3-Mercaptopyruvate sulfurtransferase represses tumour progression and predicts prognosis in hepatocellular carcinoma

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Abstract

Background and Aims: The prognosis of hepatocellular carcinoma (HCC) remains dismal, and its molecular pathogenesis has not been completely defined. The enzyme 3-mercaptopyruvate sulfurtransferase (MPST) regulates endogenous hydrogen sulfide (H2S) biosynthesis. However, the role of MPST in HCC has never been intensively investigated.

Methods: MPST protein expression was analysed in HCC tumour tissues and matched adjacent tissues. The effect of MPST on HCC progression was studied in vitro and in vivo.

Results: The mRNA and protein expression of MPST was significantly downregulated in HCC samples compared with their paired nontumour counterparts. A low MPST expression was associated with larger tumour size and a worse overall survival. Overexpression of MPST in HCC cells inhibited cell proliferation and induced apoptosis. MPST overexpression also significantly suppressed the growth of tumour xenografts in nude mice, whereas silencing MPST by intratumour delivery of siRNA substantially promoted tumour growth. Moreover, diethylnitrosamine-induced mouse HCC was aggravated by MPST gene knockout. Mechanistically, MPST suppressed the cell cycle associated with H2S production and inhibition of the AKT/FOXO3a/Rb signalling pathway in HCC development. In addition, MPST expression negatively correlated with that of pRb in HCC specimens and the combination of these two parameters is a more powerful predictor of poor prognosis.

Conclusions: MPST may function as a tumour suppressor gene that plays an essential role in HCC proliferation and liver tumorigenesis. It is a candidate predictor of clinical outcome in patients with HCC and may be used as a biomarker and intervention target for new therapeutic strategies.

Abbreviations: CBS, cystathionine β-synthase; CCND1, cyclin D1; CSE, cystathionine γ-lyase; DEN, diethylnitrosamine; FOXO, Forkhead box O; HCC, hepatocellular carcinoma; H2S, hydrogen sulfide; IHC, immunohistochemistry; IOD, integral optical density; KO, knockout; MMP, mitochondrial membrane potential; MPST, 3-mercaptopyruvate sulfurtransferase; NAFLD, nonalcoholic fatty liver disease; OS, overall survival; PBS, phosphate-buffered saline; Rb, retinoblastoma; ROS, reactive oxygen species; siRNA, small interfering RNA; TMA, tissue microarray; WT, wild type.

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Lay Summary

The enzyme 3-mercaptopurvate sulfurtransferase regulates endogenous hydrogen sulfide biosynthesis. Our findings suggest that MPST may suppress cell proliferation associated with hydrogen sulfide production and inhibition of retinoblastoma protein signalling pathway in the development of hepatocellular carcinoma.

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, and the second leading cause in China. Despite major technical improvements in curative treatments such as medicine, surgical resection and transplantation, the clinical course of HCC is variable and the prognosis of HCC patients remains poor. The molecular pathogenesis of HCC is extremely complex and heterogeneous. A better understanding of the molecular mechanism of this disease may help identify novel therapeutic targets to improve the outcome of patients with HCC.

Evolving information suggests that nonalcoholic fatty liver disease (NAFLD) may be an important cause of HCC in addition to viral hepatitis and alcohol-induced liver disease. Recently, we demonstrated a role for 3-mercaptopurvate sulfurtransferase (MPST), a key enzyme that regulates endogenous hydrogen sulfide (H\textsubscript{2}S) biosynthesis, in regulating lipid metabolism involved in the pathogenesis and development of NAFLD. However, the information currently available on the functional role of MPST in HCC remains very limited.

Recent studies have indicated that H\textsubscript{2}S, acknowledged to be an important gasotransmitter, is involved in cancer biological processes. H\textsubscript{2}S is produced in mammalian cells by three major enzymes including cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and MPST. Numerous studies have shown altered expression of these enzymes in the progression of various types of cancer cells. Notably, accumulating evidence indicates that H\textsubscript{2}S-generating enzymes play important roles in HCC. CBS mRNA content was markedly decreased in HCC tissues. Another study demonstrated that reduced CBS expression was associated with poor prognosis in HCC patients. CSE protein is strongly expressed in tumour cell lines, and CSE/H\textsubscript{2}S promotes cell proliferation via cell cycle progression regulation in HCC.

Several studies have investigated the role of MPST in the context of cancer biology. One of the early studies focusing on the expression of MPST in cancer was conducted in 2006 in which MPST expression was markedly reduced in colon cancer and the decrease in expression was correlated with the depth of infiltration, suggesting that MPST is a tumour marker for colon cancer. The expression and activity of MPST were also investigated in the human neoplastic cell lines, including astrocytoma U373, neuroblastoma SH-SY5Y and melanoma cell lines. However, to date, studies that have shown the consequences of MPST modulation in cancer are limited, and the exact role of MPST in the development of HCC remains largely unknown.

In this study, we investigated the MPST expression pattern and determined its contribution to HCC progression. We also dissected the molecular mechanisms by which MPST promoted tumorigenesis.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

Samples from 42 HCC patients receiving hepatic resection at our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China) were collected in this study. All experimental protocols were approved by the local ethics committee of the Clinical Specimens Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine. Additionally, a commercial human HCC tissue microarray (TMA) was used for immunostaining analysis of MPST and p-Rb protein expression purchased from Shanghai Outdo Biotech. The overall survival (OS) for the corresponding patients was calculated from the day of surgery to the day of death or to the last follow-up.

2.2 | Immunohistochemistry staining

Immunohistochemistry (IHC) staining was conducted as described previously. Briefly, tissue sections were dewaxed and rehydrated before performing antigen retrieval. The slides were incubated with anti-MPST (GeneTex), anti-p-Rb (CST) or anti-Ki67 (CST) overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C in an incubator. Immunoreactivity was detected using a DAB kit (ZSGB-BIO) and visualized as brown staining. Slides were counterstained with haematoxylin. The strength of positivity was semiquantified by taking into account the staining intensity and the percentage of positive cells measured by Image-Pro Plus 6.0 software. Paraffin sections were scored semiquantitatively as follows: Grade 0: 0% immunoreactive cells; Grade 1: ≤5% immunoreactive cells; Grade 2: >5%–50% immunoreactive cells; Grade 3: ≥50 immunoreactive cells. For statistical purposes, cases with Grade 0 and 1 were considered to have low expression, and those with Grade 2 and 3 were considered to have high expression.

2.3 | Cell lines and cell culture

Human HCC cell lines (HepG2, MHCC-LM3, Huh7 and Hep3B) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences and were cultivated as described by the suppliers.
2.4 | H₂S donor treatment, H₂S and reactive oxygen species measurement

The cells were exposed to different concentrations of NaHS (an H₂S donor; Sigma-Aldrich) for 24 h. The phosphate-buffered saline (PBS) group served as a control. The measurement of H₂S production was performed as previously described. The assay of reactive oxygen species (ROS) was described in Appendix S1.

2.5 | Plasmid transfection and cell treatment

An overexpression plasmid containing full-length MPST DNA was transfected into HCC cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, 0.9 mg/ml G418 solution (Sigma-Aldrich) was added to cells for selection of stable clones. The sequences of MPST siRNA used in this study and details regarding siRNA transfection were described in Appendix S1.

2.6 | Cell cycle analysis

Cells were collected 48 h after siRNA or vector transfection treatment and fixed in 75% ethanol overnight at −20°C. Then, the cells were washed twice with PBS, followed by RNase A and propidium iodide treatment for 30 min at 4°C in the dark. Finally, cell cycle analyses were performed with a flow cytometer (BD Bioscience). The raw data were analysed by ModFit LT 3.2 software (Verity Software House).

2.7 | Cell proliferation assay

Cell proliferation was detected by Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer’s instructions. After the supernatant was removed, 100 μl of medium and 10 μl of WST-8 solution were added to every well, and then incubated for 1 h. The absorbance of the solution was measured spectrophotometrically at 450 nm with an MRX II absorbance reader (Dynex Technologies).

2.8 | Cell colony formation assay

Cells were seeded at 40 cells/cm² in 6-well plates and incubated for 2 weeks. Cells were fixed with methanol and stained with 0.1% crystal violet. Visible clones containing over 50 cells were counted.

2.9 | Cell apoptosis assessment

Cell apoptosis was assessed with a PI and Annexin V-APC apoptosis detection kit (KeyGen BioTECH). Briefly, the cells were trypsinized, resuspended in AnnexinV-binding buffer and incubated with AnnexinV-APC/PI in the dark for 20 min. The samples were detected using a FACS Calibur flow cytometer (BD Biosciences) and analysed by FloMax software. The measurement of mitochondrial membrane potential (MMP), associated with early apoptotic process, was described in detail in Appendix S1.

2.10 | RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA isolation and qRT-PCR analysis were carried out as previously described. Levels of relative expression were calculated and quantified with the 2^−ΔΔCT method after normalization with reference to the expression of GAPDH.

2.11 | Western blot analysis

Western blot analysis was carried out as previously described with the following appropriate primary immunoblotting antibodies: anti-GAPDH (ab8245) and anti-Tubulin (ab6160), which were purchased from Abcam; anti-MPST (Abcam ab85211 and Novus NB81-82617 for human and mouse respectively); anti-E2F1 (3742), anti-CDK4 (12790), anti-FOXO3a (12829), anti-p-FOXO3a (phospho S253, 13129), anti-RB (9309), anti-p-RB (8516), anti-p-AKT (phosphor S473) (4060), anti-AKT (4691), anti-CCND1 (2978), anti-p27 (3686), anti-cleaved-caspase 3 (9664), and anti-PARP (9532), which were purchased from Cell Signalling Technology.

2.12 | Tumour xenograft experiments

A total of 1 × 10⁷ LM3 cells were resuspended in 100 μl of PBS and injected subcutaneously into the lateral flanks of immunodeficient mice. Tumour volumes were measured every third day after 1 week using the equation: V (cm³) = width² (cm²) × length (cm)/2. Tumours were harvested for immunostaining 4 weeks after tumour implantation. For evaluation of the effect of MPST knockdown in vivo, after the appearance of a palpable tumour, nude mice bearing LM3 xenografts were randomized to receive NC or chemically modified MPST siRNA (RiboBio) locally injected into the tumour mass every 4 days for 2 weeks.

2.13 | Diethylnitrosamine-induced hepatocarcinogenesis

3-Mercaptopuruvate sulfurtransferase-knockout (KO) mice were generated and maintained as described previously. All mice were housed under pathogen-free conditions in a temperature-controlled room (23°C) on a 12-h light/dark cycle and consumed water ad libitum. At postnatal day 14, male MPST-KO and the littermate wild-type (WT) control mice were given a single intraperitoneal injection of diethylnitrosamine (DEN, 25 mg/kg body weight; Sigma-Aldrich). Nine months later, the mice were sacrificed and necropsied. Tumour
number and largest tumour size were determined by counting the number of visible tumours (at least 1 mm in diameter) and measuring the size of the largest tumour with a calliper. The livers were then separated into individual lobes, analysed for the presence of tumours, and subjected to histological analysis. All of the animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

2.14 | Statistical analysis

SPSS 22.0 software was used for statistical analysis. The experimental data are expressed as the mean ± SD and were assessed by a two-tailed Student’s t test or Mann–Whitney U tests. The OS rate was calculated with the Kaplan–Meier method, and the significant difference between survival curves was determined with the log-rank test. Statistical significance was accepted if $p < .05$.

3 | RESULTS

3.1 | Downregulation and clinical significance of 3-mercaptopyrurate sulfurtransferase expression in hepatocellular carcinoma

Based on the data from gene expression omnibus (GEO) database (GSE62232), compared to normal liver tissues, MPST was identified to be downregulated in HCC (Figure 1A) caused by various aetiologies (Figure S1A). We then performed q-PCR to verify the results and found that the mRNA expression of MPST was significantly downregulated in 30 primary HCC tissues compared with their adjacent nontumour tissues (Figure 1B). Besides, the relative MPST mRNA level was negatively correlated with the tumour size (Figure 1C). Next, we found protein level of MPST expression is markedly decreased in HCC caused by common aetiologies, among which hepatitis B virus (HBV)-related HCC is the most notable (Figure S1B). This finding was further validated by western blot analysis of MPST.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Downregulation and clinical significance of MPST expression in HCC. (A) MPST expression is significantly decreased in HCC patients compared with normal liver tissues according to the analysis of GEO database. GSE62232, normal, $n = 10$; HCC, $n = 81$. (B) Significant downregulation of MPST was detected in clinical HCC tissues by qPCR ($p < .0001$). (C) Correlation analysis of relative MPST mRNA expression and tumour size in clinical HCC tumour tissues. (D) Downregulation of MPST expression was frequently detected in primary HCC tissues by western blot analysis. (E) Representative MPST expression in a pair of HCC (middle) and adjacent nontumour tissues (left) detected by immunostaining with anti-MPST antibody (brown). Negative expression of MPST (right) was detected in one HCC case. (original magnification, top 100×, bottom 400×). Semiquantitative analysis for the IOD of MPST positive areas is shown on the right of the images. (F) Kaplan–Meier curves for the overall survival rate of patients with HCC according to the expression status of MPST. Red, patients with high expression of MPST ($n = 43$, median survival, 43 months); blue, patients with downregulation of MPST expression ($n = 47$, median survival, 16 months). **$p < .01$, ****$p < .0001$. GEO, gene expression omnibus; HCC, hepatocellular carcinoma; IOD, integral optical density; MPST, 3-mercaptopyrurate sulfurtransferase
protein expression in an additional 12 paired HBV-associated HCC samples (Figure 1D).

To further explore the prognostic value of MPST in HCC, IHC studies were performed in TMA containing paired tumour and adjacent tissue specimens from 90 HCC patients. The protein expression level of MPST in HCC tissues was weak in 80 cases compared with their adjacent tissue specimens, including one with negative staining (Figure 1E). And lower MPST expression was significantly associated with larger tumour size (Table 1). In addition, Kaplan–Meier analysis revealed that HCC patients with low expression of MPST had worse OS (Figure 1F).

### 3.2 Overexpression of 3-mercaptopyruvate sulfurtransferase represses hepatocellular carcinoma cell proliferation

The association of lower MPST expression with larger tumour size and increased OS suggests its potential inhibitory role in HCC progression. To test this hypothesis, we assessed the effect of MPST on the proliferation and growth of HCC cells. As shown in Figure 2A, MPST was overexpressed by plasmid-mediated transduction in HCC cell lines, including LM3 and HepG2 cells, as determined by western blot. The results showed that overexpression of MPST significantly inhibited cell proliferation in both LM3 and HepG2 cells (Figure 2B). The colony formation assay confirmed the inhibitory effect of MPST on cell growth, showing a significant decrease in the colony number of the MPST-overexpressing cells compared with the control cells (Figure 2C). These in vitro data indicated the suppressive function of MPST in HCC.

| Variable                  | MPST density |  | p value |
|---------------------------|--------------|---|---------|
|                           | Low-MPST | High-MPST |
| In general                |           |         |         |
| Peritumoural tissue       | 0        | 90      | <.0001* |
| Tumour tissue             | 47       | 43      |         |
| Sex                       |           |         |         |
| Male                      | 43       | 35      | .159    |
| Female                    | 4        | 8       |         |
| Age (years)               |           |         |         |
| ≤50                       | 17       | 14      | .719    |
| >50                       | 30       | 29      |         |
| Tumour size (cm)          |           |         |         |
| ≤5                        | 14       | 24      | .016*   |
| >5                        | 32       | 19      |         |
| TNM stage                 |           |         |         |
| I–II                      | 20       | 24      | .209    |
| III–IV                    | 27       | 19      |         |

*Statistical significance (p < .05) is shown.
3.3 3-Mercaptopuruvate sulfurtransferase inhibits the tumour formation and growth in vivo

To further investigate the role of MPST in the tumour growth of HCC in vivo, we extended our investigation by subcutaneous implantation of MPST-overexpressing and control HCC cells in nude mice. Tumour growth was significantly suppressed in the cells overexpressing MPST compared with the control cells, resulting in a significant reduction in tumour volume (Figure 3A). Moreover, an improved histological pattern of HCC tumours was shown in the MPST-overexpressing group compared with the control group (Figure 3B). IHC staining showed that the tumours derived from the MPST-overexpressing cells displayed much lower expression of Ki-67, a marker of cell proliferation, than the tumours from
the control group (Figure 3C). We next explored the effects of MPST knockdown on the growth of established HCC xenografts. After the appearance of a palpable tumour at day 14, nude mice bearing LM3 xenografts were randomized to receive cholesterol-conjugated MPST siRNA or negative control for the following 16 days. Significant differences in tumour volumes between the MPST-siRNA-treated and control groups were observed from 4 days after the first initiation of treatment (Figure 3D). At the end of treatment, knockdown of MPST expression significantly promoted tumour growth, with a more than 1.5-fold higher in final volume than that of the control mice (Figure 3E). To further address the role of MPST in hepatocarcinogenesis, we constructed a classical DEN-induced HCC mouse model with C57BL/6 mice comprised of MPST-KO and WT male mice. Nine months after DEN treatment, the livers of each group of mice were separated, and the tumour number and size were recorded. All mice presented tumour growth, and the MPST-KO mice exhibited markedly more tumour number than the WT mice (Figure 3F). Histological assessment of the livers revealed that the MPST-KO mice developed more severe steatosis, focal dysplasia and HCC than the WT mice (Figure S2A). These findings indicated that MPST suppressed the tumour growth while MPST deficiency promotes HCC development in vivo.

3.4 Hydrogen sulfide is involved in the regulation induced by 3-mercaptopyrutarate sulfurtransferase in hepatocellular carcinoma

3-Mercaptopyrutarate sulfurtransferase is an important enzyme that regulates \( \text{H}_2\text{S} \) synthesis, which is involved in the development and progression of many types of cancer. To examine the effect of \( \text{H}_2\text{S} \) on HCC, we treated LM3 cells with NaHS, the \( \text{H}_2\text{S} \) donor, for 24 h. \( \text{H}_2\text{S} \) significantly promoted cell proliferation in LM3 cells in a dose-dependent manner (Figure 4A). Therefore, we hypothesized that MPST regulated HCC development partly through the regulation of \( \text{H}_2\text{S} \) production. As illustrated in Figure 4B, overexpression of MPST led to a significant decrease in \( \text{H}_2\text{S} \), while siRNA-mediated downregulation of MPST expression resulted in increased levels of \( \text{H}_2\text{S} \) in a pattern of negative regulation in HCC cells. Mechanistically, treatment with NaHS accelerated cell cycle progression by decreasing the G0-G1 population and increasing the G2/M phase cell population (Figure 4C). In addition, increasing evidence shows that \( \text{H}_2\text{S} \) is involved in the regulation of mitochondrial function and cellular bioenergetics, involving the generation of ROS. The results showed that MPST overexpression led to a significant reduction in intracellular ROS levels in both LM3 and HepG2 cells (Figure 4D). In line with the in vitro data, we detected a reduction in ROS in tumours derived

![Image](https://example.com/image1.png)

**FIGURE 4** Hydrogen sulfide (\( \text{H}_2\text{S} \)) is involved in the regulation induced by MPST in HCC. (A) Effect of \( \text{H}_2\text{S} \) on cell proliferation. NaHS treatment induced for 24 h in HCC LM3 cells in a dose-dependent manner. Cell proliferation was measured by using a CCK-8 kit. (B) Negative regulation of the level of \( \text{H}_2\text{S} \) induced by MPST in HCC cells. (left panel) Overexpression of MPST led to a significant decrease in \( \text{H}_2\text{S} \), while siRNA-mediated inhibition of MPST resulted in increased levels of \( \text{H}_2\text{S} \) (right panel). Experiments were performed in \( n=3–6 \) per group from three independent experiments. (C) Effect of \( \text{H}_2\text{S} \) on cell cycle distribution in HCC cells. (D) Determination of ROS generation in HCC cells after the overexpression of MPST. (E) Decreased ROS generation was detected in the MPST-overexpressing HCC cell-generated xenografts in nude mice. Experiments were performed in \( n=5–6 \) per group from two experiments. Error bars represent the SD. *\( p < .05 \), **\( p < .01 \) compared with the corresponding negative controls. HCC, hepatocellular carcinoma; MPST, 3-mercaptopyrutarate sulfurtransferase; ROS, reactive oxygen species.
from the MPST-overexpressing cells (Figure 4E), indicating its contribution to slowing HCC cell proliferation and tumour growth. These data collectively suggest that H$_2$S plays an important role in MPST-regulated HCC development.

3.5 Overexpression of 3-mercaptopropionate sulfurtransferase induces G1-phase cell cycle arrest and regulates the phosphorylation of Rb in hepatocellular carcinoma

To further determine the mechanism associated with growth inhibition by MPST, we evaluated the cell cycle, and the results showed that overexpression of MPST increased the number of cells in G1 phase in HCC cells (Figure 5A). Our above data revealed the effect of MPST regulation on the generation of ROS, which act as signalling molecules which activate oncogenic pathways like PI3K/AKT that plays an important role in cell cycle progression through the G1 phase. We found that the phosphorylation levels of AKT were decreased in the MPST-overexpressing HCC cells (Figure 5B). The phosphorylation of FOXO3a, a major PI3K/AKT effector involved in the regulation of the cell cycle and apoptosis in human cancers, was also inhibited in response to MPST overexpression (Figure 5B). In addition, MPST overexpression significantly enhanced the protein level of p27, a downstream protein of AKT/FOXO3a pathway, in HCC cells compared with the control cells (Figure 5B). Retinoblastoma protein (Rb), an important transcriptional repressor of G1-S progression, is also transcriptionally regulated by the AKT/FOXO3a pathway.\textsuperscript{15} The activity of Rb was controlled by the phosphorylation by CDK complexes. Significant reductions in cyclin D1 (CCND1) and CDK4 levels were observed upon the overexpression of MPST in LM3 and HepG2 cells (Figure 5C). And we found that MPST overexpression markedly suppressed the phosphorylation level of Rb (p-Rb), resulting in an inhibitory effect on E2F-mediated gene expression, which is crucial for the initiation of DNA replication, in LM3 and HepG2 cells (Figure 5C). Analysis of tumour tissues by IHC verified that MPST overexpression inhibited p-Rb, while knockdown of MPST induced by intratumoral siRNA injection enhanced the expression of p-Rb (Figure 5D). These findings suggest that the upregulation of MPST expression may suppress HCC cell growth by inhibiting AKT/FOXO3a/Rb signalling.

3.6 3-Mercaptopropionate sulfurtransferase promotes apoptosis

In addition to cell cycle regulation, it is well established that Rb affects tumour progression by regulating apoptosis, which plays a critical role in tumorigenesis.\textsuperscript{16} Flow-cytometric analysis revealed significantly higher percentage of apoptosis in the MPST-overexpressing HCC cells (Figure 6A). Mitochondrial dysfunction was demonstrated to be an important early step in the regulation of the apoptotic process, in which a reduction in MMP is an early event.\textsuperscript{17} We found that overexpression of MPST led to a significant decrease in MMP in HCC cells (Figure 6B).

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Overexpression of MPST induces G1-phase cell cycle arrest and regulates the AKT/FOXO3a/Rb pathway in HCC. (A) Flow cytometric analysis of cell cycle distribution. Overexpression of MPST induced a significant accumulation of cells in G1 phase. Error bars represent the SD from three independent experiments. (B) The protein levels of p-AKT, AKT, p-FOXO3a, FOXO3a and p27 after the overexpression of MPST are shown by western blot analysis. GAPDH was used as a loading control. (C) Detection of protein levels of CCND1, CDK4, pRb, Rb and E2F1 after the overexpression of MPST. Tubulin was used as a loading control. (D) Immunohistochemistry analysis of p-Rb in MPST-overexpressing HCC cell-generated xenografts or tumours with intratumour delivery of MPST siRNA. *p < .05, **p < .01 compared with the corresponding negative controls. HCC, hepatocellular carcinoma; MPST, 3-mercaptopropionate sulfurtransferase.}
\end{figure}
Since apoptosis is often mediated by the activation of caspases that lead to PARP binding to fragmented DNA, western blot analysis was then used to detect caspase activation. The results showed that cleavages of caspase-3, and PARP were dramatically increased in the MPST-overexpressing cells compared with the control cells (Figure 6C).

3.7 | The combination of 3-mercaptopyruvate sulfurtransferase and p-Rb levels has better prognostic value for hepatocellular carcinoma

The correlation between p-Rb expression and survival data of HCC was further evaluated. The results of Kaplan–Meier analysis showed that OS was significantly lower in HCC patients with high expression of p-Rb (Figure 7A). We further analysed the association between the expression levels of MPST and p-Rb in 85 pairs of HCC samples by IHC. TMA analysis revealed a significantly negative correlation between MPST and p-Rb levels (Figure 7B,C).

Patients whose tumours had low levels of MPST and high p-Rb had worse OS (Figure 7D). In summary, evaluation of MPST and p-Rb expression is a powerful predictor of poor prognosis for HCC patients.

4 | DISCUSSION

The expression pattern and role of MPST in chronic liver disease progression is what we are of interest and have long been focusing on. By analysis of the data set from GEO database, we found the expression of MPST is significantly increased in NAFLD and NASH (Figure S3A), while significantly downregulated in patients with cirrhosis (Figure S3B), considered a premalignant condition and pre-disposing to HCC. Despite the development in the diagnosis and treatment, HCC remains a “difficult to treat” cancer, and the prognosis of the patients remains poor in a large proportion of cases. There is an urgent need to identify novel molecular targets, possibly with
the potential to be translated into clinical practice. The expression and function of MPST in HCC is intriguing.

In the present study, the MPST expression level was significantly downregulated in the HCC tissues compared with the corresponding nontumour tissues. Moreover, our data showed that higher MPST expression is associated with better clinical outcome in HCC patients. Next, gain-of-function analyses of MPST in vitro and in vivo suggested that MPST could suppress cell proliferation, promote apoptosis and inhibit HCC tumour growth in nude mice. In addition, MPST deficiency promotes DEN-induced hepatocarcinogenesis in a genetic model. Mechanistically, inhibition of H$_2$S production and the AKT/FOXO3a/Rb signalling pathway played important roles in the process of MPST-induced HCC suppression. Furthermore, in a clinical HCC tissue microarray, we found that MPST expression was inversely correlated with phosphorylated Rb expression in tumour cells, and the combination of low MPST and high pRb levels enhanced the correlation with poor prognosis in HCC. Taken together, our findings revealed that MPST may serve as an important tumour suppressor in HCC development.

Evolving information suggests that the dysregulation of H$_2$S-generating enzymes might contribute to the development of HCC, predominantly with a focus on the pathogenic roles of CBS or CSE. MPST is localized in both the cytosol and mitochondria. High levels of MPST have been discovered in numerous cancer cell lines or tissues, including the astrocytoma U373, the neuroblastoma SH-SY5Y, the melanoma cell lines A375 and WM35, human lung adenocarcinoma cells and gliomas tissue. In contrast, MPST was only slightly expressed in papillary thyroid cancer tissues, similar to that in adjacent normal tissues, while in renal cell carcinoma resections, the MPST expression was highly variable. Ramasamy et al. identified MPST as a potential tumour marker for colorectal cancer since its expression was markedly reduced in advanced colon cancer and correlated with disease progression. Interestingly, MPST expression was found to be upregulated in HepG2 cells that exhibit stem-like properties when they recover from a cytotoxic stimulus. Here, we observed that the expression of MPST was decreased in most of our HCC tissues; moreover, low MPST expression was associated with worse OS and larger tumour size in HCC patients. This discrepancy might be attributed to the fact that the MPST system in cancer cells appears to be highly complex and may well be highly cell-type and context-dependent.

However, to date, the modulatory effect of MPST on cancer progression remains largely unknown. A recent study showed that silencing of MPST reduced the proliferation rate of the human lung adenocarcinoma cell and attenuated the mitochondrial DNA repair rate. In the murine hepatoma cell line Hepa1c1c7, siRNA-mediated inhibition of MPST reduced bioenergetic parameters; however, the mechanism of how MPST regulates HCC development remains unclear. In this study, we focused on the biological function of MPST in HCC cell growth and proliferation. The results showed that overexpression of MPST significantly decreased HCC cell viability and inhibited cell proliferation in LM3 (high-grade malignant)
and HepG2 (low-grade malignant) cells. Besides, we detected a similar phenotype after MPST overexpression in two different HCC cell lines, Huh7 and Hep3B cells (Figure S4), whereas in not quite the same manner because of the different degrees of differentiation and grades of malignant properties that HCC cells have. We also verified the inhibitory effects of MPST on hepatocarcinogenesis and tumour development in both a mouse model of HCC xenografts and a DEN-induced HCC model in MPST-KO mice.

Recent studies suggest that the MPST/H2S system plays a functional role in cancer progression through several mechanisms.24 In the current study, we found that H2S significantly promoted cell proliferation in HCC cells. MPST overexpression induced inhibition of H2S, while siRNA-mediated downregulation of MPST expression resulted in increased levels of H2S in HCC cells, indicating the role of H2S production in regulating the proliferation of MPST-regulated HCC development. We speculate that this negative regulatory effect on H2S level should be attributed to the feedback between MPST and CBS/CSE system involved in H2S-metabolism. Our further study confirmed that H2S could serve as a pro-proliferative factor by accelerating cell cycle progression in HCC cells, consistent with previous reports.8 The regulation of mitochondrial bioenergetics also contributes to the pro-cancer effect of H2S,26 and we detected parallel changes in H2S and ROS levels after MPST overexpression, verifying the involvement of cellular bioenergetics and potential oxidative stress in MPST/H2S-regulated HCC development.

Our data also indicated that the molecular mechanism by which MPST inhibited HCC cell proliferation and tumorigenesis involves triggering G1-phase arrest and inducing apoptosis via suppression of the AKT/FOXO3a signalling pathway, hypophosphorylation of Rb and accumulation of p27. PI3K/AKT is an important signalling pathway involved in cell survival, inhibition of apoptosis, cell cycle progression and proliferation in human cancers.27 Activated AKT is known to phosphorylate specific targets such as FOXO3a and leads to its cytoplasmic translocation, while hypophosphorylated FOXO3a is released from 14-3-3 protein and translocates into the nucleus, where it transactivates target genes that control cell death and thereby induce apoptosis.28,29 Besides, the AKT/FOXO pathway has been shown to promote cell growth by inactivating the negative cell-cycle regulator p27.30

Our study also suggested an important role of MPST in the regulation of apoptosis involved in HCC development. Mitochondria manage apoptotic signals that include changes in electron transport, loss of MMP and release of caspase activators.31 A breakdown in the MMP is an invariant feature of early apoptosis. In the current study, we found that overexpression of MPST induced a significant decrease in MMP in HCC cells. In view of this, the apoptosis promoted by MPST is partly mediated by the mitochondrial pathway. The Rb pathway acts as a master checkpoint in cell cycle progression, and regulates cell apoptosis. It is mediated by the interaction of Rb with other cellular proteins, especially E2F transcription factors.32 Phosphorylation activated by CDK4-cyclin D1 complexes enables E2F release from Rb, resulting in the transcription of a number of genes that are necessary for DNA synthesis.33 However, hypophosphorylated Rb actively inhibits cell cycle progression from the G1 to S phase and induces premature senescence.34 Further studies will be necessary to elucidate the regulatory mechanism of MPST on Rb signalling pathways. Notably, the predictive value of MPST expression levels combined with the p-Rb signal was more sensitive than that of MPST alone for OS rate. This result identified the combined evaluation of MPST and p-Rb levels as a new prognostic marker in patients with HCC. This finding is important because these factors provide not only a new criterion for prognosis but also a potential therapeutic target.

In summary, our findings showed that MPST plays an important role in suppressing proliferation and tumorigenesis in HCC and is associated with H2S production and the AKT/FOXO3a/Rb signalling pathway. Therefore, we propose that MPST may function as a potential tumour suppressor and serve as a candidate predictor of outcome in HCC patients. Further studies are warranted to determine the molecular mechanism driving the loss of MPST during HCC progression and how H2S interacts with the various constituents of the cellular microenvironment so as to regulate cell signalling functions in HCC development. The restoration of MPST could be an effective therapeutic strategy for HCC treatment.

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CONFLICT OF INTEREST
The authors declare no competing interests.

ETHICS APPROVAL STATEMENT
This study was approved by the local ethics committee, the Clinical Specimens Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine. The principles of the Declaration of Helsinki were followed. All of the animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

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SUPPORTING INFORMATION

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