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.

<table>
<thead>
<tr>
<th>Target</th>
<th>Direction</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1M</td>
<td>Forward</td>
<td>TAGCAGTCGTCATTTATC</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTTCCCACATCAGGCA</td>
<td></td>
</tr>
</tbody>
</table>

### 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.
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A group (median of 20.39 vs 14.35 months, respectively; P = 0.002; Figure 3). The area under the curve for MT1M level (2^{−ΔΔCt} = 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.

![Differentially expressed genes](image)

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

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>MT1M expression in HCC (2^{−ΔΔCt})</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (&lt;0.066)</td>
<td>Moderate and high (&gt;0.066)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Male</td>
<td>46</td>
<td>29</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>&gt;50</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5 cm</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>Multiple</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Alpha-fetoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤400 ng/mL</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>&gt;400 ng/mL</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>BCLC stage</td>
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<td></td>
</tr>
<tr>
<td>0/A</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>B/C</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>Yes</td>
<td>28</td>
<td>11</td>
</tr>
</tbody>
</table>

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

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Figure 2. Recurrence rates in low- and high-MT1M groups were significantly different ($MT1M \Delta\Delta C_t$ 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 $\leq$ 5 cm (40.7 vs 26.1%), a single tumor (42.5 vs 22.2%), and an AFP level $\leq$ 400 ng/mL (42.4 vs 31.3%; Figure 5).

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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\textsuperscript{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
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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.

ACKNOWLEDGMENTS

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REFERENCES


