Monitoring Hepatocellular Carcinoma by Using a Monoclonal Immunoenzymometric Assay for Alpha-Fetoprotein

Martin L. Keilsten,1 Daniel W. Chan,2 Debra J. Bruzek, and Robert C. Rock

A monoclonal immunoenzymometric assay for alpha-fetoprotein (M-AFP) was evaluated with respect to its utility in monitoring hepatocellular carcinoma (HCC) patients. Earlier (Clin Chem 1986;32:1318–22), we found this immunoassay to demonstrate abilities similar to polyclonal AFP assays, and we suggested that changes in M-AFP correlated with changes in intrahepatic tumor volume in most HCC patients. In the present study, 107 HCC patients were evaluated between 1978 and 1986. Patient demographics characterized this study population as being similar to those seen in regions with low incidence of HCC. Changes in serum M-AFP concentration correlated moderately (r = 0.55) with changes in intrahepatic tumor volume. The AFP concentration in serum was found to be a statistically significant independent predictor of survival; patients with above-normal M-AFP (AFP[+]) at presentation demonstrated a median survival time of 10 months, compared with 16 months for patients with “normal” values for M-AFP (AFP[−]) (P = 0.008). This prognostic pattern persisted when adjusted for serum bilirubin concentration (AFP[+] 12 months vs AFP[−] 29 months, P = 0.01).

Hepatocellular carcinoma (HCC) is a disease of major epidemiological importance because of its high prevalence in certain parts of the world and its association with hepatitis B virus (HBV) (1). In addition, its high mortality rates make prevention an especially desirable goal (2). The incidence of HCC varies considerably in different parts of the world. It is most common in Africa and the Far East (3–8), and there are geographic variations in incidence by sex and age (5, 9, 10). The various factors associated with HCC—HBV, aflatoxin, and cirrhosis (of any cause)—in different parts of the world suggest a multifactorial etiology.

Serum alpha-fetoprotein (AFP), the major protein in fetal circulation during early life (11), increases above the low concentrations seen in normal adults (12) in various disease states, including hepatitis neoplasms (primary and metastatic), germ-cell tumors (12, 13), hepatitis, and cirrhosis (14). The development of quantitative immunoasays for AFP that are sufficiently sensitive to allow detection of AFP in normal serum has had one unfortunate consequence: nonspecificity in distinguishing between malignant and nonmalignant causes of moderately increased AFP concentrations.

Recently, the first FDA-approved monoclonal AFP reagent involving a two-site immunoassay was evaluated by Chan et al. (19) in patients with HCC, other malignant tumors, and various non-malignant conditions. As previously demonstrated with polyclonal-antibody AFP assays (15–18), most HCC patients had increased AFP values, and in most other diseases concentrations were lower, but there was much overlap among the disease groups.

Though sensitivity for early detection of HCC with AFP was best when the upper limit of “normal” (9 μg/L) was used as the decision level, discrimination between HCC and other diseases was most efficient when a higher decision level (100 μg/L) was used. Discrepancies seen between results for the monoclonal and the polyclonal assay may be accounted for in part by the presence of AFP variants (19, 20).

In addition to its use in the diagnosis of HCC, assay of serum AFP has also been used to monitor the course of HCC (21, 22). In our previous study in which we used a monoclonal assay (19), preliminary data suggested that changes in serum AFP correlated with changes in intrahepatic tumor volume in most HCC patients. Here, we further evaluate the utility of monitoring HCC patients with this monoclonal AFP immunoassay (M-AFP) during treatment.

Materials and Methods

Methods

Serum AFP was determined with the TANDEM®-E AFP (Hybritech Inc., San Diego, CA 92121)—a solid-phase, two-site immunoenzymometric assay (M-AFP)—as previously described (19). Liver-function tests (SMAC continuous-flow analyzer; Technicon Corp., Tarrytown, NY 10591) and hepatitis B serologic studies (“Abbott EIA”; Abbott Labs., North Chicago, IL 60064) were also done. Liver tumor volumes were calculated from computer-assisted tomographic (CT) scans as described by Order et al. (23). Statistical analysis of discrete data was performed by chi-square computations. Kaplan–Meier survival analyses (24) were generated from computer software provided by the Johns Hopkins Oncology Center, which included log rank and generalized Wilcoxon calculations, as well as determination of median survival times with confidence intervals estimated by the Brookmeyer–Crowley method. Time of survival was defined as the interval from the date of initial presentation until the date of death.

Patients

Serum AFP concentrations were determined on specimens from 111 “normal” blood-bank donors, from 107 HCC patients, from 211 patients with non-HCC neoplastic diseases, from 156 patients with non-neoplastic liver disease, and from 174 patients with various other disorders (19).

The study population consisted of 107 HCC patients seen between 1978 and 1986, who averaged four specimens per patient. These patients either received their primary tissue diagnosis of HCC at The Johns Hopkins Hospital or had the diagnosis confirmed following referral. They were divided
into two subgroups (pre- and post-treatment), defined by the initiation of any treatment regimen, e.g., surgical excision, radiotherapy, chemotherapy—including 5-fluorouracil, doxorubicin, mitomycin C, vincristine, and cis-platinum—and (or) immunotherapy with $^{131}$I-labeled anti-ferritin. The histories of these patients were systematically reviewed. Liver function and hepatitis serologic status were assessed, as well as AFP. To more clearly delineate the relationships between tumor volume, serum AFP value, and therapy, each time period between successive determinations was analyzed separately.

Results

AFP Distribution among Different Disease Groups

A total of 1343 samples from 759 patients were analyzed for M-AFP. Figure 1 shows the distribution of the results among these patients, published previously (19). For most (77%) of the HCC cases M-AFP results were abnormal (>9 μg/L), but overlapped with the normal reference interval. Most M-AFP values that were >20 μg/L and <300 μg/L in patients with benign liver disease were associated with hepatitis B virus (HBV) infection.

Figure 2 expands upon the relationship between AFP and hepatitis serology in patients without HCC. The patients with HBV-related hepatitis and cirrhosis had significantly supranormal values for serum AFP compared with non-HBV-related liver disease. Those with hepatitis (especially associated with HB$_B$Ag and anti-HB$_B$) showed greater increases than those with cirrhosis. No other patterns in serology were observed. On the other hand, as shown in Figure 3, there was no obvious relationship between M-AFP and HCC with respect to hepatitis B serology. This was true regardless of type and extent of liver disease, as well as therapeutic intervention. A possible explanation for these findings is discussed later.

Hepatocellular Carcinoma (HCC)

Patient demographics. In the 107 HCC cases, the ages at presentation ranged from five to 80 years (average 50.6) with 1.7 males seen for each female. The racial distribution was 80 (76%) white patients, 14 (13.5%) black patients, eight (7.5%) Oriental patients, two (1.5%) Hispanic patients, and three (2.5%) other patients.

As shown in Table 1, 82 (77%) of these 107 HCC patients had at least one above-normal value for M-AFP in serum (AFP+1) during the course of their disease, with 38 (46%) of these at some time exhibiting metastatic disease. There were 74 patients with pre-treatment AFP values. Within this group of patients, 63 had supranormal values for AFP. On average, there were four specimens per patient analyzed throughout the course of the disease.

Association with liver diseases. The relationship between M-AFP and hepatitis B serology or the presence of cirrhosis in these patients is seen in Table 2. Thirty-two (39%) of the AFP+ patients exhibited serologic evidence of exposure to the hepatitis B virus (HBV)—15 (18%) HB$_B$Ag-positive and 17 (21%) HB$_B$-antibody-positive—compared with six (24%) patients with normal values for AFP (AFP–) (P > 0.05)—two (8%) HB$_B$Ag+1 (P > 0.05) and four (16%) antibody-positive (P > 0.05). Therefore, none of these groups differed significantly from one another. About a quarter of each group had an incomplete laboratory evaluation with respect to hepatitis B status. Most of the patients had no documentation as to the presence or absence of cirrhosis; however, of the 39 (48%) AFP+1 patients with this information, 34 (87%) were cirrhotic and five (13%) were non-cirrhotic.
Table 1. HCC Patient Categories (107 Patients)

<table>
<thead>
<tr>
<th>AFP*</th>
<th>Metastases</th>
<th>Treatment^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)  53(65%)</td>
<td>(-)  38(46%)</td>
<td></td>
</tr>
<tr>
<td>(+)  82(77%)</td>
<td>(+)  53(65%)</td>
<td></td>
</tr>
<tr>
<td>(+)  38(46%)</td>
<td>(+)  38(46%)</td>
<td></td>
</tr>
<tr>
<td>(-)   9(36%)</td>
<td>(-)   4(16%)</td>
<td></td>
</tr>
<tr>
<td>(-)  25(23%)</td>
<td>(-)   7(30%)</td>
<td></td>
</tr>
<tr>
<td>(+)  16(64%)</td>
<td>(+)  16(64%)</td>
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*AFP as determined by a monoclonal immunoenzymometric assay (M-AFP). Note: a patient may be represented more than once in this table.

^b - , pre-treatment; +, post-treatment.

Survival statistics. Figures 4 and 5 show the Kaplan-Meier patient-survival curves with respect to initial AFP and bilirubin status. The AFP and bilirubin status each alone demonstrated significant differences in survival. Regarding AFP status (Figure 4), the median survival of AFP(-) patients, 16 months, is statistically significantly longer than the 10 months for AFP(+ patients (P = 0.008). However, conversion of AFP status from negative to positive had no significant association with survival (P >0.05). Patients with normal values for bilirubin at presentation (Figure 5) showed a 14-month median survival, compared with six months for those with values >20 mg/L, a statistically significant difference (P <0.001). By adjusting for potential interferences in survival owing to an increased bilirubin concentration, AFP(-) status continued to predict improved survival (Figure 6, 29 vs 12 months, P = 0.01). Hepatitis B status and cirrhosis status were not associated with prolonged survival (data not shown).

Monitoring HCC with M-AFP

Correlation with tumor volume. Liver tumor volume (TV) and serum M-AFP concentration are roughly correlated (r = 0.55), as shown in Figure 7. This plot contains points corresponding to each time interval between clinical visits for patients with an increased M-AFP and metastatic disease who had received treatment (AFP(+)/MET(+)/TX(+)). Such plots for AFP+/MET(-)/TX(-) and AFP+MET(-)/TX+ showed even weaker correlation (not shown). For the same patient groups, correlations were poor between M-AFP and serum alkaline phosphatase and between TV and alkaline phosphatase. No significant correlations were found among the other variables examined, including serum bilirubin, clinical condition, and metastatic status.

We define a significant change in a value as one ≥10%. Of the 67 HCC patients with values from two or more points in time, 15 (22%) showed complete agreement in direction of change of M-AFP and TV for all points and 40 (59%) had at least 50% agreement. Complete disagreement was seen in 17 (25%) of the patients. These results are statistically significant (P <0.025).

Effect of a change in metastatic status. During the course of this study, eight (7%) patients experienced a change in their metastatic disease status. Seven developed metastases, and one was apparently clinically cleared of all metastatic, as well as primary disease. In an attempt to use the development of metastases to explain an increase in M-AFP, we evaluated AFP+ patients without metastases and receiving treatment (AFP+MET(-)/TX+). Of the seven

Table 2. Relationship among AFP Status, HBV Serology, and Cirrhosis Status

<table>
<thead>
<tr>
<th>Hepatitis B serology</th>
<th>Cirrhosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td><strong>Neg</strong></td>
</tr>
<tr>
<td><strong>AFP[+]</strong></td>
<td></td>
</tr>
<tr>
<td>82(77%)</td>
<td>37(45%)</td>
</tr>
<tr>
<td><strong>AFP[-]</strong></td>
<td>25(23%)</td>
</tr>
<tr>
<td><strong>Anti-HB</strong></td>
<td>12(16%)</td>
</tr>
<tr>
<td><strong>Unknown</strong></td>
<td>16(20%)</td>
</tr>
</tbody>
</table>

^Anti-HB: presence of antibodies to any of the different hepatitis B antigens.
patients who subsequently developed metastases, six showed a significant increase in M-AFP, compared with 46 increases among 110 patients with known metastases. Although this involves small numbers and was not of statistical significance (P > 0.05), these results suggest that, in patients without metastases, an increase in M-AFP could be predictive of conversion to positive metastatic status.

Discussion

Characteristics of HCC Study Populations

Epidemiologic studies of hepatocellular carcinoma (HCC) have suggested that low-incidence and high-incidence populations exhibit many demographic and environmental differences (2). In the present population, the age incidence, sex ratio, racial distribution, and associated diseases were, as expected, consistent with that seen in low-incidence populations. The significance of these differences seen in various populations is unclear, and may represent varying environmental exposures, host susceptibility, or intrinsic differences in the disease itself. Nevertheless, the importance of knowing the characteristics of the study population in HCC is not completely understood, so such data should be included in any report of this disease.

Prognostic Indicators

The product limit method of Kaplan and Meier (24) was chosen over the life-table method for estimating the survival distribution. Although both methods are similar in concept, the Kaplan–Meier method is appropriate for any number of patients, but the life-table method requires a large patient population (25).

Comparisons of survival rates (Figures 4–6) revealed that alpha-fetoprotein (AFP) status and serum bilirubin concentration are significant factors in prognosis. A bilirubin >20 mg/L upon presentation is associated with a grave prognosis (26). HBV status was statistically insignificant as a prognosticator, but has shown varying trends (26, 27). The presence of cirrhosis, usually thought to be a necessary feature of HCC in a low-incidence population, was not present in six patients and had no significant effect upon survival. Other factors that have been investigated include sex, age, performance status, alkaline phosphatase, gamma globulins (27), metastatic lung involvement (26), and cirrhosis (28); only age and lung metastases have proved to be of statistical importance. On the other hand, this study reveals that an increased serum AFP concentration suggests a worse prognosis, independent of bilirubin concentration. Falkson et al. (27) found an opposite trend for AFP, which was statistically insignificant; however, in their study they used a polyclonal radioimmunoassay (27). The importance of this finding is not known at this time, but it could serve to determine which patients are at higher risk of recurrence and should be treated more aggressively.

Monitoring HCC with M-AFP

As shown previously by Chan et al. (19), a monoclonal AFP immunoassay (M-AFP) can detect HCC with maximum efficiency at a decision level of 100 µg/L. In addition, it was suggested that, in most HCC patients who exhibit significant changes in AFP and tumor volume, monitoring serum AFP over time would reveal concordance between changes in serum AFP and tumor volume. Further analysis of these and other patients was performed in the present study.

If one considers each time period between laboratory studies as a separate event, there is some correlation between M-AFP and liver tumor volume (TV), and weaker correlations between M-AFP and alkaline phosphatase and between TV and alkaline phosphatase. In addition, 75% of the patients agreed at least once simply in direction of change of M-AFP and TV, with 59% exhibiting agreement at least 50% of the time. This correlation was not significantly affected by the presence of metastases. In fact, although it was not statistically significant, there was a suggestion that, in a small group of patients, metastases were heralded by an M-AFP increase.

Most clinical studies in which AFP was used as a monitoring tool compare different therapeutic modalities (21, 22, 29–33). The present data corroborate most of the earlier data obtained with polyclonal assays. For example, Melia et al. (32) evaluated 35 HCC patients for changes in AFP and ferritin in response to intravenous doxorubicin and (or) gel-foam embolization. The average decrease and range of decrease in these markers was provided, and it was concluded that the markers were useful in gauging therapeutic response. Other authors make similar semiquantitative observations.

A new test is useful if it contributes some new attribute to currently available studies by serving a prognostic purpose, by demonstrating clinical changes in a timely fashion, or by monitoring the effects of therapy—all involving as little time, pain, and risk to the patient as possible.
The present study has detailed information of general statistical, as well as more specific, importance with regard to the above issues. First of all, the test involves a relatively simple serum immunoassay. Next, the direction of change in M-AFP concentration roughly correlates with change in liver tumor volume, with or without treatment. Also suggested is prediction of metastases. Both of these are important for clinical monitoring. The findings related to survival—i.e., decreased with increased bilirubin and increased values for M-AFP—serve a general prognostic purpose at the time of diagnosis. The utility of the test in an individual patient is that test results for the most part mirror the clinical progress of the patient regardless of treatment. During periods of discrepancy between M-AFP concentration and TV or clinical status, one should look for an explanation; for example, inflammatory liver disease, metastases, and terminal disease were suspected in approximately half of our cases. Furthermore, some disagreement between AFP and TV may not be unexpected, because severely necrotic liver tumor may not produce as much AFP. It has been suggested that AFP is synthesized by the regenerating liver cell (35).

The Association between AFP Production and Hepatitis B Virus Infection

It was of interest to note, as demonstrated in Figures 1 and 2, that most of the significant increases in serum AFP (20 µg/L) observed in patients with benign liver disease occur in diseases associated with hepatitis B virus (15, 16, 34). In the present data, approximately two-thirds of the "benign" increases (see Figure 1) were directly associated with active hepatitis, both acute and chronic, and approximately two-thirds of those cases (see Figure 2) were directly associated with the presence of HBsAg and anti-HBc. No similar association was demonstrated between AFP and hepatitis serology in HCC patients. However, retrospective data-gathering showed that 20% of the samples were from patients with incomplete hepatitis serology.

It is often stated that AFP synthesis is associated with liver cell regeneration (35). However, Alpert and Feller (36) did not find this to be supported in post-hepatectomy samples, and they suggested that viruses and other toxins may induce a "distinctive type of cell injury or altered hepatocyte regeneration" resulting in AFP synthesis. These data, together with other literature (34, 37, 38), suggest some association between hepatitis B infection and AFP synthesis.

The mechanism of such an association is unknown. One possibility, suggested by Shafritz et al. (39), recognizes the ability of integrated DNA to cause cellular transformation in vitro of cultured cells that produce solid tumors after they are injected into animals. This could therefore imply that integration of HBV-DNA in hepatocytes may be one event preceding the development of gross neoplasia. On the other hand, the presence of virus may also result in altered cell growth, which could then be responsible either directly or indirectly for the expression of AFP. Because these changes may precede presentation of gross neoplasia by many years, a prospective study would be needed to establish any possible relationship between progression of serum AFP or HBV serologies, HBV-DNA integration, and tumor development.

In addition, with the present data associating seronegativity, expression of HBV-DNA in hepatocytes, and development of hepatocellular carcinoma (40, 41), one would like to assess if a seronegative patient with a significantly increased serum AFP, whether associated with HCC or not, exhibits HBV-DNA in hepatocytes and whether this could have any relationship to the disease prognosis. This could also provide a possible explanation for the observed discrepancy between AFP and HBV serologic status and survival seen above. That is to say, a worse prognosis may be associated with AFP synthesis related to HBV, but this association is not necessarily evident in the HBV serology because of variable expression of these serologic markers.

In conclusion, the highly lethal disease of hepatocellular carcinoma was studied in a low-incidence population with a monoclonal immunoenzymometric serum assay for AFP. Some correlation was seen between M-AFP concentration changes and intrahepatic tumor volume changes, and it was demonstrated to be a fairly good index to the clinical course. Most importantly, serum AFP concentration was found to be an independent predictor of survival. The association between hepatitis B virus and AFP synthesis is unclear. With greater understanding of this relationship, better tests can perhaps be developed for therapeutic purposes and there can be more investigative progress in prevention and cure.

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References