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Low metallothionein 1M expression association with poor hepatocellular carcinoma prognosis after curative resection

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ABSTRACT. According to the typical clinical characteristics of hepatocellular carcinoma (HCC), recurrence and prognosis can differ dramatically between patients. Using RNA sequencing, we identified differential expression of the gene metallothionein 1M (*MT1M*) by comparing early-recurrence HCC (N = 11), no-recurrence HCC (N = 10), and normal liver tissues (N = 5). Reverse transcription-polymerase chain reaction was employed to test *MT1M* expression levels in 92 HCC tissue samples from a cohort of patients with whom contact was established for post-operative follow-up. Low *MT1M* expression correlated with high alpha-fetoprotein levels (P = 0.017) and tumor recurrence within 24 months after surgery (P = 0.029). Recurrence rates in high- and low-

MT1M groups were significantly different (*MT1M* cutoff point = 0.066; P = 0.008). Moreover, the disease-free survival time of patients in the former was longer than that of those in the latter (median of 20.39 *vs* 14.35 months, respectively; P = 0.002). Among early-stage HCC patients (Barcelona Clinic Liver Cancer stage 0/A), those with reduced *MT1M* expression exhibited higher recurrence rates (37.5 *vs* 12.1%; P = 0.023). Low *MT1M* expression is associated with poor HCC prognosis following curative resection, and this also applies to the early stage of this disease.

Key words: Metallothionein; Hepatocellular carcinoma; RNA sequencing

INTRODUCTION

Primary liver cancer is one of the most prevalent malignancies worldwide (Jemal et al., 2010), and demonstrates the second highest mortality rate of any cancer. Hepatocellular carcinoma (HCC) accounts for 90% of liver cancers (Ferlay et al., 2010). Despite improvements in surveillance and clinical treatment strategies, HCC prognosis remains very poor due to the high incidence of recurrence and metastasis (Cha et al., 2003). As a result, it is challenging to predict the course of tumor development, although standardized guidelines exist for the treatment of HCC (Llovet et al., 1999).

Currently, HCC tumor staging is mainly based on tumor involvement, number of nodules, tumor size, and vascular invasion, among other factors [Okuda et al., 1985; The Cancer of the Liver Italian Program (CLIP) investigators, 1998; Chevret et al., 1999; Leung et al., 2002; Vauthey et al., 2002; Kudo et al., 2003; Omagari et al., 2004; Tateishi et al., 2005; Hsu et al., 2010; Yau et al., 2014]. The development of next generation sequencing (NGS) has provided a promising new approach to tumor prognosis. RNA sequencing (RNA-seq) can be a powerful tool to explore the genetic features of malignant growths, and has the potential to identify novel genetic markers for tumor staging and evaluation for HCC prognosis.

The metallothioneins (MTs) are a family of low molecular weight, intracellular proteins, consisting of at least 10 functional members (MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A, MT3, and MT; West et al., 1990). MT1 and MT2 are frequently down-regulated in HCC (Stennard et al., 1994; Cai et al., 1998; Huang and Yang, 2002; Jacob et al., 2002; Datta et al., 2007; Tao et al., 2007). In particular, *MT1M* is often present at lower levels and its promoter is highly methylated in human HCC tumors and cell lines (Mao et al., 2012).

We hypothesized that MT1M may be implicated in HCC recurrence and metastasis, correlating with adverse clinical outcome. Therefore, in this study, RNA-seq was performed on several groups of representative liver tissues to detect differentially expressed genes. In addition, to verify its predictive value, the expression levels of a gene identified in this manner and prognoses in a patient cohort were assessed.

MATERIAL AND METHODS

Human tissue samples

For RNA-seq, three groups of representative liver tissues were collected between July

2010 and December 2011 at Beijing You-An Hospital. These consisted of case, control, and normal samples, as follows: case, HCC tissues from patients with tumor recurrence within 6 months after curative hepatectomy (N = 11); control, HCC tissues from patients with no recurrence for at least 2 years after surgery (N = 10); and normal, normal liver tissues (N = 5). All tumors were at stage 0 or A according to the Barcelona Clinic Liver Cancer (BCLC) staging system.

For the validation study, 92 specimens of HCC tissue were collected from a cohort comprising all patients having undergone curative hepatectomy at the Department of General Surgery of Beijing You-An Hospital between January 2012 and October 2013.

To be included in this study, patients had to: 1) have a definitive pathological diagnosis of HCC based on World Health Organization criteria; and 2) have undergone curative hepatectomy, defined as complete macroscopic removal of the tumor (Poon et al., 2002). All samples were stored in liquid nitrogen at the Bioinformatics Center of Beijing You-An Hospital until needed. This project was approved by the Ethics Committee of Beijing You-An Hospital, and written consent was obtained from all study participants.

RNA-seq analysis

Total RNA samples were firstly treated with DNase I to eliminate any DNA contamination. Then, mRNA was enriched using oligo (dT) magnetic beads for eukaryotic transcripts, before being mixed with fragmentation buffer and split into short sequences of approximately 200 bp. First-strand complementary DNA (cDNA) synthesis was then carried out with random hexamer primers. Buffer, deoxynucleotides (dNTPs), RNase H, and DNA polymerase I were added to synthesize the second strand. Double-stranded cDNA was subsequently purified with magnetic beads, and end repair and addition of a 3'-end single adenine residue was performed. Finally, sequencing adaptors were ligated to the fragments, which were amplified by polymerase chain reaction (PCR). Ribo-Zero (Illumina, San Diego, CA, USA) and a TruSeq Stranded Total RNA Library Prep Kit (Illumina) were used in the above procedure. During quality control, an Agilent (Santa Clara, CA, USA) 2100 Bioanalyzer and the Applied Biosystems (Foster City, CA, USA) StepOnePlus Real-Time PCR System were used for qualitative and quantitative analysis of the sample library. The library was sequenced on an Illumina HiSeq 2000 platform.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using a PikoReal PCR instrument (Thermo Scientific, Waltham, MA, USA) and the SYBR Green system (Xiamen Zeesan Biotech Co., Xiamen, China), following manufacturer protocols.

Hot start *Taq* enzyme (TaKaRa Biological Engineering Co., Dalian, China), PCR buffer (Invitrogen, Carlsbad, CA, USA), SYBR Green I (Xiamen Zeesan Biotech Co.), dNTPs (TOYOBO Co., Osaka, Japan), a MinElute Purification Kit (Qiagen, Hilden, Germany), a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), and carrier RNA (Omega Bio-tek, Norcross, GA, USA) were used when needed.

Each sample was tested in triplicate. Cycle threshold (Ct) values were recorded during the reaction under the corrected threshold setting. The β-globin gene was used as an internal control, and normal human placental cDNA, made by reverse transcription of RNA,

functioned as a reference control group. Relative quantification was carried out with the $2^{-\Delta\Delta Ct}$ method. The primers used are shown in Table 1.

Table 1. Primers used for quantitative real time-polymerase chain reaction.

Target	Direction	Sequence	Product size (bp)
MT1M	Forward	TAGCAGTCGCTCCATTTATC	244
	Reverse	TGTTCCCACATCAGGCA	

Data analyses

NGS data were comprehensively analyzed using appropriate analytical tools. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). For statistical comparisons, one-way analysis of variance, the chi-square test, the Fisher exact test, the Student *t*-test, or the Kruskal-Wallis test were performed as appropriate. Recurrence-free survival (RFS) was calculated from the date of tumor resection until detection of the first HCC recurrence, death, or the last follow-up. Overall survival (OS) was defined as the time between surgery and death or the last follow-up examination. Patients lost to follow-up or who died from causes unrelated to HCC were considered censored events. Survival curves were analyzed by the Kaplan-Meier method and compared with the log-rank test. Independent factors influencing RFS and OS were evaluated by multivariate Cox proportional hazards regression analysis. P values < 0.05 were considered significant.

RESULTS

Significant difference in *MT1M* RNA expression levels (control *vs* case and normal *vs* case groups)

We applied the NOISeq method detailed in an article published in Genome Research in 2011 (Tarazona et al., 2011) to screen differentially expressed genes. Target genes were considered to be those differentially expressed in both control *vs* case and normal *vs* case comparisons. As Figure 1 shows, 18 target genes were identified, including 11 that were up-regulated and five that were down-regulated. It is of note that all five down-regulated genes belonged to the same family, of which, the differential expression of *MT1H* and *MT1M* was most obvious. *MT1H* will be considered in a future article; the present study focuses on *MT1M*.

Low *MT1M* expression in HCC patients may indicate poorer prognosis

To further explore the clinicopathological correlation between *MT1M* and HCC, we analyzed its expression in samples from a cohort of 92 HCC patients using qRT-PCR. As shown in Table 2, low *MT1M* expression correlated with high alpha-fetoprotein (AFP) levels ($P = 0.017$) and tumor recurrence within 24 months after surgery ($P = 0.029$). Recurrence was observed in 28 of 54 patients in the low-*MT1M* expression group, and only 11 of 38 patients in the high-expression group. Categorization of patients based on *MT1M* levels resulted in significantly different recurrence rates (*MT1M* $2^{-\Delta\Delta Ct}$ cutoff point = 0.066, $P = 0.008$; Figure 2). Disease-free survival time in the high-*MT1M* group was longer than that in the low-expression

group (median of 20.39 *vs* 14.35 months, respectively; P = 0.002; Figure 3). The area under the curve for *MT1M* level ($2^{-\Delta\Delta Ct}$ = 0.066) was 0.614, with a sensitivity of 71.8% and a specificity of 50.9% (Figure 4). Compared with other clinical indices, *MT1M* expression was found to be an effective factor for predicting early HCC recurrence.

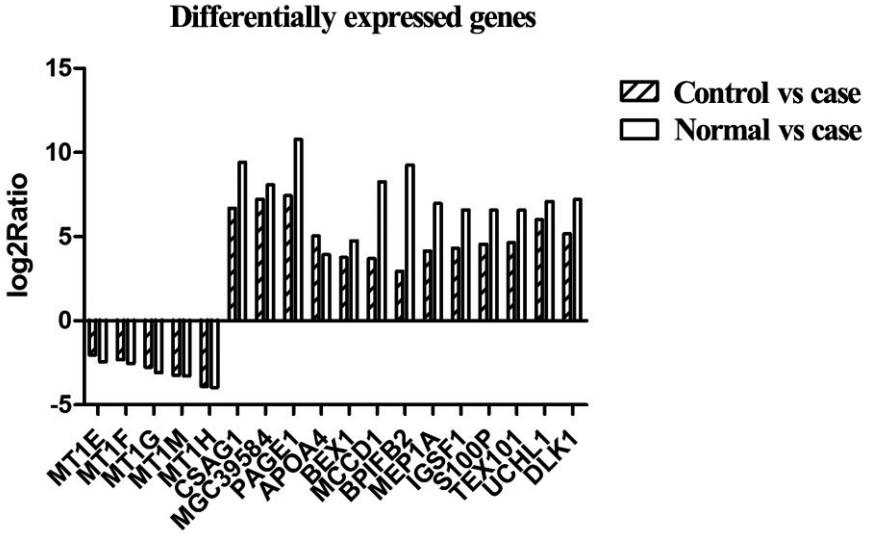

Figure 1. Eighteen target genes were identified, of which, 13 were up-regulated and 5 down-regulated.

Table 2. Correlation between clinical characteristics and *MT1M* expression among hepatocellular carcinoma patients.

Clinical characteristic	*MT1M* expression in HCC ($2^{-\Delta\Delta Ct}$)		P value
	Low (<0.066)	Moderate and high (>0.066)	
Gender			
Female	8	9	0.282
Male	46	29	
Age, years			
≤50	26	25	0.094
>50	28	13	
Tumor size			
≤5 cm	27	23	0.318
>5 cm	27	15	
Tumor number			
Single	40	27	0.748
Multiple	14	11	
Alpha-fetoprotein			
≤400 ng/mL	33	32	0.017
>400 ng/mL	21	6	
BCLC stage			
0/A	32	23	0.903
B/C	22	15	
Recurrence			
No	26	27	0.029
Yes	28	11	

HCC = hepatocellular carcinoma; BCLC = Barcelona Clinic Liver Cancer.

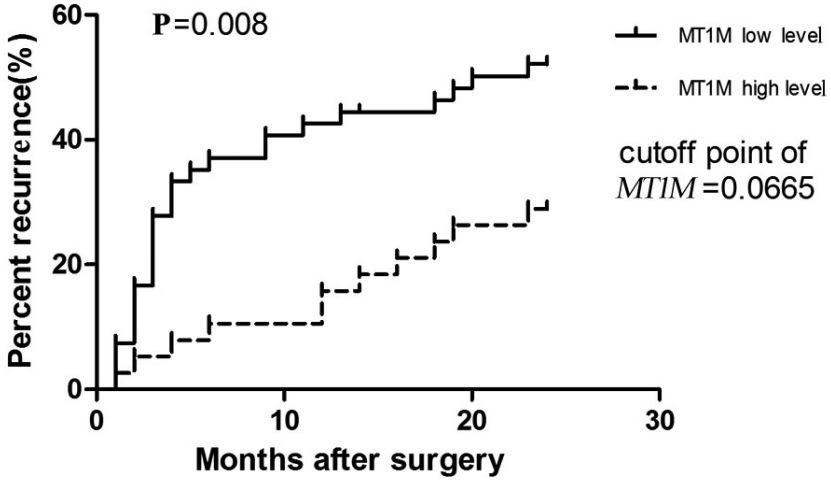

Figure 2. Recurrence rates in low- and high-*MT1M* groups were significantly different (*MT1M* $2^{-\Delta\Delta Ct}$ cutoff point = 0.066, P = 0.008).

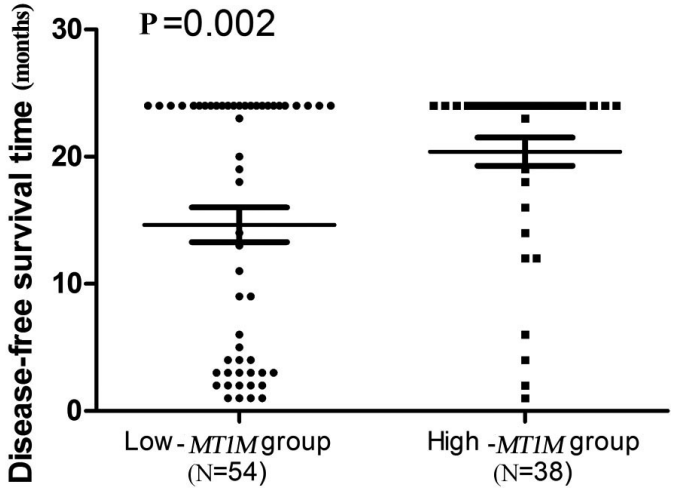

Figure 3. Disease-free survival time was longer in the high- than in the low-*MT1M* group (median of 20.39 *vs* 14.35 months, respectively; P = 0.002).

Low *MT1M* expression is associated with higher recurrence rate in early-stage HCC

The prognostic significance of *MT1M* within specific clinical subgroups was further investigated. Among patients with BCLC stage 0/A HCC, those with low *MT1M* levels demonstrated higher recurrence rates (37.5 *vs* 12.1%, P = 0.023). Postoperative recurrence was also more frequent in the low-*MT1M* expression group among patients with a tumor ≤5 cm (40.7 *vs* 26.1%), a single tumor (42.5 *vs* 22.2%), and an AFP level ≤400 ng/mL (42.4 *vs* 31.3%; Figure 5).

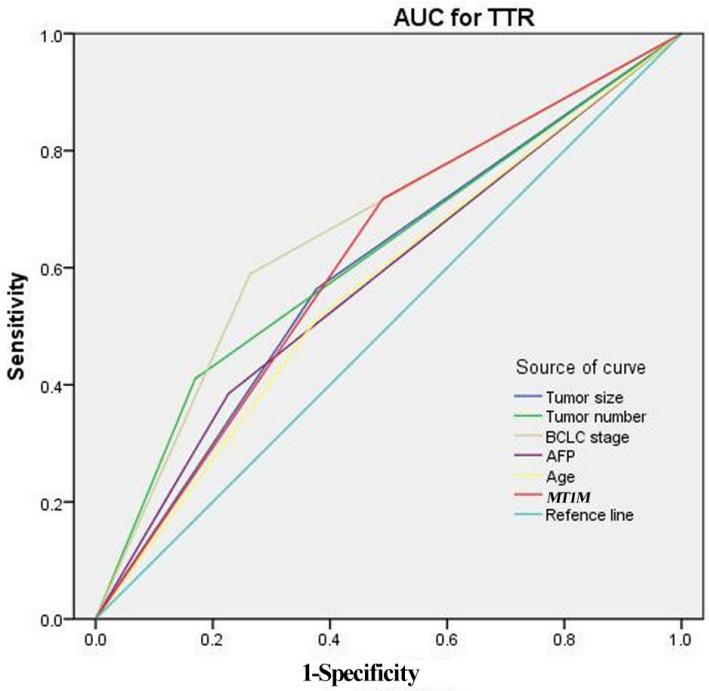

Figure 4. Area under the curve (AUC) for *MT1M* level ($2^{-\Delta\Delta Ct}$ = 0.066) was 0.614, with a sensitivity of 71.8%, and a specificity of 50.9%. BCLC = Barcelona Clinic Liver Cancer; AFP = alpha-fetoprotein; TTR = time to recurrence.

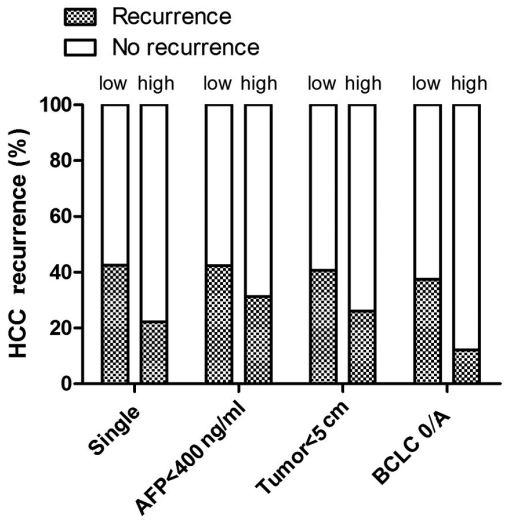

Figure 5. Among patients at Barcelona Clinic Liver Cancer (BCLC) stage 0/A, those with low *MT1M* expression had higher recurrence rates (37.5 *vs* 12.1%, P = 0.023). Postoperative recurrence was also relatively elevated in the low-*MT1M* group among patients with tumors <5 cm (40.7 *vs* 26.1%), single tumors (42.5 *vs* 22.2%), and alpha-fetoprotein (AFP) levels ≤400 ng/mL (42.4 *vs* 31.3%). HCC = hepatocellular carcinoma.

DISCUSSION

As a curative treatment for HCC, hepatectomy and liver transplantation cannot guarantee full recovery, owing to the high incidence of recurrence (50-70% at 5 years; Cha et al., 2003). In the present study, we performed RNA-seq on three groups of representative HCC tissue samples. The case group consisted of early-recurrence HCC samples, the control group comprised HCC specimens for which no recurrence was observed, and the normal group was composed of normal liver tissues. Two sets of differentially expressed genes were identified when the case group was compared separately with the control and normal groups. At the intersection of these two sets were 18 genes, which may, to some extent, be the key to explaining early tumor recurrence. All of the five down-regulated genes among these differentially expressed sequences were subtypes of *MT1*. This is consistent with previous studies, in which the overall expression of MT proteins has been found by immunohistochemistry to be down-regulated in HCC (Cai et al., 1998; Huang and Yang, 2002; Lu et al., 2003; Datta et al., 2007; Tao et al., 2007), and decreased *MT* expression has been reported as an early event in HCC progression (Jacob et al., 2002). Therefore, the differential expression of *MT1* genes in tumor tissue and normal liver tissue is closely related to HCC clinical characteristics, such that it may be used as a marker to evaluate the prognosis of this disease.

MT1M appeared to be one of the most frequently down-regulated genes in this study. To validate this finding, we tested HCC tissues from a cohort of patients. Using qRT-PCR, we demonstrated a significant difference between the high- and low-*MT1M* groups in terms of disease-free period. Elevated expression of *MT1M* tended to indicate a longer disease-free survival time, and this also applied to HCC patients at BCLC stage 0/A. On the one hand, it has been reported that *MT1M* is decreased in human HCC tissues owing to promoter hypermethylation (Mao et al., 2012); on the other hand, this gene may be associated with the regulation of p53. Fu et al. (2013) found that MT1G, a similar molecule to MT1M, appears to be a functional tumor suppressor, mainly due to its modulation of the phosphatidylinositol 3-kinase/Akt pathway. This team established that restoration of MT1G expression increases the stability of p53 and expression of its downstream targets, including p21, Bak, and Smac, in K1 cells. Of the genes transcriptionally regulated by p53, $p21^{WAF/CIP1}$ is necessary for p53-mediated G1 arrest (Waldman et al., 1995). Bak, which is involved in p53-mediated mitochondrial apoptosis, is a pro-apoptotic protein of the Bcl-2 family that induces the release of apoptogenic factors, such as cytochrome c and Smac/DIABLO (Schuler and Green, 2001; Leu et al., 2004). Taking this into account, it is possible that the effects of MT1M on cell-cycle arrest and apoptosis might be at least partially mediated by p53. In addition, it has previously been reported that MT1 and MT2 may be useful as prognostic markers for HCC (Park and Yu, 2013). As MT1M is a subtype of MT1, this supports the results of our study to some extent.

The limitations of the present study are its relatively small cohort size, short follow-up period, and single-center nature. A prospective, multicenter, randomized clinical trial should be carried out to further validate the prognostic significance of MT1M in HCC.

CONCLUSION

In summary, our data showed that low expression of *MT1M* indicates poor HCC prognosis after curative resection, and that this relationship also applies during the early stage of this disease. The *MT1M* gene can be considered a marker of tumor prognosis. Although the

exact pathomechanisms responsible remain unclear, many investigations have indicated that MT1M regulates the expression of p53. This suggests the direction that future research might take to clarify the role of MT1M in this process.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Cai L, Wang GJ, Xu ZL, Deng DX, et al. (1998). Metallothionein and apoptosis in primary human hepatocellular carcinoma (HCC) from northern China. *Anticancer Res.* 18 (6B): 4667-4672.

Cha C, Fong Y, Jarnagin WR, Blumgart LH, et al. (2003). Predictors and patterns of recurrence after resection of hepatocellular carcinoma. *J. Am. Coll. Surg.* 197: 753-758. http://dx.doi.org/10.1016/j.jamcollsurg.2003.07.003

Chevret S, Trinchet JC, Mathieu D, Rached AA, et al. (1999). A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. *J. Hepatol.* 31: 133-141. http://dx.doi.org/10.1016/S0168-8278(99)80173-1

Datta J, Majumder S, Kutay H, Motiwala T, et al. (2007). Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/enhancer binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. *Cancer Res.* 67: 2736-2746. http://dx.doi.org/10.1158/0008-5472.CAN-06-4433

Ferlay J, Shin HR, Bray F, Forman D, et al. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127: 2893-2917. http://dx.doi.org/10.1002/ijc.25516

Fu J, Lv H, Guan H, Ma X, et al. (2013). Metallothionein 1G functions as a tumor suppressor in thyroid cancer through modulating the PI3K/Akt signaling pathway. *BMC Cancer* 13: 462. http://dx.doi.org/10.1186/1471-2407-13-462

Hsu CY, Huang YH, Hsia CY, Su CW, et al. (2010). A new prognostic model for hepatocellular carcinoma based on total tumor volume: the Taipei Integrated Scoring System. *J. Hepatol.* 53: 108-117. http://dx.doi.org/10.1016/j.jhep.2010.01.038

Huang GW and Yang LY (2002). Metallothionein expression in hepatocellular carcinoma. *World J. Gastroenterol.* 8: 650-653. http://dx.doi.org/10.3748/wjg.v8.i4.650

Jacob ST, Majumder S and Ghoshal K (2002). Suppression of metallothionein-I/II expression and its probable molecular mechanisms. *Environ. Health Perspect.* 110 (Suppl 5): 827-830. http://dx.doi.org/10.1289/ehp.02110s5827

Jemal A, Siegel R, Xu J and Ward E (2010). Cancer statistics, 2010. *CA Cancer J. Clin.* 60: 277-300. http://dx.doi.org/10.3322/caac.20073

Kudo M, Chung H and Osaki Y (2003). Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). *J. Gastroenterol.* 38: 207-215. http://dx.doi.org/10.1007/s005350300038

Leu JI, Dumont P, Hafey M, Murphy ME, et al. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat. Cell Biol.* 6: 443-450. http://dx.doi.org/10.1038/ncb1123

Leung TW, Tang AM, Zee B, Lau WY, et al. (2002). Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system, and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. *Cancer* 94: 1760-1769. http://dx.doi.org/10.1002/cncr.10384

Llovet JM, Brú C and Bruix J (1999). Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin. Liver Dis.* 19: 329-338. http://dx.doi.org/10.1055/s-2007-1007122

Lu DD, Chen YC, Zhang XR, Cao XR, et al. (2003). The relationship between metallothionein-1F (*MT1F*) gene and hepatocellular carcinoma. *Yale J. Biol. Med.* 76: 55-62.

Mao J, Yu H, Wang C, Sun L, et al. (2012). Metallothionein MT1M is a tumor suppressor of human hepatocellular carcinomas. *Carcinogenesis* 33: 2568-2577. http://dx.doi.org/10.1093/carcin/bgs287

Okuda K, Ohtsuki T, Obata H, Tomimatsu M, et al. (1985). Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. *Cancer* 56: 918-928. http://dx.doi.org/10.1002/1097-0142(19850815)56:4<918::AID-CNCR2820560437>3.0.CO;2-E

Omagari K, Honda S, Kadokawa Y, Isomoto H, et al. (2004). Preliminary analysis of a newly proposed prognostic scoring system (SLiDe score) for hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 19: 805-811. http://dx.doi.org/10.1111/j.1440-1746.2004.03350.x

Park Y and Yu E (2013). Expression of metallothionein-1 and metallothionein-2 as a prognostic marker in hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 28: 1565-1572. http://dx.doi.org/10.1111/jgh.12261

Poon RT, Ng IO, Lau C, Yu WC, et al. (2002). Tumor microvessel density as a predictor of recurrence after resection of hepatocellular carcinoma: a prospective study. *J. Clin. Oncol.* 20: 1775-1785. http://dx.doi.org/10.1200/JCO.2002.07.089

Schuler M and Green DR (2001). Mechanisms of p53-dependent apoptosis. *Biochem. Soc. Trans.* 29: 684-688. http://dx.doi.org/10.1042/bst0290684

Stennard FA, Holloway AF, Hamilton J and West AK (1994). Characterisation of six additional human metallothionein genes. *Biochim. Biophys. Acta* 1218: 357-365. http://dx.doi.org/10.1016/0167-4781(94)90189-9

Tao X, Zheng JM, Xu AM, Chen XF, et al. (2007). Downregulated expression of metallothionein and its clinicopathological significance in hepatocellular carcinoma. *Hepatol. Res.* 37: 820-827. http://dx.doi.org/10.1111/j.1872-034X.2007.00113.x

Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, et al. (2011). Differential expression in RNA-seq: a matter of depth. *Genome Res.* 21: 2213-2223. http://dx.doi.org/10.1101/gr.124321.111

Tateishi R, Yoshida H, Shiina S, Imamura H, et al. (2005). Proposal of a new prognostic model for hepatocellular carcinoma: an analysis of 403 patients. *Gut* 54: 419-425. http://dx.doi.org/10.1136/gut.2003.035055

The Cancer of the Liver Italian Program (CLIP) investigators (1998). A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology* 28: 751-755. http://dx.doi.org/10.1002/hep.510280322

Vauthey JN, Lauwers GY, Esnaola NF, Do KA, et al. (2002). Simplified staging for hepatocellular carcinoma. *J. Clin. Oncol.* 20: 1527-1536. http://dx.doi.org/10.1200/JCO.20.6.1527

Waldman T, Kinzler KW and Vogelstein B (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* 55: 5187-5190.

West AK, Stallings R, Hildebrand CE, Chiu R, et al. (1990). Human metallothionein genes: structure of the functional locus at 16q13. *Genomics* 8: 513-518. http://dx.doi.org/10.1016/0888-7543(90)90038-V

Yau T, Tang VY, Yao TJ, Fan ST, et al. (2014). Development of Hong Kong Liver Cancer staging system with treatment stratification for patients with hepatocellular carcinoma. *Gastroenterology* 146: 1691-700.e3. http://dx.doi.org/10.1053/j.gastro.2014.02.032