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DTYMK promote hepatocellular carcinoma proliferation by regulating cell cycle

Tianhao Zhou[a,b,#], Rui Qin[c,#], Susu Shi[d], Hua Zhang[c], Chuanling Niu[c], Gaoda Ju[d], and Sen Miao[c]

[a]Key Laboratory of Breast Cancer Prevention and Treatment of the Ministry of Education, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center of Cancer, Tianjin, China; [b]Department of Oncology, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [c]Department of Pathology, Affiliated Hospital of Jining Medical University, Jining, China; [d]Department of Oncology, Beijing Cancer Hospital, Peking University, Beijing, China

ABSTRACT

Overexpression of DTYMK is related with tumorigenesis and progression in several human tumors. However, the role of upregulated DTYMK in hepatocellular carcinoma (HCC) patients still remains unclear. In this study, the DTYMK expression in HCC tumors was evaluated in three GEO series (GSE14520, GSE54236, GSE63898), TCGA-LIHC, and ICGC-IRLR-JP cohorts. Survival analysis of DTYMK based on TCGA-LIHC and ICGC-LIRI-JP cohorts was conducted. We found that DTYMK was dramatically upregulated in tumor tissue compared with that in adjacent liver tissue. Kaplan-Meier analysis revealed that high expression of DTYMK in HCC patients' tumor tissue was significantly corresponded to worse overall survival (OS) (P < 0.05). Further analysis showed that overexpressing DTYMK led to poor relapse free survival (RFS) and disease-specific survival (DSS) (all P < 0.05). In conclusion, DTYMK is upregulated in tumors and correlated with poor prognosis in HCC patients. In our report, DTYMK is higher expression in HCC cancer tissue and cell line than tumor adjacent tissue and normal liver cell line. Knocking down DTYMK can inhabit tumor cell proliferation by interfering cell cycle, whereas overexpression of DTYMK can promote tumor cell proliferation. These findings indicate that upregulation of DTYMK enhances tumor growth and proliferation by promoting cell cycle.

Introduction

Primary liver cancer is one of the most common malignant tumors in the world, and approximately 90% of liver cancer is hepatocellular carcinoma (HCC). Every year more than 800,000 new cases and deaths are reported. HCC is highly proliferative, invasive and metastatic potential with a low survival rate [1,2]. A large number of studies have explored the mechanism of occurrence, recurrence and metastasis, tumorigenesis, and treatment of HCC. However, many problems remain to be clarified [3,4]. One group found that genetic aberrations have a high correlation with the occurrence, development, and progression of HCC[5]. It'= is urgent to find the original drive force and identify promising prognostic biomarkers for clinical diagnosis as well as targets for drug design [6].

DTYMK is known to catalyze the phosphorylation of dTMP to dTDP [7]. This enzyme plays a vital role in DNA synthesis *in vivo* and is a main intermediate enzyme in many pyrimidines analog drugs pathways [8,9]. DTYMK is highly expressed in the bone marrow, lymph node, colon, and appendix which cell proliferation and division are vigorous. Therefore, it may also be essential for the proliferation and division of tumor cells. Upregulation of DTYMK has been reported in nonsmall cell lung carcinoma [10], correlated with cell proliferation and LKB1-mutant related lung cancer metabolic disorder [11]. Other studies indicate that DTYMK is highly expressed in oral cancer, 5-FU relative drug-resistance colon cancer has functions in tumor occurrence and development. The role DTYMK plays in HCC, however, is controversially discussed. One research revealed that DTYMK is a poor predictor for well-differential HCC and promotes cancer stem cell character of

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CONTACT Gaoda Ju ✉ jgd0412@163.com; Sen Miao ✉ miaosen128@163.com

HCC cells [12]. Another study shows that DTYMK participates in immune cell infiltration and the high expression of DTYMK is a wicked factor of overall survival (OS) and disease-free survival (DFS) in HCC patients [13]. However, the specific mechanism of DTYMK in HCC patients is still required to further identification.

For the purpose of interpreting the relationship between DTYMK expression level and HCC outcomes, we identified the *DTYMK* mRNA expression in **Gene Expression Omnibus (GEO), Liver Cancer – RIKEN, JP Project from International Cancer Genome Consortium (ICGC-LIRI-JP)**, and **The Cancer Genome Atlas (**TCGA) databases and conducted a survival analysis based on TCGA, ICGC-LIRI-JP cohorts, and Kaplan-Meier Plotter database. We draw a conclusion that DTYMK is highly expressed in HCC patients and is a certain poor indicator of OS and PFS. Subsequent analysis reveals that DTYMK is correlated with HCC cell division and cell proliferation of P53-related patients. We examined the expression of DTYMK by immunohistochemistry in tumor tissues and paired precancerous liver tissue in 60 HCC patients. Meanwhile we conducted CCK8 proliferation assay in different cellar cycles to verify the subsequent outcomes. Our findings prove that DTYMK is a HCC proliferation malignant biomarker and a potent target for HCC diagnosis and therapy.

Materials and methods

Data acquisition and process

The expression matrix and clinical characters of cohorts included TCGA-LIHC, ICGC-LIRI-JP, GSE14520, GSE54236, and GSE63898 were downloaded from HCCDB database (http://lifeome.net/database/hccdb/home.html) [14]. For GEO cohorts, the probe signal intensity values were transformed by log2. For TCGA-LIHC and ICGC LIRI-JP cohorts, the normalized read counts were transformed by log2. The comparisons of mRNA expression level between cancer and noncancer tissues in 5 cohorts were conducted with unpaired *t* test with Welch's correction by GraphPad Prism 7.04 software. $P < 0.05$ was considered to be statistically significant.

Survival analysis

The mRNA expression of *DTYMK* and survival characters of patients in TCGA-LIHC and ICGC-LIRI-JP cohorts were extracted for Kaplan-Meier overall survival (OS) analysis. The liver cancer patients were divided into *DTYMK*-high and *DTYMK*-low group by the median mRNA expression value of DTYMK in cohorts independently. In addition, Kaplan-Meier Plotter [liver cancer] (http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq) was used to identify the correlation of DTYMK and OS, Relapse free survival (RFS), and disease specific survival (DSS) in liver cancer patients [15]. The log-rank test was applied for survival analysis and $P < 0.05$ was considered to be statistically significant.

The correlation of clinical parameters and DTYMK in liver cancer

The mRNA expression of *DTYMK* and clinical parameters of patients in TCGA-LIHC, ICGC-LIRI-JP cohorts were extracted. The patients were divided into two groups according to the single clinical parameter. The mRNA level of *DTYMK* was compared between two groups. In addition, tumor double time was investigated between *DTYMK*-high and *DTYMK*-low liver cancer patients in GSE54236 cohort. Unpaired *t* test with Welch's correction was applied for static analysis and $P < 0.05$ was considered to be statistically significant.

Functional enrichment analysis

Coexpressed genes with DTYMK were computed and displayed according to the guideline in the HCCDB database website. Meta scape (http://metascape.org) was used for the annotation and functional and disease-related enriched analysis of coexpressed genes, such as gene ontology and Kyoto encyclopedia of genes [16]. The Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/) was used for confirming the correlation of DTYMK with PCNA and MKi67[17].

Human samples

The experiment with human liver cancer tissues was authorized by the Human Ethics Committee.

All subjects were provided written informed consent.

Cell lines and culture conditions

Human HCC cell lines HepG2, HepG3B, LM3, and Huh7 and human normal liver cell line LO-2 were acquired from ATCC and cultivated in DMEM medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO_2. All cell lines were certificated by STR profiling and there was no mycoplasma contamination.

Western blotting

Cell proteins were extracted by denatured buffer and then quantified by pierce BCA protein assay (Thermo Scientific). The HCC cell line protein lysate were separated on SDS-PAGE, transferred to NC membranes (Millipore), blocked, and then detected by primary antibody DTYMK (Abcam, ab154867, 1:2000) and HRP-conjugated secondary antibody (Sigma), followed by being exposed with enhanced chemiluminescence (ECL). Housekeep gene β-Actin (Abcam, ab8226,1:5000) was used as a loading control.

Plasmids and lentivirus production

DTYMK cDNA were cloned into the viral skeleton plasmid PCDH-3*flag-tagged vector for stable expression in HCC cell lines. Annealing and connection of the shRNA were performed and then constructed into the modified plasmid pLKO.1. The well-constructed vectors were transinfected into HEK293T cells by lentivirus packaging plasmids psPAX and pMD2.0 G. The shRNA sequences and DTYMK primer sequence and DTYMK plasmid PCR primer in this research are shown below: DYMKT-sh1-F: CCGGTGGGAACAAGTGCCGTTAATTCTCGA-GAATTAACGGCACTTGTTCCCATTTTTTG; DYMKT-sh1-R: AATTCAAAAAATGGGAACAAGTGCCGTT-AATTCTCGAGAATTAACGGCACTTGTTCCCA; DYMKT-sh2-F: CCGGGTCGTGGACAGATACGCATTTCTCGA-GCAAATGCGTATCTGTCCACGACTTTTTTG;

DYMKT-sh2-R: AATTCAAAAAAGTCGTGGACAGATACGCA-TTTCTCGAGAAATGCGTATCTGTCCACGAC; DTYMK-F: TCGAGCTCAAGCTTCGAATTCATGGCGG-CCCGGCGCGGG; DTYMK-R: GTCATCCTTGTAGTCGGATCCCTTCCATA-GCTCCCCCAGCG.

Cell growth assay

Lentivirus-infected stable cells were seeded into 96-well plates and cultured in 10% FBS DMEM (2000 cells per well, five parallel wells). Then, the cells were collected at different points in time, and cell number in each well was counted by CCK-8 reagent. Absorbance at 450 nm was employed to determine of the number of viable cells.

Immunohistochemistry and tissue array

The human HCC tissue samples were harvested by pathology department after obtaining consent. Tumor and normal adjacent tissues 2 cm away from the tumor was prepared. The detailed clinicopathological data (show in supplementary table 2) were scored according to the tumor classification of the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) tumor staging system. For the tissue microarray (TMA) construction, all cancers and adjacent tissue samples were reevaluated and the representative areas were chosen. IHC was operated by standard IHC protocol as described before. Anti-DTYMK rabbit polyclonal antibody (1:200, Abcam, ab154867) and anti-PCNA rabbit polyclonal antibody (1:2000, ProteinTech, 15,910-1-AP) were used in this study. DTYMK expression level in tissues was evaluated by two pathologists independently. The methods of scores for staining frequency and intensity were added to obtain an overall staining score (OSS) was described previously [18]. A staining index (values, 0–12) was determined by multiplying the score for staining intensity with the score for both cytoplasmic and nuclear positive. Staining intensity grade is classified as: 0, negative; 1, weak; 2, moderate; 3, strong. The frequency of positive cells is defined as: less

than 5%, 0 point; 5%–25%, 1 point; 26%–50%, two points; 51%–75%, 3 points; More than 75%, 4 points 1[9].

Cell cycle assay

Cell Cycle and Apoptosis Analysis Kit was used to determine cell cycle based on the specifications. HepG2 and Huh-7 cells were cultured in 24 wells plate for 24 h. Next, cells were digested with trypsin and suspended into single cell, and fixed with 70% ethanol at 4°C at least 2 hours. Then cells were resuspended and washed by PBS. 0.2 mg/mL RNase A and 50 µg/mL PI in PBS was applied for staining cells for 30 min. The percentages of cells of G0/G1, S, and G2/M were counted by Beckman Cytoflex.

Colony formation assay

Cells were seeded into 6-well plate at a density of 100 cells per well with three parallel wells. After 3 weeks of cultivation DMEM with 10%FBS, formed clones were fixed by 10% formalin then stained with 0.1% crystal violet. Numbers of colonies were counted attentively.

Statistical analysis

All data were statistically analyzed with GraphPad Prism 8.0. Two-tailed t test was applied to analyze the difference between the two groups. Data were presented as mean ± SD or SEM. Differences at $P < 0.05$ were considered statistically significant.

Results

Expression pattern and prognostic implication of DTYMK in HCC

In our study, TCGA-LIHC, ICGC-LIRI-JP, and three GEO (GSE14520, GSE54236, and GSE63898) cohorts were utilized to analyzed the difference of *DTYMK* mRNA expression between HCC and normal hepatic tissues. It showed that *DTYMK* mRNA expression level was higher in HCC samples than corresponding adjacent normal samples with $P < 0.05$ (Figure 1(a-e)). Moreover,

we found that high *DTYMK* expression was significantly associated with later stages (stages III + IV) (Figure 1(f,g)), vascular invasion (Figure 1(h, i)), more severe fibrosis (F3-4) (Figure 1(j)), and shorter tumor doubling time (Figure 1(k)). Besides, TCGA-LIHC and ICGC-LIRI-JP cohorts and Kaplan-Meier Plotter [liver cancer] online tool were utilized to explore the correlation between *DTYMK* and survival in HCC patients. Our results indicated that HCC patients with high *DTYMK* expression had poor OS (Figure 1(l,n)), RFS (Figure 1(o)), and DSS (Figure 1(p)) compared to those with low *DTYMK* expression.

DTYMK is correlated with proliferation and cell cycle of HCC

Co-expressed genes with *DTYMK* were annotated and functional enriched analyzed. Our results revealed that *DTYMK* and its coexpressed genes play a critical role in cell cycle, mitotic nuclear division and signal transduction driven by p53 class mediator etc. (Figure 2(a,b)). In addition, we utilized GEPIA online tool to explore the correlation of *DTYMK* with *MKI67* and *PCNA* based on the TCGA-LIHC cohort. The results showed that *DTYMK* mRNA expression was positively correlated with *MKI67* expression and *PCNA* expression in HCC samples (Figure 2(c,d)). Besides, we found that *DTYMK* expression was upregulated in *TP53* mutated HCC samples compared to that without *TP53* mutation (Figure 2(e)).

Validation the DTYMK protein expression in HCC cell lines and HCC patients

We tested protein expression level of DTYMK by Western blot in HCC cell lines compared to normal liver cell line L-02. IHC was performed in tissue sections with HCC (TMA) containing 60 pairs of primary liver samples. The results showed that DTYMK protein expression was mainly confined to the cell plasma and dramatically upregulated in HCC samples when compared to the adjacent normal liver cells (Figure 3(a,c)). We next investigated DTYMK expression levels in patients with different TNM stages. We found that there was an increase in DTYMK expression
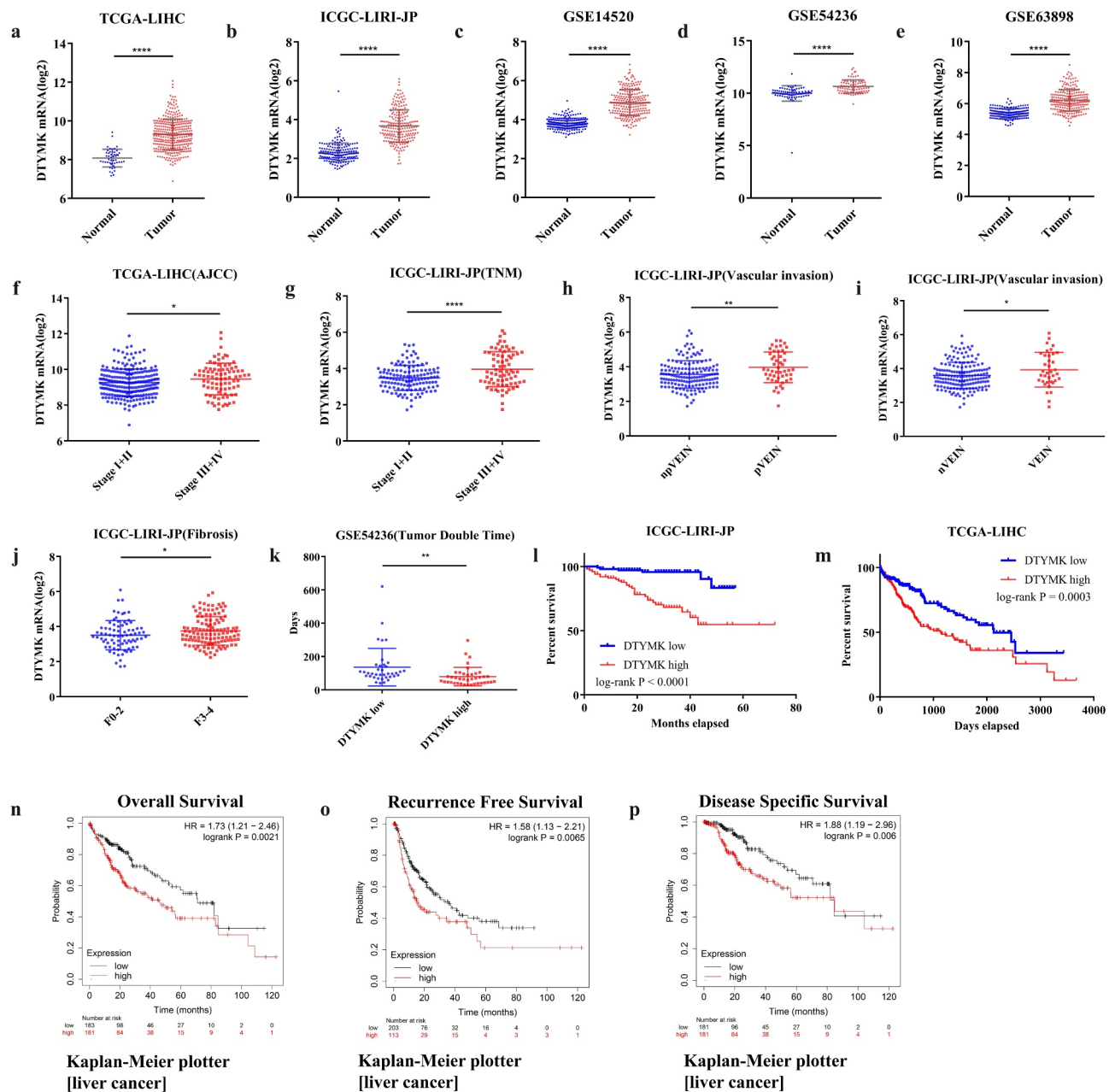
Figure 1. Expression pattern and prognostic implication of *DTYMK* in HCC.

(a) *DTYMK* mRNA expression between HCC and normal hepatic tissues in TCGA-LIHC cohort; (b) *DTYMK* mRNA expression between HCC and normal hepatic tissues in ICGC-LIRI-JP cohort; (c) *DTYMK* mRNA expression between HCC and normal hepatic tissues in GSE14520 cohort; (d) *DTYMK* mRNA expression between HCC and normal hepatic tissues in GSE54236 cohort; (e) *DTYMK* mRNA expression between HCC and normal hepatic tissues in GSE63898 cohort; (f) *DTYMK* mRNA expression between stage I+ II HCC and stage III+IV HCC samples in TCGA-LIHC cohort; (g) *DTYMK* mRNA expression between stage I+ II HCC and stage III+IV HCC samples in ICGC-LIRI-JP cohort; (h) *DTYMK* mRNA expression between HCC without portal vein invasion and HCC with portal vein invasion samples in ICGC-LIRI-JP cohort; (i) *DTYMK* mRNA expression between HCC without vascular invasion and HCC with vascular invasion samples in ICGC-LIRI-JP cohort; (j) *DTYMK* mRNA expression between fibrosis grade F0-2 HCC and fibrosis grade F3-4 HCC samples in ICGC-LIRI-JP cohort; (k) tumor doubling time between high *DTYMK* mRNA expression samples and low *DTYMK* mRNA expression samples in GSE54326 cohort; (l) Kaplan-Meier curve of overall survival for *DTYMK* expression in ICGC-LIRI-JP cohort; (M) Kaplan-Meier curve of overall survival for *DTYMK* expression in TCGA-LIHC cohort; (N) Kaplan-Meier curve of overall survival for *DTYMK* expression in Kaplan-Meier Plotter; (o) Kaplan-Meier curve of recurrence free survival for *DTYMK* expression in Kaplan-Meier Plotter; (p) Kaplan-Meier curve of disease specific survival for *DTYMK* expression in Kaplan-Meier Plotter.
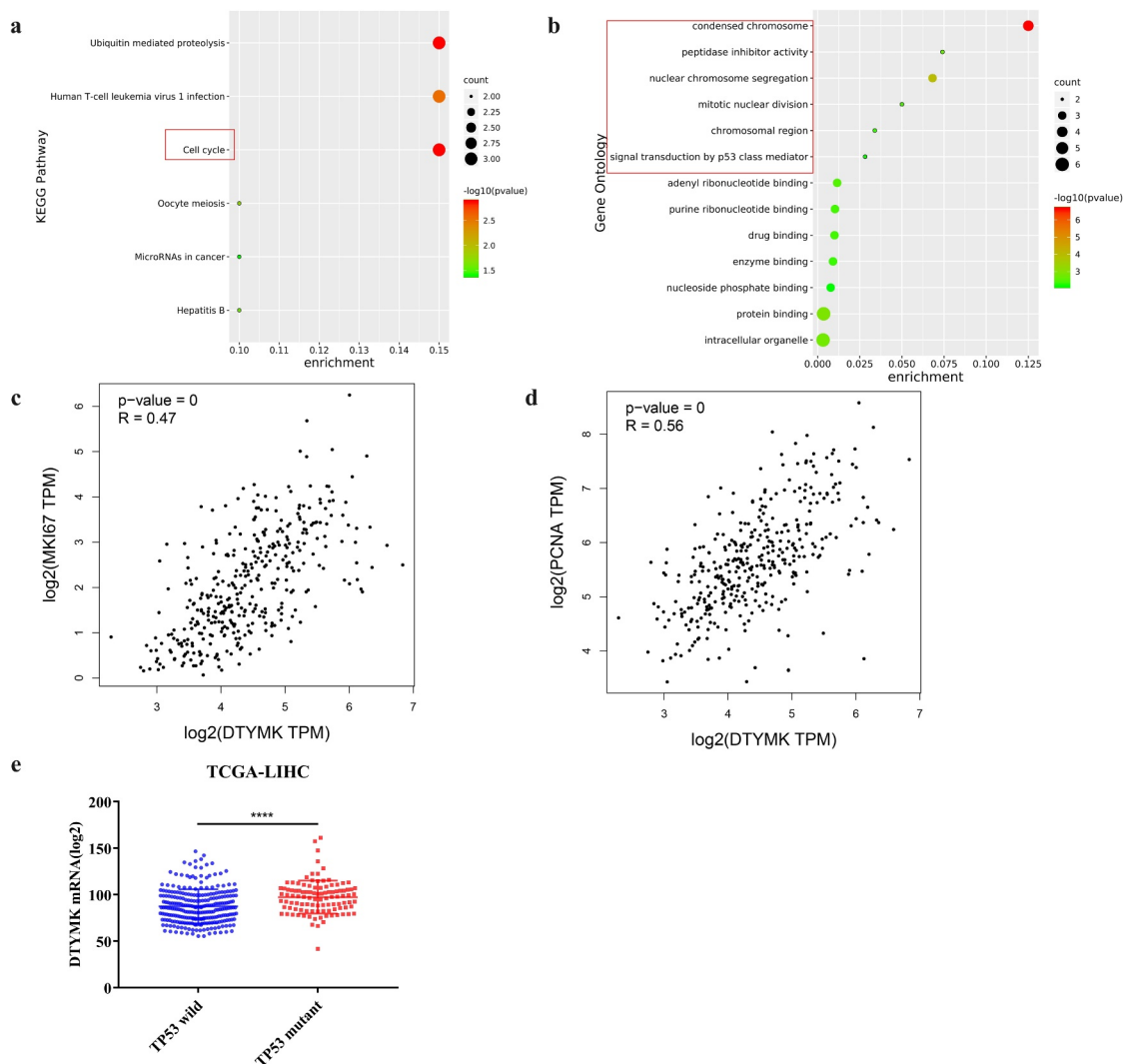
Figure 2. Functional analysis of *DTYMK* and its coexpressed genes.
(a) Functional enriched analysis of *DTYMK* and its coexpressed genes (KEGG); (b) functional enriched analysis of *DTYMK* and its coexpressed genes (GO); (c) correlation of *DTYMK* with MKI67; (d) correlation of *DTYMK* with PCNA; (e) *DTYMK* mRNA expression between TP53 mutant and TP53 wild HCC patients. KEGG, Kyoto encyclopedia of genes and genomes; GO; gene ontology.

gradually with higher HCC grades. The average DTYMK expression level increased in the term of TNM stage I to IV and pathology grade I to III (Figure 3(d,e)). This finding implied a possible role of DTYMK upregulation in promoting HCC development. To verify the potential clinical meaning of DTYMK upregulation according to our TCGA analysis, we investigated the correlation of DTYMK expression levels and the overall survival time of HCC patients. Importantly, upregulation of DTYMK was observed remarkably associated with a worse OS rate of the HCC patients (Figure 3(f)).

DTYMK promotes HCC cell proliferation

To investigate functional roles of DTYMK in HCC, we constructed DTYMK overexpression cell line HepG2 and the DTYMK stable knockdown Huh7 cell lines with two independent shRNA sequences (shSETDB#1 and #2). Successful overexpression and knockdown of DTYMK was confirmed with western blotting (Figure 4(a,b)). It was shown that the overexpression of DTYMK promoted the proliferation of HepG2 cells while the knockdown of DTYMK sig-
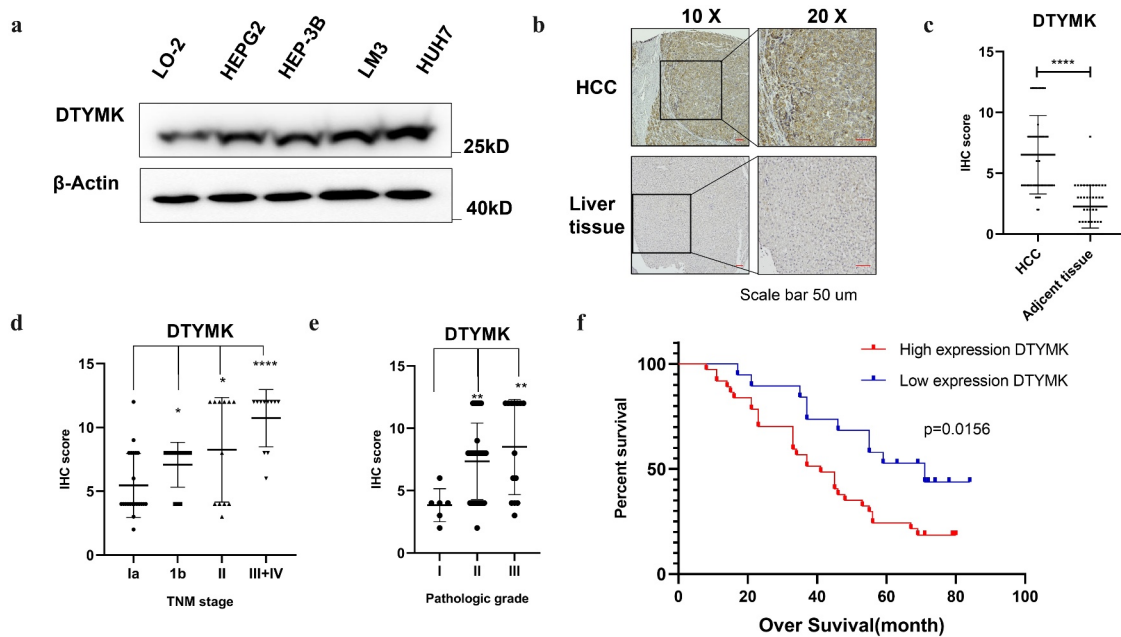
Figure 3. Validation the DTYMK expression in HCC cell lines and HCC patients.
(a) Western blot analysis of DTYMK in HCC cells including LO-2, HEPG2, HEP3B, LM-3, and Huh-7. β-Actin was used as a loading control; (b) expression of DTYMK protein by IHC. DTYMK protein was predominantly detected at the cytoplasm. Arepresentative case shows the upregulation of DTYMK protein in HCC compared to that in its adjacent normal liver tissue; (c) semi-quantitative analyses of IHC staining of DTYMK in 60 HCC patients TMAs with tumor sample and adjacent tissue; (D) semi-quantitative analyses of IHC staining of DTYMK in 60 HCC patients TMAs with different TNM-stage patient; (e) semi-quantitative analyses of IHC staining of DTYMK in 60 HCC patients TMAs with different pathology grade patients (f) Kaplan–Meier of survival of 60 patients with HCC (two groups stratified by DTYMK expression level. Differences between the groups were shown by a log-rank test.)

nificantly led to the suppression of Huh7 cell proliferation, which was demonstrated by CCK8 growth curve analysis (Figure 4(c,d)). Consistent results were obtained that the overexpression of DTYMK promoted the ability of colony formation of HCC cells while the knockdown of DTYMK suppressed that. (Figure 4(e,g)).

## DTYMK regulate HCC cell cycle progression

The TCGA database analysis results showed that DTYMK participates in cell cycles in HCC cells. To further validate this idea, we knocked down DTYMK by shRNA in stable Huh-7 cell line which highly expressed DTYMK. We applied a standard cell cycle assay by flow cytometry using propidium iodide staining. An approximately 20% increase of cells in the G0/G1 phase was observed in Huh-7 cells. Meanwhile, the knockdown of DTYMK led to a 14% decrease in the number of cells in the G2/M phase, compared to cells transfected with the scramble shRNA (Figures 5(a,b)). Besides, overexpression of

DTYMK decreased HepG2 cells in the G0/G1 phase while increased the number of cells in the G2/M phase (Figure 5(c,d)).

Discussion

In this research, we first systematically analyzed the expression pattern and prognostic prediction value of DTYMK in HCC patients by integrating cohorts from three GEO series, TCGA, and ICGC. The results show that DTYMK is upregulated in tumor tissue compared to adjacent normal hepatic tissues in all cohorts, and a higher level of DTYMK indicates a poorer prognosis in HCC patients. In addition, we found that DTYMK may play a critical role in tumorigenesis and progression by regulating cell cycle and cell proliferation.

DTYMK functions in *de novo* pyrimidine pathway, which is related to the dTTP biosynthesis, part of Pyrimidine metabolism [7,8]. Overexpression of DTYMK *in vivo* leads to
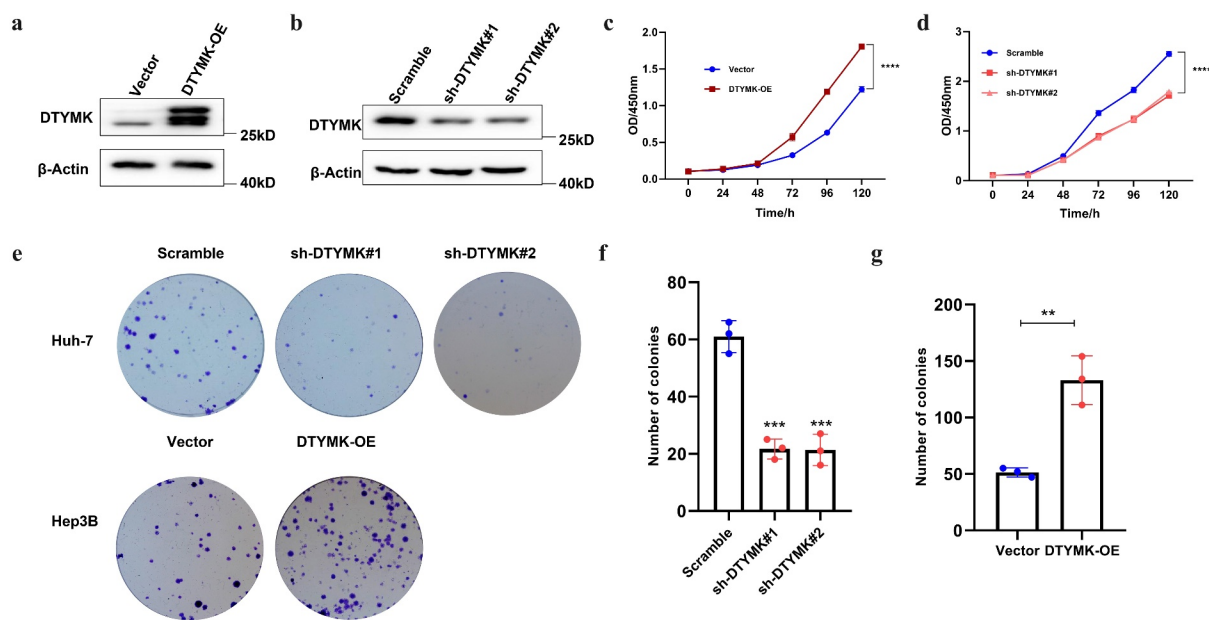
Figure 4. DTYMK promotes HCC cell proliferation.
(a) DTYMK-overexpressed HepG2 cells was confirmed with Western blot; (b) DTYMK-knockdowned Huh7 cells was confirmed with western blot; (c) CCK-8 assay was performed in DTYMK-overexpressed HepG2 cells; (d) CCK-8 assay was performed in DTYMK-knockdowned Huh7 cells; (e) colony formation assay with DTYMK overexpression or DTYMK knock down of two indicated cell lines; (f) histograms show the mean numbers of formed colon in DTYMK-overexpressed HepG2 cells; (g) histograms show the mean numbers of formed colon DTYMK-knockdowned Huh7 cells.

increasing the production of nucleotides which is necessary for DNA synthesis and repair [20]. It can be inferred that DTYMK could be related with cell cycle and drug resistant in physiological or pathological process by influencing thymidylate synthesis [21–24]. In recent years, DTYMK is reported to be upregulated accompanied by drug resistance in several cancers such as lung cancer, colon cancer, hepatic carcinoma and esophagus cancer [25], but there is no further investigation for the detailed mechanism.

In another experiment in HCC, DTYMK is dramatically highly expressed in HCC than in adjacent liver tissue. We demonstrated that higher expression of DTYMK in tumor is significantly associated with worse over survival in HCC patients. Besides, the expression level of DTYMK is also in correlated with TNM stage and pathology grade in HCC patients. The expression of DTYMK was validated in HCC cell lines HepG2, Hep3B, LM-3, and Huh-7 and human normal liver cell line LO-2. Knocking down DTYMK by shRNA silencing in Huh-7 cells

decreased cell proliferation and colony formation ability. Conversely, the overexpression of DTYMK in HepG2 increased cell proliferation and colony formation ability. The ontogenetic functional study in HCC cell line was consistent with the result in clinical analysis that the expression of DTYMK was positively correlated with proliferation marker PCNA. PCNA is known as a biomarker of cell proliferation, which can effectively reflect the proliferation level of tumor cells, especially in liver cancer, nose pharyngeal cancer, and other poorly differentiated malignant tumors [26,27]. Progression of the cell cycle is tightly related with cell proliferation and growth [28,29]. The dysfunction of DTYMK induces cell cycle arrest in Huh-7 cell line while overexpressing DTYMK increase M phase cell percent, which demonstrated that DTYMK induced cancer cell proliferation by promoting cell division.

Finally, we proposed that DTYMK presented oncogenic properties in HCC cells. The overexpression of DTYMK promotes HCC cell
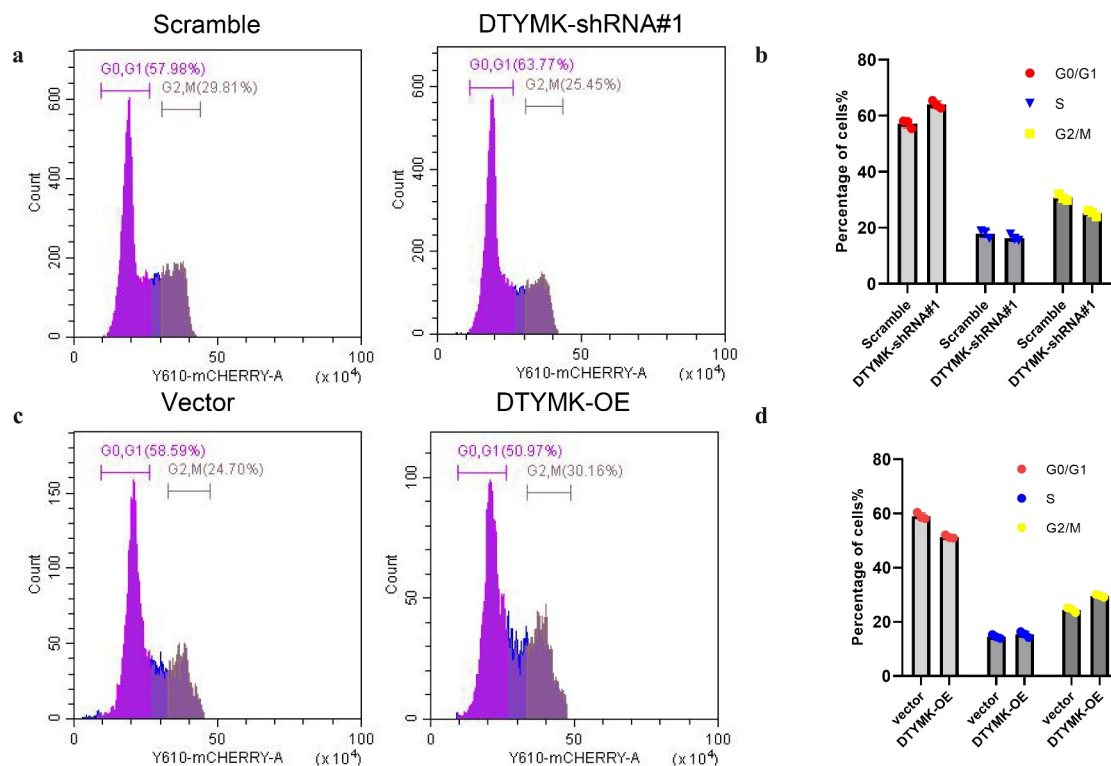
Figure 5. DTYMK regulate HCC cell cycle progression.
(a) Flow cytometry assays were conducted to analyze cell cycle in Huh-7-NC and Huh-7 DTYMK-shRNA cells; (b) quantification of G0/1, S and G2/M phase in Huh-7-NC and Huh-7 DTYMK-shRNA cells from three independent experiments; (c) flow cytometry assays were conducted to analyze cell cycle in HepG2-NC and HepG2 DTYMK-overexpression cells; (d) quantification of G0/1, S, and G2/M phase in HepG2-NC and HepG2 DTYMK-overexpression cells from three independent experiments.

proliferation. Specifically, knocking down DTYMK led to cell cycle arrest while overexpressing promoting cell cycle. As a result, DTYMK is a promising target for HCC diagnosis and treatment [30–32]. Whereas some limitations in this study need consideration as well. First, we did not explore the explicit mechanism that DTYMK drives HCC cell growth and changes cell cycle division, there were evidences that DTYMK promoting the cell cycle progression by increasing the pool of dTTP which was the raw material of DNA synthesis in colon cancer cells [33]. During the cell cycle progression, the production of dTTP is highly regulated to coordinate with DNA replication [34]. Besides, it was reported that Raltitrexed (RTX), an inhibitor of thymidylate synthase (TS), arrested HepG2 cells at G0/G1 via downregulation of cyclin A and CDK2 [35]. Second, the reason why DTYMK is upregulated in HCC cells is still unknown. Future research is required to explore the detailed mechanism between distinct DTYMK

and carcinogenesis of HCC and to reveal the mechanism of DTYMK in other carcinomas, which may be critical to the development of inhibitors in liver cancer patients.

Acknowledgments

Disclosure statement

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