A New Diagnostic Method for Chronic Hepatitis, Liver Cirrhosis, and Hepatocellular Carcinoma Based on Serum Metallothionein, Copper, and Zinc Levels

Akihiro Nakayama, Hiroyuki Fukuda, Masaaki Ebara, Hiroshi Hamasaki, Katsuyuki Nakajima, and Hiromu Sakurai*

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University.* 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan, First Department of Medicine, School of Medicine, Chiba University,* 1–8–1 Inohana, Chuō-ku, Chiba 260–8677, Japan, Department of Health Science, Kyoto Pharmaceutical University,* 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan, and Japan Immunoresearch Laboratories Co., Ltd.,* 351–1 Nishiyokote-machi, Takasaki, Gunma 370–0021, Japan. Received November 14, 2001; accepted January 28, 2002

Serum metal levels and their ratios are frequently reported to be good signals for diagnosing various diseases. These parameters are not always specific to the disease, however, it is necessary to use other serum parameters for an exact diagnosis. We examined whether the monitoring of these serum parameters such as metallothionein, copper, and zinc levels are useful in diagnosing hepatic disorders. Metallothionein levels of patients with liver cirrhosis and hepatocellular carcinoma were found to be significantly lower than those of patients with chronic hepatitis and those of controls. In contrast, copper levels of the patients with liver cirrhosis and hepatocellular carcinoma were significantly higher than those with chronic hepatitis and controls. Zinc levels of the patients with chronic hepatitis and hepatocellular carcinoma were lower than those of controls. Using these three parameters, we are introducing a new parameter, (Cu/Zn)/MT, by which we can discriminate between patients in the [control+miscellaneous diseases+chronic hepatitis] group and those in the [liver cirrhosis+hepatocellular carcinoma] group. The new parameter does not, however, allow us to clearly distinguish between the liver cirrhosis and hepatocellular carcinoma groups. Multivariate discriminant analysis was found to be very useful, with combinations of two discriminant functions having been designed to discriminate both between chronic hepatitis and liver cirrhosis and between liver cirrhosis and hepatocellular carcinoma. This method recognizes the differences between hepatic disorder, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma groups. On the basis of these results, we propose here that the diagnosis of hepatic disorders should be made based on a combination of three serum levels such as those of metallothionein, copper, and zinc.

Key words metallothionein; hepatic disorder; copper; zinc; Mahalanobis distance; discriminant analysis

Hepatocellular carcinoma (HCC) is one of the most frequent human tumors worldwide, and commonly evolves from chronic hepatitis (CH) and liver cirrhosis (LC). It is therefore very important to detect and evaluate the progressing state of chronic hepatic disorders. Several diagnostic procedures, including computed tomography, magnetic resonance imaging, and biochemical signals have been used, but they have proven to be less than satisfactory. On the other hand, serum metal levels such as those of copper (Cu) and zinc (Zn) have been reported to be highly sensitive in the diagnosis of some diseases. Hypozincemia and marked hypercupremia have been reported in patients with digestive, hepatic, breast, and lung cancers. Although the role of trace elements and the mechanism of their kinetics in relation to these diseases remain controversial, the serum copper/zinc ratio (Cu/Zn) has been claimed to be highly sensitive in the diagnosis of the diseases in question.

We have previously reported that unusual accumulations of Cu and metallothionein (MT) occur in the livers of Long– Evans Cinnamon rats and that these accumulations are deeply related to the development of jaundice and hepatoma. Similar observations have been reported with regard to the livers of human hepatocellular carcinoma, although the observed metal and MT accumulations have differs between studies. Based on these observations, we attempted in the present study to investigate whether monitoring of serum metals and MT levels is useful for diagnosing human liver disorders.

Based on the results of several trials, we propose here that the diagnosis of hepatic disorders can be achieved by the monitoring of serum levels of MT, Cu, and Zn and subsequent multivariate discriminant analysis based on the Mahalanobis distance.

MATERIALS AND METHODS

Materials Standard metallothionein-I (MT-I) from rabbit liver was purchased from Sigma (St. Louis, MO, U.S.A.). 3,3’,5,5’-Tetramethylbenzidine (TMB) was purchased from MOSS, Inc. (Pasadena, MD, U.S.A.). Monoclonal mouse anti-metallothionein (used as primary antibody), which recognizes human, horse, sheep, and rat MT-I and MT-II, was purchased from DAKO Corporation (Carpinteria, CA, U.S.A.). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (used as secondary antibody) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). Nitric acid (for poisonous metals determination), hydrogen peroxide (for atomic absorption spectrochemical analysis), perchloric acid (for poisonous metals determination), and standard solutions of Cu and Zn for the measurement of inductively coupled plasma-mass spectrometry (ICP-MS) were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Subjects Human sera of the patients with diseases were collected at Chiba University, Toyo and Yokohama East Hospitals. Healthy sera were collected from students of Kyoto
Pharmaceutical University. All the sera were collected in the morning after fasting for more than 9 h when the informed consent was obtained at the University hospital and other related hospitals. The sera from the patients with diseases were collected before surgical or drug treatment. The features of the subjects in this study are shown in Table 1. The control (Ctrl) group was composed of healthy students ($n=33$) and researchers without diseases ($n=10$). The miscellaneous group (Misc) ($n=49$) included the patients without diseases in the lesion of liver parenchyma, including fatty liver, hepatic hemangioma, gallbladder polyps, gastric cancer, malignant melanoma, diabetes mellitus, hypertension, angina pectoris, congestive heart failure, apoplexie, transient ischemic attack, and bronchitis.

Measurement of MT Levels Serum MT levels were determined by an enzyme-linked immunosorbent assay (ELISA) after acid (1 M HCl) and heat (100°C, 10 min) treatments to eliminate the coexistent antibody-reactive protein. In brief, 10 μg of standard MT-I was dissolved in 10 ml of phosphate buffered saline (PBS) containing 1% 2-mercaptoethanol, and the resulting solution was diluted 10 times with 0.1% carbonate buffer (pH 9.6). Each well containing 100 μl of standard MT-I solution in a 96-well microtiter plate was incubated for 1 h at 37°C and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Each well was blocked with 180 μl of 1% bovine serum albumin (BSA) in PBS-T for 30 min at room temperature and washed three times with PBS-T. An acid (1 M HCl)- and heat-treated sample to be tested and primary antibody were added to each well and incubated for 3 h at room temperature, then washed three times with PBS-T. Each well was incubated with 100 μl of secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature and washed three times with PBS-T. Next, 50 μl of TMB solution, as a substrate for horseradish peroxidase, was added to each well. After sufficient color development, 50 μl of 1 M HCl was added, and the absorbance at 490 nm was measured. The range of the calibration curve was 0.313—40 ng MT/μl, with the correlation coefficient for the calibration curve being $r>0.99$ for a total of five MT concentrations.

Measurement of Metal Levels Serum metal levels were determined by ICP-MS. In brief, 300 μl of sera were heated at 200°C with 60% HNO$_3$, 30% H$_2$O$_2$, and 60% HClO$_4$ in 50-ml beakers. When the residue became white, the dried samples were dissolved in 10 ml of 6% HNO$_3$. Then the metal levels were measured by ICP-MS (ICPM-8500, Shimadzu, Japan). Cu and Zn levels were calculated using calibration curves at a concentration range of 5—500 ppb for each metal. The detection limits of each metal were approximately 0.2 ppb for Cu and 2 ppb for Zn concentrations. The correlation coefficients of linear regression for Cu and Zn were $r>0.999$ for a total of five metal concentrations. Plasma conditions were as follows: coolant gas-flow, plasma gas-flow and carrier gas-flow rates were 7.0, 1.5, and 0.6 l/min, respectively, and the sampling depth was 6 mm.

Multivariate Discriminant Analysis Multivariate discriminant analysis (MDA) was carried out by using the Mahalanobis distance ($MD^2$). $MD^2$ is defined as the weighted distance between an unknown data vector and the mean data vector for a known class of samples. The $MD^2$ was calculated as follows:

$$MD^2=(X-x \cdot)^TS^{-1}(X-x \cdot)$$

where $T$ is the transposition operator, and $S^{-1}$ is the inverse of the variance-covariance matrix. $X$, $\bar{x}$, and $S$ are given by the following equations:

$$X=egin{pmatrix} x_1 \\ x_2 \\ \cdots \\ x_n \end{pmatrix}, \quad \bar{x}=egin{pmatrix} \bar{x}_1 \\ \bar{x}_2 \\ \cdots \\ \bar{x}_n \end{pmatrix}, \quad S=egin{pmatrix} s_{11} & s_{12} & s_{13} \\ s_{21} & s_{22} & s_{23} \\ \cdots & \cdots & \cdots \\ s_{n1} & s_{n2} & s_{n3} \end{pmatrix}$$

where $x_i$, $\bar{x}_i$, and $s_j$ are serum MT, Cu, and Zn levels, and $\bar{x}_i$ and $s_j$ are the mean and covariance, which are given by the equations as follows:

$$\bar{x}_i=\frac{1}{N} \sum_{k=1}^{N} x_{ik} \quad [i=1, 2 \text{ and } 3]$$

$$s_{ij}=\frac{1}{N-1} \sum_{k=1}^{N} (x_{ik}-\bar{x}_i)(x_{jk}-\bar{x}_j) \quad [i, j=1, 2 \text{ and } 3]$$

where $x_{ii}$, $x_{ij}$ and $x_{ij}$ are serum MT, Cu, and Zn levels of the $i$th subject, respectively, and $N$ is the total number of subjects.

The MDA function used in this study was calculated as follows:

$$MD^2=(x_i-x_i \cdot)^TS^{-1}(x_i-x_i \cdot)$$

If the inverse of variance-covariance matrix

$$\begin{pmatrix} s_{11} & s_{12} & s_{13} \\ s_{21} & s_{22} & s_{23} \\ s_{31} & s_{32} & s_{33} \end{pmatrix}$$

is defined as

$$\begin{pmatrix} \hat{s}_{11} & \hat{s}_{12} & \hat{s}_{13} \\ \hat{s}_{21} & \hat{s}_{22} & \hat{s}_{23} \\ \hat{s}_{31} & \hat{s}_{32} & \hat{s}_{33} \end{pmatrix},$$

the equation can be given as follows:

$$MD^2=\hat{s}_{11}(x_i-x_{i 1})^2+\hat{s}_{21}(x_i-x_{i 2})^2+\hat{s}_{31}(x_i-x_{i 3})^2+2\hat{s}_{12}(x_i-x_{i 1})(x_i-x_{i 2})+2\hat{s}_{13}(x_i-x_{i 1})(x_i-x_{i 3})+2\hat{s}_{23}(x_i-x_{i 2})(x_i-x_{i 3})$$

In the above equation, $s_{ii}$ is equal to $\hat{s}_{ii}$, so the equation can be rewritten as follows:

$$MD^2=\hat{s}_{11}(x_i-x_{i 1})^2+\hat{s}_{21}(x_i-x_{i 2})^2+\hat{s}_{31}(x_i-x_{i 3})^2+2\hat{s}_{12}(x_i-x_{i 1})(x_i-x_{i 2})+2\hat{s}_{13}(x_i-x_{i 1})(x_i-x_{i 3})+2\hat{s}_{23}(x_i-x_{i 2})(x_i-x_{i 3})$$

$$=s_{11}(x_i-x_{i 1})^2+s_{21}(x_i-x_{i 2})^2+s_{31}(x_i-x_{i 3})^2+2s_{12}(x_i-x_{i 1})(x_i-x_{i 2})+2s_{13}(x_i-x_{i 1})(x_i-x_{i 3})+2s_{23}(x_i-x_{i 2})(x_i-x_{i 3})$$

In the above equation, $s^2$ is equal to $s^2$, so the equation can be rewritten as follows:

$$MD^2=\hat{s}_{11}(x_i-x_{i 1})^2+\hat{s}_{21}(x_i-x_{i 2})^2+\hat{s}_{31}(x_i-x_{i 3})^2+2\hat{s}_{12}(x_i-x_{i 1})(x_i-x_{i 2})+2\hat{s}_{13}(x_i-x_{i 1})(x_i-x_{i 3})$$

$MD^2$, $MD^2_C$, and $MD^2_{LC}$ are the Mahalanobis distance from the Ctrl group (MDA$_1$; [Ctrl+Mics+CH] group, MDA$_2$; [Ctrl+Mics+CH+LC] group, and $MD^2_{1}$ is the Mahalanobis distance from the HCC group (MDA$_3$; [LC+HCC])
group, MDA2; [HCC] group).

**Others** All purified water was manufactured using Elix 5 and Milli Q Laboratory Water Purification Systems (Millipore Co., Bedford, MA, U.S.A.).

**Statistics** All experiments were repeated in triplicate, and the data are expressed as the means ± standard deviations (S.D.). The statistical significance of differences between the groups was evaluated using the Mann–Whitney U-test.²⁷) 

**RESULTS**

A total of 147 human sera samples (Table 1) were collected, and the MT, Cu, and Zn levels were measured. The resulting serum MT, metal levels, Cu/Zn ratio, and (Cu/Zn)/MT ratio are shown in Fig. 1. Mean serum MT levels of the patients with Misc and CH did not differ from those of the Ctrl; in contrast, serum MT levels of the patients with LC and HCC were lower than those of the Ctrl, Misc, and CH (Fig. 1). Serum Cu levels of the Ctrl and CH patients were significantly lower than those of the Misc, LC, and HCC patients (Fig. 1). In contrast, serum Zn levels of the Ctrl were the highest of all subjects, and those of the LC were higher than those of the Misc, CH, and HCC patients (Fig. 1). The serum Cu/Zn ratio of the Ctrl was the lowest, and that of the HCC was the highest among all subjects (Fig. 1). From the Cu/Zn ratio, it was not possible to distinguish between patients with CH, LC, and HCC, so a new parameter, (Cu/Zn)/MT, was introduced. As seen in Fig. 1, the (Cu/Zn)/MT ratio was found to be sensitive to differences between two groups, namely, the [Ctrl+Misc+CH] and [LC+HCC] groups. It could distinguish between the two groups with high accuracy when the threshold was set at 2.60 μM⁻¹. If the (Cu/Zn)/MT ratio is higher than 2.60 μM⁻¹, our results indicate that the patient should probably be included in the [LC+HCC] group (Fig. 2). As such 88.6% (=31/(31+4)) of the patients with HCC and LC were classified as members of the [LC+HCC] group based on the (Cu/Zn)/MT ratio. On the basis of the results using (Cu/Zn)/MT, it might be possible to diagnose the pathophysiological change from CH to LC. However, discrimination between patients with LC and HCC was difficult even when using both the Cu/Zn and (Cu/Zn)/MT ratios, as the differences between the ratios of LC and HCC patients were quite small (Fig. 1). Consequently, other discrimination methods were determined to be needed.

After several trials, we found that multivariate discriminant analysis (MDA)²⁶) is very useful in distinguishing between LC and HCC. We examined whether the MDA can identify differences between the two groups, [Ctrl+Misc+CH] and [LC+HCC], as the (Cu/Zn)/MT ratio could make this distinction. The first MDA function, named MDA₁, was designed to discriminate between [Ctrl+Misc+CH] and [LC+HCC] patients. The equation for the MDA₁ function was calculated according to the method described in the Materials and Methods, giving the following results.

\[
MDA_1 = -19.255X_1 + 0.012X_2 + 0.113X_3 + 0.642X_4 - 1.676X_5 + 0.006X_6 + 15.686X_7 + 0.019X_8 + 1.604X_9 - 10.383
\]

(1)

where \(X_1, X_2, \text{ and } X_3\) are the serum MT, Cu, and Zn levels, respectively. The resulting classification of the diseases between the [Ctrl+Misc+CH] and [LC+HCC] groups by MDA₁ score is shown in Table 2A. Based on the MDA₁ function, 85.7% (=30/(30+5)) of the patients with LC and HCC...
A total of 79.2% of the HCC patients was classified into the [HCC] group by MDA2 score, suggesting the MDA2 function can distinguish the HCC from other disease groups including the Ctrl group.

In addition, the MDA analysis was found to have high diagnostic accuracy, as evaluated by other parameters such as sensitivity and specificity. The sensitivity indicates how exactly the parameter predicts the concerned disease by using the equation: sensitivity=true positive fraction/(true positive fraction+false negative fraction). True positive and negative fractions indicate that the classifications based on MDA score correspond with those based on histological techniques, while false positive and negative fractions indicate that the classifications based on MDA score do not correspond with those based on histological techniques, where positive and negative fractions indicate the number of patients for whom the MDA score is >0 (positive) and <0 (negative), respectively. The sensitivity for the MDA1 and MDA2 functions were calculated to be 0.857 (=30/(30+5)) and 0.792 (=19/(19+5)), respectively. In contrast, the specificity indicates how exactly the parameter predicts the patients without the concerned disease [specificity=true negative fraction/(true negative fraction+false positive fraction)]. The specificities for the MDA1 and MDA2 functions were calculated to be 0.884 (=99/(99+13)) and 0.935 (=115/115), respectively.

The results suggest that the MDA1 and MDA2 functions were able to discriminate between the hepatic disorders examined. Then, to examine the MDA functions in detail, we combined the MDA1 and MDA2 scores to distinguish between the following groups, [Ctrl+Misc+CH], [LC], and [HCC]. A scattergram of MDA1 vs. MDA2 scores as well as a classification of the three groups by MDA1 and MDA2 scores are shown in Fig. 3. If both MDA1 and MDA2 scores are >0, the patients are classified into the [HCC] group. If the MDA1 score is >0 and the MDA2 score is <0, the patients are classified into the [LC] group. If both MDA1 and MDA2 scores are <0, the patients are classified into the [Ctrl+Misc+CH] group. Thus, the combination of MDA1, and MDA2 scores is able to discriminate between the [Ctrl+Misc+CH], [LC], and [HCC] groups (Figs. 3A, B). By using both MDA scores, the sensitivities for HCC and LC were calculated to be 0.791 (=19/(19+3+2)) and 0.636 (=7/7+1+3), respectively, and the specificity was calculated to be 0.884 (=99/(99+7+6)).

Based on the results, serum MT, Cu, and Zn levels and the subsequently obtained MDA scores were found to distinguish between the CH, LC, and HCC hepatic disorders.

### DISCUSSION

Many researchers have reported that the serum Cu/Zn ratio is sensitive enough for the diagnosis of various diseases. In the present study, the serum Cu/Zn ratio of patients with different hepatic diseases (Table 1) was also confirmed to be significantly higher than that of the Ctrl (Fig. 1). But some patients with congestive heart failure, diabetes mellitus, and hypertension exhibited markedly high Cu/Zn ratios (Cu/Zn ratio >2.0), indicating that the ratio was not selective for diagnosis of the diseases. In contrast, serum MT levels of patients with LC and HCC were significantly lower than those of other subjects. Thus, we combined the Cu/Zn ratio and MT levels and introduced a new parameter, the (Cu/Zn)/MT ratio.

**Table 2.** MDA1 and MDA2 Scores of A) [HCC+LC] Group and [Ctrl+Misc+CH] Group and B) [HCC] Group and [Ctrl+Misc+CH+LC] Group, Respectively

**A) MDA1 score**

<table>
<thead>
<tr>
<th>MDA1</th>
<th>HCC+LC</th>
<th>Ctrl+Misc+CH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA1&gt;0</td>
<td>30 (85.7)</td>
<td>13 (11.6)</td>
<td>43 (29.2)</td>
</tr>
<tr>
<td>MDA1&lt;0</td>
<td>5 (14.3)</td>
<td>99 (88.4)</td>
<td>104 (70.8)</td>
</tr>
<tr>
<td>Total</td>
<td>35 (100)</td>
<td>112 (100)</td>
<td>147 (100)</td>
</tr>
</tbody>
</table>

**B) MDA2 score**

<table>
<thead>
<tr>
<th>MDA2</th>
<th>HCC</th>
<th>Ctrl+Misc+CH+LC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA2&gt;0</td>
<td>19 (79.2)</td>
<td>8 (6.5)</td>
<td>27 (18.4)</td>
</tr>
<tr>
<td>MDA2&lt;0</td>
<td>5 (20.8)</td>
<td>115 (93.5)</td>
<td>120 (81.6)</td>
</tr>
<tr>
<td>Total</td>
<td>24 (100)</td>
<td>123 (100)</td>
<td>147 (100)</td>
</tr>
</tbody>
</table>
ratio, to examine a new possibility for diagnosing hepatic disorders. The serum (Cu/Zn)/MT ratio was found to distinguish between [Ctrl+Misc+CH] and [LC] groups when, for example, a cutoff value 2.60 \( \mu M^{-1} \) was used (Fig. 2). However, the ratio could not distinguish between LC and HCC. Therefore, we attempted to examine more reliable methods and found multivariate discriminant analysis (MDA) based on the Mahalanobis distance to be effective for our purpose.

In general, MDA was often computed by linear MDA. However, the linear MDA was applicable only if the variates of each group to be discriminated were statistically equal. In this study, group variances for discrimination were not statistically equal, and we therefore used the Mahalanobis distance to be effective for our purpose.

Fig. 3. (A) Multivariate Discriminant Analysis of Serum MT, Cu, and Zn Levels in the Ctrl and the Patients with Misc, CH, LC, and HCC. The insert is the expanded figure near the origin. (B) Classification of Diseases among the [Ctrl+Misc+CH], [LC], and [HCC] Groups by MDA1 and MDA2 Scores, as Calculated by Eqs. 1 and 2 in the Results Section.

to discriminate between the [Ctrl+Misc+CH], [LC] and [HCC] groups by the combination of MDA1 and MDA2 scores (Table 2, Fig. 3). The obtained percentages of 88.4, 63.6 and 79.2% for the [Ctrl+Misc+CH], [LC], and [HCC] groups corresponded to the histologically determined diagnosis. Although the diagnostic accuracy in predicting LC patients was not high, it was considered to be useful in diagnosing the hepatic disorders.

It was thought to be difficult to distinguish between HCC and LC patients by serum biochemical signals, because most of the HCC patients possess LC in their livers. Table 1 shows that serum Cu and MT levels of HCC patients were similar to those of LC patients, but serum Zn levels were different between them. In this study, (Cu/Zn)/MT ratio was less effective to distinguish the difference between HCC and LC group, because high levels of serum Zn found in LC patients were canceled by low levels of serum MT. In contrast, MDA distinguished the difference between HCC and LC by using 3 independent parameters, thus the MDA2 score was useful to distinguish them.

Castaldo G. et al. have successfully discriminated between HCC and LC using MDA.32) The MDA score showed a diagnostic sensitivity of 85% for HCC, which was higher than or comparable to that of other diagnose. In our results, 79.2% of HCC patients and 63.6% of LC patients were discriminated from other patients with or without hepatic disorders by using only three parameters, serum MT, Cu, and Zn levels (Fig. 3). Although the sensitivities for HCC and LC in our present results were lower than those of Castaldo et al., our MDA scores could discriminate between HCC, LC and CH patients.

The reported serum MT levels have varied among researchers.33—37) The reason for these differences might relate to the specificity of the antibody used. Determination of the serum protein by immunoassay is often involved in differences among results due to cross-reactions with other proteins. The monoclonal antibody used in this study reacted with not only MT-I, but also MT-II. Because some researchers have used MT-I specific antibodies, serum MT levels have been estimated as being relatively low. In the present study, we pretreated the sample with both hydrochloric acid and heat to eliminate the cross-reaction, and quantitated both serum MT-I and MT-II levels.

On the basis of our present results, we propose here that monitoring of serum MT and metal levels and subsequent MDA analysis are very useful in discriminating between hepatic diseases, providing important information for the evaluation of pathophysiological changes in chronic hepatic disorders.

REFERENCES

7) Febris C., Pirizi M., Soardo G., Falleti E., Pezzetta F., Vitulli D., J.