Relationship between *Lens Culinaris* Agglutinin Reactive α-Fetoprotein and Biological Features of Hepatocellular Carcinoma

TAKU KUSABA

The Second Department of Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan

Summary: *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3), which is a fucosylated variation of AFP, is not only sensitive and specific for localization of hepatocellular carcinoma (HCC) but also a prognostic factor for patients with HCC. The relationship between status of AFP-L3% in serum and pathological findings was studied using 48 resected HCC specimens. AFP-L3 fraction was measured by lectin-affinity blotting using an AFP Differentiation Kit L (Wako, Osaka, Japan), and was expressed as AFP-L3% (AFP-L3 /total AFP × 100%). A cut-off level of 15% was used. Pathological findings of HCC such as histological grade (well, moderately and poorly differentiated HCC), vascular invasion, and Ki67 (MIB1), p53 (DO7) and α-catenin immunohistochemical staining were studied. According to the results of serum AFP concentrations and AFP-L3%, the 48 patients were divided into the following three groups: AFP greater than or equal to 20 ng/ml and AFP-L3 positive (group A, n=14), AFP greater than or equal to 20 ng/ml and AFP-L3 negative (group B, n=14) and AFP less than 20 ng/ml (group C, n=20). Ki67 labeling index of HCC tissue in group A was 27.8±18.9%, which was significantly higher than those of group B (9.6±10.1%, p<0.024) and group C (11.1±11.2%, p<0.03). In group A, p53 expression was higher and α-catenin staining was reduced significantly compared with those of group B or C, respectively. The results of the study suggest that the proportion of AFP-L3% in serum reflects some biological features of HCC.

Key words hepatocellular carcinoma, AFP-L3, Ki67, p53, α-catenin

INTRODUCTION

α-Fetoprotein (AFP), which is a tumor marker of hepatocellular carcinoma (HCC), has a microheterogeneity due to structural variations in its sugar chain [1]. AFP-L3, an isoform of AFP, is reactive with *Lens culinaris* agglutinin and is known to be a highly specific and sensitive tumor marker of HCC [2,3]. Taketa et al. [3] and Kuromatsu et al. [4] reported that the proportion of AFP-L3 was significantly elevated in patients with moderately or poorly differentiated HCC as compared with well-differentiated HCC. Recently, Yamashita et al. [5] have reported that the proportion of AFP-L3 is closely correlated with the clinical aggressiveness of HCC, and that a determination of AFP-L3 levels in serum of patients with HCC could be used as a prognostic indicator.

Pathological findings such as vascular invasion and intrahepatic metastasis have been evaluated as factors in the progression and recurrence of HCC [6,7]. These major prognostic factors are, however, sometimes difficult to distinguish before pathological examination. To determine the therapeutic approach to HCC, evaluation of parameters reflecting aspects of the biological behavior of HCC such as proliferative activity [8,9], probability of vessel invasion [10] and genetic alterations [11,12] has become necessary.

In this study, AFP-L3 levels and those patho-
logical factors that can be evaluated by immunohistochemical staining of antigens such as Ki67 [8], p53 [11] and α-catenin [10] in formalin-fixed paraffin-embedded tumor tissue were examined to clarify the relationship between AFP-L3 and aggressiveness of HCC cells.

PATIENTS AND METHODS

Patients
Forty-eight patients with 48 HCC nodules (tumor size; 19-95 mm, mean ± SD; 43.6± 8.6 mm) who were referred to the Department of Surgery of Kurume University Hospital from February 25, 1993 to June 31, 1997 were enrolled in this study. Patients who had previously received physical or surgical therapy were excluded from the study. As shown in Table 1, the patients were comprised of 33 men and 15 women, aged 36 to 76 (mean ± SD; 61.8±6.4). In terms of the virus markers, 7 patients were HBs antigen positive and HCV antibody (second-generation antibody) negative, 37 patients were HBs antigen negative and HCV antibody positive, 2 patients were positive for both, and 2 patients were negative for both. According to Child-Pugh classification, 44 patients were Child A and 4 patients were Child B (Table 1).

Measurement of total AFP concentrations and AFP-L3%
We used serum samples of patients within 1 month before operation. Total AFP concentrations in serum samples were determined by radio-immunoassay. Among the samples with greater than or equal to 20 ng/ml of total AFP concentrations, the proportion of AFP-L3 was determined using the AFP Differentiation Kit L (Wako Pure Chemical Industries Ltd, Osaka, Japan). The proportion of AFP-L3 was calculated as (AFP-L3 Concentration/ Total AFP Concentration)×100 (%). A cut-off level of 15% was used on the basis of a previous study [2]. A positive AFP-L3 level was defined as an AFP-L3 level greater than the cut-off level.

Histological examination
All of the resected specimens were cut into serial slices 5 to 10 mm thick and fixed with 10% formalin. After macroscopic examination, the slice with the greatest dimensions and other slices containing areas of possible metastasis or vascular invasion were trimmed for paraffin blocks and cut into 4 μm microscopic sections which were then stained with hematoxylin and eosin. The histologic grades of tumor differentiation were assigned as well-differentiated HCC, moderately differentiated HCC and poorly differentiated HCC according to the general rules for the clinical and pathological study of primary liver cancer [13]. For tumors showing histologic diversity, the less differentiated histologic grade was taken into consideration when assigning a grade. The samples were then examined for microscopic vascular invasion(s) and intrahepatic metastasis and were subjected to immunohistochemical study.

Immunohistochemistry of Ki67, p53 and α-catenin
For immunohistochemical demonstration of Ki67 [9], p53 [11] and α-catenin [10], the antibodies used were (1) rabbit polyclonal antibody MIB-1 (IgG1, DAKO, Denmark) that recognizes native Ki67 antigen and recombinant fragments of the Ki67 molecule, (2) mouse monoclonal antibody DO7 (IgG2b, Novocastra Laboratories, Newcastle, UK)

<table>
<thead>
<tr>
<th></th>
<th>Number of Patients</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AFP-L3 ≥ 15%</td>
<td>AFP-L3 &lt; 15%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
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<td>62.8 ± 8.8</td>
<td>64.5 ± 7.2</td>
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<td>12</td>
<td>17</td>
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<td>3/11</td>
<td>12/8</td>
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<tr>
<td>Child-Pugh grade</td>
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<td>13/1/0</td>
<td>19/1/0</td>
<td>n.s.</td>
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<tr>
<td>Tumor size (mm, mean ± SD)</td>
<td>49.5 ± 22.2</td>
<td>41.4 ± 17.4</td>
<td>40.9 ± 19.1</td>
<td></td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Fig. 1. Immunohistochemical staining of MIB1 (Ki67) in moderately differentiated hepatocellular carcinoma showing nuclear staining. Positive nuclear staining is seen in half of the tumor cells. (original magnification ×200)

Fig. 2. Immunohistochemical staining of DO7 (p53) in moderately differentiated hepatocellular carcinoma showing nuclear staining. (original magnification ×200)
Fig. 3. Immunohistochemical staining of α-catenin in non-tumoral livers (a) and moderately differentiated hepatocellular carcinoma (b). In normal liver, α-catenin was expressed at the lateral membranes of the hepatocytes and the interlobular bile duct epithelia (a). (original magnification ×200)

In moderately differentiated hepatocellular carcinoma tissues, α-catenin expression was reduced in right nodule and was unchanged in the left nodule (b). Such cases were classified as negative expression of α-catenin. (original magnification ×100)
that recognizes a denaturation-resistant epitope at the N terminus of p53, and that was capable of detecting both wild and mutant type p53 protein and (3) mouse monoclonal antibody of α-catenin (IgGi, Transduction Laboratories, Lexington, KY) which reacts with the human homologue.

Paraffin-embedded blocks were cut at 4-μm thickness and put on 3-aminopropyl aminopropyl-triethyloxylane (APES)-coated slides. Paraffin sections were dewaxed in xylene and rehydrated in a series of graded alcohols according to histopathological standards. Deparaffined tissue sections were washed in distilled water, and immersed in 10 mM citrate buffer (pH 6.0) at 120°C for 5 min in an autoclave [14]. The sections were allowed to cool to room temperature (approximately 3 hs). Next, to block the endogenous peroxidase activity, the slides were immersed in methanol with 0.3% hydrogen peroxide for 30 min. The slides were rinsed twice in phosphate-buffered saline (PBS)-Tween20, and blocked with appropriate normal (goat or horse) serum in PBS for 30 min to minimize non-specific absorption of the antibodies to the slides. The slides were incubated with the primary antibody for 15 to 18 hs at 4°C. The optimal dilution for each antibody was 1:50 for Ki67 (MIB-1), p53 (D07) and α-catenin. After three further rinses in PBS-Tween20, the slides were incubated for 30 min with biotinylated secondary antibodies (ABC Vectastain Kit, Vector Laboratories, CA). They were subsequently incubated with streptavidin-biotin peroxidase complex (ABC Vectastain Kit, Vector Laboratories, CA). Sections were washed twice for 5 min with PBS, developed with 3,3-diaminobenzine tetrahydrochloride (DAB) substrates (Sigma, UK), lightly counterstained with hematoxylin, dehydrated, cleared, and mounted.

Evaluation of immunostaining of Ki67, p53 and α-catenin

In HCC tissues, the immunoreactive staining of Ki67 (MIB-1) was observed in a granular pattern and appeared to be located at the periphery of the nuclei and in perinucleolar regions (Fig. 1). All labeled nuclei with brown-colored staining were considered to be positively stained regardless of staining intensity or pattern. In each case, more than 1000 cells were actually counted at a final magnification of ×200 in each section to determine the labeling index (L.I.), which is the number (%) of positive cells. As positive controls, specimens of normal tonsil, which had been proven to be positive for Ki67 (MIB-1) in more than 80% of cells, were applied. For negative controls, the antibody was replaced by equivalent amounts of normal rabbit IgG.

The immunoreactive staining of p53 (D07) was seen in nuclei of HCC cells (Fig. 2). Weak cytoplasmic staining was sometimes observed, but exclusive cytoplasmic staining was scored as negative. Only nuclear staining was regarded as positive. The percentage of positive HCC cells was estimated by counting more than 1000 cells. The tumors were semiquantitatively categorized into two groups as positive group (more than or equal to 5% of HCC cells) and negative group (less than 5% of HCC cells). As a positive control, specimens of gastric carcinoma, which had been proven to be positive for p53 (D07) in more than 80% of tumor cells, were applied. For negative controls, the antibody was replaced by equivalent amounts of normal mouse IgG.

The immunostaining of α-catenin was semiquantitatively evaluated as reported by Ihara et al. [10] by comparing the immunostaining intensity of HCC cells with that of the normal hepatocytes in the same section as a positive control. In brief, the lateral surfaces of hepatocytes and interlobular bile ducts epithelia stained positively for the α-catenin (Fig. 3a). If the intensity of α-catenin staining in HCC cells was the same or enhanced compared with normal hepatocytes, the HCC cells were evaluated as α-catenin positive. When the intensity of α-catenin staining in HCC cells was lower than that of the control, the HCC cells were evaluated as negative (Fig. 3b).

Statistical analysis

Data are expressed as mean±SD. Mann-Whitney U test and chi-square test were used as appropriate to compare factors. Statistical significance was defined as a p-value of <0.05.

RESULTS

Comparisons of clinical features with total AFP concentrations and AFP-L3%

Twenty-eight (58.3%) of the 48 serum samples showed serum AFP concentrations greater than equal to 20 ng/ml, and the proportion of AFP-L3 in these 28 samples was analyzed using the kit described above. Among these 28 samples, AFP-L3% was greater than or equal to 15% in 14 samples, and these samples were classified as AFP-L3 positive.
According to the results of serum AFP concentrations and AFP-L3%, the 48 patients with HCC were classified into the following groups: AFP greater than or equal to 20 ng/ml and AFP-L3 positive (group A, n=14), AFP greater than or equal to 20 ng/ml and AFP-L3 negative (group B, n=14) and AFP less than 20 ng/ml (group C, n=20). The clinical features of the three groups are shown in Table 1. No significant difference was found between any of the groups in age, sex, tumor size, virus marker, background liver disease or Child-Pugh classification.

Comparisons of pathological examination with total AFP concentrations and AFP-L3%

Tumor differentiation in the 48 tumors was as followed: 2 well, 39 moderately and 7 poorly differentiated HCC. Microscopic vascular invasion was diagnosed in 26 tumors (54.2%) and microscopic metastasis in 13 (27.1%). Because 39 of the 48 tumors (81.3%) were moderately differentiated HCC, there was no significant difference between the three groups (Table 2).

Comparisons of immunohistochemical examination with total AFP concentrations and AFP-L3% (Table 3)

The Ki67 (MIB-1) L.I. ranged from 0%-78%. Association of Ki67 L.I. with total AFP concentrations and AFP-L3% is presented in Table 3. The Ki67 L.I. of groups A, B and C were 27.8±18.9, 9.6±10.1 and 11.1±11.2, respectively. The Ki67 L.I. of group A was significantly higher than that of group B (p<0.024) and group C (p<0.03) by Mann-Whitney U test. There was no significant difference between group B and group C.

The association of p53 staining with total AFP concentration and AFP-L3% showed the positive rate of p53 staining in group A, B and C to be 50%, 28.6% and 17.6%, respectively. The p53 positive rate in group A (50.0%) was significantly higher than that in group C (15.0%) (p<0.05, chi-square test).

Reduced a-catenin expression was observed in 27 (56.5%) of the 48 tumors. The a-catenin expression in group A (7.1%) was significantly lower than that in group B (85.7%, p<0.001) and group C (40.0%, p<0.05) by chi-square test.

DISCUSSION

The biologic features that are indicative of the clinical aggressiveness of cancer cells and that are useful for identification of patients at low and high risk have also been found to aid in the prediction of...
therapeutic effect and patient survival. Such putative predictors of HCC in diagnosis and treatment include tumor markers, pathological indices (histologic grade, vascular invasion and intrahepatic metastasis), proliferation indices (proliferating cell nuclear antigen [15], silver staining of cells of the nucleolar organizer region [16], bromodeoxyuridine [17], DNA ploidy [8], Ki67 [9,18] and mitotic index [19]), genetic alterations [12,20] and factors of intercellular adhesion molecules [10,21].

In this study, pathological findings such as histologic grade, vascular invasion and intrahepatic metastasis were evaluated. No significant difference was found between the groups studied in histologic grade, vascular invasion and intrahepatic metastasis. However, 39 (81.3%) out of 48 tumors examined were moderately differentiated HCC, and this preponderance might be a reason that no statistically significant differences were observed.

As for proliferative activity, Yoshimoto et al. [9] reported that Ki67 staining is useful for evaluation of the malignant potential of HCC. The Ki67 L.I. of AFP-L3 positive patients (group A) was statistically higher than those of AFP-L3 negative patients (group B) and AFP negative patients (group C). The value of AFP-L3 in patients with HCC may reflect the proliferative activity of HCC.

Although the normal function of the p53 gene has not been clarified completely, mutant p53 gene plays an important role in carcinogenesis and cancer progression. Oda et al. [12] reported the occurrence of p53 mutations in moderately and poorly differentiated HCC. Hayashi et al. [20] reported that mutations of the p53 gene in moderately and poorly differentiated HCC. Hayashi et al. [20] reported that mutations of the p53 gene in moderately and poorly differentiated HCC.

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