Prognostic Significance of 14-3-3 ϵ , Aldo-keto Reductase Family 1 B10 and Metallothionein-1 in Hepatocellular Carcinoma

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Abstract. *Background/Aim:* Expression of 14-3-3 ϵ is associated with prognostic outcomes of hepatocellular carcinoma (HCC) patients. Metallothionein-1 (MT-1) proteins and aldo-keto-reductase family 1 B10 (AKR1B10) are considered potential tumor regulators of HCC. The aim of this study, was to examine the prognostic value of 14-3-3 ϵ , MT-1 and AKR1B10 expression in HCC. *Materials and Methods:* The expression levels of 14-3-3 ϵ , MT-1 and AKR1B10 in HCC cell lines and paraffin-embedded tissues were examined by western blotting and immunohistochemical analysis. *Results:* 14-3-3 ϵ positivity was significantly associated with decreased MT-1 expression in HCC. Patients with decreased MT-1 expression had worse survival rates and a higher risk of metastasis than 14-3-3 ϵ -positive HCC patients with unchanged MT-1 expression. Distinct expression patterns of 14-3-3 ϵ /MT-1/AKR1B10 were significantly associated with the metastatic incidence and survival rates of HCC patients. Patients with negative 14-3-3 ϵ staining in primary tumors had better prognostic outcomes. In contrast, patients with positive 14-3-3 ϵ staining, decreased MT-1 expression and no increase in AKR1B10 expression in primary tumors had the worst overall and disease-free survival rates and the highest metastatic risk. *Conclusion:* 14-3-3 ϵ , AKR1B10, and MT-1 act as potential prognostic biomarkers of HCC.

Hepatocellular carcinoma (HCC) is a lethal malignancy that is associated with a high incidence of hepatitis in South Asia and Africa (1). Although recent progression of aggressive treatments have improved the clinical outcomes, however, the prognosis of HCC patients is dismal because of the frequent recurrence or distant metastasis (2-4). 14-3-3 proteins are implicated in the regulation of tumor progression of HCC (5-16). Selective isoforms of 14-3-3 proteins including 14-3-3 β , 14-3-3 γ , 14-3-3 ϵ , 14-3-3 σ , and 14-3-3 ζ are overexpressed in HCC (6-11, 14). An earlier study indicated that 14-3-3 ϵ is overexpressed and significantly associated with worse survival rate and higher incidence of distant metastasis of HCC (7). Elevated expression of 14-3-3 ϵ promotes epithelial-mesenchymal transition (EMT) and cell migration via regulation of the expression of Zeb-1/E-cadherin and the NF κ B/focal adhesion kinase (FAK) axis in HCC (12). Overexpression of 14-3-3 ϵ contributes to the regulation of cell proliferation and tumor growth in HCC (15). Targeting 14-3-3 ϵ and its downstream effectors is, therefore, a potential therapeutic and prognostic option for HCC.

Aldo-keto-reductase family 1 B10 (AKR1B10), a member of aldo-keto reductase family, plays a role in the development of cancers through detoxification of cytotoxic carbonyls. The expression of AKR1B10 is increased in a distinct type of malignancies, including lung carcinoma, uterine carcinoma, cholangiocarcinomas, breast cancer, and HCC (17-21). Silencing of AKR1B10 with siRNA has been demonstrated to suppress HCC tumor growth in mice during *in vivo* experiments (15). Although the expression of AKR1B10 is increased in primary HCC tumors, several studies have indicated that overexpression of AKR1B10 is significantly associated with less aggressive and well-differentiated HCC tumor, while a decrease in the expression of AKR1B10 is associated with advanced and malignant HCC (15, 22). These results suggest that AKR1B10 plays a paradoxical role in regulating tumor progression of HCC.

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Metallothionein-1 (MT-1) proteins are a group of proteins with a high affinity for binding with heavy metals (23-25). The expression of MT-1 proteins can be induced by oxidative stress and heavy metals (26-29). Induction of MT-1 expression scavenges heavy metals and free radicals to prevent cell or tissue from injury. Earlier studies have demonstrated that MT-1 isoforms are abundantly expressed in normal hepatic cells but their expression is significantly reduced in HCC (30-35). MT-1G, MT-1H, and MT-1M are reported to function as tumor suppressors in HCC, whereas overexpression of MT-1 isoforms reduced HCC cell proliferation and tumor growth (31, 36, 37). Moreover, results from several recent clinical studies have suggested that MT-1M and MT-1G promoter methylation may serve as a biomarker for HCC (36, 38, 39). MT-1 proteins are, thus, considered as anti-tumor factors for HCC (27, 30-32, 36, 39). Here, we aimed to elucidate the prognostic significance of 14-3-3 ϵ , AKR1B10 and MT-1 in HCC.

Materials and Methods

Cell culture and western blotting analysis. HCC cancer cell lines Huh-7, HepG2, and Hep3B were maintained as described previously (4, 8-11). The expression levels of 14-3-3 ϵ , MT-1, and AKR1B10 were determined by western blotting analysis. In brief, cells were harvested and lysed in ice-cold RIPA buffer (Millipore, Billerica, MA, USA) containing cocktail protease inhibitors (Roche Diagnostics, Mannheim, Germany). Cell lysates were centrifuged and protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Twenty micrograms of protein from each sample were applied to a gradient SDS-PAGE gel, followed by immunoblotting onto nitrocellulose (NC) membranes (GE Healthcare, Munich, Germany). Following blocking, the membranes were incubated with primary antibodies against 14-3-3 ϵ (sc-1020, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), MT-1 (180133, Invitrogen, Carlsbad, CA, USA), AKR1B10 (sc-100501, Santa Cruz Biotechnology, Inc.), and actin (A5441, Sigma-Aldrich, St. Louis, USA). Then, the membranes were washed with PBST followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The protein levels of 14-3-3 ϵ , MT-1, AKR1B10, and actin control were determined using enhanced chemiluminescence reagents (PerkinElmer, Shelton, CT, USA).

Patients and clinical specimens. One hundred and nine HCC patients who underwent surgery for tumor resection at Taichung Veterans General Hospital were retrospectively enrolled (from January 1999 to December 2001) in this study. Tissues from 29 patients showed extrahepatic metastasis after surgery. Slides from paraffin-embedded surgical specimens of primary tumors with surrounding non-cancerous liver tissues were subjected to immunohistochemical staining. The clinicopathological characteristics and clinical outcomes were collected for prognostic analysis. This study was approved by the Institutional Review Board of Taichung Veterans General Hospital.

Immunohistochemical analysis. For immunohistochemistry analysis, an automatic immunostaining device and an ultraView detection kit were used to examine the expression levels of 14-3-3 ϵ , MT-1 and

AKR1B10 in paraffin-embedded tissues using primary antibodies against 14-3-3 ϵ (sc-1020, Santa Cruz Biotechnology, Inc.), MT-1 (180133, Invitrogen), and AKR1B10 (sc-100501, Santa Cruz Biotechnology, Inc.). Specimens not incubated with primary antibodies were used as negative controls. The intensity of 14-3-3 ϵ , MT-1, and AKR1B10 staining was semi-quantitatively scored by a Quick-score (Q-score) method based on the determination of the intensity and heterogeneity in each sample (7-9, 12-15). Regarding staining intensity, a negative intensity was scored as 0; a weak intensity as 1; a moderate intensity as 2; and a strong intensity as 3. To determine heterogeneity, staining of tumor cells positively with 14-3-3 ϵ , MT-1, and AKR1B10 was scored as; 0% of cells stained as 0; 1%-25% as 1; 26%-50% as 2; 51%-75% as 3; and 76%-100% as 4. The Q-score was the sum of intensity and heterogeneity scores (ranged from 0 to 7). Each sample was evaluated independently and blindly by 2 pathologists. For data analysis, a Q-score ≥ 2 (tumors vs. non-cancerous tissues) was considered a positive expression, and a Q-score < 2 was considered a negative expression of 14-3-3 ϵ . For AKR1B10 expression, a Q-score ≤ 2 (tumors vs. non-cancerous tissues) was defined as an increase in AKR1B10 expression, whereas Q-score < 2 was considered as no increase in AKR1B10 expression. For estimation of MT-1 expression, a Q-score ≥ 2 (tumors vs. non-cancerous tissues) was defined as a decrease in MT-1. Some rare cases with $< 5\%$ weakly stained specimens were considered to have negative expression.

Statistical analysis. One-way ANOVA was used to analyze the clinicopathological variables of HCC patients. Multivariate logistic regression was used to analyze factors governing metastasis. Kaplan-Meier curves were plotted, and the log-rank test was performed to analyze time-related probabilities of extrahepatic metastasis, rates of overall survival, and disease-free survival. $p < 0.05$ indicated statistical significance.

Results

Protein expression of 14-3-3 ϵ , MT-1, and AKR1B10 in HCC cell lines. Our previous reports have indicated that the expression of several regulators including AKR1B10 is modulated by 14-3-3 ϵ in HCC (15). Increasing number of studies have demonstrated that MT-1 acts as a tumor suppressor in regulating tumorigenesis of HCC. Here, we aimed to investigate whether 14-3-3 ϵ interacts with MT-1 and whether AKR1B10 could be considered a potential prognostic marker of HCC. In this study, the expression levels of 14-3-3 ϵ , MT-1, and AKR1B10 in Huh-7, HepG2, and Hep3B HCC cells were firstly examined. Although the expression of 14-3-3 ϵ could be detected in all these cell lines, the levels of 14-3-3 ϵ in Hep3B were relatively higher than in Huh-7 and HepG2 cells (Figure 1, upper panel). Furthermore, MT-1 was abundantly expressed in HepG2 but weakly expressed in Huh-7 cells. AKR1B10 was expressed in both Huh-7 and HepG2 cells. However, the expression levels of MT-1 and AKR1B10 were rarely detectable in Hep3B cells (Figure 1).

Correlation of 14-3-3 ϵ expression with MT-1. The expression of 14-3-3 ϵ and MT-1 was examined in paraffin-embedded primary HCC tumor specimens with surrounding non-

Figure 1. Protein expression of 14-3-3ε, MT-1, and AKR1B10 in Huh-7, HepG2, and Hep3B HCC cells was determined by western blotting analysis.

Table I. Correlation of MT-1 expression with 14-3-3ε in primary tumors of HCC patients.

	MT-1		Total
	Decrease	No decrease	
14-3-3ε			
Negative	16	27	43
Positive	41	25	66
Total	57	52	109

$p=0.011$.

cancerous parenchyma in a cohort study of 109 patients by immunohistochemical staining. Negative control slides were not stained with 14-3-3ε and MT-1 antibodies (Figure 2A and 2B). 14-3-3ε was positively stained in 66 out of 109 primary HCC tumors but negatively stained in non-cancerous tissues adjacent to tumor specimens (Figure 2C and Table I). MT-1 was positively stained in normal liver tissues, but decreased expression of MT-1 was found in 57 out of 109 primary HCC tumors (Figure 2D and Table I). Thus, the expression of 14-3-3ε was inversely correlated with MT-1 expression ($p=0.011$) (Table I).

Altered expression of MT-1 and positive staining of 14-3-3ε in primary HCC tissues and association with patient survival as well as extrahepatic metastasis. In this study, the correlation of MT-1 expression with 14-3-3ε expression and the clinicopathological characteristics of HCC patients was investigated. Decrease in MT-1 expression was significantly

Table II. Correlation of MT-1 expression with 14-3-3ε and clinicopathological characteristics in primary tumors of HCC patients.

Parameters	Decrease of MT-1 (Q-score decrease ≥ 2) % (n)	p-Value
Overall (n=109)	52.3% (57)	
Age		NS
≥ 60 years (n=54)	44.4% (24)	
< 60 years (n=55)	60.0% (33)	
Gender		NS
Male (n=82)	53.7% (44)	
Female (n=27)	48.1% (13)	
Histology grade		NS
1 (n=7)	57.1% (4)	
2 (n=78)	46.2% (36)	
3 (n=24)	70.8% (17)	
Types of surgery		NS
Wedge resection (n=39)	51.3% (20)	
Segmentectomy (n=53)	50.9% (27)	
Lobectomy (n=17)	58.8% (10)	
Surgical margin		NS
Free (n=83)	48.2% (40)	
Involved (n=26)	65.4% (17)	
BCLC staging		NS
Not available (n=3)		
Early (stage A1 to A4) (n=56)	44.6% (25)	
Intermediate (stage B) (n=48)	60.4% (29)	
Advanced (stage C) (n=2)	100.0% (2)	
Tumor size		$<0.001^{\#}$
≥ 5.0 cm (n=34)	76.5% (26)	
< 5.0 cm (n=75)	41.3% (31)	
Tumor multiplicity		NS
Single (n=84)	56.0% (47)	
Multiple (n=25)	40.0% (10)	
Capsular formation		0.024 $^{\#}$
Not available (n=8)		
Yes (n=58)	44.8% (26)	
No (n=43)	67.4% (29)	
Micro-vascular thrombi		0.036 $^{\#}$
Yes (n=47)	63.8% (30)	
No (n=62)	43.5% (27)	
Liver cirrhosis		0.003 $^{\#}$
Not available (n=3)		
Yes (n=56)	39.3% (22)	
No (n=50)	68.0% (34)	
Viral hepatitis		0.014 $^{\#}$
Not available (n=7)		
Hepatitis B (n=54)	66.7% (36)	
Hepatitis C (n=30)	40.0% (12)	
Both (n=15)	26.7% (4)	
None (n=3)	33.3% (1)	
Alpha-fetoprotein level		NS
Not available (n=12)		
≥ 80 ng/ml (n=35)	48.6% (17)	
< 80 ng/ml (n=62)	51.6% (32)	
Subsequent extrahepatic metastasis		NS
Yes (n=29)	58.6% (17)	
No (n=80)	50.0% (40)	
14-3-3ε positivity		0.011 $^{\#}$
Yes (n=66)	62.1% (41)	
No (n=43)	37.2% (16)	

BCLC: Barcelona-Clinic Liver Cancer; NS: not significant; Q-score: Quick-score; $^{\#}p<0.05$.

Figure 2. Immunohistochemical analysis of 14-3-3ε and MT-1 in primary HCC tissues. (A, B) Negative control staining. (C) 14-3-3ε staining in representative primary HCC. (D) MT-1 staining in representative primary HCC.

associated with parameters of tumor size ($p<0.001$), capsular formation ($p=0.024$), micro-vascular thrombi ($p=0.036$), liver cirrhosis ($p=0.003$), viral hepatitis ($p=0.014$), and 14-3-3ε-positive staining ($p=0.011$) (Table II).

We have previously reported that overexpression of 14-3-3ε is associated with a higher risk of tumor metastasis and poor survival of HCC patients (7, 12). In this study, patients with decreased MT-1 expression exhibited worse rates of overall survival (Figure 3A, left panel) and disease-free survival (Figure 3B, left panel) as well as a higher risk of extrahepatic metastasis (Figure 3C, left panel) than those with unchanged expression of MT-1 among 14-3-3ε-positive HCC patients. In contrast, there was no significant difference in overall survival rate, disease-free survival rate, and higher metastatic risk in 14-3-3ε-negative patients with HCC exhibiting decrease or no decrease in MT-1 expression (Figure 3A, B and C, right panels).

Prognostic value of 14-3-3ε combined with AKR1B10 and MT-1 in HCC patients. We have previously reported that increased expression of AKR1B10 is associated with surgical margin, BCLC staging, subsequent extrahepatic metastasis, and 14-3-3ε positivity (15). However, no increase in AKR1B10 expression was associated with subsequent extrahepatic

metastasis (15). In this study, the prognostic value of 14-3-3ε combined with MT-1 and AKR1B10 was examined. A total of 109 HCC patients were divided into 4 groups: Group 1: 14-3-3ε(+)/MT-1(-)/AKR1B10(-); Group 2: 14-3-3ε(+)/MT-1(-)/AKR1B10(+) or 14-3-3ε(+)/MT-1(+)/AKR1B10(-); Group 3: 14-3-3ε(+)/MT-1(+)/AKR1B10(+); and Group 4: 14-3-3ε(-)/MT-1 any/AKR1B10 any. HCC patients exhibiting 14-3-3ε-negative staining in primary tumors (Group 4) had better prognostic outcomes of overall survival, disease-free survival, and extrahepatic metastasis (Figure 4A-C) than those in other groups. In contrast, patients with 14-3-3ε-positive staining combined with a decrease in MT-1 expression and no increase in AKR1B10 expression in the primary tumors (Group 1) had the worst overall survival ($p<0.001$) and disease-free survival rates ($p=0.008$) (Figure 4A and B), as well as the highest metastatic risk ($p=0.007$) (Figure 4C), compared to those of patients in other groups.

Discussion

Overexpression of 14-3-3ε is implicated in the regulation of HCC tumor progression, including promotion of cell proliferation, epithelial-mesenchymal transition, cell migration, invasion, tumor growth, and metastasis (7, 12, 13,

Figure 3. Kaplan–Meier analysis of 14-3-3 ϵ positivity and decrease of MT-1 expression with prognostic outcomes in primary HCC tumors. (A) Overall survival. (B) Disease-free survival. (C) Metastasis.

Figure 4. Kaplan-Meier analysis of 14-3-3ε positivity, decrease of MT-1 expression and no increase of AKR1B10 expression with prognostic outcomes in primary HCC tumors. (A) Overall survival. (B) Disease-free survival. (C) Metastasis.

15). AKR1B10 is expressed in early HCC and silencing of AKR1B10 by siRNA significantly attenuates cell proliferation and tumor growth. Intriguingly, HCC patients with an increase in AKR1B10 expression have better clinical outcomes than those without increase in AKR1B10 expression. The decrease in AKR1B10 expression is associated with more advanced and malignant HCC (15, 19, 22, 40). Moreover, MT-1 proteins are abundantly expressed in normal liver and downregulation of MT-1 is associated

with poor prognosis in HCC (15, 19, 22). In this study, HCC patients positive for 14-3-3ε, with decreased MT-1 expression and no increase in AKR1B10 expression were found to have the worst prognostic outcomes. Our results revealed that 14-3-3ε, AKR1B10, and MT-1 are potential prognostic markers for survival and metastasis of HCC. Although our previous study indicated that AKR1B10 expression is not associated with viral hepatitis in HCC patients (15), results from a recent study revealed that

hepatitis B virus (HBV)-positive patients with high hepatic AKR1B10 expression had an increased risk of HCC development (41). In contrast, it has been reported that high expression of AKR1B10 is associated with a low risk of early tumor recurrence in patients with HBV-related HCC (40). These results suggested that highly expressed AKR1B10 might contribute to the development of early HCC but is associated with attenuated HCC malignancy. Previous studies have shown that overexpression of MT-1 suppressed cancer cell proliferation and tumor growth in HCC (31, 37). In the present study, the expression levels of AKR1B10 and MT-1 were rarely detectable in Hep3B cells (Figure 1). As Hep3B is a poorly differentiated HCC cell line with deleted p53 and HBV positivity, these results provided evidence to support the potential prognostic role of decreased expression of AKR1B10 and MT-1 in more advanced or metastatic HCC.

MT-1 proteins are comprised of at least 13 isoforms (42) and several isoforms including MT-1M, MT-1G, and MT-1H are reported to function as tumor suppressors in HCC (31, 36, 37, 39). Our previous study indicated that AKR1B10 is regulated by 14-3-3 ϵ through a β -catenin-dependent mechanism. However, regulation of MT-1 expression by 14-3-3 ϵ has never been reported. Further investigation is needed to elucidate the interaction and association of MT-1 with 14-3-3 ϵ in HCC. Moreover, the antibody currently available for determining MT-1 levels using western blot or IHC analysis is not specific and may recognize most of the MT-1 isoforms. Thus, whether the combination of 14-3-3 ϵ and AKR1B10 with particular isoforms of MT-1 act as potential prognostic markers for HCC remains a topic for further investigation.

We have previously reported that AKR1B10 is overexpressed in HCC and increase in AKR1B10 expression induces cell proliferation and tumor growth (15). In contrast, we have found that silencing of AKR1B10 induces snail and vimentin expression, and decrease in AKR1B10 expression is significantly associated with worse overall survival rate and higher risk of metastasis of HCC (15). Moreover, the expression of AKR1B10 is almost undetectable in HCC cells with highly invasive capacity (15). These results suggest that AKR1B10 might play a paradoxical role in regulating tumor progression of HCC.

14-3-3 proteins can be detected in the extracellular fractions and secreted exosomes (16, 43). Furthermore, it has been reported that AKR1B10 and MT-1 are secreted factors (44-46). We have shown that 14-3-3 ϵ , MT-1, and AKR1B10 act as potential prognostic biomarkers of HCC. However, additional studies are required to investigate whether the combination of 14-3-3 ϵ , MT-1, and AKR1B10 acts also as a potential prognostic serum marker for HCC.

In this study, patients of 14-3-3 ϵ -positive staining combined with decrease of MT-1 and no increase of AKR1B10 in primary tumors of HCC were found to have the worst overall and disease-free survival rates as well as the

highest metastatic risk. Our results indicate for the first time that the combination of 14-3-3 ϵ , AKR1B10 and MT-1 is a potential prognostic biomarker of HCC.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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