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Elevation of Serum MAGE-4 Protein Levels and Prediction of Hepatocellular Carcinogenesis in Patients with Liver Cirrhosis

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Early detection of hepatocellular carcinoma (HCC) is clinically important because advanced HCC limits treatment modalities for the cancer. We have previously reported that serum levels of MAGE-4 protein are strongly associated with the development of HCC. The present study was designed to determine whether elevated serum MAGE-4 protein levels can predict hepatocellular carcinogenesis in patients with liver cirrhosis before clinical diagnosis. Among 62 cirrhotic patients, 28 patients were diagnosed with HCC during the follow-up period. The levels of MAGE-4 protein and α-fetoprotein (AFP) were significantly elevated in cirrhotic patients with HCC. Univariate and multivariate analyses suggest that elevated serum MAGE-4 protein is more significant than AFP. Importantly, retrospective analysis of prefrozen sera of cirrhotic patients revealed a transient or continuous elevation of serum MAGE-4 protein levels in 14 of 28 cirrhotic patients with HCC (50%) before clinical diagnosis. In contrast, elevated serum MAGE-4 protein levels were observed in 3 of 33 cirrhotic patients without HCC (9%), and in 2 of 34 hepatitic patients (6%). These results indicate that elevated serum MAGE-4 protein levels can be a predictive marker of hepatocellular carcinogenesis in cirrhotic patients, thereby enabling us to treat patients at an earlier stage.

Key words: MAGE-4 — hepatocellular carcinoma — liver cirrhosis — marker

Hepatocellular carcinoma (HCC) is usually caused by chronic inflammation of hepatocytes infected with hepatitis viruses.[1–3] Because HCC at an advanced stage with intrahepatic metastasis and/or vascular invasion is a serious graveyard disease, earlier detection of the development of HCC is clinically important. Although there are several markers of HCC, including α-fetoprotein (AFP)[4, 5] and aspartate aminotransferase (ALT),[6, 7] additional markers would be useful in managing high-risk groups, including those with chronic liver disease.

The MAGE gene family consists of at least 12 closely related genes located on the long arm of chromosome X.[8, 9] The MAGE-1, -2, -3, -4, -6, and -12 genes are preferentially expressed at the mRNA level in many different cancers.[8–13] Normal cells, except for testicular cells, do not express the MAGE genes.[10, 11] We have previously reported that the MAGE-4 protein is detectable in patients with several types of cancers, including head and neck cancer,[14] lung cancer,[15] and ovarian cancer.[16] The protein is also detectable in certain liver cirrhosis (LC) patients with hepatitis C virus (HCV) infection,[17] suggesting that detection of the MAGE-4 protein in sera of LC patients could also be useful for early prediction of the development of HCC. In the present study, we compared MAGE-4 with AFP as a marker for HCC, and tested whether elevated serum MAGE-4 protein levels can be used to predict hepatocellular carcinogenesis in LC patients before clinical diagnosis.

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MATERIALS AND METHODS

Patients and sample collection Sixty-two LC patients and 34 chronic hepatitis (CH) patients were enrolled in this study, and their profiles are shown in Table I. All patients were followed at either the Social Health Saga Hospital or the Kurume University Hospital. Sera of these patients was obtained from 1990 to 1999, and was frozen at −20°C until use. Diagnosis of HCC, LC, and CH was based on histological findings, ultrasonography, and computed tomography. Serum HCV markers were measured by enzyme-linked immunosorbent assay (ELISA) using anti-HCV antibody (PHA, Dinabot, Tokyo). Hepatitis B surface antigen (HBs-Ag) and HBc Ab were measured by enzyme-immunoassay (Misuho Medi, Tosu). Serum AFP levels were measured by radioimmunoassay (Dinabot).

Detection of the MAGE-4 protein by ELISA Detection of the MAGE-4 protein in human sera was carried out by an ELISA using a mouse monoclonal antibody (mAb) (immunoglobulin (Ig) G1, R5) and a polyclonal rabbit affinity-purified IgG antibody against MAGE-4 protein, as previously reported.[11] In brief, the plate, which was coated

Table I. Patient Profiles

Factor	LC with HCC	LC without HCC	CH
Number of patients	28	34	34
Age at entry	63.1±5.4	59.3±8.6	55.2±7.9
Gender (male/female)	18/10	19/15	19/15
HBs-Ag (+)	7	7	9
HCV-Ab (+)	22	25	26
HBs-Ag (–), HCV-Ab (–)	1	3	1

Table II. Comparison between HCC and LC Patients

Factor	LC with HCC	LC without HCC	*P* value
Follow-up period (day)	1176±444	1909±458	<0.0001[a)]
MAGE-4 (ng/ml)	1.47±1.89	0.32±0.48	0.0002[b)]
AFP (ng/ml)	47.3±43.6	40.2±53.9	0.0161[b)]

a) Fisher's exact test.
b) Mann-Whitney *U*-test.

with 10 μg/ml R5 mAb, was washed twice in TBS (100 m*M* Tris, 0.9% NaCl and 0.1% Tween 20, pH 7.5), and treated with 150 μl of 25% Block Ace (Dai-Nippon Seiyaku, Osaka) in phosphate-buffered saline (PBS). The plate-bound antibodies were then reacted with antigens in 40 μl of serum. Following a 3-h incubation, the plate was washed six times with 300 μl of 0.1% Tween 20 in PBS and incubated for 1 h with 100 μl of biotinylated rabbit antibody. Thereafter, the plate was washed, then incubated with 1000-fold-diluted avidin-conjugated peroxidase (Sigma, St. Louis, MO) for 20 min at room temperature. Detection was accomplished using 100 μl of 1.0 mg/ml solution of *o*-phenylenediamine in 0.1 *M* citrate buffer (pH 4.0) with 0.015% H_2O_2. After incubation for 10 min in the dark, the reaction was stopped by the addition of 25 μl of 4 *M* H_2SO_4 and the absorbance at 492 nm was measured. Recombinant MAGE-4 protein was used as a standard to obtain a calibration curve. A sample was considered positive when MAGE-4 protein levels were higher than 1.15 ng/ml (the mean±3SD of sera from health donors).

Statistical analysis The Mann-Whitney *U*-test was used for analysis of serum MAGE-4 levels of patients with chronic liver disease. Univariate analysis of risk factors was performed by the Kruskal-Wallis test, in which age was classified as under or over 65 years, serum ALT levels were classified as under or over 80 IU/liter, and serum AFP levels were classified as under or over 20 ng/ml. Multivariate analysis was performed by the logistic regression model. A *P* value of less than 0.05 was considered statistically significant.

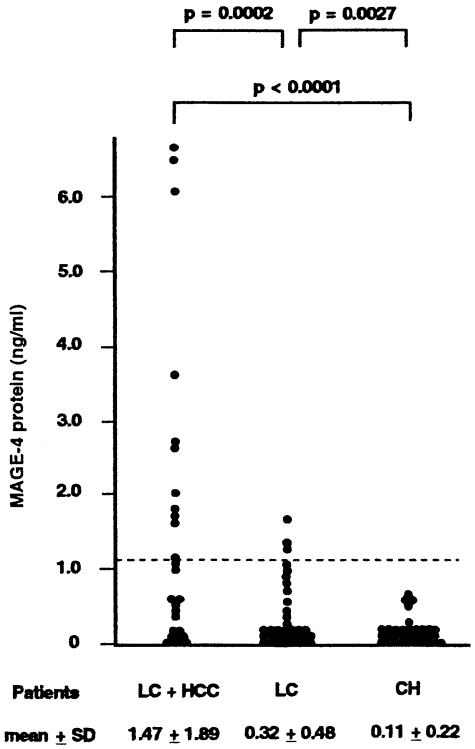

Fig. 1. MAGE-4 protein levels in sera of patients with HCC, LC, and CH. The levels of serum MAGE-4 protein are shown separately for each liver disease. The cut-off value of 1.15 ng/ml is the mean plus three SD in healthy controls, and is shown as a dotted line.

RESULTS

Ninety-six patients with chronic liver disease were followed from 1990 to 1999. During this period, 28 of 62 LC patients (45%) were diagnosed as having developed HCC, while the other 34 CH patients did not develop the disease. These patients were classified into three groups: 28 LC patients with HCC, 34 LC patients without HCC, and 34 CH patients (Table I). There was no statistical difference in age, gender, or percentage of HBs-Ag positive or HCV-Ab positive patients when these patients were enrolled in the study.

Because AFP is regarded as a marker for the development of HCC,[4, 5)] we next compared the AFP and MAGE-4 levels of LC patients with or without HCC (Table II). The follow-up period of the LC patients without HCC was significantly longer than that of the LC patients with HCC. The serum levels of MAGE-4 and AFP for the LC patients with HCC were significantly higher than those for the LC patients without HCC (*P*=0.0002 and 0.0161, respectively). Fig. 1 shows the MAGE-4 protein levels of the LC

patients with or without HCC, as well as those of CH patients. Among 28 LC patients with HCC, 12 patients (43%) showed significantly high serum MAGE-4 protein levels. Among 34 LC patients without HCC, 3 (9%) showed high serum MAGE-4 protein levels. No increase in serum MAGE-4 protein levels was detected in the 34 CH patients.

We next carried out univariate analysis to evaluate the usefulness of the measurement of ALT, AFP, and MAGE-4 in patients with or without HCC in predicting hepatocellular carcinogenesis (Table III). There was no difference in ALT levels between the two groups. With regard to AFP, high AFP levels were detected in 16 of 28 LC patients with HCC (57%) and 9 of the 34 LC patients without HCC (26%) (P=0.02). In contrast, 12 of 28 LC patients with HCC (43%) showed high MAGE-4 protein levels, and a significant increase in MAGE-4 protein levels was observed only in 2 of 34 LC patients (6%) without HCC (P=0.01). In addition, multivariate analysis using the logistic regression model was carried out to estimate the usefulness of the measurement of MAGE-4 (Table IV). It was found that MAGE-4 protein levels are more significant than AFP levels. These results indicate that serum MAGE-4 protein levels could be a useful marker of HCC, even in comparison with AFP.

Furthermore, to determine whether measurement of serum MAGE-4 protein levels could be of use in predicting the development of hepatocellular carcinogenesis in cirrhotic patients, the kinetics of serum MAGE-4 protein in LC and CH patients were examined retrospectively (Fig. 2). A continuous or transient increase in serum MAGE-4 protein levels was detected in 14 of the 28 LC patients who were subsequently diagnosed with HCC (50%) during the follow-up period (Fig. 2A). Interestingly, tumor-reduction therapies, including percutaneous ethanol injection and transcatheter arterial embolization, resulted in a dramatic decrease in those protein levels in all six patients. In contrast, MAGE-4 levels increased up to a cut-off level only in 3 of 34 LC patients without HCC (Fig. 2B) and in 2 of 34 CH patients (Fig. 2C). Among the LC patients with HCC showing increased serum MAGE-4 levels, this increase was detected in most patients as long as 1 or 2 years before clinical diagnosis. These results suggest that increased serum MAGE-4 protein levels in LC patients can efficiently predict development of hepatocellular carcinogenesis.

able III. Results of Univariate Analysis

Factor		LC with HCC	LC without HCC	P value
Gender	male	18	19	0.6
	female	10	15	
Age	<65	14	24	0.11
	≥65	14	10	
ALT (IU/liter)	<80	17	26	0.2
	≥80	11	8	
AFP (ng/ml)	<20	12	25	0.02
	≥20	16	9	
MAGE-4 (ng/ml)	<1.15	16	32	0.01
	≥1.15	12	2	

Table IV. Results of Multivariate Analysis

Factor		ODDS ratio	95%CI	P value
AFP (ng/ml)	<20	1	0.9–10.9	0.06
	≥20	3.2		
MAGE-4 (ng/ml)	<1.15	1	2.8–75.9	0.002
	≥1.15	14.6		

DISCUSSION

MAGE genes are preferentially expressed in many different cancers. Other than testicular cells, however, no normal cells express the MAGE genes. We have recently shown that MAGE-4 protein is detectable in sera of patients with head-and-neck squamous-cell carcinoma,[14] with lung cancer,[15] and with ovarian cancer.[16] In addition, we have also found that elevated serum MAGE-4 protein levels are detectable in patients with HCV-related HCC.[17] Based on these findings, we tested the possibility that elevated serum MAGE-4 protein levels can predict development of hepatocellular carcinogenesis in cirrhotic patients before clinical diagnosis.

We first compared the usefulness of MAGE-4 and AFP as tumor markers. Although elevated serum AFP levels have been routinely applied as a parameter for HCC diagnosis, AFP transcripts are also detected in normal liver cells, in liver cirrhosis, and in liver infectious diseases such as HBV/HCV.[18] Indeed, fluctuating AFP levels in cirrhotic patients and the low specificity of AFP to HCC patients have been documented.[19] As shown in the present study, both AFP and MAGE-4 levels were significantly elevated in LC patients with HCC compared with those without HCC. However, MAGE-4 elevations were more significant than those of AFP in both univariate and multivariate analyses. These lines of evidence indicate that the serum MAGE-4 protein level could be a useful tumor marker to predict HCC, and that accurate diagnosis would be possible by combined measurement of serum MAGE-4 and AFP levels. However, a protein induced by vitamin K absence or antagonist-II (PIVKA-II) and AFP-L_3 were also reported as good indicators of HCC.[20, 21] We are planning to compare the usefulness of PIVKA-II, AFP-L_3, and MAGE-4 as indicators of HCC.

We next tested the possibility that elevated serum MAGE-4 protein levels can predict hepatocellular carcino-

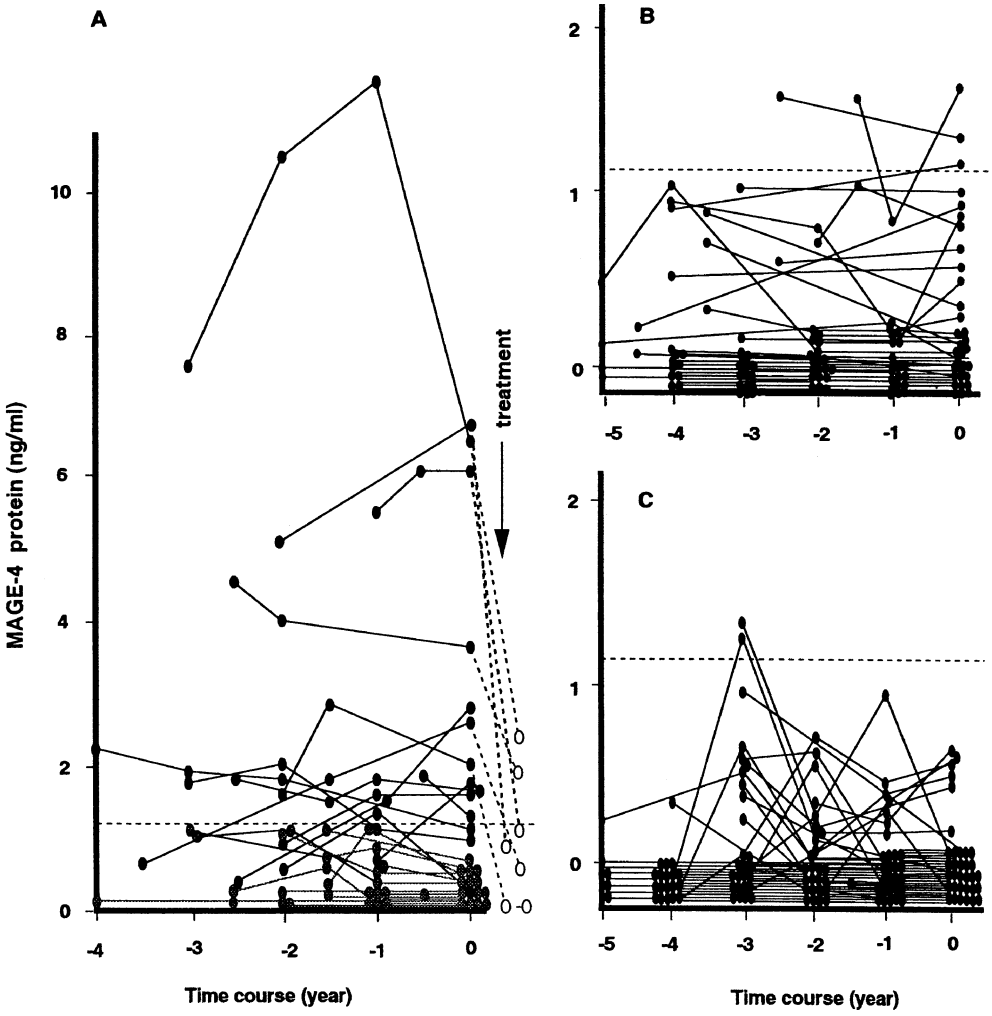

Fig. 2. Kinetic analysis of MAGE-4 protein levels in sera of patients with HCC, LC, and CH. The serum MAGE-4 protein levels of patients with HCC (A), LC (B), and CH (C) are shown. The kinetics of each patient are represented by a line. The cut-off value of 1.15 ng/ml is the mean plus three SD in healthy controls, and is shown as a dotted line. The gray symbols represent the LC patients who showed no increase in MAGE-4 protein levels during the follow-up period. The open symbols represent the MAGE-4 protein levels after tumor-reduction therapies. Concerning the HCC patients, the time of clinical diagnosis is shown by the time course (year) "0" and their prefrozen sera were analyzed retrospectively.

genesis in cirrhotic patients before clinical diagnosis by retrospectively analyzing prefrozen sera of cirrhotic patients. As shown in Fig. 2, a continuous or transient increase in serum MAGE-4 protein levels was detected in 50% of the LC patients with HCC. MAGE-4 levels increased in 9% and 6% of LC patients without HCC and in CH patients, respectively. It is of note that tumor-reduction therapies, including percutaneous ethanol injection and transcatheter arterial embolization, resulted in a dramatic decrease in MAGE-4 protein levels in HCC patients. A similar result was observed in the patients with head-and-neck squamous-cell carcinomas.[14] Although we have not calculated the tumor volume of HCC, we speculate that the tumor size and the serum MAGE-4 protein level could be correlated. These results suggest that measurement of serum MAGE-4 levels could provide useful information for management of cirrhotic patients.

In addressing why elevated serum MAGE-4 protein levels are detectable even a couple of years before clinical diagnosis, several explanations can be proposed. First, HCC producing MAGE-4 protein may already exist in patients whose tumor mass is too small for diagnosis by presently available methods. Second, HCV infection, which causes constitutive destruction of liver cells, may

induce reproliferation of liver cells, and proliferating hepatocytes may produce MAGE-4 protein. This possibility is supported in part by the observation that the MAGE-4 gene is transiently expressed in the basal layer of the skin during would healing.[22] Further study, however, is needed to elucidate the precise mechanism.

In conclusion, we have demonstrated that measurement of serum MAGE-4 protein levels in LC patients is useful not only as a tumor marker, but also as a predictive marker of HCC. Measurement of serum MAGE-4 protein in high-risk groups, including LC patients, could facilitate prediction of HCC at an early stage when presently available methods are unable to detect HCC.

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REFERENCES

1) Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M. and Ohta, Y. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, **87**, 6547–6549 (1990).
2) Nishioka, K., Watanabe, J., Furuta, S., Tanaka, E., Iino, S., Suzuki, H., Tsuji, T., Yano, M., Kuo, G. and Choo, Q. L. A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan. *Cancer*, **67**, 429–433 (1991).
3) Tsukuma, H., Hiyama, T., Tanaka, S., Nakao, M., Yabuuchi, T., Kitamura, T., Nakanishi, K., Fujimoto, I., Inoue, A. and Yamazaki, H. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N. Engl. J. Med.*, **328**, 1797–1801 (1993).
4) Oka, H., Tamori, A., Kuroki, T., Kobayashi, K. and Yamamoto, S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology*, **19**, 61–66 (1994).
5) Kuromatsu, R., Tanaka, M. and Tanikawa, K. Serum alpha-fetoprotein and *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein in patients with hepatocellular carcinoma. *Liver*, **13**, 177–182 (1993).
6) Tarao, K., Takemiya, S., Tamai, S., Sugimasa, Y., Ohkawa, S., Akaike, M., Tanabe, H., Shimizu, A., Yoshida, M. and Kakita, A. Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in the hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. *Cancer*, **79**, 688–694 (1997).
7) Tarao, K., Rino, Y., Ohkawa, S., Shimizu, A., Tamai, S., Miyakawa, K., Aoki, H., Imada, T., Shindo, K., Okamoto, N. and Totsuka, S. Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatic C virus-associated cirrhosis. *Cancer*, **86**, 589–595 (1999).
8) van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van der Eynde, B., Knuth, A. and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **254**, 1643–1647 (1991).
9) De Plaen, E., Arden, K., Traversari, C., Gaforio, J. J., Szikora, J. P., De Smet, C., Brasseur, F., van der Bruggen, P., Lethe, B., Hurquin, C., Brasseur, R., Chomez, P., De Backer, O., Cavenee, W. and Boon, T. Structure, chromosomal localization, and expression of 12 genes of the *MAGE* family. *Immunogenetics*, **49**, 360–369 (1994).
10) Weynants, P., Lethe, B., Brasseur, F., Marchand, M. and Boon, T. Expression of MAGE genes by non-small-cell lung carcinomas. *Int. J. Cancer*, **56**, 826–829 (1994).
11) Shichijo, S., Tsunosue, R., Kubo, K., Kuramoto, T., Tanaka, Y., Kayashi, A. and Itoh, K. Establishment of an enzyme-linked immunosorbent assay (ELISA) for measuring cellular MAGE-4 protein on human cancers. *J. Immunol. Methods*, **186**, 137–149 (1995).
12) Takahashi, K., Shichijo, S., Noguchi, M., Hirohata, M. and Itoh, K. Identification of MAGE-1 and MAGE-5 proteins in spermatogonia and primary spermatocytes of testis. *Cancer Res.*, **55**, 3478–3482 (1995).
13) Toh, Y., Yamana, H., Shichijo, S., Fujita, H., Tou, U., Sakaguchi, M., Kakegawa, T. and Itoh, K. Expression of MAGE-1 gene by esophageal carcinomas. *Jpn. J. Cancer Res.*, **86**, 714–717 (1995).
14) Iwamoto, O., Nagao, Y., Shichijo, S., Eura, M., Kameyama, T. and Itoh, K. Detection of MAGE-4 protein in sera of patients with head and neck squamous cell carcinoma. *Int. J. Cancer*, **70**, 287–290 (1997).
15) Shichijo, S., Hoshino, T., Koufuji, K., Hayashi, A., Kawamoto, M., Kikuchi, M., Higuchi, T., Ichiki, M., Oizumi, K. and Itoh, K. Detection of MAGE-4 protein in sera of lung cancer patients. *Jpn. J. Cancer Res.*, **88**, 414–419 (1997).
16) Kawagoe, H., Yamada, A., Matsumoto, H., Ito, M., Ushijima, K., Nishida, T., Yakushiji, M. and Itoh, K. Serum MAGE-4 protein in ovarian cancer patients. *Gynecol. Oncol.*, **76**, 336–339 (2000).
17) Tsuzurahara, S., Sata, M., Iwamoto, O., Shichijo, S., Kojiro, M., Tanikawa, K. and Itoh, K. Detection of MAGE-4 protein in the sera of patients with hepatitis-C virus-associated hepatocellular carcinoma and liver cirrhosis. *Jpn. J. Cancer Res.*, **88**, 915–918 (1997).

18) Jiang, S. Y., Shyu, R. Y., Huang, M. F., Tang, H. S., Young, T. H., Roffler, S. R., Chiou, Y. S. and Yeh, M. Y. Detection of alphafetoprotein-expressing cells in the blood of patients with hepatoma and hepatitis. *Br. J. Cancer*, **75**, 928–933 (1997).
19) Yamashita, F., Tanaka, M., Satomura, S. and Tanikawa, K. Prognostic significance of *Lens culinaris* agglutinin A-reactive alpha-fetoprotein in small hepatocellular carcinoma. *Gastroenterology*, **111**, 996–1001 (1996).
20) Fujiyama, S., Morishita, T., Sagara, K., Sato, T., Motohara, K. and Matsuda, I. Clinical elevation of plasma abnormal prothrombin (PIVKA-II) in patients with hepatocellular carcinoma. *Hepatogastroenterology*, **33**, 201–205 (1986).
21) Taketa, K., Endo, Y., Sekiya, C., Tanikawa, K., Koji, T., Taga, H., Satomura, S., Matsuura, S., Kawai, T. and Hirai, H. A collaborative study for the evaluation of lectin-reactive alpha-fetoproteins in early detection of hepatocellular carcinoma. *Cancer Res.*, **53**, 5419–5423 (1993).
22) Becker, J. C., Gillitzer, R. and Brocker, E. B. A number of the melanoma antigen-encoding gene (MAGE) family is expressed in human skin during wound healing. *Int. J. Cancer*, **58**, 346–348 (1994).