

**MT1M and MT1G promoter methylation as biomarkers for hepatocellular carcinoma**

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**AIM:** To investigate the potential of promoter methylation of two tumor suppressor genes (TSGs) as biomarkers for hepatocellular carcinoma (HCC).

**METHODS:** A total of 189 subjects were included in this retrospective cohort, which contained 121 HCC patients without any history of curative treatment, 37 patients with chronic hepatitis B (CHB), and 31 normal controls (NCs). DNA samples were extracted from 400 μL of serum of each subject and then modified using bisulfite treatment. Methylation of the promoters of the TSGs (metallothionein 1M, \(\text{MT1M}\); and metallothionein 1G, \(\text{MT1G}\)) was determined using methylation-specific polymerase chain reaction. The diagnostic value of combined \(\text{MT1M}\) and \(\text{MT1G}\) promoter methylation was evaluated using the area under the receiver operating characteristic curves.

**RESULTS:** Our results indicated that the methylation status of serum \(\text{MT1M}\) (48.8%, 59/121) and \(\text{MT1G}\) (70.2%, 85/121) promoters in the HCC group was significantly higher than that in the CHB group (\(\text{MT1M}\) 5.4%, 2/37, \(P < 0.001\); \(\text{MT1G}\) 16.2%, 6/37, \(P < 0.001\)) and NC group (\(\text{MT1M}\) 6.5%, 2/31, \(P < 0.001\); \(\text{MT1G}\) 12.9%, 4/31, \(P < 0.001\)). Aberrant serum \(\text{MT1M}\) promoter methylation gave higher specificity to discriminate HCC from CHB (94.6%) and NCs (93.5%), whereas combined methylation of serum \(\text{MT1M}\) and \(\text{MT1G}\) promoters showed higher diagnostic sensitivity (90.9%), suggesting that they are potential markers for noninvasive detection of HCC. Furthermore, \(\text{MT1M}\) promoter methylation was positively correlated with tumor size (\(r_s = 0.321, P < 0.001\)), and HCC patients with both \(\text{MT1M}\) and \(\text{MT1G}\) promoter methylation tended to show a higher incidence of vascular invasion or metastasis (\(P = 0.018\)).

**CONCLUSION:** \(\text{MT1M}\) and \(\text{MT1G}\) promoter methylation may be used as serum biomarkers for noninvasive detection of HCC.

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**Key words:** \(\text{MT1M}\); \(\text{MT1G}\); Methylation; Serum biomarker; Hepatocellular carcinoma

**Core tip:** DNA methylation of tumor suppressor gene promoter regions appears to be a valuable biomarker in many tumors, including hepatocellular carcinoma (HCC). We found that aberrant serum metallothionein 1M (\(\text{MT1M}\)) promoter methylation gave higher specificity to discriminate HCC from chronic hepatitis B and normal controls. In contrast, combined methylation of serum \(\text{MT1M}\) and metallothionein 1G promoters showed higher diagnostic sensitivity. This indicates that they may be used as potential biomarkers for noninvasive detection of HCC.
**INTRODUCTION**

Hepatocellular carcinoma (HCC) is the sixth most common tumor and has the third highest mortality\[15\]. The areas of highest incidence are Asia and Africa, which are linked to the wide prevalence of hepatitis B virus (HBV) infection\[16\]. However, the incidence of HCC has been rapidly increasing in the United States and United Kingdom over the past 20 years, which is attributed to increased hepatitis C virus (HCV) infection\[17\]. In addition, aflatoxin B1 exposure and alcohol addiction are also associated with hepatocarcinogenesis. Despite advanced treatment, patients with HCC have a dismal 5-year survival rate of about 5\%, as a result of late diagnosis\[18\].

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**MATERIALS AND METHODS**

**Collection of serum specimens**

After obtaining informed consent, we collected 189 serum samples from 121 patients with HCC, 37 patients with chronic hepatitis B (CHB), and 31 normal controls (NCs), based on clinical and laboratory examinations. Patients with HCC and CHB were recruited from those enrolled from July 2011 to March 2013 at Qilu Hospital, Shandong University in accordance with American Association for the Study of Liver Diseases Practice Guidelines for HCC and CHB, respectively\[29,30\]. All cases of HCC included in our study were confirmed by pathological data. Serum samples were collected from HCC patients who did not receive curative treatments such as surgical resection, transcatheter arterial chemoembolization (TACE), or radiofrequency ablation before and during the study. Exclusion criteria included other tumors, co-infection with HCV or human immunodeficiency virus, and other causes of chronic liver diseases. The patient selection process is shown in Figure 1.

Tumor size was calibrated by computed tomography and presented as the longest diameter. AFP concentration > 20 ng/mL was regarded as abnormal\[31\]. The study protocol was approved by the Ethics Committee of Qilu Hospital.

**Serum DNA extraction and sodium bisulfite modification**

DNA was extracted from 400 μL of serum with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the DNA Purification from Blood or Body Fluids protocol. Bisulfite modification was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, United States) according to the manufacturer’s instructions. After bisulfite treatment, all unmethylated cytosine residues were converted to uracil, whereas the methylated residues would have been resistant to this modification and remained as cytosine. The modified DNA was finally stored at -20 °C before methylation-specific polymerase chain reaction (MSP).

**MSP**

The primer pairs of MT1M and MT1G for MSP analysis were as described previously\[27,28\] (Table 1). One microliter of bisulfite-treated DNA, 0.5 μL each primer (10 μmol/L), 10.5 μL nuclease-free water, and 12.5 μL Pre-mix Taq (Zymo Research) were mixed together to form a 25-μL MSP reaction mixture. The PCR protocol included an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at the respective temperature (54 °C for MT1M, 59 °C for MT1G-U, and 50 °C for MT1G-M) for 40 s, primer ex-
tension at 72 °C for 40 s, and a final extension at 72 °C for 10 min (Table 1). Water without DNA was used as a negative control. PCR products were electrophoresed on 2% agarose gels, stained with Gel Red, and visualized under UV illumination.

Statistical analysis
The differences in DNA methylation status of $MT1M$ and $MT1G$ promoters between different groups and the associations between gene methylation in HCC patients and clinical pathological variables were analyzed using the $\chi^2$ test. Correlation between $MT1M$ and $MT1G$ promoter methylation and tumor size was calculated by Spearman rank correlation. Diagnostic value of combined methylation of $MT1M$ and $MT1G$ promoters and serum AFP level was evaluated by the area under the receiver operating characteristic curves (AUC). Differences were considered significant at $P < 0.05$. All statistical analyses were conducted with SPSS 16.0 software.

RESULTS

Methylation status in serum
The methylation status of $MT1M$ or $MT1G$ promoter in 121 patients with HCC, 37 patients with CHB and 31 NCs was compared (Figure 2). The methylation percentages were higher in HCC (48.8% for $MT1M$ and 70.2% for $MT1G$) than in CHB (5.4% for $MT1M$ and 16.2% for $MT1G$) or NCs (6.5% for $MT1M$ and 12.9% for $MT1G$ ($P < 0.001$). However, no differences were found for either of them between the CHB and NC groups. Representative MSP results for methylated $MT1M$ and $MT1G$
promoters are shown in Figure 3.

**Correlation with clinicopathological parameters**

For analysis of the correlation between methylation status of a single gene promoter in serum and clinicopathological features, there was a significant association between the methylation ratio of MT1M promoter and tumor size ($P = 0.001$) (Table 2). Further analysis revealed that the correlation was positive ($\rho = 0.321$, $P < 0.001$) (Table 3). Moreover, advanced TNM stage (III-IV) was associated with a more elevated percentage of serum MT1M promoter methylation than early TNM stage (I-II), although the difference was not significant ($P = 0.058$) (Table 2). In addition, HCC patients with both MT1M and MT1G promoters methylated (18/44) tended to show a higher incidence of vascular invasion or metastasis than those with only one or neither gene methylated (16/77) ($P = 0.018$) (Table 4). However, no significant relationships were observed between the methylation levels of MT1M and MT1G promoters and other parameters, such as sex, age, HBV infection, serum AFP levels, tumor multiplicity or TNM stage ($P > 0.05$).

**Sensitivity and specificity for single or combination methylation**

There were 100 HCC patients with HBV infection (Table 2). To discriminate HBV-associated HCC from CHB, MT1M and MT1G promoter methylation showed a
Table 6 Sensitivity and specificity of gene sets for hepatocellular carcinoma detection in normal controls group

| No. | Gene   | TP/FN | FP/TN | Sensitivity (%) | Specificity (%) |
|-----|--------|-------|-------|----------------|----------------|
| 1   | MT1M   | 59/62 | 2/29  | 48.8           | 93.5           |
| 2   | MT1G   | 85/36 | 4/27  | 70.2           | 87.1           |
| 3   | MT1M/MT1G | 110/11 | 5/26  | 90.9           | 83.9           |

Sensitivity (%), TP/(TP + FN) and specificity (%), TN/(TN + FP) of each gene set were calculated and plotted. MT1M/MT1G, MT1M or MT1G promoter methylation. TP: True positive; FN: False negative; FP: False positive; TN: True negative; MT1M: Metallothionein 1M; MT1G: Metallothionein 1G.

moderate sensitivity (MT1M, 50%; 50/100; MT1G, 69%; 69/100) but a high specificity (MT1M, 94.6%; 2/37; MT1G, 83.8%; 6/37), whereas the sensitivity and specificity of AFP were 56% (56/100) and 62.1% (23/37), respectively (Table 5). To discriminate HCC from the NC group, the specificity was still high (MT1M, 93.5%; 2/31; MT1G, 87.1%; 4/31) (Table 6). Otherwise, combined methylation of MT1M and MT1G promoters gave a sensitivity up to 90.9% (110/121) but a lower specificity to discriminate HCC from the NC (83.9%, 5/31) or CHB (81.1%, 7/37) groups (Tables 5 and 6). Moreover, the AUC of combined methylation of MT1M and MT1G promoters was 0.855 (95%CI: 0.785-0.910), which was significantly higher than that of AFP (0.754; 95%CI: 0.673-0.824) (P = 0.0446) (Figure 4).

DISCUSSION

DNA methylation is suggested as a promising biomarker for cancer detection. However, most studies about DNA methylation have concentrated on the analysis of tumor tissue, which is invasive and not always available, as well as one single gene, which cannot provide enough diagnostic sensitivity. In the present study, we first demonstrated that aberrant methylation status of MT1M and MT1G promoters could be detected in the serum of patients with HCC, and the frequencies were 48.8% (59/121) and 70.2% (85/121) using MSP, which were significantly higher than those in the CHB and NC groups. This was consistent with previous studies in which MT1M and MT1G promoters were methylated in HCC tissues[27,28]. From a diagnostic point of view, assaying a single gene, MT1G promoter methylation, showed a higher sensitivity of 70.2%, whereas MT1M promoter methylation gave a higher specificity to discriminate HCC from CHB (94.6%) and NCs (93.5%). However, combined methylation of MT1M and MT1G promoters significantly elevated the diagnostic sensitivity for HCC (90.9%). In addition, aberrant methylation status of MT1M and MT1G promoters was also observed in early HCC, including TNM stage I, well differentiated and small in size, as well as in patients with negative AFP. Thus, analysis of MT1M and MT1G promoter methylation showed potential value in early detection of HCC.

MT was first isolated in 1957. In addition to its function in metal homeostasis and protection against oxidative damage, several studies have focused on its role in tumors. However, large discrepancies in MT exist between different tumor types. MT expression in tumors of the lung, nasopharynx, breast, kidney, ovary, testes, thyroid, salivary gland, and urinary bladder is increased[20,21], but it is decreased in other tumors such as prostate cancer, colorectal cancer and HCC[22-25,32-34]. Compared with overall MT expression in tumors, its isoforms appear more specific and play distinct roles in different tumor types, such as breast cancer, urological malignancies, and nasopharyngeal cancer[35]. However, there are few reports on the expression of different isoforms of MT in HCC. MT1M and MT1G are two major isoforms that were recently reported to be downregulated in HCC tissues by promoter hypermethylation. Restored expression of MT1M in HCC cells impedes HCC cell growth, and low levels of MT1M are correlated with clinical TNM grade[27]. MT1G acts as a TSG in HCC and patients with MT1G promoter methylation have a poorer prognosis, although the difference is not significant[28].

In our present study, we also evaluated whether methylation status of serum MT1M and MT1G promoters in patients with HCC was associated with any clinicopathological parameter. MT1M promoter methylation was positively correlated with tumor size (r = 0.321, P < 0.001), suggesting that methylated MT1M promoter could reflect tumor load. In addition, patients with advanced TNM stage (III-IV) showed a higher elevated percentage of serum MT1M promoter methylation than those with early TNM stage (I - II), although the difference was not significant (P = 0.058). These differences from the previous study[27] may have been due to the use of different biological samples of HCC in different regions. Surprisingly, HCC patients with combined methylation of MT1M and MT1G promoters tended to show a higher incidence of vascular invasion and lymph node or extrahepatic metastasis (P = 0.018). Tumor invasion in the portal vein is the main route for intrahepatic meta-

Figure 4 Receiver operating characteristic curves of α-fetoprotein and combined methylation of metallothionein 1M and metallothionein 1G promoters. MT1M/MT1G, MT1M or MT1G promoter methylation. AUC: Area under the ROC curve; MT1M: Metallothionein 1M; MT1G: Metallothionein 1G.
tasis, which is regarded as the most frequent metastatic site of HCC\(^{20,21}\). Lymph node or extrahepatic metastasis is less common. Although curative resection remains a major effective method for HCC, the possibility of tumor recurrence, caused mainly by metastasis, leads to dismal prognosis. Therefore, combined methylation of serum MT\(_1M\) and MT\(_1G\) promoters may be a valuable prognostic marker for HCC. Also, our findings indicated that MT\(_1M\) and MT\(_1G\) may not only be tumor suppressors but also metastatic suppressors in HCC. However, the molecular mechanisms of this remain unclear. In previous studies, it was reported that MT\(_1G\) methylation contributes to tumor invasion in prostate cancer and peripheral pulmonary adenocarcinoma\(^{37,38}\). However, to the best of our knowledge, no studies have investigated MT\(_1M\) and tumor invasion. Further study is necessary to elucidate the mechanism of how MT\(_1M\) and MT\(_1G\) promoter methylation synergistically acts on metastasis in HCC. However, no significant differences between serum MT\(_1M\) and MT\(_1G\) promoter methylation and sex, age and history of HBV infection were observed, thus the analysis of serum MT\(_1M\) and MT\(_1G\) promoter methylation enabled the detection of HCC independent of patient settings.

Our findings demonstrated that MT isofrom gene expression may be specific and reciprocal in carcinogenesis and progression of HCC. They also support the concept that the clinical significance of MT expression in HCC might be further defined if specific MT isoforms were known for individual tumors\(^{21}\).

Our study had some limitations. First, the small number of HCC patients and NCs may have led to bias. Second, we do not have long-term follow-up data for HCC patients, which may reveal the predictive value of MT\(_1M\) and MT\(_1G\) promoter methylation in prognosis. Further study with a larger number of cases and longer follow-up is needed.

In conclusion, we demonstrated that MT\(_1M\) and MT\(_1G\) promoter methylation was frequently detected in serum of patients with HCC, and appeared to be a valuable diagnostic marker for noninvasive detection of HCC. Furthermore, we observed that MT\(_1M\) promoter methylation was associated with tumor size and combined MT\(_1M\) and MT\(_1G\) promoter methylation in serum was easily detected in HCC patients with vascular invasion or metastasis, suggesting that it may be a useful prognostic marker as well.

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