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# Detection of MAGE-4 Protein in the Sera of Patients with Hepatitis-C Virus-associated Hepatocellular Carcinoma and Liver Cirrhosis

Shin-ichi Tsuzurahara,[1] Michio Sata,[1,4] Osamu Iwamoto,[2] Shigeki Shichijo,[2] Masamichi Kojiro,[3] Kyuichi Tanikawa[1] and Kyogo Itoh[2]

*[1]Second Department of Medicine, [2]Department of Immunology and [3]First Department of Pathology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830*

**The aim of this study was to determine whether MAGE-4 protein is detectable in sera of patients with hepatocellular carcinoma and other liver diseases. An enzyme-linked immunosorbent assay was employed for detection of MAGE-4 protein in sera of liver disease patients, healthy men and women (control I) and those undergoing prostatic cancer screening (control II). MAGE-4 protein levels in sera of patients with hepatitis C virus-associated HCC (HCC-C) ($n=45$, mean=2.160 ng/ml) and HCV-associated cirrhosis (LC-C) ($n=55$, 1.072 ng/ml) were significantly higher ($P<0.0001$) than those of control I (0.327 ng/ml) or control II (0.394 ng/ml). MAGE-4 protein was positive in 21/45 (46.7%) HCC-C patients and 18/55 (32.7%) LC-C patients (cut-off, mean plus 2 SD in healthy controls) but in 0/12 (0%) hepatitis B virus-associated HCC (HCC-B) patients, 3/49 (6.1%) hepatitis B virus-associated LC (LC-B) patients, 4/47 (8.5%) alcoholic liver disease patients, and 1/49 (2.0%) controls. Serum MAGE-4 protein level may be useful as a marker for identification of LC-C patients suffering from HCC that is undetectable by presently available methods.**

Key words: MAGE-4 protein — Hepatitis C virus — Liver cirrhosis — Hepatocellular carcinoma

The *MAGE-1* gene is comprised of three exons spread over 4.5 kb, and is a member of a family of at least 12 closely related genes located on chromosome X.[1,2] Among them, the *MAGE-1* or *-3* gene codes for tumor antigens on HLA-A1 and -Cw1601 or -A1 and -A2 recognized by cytotoxic T lymphocytes, respectively.[2–4] The *MAGE-1*, *-2*, *-3*, *-4*, *-6* and *-12* genes are preferentially expressed at the mRNA level in many different cancers.[1–7] However, other than testicular cells, no normal cells express the *MAGE* genes.[5,6] We recently showed that MAGE-4 protein was detectable in sera of patients with MAGE-4 mRNA-positive head and neck squamous cell carcinomas by ELISA.[8] The present study addressed whether MAGE-4 protein is present in sera of patients with chronic liver disease.

## PATIENTS AND METHODS

**Sample collection** Peripheral blood was obtained from liver disease patients who underwent therapy at the Kurume University Hospital. Brief profiles are shown in Table I. The diagnosis of liver disease was based on histological findings. The histological gradings and stagings were matched among different etiologies to avoid the influence of histological differences. Biochemical and serological markers, serum HBV and HCV markers, histological findings of the liver, and/or the data from imaging techniques including ultrasonography, computed tomography, magnetic resonance, and angiography were also taken into consideration. In all HCC patients the MAGE-4 protein levels were measured in the pretreatment phase.

Serum HCV marker was measured using HCV antibody (PHA, Dinabot, Tokyo) and the RT-nested PCR method with the 5′ non-coding region as a primer. Serum HCV RNA was measured using the branched DNA signal amplification assay (Quantiplex, Chiron Corp., Emeryville, CA). HBsAg and HBcAb were measured by EIA (Mizuho Medi, Tosu).

Healthy volunteers with no history of viral hepatitis and with normal liver function, as judged from biochemical and serological markers, were the primary control group (control I). The mean age of this group was much younger than that of some of the patient groups (Table I). Furthermore, this group consisted of 21 males and 28 females, while some of the patient groups mainly consisted of males (Table I). So, we provided a second control group (control II) which consisted of 92 volunteers undergoing prostatic cancer screening in the Department of Urology in the Kurume University Hospital. They were also negative for each viral marker and had

[4] To whom correspondence should be addressed.

Abbreviations: ALD, alcoholic liver disease; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; -C, HCV-associated; -B, HBV-associated; ELISA, enzyme-linked immunosorbent assay; RT-nested PCR, reverse transcription-nested polymerase chain reaction; bDNA, branched DNA signal amplification assay; HBsAg, hepatitis B surface antigen; HBcAb, hepatitis B core antibody; EIA, enzyme-immuno assay; FBS, fetal bovine serum; mAb, monoclonal antibody; TBS, tris-buffered saline; PBS, phosphate-buffered saline.

Table I. Profile of Patients

| Diagnosis | No. | Age (mean±SD) | Sex (M/F) |
|---|---|---|---|
| CH-C | 70 | 51.2±11.8 | 34/36 |
| LC-C | 55 | 61.5±9.1 | 24/31 |
| HCC-C | 45 | 63.4±6.3 | 37/8 |
| CH-B | 49 | 38.9±10.2 | 33/16 |
| LC-B | 49 | 50.1±11.9 | 34/15 |
| HCC-B | 12 | 51.2±10.6 | 8/4 |
| ALD | 47 | 50.7±10.1 | 46/1 |
| Control I | 49 | 43.3±13.7 | 21/28 |
| Control II | 92 | 66.4±5.8 | 92/0 |
| Total | 468 | 54.3±12.8 | 329/139 |

No, number of patients or donors; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALD, alcoholic liver disease; C, HCV (+); B, HBV (+).

normal results of liver function tests. The study protocol was approved by the ethics committee of our hospital, and the volunteers received a full explanation of the purpose of this study and gave their written informed consent. Sera of umbilical cord blood were obtained from five newborns for measurement of MAGE-4 protein. All sera were cryopreserved until use. The stage of HCC was judged in accordance with Union Internationale Contre le Cancer criteria.[9]

**Detection of soluble MAGE-4 protein** Detection of MAGE-4 protein in human sera or culture supernatants of cell lines was carried out by a sandwich ELISA using a mouse mAb (IgG1, R5 mAb) and a biotinylated polyclonal rabbit affinity-purified IgG antibody against MAGE-4 protein as reported.[8] Briefly, 10 $\mu$g/ml R5 mAb solution (100 $\mu$l per well) was dispensed into a 96-well plate and incubated overnight at 4°C. The plate was washed twice in TBS (100 m*M* Tris, 0.9% NaCl and 0.1% Tween 20, pH 7.5), and 150 $\mu$l of 25% Block Ace (Dai-Nippon Seiyaku, Osaka) in PBS was added to each well to block the remaining protein-binding sites. The plate-bound antibodies were then reacted with antigens in 40 $\mu$l of serum and 100 $\mu$l of cell lysates. Following a 3 h incubation, the samples were removed and the plate was washed six times with 300 $\mu$l of 0.1% Tween 20 in PBS and incubated for 1 h with 100 $\mu$l of biotinylated rabbit antibody (5 $\mu$g/ml). After removal of the antibody, the plate was washed six times with 0.1% Tween 20 in TBS, then incubated with 1000-fold-diluted avidin-conjugated peroxidase (Sigma, St. Louis, MO) for 20 min at room temperature. Detection was accomplished using 100 $\mu$l of 1.0 mg/ml solution of *o*-phenylene diamine in 0.1 *M* citrate buffer (pH 4.0) with 0.015% $H_2O_2$. After 10 min of incubation in the dark, the reaction was stopped by the addition of 25 $\mu$l of 4 *M* $H_2SO_4$ and the absorbance at 492 nm was measured. Recombinant MAGE-4 was used as the standard to obtain a calibration curve. The concentrations of MAGE-4 protein are shown as ng/ml. A sample was considered as positive when the MAGE-4 protein level was higher than 1.04 ng/ml (the mean plus 2SD in healthy controls).

**Statistics** Statistical analyses were performed using the Mann-Whitney U test, the $\chi^2$ test, and the Spearman rank correlation coefficient. A $P$ value of less than 0.05 was considered as statistically significant.

## RESULTS

MAGE-4 protein levels were measured in the sera of 468 samples (463 from peripheral blood and 5 from umbilical cord blood) (Table II). Serum levels of MAGE-4 protein in patients with HCV-associated HCC (HCC-C) ($n=45$, mean±SD=2.160±2.682 ng/ml) were significantly higher than those in controls I and II, and in patients with ALD, CH-B, CH-C, and LC-B (at least $P<0.005$), but not the other groups. MAGE-4 protein levels of LC-C patients ($n=55$, 1. 072±1.096 ng/ml) were significantly higher than in controls I and II, and in patients with ALD, CH-B, CH-C, and LC-B (at least $P<0.05$), but not the other groups. CH-C patients ($n=70$, 0.755±1.125 ng/ml) had significantly higher levels than in controls I and II, and in patients with ALD, CH-B, and LC-B (at least $P<0.05$), but not the other groups. The value of MAGE-4 protein in each of these patients with HCV infection is shown in Fig. 1. There was no difference in MAGE-4 protein levels among any of the other groups (Table II).

Sera from 21 of 45 of HCC-C patients (46.7%, $P<0.0001$ vs. controls I and II) or 18 of 55 of LC-C patients (32.7%, $P<0.0001$ vs. controls I and II) were positive for MAGE-4 protein when levels were above the cut-off value of 1.04 ng/ml (mean plus 2 SD in healthy controls) (Table II). MAGE-4 protein was also positive in 10 of 70 CH-C patients (14%, $P=0.03$ vs. control I, $P=0.05$ vs. control II). However, MAGE-4 protein was positive in none of 12 HCC-B, 3 of 49 LC-B, 6 of 49 CH-B, 4 of 47 patients with ALD, 1 of 49 of control I and 4 of 92 of control II.

Forty-five HCC-C patients were divided according to clinical stage, based on the Union Internationale Contre le Cancer criteria. The mean level of serum MAGE-4 protein was 2.229 ng/ml in stage I patients ($n=7$), 2.595 ng/ml in stage II ($n=17$), 1.693 ng/ml in stage III ($n=4$), and 1.806 ng/ml in stage IV ($n=17$). There were no significant differences among these groups. The serum protein level of all five umbilical cord blood samples was below the detectable level (1.04 ng/ml).

MAGE-4 protein level did not correlate with asparate aminotransferase, alanine aminotransferase, or HCV RNA level (data not shown).

Table II. Expression Rate and Levels of MAGE-4 Protein

| Diagnosis | MAGE-4 Protein | | | | | |
|---|---|---|---|---|---|---|
| | Level (ng/ml) | *P* value vs.[a] | | Expression rate | *P* value vs.[b] | |
| | | Control I | Control II | | Control I | Control II |
| CH-C | 0.755±1.125 | 0.0005 | 0.0013 | 10/70 (14.3%) | 0.0258 | 0.0488 |
| LC-C | 1.072±1.096 | <0.0001 | <0.0001 | 18/55 (32.7%) | <0.0001 | <0.0001 |
| HCC-C | 2.160±2.682 | <0.0001 | <0.0001 | 21/45 (46.7%) | <0.0001 | <0.0001 |
| CH-B | 0.415±0.560 | NS | NS | 6/49 (12.2%) | NS | NS |
| LC-B | 0.339±0.392 | NS | NS | 3/49 (6.1%) | NS | NS |
| HCC-B | 0.325±0.681 | NS | NS | 0/12 (0%) | NS | NS |
| ALD | 0.423±0.441 | NS | NS | 4/47 (8.5%) | NS | NS |
| Control I | 0.327±0.238 | — | NS | 1/49 (2%) | — | NS |
| Control II | 0.394±0.332 | NS | — | 4/92 (4.3%) | NS | — |
| Total | 0.708±1.235 | | | 67/468 (14.3%) | | |

Expression rate was calculated as number of patients who were positive at the cut-off value of 1.04 ng/ml per total number of patients. CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALD, alcoholic liver disease; C, HCV (+); B, HBV(+).
*a*) Difference vs. control I and II (Mann-Whitney U test).
*b*) Difference vs. control I and II ($\chi^2$ test).

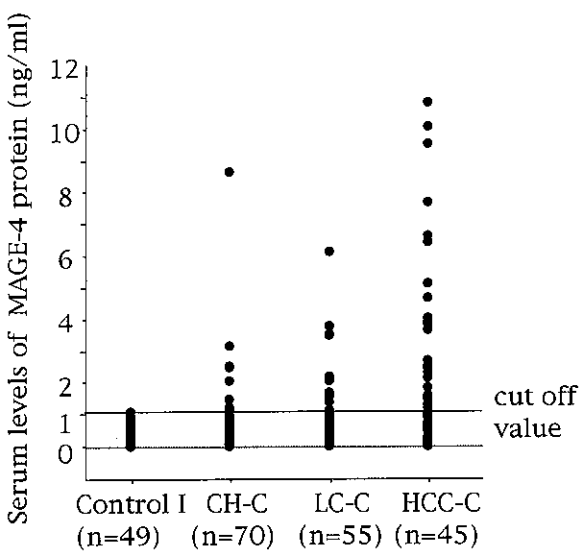


Fig. 1. MAGE-4 protein levels in sera of patients with HCV infection. The levels of serum MAGE-4 protein are shown separately for each liver disease diagnosis. The cut-off value of 1.04 ng/ml is the mean plus two SD in healthy controls.

## DISCUSSION

This study found that the serum level of MAGE-4 protein in HCC-C patients was significantly higher than in the controls, or in patients with ALD, CH-B, CH-C, or LC-B. Similarly, MAGE-4 protein levels of sera from patients with MAGE-4 mRNA-positive head and neck squamous cell carcinomas[8, 10] and lung cancers (Shichijo *et al.*, unpublished results) were significantly higher than those of patients with benign diseases or healthy controls. MAGE-4 mRNA and proteins are expressed in many cancers, including head and neck squamous cell carcinoma,[6] ovarian carcinoma,[11] esophageal carcinoma,[12] and HCC (Nakao *et al.*, unpublished results). However, except for testis and placenta, no normal tissues, including liver, express MAGE-4 mRNA or protein. These findings suggest HCC cells produces MAGE-4 protein in a significant number of HCC patients.

A higher level of MAGE-4 protein was also detected in LC patients with chronic HCV infection. These patients are a high-risk group for HCC.[13–15] Furthermore, CH-C patients had higher levels than controls. But those with HBV-associated liver diseases or ALD did not differ from the controls. Thus, both the mean level of serum MAGE-4 protein and its positive rate in patients with HCV infection, but not HBV infection, increased with disease progression. There are several possible explanations for these phenomena, although the mechanisms remain unclear. First, cancer cells producing MAGE-4 protein may already exist in some CH-C or LC-C patients in whom the cancer mass is too small for diagnosis by presently available methods. Second, MAGE-4 protein may be produced by progression of liver disease or may be associated with hepatic fibrosis. However, this hypothesis is not fully supported by the evidence available, because the serum levels of MAGE-4 protein in LC-B and ALD patients were lower than in those with HCV-associated liver diseases. Third, liver cells infected with HCV, but not HBV, may produce MAGE-4 protein. Alternatively,

HCV infection, causing the constitutive destruction of liver cells, may induce re-proliferation of liver cells. Proliferating hepatocytes may produce MAGE-4 protein. This hypothesis is supported in part by the observation that *MAGE-1* gene is transiently expressed in the basal layer of the skin during wound healing.[16] However, MAGE-4 protein levels in umbilical cord blood were low, which suggests that proliferation of hepatocytes in infants is unrelated to the production of MAGE-4 protein.

HCC is increasing in Japan, and its prevention and therapy at an early stage are very important. Since MAGE-4 protein was detectable in sera of a significant number of HCV-associated HCC and LC patients, its measurement could facilitate detection of HCCs that are undetectable by other presently available methods.

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