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PII: S0021-9258(22)01121-8
DOI: https://doi.org/10.1016/j.jbc.2022.102678
Reference: JBC 102678

To appear in: Journal of Biological Chemistry

Received Date: 23 April 2022
Revised Date: 23 October 2022
Accepted Date: 25 October 2022


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Anti-diabetic drug metformin suppresses tumorigenesis through inhibition of mevalonate pathway enzyme HMGCS1

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Abstract

Metformin, an antidiabetic drug, shows some potent anti-tumor effects. However, the molecular mechanism of metformin in tumor suppression has not been clarified. Here we provided evidence using in vitro and in vivo data that metformin inhibited mevalonate pathway by downregulation of HMGCS1, a key enzyme in this pathway. Our results further demonstrated that metformin downregulated HMGCS1 expression through inhibition of transcription factor NRF2. Additionally, we determined that HMGCS1 was highly expressed in human liver and lung cancer tissues and associated with lower survival rates. In summary, our study indicated that metformin suppresses tumorigenesis through inhibition of the NRF2/HMGCS1 axis, which might be a potential target in cancer prevention and treatment.
Introduction

Tumor growth and metastases need a constant and excessive supply of nutrients as building bricks, fuels and signals so that altered nutrient metabolism is widely accepted to be added to the list of hallmarks of cancer. Among those nutrient sources, mevalonate pathway intermediates have been reported significantly accumulated in multiple tumors. Recent studies reported that mevalonate pathway and its key enzyme HMGCR had additional potent tumor-supportive effects besides sterols biosynthesis. Driven by oncogenes such as mutant p53 and MYC, the mevalonate flux plays a favorable role in several types of cancers. Several studies focus on the mevalonate flux's role in the misfolded state and stability of tumor suppressor gene p53, thus forming a positive feedback loop in tumor formation. The mevalonate pathway provides several intermediate products that are important to tumor growth, for example, ubiquinone to adjust redox control and mitochondrial activity during nutrient restriction, and squalene for prevention of oxidative cell death, bile acid signaling and its crosstalk with microbiota, let alone cholesterol, which is important for cell membrane formation and signal transduction in cancer cells.

Metformin, an oral hypoglycemic agent mainly prescribed to patients with type 2 diabetes, is known for its pleiotropic functions and safety. Besides the antidiabetic effects, metformin also shows the latent capacity to prevent and improve the prognosis of multiple cancer such as breast cancer and prostate cancer. According to ClinicalTrials.gov, 393 clinical trials are registered using metformin in the treatment of cancer till February 2022. Diabetes Prevention Program Outcomes Study (DPPOS) demonstrated that metformin was associated with a 12% lower risk of cancer in a 22-year follow-up. After adjusting for age, gender, viral hepatitis and other variables, metformin still reduced the incidence of hepatocellular carcinoma (HCC). Since liver is a key organ for cholesterol homeostasis and bile production, it would be of interest to explore the effect of metformin on mevalonate pathway and HCC development. HCC accounts for nearly 90% of all liver cancers, casting heavy burden on public health and global economy. Therefore, there is an urgent need to identify the critical signaling pathways and molecular targets in the HCC for the prevention of progression and metastasis.

To date, there is no report about metformin-induced suppression of tumorigenesis through inhibition of mevalonate pathway. Nuclear factor E2-related factor 2 (NRF2) is a transcription factor in oxidative stress response. NRF2 activation is reported to be associated
with poor survival and tumor invasion of HCC. In this study, we investigated the role of mevalonate pathway enzyme HMGCS1 in tumorigenesis and hypothesized that metformin functioned through inhibition of the NRF2-HMGCS1 axis to diminish tumor proliferation.

**Results**

**Metformin inhibits mevalonate pathway enzyme HMGCS1 expression in cancer cells**

Some pieces of research showed that metformin improved survival rate in patients with HCC through pleiotropic effects including the influence of cholesterol biosynthesis in cancer cells. We postulated that metformin may achieve this effect through the regulation of mevalonate pathway.

We first checked the enzymes in the mevalonate pathway for their mRNA levels in HepG2 cells treated with metformin, and verified that HMGCS1, rather than ACAT, HMGCR, HMGCS2, was the mere enzyme that underwent an expression change (Fig. 1A, D). HepG2 cells treated with 20mmol/L metformin for 48 hours showed a time-dependent reduction (Fig. 1A) in mRNA level of HMGCS1 and was significantly reduced from 5 mmol/L to 20 mmol/L of metformin (Fig. 1B). There was a sharp decrease in the protein level of HMGCS1 while treated with 20mmol/L metformin for 48 hours (Fig. 1C). We used the UPLC-MS to measure the contents of metabolites in the mevalonate pathway in hepG2 cell after metformin treatment (Fig. 1E). The concentrations of HMG-CoA and mevalonic acid showed a significant decrease, while there was no significance change in acetyl-CoA and acetoacetyl-CoA concentrations. We then put emphasis on HMGCS1, the enzyme for HMG-CoA production. The inhibition of HMGCS1 expression by metformin was also observed in lung cancer cells A549 (Fig. S1A) and H1299 (Fig. S1B). These results show that metformin inhibits HMGCS1 expression in both mRNA and protein levels in liver cancer and lung cancer cell lines.

**Metformin regulates HMGCS1 expression through NRF2**

To determine whether metformin regulates the expression of HMGCS1 through transcription factors, we conducted luciferase promoter assays in hepG2 cells and HEK293T cells under the treatment of metformin (Fig. 2A, B). We shortened the promoter activity region of HMGCS1 and narrowed down the binding sites of metformin on HMGCS1 to -267 to -838 (Fig. 2C). With the
help of the PROMO website\textsuperscript{19}, we found out several transcription factors that may work on the
binding sites, including YY1, C/EBPalpha, C/EBPbeta, GR, and NRF2. By overexpressing each of
these transcription factors in HEK 293T cells, we found out only NRF2 upregulated the
luciferase promoter assay of HMGCS1 (Fig. 2D, E). Mutations of the two binding sites of NRF2
ceased its inhibition of HMGCS1 promoter by metformin (Fig. 2F). ChIP provided further evidence
between metformin treatment and the reduction in NRF2 and HMGCS1 combination (Fig. 2G).
Since metformin did not change the mRNA expression of NRF2 (Fig. 2H), we examined the
phosphorylation level of NRF2 to investigate the mechanism through which metformin inhibits
NRF2. After treatment of metformin, phosphorylation levels of NRF2 were reduced in
nuclear and cytosolic extracts of HepG2 cells, especially in the nucleus (Fig. 2I).

We established NRF2-overexpressed A549 cells (Fig. S2A) and witnessed an increase in
HMGCS1 mRNA levels (Fig. S2B). To elucidate whether NRF2 regulates the expression of
endogenous HMGCS1, we used NRF2 activator Oltipraz or NRF2 inhibitor ML385 (Fig. S2C, D,
E) in cancer cell lines. Oltipraz upregulated the expression of NRF2 downstream genes Npnt,
Bmpr1a, and Igf1 as well as HMGCS1 (Fig. S2C), while ML385 led to the opposite trend,
causing downregulation of Npnt and Igf1 together with HMGCS1 (Fig. S2D). Protein levels of
endogenous HMGCS1 expression also changed synchronously with NRF2 (Fig. S2E). These
results are consistent with the former data that Oltipraz\textsuperscript{20} and ML385\textsuperscript{21}, although known as
activator and inhibitor of NRF2, which could also change the protein expression of NRF2.
These results suggest that endogenous HMGCS1 can be transcriptionally regulated by NRF2
in response to metformin treatment.

HMGCS1 overexpression reverses tumor suppressive effect of metformin
To determine whether the suppression of tumorigenesis by metformin depends on the
inhibition of HMGCS1, we constructed overexpression plasmid of HMGCS1. Cell viability
assessed by CCK8 was markedly restored after overexpressing HMGCS1 in HepG2 cells (Fig.
3A) and A549 cells (Fig. S3A). The percentage of apoptotic cells was soared with the treatment
of metformin and returned to the baseline level by add-on of overexpression of HMGCS1
(Fig. S3B,C). We further explored the anti-tumor activity of metformin in vivo.
Transplanted tumor models in nude mice were established, using HepG2 cells and A549 cells
transfected with HMGCS1 and control vectors, and treated with metformin (200mg/kg) or solvent control by gastric perfusion and sacrificed to measure the volume and weight of tumors and relative gene expression. The first day of cell injection was recorded as day 0, and metformin was introduced from day 9, with a total observation period of 15 days. Tumor growth was suppressed by metformin in liver cancer(Fig.3B,D,E) and lung cancer(Fig.3D,E,F) and the tumor-suppressing effect of metformin was reversed by HMGCS1 overexpression in nude mice xenograft models of both liver cancer (Fig. 3C,D,E) and lung cancer (Fig. S3D,E,G) without changing the weight of the mice (Fig. 3F, Fig.S3H).

Based on these facts, we verify that the tumor suppressive effect of metformin is mediated by mevalonate pathway key enzyme HMGCS1.

**RNA interference of HMGCS1 exhibits tumor suppressive effect**

To test the oncogenic role of HMGCS1, we used RNA interference of HMGCS1 in tumorigenicity assays. HepG2 cells (Fig. 4A) and A549 cells (Fig. S4A) were transfected with three different siRNA to knock down HMGCS1 for 4 days, respectively. RNA interference of HMGCS1 in both cell lines using potent KD2 siRNA demonstrated a marked tumor suppressive effect, as shown in cell viability (Fig. 4B, Fig.S4B), BrdU proliferation assay (Fig. 4C, Fig.S4C), cell cycle (Fig. 4D, Fig.S4D). RNA interference also led to tumor cell apoptosis (Fig.4E, Fig.S4E). These findings collectively indicate that RNA interference of HMGCS1 imitates the effect of metformin.

**Upregulation of HMGCS1 is associated with poor survival of liver and lung cancer patients**

We have investigated the oncogenic role of HMGCS1 in liver cancer cells and animals. We further explored the role of HMGCS1 in human liver cancer. In human HCC samples, expression of HMGCS1 was markedly increased in mRNA levels (Fig. 5A) and in protein levels by immunohistochemistry staining compared with the paired adjacent non-cancerous tissues (Fig.5B). Higher HMGCS1 expression in HCC tissues leads to a poor overall survival rate in hepatocellular carcinoma patients (Fig. 5C). Similar results were observed in the patients with lung cancer (Fig.S5A,B,C).
Discussion

Several previous studies revealed the relationship between mevalonate pathway and tumor formation. In this study, we shed light on the tumor suppressive role of metformin mediated by inhibition of mevalonate pathway enzyme HMGCS1. The mevalonate pathway exists in almost all cell types so its oncogenic role and possibility as a drug target are worth further investigation. HMGCS1, the first key enzyme in mevalonate pathway, turns acetoacetyl-CoA into HMG-CoA, which further undergoes the catalyze of HMGCR to generate mevalonic acid. Jiang et al.\(^2\) reported that HMGCS1 expression could be suppressed by metformin. Our results verified that HMGCS1 is an important target to mediate the anti-tumor activities of metformin. The expression levels of HMGCS1 and its downstream metabolite mevalonate decrease after treatment with metformin (Fig.1) and this decrease is synchronizing with the cell viability and tumor growth (Fig3). We also confirm that HMGCS1 is highly expressed in human liver cancer and lung cancer tissues and related to poor prognosis and overall survival rate (Fig5). It is possible that increased HMGCS1 expression in tumor tissues might contribute to cell division, proliferation and signal transduction.

The mevalonate pathway contributes to the progression of several types of cancers through its key enzymes and metabolites. Some researches were consistent with our point of view by pointing out that HMGCS1 is highly expressed in most cancer types and it is also related to the poor prognosis and drug resistance.\(^{23}\) Other studies found out that HMGCR-driven MYC activation\(^{24}\) and cholesterol production\(^{25}\) supports the growth of liver cancer. Lipid reprogramming including regulation of HMGCS1 and HMGCR contributes to epithelial-to-mesenchymal transition in hepatocellular carcinoma and invasive phenotype like invadopodia formation. In a mouse xenograft model of HT29 human colon cancer cell line, mevalonate replenish experiment reversed the tumor-suppressing effect of metformin.\(^{26}\) This result is consistent with our HPLC/MS results (Fig.1E) and verified our speculation that the presence of mevalonate is essential in tumor proliferation. Cell viability restored by mevalonic acid replenishment could be inhibited by metformin (Fig.S6). These facts all provide reasons why the inhibitors of the mevalonate pathway are under heated investigation for their anti-tumor activities.\(^{27, 28}\)

Studies over the past decade revealed that metformin functions through inhibition of mTOR to a great extent\(^{29}\). It happened that an observation gave a clue for the metabolite mevalonate’s role in activation of mTOR\(^{30}\). Chances are that there is a convergence of the
anti-tumor role of metformin and the mevalonate pathway, and mTOR is their shared downstream target. A study on renal cell carcinoma showed the additive effect of mevalonate pathway inhibitor statins and mTOR inhibitor [31].

Our study uncovers that the mevalonate pathway dependence is a common feature in liver cancer and lung cancer. Between the two lung cancer cell lines we used, A549 cells have more epithelial characteristics while H1299 cells are derived from mesenchymal connective tissues. We also used hepatocellular carcinoma cell line SNU182 and hepatoblastoma cell line HepG2. These four tumor cell lines are from different tissues and origins, all demonstrating the same trend that mevalonate pathway together with cell activity can be inhibited by metformin. These results validate our hypothesis that the oncogenic role of mevalonate pathway and HMGCS1 is universal in multiple tumors. We speculate that the anti-tumor effect of metformin could be reproduced in other tumor cell lines and even tumors in vivo.

Here, our results showed that HMGCS1 could be transcriptionally upregulated by NRF2. Previous studies reported NRF2 mutations and NRF2-Keap1 pathway activation throughout the hepatocarcinogenesis. [32] Constitutive NRF2 activation was observed in human cancer tissues. [33] The elevated expression of NRF2 may serve as a compensatory mechanism to resist ischemia and subsequent hypoxia. [34] We discovered that a large dose of metformin weakened the transcription activity of NRF2 by reducing its protein phosphorylation, leading to a decrease of HMGCS1 expression and tumor arrest. These observations are consistent with the established theory that NRF2 inhibitors are effective in treatment of aggressive tumors that grow rapidly and are resistant to treatment, while NRF2 inducers are used to protect normal cells from carcinogens through anti-oxidative effects. [35]

A piece of previous study showed that hmgcs1 gene was negatively regulated by Nrf2 in mice. [36] To our knowledge, it is the first time to demonstrate that NRF2 regulates HMGCS1 through transcriptional activity in controlling cancer progression. We postulate that the change of phospho-NRF2 under the treatment of metformin is probably induced by protein kinase C (PKC) λ/ι [37, 38] because we used an antibody specific for Nrf2 phosphorylated at S40. In previous studies, metformin not only inhibits the phosphorylation levels of Nrf2 [39] but also mediates Nrf2 degradation. [40]

To sum up, this present study revealed the key role of the NRF2-HMGCS1 axis in the anti-tumor activity of metformin. Consistent with this notion, we assume that HMGCS1 might
serve as a novel therapeutic target for liver cancer, which adds another piece to the puzzle of the relationship between metformin, cancer and metabolism.

Limitations of the study

In this research, mice that underwent a high dose of metformin remained the same weight, indicating that metformin suppresses the tumor progression at a safe dose without disturbance of energy metabolism or aggravation of cachexia. However, it should be noted that we used metformin with a dose beyond the physiological amount.\(^\text{[41]}\) In this regard, more investigations are needed to show the potential of metformin within the regular dosage for patients. Targeting the delivery system of metformin to improve its therapeutic efficacy is worth equal attention. The targets we predicted using the PROMO website may not include all possible scenarios. Based on the transcriptional regulation role of GATA 1 on HMGCS1 in the process of erythropoiesis, further investigations can be made to determine the role of GATA 1 in regulation of HMGCS1 in the development of solid tumors.\(^\text{[42]}\) Noteworthy, the downstream mechanism of HMGCS1-mediated tumor promotion function needs further investigation.

Conclusions

In summary, we unveil that metformin suppresses tumor growth and progression through inhibition of mevalonate pathway enzyme HMGCS1 (Fig.6). HMGCS1 also serves as a predictive biomarker of poor prognosis in patients with liver cancer and lung cancer.

Experimental Procedures

Reagents

Metformin was purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). Nrf2 pathway activator oltipraz and inhibitor ML385 were obtained from MedChemExpress (Monmouth Junction, NJ, USA). Antibody for HMGCS1 was purchased from Abcam (ab194971, Shanghai, China). The anti-NRF2 antibody was purchased from Proteintech (16396-1-AP, Wuhan, China) and Abcam (ab62352, Cambridge, MA, USA). The anti-phosphor-NRF2 antibody was purchased from Abcam (ab76026, Shanghai, China). The antibodies against GAPDH (#3683,
Cell Signaling Technology, Danvers, MA, USA), tubulin (#5346, Cell Signaling Technology, Danvers, MA, USA), Histone H3 (AH433, Beyotime, Shanghai, China) served as internal references for western blots.

Tumor xenograft model

All animal protocols were reviewed and approved by the Animal Care Committee of Zhongshan Hospital, Fudan University. All mice were housed in a temperature-controlled environment (20–22°C) with 12:12-h light/dark cycles with free access to food and drinking water. HepG2 and A549 cells with HMGCS1 overexpression for 48 hours or vehicle control were transplanted to nude mice. Each mouse was subcutaneously inoculated with HepG2 or A549 tumor cells at a volume of 5×10⁵/200μL. In two weeks, tumor bearing nude mice were allocated to metformin and PBS control groups, respectively. A 15-day treatment with metformin was applied then. Metformin (200mg/kg) or PBS control were administrated by gastric perfusion. Four mice were used in each group. Tumor volume was measured every 3 days according to the diameters. Body weight are assessed every 3 days.

Cell lines and culture conditions

Cell lines and the origins are listed as follows: HepG2 (hepatocellular carcinoma), SNU182 (hepatocellular carcinoma), A549 (lung cancer), H1299 (lung cancer), HEK 293T (human embryonic kidney).

Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin and streptomycin at 37°C and 5% CO2.

Transfection and agents

siRNA targeted to HMGCS1 and negative control were purchased from Jima Biotechnology Co., Ltd. (Shanghai, China). Overexpressed plasmids (HMGCS1, NRF2, YY1, C/EBPα, C/EBPβ, GR) were purchased from JiKai Gene Co., Ltd (Shanghai, China). Cell transfection was performed on HEK 293T cells, HepG2, and A549 using the Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions.

Cell Growth and Viability Assays
Cells were seeded at 5000 per well in 96-well plates and incubated overnight before drug treatment. 10uL CCK8 (Dojindo, Kyushu, Japan) was added to the 90uL cell culture system and cells were incubated for 1-4 h at 37°C and 5% CO2 before measurement.

Cell cycle analysis
Flow cytometry (Accuri C6 FACS, MA, USA) was used to analyze the cell cycle through Cell Cycle Analysis kit (BD Biosciences, USA). The FlowJo software (BD FACSCalibur, USA) was used for data analysis in flow cytometry.

Reporter Gene Assays
293T cells were seeded in 24-well plates, co-transfected with siRNA targeting different active regions of HMGCS1, pcDH-NRF2 plasmids or its control plasmids. The luciferase activity was assessed 24 hours after transfection.

Quantitative Real-Time PCR Analysis
Total RNA was extracted using the TRizol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Takara PrimeScript RT reagent kit was used to obtain cDNA (Takara, Tokyo, Japan). Primers for qPCR were as follows: HMGCS1 forward: 5' - TGTACACATCTCTCAGTATATGGTTCCC -3', reverse: 5' - AAGAAAAACCTCAAATCTTCCCT - 3', HMGCR forward: 5' - TGATTGACCTTTCCAGAGCAAG -3', reverse: 5' - CTAAATTGCCCATTCCACAGGC -3', ACAT1 forward: 5' - AAGCAGGCGATATGGGTG -3', reverse: 5' - ACATCAGTTAGCCCCTTTTAC -3', HMGCS2 forward: 5' - GCCCAATATGTGGACCAACT -3', reverse: 5' - GAAAGCCATAACGGGTCTGG -3', NRF2 forward:5' - TCAGCGACGAAAGATGATGA -3', reverse: 5' - CCACTGGTTTCTGACTGGATGT -3', NPNT forward: 5' - CTAATACGACGACGTATCA -3', reverse: 5' - AACAATCTCCGCATGAGCATA -3', BMPR1A forward: 5' - TGGCAAAGCAATGGCTATTAGTT -3', reverse: 5' - GCCTCTAGTCGTCATGAGCATA -3', IGF1 forward: 5' - GCTCTTCAGTCGTCATGAGCATA -3', reverse: 5' - GCCTCTAGTCGTCATGAGCATA -3'. All data were normalized to 36B4, and the expression levels were calculated by the 2−DDCT method.

Western Blot Analysis
Total proteins were extracted, separated by 10% SDS-polyacrylamide gels, and transferred to
PVDF membranes (Millipore, Bedford, MA). The membrane was blocked for 1 hour with 5%BSA, washed with TBST, and incubated overnight at 4°C with a primary antibody listed as before. Then the membrane was incubated with secondary antibodies for 1 hour at room temperature and detected using ECL reagents.

Chromatin immunoprecipitation-quantitative real time PCR (ChIP-qPCR)

Control IgG- and NRF2-ChIPed DNA libraries were prepared using MAGnify Chromatin immunoprecipitation system (Thermo Fisher Scientific). qPCR was performed using the same procedure described above in the qRT-PCR methods.

UPLC-MS/MS for metabolite detection

Cells are seeded in 6-well plates and cultured for 48 hours until the confluence of 80%-90%. Cells in each well were washed with 1mL PBS buffer (pre-cooled at 4°C) twice and added with 0.4mL HPLC grade methanol (pre-cooled at -80°C) containing 1μg/mL tridecanoic acid (91988-5G, Sigma-Aldrich). Cells were harvested by scraping and gently transferred to Eppendorf(EP) tubes. Samples were stored at -80°C for late assessment by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS).

Histological analysis

Liver cancer and lung cancer specimen and the corresponding adjacent tissues were obtained by surgery. For histological analysis, the tissues were fixed in 4% neutral buffered formalin overnight. Paraffin embedding, sectioning, blocking, incubation, and antigen retrieval were performed according to the standard protocols. The human study was approved by the Human Research Ethics Committees of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each subject.

Human studies

A total of 90 subjects with liver cancers and lung cancers, respectively were recruited for
survival analysis. The human study was approved by the Human Research Ethics Committees of Zhongshan Hospital, Fudan University. Written, informed consent was obtained from each subject.

Statistical Analysis

Data are presented as the mean ± S.D. Differences among groups were analyzed by one-way ANOVA followed by a t-test. A two-sided P < 0.05 was considered significantly different.

Acknowledgement

This study is supported by the grants from the National Nature Science Foundation (Nos.81820108008, 31830041) and the National Key Research and Development Program of China(No.2021YFC2700400)

Data Availability

All data discussed here are presented in the manuscript and supporting information.

References


Figure Legends

Fig.1: Metformin downregulates mevalonate pathway enzyme HMGCS1 in liver cancer cells (A) Time-dependent mRNA expression of HMGCS1 in metformin-treated HepG2 cells. Shown are mean ± SD (n=3). Cells were treated by 20 mmol/L metformin for 0, 12, 24, 36, 48 hours. (B) Dose-independent mRNA expression of HMGCS1 in metformin-treated HepG2 cells. Shown are mean ± SD (n=3). Cells were treated for 48 hours by 0, 5, 10, 20 mmol/L metformin.

(C) The levels of expression of HMGCS1 proteins in HepG2 cells, after 20 mmol/L metformin treatment for 48 hours, normalized with GAPDH.

(D) Real-time PCR comparing expression of ACAT1, HMGCR, and HMGCS2 in metformin-
treated HepG2 cells. Shown are mean ± SD (n=3).

(E) UPLC/MS analysis of metabolites in the mevalonate pathway. Cells were treated by 20 mmol/L metformin for 48 hours.

Fig.2: Metformin regulates HMGCS1 through NRF2
(A) Luciferase results for HMGCS1 promoter activity in HepG2 cells when treated with different concentrations of metformin for 48 hours. Shown are mean ± SD (n=3).
(B) Luciferase results for HMGCS1 promoter activity in HEK 293T cells treated with different concentrations of metformin for 48 hours. Shown are mean ± SD (n=3).
(C) Luciferase results for HMGCS1 promoter activity in HEK 293T cells in promoter deletion analysis of HMGCS1 when transfected with pcDH-NRF2 plasmid or its control plasmid for 48 hours, with or without 20 mmol/L metformin. Shown are mean ± SD (n=3).
(D) Luciferase results for HMGCS1 promoter activity in HEK 293T cells overexpressing each of these transcription factors (YY1, C/EBPalpha, C/EBPbeta, and GR) predicted by PROMO. Shown are mean ± SD (n=3).
(E) Luciferase results for HMGCS1 promoter activity in HEK 293T cells overexpressing NRF2. Shown are mean ± SD (n=3).
(F) Luciferase results for HMGCS1 promoter activity in HEK 293T cells with mutations in one, two binding sites, and control. Shown are mean ± SD (n=3) (above). A schematic graph about the mutation strategy of the NRF2 core binding site (TCAG) on Hmgcs1 promoter(below).
(G) Relative enrichment of ChIP obtained with NRF2 antibody over the IgG control in HEK 293T cells with 20mmol/L metformin treatment for 72 hours.
(H) Real-time PCR comparing expression of NRF2 in 20 mmol/L metformin-treated HepG2 cells. Shown are mean ± SD (n=3).
(I) The levels of expression of phospho-NRF2 and NRF2 proteins in the cytosol and nuclear of SNU182 cells, after 20 mmol/L metformin treatment, normalized with tubulin and histone H3. *p<0.05, **p<0.005, and ***p<0.0005

Fig.3: HMGCS1 overexpression partially reversed tumor-suppressive effect of metformin
(A) Effects of HMGCS1 overexpression on cell viability by CCK8 in HepG2 cells with or without metformin at 20 mmol/L for 48 hours. Shown are mean ± SD (n=6).
(B) Tumor volume (mm$^3$) over time (days) in nude mice subcutaneously inoculated with HepG2 cells, treated with metformin or vehicle control. (n=4)

(C) Tumor volume (mm$^3$) over time (days) in nude mice subcutaneously inoculated with HepG2 cells overexpressed HMGCS1, treated with metformin or vehicle control. (n=4)

(D) Effect of HMGCS1 overexpression on the growth of HepG2 cells inoculated into nude mice, with or without metformin treatment. (n=4)

(E) Effect of HMGCS1 overexpression on the tumor weights of tumor-forming experiment in nude mice using HepG2 cells, with or without metformin treatment. Shown are mean ± SD (n=4).

(F) Mice weight (g) of the four groups inoculated with HepG2 cells, presented as the mean ± SEM. (n=4) presented as the mean ± SEM. (n=4) *p<0.05, **p<0.005, and ***p<0.0005

Fig.4: RNA interference of HMGCS1 imitated tumor-suppressive effect of metformin
(A) The levels of HMGCS1 protein in HepG2 cells transfected with siRNA for HMGCS1 for 4 days, normalized with GAPDH.
(B) Effects of different incubation time of siRNA for HMGCS1 (1-5 days) on the cell viability by CCK8 of HepG2 cells. Shown are mean ± SD (n=6).
(C) BrdU incorporation assay in HepG2 cells, 2 and 4 days after transfected with siRNA for HMGCS1. Shown are mean ± SD (n=3).
(D) Cell cycle distribution measured by flow cytometry using a BrdU Flow Kit in HepG2 cells transfected with siRNA for HMGCS1 for 4 days.
(E) Percentage of apoptotic HepG2 cells transfected with siRNA for HMGCS1 for 4 days. Apoptotic cells are labeled with annexinV-FITC and measured by flow cytometry. Shown are mean ± SD (n=3). *p<0.05, **p<0.005, and ***p<0.0005

Fig.5: Upregulation of HMGCS1 is associated with poor prognoses of liver cancer
(A) Real-time PCR analysis of the HMGCS1 expression in hepatocellular carcinoma samples and the paired non-cancerous adjacent tissues. Shown are mean ± SD (n=14).
(B) HMGCS1 immunostaining in hepatocellular carcinoma and adjacent non-cancerous tissues.
(C) Kaplan-Meier curve showing the percent survival in hepatocellular carcinoma patients with high (n=57) or low (n=33) HMGCS1 expression (p=0.031). *p<0.05, **p<0.005, and ***p<0.0005
Fig.6: Schematic depiction of metformin’s role in tumor suppression through mevalonate pathway

Supplementary Fig.1: Metformin downregulates mevalonate pathway enzyme HMGCS1 in lung cancer cells
(A) (above) Real-time PCR analysis of HMGCS1 expression in A549 cells after 20 mmol/L metformin treatment for 48 hours. Shown are mean ± SD (n=3). (below) Western blots of HMGCS1 and GAPDH in lysates of metformin-treated A549 cells.
(B) (above) Real-time PCR analysis of HMGCS1 expression in H1299 cells after 20 mmol/L metformin treatment for 48 hours. Shown are mean ± SD (n=3). (below) Western blots of HMGCS1 and GAPDH in lysates of metformin-treated H1299 cells. Shown are mean ± SD (n=3). *p<0.05, **p<0.005, and ***p<0.0005

Supplementary Fig.2: Metformin regulates HMGCS1 through NRF2.
(A) The level of NRF2 proteins in A549 cells after transfected with PCDH-NRF2, normalized with GAPDH.
(B) Real-time PCR measuring HMGCS1 mRNA levels in A549 cells transfected with either empty vector or PCDH-NRF2 plasmid. Shown are mean ± SD (n=3).
(C) Real-time PCR measuring NRF2, NPNT, BMPR1A, IGF1, and HMGCS1 mRNA levels in A549 cells treated with 30uM Oltipraz for 48 hours. Shown are mean ± SD (n=3).
(D) Real-time PCR measuring NRF2, NPNT, IGF1, and HMGCS1 mRNA levels in A549 cells treated with 10uM ML385 for 48 hours. Shown are mean ± SD (n=3).
(E) Protein expression levels of NRF2 and HMGCS1 in SNU182 cells treated with 30uM Oltipraz or 10uM ML385 for 48 hours, normalized with GAPDH. *p<0.05, **p<0.005, and ***p<0.0005

Supplementary Fig.3: HMGCS1 overexpression partially reverses tumor-suppressive effect of metformin
(A) Effects of HMGCS1 overexpression on cell viability by CCK8 in A549 cells with or without metformin at 20 mmol/L for 48 hours. Shown are mean ± SD (n=6)
(B) Effects of HMGCS1 overexpression on the percentage of apoptotic A549 cells with or without metformin at 20 mmol/L for 48 hours. Apoptotic cells were labeled with annexin V-
FITC and appeared in the Q2 and Q3 quadrants.

(C) Effects of HMGCS1 overexpression on the percentage of apoptotic A549 cells with or without metformin at 20 mmol/L for 48 hours. Shown are mean ± SD (n=3)

(D) Effect of HMGCS1 overexpression on the growth of A549 cells inoculated into nude mice, with or without metformin treatment. (n=4)

(E) Effect of HMGCS1 overexpression on the tumor weights of tumor-forming experiment in nude mice using A549 cells, with or without metformin treatment. Shown are mean ± SD (n=4).

(F) Tumor volume (mm3) over time (days) in nude mice subcutaneously inoculated with A549 cells, treated with metformin or vehicle control. (n=4)

(G) Tumor volume (mm3) over time (days) in nude mice subcutaneously inoculated with A549 cells overexpressed HMGCS1, treated with metformin or vehicle control. (n=4)

(H) Mice weight (g) of the four groups inoculated with A549 cells, presented as the mean ± SEM. (n=4) *p<0.05, **p<0.005, and ***p<0.0005

Supplementary Fig.4: RNA interference of HMGCS1 imitated tumor-suppressive effect of metformin

(A) The levels of expression of HMGCS1 proteins in A549 cells transfected with siRNA for HMGCS1 for 4 days and normalized with GAPDH.

(B) Effects of different incubation time of siRNA for HMGCS1 (1-5 days) on cell viability of A549 cells by CCK8. Shown are mean ± SD (n=6).

(C) BrdU incorporation assay in A549 cells, 2 and 4 days after transfected with siRNA for HMGCS1. Shown are mean ± SD (n=3).

(D) Cell cycle distribution measured by flow cytometry using a BrdU Flow Kit in A549 cells transfected with siRNA for HMGCS1 for 4 days.

(E) Percentage of apoptotic A549 cells transfected with siRNA for HMGCS1 for 4 days. Apoptotic cells are labeled with annexin V-FITC and measured by flow cytometry. Shown are mean ± SD (n=3). *p<0.05, **p<0.005, and ***p<0.0005

Supplementary Fig.5: Upregulation of HMGCS1 is associated with poor prognoses of lung cancer

(A) HMGCS1 immunostaining in lung cancer and adjacent non-cancerous tissues.

(B) Quantification of immunohistochemistry staining of lung cancer and adjacent non-
cancerous tissues.

(C) Kaplan-Meier curve showing the percent survival in lung cancer patients with high (n=60) or low (n=30) HMGCS1 expression. *p<0.05, **p<0.005, and ***p<0.0005

Supplementary Fig.6: Mevalonate supplementation restores viability of HepG2 cells

(A) Effects of 5mM mevalonic acid lithium salt on cell viability by CCK8 in HepG2 cells with or without metformin at 20 mmol/L for 48 hours. Shown are mean ± SD (n=6)

(B) The levels of expression of AMPK-mTOR pathway proteins in HepG2 cells, after 20 mmol/L metformin treatment for 48 hours, with or without addition of 5mM mevalonic acid lithium salt, normalized with HSP90.
Declaration of interest: We have no conflicts of interests.
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