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Carbohydrate-based measurements on alpha-fetoprotein in the early diagnosis of hepatocellular carcinoma

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Serum alpha-fetoprotein (AFP) is a useful marker for the diagnosis of hepatocellular carcinoma (HCC), although this protein also increases moderately in benign liver diseases. The serum concentration of AFP in HCC at the time of initial diagnosis is now lower than before because of advancements in techniques for imaging the liver. The AFP concentration alone cannot distinguish between HCC and benign liver diseases, especially when it is less than 1000 ng ml^{-1}. These circumstances lead to the need to discriminate between these diseases. This has been achieved by determining the carbohydrate structures of AFP by its reactivity with *Lens culinaris* agglutinin (LCA). The percentage of LCA-reactive species of AFP is significantly higher in HCC than in benign liver diseases. The fucosylation of the sugar chain at the innermost *N*-acetylglucosamine is the molecular basis of this variation. Therefore, the term 'fucosylation index' has been introduced to express the percentage of LCA-reactive species of AFP. This index is useful for the diagnosis of HCC even if the carcinoma is at an early stage. Furthermore, it can predict the development of HCC in the follow-up of chronic liver diseases. Thus, the qualitative and quantitative measurements of carbohydrate in AFP provide us with very valuable information for the differential diagnosis of various liver diseases.

Keywords: hepatocellular carcinoma, alpha-fetoprotein, fucosylation index, *Lens culinaris* agglutinin

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human cancers, and early detection of HCC is very important during the follow-up of patients with chronic liver diseases, because there is a very high incidence of HCC in association with liver cirrhosis [1, 2]. The measurement of serum concentration of alpha-fetoprotein (AFP) has been extensively used for the detection of HCC [3–8]. The serum concentration of AFP also, however, increases in benign liver diseases such as chronic hepatitis and liver cirrhosis [9–11], although the degree of elevation is low. Furthermore, the serum concentration of AFP at the time of clinical diagnosis is now lower than before in patients with HCC because of progress in several imaging modalities, such as ultrasonographic examination, computed tomography and magnetic resonance imaging [12–14]. Therefore, a value which discriminates an AFP species associated with HCC from one with benign liver disease becomes very useful as a diagnostic tool, especially when there is only a slight increase in the serum AFP concentration during the course of chronic liver diseases.

Molecular heterogeneity of human AFP was first demonstrated by Purves *et al.* in 1963 [15], and since then many reports have dealt with charge and molecular size heterogeneity of human AFP [16–21] and the lectin-binding heterogeneity of human AFP associated with fetal development and its anomalies [22–26]. In 1978, Ruoslahti *et al.* reported that AFP produced by the yolk sac and the liver are glycosylated differently because of their reactivity with an immobilized Concanavalin A (Con A) column. It was originally proposed that these variants might prove to be diagnostically useful [27]. Subsequently, several investigators have applied this concept of differential reactivities with lectins in order to discriminate between AFP species originating from HCC and those from benign liver diseases [28–31].

In this review, the author will focus mainly on the carbohydrate-based molecular heterogeneity of AFP, which can be used for the early diagnosis of HCC. Other related areas of molecular species of AFP have been covered by previous articles [32–34].

Comparison of the serum AFP concentration in patients with HCC and benign liver diseases

The 11th Report by the Liver Cancer Study Group of Japan (1994) found that 7 577 sera (71%) from 10 662 patients with HCC had an increased AFP concentration of more than 20 ng ml^{-1}. An elevated AFP level has been noted in 81%, 77% and 74% of HCC sera according to the 6th, the 8th and the 10th Reports respectively [35–37], when the cutoff value has been set at 20 ng ml^{-1}. Additionally, the incidence of HCC sera with a concentration of AFP of more than 1000 ng ml^{-1} decreased from 46% (6th Report) to 33% (11th Report). Thus, a positive serum AFP result at the time of initial diagnosis in HCC patients has become less likely with the dramatic advances in imaging.

On the other hand, a slight increase in AFP concentration has sometimes been observed in patients with chronic liver diseases without any evidence of HCC. Several investigators have reported that the frequency of a positive AFP result can rise from 13 to 58% in chronic hepatitis, and from 11 to 34% in liver cirrhosis [10, 11, 38–40]. However, the increased AFP concentration in most cases in these studies was less than 500 ng ml^{-1}. If the cutoff value of AFP is set at 200 or 400 ng ml^{-1}, in order to increase the specificity in the diagnosis of HCC, the false positive rate of AFP in chronic hepatitis and liver cirrhosis decreases to less than 5%. However, the sensitivity of AFP in detecting HCC would also decrease from 71% to 40% at a cutoff value of 200 ng ml^{-1} and to 37% at a value of 400 ng ml^{-1}, according to the data of the 11th Report. Consequently, 31% (71% minus 40%) or 34% (71% minus 37%) of patients with HCC would fail to be diagnosed as a result of these changes in the cutoff values.

To date our department has collected sera from 493 patients with HCC and 259 with benign liver diseases with a serum concentration of AFP of more than 20 ng ml^{-1}. Figure 1 shows the serum AFP concentrations of less than 1000 ng ml^{-1} in patients with HCC or benign liver diseases at the time of initial clinical diagnosis. The data indicate that there is no clear distinction in serum AFP concentrations of HCC and the benign group. Thus, the concentration of AFP alone appears to be of little practical value in the differential diagnosis of HCC from the benign group especially when it is below 1000 ng ml^{-1}.

AFP species detected by crossed immuno-affino-electrophoresis

Two molecular species of AFP were detected on crossed immunoaffino-electrophoresis in the presence of Con A or LCA. Migration of one of these species is retarded (reactive species), whilst the other is unaffected (nonreactive species). In almost all the patients with benign liver diseases, AFP consists mainly of the Con A-reactive species, whereas the percentage of the LCA-reactive species is very low. On the other hand, AFP from patients with HCC predominantly consists of the Con A-reactive species similar to AFP in the benign group.

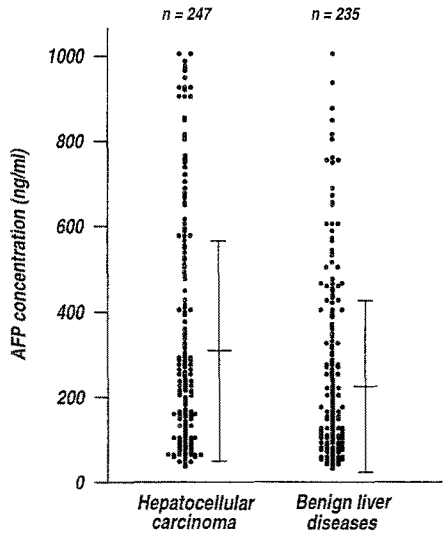

Figure 1. Comparison of the serum AFP concentration (less than 1000 ng ml^{-1}) in patients with HCC and benign liver diseases. For Figures 1–5 vertical lines in each column indicate mean ± SD.

However, the LCA-binding pattern is quite different from that of the benign group, showing an increase of the LCA-reactive species [29].

Carbohydrate structures on AFP with reference to lectin reactivity

The complete amino acid sequence of human AFP deduced from cDNA sequencing [41–43] indicates that the 233rd asparagine is a possible *N*-glycosylation site because of the presence of the characteristic sequence of Asn-Phe-Thr.

The carbohydrate structure of human AFP from ascites fluid of patients with HCC was reported by Yoshima *et al.* [44]. A combination of sequential exoglycosidase digestion and methylation analysis showed that the molecule had one asparagine-linked sugar chain, the structure of which is NeuAc α2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(± Fucα1-6)GlcNAc. The carbohydrate structure of AFP from human yolk sac tumour was determined by Yamashita *et al.* [45]. The structure is essentially identical to that of AFP from HCC except for the presence of bisecting-*N*-acetylglucosamine, *i.e.* NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(*GlcNAcβ1-4*)(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fuc α1-6)GlcNAc.

We demonstrated that the mean percentage of LCA-reactive species of AFP in HCC was significantly higher than that in benign liver diseases, and that measurement of the LCA-reactive species of AFP was useful in differentiating the malignant from the benign conditions [29]. Subsequently, we attempted to determine the carbohydrate structures on these AFP species,

and showed that purified LCA-reactive species contained 1 mol fucose per mol AFP [46]. No fucose was detected in the LCA-nonreactive species and there was a direct relationship between the fucose content and the percentage of LCA-reactive species. The radiolabelled glycopeptides from the LCA-reactive species were unretained on an LCA-Sepharose column after α-L fucosidase digestion, while still preserving their ability to bind Con A. Kornfield *et al.* (47) showed that LCA binds specifically to the biantennary sugar chain with the fucose residue at the innermost *N*–acetylglucosamine (GlcNAc) of the trimannosyl core (fucosylated biantennary sugar chain) or binds to chains having both fucose and bisecting GlcNAc (fucosylated and *N*-acetylglucosaminylated biantennary sugar chain) [47]. On the other hand, Con A binds to fucosylated and non-fucosylated biantennary sugar chains. This binding does not occur, however, if the biantennary sugar chain structure acquires a bisecting GlcNAc and/or further branching leading to the formation of triantennary and tetraantennary structures [48]. Our data together with the carbohydrate specificity for LCA and Con A, suggest that the carbohydrate structure of the LCA-reactive species of AFP is of a fucosylated biantennary sugar type [46].

A very sensitive and convenient method has been recently developed to study carbohydrate fine structure by separating fluorescent-labelled oligosaccharides by high performance liquid chromatography [49–51]. Since a number of fluorescent-labelled oligosaccharides with known structures (determined by ^{1}H-nuclear magnetic resonance) have become commercially available, we have applied this method in combination with exoglycosidase digestion to study the carbohydrate structure of various AFP preparations. The AFPs were purified from sera of two patients with HCC and a patient with gallbladder carcinoma which metastasized to the liver. Subsequently, the carbohydrate structures of four other AFP species were determined, Con A-reactive and LCA-reactive, Con A-reactive and LCA-nonreactive, Con A-nonreactive and LCA-reactive, and Con A-nonreactive and LCA-nonreactive [52]. The conclusions from this study are as follows. The Con A-reactive and LCA-nonreactive species of AFP was a biantennary sugar chain, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, the basic carbohydrate unit of AFP. The Con A-reactive and LCA-reactive species was a biantennary chain with a α1-6 fucose residue at the innermost GlcNAc. The Con A-nonreactive and LCA-reactive species had a biantennary chain with a bisecting β1-4GlcNAc attached to the trimannosyl core and an internal α1-6 fucose. The Con A-nonreactive and LCA-nonreac tive species had a α1-6 fucosylated triantennary sugar - chain, Galβ1-4GlcNAcβ1-4(Galβ1-4GlcNAcβ1-2)Manα1-3 (Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc, as the major component, and two minor components which were a triantennary sugar chain, and a biantennary sugar chain with a bisecting-GlcNAc. The fu cosylated and non-fucosylated triantennary sugar chains are newly identified on human AFP [52].

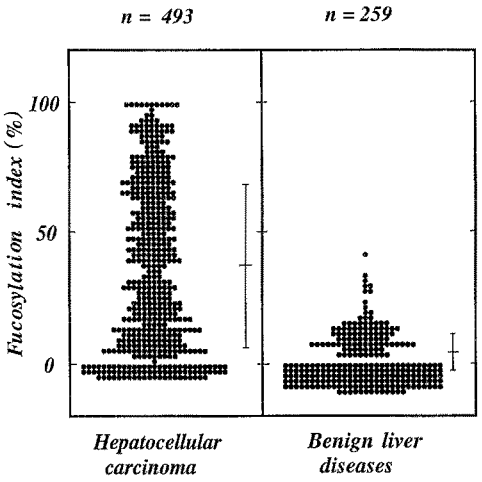

Figure 2. The fucosylation index of AFP in HCC patients and patients with benign liver diseases.

Discrimination between HCC and benign liver diseases by fucosylation index

Our previous study on the carbohydrate structures of lectin-separated species of AFP indicated that the AFP reactive with LCA carried fucosylated sugar chains [46, 52]. Thus, we proposed the 'fucosylation index' of AFP as the percentage of the LCA-reactive species in total AFP [53–55].

Currently, we have determined the index in sera from 493 patients with HCC and 259 patients with benign liver diseases (24 patients with acute viral hepatitis, 109 patients with chronic hepatitis and 126 patients with liver cirrhosis). There was no difference in serum AFP concentration between HCC and the benign group, as indicated in Fig. 1. The index, however, in all patients with HCC (mean = 38% ± SD = 31%) was significantly higher than that in the benign group (4 ± 7%), as shown in Fig. 2.

When the mean index + 2 SD (18.6%) of the benign group was used to discriminate between the HCC and the benign group; 308 of the 493 patients with HCC (62%) had values above this level. This is the sensitivity for the diagnosis of HCC using the index. However, in patients with benign liver diseases, only 11 patients had values above this level. Eight of these patients were the severe type of acute viral hepatitis, and two more were acute exacerbations of chronic hepatitis. Only one had liver cirrhosis, the etiology of which was autoimmune hepatitis. Most of these patients could be readily discriminated from HCC by routine liver function tests and clinical features [54].

When a fucosylation index of 15.0% (mean index + 2 SD in liver cirrhosis) was used to discriminate between the benign and malignant groups, the sensitivity increased from 62% (308/493) to 66% (327/493). Thus, the percentage of patients with liver cirrhosis (126 cases) and chronic hepatitis (109 cases) with an index less than 15.0% was 96% (225/235)

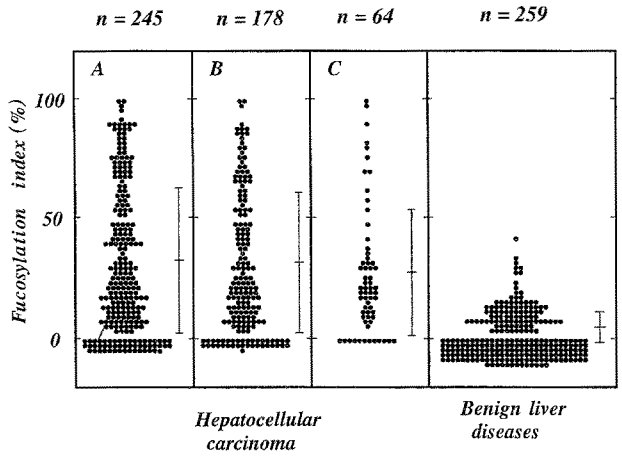

Figure 3. The fucosylation index of AFP in HCC patients with serum AFP concentration of less than 1000 ng ml^{-1} (A), less than 400 ng ml^{-1} (B) and less than 100 ng ml^{-1} (C), and in patients with benign liver diseases.

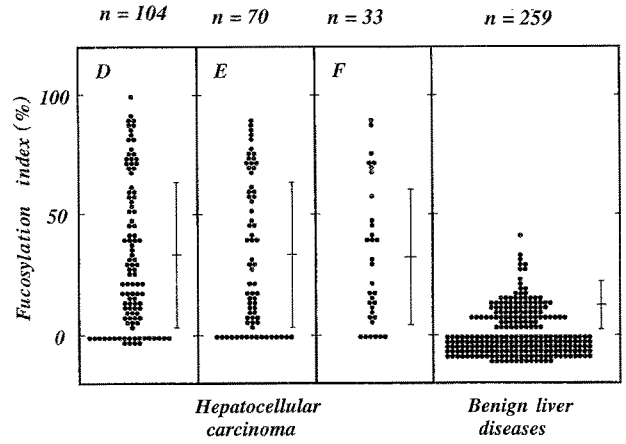

Figure 4. The fucosylation index of AFP in HCC patients with a tumour diameter less than 5 cm (D), less than 3 cm (E) and less than 2 cm (F), and in patients with benign liver diseases.

specificity of this test. The total accuracy of the analysis using the fucosylation index was 76% (327 + 225/493 + 235). In all the patients with HCC, there was no significant correlation between the fucosylation index and the serum concentration of AFP [54].

Fucosylation index in early HCC

To obtain more detailed information on the relationship between the fucosylation index and the clinical stage of HCC, we performed statistical analysis on serum AFP concentration and tumour diameter [54].

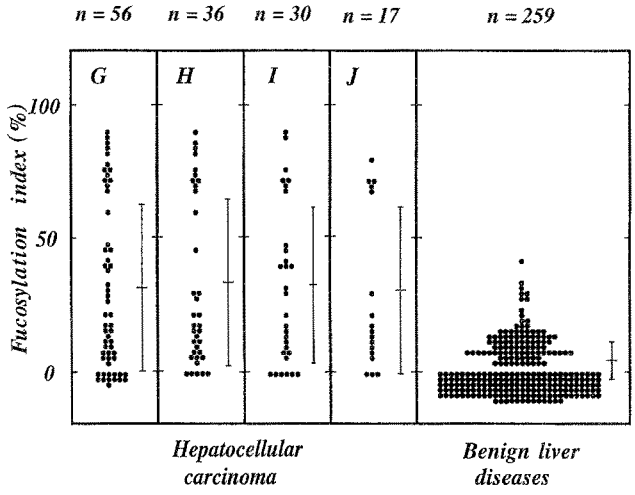

Figure 5. The fucosylation index of AFP in HCC patients with a serum concentration of AFP below 1000 ng ml^{-1} and a tumour diameter less than 3 cm (G), with an AFP concentration below 400 ng ml^{-1} and a tumour size of less than 3 cm (H), with an AFP concentration below 1000 ng ml^{-1} and a tumour size less than 2 cm (I), with an AFP concentration below 400 ng ml^{-1} and a tumour size less than 2 cm (J), and in patients with benign liver diseases.

When graded according to the serum concentration of AFP, the HCC group included 245 patients with concentrations less than 1000 ng ml^{-1}, 178 patients with less than 400 ng ml^{-1} and 64 patients with less than 100 ng ml^{-1}. The mean indices of these groups were 32 ± 30% (Fig. 3A), 31 ± 29% (3B) and 27 ± 26% (3C), respectively. All these values were significantly higher than those for patients with benign liver diseases (4 ± 7%).

When tumours were graded by size, the HCC group contained 104 patients with a tumour diameter less than 5 cm, 70 with a diameter less than 3 cm, and 33 with a diameter less than 2 cm. The mean indices of these groups were 33 ± 30%, 33 ± 30%, and 32 ± 28%, respectively (Fig. 4D, E, F). The difference between these values and those of benign groups was also significant.

When graded according to a combination of tumour size and serum AFP concentration, 56 patients with HCC had serum concentrations of AFP below 1000 ng ml^{-1} and a tumour diameter of less than 3 cm, and 36 patients had AFP concentrations below 400 ng ml^{-1} and tumour diameter of less than 3 cm. Thirty patients had AFP concentrations below 1000 ng ml^{-1} and a tumour diameter of less than 2 cm, and 17 below 400 ng ml^{-1} and a tumour diameter of less than 2 cm. The mean indices of these groups were 31 ± 31%, 33 ± 31%, 32 ± 29% and 30 ± 31%, respectively (Fig. 5G, H, I, J). These values were significantly higher than those in patients with benign diseases (4 ± 7%).

Switching from nonfucosylated to fucosylated AFP after the malignant transformation of hepatocytes

Previously, we have reported on clinical cases in which the fucosylation index of AFP rose in association with a development of HCC from liver cirrhosis during long-term follow-up of patients [56].

In one case, a 66-year-old woman was admitted to hospital in May 1979 because of an increased concentration of serum AFP of 530 ng ml^{-1}. However, several imaging modalities, such as ultrasonography and selective celiac arteriography showed no evidence of HCC. Histological examination of a specimen obtained by peritoneoscopy-guided needle biopsy indicated early cirrhosis. Her serum AFP then fell gradually to below 20 ng ml^{-1} and she was discharged. The serum concentration of AFP rose again to 390 ng ml^{-1} in February 1984. Arteriography revealed an oval-shaped HCC (4.7×2.7 cm) in segment 6 of the right lobe. The AFP fucosylation index was 7% at her first hospital admission, when early cirrhosis was diagnosed, but was 89% when she had clear evidence of HCC. Five other similar cases were also described in which a distinct change in the AFP index was observed over 2–9 years.

Early recognition of HCC by fucosylation index

It is most desirable for the early diagnosis of HCC to monitor patients with chronic liver diseases and predict the development of HCC. We previously described a prospective study of three patients with liver cirrhosis in which the prediction of HCC was made possible by the measurement of the fucosylation index of AFP [57]. Increased levels of the index were associated with a moderate increase in the serum concentration of AFP when there was no evidence of HCC by several imaging modalities. In one case a small HCC with a tumour diameter of less than 2 cm was detected four years after the increase in the index. In the other two cases, small HCCs were detected 9 months and 24 months later. However, the development of HCC was not detected in five cases with liver cirrhosis in which there was a continuous increase in the concentration of serum AFP, but a low index during 3–9 years follow-up. These results indicate that the fucosylation index of AFP can be used for the prediction of HCC especially in patients with liver cirrhosis during long-term follow-up in which there is a moderate increase in the serum concentration of AFP.

Similar observations were reported by Sato *et al.* [58] and Endo [59] for the LCA-reactive species and the *Phaseolus vulgaris*-agglutinin reactive species of AFP.

Altered glycosylation of other serum glycoproteins in HCC

Altered glycosylation of other serum glycoproteins has been recognized in patients with HCC. We determined the reactivity of alpha-1-antitrypsin (AAT) with LCA in 71 patients with HCC, 23 patients with carcinoma metastatic to the liver from the digestive organs, 120 patients with non-neoplastic liver diseases (33 with acute hepatitis, 33 with chronic hepatitis and 54 with liver cirrhosis) and 32 normal controls [60]. Two species of AAT, LCA-reactive and LCA-nonreactive species were detected on crossed immunoaffinity electrophoresis which is a similar result to that of AFP. The percentage of LCA-reactive species and the serum concentration of AAT in HCC and carcinoma metastatic to the liver, were significantly higher than those in non-neoplastic liver diseases and normal controls. We also tested 15 pairs of sera from patients with a long history of chronic liver diseases, before and after subsequent development of HCC. Although there was no significant difference in the serum AAT concentration before and after, the percentage of the LCA-reactive species of AAT after development of HCC was significantly higher than before. Recently, we determined the chemical structures of the sugar chains of the AAT species separated by LCA [61]. Alpha 1-6 fucosylation of the biantennary complex type sugar chain at the innermost GlcNAc residue was found to be the basis for the reaction of LCA with AAT, a similar finding to that observed from AFP from HCC patients.

Altered glycosylation has also been observed in serum transferrin (Tf) from patients with HCC [62] and from culture medium of the HCC cell line Hep G2 [63]. The presence of fucosylated triantennary, tetraantennary and pentaantennary glycans has been described. Furthermore, there is evidence to indicate an increase in the fucosylation of the sugar chains of Tf, not only in the trimannosyl core, but also in the outer chain [62].

Concluding remarks

In this review article, the author has shown the usefulness of measuring lectin-reactive species of AFP in the differential diagnosis of HCC and benign liver diseases, even if the disease is at an early stage. However, crossed immunoaffinoelectrophoresis has certain limitations. It is our aim therefore to provide a more convenient method which distinguishes between fucosylated and non-fucosylated AFP species in routine clinical work [64, 65]. A similar approach is being taken by Bellet *et al.* [66] in the development of a monoclonal radioimmunoassay for AFP; however antibodies utilized so far are all against the protein portion of AFP. Accordingly, it is hoped to develop the enzyme immunoassay using an antibody which directly recognizes the carbohydrate moiety of AFP should be developed.

References

1. Okuda K and The liver cancer study group of Japan (1980). *Cancer* **45**:2663–69.
2. Miyaji T (1977) *Gann Monogr* **14**:179–83.
3. Abelev GI, Perova S, Khramkova N, Postinikova Z, Irlin Y (1963) *Transplantation* **1**:174–80.
4. Tatarinov YS (1965) *Vopr Med Khim* **11**:20–24.
5. Masopust J, Kithier K, Radl J, Koutecky J, Kotal L (1968) *Int J Cancer* **3**:364–73.

6. Alpert ME, Uriel J, de Nechaud B (1968) *N Engl J Med* **278**:984–86.
7. O'Conor GI, Tatarinov YS, Abelev GI, Uriel J (1970) *Cancer* **25**:1091–98.
8. Nishi S, Hirai H (1973) *Gann Monogr* **14**:79–87.
9. Karvountzis GG, Redeker AG (1974) *Intern Med* **80**:156–60.
10. Chen D-S, Sung J-L (1977) *Cancer* **40**:779–83.
11. Alpert E, Feller ER (1978) *Gastroenterology* **74**:856–58.
12. Yumoto Y, Jinno K, Tokuyama K, Ishimitsu T, Maeda H, Konno T, Iwamoto S, Okuda K (1985) *Radiology* **154**:19–24.
13. Schwerk WB, Schmitz–Moormann P (1981) *Cancer* **48**:1469–77.
14. Shinagawa T, Ohto M, Kimura K, Tsunetomi S, Morita M, Saisho H, Tsuchiya Y, Saotome N, Karasawa E, Miki M, Ueno T, Okuda K (1984) *Gastroenterology* **86**:495–502.
15. Purves LR, MacNab M, Rolle M, Bersohn I (1969) *S Afr Med J* **43**:1194–96.
16. Smith CJ, Kelleher PC, (1973) *Biochim Biophys Acta* **317**:231–35.
17. Alpert E, Drysdale JW, Isselbacher KJ, Schur PH (1972) *J Biol Chem* **247**:3792–98.
18. Gustine DL, Zimmerman EF (1973) *Biochem J* **132**:541–51.
19. Purves LR, Merwe EVD, Bersohn I (1970) *Lancet* **2**:464–65.
20. Bayard B, Kerchaert J–P (1977) *Biochem Biophys Res Commun* **77**:489–95.
21. Smith CJ, Morris HP, Kelleher PC (1977) *Cancer Res* **37**:2651–56.
22. Smith CJP, Kelleher PC, Belanger L, Dallaire L (1979) *Br Med J* **i**:920–21.
23. Ruoslahti E, Pekkala A, Comings DE (1979) *Br Med J* **ii**:768–69.
24. Toftager-Larsen K, Kjaersgaard E, Jacobsen JC, Nøgaard–Pedersen B (1980) *Clin Chem* **26**:1656–59.
25. Buamah PK, Taylor P, Ward AM (1981) *Clin Chem* **27**:1658–60.
26. Ishiguro T, Sakaguchi H, Sugitachi I (1983) *Am J Reprod Immunol* **3**:61–64.
27. Ruoslahti E, Engvall E, Pekkala A, Seppälä M (1978) *Int J Cancer* **22**:515–20.
28. Breborowicz J, Mackiewicz A, Breborowicz D (1981) *Scand J Immunol* **14**:15–20.
29. Aoyagi Y, Suzuki Y, Isemura M, Soga K, Ozaki T, Ichida T, Inoue K, Sasaki H, Ichida F (1984) *Gann* **75**:809–15.
30. Buamah PK, Harris R, James OFW, Skillen AW (1986) *Clin Chem* **32**:2083–84.
31. Taketa K, Hirai H (1989) *Electrophoresis* **10**:562–67.
32. Smith CJP, Kelleher PC (1980). *Biochim Biophys Acta* **605**:1–32.
33. Taketa K (1990) *Hepatology* **12**:1420–32.
34. Aoyagi Y (1994) *Int J Oncology* **4**:369–83.
35. Liver Cancer Study Group of Japan (1987) *Cancer* **60**:1400–11.
36. Liver Cancer Study Group of Japan (1988) *Acta Hepatol Jpn* **29**:1619–26.
37. Liver Cancer Study Group of Japan (1993) *Acta Hepatol Jpn* **30**:805–13.
38. Alpert E (1975) *Clin Chim Acta* **58**:77–83.
39. Bisceglie AMD, Hoofnagle JH (1989) *Cancer* **64**; 2117–20.
40. Lehmann F-G, Wegener T (1979) In *Carcino-Embryonic Proteins, Vol I*, (Lehmann F-G ed.), pp. 219–31. Elsevier/North–Holland Biomedical Press.
41. Morinaga T, Sakai M, Wegmann TG, Tamaoki T (1983) *Proc Natl Acad Sci USA* **80**:4604–8.
42. Gibbs PEM, Zielinski R, Boyd C, Dugaiczyk A (1987) *Biochemistry* **26**:1332–43.
43. Pucci P, Siciliano R, Malorni A, Marino A, Marino G, Tecce MF, Ceccarini C, Terrana B (1990) *Biochemistry* **30**:5061–66.
44. Yoshima H, Mizuochi T, Ishii M, Kobata A (1980) *Cancer Res* **40**:4276–81.
45. Yamashita K, Hitoi A, Tsuchida Y, Nishi S, Kobata A (1983) *Cancer Res* **43**:4691–95.
46. Aoyagi Y, Isemura M, Yosizawa Z, Suzuki Y, Sekine C, Ono T, Ichida F (1985) *Biochim Biophys Acta* **830**:217–23.
47. Kornfeld K, Reitman ML, Kornfeld R (1981) *J Biol Chem* **256**:6633–40.
48. Baenziger JU, Fiete D (1979) *J Biol Chem* **254**:2400–7.
49. Hase S, Ibuki T, Ikenaka T (1984) *J Biochem* **95**:197–203.
50. Yamamoto S, Hase S, Fukuda S, Sano O, Ikenaka T (1989) *J Biochem* **105**:547–55.
51. Tomiya N, Awaya J, Kurono M, Endo S, Arata T, Takahashi N (1988) *Anal Biochem* **171**:73–90.
52. Aoyagi Y, Suzuki Y, Igarashi K, Saitoh A, Oguro M, Yokota T, Mori S, Suda T, Isemura M, Asakura H (1993) *Br J Cancer* **67**:486–92.
53. Aoyagi Y, Isemura M, Suzuki Y, Sekine C, Soga K, Ozaki T, Ichida F (1985) *Lancet* **ii**:1353–54.
54. Aoyagi Y, Suzuki Y, Isemura M, Nomoto M, Sekine C, Igarashi K, Ichida F (1988) *Cancer* **61**:769–74.
55. Aoyagi Y, Suzuki Y, Igarashi K, Saitoh A, Ogura M, Yokota T, Mori S, Nomoto M, Isemura M, Asakura H (1991) *Cancer* **67**:2390–94.
56. Aoyagi Y, Isemura M, Suzuki Y, Sekine C, Soga K, Ozaki T, Ichida F (1986) *Lancet* **i**:210.
57. Aoyagi Y, Saitoh A, Suzuki Y, Igarashi K, Ogura M, Yokota T, Mori S, Suda T, Isemura M, Asakura H (1993) *Hepatology* **17**:50–52.
58. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S (1993) *N Engl J Med* **328**:1802–6.
59. Endo Y (1992) In *Primary liver cancer in Japan*, (Tobe T, Kameda H, Okudaira M, Ohto M, Endo Y, Mito M, Okamoto E, Tanikawa K, Kojiro M eds):Springer–Verlag pp. 163–173.
60. Sekine C, Aoyagi Y, Suzuki Y, Ichida F (1987) *Br J Cancer* **56**:371–75.
61. Saitoh A, Aoyagi Y, Asakura H (1993) *Arch Biochem Biophys* **303**:281–87.
62. Yamashita K, Koide N, Endo T, Iwaki Y, Kobata A (1989) *J Biol Chem* **264**:2415–23.
63. Campion B, Leger D, Wieruszeski JM, Montreuil J, Spik G (1989). *Eur J Biochem* **184**:405–13.
64. Suzuki Y, Aoyagi Y, Muramatsu M, Isemura M, Ichida F (1987) *Br J Cancer* **55**:147–52.
65. Suzuki Y, Aoyagi Y, Muramatsu M, Igarashi K, Saitoh A, Oguro M, Isemura M, Asakura H (1990) *Ann Clin Biochem* **27**:121–28.
66. Bellet DH, Wands JR, Isselbacher KJ, Bohuon C (1984) *Proc Natl Acad Sci USA* **81**:3869–73.