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Original Article

The essential role of MTDH in the progression of HCC: a study with immunohistochemistry, TCGA, meta-analysis and *in vitro* investigation

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Abstract: Recent studies found that metadherin (MTDH) played an essential role in hepatocellular carcinoma (HCC). Nevertheless, the exact function of MTDH in the pathogenesis of HCC was unclarified. In the present study, we aimed to investigate the clinical significance of MTDH in HCC and its effect on HCC cells. Immunohistochemistry (IHC) was performed to detect MTDH expression in HCC tissues. Data from The Cancer Genome Atlas (TCGA) and ONCOMINE was obtained to examine MTDH expression in HCC and its clinical significance. Meta-analysis was conducted to assess the correlation between MTDH expression and both the prognosis (Overall Survival (OS) or Disease-free Survival (DFS)) and clinicopathological features of HCC via STATA 12.0. *In vitro* experiments were performed to investigate the role of MTDH in cell growth, caspase-3/7 activity and apoptosis in HCC cells. The MTDH staining was remarkably stronger in HCC tissues than in non-cancer tissues from IHC, TCGA and ONCOMINE data. Moreover, MTDH-positive expression was significantly correlated with pathological grade, distant metastasis and hepatitis B virus (HBV) infection by IHC. For meta-analysis, MTDH expression was indicative of poor OS without heterogeneity in HCC patients. Additionally, MTDH expression was correlated with high-grade histological differentiation, non-vascular invasion and metastasis in HCC. *In vitro* experiments revealed that MTDH could the inhibit cell growth and activate caspase-3/7 activity and apoptosis in the four HCC cell lines. In conclusion, MTDH expression may serve as a novel targeting strategy for HCC due to its clinical significance and oncogenic function in HCC cells.

Keywords: Hepatocellular carcinoma (HCC), metadherin (MTDH), TCGA, *in vitro*, clinical significance

Introduction

Hepatocellular carcinoma (HCC) ranks second among digestive cancers [1]. The distribution of HCC is unbalanced, with a high prevalence in Asia and Africa [2-5]. Liver cirrhosis, the infection of hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, smoking, and exposure to aflatoxins are the major etiologic factors for the initiation of HCC [6-10]. Thus far, there have been limited options for the treatment of HCC, resulting in a poor prognosis for HCC patients [11-15]. Therefore, it is urgently necessary to seek novel diagnostic and prognostic targets for the treatment of HCC. The carcinogenesis of HCC is believed to be a multi-step process that involves various genes and proteins [16-20]. A comprehensive understanding of the molecular mechanisms of HCC may help facilitate the improvement of the clinical treatment of HCC.

Metadherin (MTDH), also identified as lysine-rich carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1-coisolated (LYRIC) protein or astrocyte elevated gene-1 (AEG-1) protein [21, 22], is a trans-membrane protein [23]. The gene encoding MTDH is located at 8q22 [24], and it is recognized as an oncogene with significant amplification in a wide type of cancers, including breast cancer [25], melanoma [26], and malignant glioma [27]. MTDH plays an essential role in the proliferation, invasion, chemo-resistance, angiogenesis, and metastasis of cancers by regulating signaling pathways related to tumorigenesis, such as the

phosphoinositide-3 kinase (PI3K)-Akt, mitogen-activated protein kinase (MAPK), Wnt, and NF-kB pathways [28-32]. We hypothesized that MTDH could be pivotal for the carcinogenesis and progression of HCC. Recently, studies focusing on the MTDH expression and HCC have emerged. For example, Jung HI et al. reported that MTDH expression served as an independent prognostic factor for the overall survival (OS) and disease-free survival (DFS) of HCC [33]. A study from Srivastava J et al. revealed that MTDH induced steatosis, inhibited senescence, and activated the coagulation pathway to promote hepatocarcinogenesis [34]. Zhou Z et al. reported that MTDH may promote HCC metastasis through the induction of the epithelial-mesenchymal transition (EMT) process [35]. Li WF et al. reported higher MTDH expression in HCC tissues than in non-cancer tissues, and the knockdown of MTDH suppressed growth and induced apoptosis in HCC cells [36]. However, the role of MTDH in the carcinogenesis of HCC has not been elucidated. Thus, we performed immunohistochemistry (IHC), TCGA and ONCOMINE data excavation, meta-analysis and *in vitro* experiments to achieve an accurate and reliable interpretation of the function of MTDH in HCC.

Materials and methods

IHC

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded HCC tissues to detect the expression pattern of MTDH in HCC cells. The MTDH rabbit polyclonal antibody was provided from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. Two pathologists (Zhenbo Feng and Gang Chen) identified the staining intensity and positive ratio of MTDH staining through blinded-reading. We subsequently graded the MTDH staining according to the following steps: First, the staining intensity was assessed--negative (0), weak (1), moderate (2), strong (3); then, the proportion of positively stained cells was evaluated: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The result would be considered as positively immuno-reactive if the score of the staining intensity multiplied by the proportion of positively stained cells was greater than 2. The relationship between MTDH expression and the clinicopathological variables of HCC was calculated by Kruskal-Wallis H test when there were more than 3 groups of specific clinicopathological variables; otherwise, χ^2 test was performed. A two-tailed *P* value <0.05 was of statistical significance.

TCGA and ONCOMINE data extraction

The TCGA and ONCOMINE data were processed to evaluate the association of MTDH expression and the clinical variables of HCC using independent sample tests. Additionally, Pearson's correlation test was performed to assess the correlation between MTDH expression and the clinical features of HCC. We also plotted a receiver operating characteristic (ROC) curve to evaluate the diagnostic significance of MTDH by analyzing the area under the curve (AUC) value. A Kaplan-Meier survival analysis was conducted to evaluate the prognostic value of MTDH in HCC patients. The alterations of MTDH-related genes were obtained using cBioPortal OncoPrint (http://www.cbioportal.org/index.do). Kaplan-Meier analysis was also conducted to evaluate the prognostic value of mRNA alterations in HCC. All of the statistical analysis were performed with SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) with a two-tailed *P* value <0.05 indicating statistical significance.

Meta-analysis

Literature searching and screening criteria: We searched in PubMed, Web of Science, Springer Link, ProQuest Health & Medical Complete (PHMC), Ebsco, the Ovid database, Chinese National Knowledge Infrastructure, VIP, and the Wanfang Database to screen eligible studies with the following search strategy: ((malignan* OR cancer OR tumor OR tumour OR neoplas* OR carcinoma) AND (hepatocellular OR liver OR hepatic OR HCC)) AND (AEG1 OR MTDH OR "astrocyte elevated gene-1" OR MTDH OR metadherin OR LYRIC OR "lysine-rich CEACAM1 co-isolated" OR "metastasis adhesion protein" OR "3D3-lyric"). The evaluation of eligible studies for our meta-analysis was based on the following inclusion and exclusion criteria. The inclusion criteria were as follows: (1) The experiment subjects should be HCC patients. (2) The eligible studies for this meta-analysis should detect the expression of MTDH in HCC tissues or serum. (3) The study investigated the relationship between MTDH expression and survival data (OS or Progression-free Survival (PFS) or

DFS), diagnostic data or clinicopathological features. (4) The study included available data for the calculation of Hazard Ratio (HR), 95% CI or sensitivity and specificity indices (True Positivity (TP), True Negativity (TN), False Positivity (FP), False Negativity (FN)). (5) The study was published in Chinese or English. (6) When several studies were conducted on the same cohort, the studies with the most complete data were selected. (7) The number of patients involved in the studies should be no less than 20. The exclusion criteria were as follows: (1) Studies with insufficient data to calculate prognostic or diagnostic indices were excluded. (2) Studies with the use of animals or cell lines were excluded. (3) Reviews, case reports, letters and conference articles were excluded. (4) Duplicated studies with the same data or cohort were excluded.

Data extraction: Survival data and clinicopathological parameters, including HR and 95% CI for OS and DFS, liver cirrhosis, tumor size, histological differentiation, TNM stage, vascular invasion and metastasis, were extracted from all of the selected articles. For articles providing incomplete survival data, Engauge Digitizer 4.1 was used to extract the HR and 95% CI indirectly from survival curves or primitive data.

Assessment of study quality: The Newcastle-Ottawa quality assessment scale for cohort studies was used to assess the incorporated studies based on three parts of the system: the selection of the studies, the comparability between study cohorts and the outcome of studies [37]. For each item in the selection and outcome sections, a maximum of one star could be given to a study, while a maximum of two stars could be given to a study in the comparability section. Hence, a study could be awarded not more than nine stars in total. The more stars a study scored, the higher the quality of the study would have.

Statistical analysis

All statistical analyses of the meta-analysis were performed with STATA 12.0 software. The HR value and 95% CI extracted from each eligible study were combined to evaluate the correlation between MTDH expression and the prognosis of HCC patients.

For the association between MTDH expression and the clinicopathological variables of HCC, clinicopathological parameters, including liver cirrhosis, tumor size, histological differentiation, TNM stage, vascular invasion and metastasis were also extracted to obtain a pooled OR value and 95% CI. During the process, Chochrane's Q test and I^2 statistic were used to estimate the heterogeneity. We considered significant heterogeneity in studies if the I^2>50% or *P* value <0.05. When there was no heterogeneity (I^2<50% or *P* value >0.05), we adopted a fixed-effects model, otherwise we chose a random-effects model [38]. A two-tailed *P* value of less than 0.05 indicated a statistically significant pooled result of studies. Begg's test and Egger's test were carried out to detect the publication bias in the studies. When the *P* value was more than 0.05, significant publication bias existed in the studies. Moreover, we performed a subgroup analysis according to the categories of country, blinded reading, quality assessment and staining for MTDH positivity to detect the source of the heterogeneity. A sensitivity analysis was performed by excluding each study from the cohorts to yield a pooled OR and 95% CI for the remaining studies for the purpose of assessing the stability of the study results.

In vitro experiments

Cell transfection: HepG2 (American Type Culture Collection, ATCC), SMMC-7221 (Chinese Academy of Medical Sciences), Hep3B (ATCC) and SNU449 (ATCC) cell lines were cultured and subsequently seeded in 96-well plates with 2.5×10^3 cells per well. The incubation of the cells was conducted at 37°C for 24 h prior to each transfection. Each *in vitro* experiment was carried out in triplicate. The cells were transfected with blank control, scrambled siRNAs or MTDH siRNAs (Ambion, Life Technologies Grand Island, NY, USA) at a concentration of 200 nmol/L for up to 96 h by CombiMag Magnetofection (OZBIOSCIENCES, Marseille Cedex 9, France) based on the manufacturer's instructions. Four siRNAs, including siRNA-1033: AACTACAACCGCATCATT, siRNA-1455: ATGATGAATGGTCTGGGTT, siRNA-1967: AAGTCAAATACCAAGCAAA and siRNA-3566: CTTATTAATGGACAGCTTT, showed the strongest effect of the knock-down of MTDH according to our previous tests for selecting suitable siRNAs for MTDH. The scrambled siRNAs were set as the negative control. RT-qPCR was used to monitor the efficiency of the transfection.

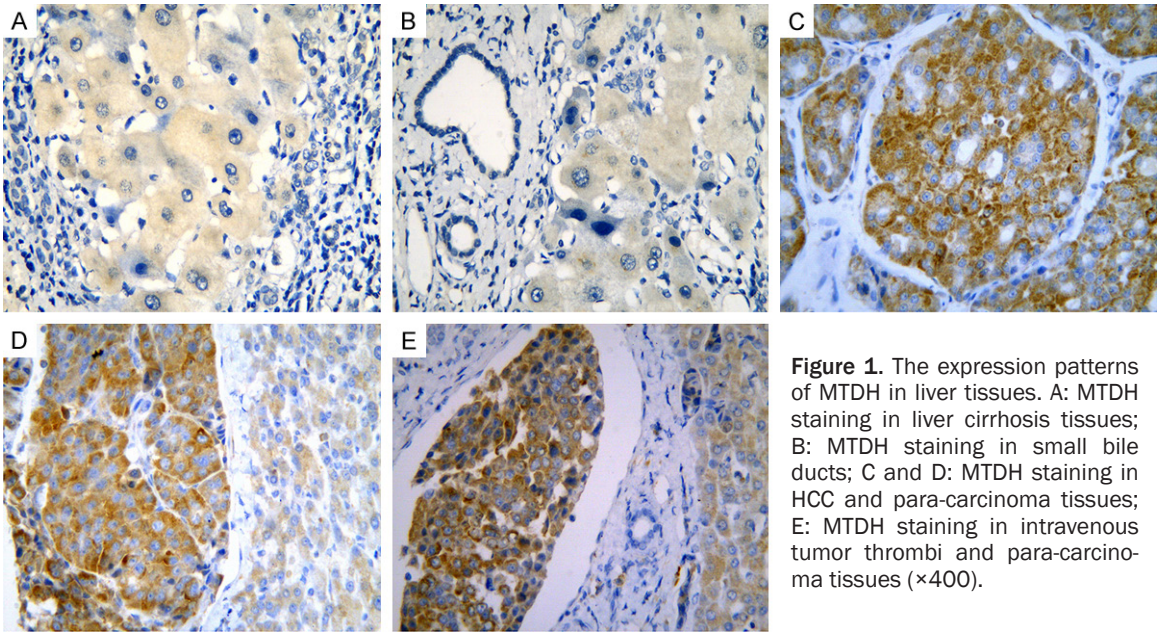

Figure 1. The expression patterns of MTDH in liver tissues. A: MTDH staining in liver cirrhosis tissues; B: MTDH staining in small bile ducts; C and D: MTDH staining in HCC and para-carcinoma tissues; E: MTDH staining in intravenous tumor thrombi and para-carcinoma tissues (×400).

Table 1. Percentage of MTDH-positive expression in various liver tissues from clinical samples in house

Liver tissue	Total	MTDH expression		χ^2	*P* value
		Negative	Positive		
HCC	89	38 (42.7%)	51 (57.3%)		
Normal liver tissue	25	19 (76.0%)	6 (24.0%)	3.798[a]	>0.05[a]
Liver cirrhosis	37	26 (70.3%)	11 (29.7%)	7.950[b]	≤0.05[b]
Para-carcinoma tissue	89	51 (57.3%)	38 (42.7%)	8.659[c]	≤0.05[c]

a: HCC tissues compared with normal liver tissues; b: HCC tissues compared with liver cirrhosis tissues; c: HCC tissues compared with para-carcinoma tissues.

The effect of MTDH expression on cell biological processes: The role of MTDH on biological processes, including cell growth, caspase-3/7 activity, apoptosis and nuclear morphology was assessed through fluorimetric resorufin viability assays, MTS tetrazolium assays, Apo-ONE Homogeneous Caspase-3/7 Assays and Hoechst 33342/propidium iodide (PI) double fluorescent chromatin staining.

Statistical analyses

Statistical analyses were conducted with SPSS software (version 22.0). The results of the experiment were generated from three repeated experiments and were presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to determine the statistical significance of the results. The least significant difference (LSD) method was used for multiple comparisons between two groups when there was statistical significance in the ANOVA tests. A *P* value <0.05 demonstrated statistical significance.

Results

IHC

As shown in **Figure 1**, five pictures represented the results of MTDH immunohistochemical staining. MTDH exhibited weak staining in liver cirrhosis tissues (**Figure 1A**) and absolutely no staining in the para-liver small bile duct (**Figure 1B**). Compared with the para-carcinoma tissues, the intensity of MTDH was notably stronger in HCC tissues, with brown staining (**Figure 1C** and **1D**). Similarly, the strong staining intensity of MTDH in intravenous tumor thrombi contrasted sharply with that of the para-carcinoma tissues (**Figure 1E**). The staining patterns of MTDH in the different liver tissues are summarized in **Table 1**. The percent of HCC tissue samples that were MTDH-positive was 57.3%, whereas MTDH-positive staining was found in 42.7% of the para-carcinoma tissues. The difference bore no statistical significance (χ^2=3.798, *P* value >0.05). These percentages of MTDH-positive staining in HCC tissues was notably higher than that in both liver cirrhosis tissue

Table 2. Relationship between MTDH expression and clinicopathological variables of HCC from clinical samples in house

Variables	Category	Total	N of positive Cases	Percentage	χ^2	*P* value
Gender	Female	17	8	47.1	0.901	0.342
	Male	72	43	59.7		
Age	<50	46	26	56.5	0.024	0.877
	≥50	43	25	58.1		
Pathological grade	I-II	19	7	36.8	4.134	0.042
	III-IV	70	44	62.9		
Grade of differentiation	High	6	2	33.3	1.906[a]	0.386[a]
	Median	57	35	61.4		
	Low	26	14	53.8		
Liver cirrhosis	No	46	27	58.7	0.075	0.784
	Yes	43	24	55.8		
Tumor size (cm)	<5	15	11	73.3	1.895	0.169
	≥5	74	40	54.1		
Distant metastasis	No	43	19	44.2	5.851	0.016
	Yes	46	32	69.6		
AFP	Negative	54	31	57.4	0.026	0.872
	Positive	35	20	57.1		
HCV	Negative	60	34	56.7	0.031	0.861
	Positive	29	17	58.6		
HBV	Negative	16	13	81.3	81.3	0.032
	Positive	73	38	52.1		

a: Kruskal-Wallis H test was used to assess the correlation between MTDH expression and pathological grade. The association between MTDH and other clinical variables were performed with χ^2 tests.

Table 3. MTDH expression in HCC and non-HCC tissues from TCGA data

Paired samples t-test				
Standard error average	95% confidence interval	T	df	*P* value (two-tailed)
0.0989263	(-0.4670398–0.0694402)	-2.712	49	0.009

A two-tailed *P* value of 0.009 indicated a distinct distribution of MTDH between HCC and para-carcinoma tissues.

(29.7%) and normal liver tissue (24.0%, *P* value <0.05).

Moreover, we also evaluated the relationship between MTDH expression and clinicopathological parameters of HCC. The results indicated that the MTDH-positive percentage was remarkably higher in groups of pathological grade III-IV, groups with distant metastases, and groups with HBV infection than that in the corresponding negative groups. The difference was of statistically significance (*P* value <0.05, **Table 2**). Spearman's correlation tests proved that MTDH expression was significantly associated with pathological grade (r=0.216, *P* value =0.043), distant metastasis (r=0.256, *P* value =0.015) and HBV infection (r=0.227, *P* value =0.033). No significant correlation was established between MTDH expression and other clinicopathological parameters, such as age, gender, differentiation grade, tumor size, liver cirrhosis, capsules, cancer embolus, vessel invasion or HCV infection (*P* value >0.05, **Table 2**).

TCGA and ONCOMINE data excavation

A total of 359 HCC cases and 50 non-HCC cases were included in the TCGA data. Among the 359 HCC cases, there were 243 male and 116 female patients. The age of the HCC patients ranged from 16-90, with a median age of 59.52. As shown in **Table 3**, the results from the paired samples t-test indicated that MTDH was over-expressed in HCC tissues compared with non-cancer tissues (*P* value =0.009). Furthermore, the consistent overexpression of MTDH could be observed in other independent cohorts from ONCOMINE, including the study termed as Chen Liver (**Figure 2**) and Roessler Liver 2 (**Figure 3**). Independent samples tests showed that MTDH expression in TCGA data exhibited significant difference in gender (Z=-4.154, *P* value <0.001) and histological type (Z=-2.163, *P* value =0.031) (**Table 4**). Pearson's correlation tests confirmed a correlation between MTDH expression and gender (r=-0.229, *P* value

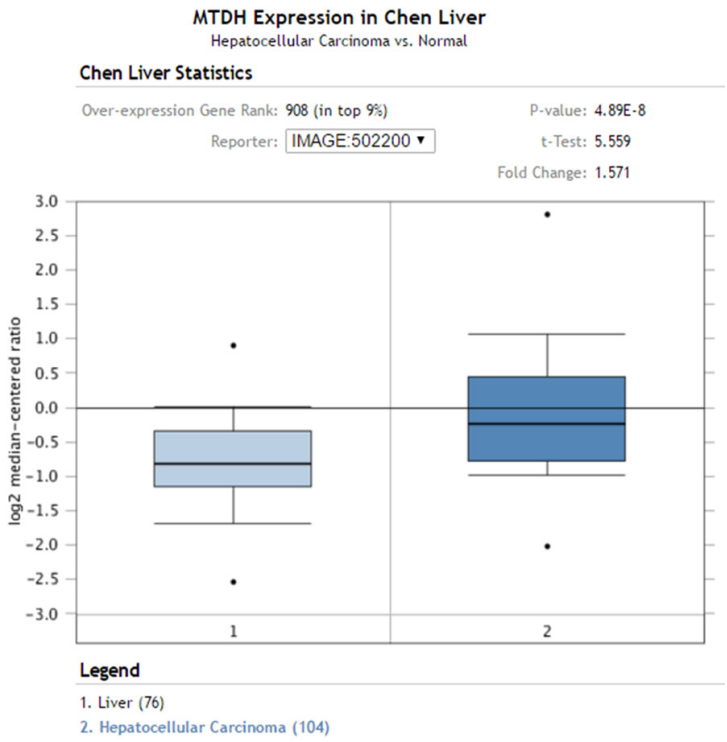

Figure 2. Validation of MTDH expression in the cohort of Chen Liver from ONCOMINE. Normal liver tissues (n=76) and hepatocellular carcinoma tissues (n=104) were included in the cohort of Chen Liver. The detecting platform was not pre-defined in ONCOMINE.

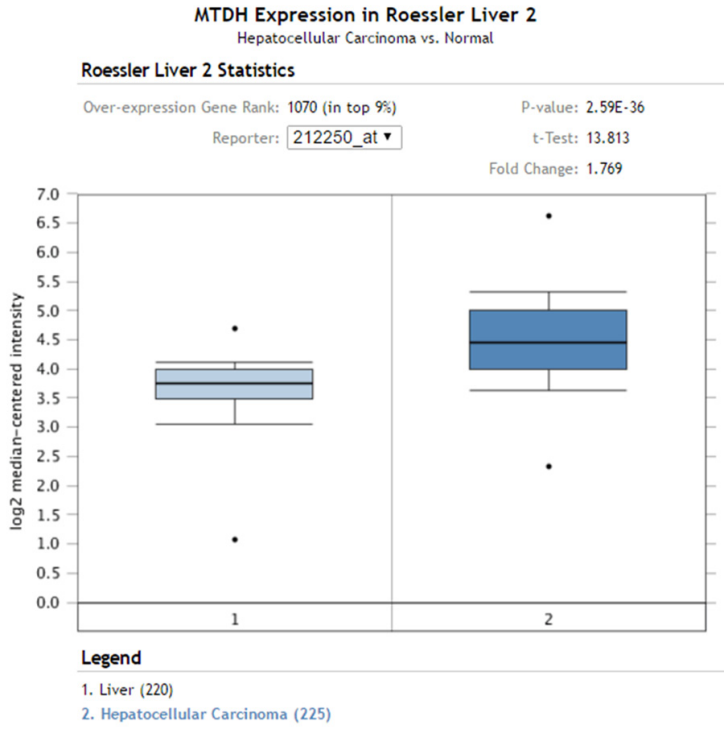

Figure 3. Validation of MTDH expression in the cohort of Roessler Liver 2 from ONCOMINE. Normal liver tissues (n=220) and hepatocellular carcinoma tissues (n=225) were included in the cohort of Roessler Liver 2 detected by the Affymetrix Human Genome HT U133A Array.

<0.001) (**Table 5**), height (r= 0.128, *P* value =0.019) or histological type (r=-0.111, *P* value =0.025) (**Table 5**). With regard to the prognostic function of MTDH expression in HCC, the results from Kaplan-Meier analysis revealed MTDH as an invalid prognostic factor for the OS and DFS of HCC patients (**Figure 4A** and **4B**). The AUC value of ROC curves was 0.594 (*P* value =0.031), which indicated MTDH owning the potential to act as a diagnostic target for HCC (**Figure 4C**). We also searched data containing the alteration of MTDH from cBioPortal OncoPrint (http://www.cbioportal.org/index.do). As shown in **Figure 5A**, there were 371 patients (373 specimens) with data of gene alterations. Subsequently, 120 of the 371 patients (32%) bore mRNA alterations. Among the alterations, 118 patients showed mRNA up-regulation, whereas 2 patients showed mRNA down-regulation. **Figure 5B** and **5C** from the Kaplan-Meier analysis revealed an insignificant prognostic value of mRNA alterations for the DFS and OS in HCC patients. In the survival analysis for DS, there were a total of 102 cases with alterations in the queried genes. Of those, 58 cases had relapsed, and the median disease free-survival was 18.33 months. A total of 218 cases had no alterations in the queried genes. Of those, 117 cases relapsed, with a median disease free survival of 21.16 months (*P* value =0.573 from Log rank test, **Figure 5B**). In the survival analysis for OS, there were a total of 119 cases with alterations in the queried genes. Of those, 42 cases relapsed, and the median disease-free survival was 48.95

Table 4. The relationship between MTDH expression and clinical variables of HCC from TCGA data

Variable	Total (n)	Z	*P* value
History of other malignancy		-1.673	0.094
Yes	35		
No	324		
History of neoadjuvant treatment		-0.307	0.758
No	357		
Yes	2		
Tumor status		-0.595	0.552
Tumor free	227		
With tumor	108		
Relative family history cancer		0.000	1.000
No	203		
Yes	107		
Radiation therapy adjuvant		-0.074	0.941
No	232		
Yes	4		
Pharmaceutical tx adjuvant		-0.338	0.736
No	218		
Yes	13		
Post-op ablation embolization		-1.350	0.177
No	224		
Yes	13		
No History of Primary Risk Factors		-1.456	0.145
"-"	255		
"+"	85		
Alcohol consumption		-0.863	0.388
"-"	223		
"+"	117		
Hemochromatosis		-0.604	0.546
"-"	336		
"+"	4		
Hepatitis B		-1.603	0.109
"-"	237		
"+"	103		
Hepatitis C		-1.093	0.274
"-"	286		
"+"	54		
Non-Alcoholic Fatty Liver Disease		-0.237	0.813
"-"	320		
"+"	20		
Smoking		-0.222	0.825
"-"	323		
"+"	17		
Cirrhosis		-0.142	0.887
"-"	334		
"+"	6		
Age		-1.043	0.297
≤60	165		

months. A total of 252 cases had no alterations in the queried genes. Of those, 88 cases relapsed, with a median DFS of 60.84 months (*P* value =0.648 from Log rank test, **Figure 5C**).

Meta-analysis

Literature research and study characteristics: An initial 793 publications were collected with the search terms mentioned above. A preliminary selection of eligible studies was conducted by screening the titles and abstracts; then, we scrutinized the full texts of the remaining articles. Eventually, 10 studies were eligible for our research. The detailed process of study selection is displayed in Supplementary Figure 1.

The characteristics of all studies are listed in Supplementary Table 1. Nine studies with 971 specimens were eligible for the evaluation of the relationship between MTDH expression and clinicopathological parameters. Seven studies with survival-related data were enrolled for assessing the correlation between MTDH expression and the OS or DFS. For the origins of the included study cohorts, eight studies were conducted in China (921 specimens), and two were conducted in Korea (373 specimens).

Correlation between MTDH expression and the clinicopathological variables of HCC: For the clinicopathological variables, the pooled data confirmed that MTDH expression was significantly associated with high-grade histological differentiation (OR=0.346, 95% CI=0.171-0.697, *P* value =0.003) with moderate heterogeneity (I^2=65.30%, *P* value =0.008, random-effects model), non-vascular invasion (OR=0.388,

≥60	193		
Gender		-4.154	<0.001
Male	243		
Female	116		
Race		4,231	0.238
Asian	156		
White	174		
Black or African American	17		
American Indian or Alaska native	2		
Ethnicity		-0.160	0.873
Hispanic or Latino	18		
Not Hispanic or Latino	325		
Histological type		-2.163	0.031
HCC	359		
Non-HCC liver cancers	40		
Grade		3.697	0.296
G1	53		
G2	171		
G3	119		
G4	11		
Grade 2		-1.548	0.122
GI-II	224		
GIII-IV	130		
AJCC Pathologic T		4.012	0.675
T1	176		
T2	90		
T3	43		
T3a	28		
T3b	6		
T4	13		
Tx	1		
AJCC Pathologic T2		3.479	0.481
T1	176		
T2-T2c	90		
T3-T3c	77		
T4-T4c	13		
Tx	1		
AJCC Pathologic T3		1.857	0.395
T0 Tx	1		
T1	176		
T2-4	180		
AJCC Pathologic N		1.177	0.555
N0	243		
N1	3		
Nx	112		
AJCC Pathologic M		0.103	0.950
M0	258		
M1	4		
Mx	97		
Pathologic Stage		5.477	0.484

95% CI=0.294-0.511, *P* value <0.001) with no heterogeneity, and metastasis (OR=3.791, 95% CI=1.958-8.051, *P* value <0.001) with moderate heterogeneity (I^2=67.80%, *P* value =0.005, random-effects model) (Supplementary Table 2).

Correlation between MTDH expression and OS or DFS: As shown in **Figure 6** and Supplementary Table 2, 7 studies were related to prognosis (OS or DFS) and MTDH expression in HCCs. Among the 7 studies, in some articles, the HR value and 95% CI of the OS or DFS were calculated with Engauge digitizers from the survival curve or primitive data [33, 39-41]. The remaining articles reported the direct survival data (HR and 95% CI) [42-44]. Meta-analysis indicated a significant correlation between MTDH expression and poor OS (HR= 2.394, 95% CI=1.956-2.931, I^2=37.60%, and *P* value =0.171) with fixed-effects model.

Publication bias: As shown in Supplementary Figure 2, a symmetrical funnel plot with results from Begg's test and Egger's test showed no publication bias for the impact of MTDH on OS of HCC patients (*P* value =0.221).

Subgroup analysis

Intriguingly, as shown in Supplementary Table 3, in Korea, MTDH was correlated with a poorer OS (HR=4.756, 95% CI=1.697-13.329) than in China (HR=2.329, 95% CI=1.895-2.863). The studies that adopted blinded-reading also exhibited a correlation between MTDH expression and poorer OS (HR=2.982, 95% CI=1.857-4.789) than studies that did not adopt blinded reading (HR= 2.280, 95% CI=1.823-2.852).

Stage I	167		
Stage II	82		
Stage III	3		
Stage IIIA	62		
Stage IIIB	8		
Stage IIIC	9		
Stage IV	2		
Pathologic Stage 2		4.077	0.253
Stage I	167		
Stage II	82		
Stage III ABC	82		
Stage IV	2		
Pathologic Stage 3		-0.788	0.430
Stage I-II	249		
Stage III-IV	84		
Vascular invasion		0.580	0.748
None	200		
Micro	89		
Macro	14		
Vascular invasion 2		-0.754	0.451
No	200		
Yes	103		
Residual tumor		4.295	0.231
R0	317		
R1	14		
R2	1		
Rx	22		
Ishak fibrosis score		3.440	0.487
0-no fibrosis	72		
1,2-portal fibrosis	31		
3,4-fibrous septa	28		
5-nodular formation and incomplete cirrhosis	9		
6-established cirrhosis	70		
Child-Pugh classification grade		0.489	0.783
A	205		
B	19		
C	1		

Independent samples tests were used to evaluate the difference of MTDH expression between groups of clinical variables. A two-tailed *P* value <0.05 was regarded to be statistically significant.

In addition, studies with an NOS score of 7 showed an association of MTDH expression with poorer OS (HR=2.677, 95% CI=1.823-2.852) than studies with an NOS score of 6 (HR=2.190, 95% CI=1.227-3.909) and 8 (HR=1.736, 95% CI=1.106-2,725).

Sensitivity analysis

For the sensitivity analysis, each study was excluded to generate a pooled OR and 95%CI from the remaining articles. No significant change was found in the results, which supported the stability of our results (Supplementary Figures 3, 4, 5, 6, 7, 8).

In vitro experiment

Effect of MTDH on HCC cell growth: The influence of MTDH on cell growth was evaluated with the fluorimetric resorufin viability assay, the MTS tetrazolium assay and Hoechst 33342/PI double fluorescent chromatin staining. As shown in **Figure 7**, for the fluorimetric resorufin viability assay, HepG2 and SMMC-7221 cells exhibited an approximately 40% decrease in cell viability 96 h post transfection with MTDH siRNAs, in comparison to blank and scrambled siRNAs controls. In Hep3B and SNU449 cell lines, cell viability experienced a slight decrease of an estimated 25 percent and 20 percent 96 h post transfection, respectively. Similarly, MTDH siRNAs induced a reduction in proliferation in all 4 cell lines, with HepG2 and SMMC-7221 showing a relatively larger decreasing amount of approximately 40 percent 96 h post-transfection (**Figure 8**). The inhibition of MTDH expression in HCC cell growth occurred in a time-dependent manner. Furthermore, the MTS tetrazolium assay and Hoechst 33342/PI double fluorescent chromatin staining confirmed the results of the fluorimetric resorufin viability assay (data not shown).

Effect of MTDH on apoptosis and caspase-3/7 activity of HCC cells: To investigate the effect of MTDH on HCC apoptosis and the activity of activated caspase, the CellTiter-Blue assay in combination with a fluorescent caspase-3/7 assay was performed. The caspase-3/7 activity was observed to be remarkably elevated in the

Table 5. Pearson's correlation of MTDH and clinical variables of HCC from TCGA data

Variables	r	*P* value
History_other_ malignancy	-0.090	0.089
History_of_neoadjuvant_treatment	0.009	0.864
Tumor_status	0.05	0.360
Relative_family_history_cancer	0.004	0.938
Radiation_therapy_adjuvant	-0.009	0.889
Pharmaceutical_tx_adjuvant	0.015	0.816
Post_op_ablation_embolization	0.080	0.220
No_History_of_Primary_Risk_Factors	-0.077	0.156
Alcohol_consumption	-0.028	0.612
Hemochromatosis	-0.040	0.461
Hepatitis_B	0.090	0.096
Hepatitis_C	0.042	0.443
Non_Alcoholic_Fatty_Liver_Disease	0.008	0.880
Smoking	0.004	0.936
Cirrhosis	0.015	0.780
Age (Z)	-0.061	0.252
Gender	-0.229	<0.001
Height	0.128	0.019
Weight	0.090	0.100
Race	-0.071	0.188
Ethnicity	-0.007	0.891
Histological_type2	-0.111	0.025
Grade	0.093	0.081
Grade2	0.078	0.142
AJCC_Pathologic_T	-0.026	0.619
AJCC_Pathologic_T2	-0.016	0.764
AJCC_Pathologic_T3	0.021	0.699
AJCC_Pathologic_N	-0.008	0.881
AJCC_Pathologic_M	0.017	0.751
Pathologic_Stage	-0.010	0.853
Pathologic_Stage2	-0.009	0.868
Pathologic_Stage3	-0.037	0.502
Vascular_invasion	0.034	0.557
Vascular_invasion2	0.032	0.584
Residual_tumor	0.052	0.331
AFP	-0.075	0.217
Platelet	-0.058	0.321
Prothrombin	-0.034	0.567
Albumin	0.058	0.327
Total_bilirubin	0.062	0.294
Creatinine	0.083	0.157
Ishak_fibrosis_score	0.099	0.153
Child_Pugh_classification_grade	-0.006	0.927

Pearson's correlation tests were used to evaluate the correlation between MTDH expression and clinical variables of HCC. A two-tailed *P* value <0.05 was regarded to be statistically significant.

four HCC cell lines 48 h after transfection with MTDH siRNAs compared with the blank control and scrambled siRNAs control, in a time-dependent manner (**Figure 9**). The Hoechst 33342 and PI double fluorescent staining provided evidence that MTDH siRNAs promoted HCC apoptosis in all four cell lines, with a solider effect detected in the HepG2 and SMMC-7221 cell lines (**Figures 10** and **11**).

Discussion

Recently, MTDH has gained increasing recognition as an oncogenic factor, with an aberrantly high level of expression in multiple types of cancers, including breast cancer, prostate cancer, gallbladder carcinoma, and HCC [23, 25, 45, 46]. Many studies have been carried out to investigate the clinical significance and the underlying molecular mechanism of MTDH expression in a broad range of cancers. Li W et al. reported that IL-8 accelerated the proliferation and migration ability of gastric cancer cells by interacting with MTDH [47]. A study by Yao Y et al. reported that cryptotanshinone (CPT) exerted an inhibitory function on the proliferation and apoptosis of DU145 prostate cancer cells through the reduction of MTDH levels in DU145 cells, which consequently suppressed the downstream PI3K/AKT signaling pathway [48]. Research from Wang Z et al. revealed that the retinoic acid receptor responder 3 (RARRES3) inhibited the metastasis of colorectal cancer (CRC) both *in vitro* and *in vivo* by suppressing the expression of MTDH [49]. To date, limited studies have been conducted to clarify the carcinogenic role of MTDH in HCC, and the underlying molecular mechanism remains far from elucidation. To facilitate a comprehensive understanding of MTDH expression in HCC, we incorporated IHC, TCGA and ONCOMINE data excavation, meta-analysis and *in vitro* experiments to achieve an accurate evaluation of MTDH expression in the carcinogenesis and progression of HCC that was objectively based on collected large samples.

We first focused on the role of MTDH in the tumorigenesis of HCC. From the results of IHC, TCGA and ONCOMINE, MTDH was significantly over-expressed in HCC tissues than in non-cancer tissues, which suggested that the aberrant expression of MTDH may play an essential role in the pathogenesis of HCC. Furthermore, an AUC value of 0.594 (*P* value =0.031) for ROC

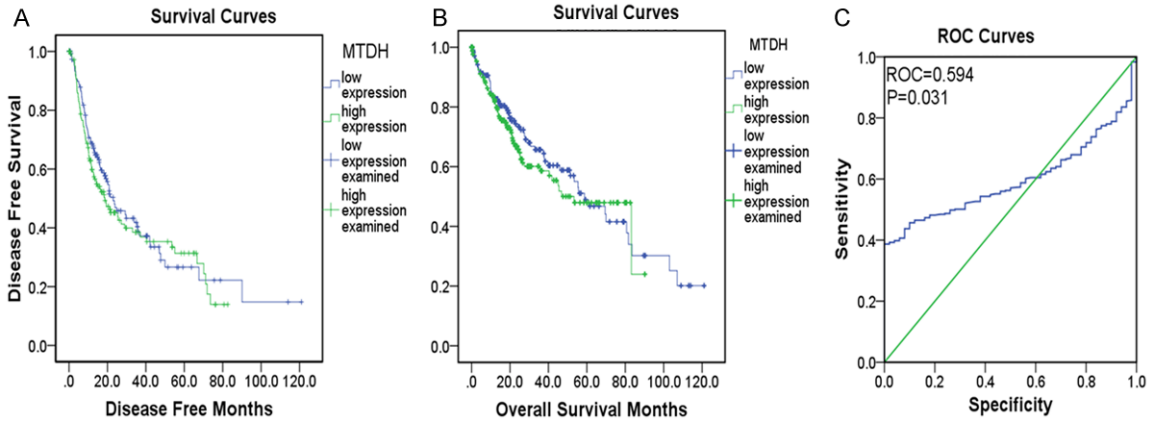

Figure 4. The prognostic and diagnostic value of MTDH in HCC. A and B. Represent the survival or recurrence status of patients with low or high MTDH expression for OS and DFS survival. The *P* values of both were >0.05. C. Represents the ROC curves of MTDH expression for the diagnosis of HCC patients. The AUC value of ROC curve was 0.594, *P* value =0.031.

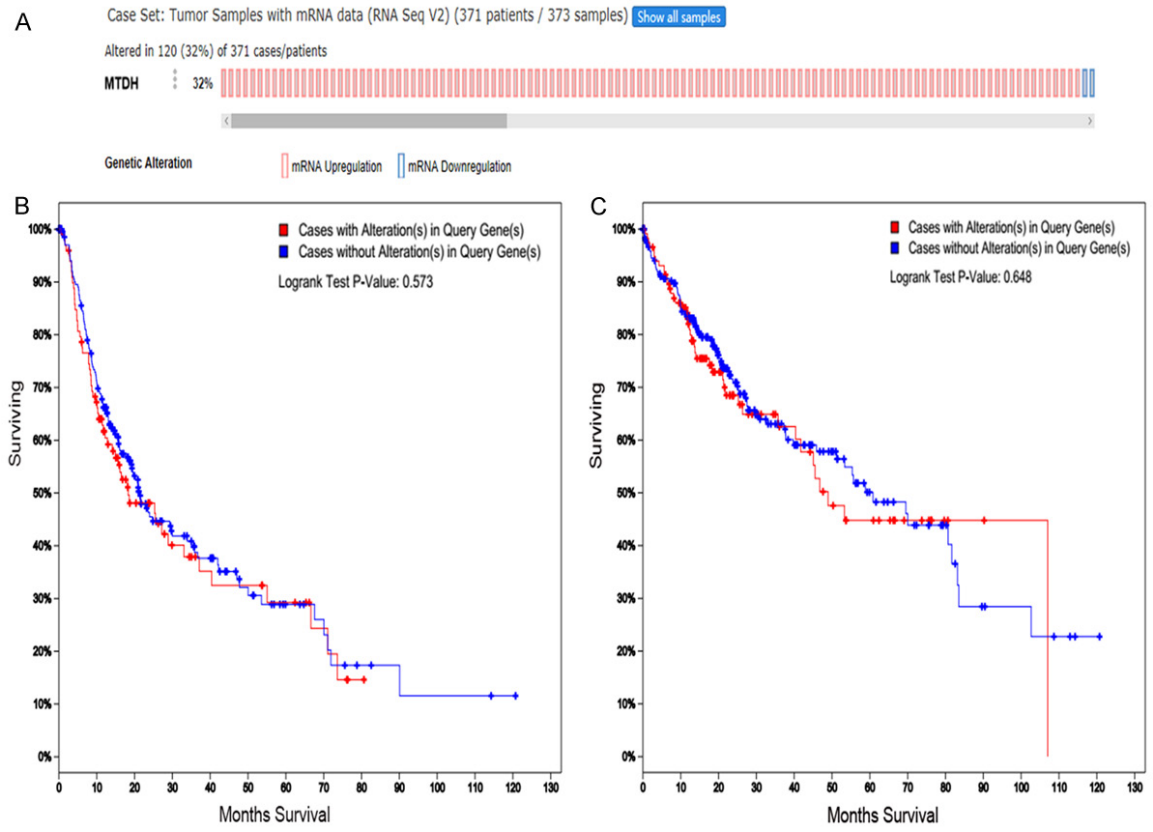

Figure 5. The alteration of MTDH-related mRNAs in HCC and its prognostic significance. A: The data on MTDH gene alteration downloaded from cBioPortal OncoPrint (http://www.cbioportal.org/index.do) indicated 118 cases with mRNA upregulation and 2 cases with mRNA down-regulation. B and C: Reflect the Kaplan-Meier survival analysis of the status of cases with alterations in MTDH genes and cases without alterations in MTDH genes for DFS and OS of HCC patients. Both *P* values >0.05.

curves from TCGA data suggested that MTDH may gain the potential to serve as a future diagnostic target for HCC. In this study, data relevant to the alteration of MTDH-related mRNAs downloaded from cBioPortal OncoPrint also suggested that approximately a third of the

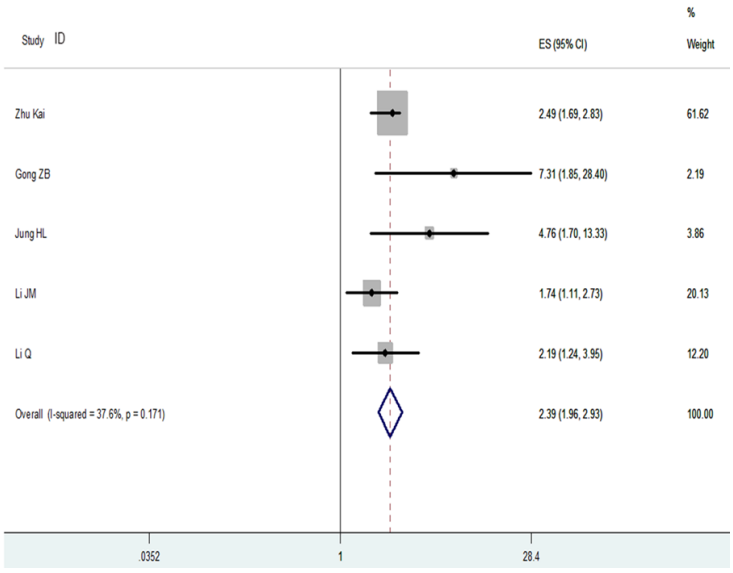

Figure 6. Forest plot of the pooled results. The forest plot reflected the correlation between MTDH expression and OS in HCC.

HCC cases possessed mRNA dysregulation, particularly mRNA up-regulation. However, the relationship between gene alteration of MTDH and the risk of HCC needs further verification.

Next, we were interested in the significance of MTDH in the progress of HCC. The results of our study indicated that MTDH was also correlated with some clinicopathological features of HCC. Meta-analysis and IHC results revealed that MTDH was associated with the metastasis of HCC. The study by LI C et al. reported that MTDH facilitates the metastatic ability of HCC cells [50], which was in accordance with our meta-analysis. The mechanism by which MTDH promotes tumor metastasis may be explained by the relationship between the epithelial-mesenchymal transition (EMT) and MTDH expression. Previous experiments with cancer cell lines demonstrated that the invasiveness, progression, and metastasis of cancer cells can be regulated by MTDH via EMT [24]. Zhang J et al. also reported that MTDH could mediate EMT development induced by CCL20/CCR6 via modulating the Erk1/2 and Akt signaling pathway in cervical cancer [51], which strengthens the correlation between MTDH expression and EMT in HCC. Moreover, the results from our IHC and meta-analysis confirmed that MTDH may facilitate vascular invasion and was significantly overexpressed in HCC tissues with advanced pathological grade and lower histological differentiation, thus we postulate that the overexpression of MTDH may facilitate the deterioration of HCC. The study from Yoo BK et al. [27] showed that MTDH regulated the expression of genes associated with invasion, metastasis, chemo-resistance, angiogenesis and senescence. MTDH also played a role in the activation of Wnt/β-catenin signaling pathway by activating ERK42/44 and inducing the up-regulation of the ultimate commander of the Wnt pathway: lymphoid-enhancing factor 1/T cell factor 1 (LEF1/TCF1), which provides a hint that MTDH may stimulate the malignant potential of MTDH by modulating related pathways including Wnt/β-catenin signaling pathway. There is also study that evaluated MTDH and its related molecular targets in HCC via IHC. For example, MTDH and DIO1 expression were inversely correlated in IHC from a previous study that validated MTDH could contributed to non-thyroidal illness syndrome-related HCC via mediating DIO1 expression [52]. We can benefit from validating the association between MTDH expression and other molecules in future studies. Additionally, our IHC and TCGA results indicated that MTDH expression was also significantly higher in patients with HBV infection and showed difference in the groups of age and height. Up to date, no study has concentrated on the association between MTDH expression and HBV infection, age or height. Whether the expression of MTDH correlated with HBV infection, age or height of HCC patients need further validation with larger samples.

Specially, we further assessed the prognostic value of MTDH in HCC. The results from the pooling HR values indicated that MTDH was of prognostic significance in HCC. Subgroup analysis revealed that the classifications of country, blinded reading and NOS scores may be the source of heterogeneity. Furthermore, the results from sensitivity analysis proved the robustness of our analysis results and no publication bias existed in our study cohorts. Nevertheless, deficiencies can be found in our meta-analysis. The selected studies were published in either Chinese or English, and studies

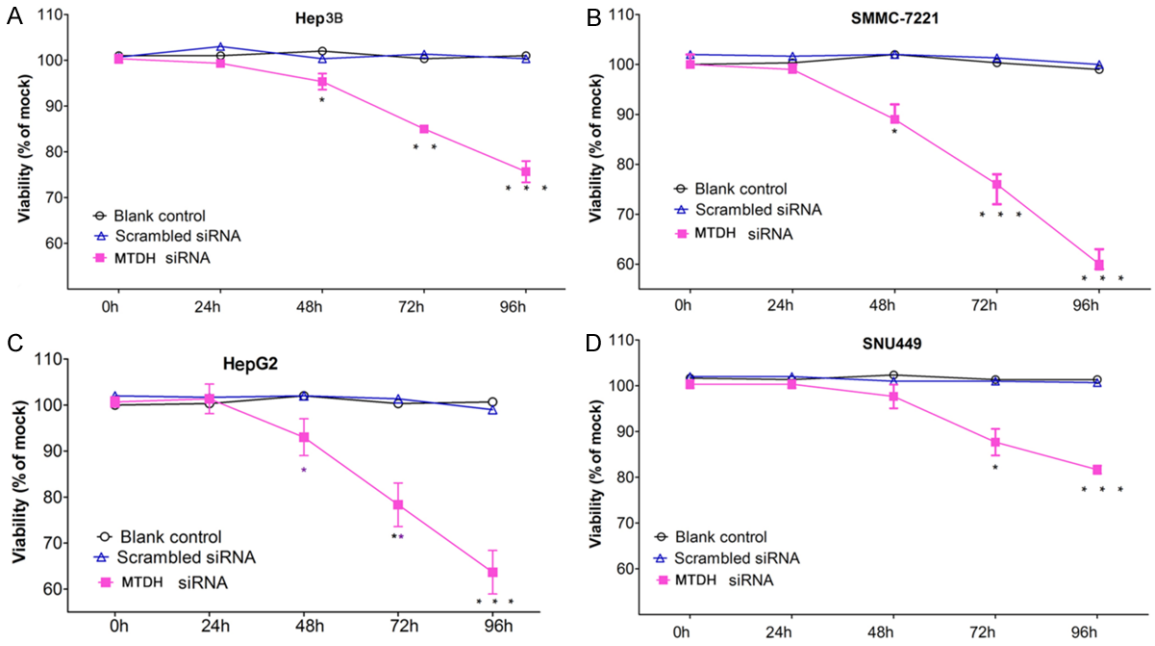

Figure 7. The effect of MTDH on cell viability in four cell lines. A: Hep3B cell lines; B: SMMC7221 cell lines; C: HepG2 cell lines; D: SNU449 cell lines. Bars represent standard deviation. **P* value <0.05, ***P* value <0.01 and ****P* value <0.001 compared to blank and scrambled RNA controls at the same time point.

related to MTDH expression in HCC in other language were not included in our study. In addition, all the samples in our meta-analysis originated from Asia. HCC patients from other regions of the world were not enrolled in our study. The racial and geographical imbalance may affect the reliability of our results. Due to the limited studies in our meta-analysis, the subgroup analysis failed to explain the significant heterogeneity in our analysis for the relationship between MTDH expression and liver cirrhosis, tumor size, TNM stage or DFS in HCC. The 95% CI of the pooling HR or OR in the above items contains 1, thus, a statistically significant relationship could not be established between MTDH and these variables. Further study with a larger study sample size is necessary for a more precise interpretation on MTDH expression in HCC tissues.

As for the biological function of MTDH in HCC cells via *in vitro* experiments, MTDH siRNAs induced a suppression of HCC cell viability and proliferation in all the cell lines tested. Additionally, all the cell lines exhibited a consistent activation of cell apoptosis and caspase-3/7 activity after transfection with MTDH siRNAs. A combined method of fluorimetric resorufin viability assay and MTS tetrazolium assay was performed to detect the influence of MTDH on HCC cells, and a combined method of Apo-ONE Homogeneous Caspase-3/7 Assay and Hoechst 33342/PI double florescent chromatin staining was carried out to identify the influence of MTDH on the apoptosis of HCC cells. The combination of various methods strengthened the reliability of our results. We can infer from the results of our experiments that MTDH may stimulate the viability as well as proliferation of HCC cells, and inhibit the apoptosis and caspase-3/7 activity of HCC cells. Li WF et al. also reported that knockdown of MTDH suppressed the growth and induced apoptosis in HCC cells through activating PTEN [53], which is consistent with our results. Another study conducted on a mouse model revealed that hepatocytes from mice transfected with MTDH exhibited chemotherapy-resistance, senescence-resistance and the augmented ability to induce angiogenesis through activating the coagulation pathway [54]. These findings inspired us that MTDH might exert an oncogenic function on hepatocyte through modulating potential targets and pathways that await further investigation.

Recently, a growing body of studies have focused on the role of miRNAs in the occur-

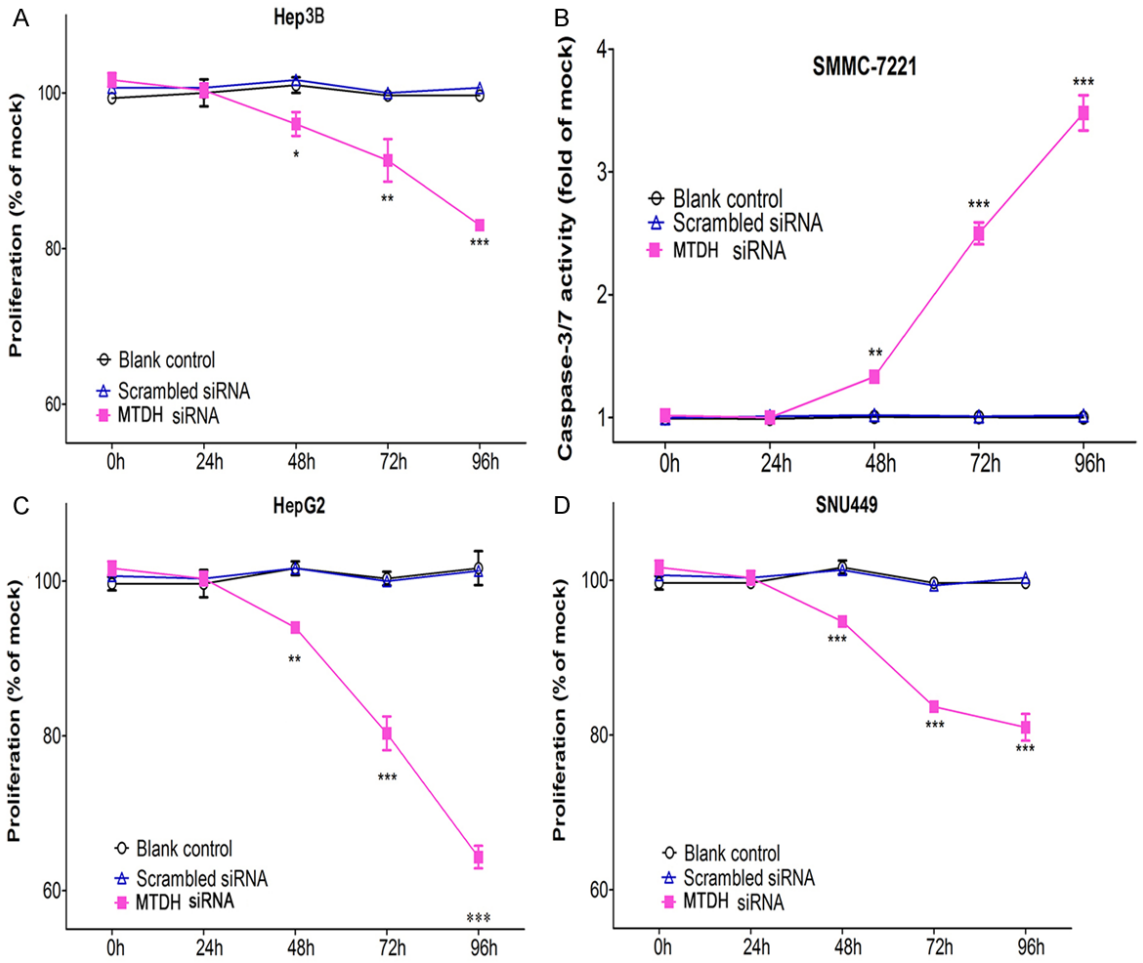

Figure 8. The effect of MTDH on cell proliferation in four cell lines. A: Hep3B cell lines; B: SMMC-7221 cell lines; C: HepG2 cell lines; D: SNU449 cell lines. Bars represented standard deviation. **P* value <0.05, ***P* value <0.01 and ****P* value <0.001 compared to blank and scrambled RNA controls at the same time point.

rence and progression of various human cancers. MiRNAs are small non-coding RNAs with a length of 22-24 nucleotides that inhibit the expression of target mRNAs by initiating the degradation or suppression of the process of translation [55] It is acknowledged that miRNAs can act as tumor suppressors or oncogenes in the development of cancer [56]. There are also some studies reported on the relationship between the expression of miRNAs and MTDH in HCC. A previous study from our research team reported that miR-30a-5p could inhibit cell growth and induce apoptosis in HCC via directly targeting MTDH [57]. In the study by Yan JJ et al., down regulation of miR-497 was validated to accelerate angiogenesis and metastasis in HCC, and miR-497 inhibited the 3'-untranslated regions of MTDH [58]. Yu J et al. reported that miR-320a suppressed cell metastasis by directly inhibiting MTDH expression [59]. Since an individual miRNA could target multiple genes and a single gene could also be a target of different miRNAs, we speculate that MTDH may be triggered by other corresponding miRNAs, thus leading to the contribution to the carcinogenesis of HCC. Therefore, seeking miRNAs targeted by MTDH may serve as a novel strategy for the diagnosis and treatment of HCC.

Nevertheless, there were limitations in our study, as can be illustrated in the following aspects. For the meta-analysis, the number of selected studies restricted us to verify the source of heterogeneity for the association between MTDH and clinical variables including liver cirrhosis, tumor size, TNM stage or DFS. The diagnostic value of MTDH in HCC was omit-

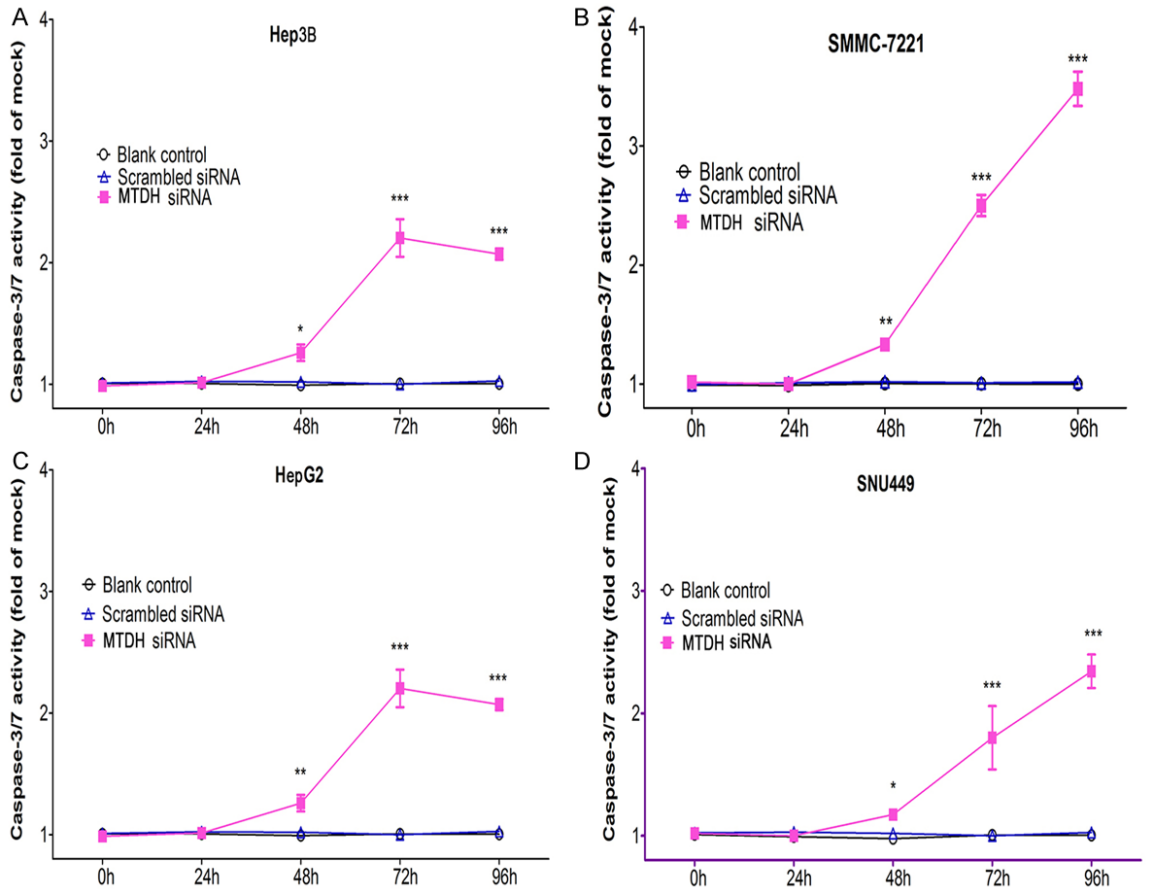

Figure 9. The effect of MTDH on caspase-3/7 activities in four cell lines. A: Hep3B cell lines; B: SMMC-7221 cell lines; C: HepG2 cell lines; D: SNU449 cell lines. Bars represent standard deviation. *P value <0.05, **P value <0.01 and ***P value <0.001 compared to blank and scrambled RNA controls at the same time point.

ted in our study because no study with diagnostic data of MTDH emerged in the literature research. Hence, further study with a larger sample is necessary to identify the clinical significance of MTDH in HCC. It should also be noted that all the samples in our IHC and TCGA data were from tissues. We failed to collect the serum samples of HCC patients to investigate the clinical implication of MTDH in HCC and currently there is no study evaluating the clinicopathological significance of MTDH from serum in HCC. Future work was needed to assess expression of serum MTDH in HCC as noninvasive methods are more valuable in the early diagnosis of HCC. In addition, *in vivo* experiments corresponding to our *in vitro* experiment were not included in our study. The results from the *in vitro* experiments need to be confirmed by *in vivo* experiment to elucidate the effect of MTDH on cell growth, caspase-3/7 activity and cell apoptosis.

In summary, our study confirms that MDTH was over-expressed in HCC tissues with a prospective diagnostic significance, and MDTH was significantly correlated with histological differentiation, vascular invasion and metastasis, which can reflect the progression of HCC. Further *in vitro* experiments verified that MDTH siRNAs can inhibit cell proliferation and induce apoptosis in HCC. The results of our studies provide evidence for promotion of MDTH in clinical applications for an improved diagnosis and treatment of HCC patients.

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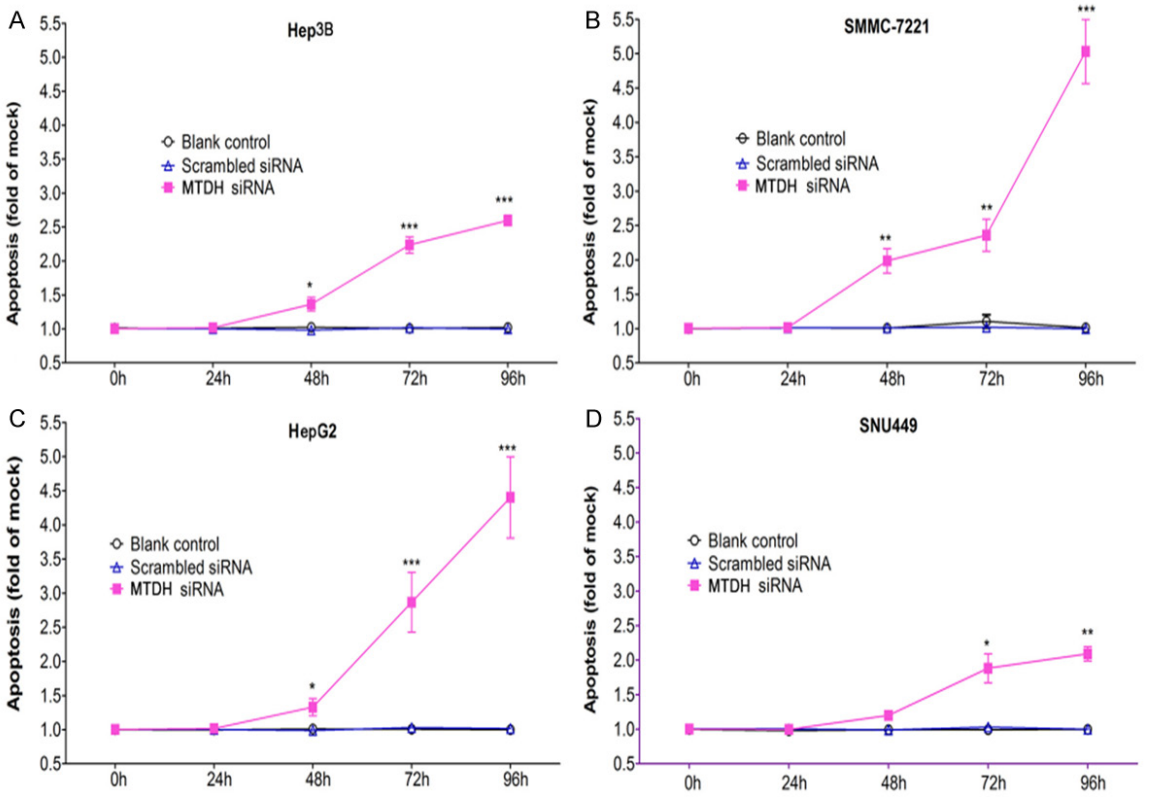

Figure 10. The effect of MTDH on apoptosis in four cell lines. A: Hep3B cell lines; B: SMMC-7221 cell lines; C: HepG2 cell lines; D: SNU449 cell lines. Bars represent standard deviation. *P value <0.05, **P value <0.01 and ***P value <0.001 compared to blank and scrambled RNA controls at the same time point.

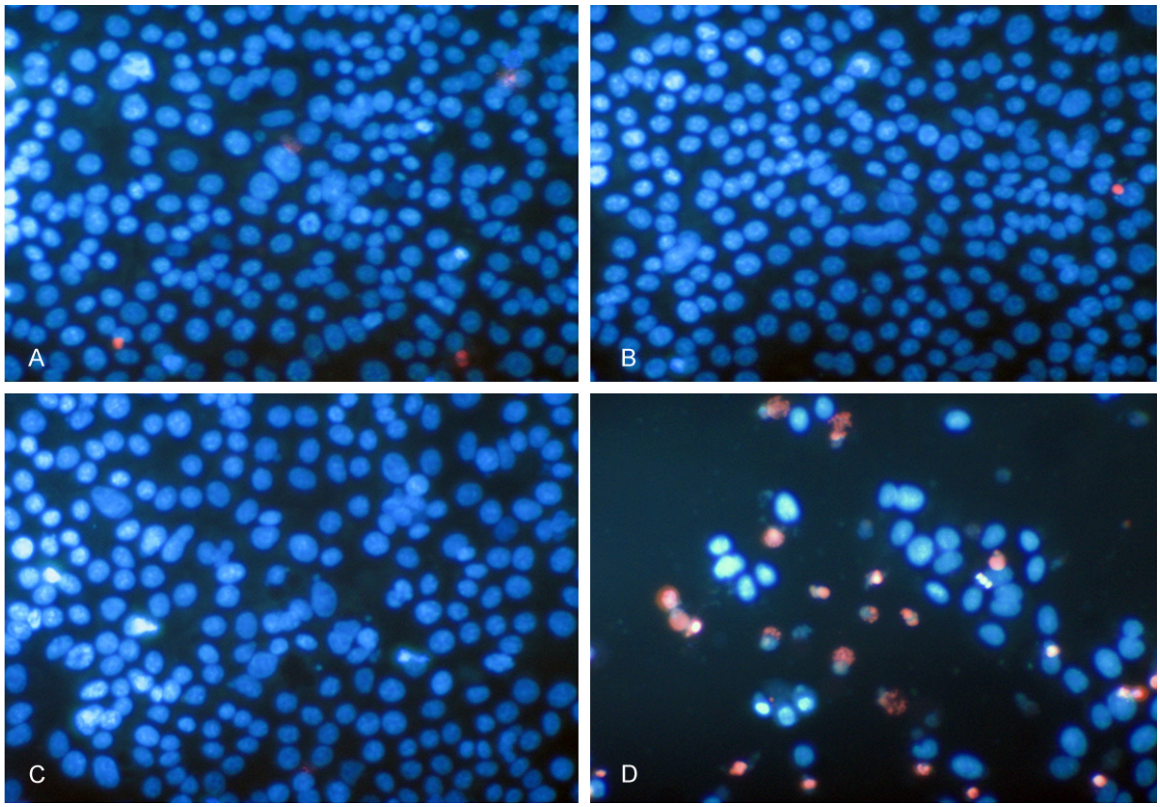

Figure 11. Four pictures present the results from Hoechst 33342 and PI double fluorescent staining. A. Blank control; B. Mock control; C. Scrambled siRNA; D. MTDH siRNA. The viable cell number of cell lines transfected with MTDH siRNA was less than the other three controls, and apoptotic cells were increasing, indicating that the MTDH siRNA could inhibit cell growth and promote apoptosis.

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Disclosure of conflict of interest

None.

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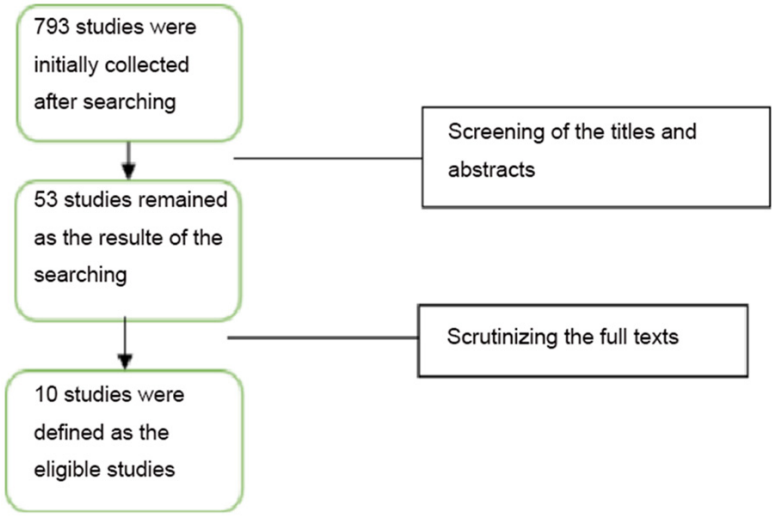


Supplementary Figure 1. The flowchart of the meta-analysis in the current paper. The flowchart described the process of selecting the eligible studies for the current meta-analysis.

Supplementary Table 1. Characteristics of the selected studies

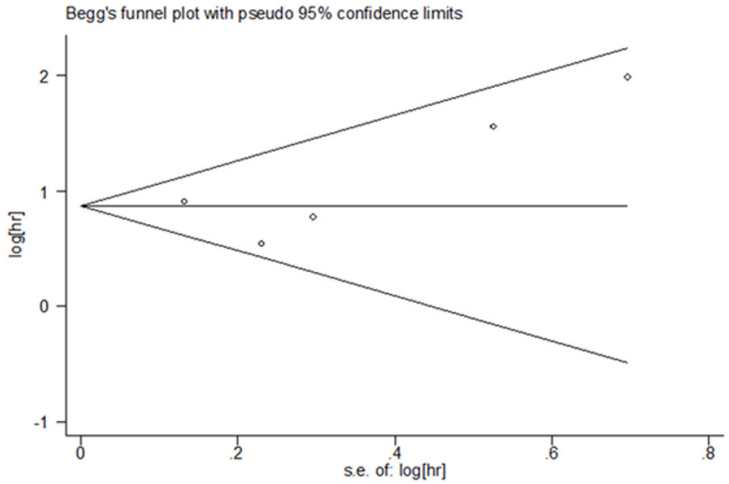
First author	Huang JS	Luo YH	Cheng YJ	Zhu K	Gong ZB	Jung HL	Ahn S	Li JM	Li Q	Chen XJ
Year	2012	2015	2015	2011	2012	2015	2013	2015	2014	2013
Country	China	China	China	China	China	Korea	Korea	China	China	China
N (M/F)	50 (44/6)	89 (72/1)	40 (30/7)	323 (277/46)	73 (62/11)	85 (69/16)	288 (237/51)	152 (132/20)	87 (44/43)	107 (73/34)
Antibody	anti-MTDH	anti-MTDH	anti-MTDH	NR	anti-MTDH	anti-MTDH	anti-MTDH	anti-MTDH	anti-MTDH	anti-MTDH
Staining for MTDH positivity	NR	Staining index score ≥3	NR	NR	Staining index score ≥6	Staining index score ≥3	Staining index score ≥7	Staining index score ≥7	Staining index score ≥2	Staining index score ≥4
Blinded-reading	NR	Yes	NR	NR	Yes	Yes	Yes	NR	Yes	NR
Follow-up (Months)				20*	60*	130*	126	80*	60	72*
Analysis types for survival				OS	OS	OS	DFS	OS	OS	DFS
Statistical method	Multivariate analysis	Multivarte analysis	Multivariate analysis	Multivarate analysis	Multivariate analysis	Multivariate analysis	Multivariate analysis	Multivariate analysis	Multivariate analysis	Multivariate analysis
HR				2.492	7.314	4.756	1.451	1.736	2.19	3.431
LL				1.69	1.848	1.697	1.082	1.106	1.24	1.254
UL				2.83	28.398	13.329	1.944	2.726	3.95	7.318
P				<0.001	0.004	0.003	0.014	0.017	<0.05	0.008
Liver cirrhosis (-/+)	15/35	46/43				25/60				30/22
Tumor size (≤5 cm/>5 cm)	10/40	15/74	22/18		27/46	52/33	190/98	101/51	68/19	35/17
Histological differentiation (high/median, low)		6/83	16/24		9/64	42/43	30/258	93/59		29/23
TNMstage (I-II/III-IV)			22/18		57/16	52/33				
Vascular invasion	32/18				62/11	56/29	129/159	69/83		
Metastasis (-/+)	20/30	43/46			67/6	59/26	220/68	99/53	62/25	
Quality assessment	7	6	4	7	7	7	8	8	6	7

NR: Not Clear.

Supplementary Table 2. Summary of pooled results in the meta-analysis

Groups	No. of studies	OR and 95% CI	*P* value	Heterogeneity		Model
				I^2	*P* value	
Liver cirrhosis	5	1.049/(0.647-1.700)	0.846	45.50%	0.119	R
Tumor size	9	0.671/(0.438-1.026)	0.065	53.70%	0.027	R
Histological differentiation	7	0.346/(0.171-0.697)	0.003	65.30%	0.008	R
TNM stage	4	0.603/(0.174-2.086)	0.424	79.30%	0.002	R
Vascular invasion	6	0.388/(0.294-0.511)	<0.001	0.00%	0.615	F
Metastasis	7	3.971/(1.959-8.051)	<0.001	67.80%	0.005	R
Groups	No. of studies	HR and 95% CI	P	Heterogeneity		Model
				I-square%	P	
OS	5	2.394 (1.956-2.931)	<0.001	37.60%	0,171	F
DFS	2	2.009 (0.887-4.554)		69.60%		R

OS: Overall Survival; DFS: Disease-free Survival; OR: Odds Ratio; HR: Hazards Ratio.

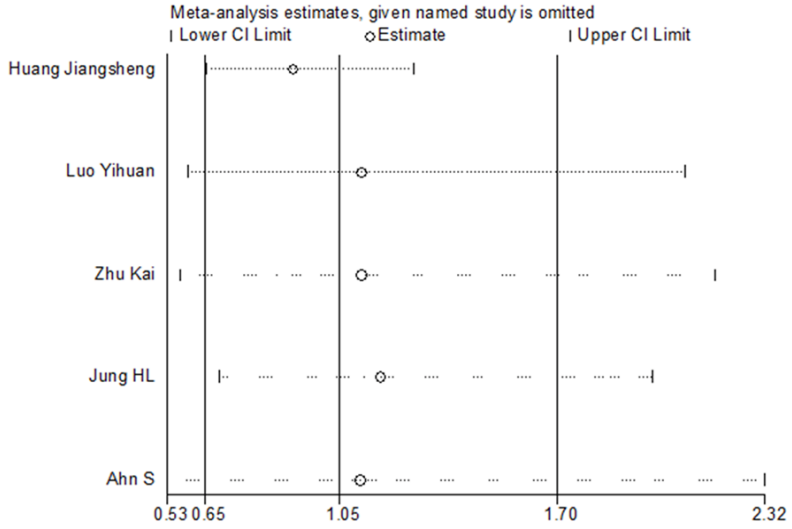


Supplementary Figure 2. Begg's test and Egger's test plus funnel plot. Publication bias in pooled results of OS was examined.

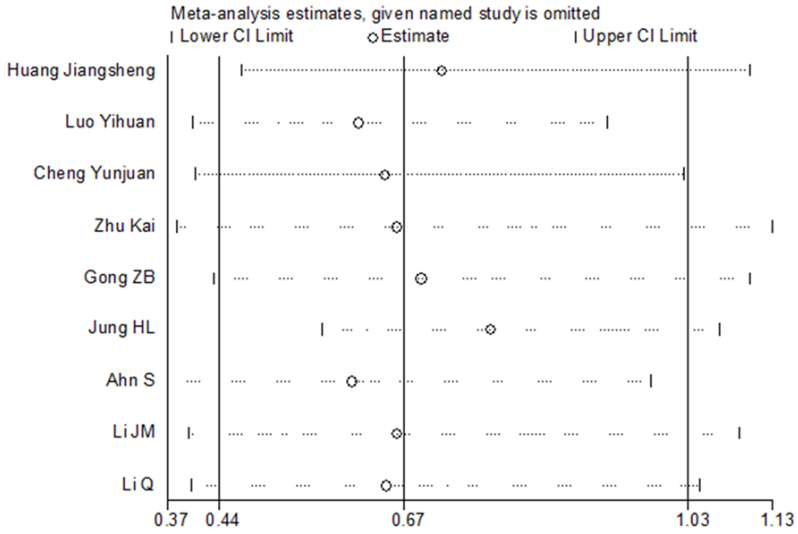
Supplementary Table 3. Subgroup analysis of MTDH expression with clinicopathological factors and survival

Subgroup	Tumor size	Histological differentiation	TNM stage	Metastasis	OS
Country					
China	OR=0.697, 95% CI=0.496-0.978	OR=0.520, 95% CI=0.247-1.095	OR=0.870, 95% CI=0.027-28.278	OR=4.295, 95% CI=1.982-9.305	HR=2.329, 95% CI=1.895-2.863
Korea	OR=0.367, 95% CI=0.038-3.513	OR=0.125, 95% CI=0.057-0.278	OR=0.467, 95% CI=0.133-1.646	OR=4.733, 95% CI=0.328-68.265	HR=4.756, 95% CI=1.697-13.329
Blinded reading					
NR	OR=0.635, 95% CI=0.447-0.904	OR=0.734, 95% CI=0.254-2.118	OR=5.067, 95% CI=1.095-23.445	OR=3.215, 95% CI=0.938-11.020	HR=2.280, 95% CI=1.823-2.852
Yes	OR=0.685, 95% CI=0.308-1.524	OR=0.164, 95% CI=0.086-0.315	OR=0.350, 95% CI=0.113-1.079	OR=4.935, 95% CI=1.714-14.206	HR=2.982, 95% CI=1.857-4.789
Quality assessment					
7	OR=0.363, 95% CI=0.169-0.782	OR=0.284, 95% CI=0.146-0.552	OR=0.196, 95% CI=0.080-0.482	OR=21.094, 95% CI=2.679-166.057	HR=2.677, 95% CI=2.093-3.424
6	OR=1.324, 95% CI=0.494-3.551	OR=0.347, 95% CI=0.060-2.002		OR=14.317, 95% CI=3.840-53.380	HR=2.190, 95% CI=1.227-3.909
8	OR=0.911, 95% CI=0.598-1.386	OR=0.237, 95% CI=0.051-1.094	OR=0.821, 95% CI=0.446-1.511	OR=1.850, 95% CI=0.911-3.755	HR=1.736, 95% CI=1.106-2.725
4	OR=1.026, 95% CI=0.254-4.136	OR=10.714, 95% CI=1.210-94.862	OR=5.067, 95% CI=1.095-23.445		
Staining for MTDH positivity					
Staining index score ≥3		OR=0.185, 95% CI=0.075-0.457		OR=6.346, 95% CI=0.853-47.189	
Staining index score ≥6		OR=0.244, 95% CI=0.056-1.074		OR=8.301, 95% CI=0.449-153.521	
Staining index score ≥7		OR=0.237, 95% CI=0.051-1.094		OR=1.636, 95% CI=1.061-2.522	
Staining index score ≥2				OR=14.317, 95% CI=3.840-53.380	

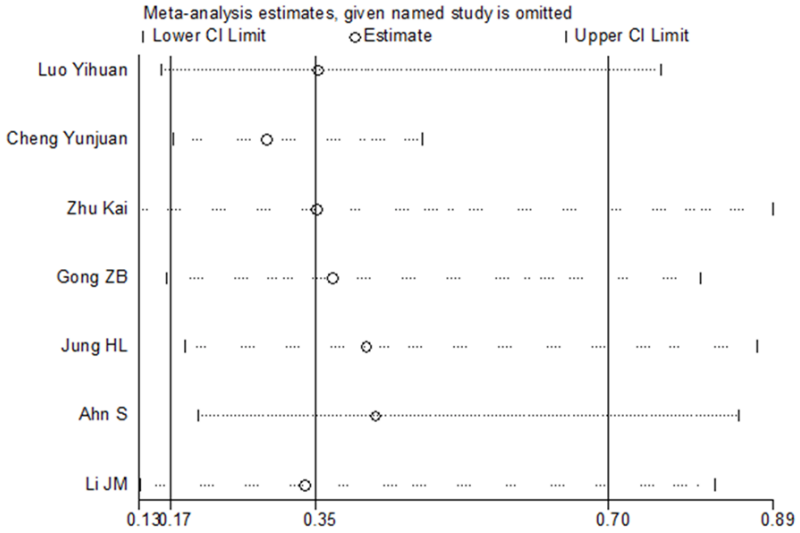
OS: Overall Survival; DFS: Disease-free Survival; OR: Odds Ratio.



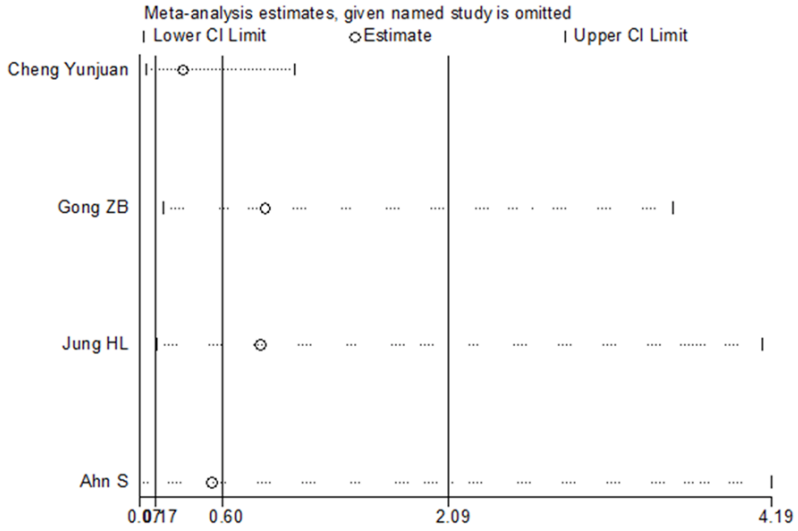
Supplementary Figure 3. Sensitivity analysis of the pooled results for liver cirrhosis. The sensitivity analysis indicated that no study significantly influenced the pooled OR.



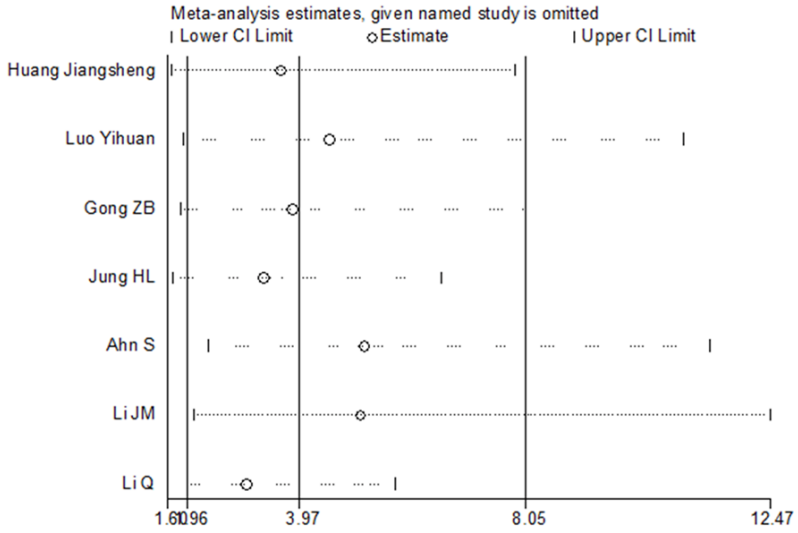
Supplementary Figure 4. Sensitivity analysis of the pooled results for tumor size. The sensitivity analysis indicated that no study significantly influenced the pooled OR.



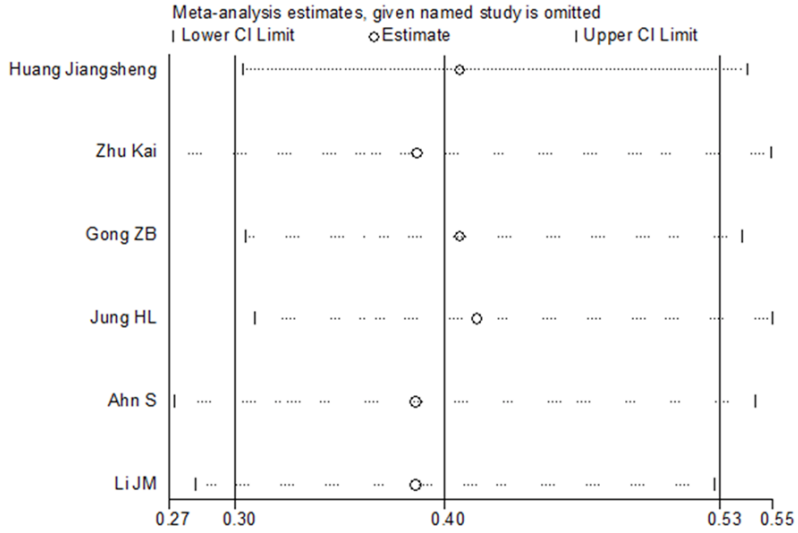
Supplementary Figure 5. Sensitivity analysis of the pooled results for histological differentiation. The sensitivity analysis indicated that no study significantly influenced the pooled OR.



Supplementary Figure 6. Sensitivity analysis of the pooled results for TNM stage. The sensitivity analysis indicated that no study significantly influenced the pooled OR.



Supplementary Figure 7. Sensitivity analysis of the pooled results for metastasis. The sensitivity analysis indicated that no study significantly influenced the pooled OR.



Supplementary Figure 8. Sensitivity analysis of the pooled results for overall survival. The sensitivity analysis indicated that no study significantly influenced the pooled OR.