Expression of cystathionine ß-synthase is downregulated in hepatocellular carcinoma and associated with poor prognosis

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Abstract. The cystathionine ß-synthase (CBS) gene encodes an enzyme that catalyzes the synthesis of cystathionine in the trans-sulfuration pathway and is subject to tight regulation because of its critical role in antioxidant and methylation metabolism. The expression level of CBS in 120 hepatocellular carcinoma (HCC) specimens evaluated by real-time reverse transcriptase PCR (RT-PCR) is markedly lower than in surrounding non-cancerous liver ($P<0.0001$). The correlation between CBS gene expression in HCC and clinicopathological parameters or survival of HCC patients was statistically analyzed in the present study. Our study demonstrated that reduced CBS expression is significantly correlated with high tumor stage ($P=0.0019$), high Edmondson grade ($P=0.00084$), and high AFP level ($P=6.2\times10^{-5}$). Interestingly, a survival analysis showed that a significantly shorter overall survival (OS) time is observed in patients with reduced CBS expression ($P=0.0022$), although CBS expression was determined not to be an independent prognostic factor for OS ($P=0.071$) after considering tumor stage, tumor size, and AFP level. However, for the 62 patients with low AFP levels (<100 ng/ml), reduced CBS expression was found to be an independent prognostic factor for OS ($P=0.0042$) after considering tumor stage and tumor size. Thus, the expression level of CBS mRNA could be useful to predict clinical outcome of HCC, especially for patients with low AFP levels.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignant tumors worldwide, and particularly in Asia and sub-Saharan Africa. Furthermore, the incidence of HCC is increasing in several developed countries, including the United States (1). HCC is caused mainly by chronic hepatitis due to hepatitis B virus, hepatitis C virus, alcohol abuse, or hemochromatosis (2). HCC is a type of cancer that is highly resistant to conventional antineoplastic medicine. Although diverse treatment modalities, including surgical, medical, and radiological measures, have been introduced in recent anticancer therapies, the clinical course of HCC is variable and the overall survival (OS) of HCC patients remains poor (3). Thus, identification of key genes involved in the molecular pathogenesis of HCC may improve therapeutic strategies.

The cystathionine ß-synthase (CBS) gene, located on chromosome 21q22.3, encodes an enzyme that catalyzes the synthesis of cystathionine in the trans-sulfuration pathway (4). CBS is highly regulated due to its role that links methionine metabolism to the biosynthesis of cellular redox-controlling molecules, like cysteine, glutathione, and taurine (5). Aberrations in methylation and redox homeostasis are common to a number of chronic diseases including pathologies of liver. In alcoholic liver disease and hepatocellular carcinoma, an increase in markers of oxidative stress has been observed (6). Indeed, CBS levels were diminished in a mouse model for chronic steatohepatitis, human liver cirrhosis, and HCC (7,8). However, only a small number of human HCC specimens were analyzed and the clinical implications of CBS expression in HCC have not been fully investigated.

The aim of the present investigation was to examine whether CBS expression could be used to predict the clinical course of HCC. Using a real-time RT-PCR analysis of CBS gene expression, we found reduced expression of CBS gene, which correlated with several clinicopathological features and poor prognosis of HCC. Thus, potential biological changes induced by reduced CBS gene expression require further study, as they may have implications in predicting clinical

outcome and choosing treatment modalities as well as understanding the molecular pathogenesis of gene expression changes in methionine metabolism observed in malignancy.

Materials and methods

Patients and tissue samples. HCC (T) and corresponding non-cancerous hepatic tissues (NT) were obtained with informed consent from 120 patients who underwent curative hepatectomy for primary HCC between 2001 and 2006 in Department of Surgery, Samsung Medical Center, Korea. The study protocol was approved by the Institutional Review Board of Samsung Medical Center. Complete clinical data were available in all 120 cases (median follow-up, 50 months; range, 3-92 months). The patients, ranging in age from 21 to 78 years (mean, 51.3 years) and having adequate liver function reserve, had survived for at least 2 months after hepatectomy, and none received treatment prior to surgery such as transarterial chemoembolization or radiofrequency ablation. Clinicopathological features of the 120 HCCs in this study are described in Table I. Surgically resected specimens were partly embedded in paraffin after fixation in 10% formalin for histological processing and partly immediately frozen in liquid nitrogen and stored at -80˚C. All available hematoxylin and eosin stained slides were reviewed. The differentiation of tumor cells was based on the criteria proposed by Edmondson and Steiner (I, well differentiated; II, moderately differentiated; III, poorly differentiated; IV, undifferentiated) (9). The conventional TNM system outlined in the cancer staging manual (6th edition) by the American Joint Committee on Cancer (AJCC) was used in tumor staging.

RNA extraction and cDNA synthesis. Total RNA was extracted from cancerous and surrounding non-cancerous frozen tissues using an RNeasy minikit (Qiagen, Germany) according to the manufacturer's instructions. The integrity of all tested total RNA samples was verified using a Bioanalyzer 2100 (Agilent Technologies, USA). DNase I treatment was routinely included in the extraction step. Residual genomic DNA contamination was assayed by a quantitative real-time PCR assay for GAPDH DNA and samples with contaminating DNA were re-subjected to DNase I treatment and assayed again. Samples containing 4 μg of total RNA were incubated with 2 μl of 1 μM oligo $d(T)_{18}$ primer (Genotech, Korea) at 70˚C for 7 min and cooled on ice for 5 min. The enzyme mix was separately prepared in a total volume of 11 μl by adding 2 μl of 0.1 M DTT (Sigma, USA), 2 μl of 10X reverse-transcription buffer, 5 μl of 2 mM dNTP, 1 μl of 200 U/μl MMLV reverse-transcriptase, and 1 μl of 40 U/μl RNase inhibitor (Enzynomics, Korea). After adding the enzyme mix to the annealed total RNA sample, the reaction was incubated for 90 min at 42˚C prior to heat inactivation of reverse-transcriptase at 80˚C for 10 min. The cDNA samples were brought up to a final volume of 400 μl by the addition of diethylpyrocarbonate (DEPC)-treated water.

Quantitative real-time PCR. Real-time PCR amplifications were carried out in 384-well plates according to the instructions of the manufacturer, using Applied Biosystems PRISM 7900HT instruments. The real-time PCR analysis was performed in a total volume of 10 μl with 5 μl of 2X TaqMan gene expression master mix (Applied Biosystems, USA), 1 μl each of 5 μM forward and reverse primers and 1 μM probe (Genotech), and 2 μl of cDNA (or water as a control, which was always included). The amplification steps were as follows: initial denaturation step, 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 15 sec; elongation at 60˚C for 1 min. The primer and probe sequences were designed using Primer Express 3.0 software (Applied Biosystems) and all probe sequences were labeled with FAM at the 5' end and with TAMRA at the 3' end. The following primer and probe sequences were used: B2M forward (5'-CAT TCG GGC CGA GAT GTC T-3'), reverse (5'-CTC CAG GCC

Table I. Relations between CBS mRNA levels and clinicopathological features in HCC.

	All patients (n=120)		
Clinicopathological parameters	High CBS (n=67) Copy number ratio ≥1.44	Low CBS (n=53) Copy number ratio <1.44	P-value
Age (years)			
<55	35	39	
≥55	32	14	0.028
Gender			
Male	51	41	
Female	16	12	0.954
HBsAg			
Absent	17	5	
Present	50	48	0.045
HCV			
Absent	61	51	
Present	6	2	0.446
Liver cirrhosis			
Absent	38	27	
Present	29	26	0.656
Tumor stage			
I	35	11	
II	19	23	
III + IV	13	19	0.0019
AFP level			
<100 ng/ml	46	16	
≥100 ng/ml	21	37	$6.2x10^{-5}$
Tumor size (cm)			
<5	43	28	
≥5	24	25	0.285
Edmondson grade			
I	24	4	
II	36	37	
III + IV	7	12	0.00084

AGA AAG AGA GAG TAG-3') and probe (5'-CCG TGG CCT TAG CTG TGC TCG C-3'); GAPDH forward (5'-CAC ATG GCC TCC AAG GAG TAA-3'), reverse (5'-TGA GGG TCT CTC TCT TCC TCT TGT-3') and probe (5'-CTG GAC CAC CAG CCC CAG CAA G-3'); HMBS forward (5'-CCA GGG ATT TGC CTC ACC TT-3'), reverse (5'-AAA GAG ATG AAG CCC CCA CAT-3') and probe (5'-CCT TGA TGA CTG CCT TGC CTC CTC AG-3'); HPRT1 forward (5'-GCT CGA GAT GTG ATG AAG GAG AT-3'), reverse (5'-CCA GCA GGT CAG CAA AGA ATT-3') and probe (5'-CCA TCA CAT TGT AGC CCT CTG TGT GCT C-3'); SDHA forward (5'-CAC CTA GTG GCT GGG AGC TT-3'), reverse (5'-GCC CAG TTT TAT CAT CTC ACA AGA-3') and probe (5'-TGG CAC TTA CCT TTG TCC CTT GCT TCA-3'); CBS forward (5'-GTT GGC AAA GTC ATC TAC AAG CA-3'), reverse (5'-GGG CGA AGT GGT CCA TCT C-3') and probe (5'-ACG CTG GGC AGG CTC TCG CAC-3'). Expression of CBS mRNA was measured (the number of cycles required to achieve a threshold, or C_T) in triplicate, and then normalized relative to a set of reference genes (B2M, GAPDH, HMBS, HPRT1 and SDHA) by subtracting the average of the expression of the 5 reference genes (10). Using the ΔC_T value (CBS C_T - average C_T of reference genes), the mRNA copy number ratio was calculated as $2^{-\Delta Ct}$. Standard curves were constructed from the results of simultaneous amplifications of serial dilutions of the cDNA samples.

Statistical analysis. All statistical analyses were done with the open source statistical programming environment R (http://www.r-project.org/). Significant differences between gene expression levels were evaluated by a Student's t-test. Correlation between gene expression and clinicopathological variables was evaluated using the χ^2 test. Kaplan-Meier survival curves were calculated using tumor recurrence (defined as the first appearance of tumor at any site following definitive treatment) or death as the end points. The difference in the time interval to overall survival or disease-free survival was calculated by means of a log-rank test. In addition, the Cox proportional hazard regression model was used to identify independent prognostic factors for overall survival and disease-free survival. For the Cox analysis, stratification for Edmondson grade was low (Edmondson grades 1 and 2) vs. high (Edmondson grades 3 and 4) and stratification for the tumor stage was low (stages 1 and 2) vs. high (stages 3 and 4). A two-tailed P-value test was used and a P-value of <0.05 was considered statistically significant.

Results

Expression of CBS gene in hepatocellular carcinoma. We performed real-time RT-PCR for CBS mRNA from frozen paired samples derived from 120 patients with HCC. A total of 120 HCCs (T) and 40 non-cancerous hepatic samples (NT) were assessed by real-time RT-PCR. Expression of CBS mRNA was measured in triplicate, and then normalized relative to a set of reference genes (B2M, GAPDH, HMBS, HPRT1, SDHA) by subtracting the average of the expression of the 5 reference genes (10). Expression of CBS mRNA was significantly lower in T than in NT (0.78 vs. 1.67; median copy number ratio, P<0.0001) (Fig. 1A). In addition, CBS mRNA levels in moderately-differentiated tumors (Edmondson grade 2) were significantly lower than in well-differentiated tumors (Edmondson grade 1), (0.69 v.s 1.34; median copy number ratio, P<0.0005) (Fig. 1B). CBS mRNA levels were also lower in poorly-differentiated or undifferentiated tumors (Edmondson grade 3 or 4) than in well-differentiated tumors (Edmondson grade 1) but the difference was not statistically significant due to the small number of poorly-differentiated or undifferentiated tumors (0.49 vs. 1.34; median copy number ratio, P>0.05) (Fig. 1B).

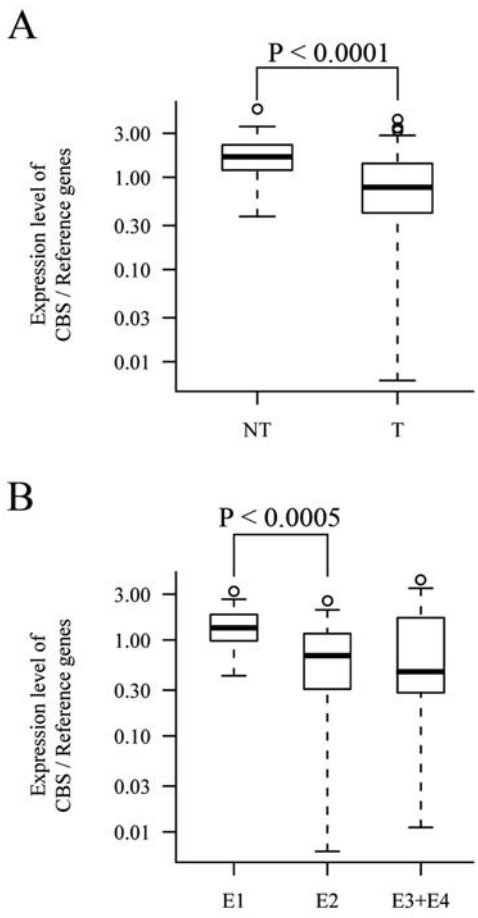

Figure 1. Expression of CBS in HCC. (A) Box and whiskers plot for CBS mRNA levels in non-cancerous liver (NT) and HCC (T) determined by real-time RT-PCR. (B) Relationship of CBS mRNA levels and Edmondson grades. E1, Edmondson grade 1; E2, Edmondson grade 2; E3+E4, Edmondson grades 3 or 4.

Relationship between tumor CBS mRNA level and clinicopathological features. To better understand the significance of decreased CBS expression in HCC, we correlated the mRNA expression level with the major clinicopathological features. As shown in Table I, decreased CBS expression (<1.44; copy number ratio) was strongly associated with high tumor stage (P=0.0019), high Edmondson grade (P=0.00084), and high AFP level ($P=6.2x10^{-5}$) in 120 HCCs. CBS mRNA level was also correlated with age (P=0.028) and presence of HBsAg (P=0.045). No correlation was observed between CBS expression and other clinicopathological parameters (tumor size, gender, HCV, and liver cirrhosis).

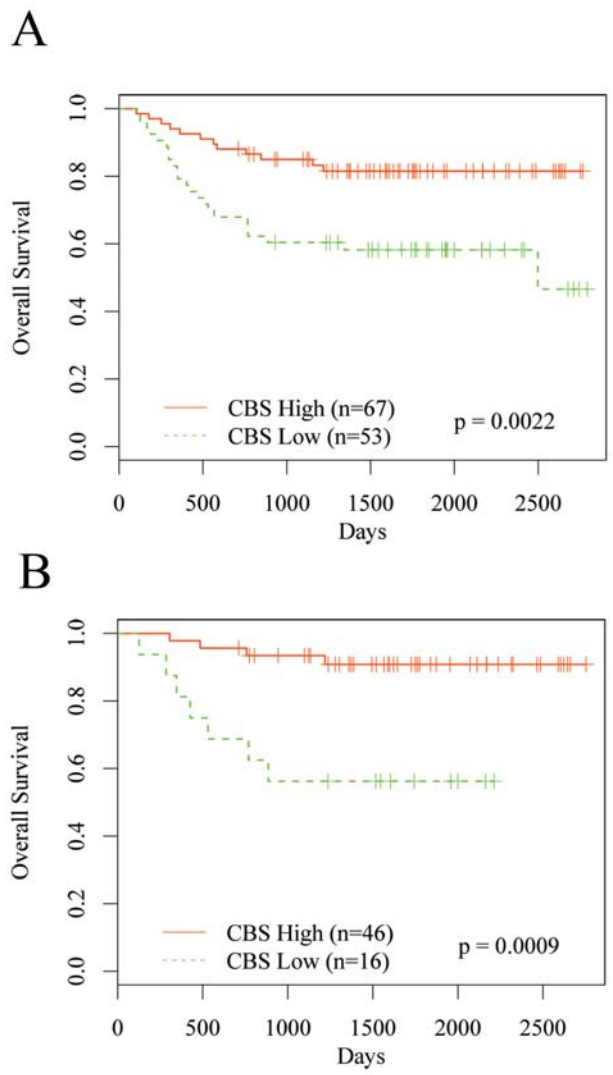

Figure 2. Kaplan-Meier curves for OS of patients with high and low CBS mRNA levels after surgery. (A) Patients with low CBS mRNA levels (<1.44; copy number ratio) had a significantly shorter OS time (P=0.0022). Broken lines, patients with low CBS mRNA levels (n=53); thin lines, patients with high CBS mRNA levels (n=67). (B) Among the patients with low AFP levels (<100 ng/ml), the patients with low CBS mRNA levels (<1.44; copy number ratio) had a significantly shorter OS time (P=0.0009). Broken lines, patients with low AFP levels and low CBS mRNA levels (n=16); thin lines, patients with low AFP levels and high CBS mRNA levels (n=46).

Impact of tumor CBS mRNA levels on OS. During the follow-up observation period of up to 92 months, loco-regional recurrence or distant metastases occurred in 72 patients (60%) and death was confirmed in 35 patients (29%). To assess the prognostic significance of CBS expression, we analyzed OS rates using the Kaplan-Meier method (Fig. 2A). At the 5-year follow-up, approximately 82% of the patients with high CBS expression (≥1.44; copy number ratio) survived, whereas 58% of the patients with low CBS expression (<1.44; copy number ratio) survived. The log-rank test showed that patients who expressed lower CBS mRNA levels have a significantly shorter OS time (P=0.0022). However, no significant difference in disease-free survival (DFS) time was observed between the patients with high CBS expression and low CBS expression (data not shown).

Table II. Univariate Cox regression analysis for overall survival.

Variable	Odds Ratio	Lower .95	Upper .95	P-value
Age (years) (<55:≥55)	0.76	0.38	1.53	0.45
Gender (Male:Female)	1.00	0.46	2.21	1.00
Edmondson grade (I-II:III-IV)	1.58	0.69	3.62	0.28
HbsAg (Absent:Present)	1.49	0.58	3.83	0.41
HCV (Absent:Present)	2.06	0.73	5.87	0.17
AFP level (ng/ml) (<100:≥100)	2.67	1.31	5.46	0.0070
Liver cirrhosis (Absent:Present)	1.50	0.77	2.93	0.23
Tumor size (cm) (<5:≥5)	4.07	1.99	8.31	0.00012
Tumor stage (I-II:III-IV)	5.74	2.92	11.28	$4x10^{-7}$
CBS (High:Low)	2.84	1.41	5.71	0.0034

Table III. Multivariate Cox regression analysis for overall survival.

Variable	Odds Ratio	Lower .95	Upper .95	P-value
CBS (High:Low)	2.03	0.94	4.38	0.071
Tumor size (cm) (<5:≥5)	2.16	0.95	4.90	0.065
Tumor stage (I–II:III–IV)	3.28	1.50	7.15	0.0028
AFP level (ng/ml) (<100:≥100)	1.48	0.67	3.29	0.33

Univariate Cox regression analysis was used to identify important prognostic factors of OS (Table II). Tumor size and tumor stage were identified as important histopathological risk factors for OS, particularly the high tumor stage ($P=4x10^{-7}$), whereas high AFP level and decreased CBS mRNA expression were the molecular risk factors, particularly decreased CBS expression (P=0.0034). In a multivariate Cox analysis, CBS expression was not a significant prognostic

Table IV. Multivariate Cox regression analysis for overall survival among patients with low AFP levels (<100 ng/ml).

Variable	Odds Ratio	Lower .95	Upper .95	P-value
CBS (High:Low)	6.20	1.78	21.62	0.0042
Tumor size (cm) (<5:≥5)	2.11	0.50	8.88	0.31
Tumor stage (I-II:III-IV)	4.95	1.20	20.41	0.027

factor for OS (P=0.071) after considering tumor stage, tumor size, and AFP level (Table III). Because the expression of CBS mRNA was significantly correlated with AFP levels, we analyzed OS rates for patients stratified by AFP levels using the Kaplan-Meier method (Fig. 2B). The log-rank test showed that patients who expressed lower CBS mRNA levels have a significantly shorter OS time among the patients with low AFP levels (P=0.0009). In a multivariate Cox analysis, CBS expression was a significant prognostic factor for OS (P=0.0042) after considering tumor stage and tumor size for patients with low AFP levels (Table IV).

Discussion

Human liver cirrhosis is known to be associated with alterations of methionine metabolism (11) and many patients with alcoholic liver cirrhosis have increased serum methionine and abnormal clearance of methionine (12) as well as diminished hepatic glutathione content (13). The CBS gene encodes a key enzyme that catalyzes the synthesis of cystathionine as a part of the trans-sulfuration pathway within methionine metabolism (4). CBS enzyme deficiencies are associated with mental retardation, elevated homocysteine levels, skeletal abnormalities, and elevated risk of blood clots and atherosclerosis (14), as well as neurodegenerative diseases such as Alzheimer's and dementia (15). In particular, the expression of CBS and other enzymes of methionine metabolism, such as glycine N-methyltransferase and betaine homocysteine methyltransferase, is confined mainly to the liver (16,17), suggesting a close link between deregulated methionine metabolism and liver diseases. Indeed, CBS levels were diminished in a mouse model for chronic steatohepatitis, human liver cirrhosis, and HCC (7,8). However, only a small number of human HCC specimens were analyzed and the clinical implications of CBS expression in HCC have not been fully investigated.

This study focused on CBS, an important enzyme involved in the trans-sulfuration cascade, as a potential molecular marker responsible for determining clinicopathological features and prognosis of HCC. With a large number of HCC specimens, the current quantitative real-time RT-PCR analysis showed that the expression of CBS gene is markedly reduced in HCCs compared to non-cancerous surrounding tissues. Furthermore, the median expression levels of CBS gene were inversely correlated with Edmondson grade. Stratification of HCC specimens based on CBS gene expression levels showed that CBS expression was significantly correlated with important clinicopathological features such as tumor stage, Edmondson grade, and AFP level. The CBS expression was also correlated with age and the presence of HBsAg. More importantly, reduced CBS expression was significantly correlated with shorter OS time in a log-rank test (P=0.0022), although a multivariate Cox analysis indicated that CBS expression was not an independent prognostic factor (P=0.071). Interestingly, CBS expression was a significant prognostic factor for OS (P=0.0042) after considering tumor stage and tumor size for patients with low AFP levels. Our analysis of CBS expression in correlation with the clinicopathological features and prognosis of HCC provided a novel finding that the CBS mRNA levels could be used as a prognostic factor, especially for patients with low AFP levels.

The trans-sulfuration pathway is a biochemical mechanism that links methionine metabolism to the biosynthesis of cellular redox-controlling molecules such as cysteine, glutathione, and taurine (18). The amount of cysteine provided through the trans-sulfuration pathway essentially determines the level of cellular redox-controlling molecules, such as glutathione and taurine, that protect cells against reactive species-induced damage (8). Approximately 50% of the cysteine used for glutathione anabolism is derived from methionine through the trans-sulfuration pathway (19). It is widely accepted that reactive species produce a broad range of DNA damage including base and sugar modifications, base-free sites, DNA-protein crosslinks, and strand breaks (20,21). In addition, a redox imbalance is shown to stimulate protein kinase and poly-(ADP ribosylation) pathways, affecting signal transduction and promoting tumor development (22). The overactivation of poly-(ADP ribose) polymerase by oxidative stress can lead to a severely compromised cellular energetic state that inhibits apoptotic cell death and results in necrotic cell death, followed by inflammatory response and tumor development (23). Thus, the suppression of CBS would result in decreased commitment to cysteine synthesis and ultimately towards glutathione and taurine synthesis, leading to oxidative damage and potentially the progression of tumor.

Many carcinomas demonstrate a methionine-dependent phenotype (24), suggesting that deregulation of methionine metabolism could play an important role in carcinogenesis. Several studies addressed the expression profiles and polymorphisms of enzymes involved in methionine metabolism, including CBS in gastric cancer (25), cancer cell lines (26), biliary tract cancer (27), bladder cancer (28), colorectal cancer (29,30), breast cancer (31), and liver cancer (7,8). However, the molecular basis of altered methionine metabolism and its role in carcinogenesis have not been clarified and the connection is likely to be complex and dependent on the type of cancer.

In conclusion, our study shows that the expression of CBS is suppressed in HCC. The deregulation of CBS and its significant association with malignant phenotype and poor prognosis of HCC could be due to its crucial role in methionine metabolism and maintaining intracellular redox

homeostasis. Thus, further investigation of CBS in a larger cohort of HCC patients could potentially verify its relevance in molecular pathogenesis of HCC and in facilitating the choice of appropriate therapeutic strategies of HCC.

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