVELD1* inhibited xenograft tumor growth. To further explore the suppressing role of *MARVELD1* on tumor growth, nude mice xenografts were modeled using HepG2 cells following expression of *MARVELD1*. HepG2/*MARVELD1* and HepG2/Vector were separately injected into nude mice, each group containing seven mice. The results showed that the wet weights of tumors excised in HepG2/*MARVELD1* group were obviously smaller than that of HepG2/Vector group after 7 weeks ($P < 0.05$) (Fig. 4a). Moreover, according to results *in vitro*, we detected the expression of p16 and p53 and results showed that the proteins were also increased, but the expression of cyclin D1 was suppressed (Fig. 4b). Collectively, these results indicated *MARVELD1* could slower the xenograft tumor growth through the same pathway in HepG2 cells.

Increasing *MARVELD1* expression promoted chemosensitivity of HCC. Previously, we found that expression of *MARVELD1* could be induced by multi-chemotherapeutic agents (Youtao Yu, Lei Yue, Fang Han and Yu Li, unpublished data, 2009). According to the result, we selected two drugs targeted to topoisomerase I and II and detected chemotherapeutic sensitivity in nude mice. Our results showed that *MARVELD1* could improve the sensitivity of ADM and HCPT combination and reduce the xenograft tumor growth and final wet weights (Fig. 5a). Furthermore, histopathology of the xenograft tumors was detected by H&E staining, and expression of p16 and p53 was revealed by immunohistochemical staining. The results

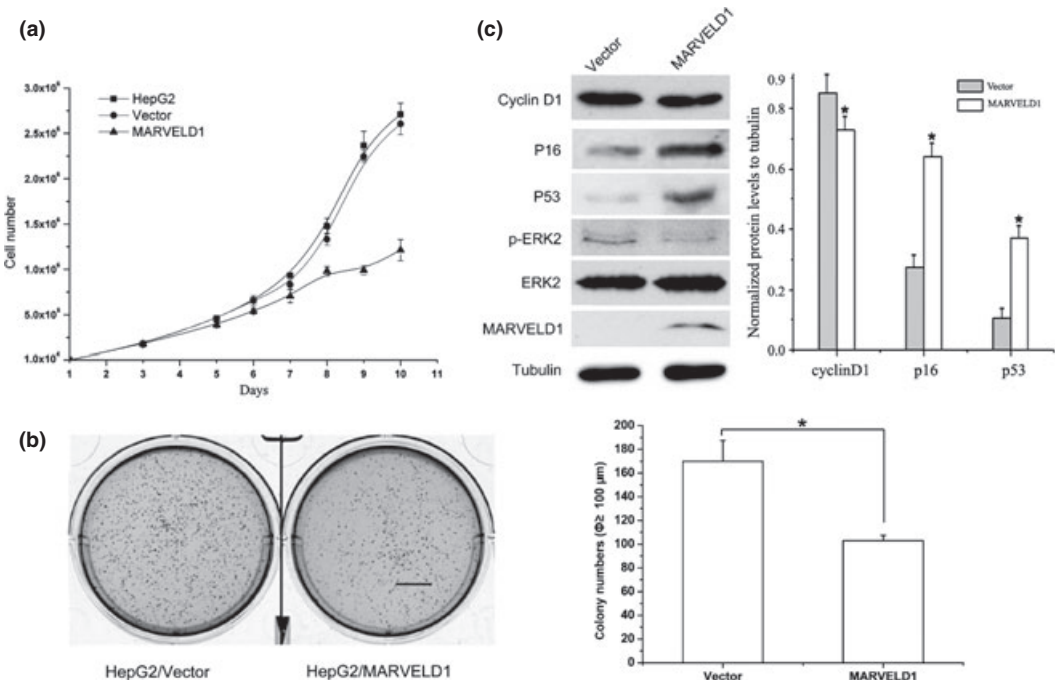

Fig. 3. Overexpression of *MARVELD1* inhibited hepatocellular carcinoma (HCC) cell proliferation *in vitro*. (a) Hep G2 cell proliferation assay. Hep G2 cells were overexpressed *MARVELD1* and control vector. The cell number was counted at the indicated time points. (b) Overexpression of *MARVELD1* inhibits the anchorage-independent growth ability of Hep G2; the colonies a well of a 6-well plate were counted and each experiment was repeated three times (*$P < 0.05$; scale bar = 50 μm). (c) Several proliferation related-genes were detected in Hep G2 cells with overexpressing of *MARVELD1* and the expression levels of these proteins were normalized to those of tubulin protein (*$P < 0.05$).

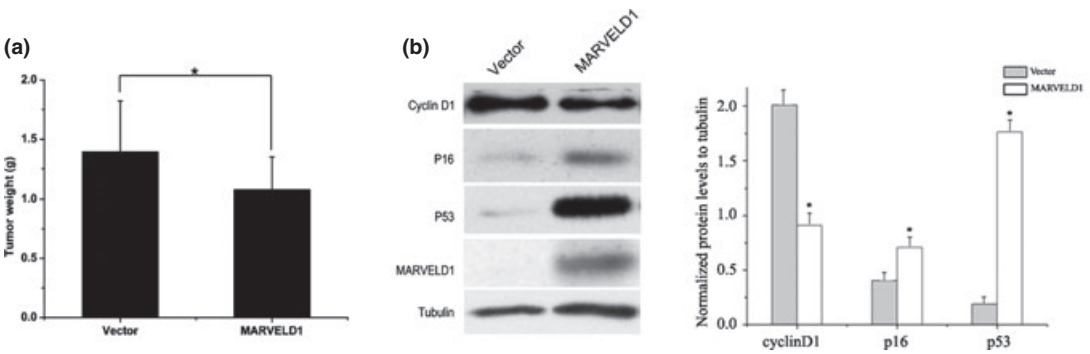

Fig. 4. *MARVELD1* inhibited hepatocellular carcinoma (HCC) cell proliferation *in vivo*. (a) HepG2/*MARVELD1* and Hep G2/Vector were injected into nude mice to generate xenograft tumor. Xenograft tumor wet weight was determined after 7 weeks following the cell inoculation. (b) Several proliferation-related genes were detected in xenograft tumor and the expression levels of these proteins were normalized to those of tubulin protein (*$P < 0.05$).

exhibited that activity of cells was ruptured in the xenograft tumor tissues of HepG2/*MARVELD1* group treated with chemotherapeutic agents (Fig. 5b), and p53 and p16 were upregulated significantly, especially in the HepG2/*MARVELD1* group with ADM combining HCPT (Fig. 5c).

Collectively, all of the results indicated that *MARVELD1* was frequently downregulated in HCCs, and promoter methylation was the main mechanism for its suppression in tumor. *MARVELD1* negatively regulated proliferation of HCC cells via increasing expression of p53 and p16, and improved the sensitivity of chemotherapeutic agents.

Discussion

MARVEL domain-containing proteins are evolutionarily conserved and widely expressed in normal adult tissues. They are involved in a variety of biological processes, including cell cycle progression, chemotactic activity, tight junction, and clathrin-mediated endocytosis.[15,18–20] It is documented that MARVEL domain-containing proteins are decreased in various types of cancer, and exert tumor-suppressive functions.[12–15] *MARVELD1* is a novel member of this family, with very limited work performed so far about its molecular function. Recently, we established that *MARVELD1* is frequently downregulated in multiple human cancers. We further identified that the reduced expression of *MARVELD1* was owing to DNA methylation and could be reversed by pharmacologic demethylation in breast cancer cell lines[16] and HCC in this paper. Based on our study and other reported findings, we propose that *MARVELD1* may act as a potential tumor suppressor in HCC.

Although development is continually made in diagnostic and therapeutic modalities, the mortality rate of HCC has remained

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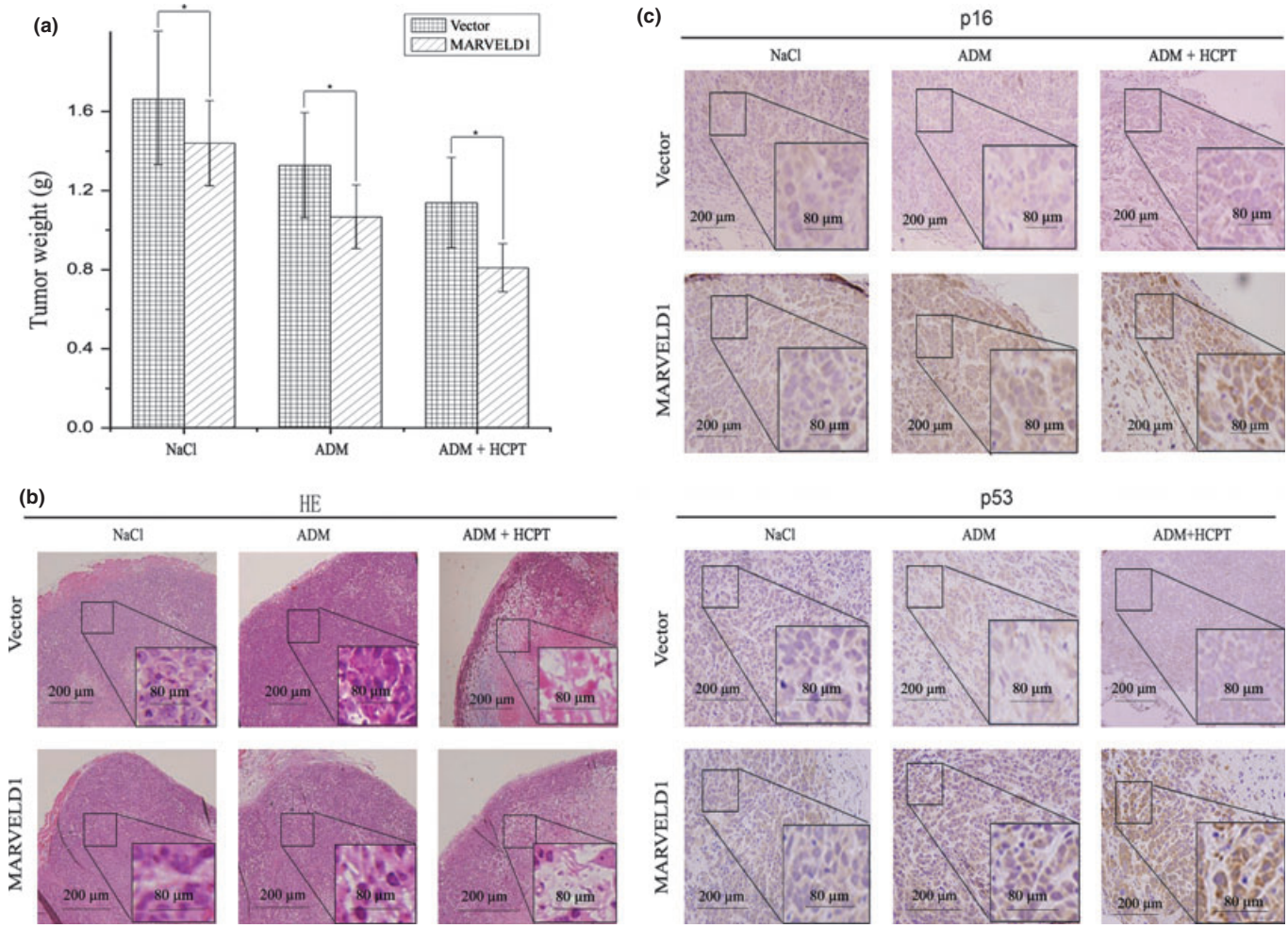

Fig. 5. *MARVELD1* enhanced the chemosensitivity of hepatocellular carcinoma (HCC). (a) Tumor wet weight was detected in NaCl, epirubicin (ADM), ADM and 10-hydroxycamptothecin (HCPT) combination groups and was presented as mean tumor weight ± standard deviation (*$P < 0.05$). (b) H&E staining of all kinds of xenografts. Magnification: 100×. (c) Immunohistochemical staining of p16 and p53 in xenografts with NaCl, ADM, ADM and HCPT combination. Magnification: 400×.

unchanged over the past decades. This is mainly due to poor understanding of its pathogenesis, thus identification and characterization of novel aberrant genes in HCC development is of great value for developing more effective treatment. Tumor suppressor gene deletion is one of the major contributors of tumorigenesis, it is reported that deletion of the p16 and p15 genes is frequent in most cell lines and such deletions are common in certain cancers.[21,22] DNA methylation is one of the important epigenetic modifications of the genome, aberrant DNA methylation is a common phenomenon in human cancer. Studies have revealed that many tumor suppressor genes such as p16, RARb, DAPK and MGMT, showed frequently hypermethylation at promoters, thus leading to gene silencing and affecting plenty of cellular processes, including apoptosis, cell adherence, DNA repair and cell-cycle control.[23,24] ERK is a critical member in Ras/Raf/MEK/ERK signaling cascade and its activation plays important roles in cell proliferation through regulating cyclin D1, thus it affects the G1/S transition. Also, ERK activation has great effects on G2/M transition.[25] It is reported that sustained activity of ERK is required for the downregulation of many antiproliferative genes, such as p53, p16, throughout the whole G1 phase of the cell cycle.[26] These molecules also play critical roles in the pathogenesis of HCC.[27–29] Overexpression of *MARVELD1* results in an increased expression of p16 and p53 and a decreased expression of cyclin D1 and p-ERK2. Knockdown of endogenous *MARVELD1* in Hela cells had an expected opposite result. These data suggest that *MARVELD1* inhibited cell proliferation may be mediated through regulating the expression of p53, p16, cyclin D1 or being regulated by them. Considering these molecules share a common effect in cell cycle G1 control, we infer that *MARVELD1* inhibits HCC proliferation by cooperating with p53, p16, cyclin D1 and p-ERK1/2, resulting in G1 phrase arresting. Moreover, in our *in vivo* mouse model study, *MARVELD1* significantly reduced tumor volume and weight and the expression of p53, p16, cyclin D1 showed the same patterns of *in vitro*. Taking *in vitro* and *in vivo* results together, we draw the conclusion that *MARVELD1* exerts tumor-suppressive functions in HCC via inhibiting cell proliferation.

Hepatocellular carcinomas are highly resistant to chemotherapeutic agents and it is urgent to discover effective therapeutic molecules to enhance liver tumor sensitivity to drugs. In this study, we found that *MARVELD1* could promote the tumor sensitivity to ADM and HCPT, drugs targeted to topoisomerase I and II, respectively. Additionally, immunohistochemical staining of p16 and p53 showed that they were further improved in Hep G2/*MARVELD1* xenograft mice and contributed to enhanced drug sensitivity in HCC. Therefore, we

postulate that transferring *MARVELD1* into these cancers may sensitize them to standard chemotherapy drugs.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DNA copy number alterations of *MARVELD1* gene in hepatocellular carcinoma (HCC).

Fig. S2. Overexpression of MARVELD1 inhibited hepatocellular carcinoma (HCC) cell proliferation *in vitro*.

Fig. S3. Overexpression of *MARVELD1* inhibited NIH3T3 cell proliferation *in vitro*.

Fig. S4. Downregulation of *MARVELD1* promoted HeLa cell proliferation *in vitro*.

Fig. S5. The effect of the decreased cell growth rate was significantly reversed when *MARVELD1*-overexpressing cells were transfected with p53 or p16 siRNA.

Table S1. The primer sequences for studies of the *MARVELD1* gene.

Table S2. Correlation between *MARVELD1* expression and clinicopathological features of HCC patients.

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