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The usefulness of anti-fucosylated antigen antibody YB-2 for diagnosis of hepatocellular carcinoma

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Levels of fucosylated antigens in sera from patients with liver diseases were examined by a newly developed sandwich-type enzyme immuno assay with the aid of anti-fucosylated antigen antibody, YB-2 which reacts simultaneously with Y, Le^b and H type 2 antigens. When the cut-off value was set arbitrarily at mean + 3 SD values of normal, 30 (69.8%) of the 43 patients with HCC, 14 (53.8%) of the 26 patients with liver cirrhosis (LC) and 24 (45.3%) of the 53 patients with chronic hepatitis (CH) were found to be positive, whereas all of the 30 samples from healthy controls were negative. The levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) in HCC were not correlated with those of YB-2 antigens. The positive rates of the combination YB-2 and AFP assay and YB-2 and PIVKA-II assay in HCC were significantly higher (83.7 and 86.0%, respectively) than that of the AFP and PIVKA-II combination (65.1%) which had been reported to be the best combination up to this time.

***Keywords*: hepatocellular carcinoma, glycoconjugates, fucosylated antigens, α-fetoprotein, YB-2 antigens**

Introduction

Dramatic changes in glycosylation are known to occur in tumour cells. Both the accumulation of fucosylated antigens and the presence of aberrant fucosylated antigens in various human cancers have been regarded as one of the tumour-associated phenomena [1]. Some of the fucosylated antigens circulating in blood, have been detected by immunological techniques, and the levels of these antigens were used as tumour markers [2–4]. All these fucosylated antigens are synthesized by a number of fucosyltransferases with different substrate specificities which must be encoded by individual genes [5].

In our previous studies, the level of $\alpha 1 \rightarrow 3$ fucosyltransferase activities has been demonstrated to be variably elevated in sera from patients with various cancers [6–8]. The increase in activities of $\alpha 1 \rightarrow 2$ fucosyltransferase as well as $\alpha 1 \rightarrow 4$ fucosyltransferase has been determined in colorectal tumours and the presence of aberrant $\alpha 1 \rightarrow 2$ fucosyltransferase was found in the same tissues [9]. With the results mainly from immunohistochemical studies on fucosylated antigens of tumour tissues it has been postulated that such fucosyltransferases must be involved in the synthesis of tumour-associated fucosylated antigens such as X, Y, sialyl X, sialyl Le^a and related antigens. It was also demonstrated that common blood group antigens such as H and Le^b antigens were detected inappropriately in colorectal tumour tissues together with Y antigen even though these fucosylated antigens were absent in adjacent normal tissues [8].

More recently, a novel monoclonal antibody YB-2, was developed against fucosylated antigens [10] and was found to react simultaneously with Y (Fuc$\alpha 1 \rightarrow$ 2Gal$\beta 1 \rightarrow$ 4[Fuc$\alpha 1 \rightarrow$ 3]GlcNAcβ), Le^b (Fuc$\alpha 1 \rightarrow$ 2Gal$\beta 1 \rightarrow$ 3[Fuc$\alpha 1$ $\rightarrow$ 4]GlcNAcβ) and H type 2 (Fuc$\alpha 1 \rightarrow$ 2Gal$\beta 1 \rightarrow$ 4GlcNAcβ) antigens to the same extent. The results of immunohistochemical studies on colorectal cancer showed that YB-2 monoclonal antibody could be useful for the diagnosis and evaluation of the progression of colorectal cancer due to the specificity of the antibody [10, 11].

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers, and the early diagnosis of HCC is most desirable during the monitoring of patients with chronic liver diseases to predict for the development to HCC.

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α-Fetoprotein (AFP) has been considered the most valuable marker for diagnosing HCC. The levels of serum AFP have been demonstrated to increase in HCC and in patients with liver diseases such as chronic hepatitis (CH) and liver cirrhosis (LC) [12]. Analyses of carbohydrate structures of AFP in such liver diseases showed that the amount of *Lens culinaris* agglutinin (LCA)-reactive species of AFP was significantly greater in HCC than in other benign liver diseases [13–15]. LCA-reactive AFP could be used to detect early HCC which developed from LC. The protein induced by vitamin K absence or antagonist-II (PIVKA-II) has also been used as a tumour marker associated with the presence of HCC [15–19]. In these reports PIVKA-II showed higher specificity for HCC, but its sensitivity was still low. The assay of AFP and PIVKA-II on the same sample has been considered the best combination to detect HCC up to this time [17]. But an additional highly sensitive HCC-associated marker is still desirable, since the positive rate is not good enough for mass screening.

The accumulation of fucosylated antigens has recently been reported in sera from patients with HCC and altered fucosylation in the same tumour tissues has also been demonstrated [20]. In this study, we developed a new sandwich-type enzyme immuno assay for serum levels of fucosylated antigens (YB-2 antigens) and measured the levels of the antigens in serum samples of HCC, LC and CH. The usefulness of this antibody was then evaluated for the diagnosis of HCC. In addition, the levels of AFP and PIVKA-II were measured in the same samples and the usefulness of two and/or three assays in combination was also examined to detect HCC.

Materials and methods

Samples

Serum specimens were obtained from patients who were admitted to the First Department of Surgery, Gunma University Hospital, Third Department of Internal Medicine, Hyogo College of Medicine Hospital and the First Department of Internal Medicine, Tokushima University Hospital. Patients with HCC ($n = 43$) consisted of 33 men and 10 women from 49 to 79 years old, LC ($n = 26$) consisted of 14 men and 12 women from 32 to 82 years old and CH ($n = 53$) consisted of 29 men and 24 women from 28 to 74 years old. Samples of HCC were taken before treatment. The diagnosis was histologically verified in all but a few cases of HCC. Stages of the HCC patients were from Stage I to IV determined by TNM classification of malignant tumours. Sera from controls ($n = 30$) were obtained from healthy volunteers who consisted of seven men and 23 women from 18 to 42 years old. They were determined to be completely free from disease by routine physical examination.

Assay protocol for YB-2 antigens

The principle of the assay was a sandwich-type enzyme linked immunosorbent assay (ELISA). Twenty microlitres of serum samples was applied to each well of an anti-YB-2 antibody coated multiwell plastic plate (0.5 μg/well) with 150 μl phosphate buffered saline (PBS) containing 2% bovine serum albumin and 0.1% Tween 20 (PBS-B-T). After incubation on a shaker at 25 °C for 2 h, the wells on the plate were washed four times with PBS containing 0.05% Tween 20, and 100 μl of horseradish peroxidase-conjugated anti-YB-2 antibody was added to each well. Then the mixture was incubated on a shaker at 25 °C for 2 h. After washing each well with the same buffer, 100 μl of citrate-phosphate buffer (pH 5.0) containing o-phenylene diamine and H_2O_2 was added to each well and incubated for 15 min at room temperature. The colour development was stopped by adding 100 μl of 1 M sulfuric acid. The absorbance at 492 nm of each well was measured with a microplate reader. The standard sample which contained Y, Le^b and H type 2 antigen was isolated from human meconium [21] and the solution of standard antigen was prepared by diluting the standard sample with the aforementioned PBS-B-T at a dilution ratio suitable for the ELISA system. The cut-off value was determined as 58.6 U ml^{-1} from the mean + 3 SD values of normal ($n = 30$). The level of the YB-2 antigens was expressed as an arbitrary value (U ml^{-1}) by comparison with the standard solution. All experiments were carried out in duplicate. To evaluate the assay method, three different serum samples whose levels of the YB-2 antigens were low, middle and high were also examined at the same time.

AFP and PIVKA-II assay

Serum levels of AFP and PIVKA-II were measured with an RIA kit for AFP (α-FETO-RIABEAD, Dainabot, Tokyo, Japan) and an EIA kit for PIVKA-II (Eitest MONO-II, Eisai, Tokyo, Japan), respectively, according to the manufacturers' instructions. The cut-off values for AFP and PIVKA-II were 20 ng ml^{-1} and 0.1 U ml^{-1}, respectively, as described in the instructions.

GOT and GPT assay

Serum levels of GOT (glutamic oxaloacetic transaminase) and GPT (glutamic pyruvic transaminase) were measured with an enzyme assay kit (Transaminase-HR II, Wako Pure Chemical Ind Ltd, Osaka, Japan) according to the manufacturers' instructions.

Statistical analysis

Statistical analyses have been made by Mann-Whitney's U-test to compare YB-2 levels in HCC, LC, CH and normal individuals and χ^2 test to compare the positive rate in combination of two or three assays. p Values less than 0.05 were considered significant.

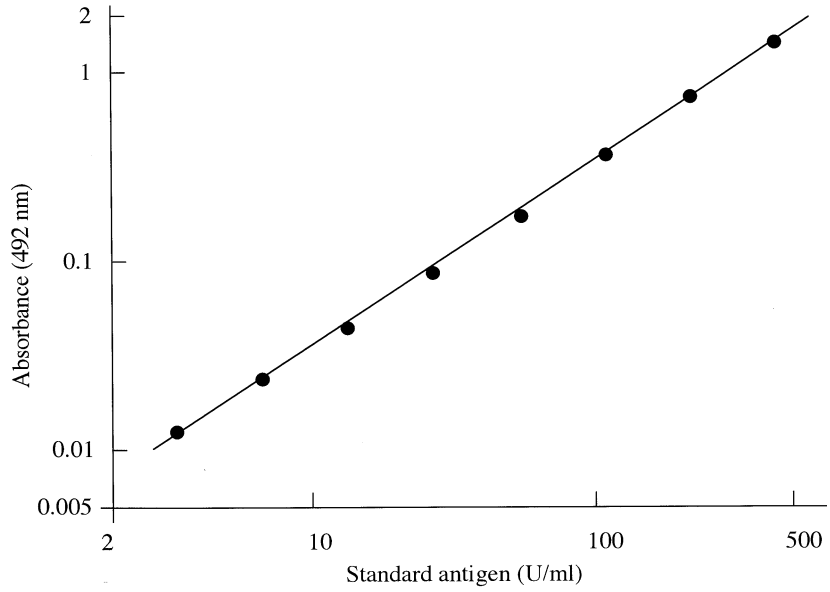

Figure 1. Standard curve for YB-2 assay.

Table 1. Precision of assay of YB-2 antigens.

Serum	*YB-2 antigens (U ml^{-1})*		
	Mean	SD	*CV (%)*
Interassay			
No. 1	9.4	0.69	7.4
No. 2	95.3	5.5	5.7
No. 3	215.2	9.6	4.4
Intra-assay			
No. 4	16.5	0.36	2.2
No. 5	59.5	1.2	2.1
No. 6	248.2	7.7	3.1
(n = 5)			

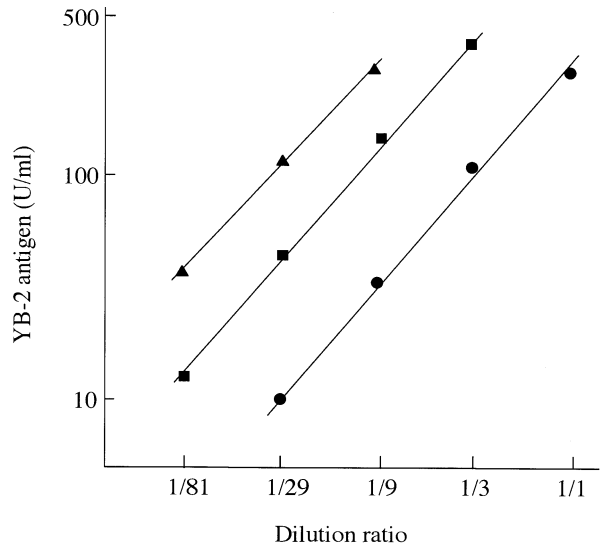

Figure 2. Levels of YB-2 antigens in patients' sera with different dilution rates. YB-2 antigen levels in serum 7 (●), 8 (■) and 9 (▲) were assayed at different concentrations.

Results

Analytical studies

As shown in Fig. 1, the absorbance in the assay was found to increase in a linear fashion with the concentration of the standard YB-2 antigens changing from 7.9 to 500 U ml^{-1}. Intra-assay reproducibility was tested with five replicates. Inter-assay reproducibility was demonstrated by duplicating the assay five times. For both inter- and intra-assays six different serum samples as mentioned above were used (Table 1). The assay reproducibility (CV) was $< 8\%$ for six different samples and indicated that the precision was adequate over the entire range of the standard curve. Dilution linearity was also determined in serum samples of three patients before and after dilution with the zero standard (Fig. 2). The ratio of the observed values to calculated ones was found to be near 100% (data not shown).

Levels of YB-2 antigens in serum samples

Figure 3 shows the levels of YB-2 antigens in sera from patients with HCC, LC and CH. The median level of YB-2 antigens in sera from patients showed to be significantly high compared with that from normal individuals

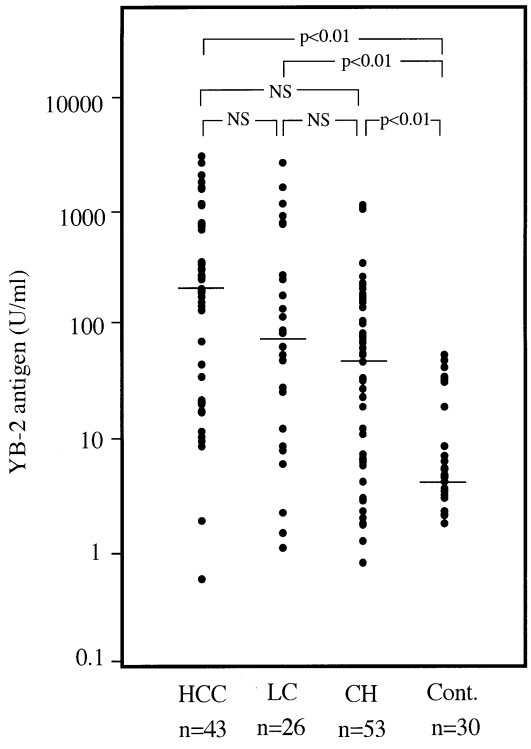

Figure 3. Levels of fucosylated antigens detected by YB-2 antibody in sera from patients with liver diseases. *p* Values are less than 0.01 in HCC and LC, and less than 0.05 in CH, compared with the normal control. Each value was plotted on a logarithmic scale.

($p < 0.01$), and the median levels in HCC, LC, CH and normal individuals were 201.8, 73.8, 46.7 and 3.9 U ml^{-1}, respectively. It was clear that the order of the median levels was HCC > LC > CH, but there were no significant differences between these groups. All samples from healthy controls were below the cut-off level.

Positive rates for YB-2 antigens, AFP and PIVKA-II in serum samples

Thirty (69.7%) of 43 sera from patients with HCC showed increased levels of YB-2 antigens, but 14 (53.8%) of 26 patients with LC and 24 (45.3%) of 53 patients with CH were found to be positive (Fig. 3). Levels of AFP and PIVKA-II in the same sera were also measured. Sixty percent (26/43) of patients with HCC, 42.3% (11/26) of patients with LC and 22.6% (12/53) of patients with CH were found to have high levels of AFP and the tendency to a positive rate in each disease seemed to be similar with that found in the levels of YB-2 antigens. On the other hand, the positive rate for PIVKA-II was found to be not so high as those of YB-2 and AFP in HCC, but it had a quite high specificity for HCC (94.7%) (Table 2). Both YB-2 antigens (44.1%) and AFP (53.1%) showed lower specificity for detecting HCC even though the sensitivity of two indexes for HCC was fairly high as mentioned above.

Table 2. Positive rates of YB-2 antigens, and tumour-associated antigens in sera of patients with liver diseases.

Patients	*Positive rate*[a]					
	YB-2		*AFP*		*PIVKA-II*	
HCC	30/43	(69.8%)	26/43	(60.5%)	18/43	(41.9%)
LC	14/26	(53.8%)	11/26	(42.3%)	1/26	(3.8%)
CH	24/53	(45.3%)	12/53	(22.6%)	0/53	(0%)

[a] Results are given as numbers of positive sera/numbers of sera tested. HCC, hepatocellular carcinoma; LC, liver cirrhosis; CH, chronic hepatitis; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

Correlations of serum levels between YB-2 antigen, AFP and PIVKA-II

No significant correlation was found between the levels of YB-2 antigens and those of AFP in all the sera examined (Fig. 4). There was also no significant correlation between YB-2 antigens and PIVKA-II ($r = -0.1083$, NS) or between AFP and PIVKA-II ($r = 0.0349$, NS). These results showed that levels of these three indexes seemed to change independently in sera from liver diseases. Levels of GOT ($r = 0.1736$, NS) and GPT ($r = 0.0609$, NS) which are commonly used as indexes for liver diseases, were different from those of YB-2 antigens and PIVKA-II in the same serum samples, but were similar with those of AFP (data not shown).

Positive frequency from serum levels of YB-2 antigens, AFP and PIVKA-II in combination

In order to increase the rate at which HCC could be detected, the elevation of YB-2 antigens, AFP and PIVKA-II in each serum was examined in combination (Table 3). If YB-2 antigens and AFP were assayed together, the combined frequency at which HCC showed levels of either AFP or YB-2 antigens above their cut-off values was increased to 83.7%. The combined frequency was also increased to 86.0%, if YB-2 antigens and PIVKA-II were assayed together. No significant increases in the detection rate for LC and CH were observed in the combinations of two assays except in the combination of YB-2 antigens and AFP assay for LC. The rate at which HCC could be detected was extremely high (88.4%), if YB-2 antigens, AFP and PIVKA-II were examined in combination.

Discussion

The alteration in the expression of glycoconjugates has been described in a variety of cancers, and the presence of aberrant glycoproteins and glycolipids which are absent or present only at low levels in normal tissues has been widely observed in various cancer tissues [1]. In particular,

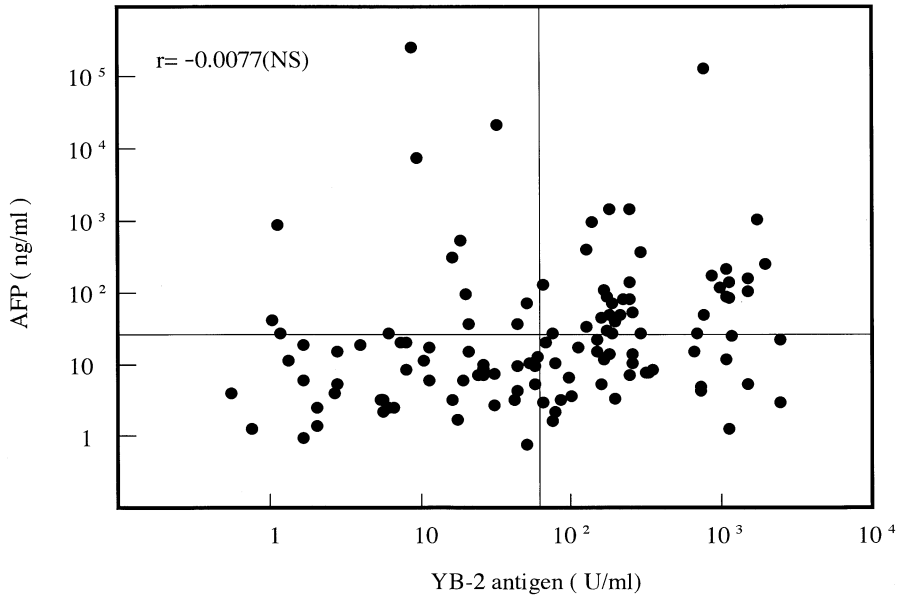

Figure 4. Correlation between YB-2 antiens and AFP in sera from patients with liver diseases. NS, not significant. The lines show cut-off levels. Each value was plotted on a logarithmic scale.

Table 3. Positive rates of combination assay of YB-2 antigen, and tumour-associated antigens in sera of patients with liver diseases.

Patients	*Positive rate*[a]							
	YB-2 + AFP[(A)]		YB-2 + PIVKA-II[(B)]		AFP + PIVKA-II[(C)]		YB-2 + AFP + PIVKA-II[(D)]	
HCC	36/43	(83.7%)**	37/43	(86.0%)**	28/43	(65.1%)	38/43	(88.4%)
LC	18/26	(69.2%)	15/26	(57.7%)	12/26	(46.2%)	19/26	(73.1%)
CH	26/53	(49.1%)	24/53	(45.3%)	12/53	(22.6%)	26/53	(49.1%)

[a] Results are given as numbers of positive sera/numbers of sera tested.
** *p* Value is less than 0.05: (A) vs (C) and (B) vs (C).
HCC, LC, CH, AFP and PIVKA-II are as shown in Table 2.

accumulation of fucosylated antigens in tumour tissues has been regarded as one of the typically cancer-associated phenomena [9], and $\alpha 1 \rightarrow 3$ fucosylated antigens such as X, Y antigens and the related fucosylated antigens such as sialyl X, sialyl Le^a antigens and their derivatives have been reported to be present mostly in tumour tissues but not in normal tissues [2]. On the other hand, in our previous study, fucosylated blood group antigens such as Le^b and H type 2 antigens were found to be specifically accumulated in colorectal tumour tissues as well as Y antigen [10, 11]. Therefore, immunohistochemical studies on the fucosylated antigens in tumour tissues were thought to be still needed to detect not only the antigens which are specifically present in tumour tissues but also those which are commonly present with some tissue-specificity.

A large number of monoclonal antibodies have been raised against fucosylated antigens, which directly recognize the fucosylated moieties [3, 4]. Many anti-fucosylated antigen monoclonal antibodies are available now to detect specifically the aforementioned antigens, but as mentioned in our previous studies [10, 11], some monoclonal antibodies showed broad patterns of reactions and cross-reactions with closely related structures. Some of the anti-fucosylated antigen antibodies were found to be useful for the detection of cancer-associated fucosylated antigens [1–3, 22].

Recently, a new anti-fucosylated antigen antibody, YB-2, was established and its characteristics were determined to react with Y, Le^b and H type 2 antigens, simultaneously [10]. Since these fucosylated antigens were found to be accumulated in colorectal tumour tissues [1], it was assumed that YB-2 antibody is useful for the histochemical diagnosis of colorectal cancer [10, 11]. In fact, 92.2% of the tissues from colorectal cancer but only 12.0% of the tissues from normal subjects were stained positively by YB-2 antibody [10].

HCC is one of the most common cancers in Japan [23]. Recently, hepatic imaging techniques such as ultrasound imaging, computed tomography and magnetic resonance imaging have made it possible to easily detect HCC legions. However, imaging techniques are not suitable for mass-screening. It has been desirable that simpler and easier diagnostic methods could be developed.

The HCC-associated alteration of glycoconjugates has been described in recent studies and changes in sugar moieties of both AFP [13–15] and cholinesterase [24] in sera from patients with HCC have been found. AFP is a component in the fetal serum of mammals and is synthesized by the fetal liver and yolk sac. It has been demonstrated that AFP is detected in adult individuals associated with primary liver carcinoma and regenerating liver tissue [12]. Serum levels of AFP are used widely for the early diagnosis of HCC because of their high specificity found in patients with HCC [23], but levels of the same antigen were also observed to be high in benign liver diseases [12]. On the other hand, AFP with differently fucosylated structures was found in HCC but not in other chronic liver diseases and metastatic liver cancers [13]. Hence, it was speculated that HCC and benign liver diseases could be distinguished by the different structures in the fucosylated sugar chains of AFP. One of the distinctive differences in such structures was detected by LCA whose binding specificity was proved to be against $\alpha 1 \rightarrow 6$ fucosyl linkage [25].

Many other HCC-associated markers have also been reported to date [26, 27]. Serum levels of PIVKA-II are used as another tumour marker for the diagnosis of HCC. Although the specificity was extremely high (90%), the sensitivity of PIVKA-II was not so high for HCC (66%) [16, 17].

Immunohistochemical studies on HCC with anti-Y antibody (BM-1) showed that Y antigen was present in 43.5% of the HCC samples [20], but serum levels of Y antigen in HCC were found to be not so high as those of YB-2 antigens in our present study (unpublished observations). Although the serum levels of YB-2 antigens did not increase significantly, the same antigen levels were found to be accumulated in colorectal tumour tissues as mentioned above. It remains to be investigated why the accumulation of YB-2 antigen in tumour tissues from patients with colorectal cancer did not follow with the elevated levels of their antigens in serum.

All the glycoconjugates have been proved to be synthesized by the action of a series of glycosyltransferases. As described above, the expression of aberrant glycoconjugates in human cancers has been regarded as one of the tumour-associated phenomena [1], and it must be of particular interest to investigate whether such glycoconjugates are synthesized by novel glycosyltransferases associated with cancers. In our previous studies, increased plasma $\alpha 1 \rightarrow 3$ fucosyltransferase activities have been demonstrated in patients with HCC as well as in various cancer patients [6–8, 28]. In addition, interestingly, the same enzyme activity in the cirrhotic liver tissue was found to be significantly higher than that in the hepatoma tissue [28]. Enzymatic studies are now in progress to investigate fucosyltransferases related to the synthesis of fucosylated antigens detected by YB-2 antibody in HCC.

Since it has already been demonstrated that increased fucosylation occurs in HCC, the assay to detect three kinds of fucosylated antigen, Y, Le^b and H type 2, at the same time should improve the sensitivity for detecting HCC. In fact, the sensitivity of YB-2 antigens for detecting HCC was highest when three assays were used. The positive rates for HCC were observed to be very high when the YB-2 and AFP assays or the YB-2 and PIVKA-II assays were used in combination. It was clear that the positive rates for such combinations were better than that of the combination assay of AFP and PIVKA-II, which has been considered the best combination to detect HCC until now [17]. In conclusion, serum levels of YB-2 antigens should be measured in patients with HCC in conjunction with AFP and/or PIVKA-II assay in the same samples.

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