Clonal Origin of Human Hepatoma Determined by Integration of Hepatitis B Virus DNA

Mariko Esumi, Tomoki Arihata, Masayuki Arii, Koyu Suzuki, Kyuchi Tanikawa, Hitoshi Mizuo, Toshiaki Mima, and Toshio Shikata

First Department of Pathology, Nihon University School of Medicine, 30, Ohyaguchi-kamimachi, Itabashi-ku, Tokyo 173 [M. E., M. A., K. S., T. S.]; Second Department of Internal Medicine, Kurume University School of Medicine, 67, Asahi-machi, Kurume 830 [T. A., K. T.]; and Kin’ikyo Chuo Hospital, 10-2, Fushiko, Higashi-ku, Sapporo 065 [H. M., T. M.], Japan

ABSTRACT

The hepatitis B virus genome is integrated in cellular DNA of human hepatocellular carcinoma from hepatitis B surface antigen-positive patients. Using this phenomenon, we determined the clonal origin of hepatocellular carcinoma from the integration mode of hepatitis B virus DNA. The molecular size and the number of restriction fragments of integrated hepatitis B virus DNA in several parts of tumors in the same liver and in metastatic tumors were compared by Southern blot analysis. Of 14 cases of hepatoma, 13 cases were monoclonal; in one case, a different clone of hepatoma was found in one part of the tumor. In three of 13 cases of monoclonal hepatoma, metastatic tumors in lymph nodes and the lung were also examined and found to be the same clone as the liver tumors. These results indicate that hepatocellular carcinomas were usually generated from a single tumor cell even though tumor cells spread in the liver and invaded other organs for a long time. Development of different clones of tumor was apparently unusual but was observed in one case of hepatocellular carcinoma.

INTRODUCTION

Information on the clonal origin of human cancers, including HCC, is important for determining the cause of carcinogenesis and suitable therapeutical treatments. HCCs seem to be polyclonal in origin in some cases, such as when two small liver cancers with a nodular septum are present in the right and left lobe, or when several tumors in the same liver show different histological features. However, there is no precise evidence or an adequate method for determining the clonal origin of HCCs. In this work we analyzed the clonal origin of HCCs using the phenomenon of integration of HBV DNA into HCC.

Hepatitis B virus is etiologically linked to the development of HCCs (1), and HBV DNA has been found to be integrated into the cellular DNA of HCCs (2-4) and HCC cell lines (2, 5-7). Integration can occur at variable sites for both the cellular and viral DNAs, following rearrangement of viral and cellular sequences (8-10) or chromosomal deletion (11). On Southern blot hybridization analysis, a restriction fragment containing HBV DNA can be seen as a definite band because of clonal integration and the integration sites in chromosomal DNA can be used to determine whether multiple HCCs in a given case are of clonal origin. Therefore, we examined the molecular sizes and numbers of integrated fragments of HBV DNA in several parts of HCCs including metastases. The clonal states of the tumors were determined by comparing the modes of integration of HBV DNA.

MATERIALS AND METHODS

Tissue Specimens. Tissue specimens were obtained at autopsy from 14 HBV surface antigen-positive cases of HCC. Thirty to 800 mg of tumor tissues were obtained from 2 to 7 parts of each HCC in different nodules. Metastatic tumors were available in 3 cases. The samples were immediately frozen and stored at -80°C. Tissues adjacent to these frozen specimens were fixed with 10% formalin and embedded in paraffin for histological examination.

DNA Extraction. Tumor tissues were minced with scissors and washed 3 times with cold 0.01 M sodium and potassium phosphate/0.14 M NaCl/0.003 M KCl. The samples were then digested overnight at 37°C with protease K (Merck, Darmstadt, West Germany) at 100 µg/ml in 0.01 M Tris-HCl (pH 8.0)/0.01 M EDTA/0.1 M NaCl/0.2% SDS and extracted with phenol and then chloroform/isooamyl alcohol (24:1). The aqueous phase was dialyzed against 0.01 M Tris-HCl (pH 8.0)/0.001 M EDTA/0.1 M NaCl, treated with RNase A (Cooper Biochemical, Inc.) at 50 µg/ml at 37°C and then with protease K (100 µg/ml) and 0.3% SDS at 37°C for 1 h, and extracted as before. Purified DNAs were precipitated twice with ethanol and dissolved in distilled water.

Southern Blot Analysis. Samples of 20 µg of cellular DNA were digested with 60 units of HindIII, EcoRI, or PvuII (Takara Shuzo Co., Kyoto, Japan) at 37°C overnight. These restriction enzymes do not cleave HBV DNA of subtype adr. Digested DNA and undigested DNA were subjected to electrophoresis in 0.6% agarose gel and transferred to a nitrocellulose filter (Schleicher & Schuell GmbH, Einbeck, West Germany) (12, 13).

The cloned whole genome of HBV DNA was prepared by cleavage of plasmid pHBl23 (14) with BamHI, followed by recovery from preparative agarose gels, and used as a hybridization probe. The probe was labeled with [γ-32P]dCTP (Amersham, England; 800 Ci/mmol) by the nick-translation procedure (15). The specific activity of the probe was 1 to 2 × 10^6 cpm/µg.

The filters were prehybridized at 42°C for 3 to 18 h in 5 × SSC (0.75 M NaCl/0.075 M sodium citrate), 5 × Denhardt’s solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin), sonicated denatured salmon sperm DNA (100 µg/ml), and 50% deionized formamide. Hybridization was performed at 42°C overnight in an identical solution containing 2 × 10^6 cpm of denatured probe DNA. After hybridization, the filters were washed 3 times at room temperature for 10 min each in 2 × SSC and 0.2% SDS, and 4 times at 50°C for 30 min each in 0.1× SSC and 0.2% SDS. The filters were dried and autoradiographed at -80°C with X-Omat AR film and intensifying screens.

Recently it was reported that human tumor samples were frequently contaminated with bacteria (16). Southern blot analysis with vector probe 322 as a probe was performed as a necessary control for all samples after removing the HBV DNA probe from the hybridized filters.

RESULTS

The clonal origin of HCCs was examined in autopsy specimens of 14 cases (Table 1).
Table I. Clonal origin of hepatocellular carcinoma

Various parts of HCC were sampled from each case of HCC, and their histological features and Southern blot analysis of their DNA were examined. The monoclonal or polyclonal origin of HCC was determined from the mode of integration of HBV DNA.

<table>
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<tr>
<th>Case</th>
<th>sAg/Ab</th>
<th>eAg/Ab</th>
<th>Sample No. of</th>
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<td>S</td>
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*a sAg/Ab, HBV surface antigen/antibody in serum; eAg/Ab, HBV e antigen/antibody in serum; ND, not determined.
*b T, trabecular type; PG, pseudoglandular type; S, solid type; II and III, grade of cellular atypism by Edmondson's classification.
*c Number of bands determined by Southern blot analysis of HindIII fragments of DNA.
*d S, the same clone of tumor in all HCC samples tested; D, different clones of tumor found in different samples tested.

Fig. 1. Clonal analysis of HCC in Case 1. A, section of liver. Squares indicate locations of test samples. B, trabecular type of HCC with moderate differentiation. H & E × 160. C, Southern blot analysis of cellular DNA from the 6 parts of HCC shown in A after digestion with PvuII or HindIII.

Fig. 2. Southern blot analysis of cellular DNA from 4 parts of the HCC in Case 2.

Monoclonal of HCC. As shown in Fig. 1A, Case 1 was a nodular type of HCC; the main tumor was in the middle of the liver, and there were a number of small tumor nodules in other areas. Six samples from different nodules showed the same histological features of moderately differentiated trabecular HCC (Fig. 1B). Hepatitis B virus DNA was integrated into tumor cells in all 6 samples. When DNA was digested with PvuII or HindIII, neither of which cut the HBV DNA sequence, a single fragment of the same size was obtained from all 6 DNAs (Fig. 1C). Therefore, HBV DNA was integrated into the same site of cellular DNA in the 6 tumor samples. Tumor cells growing in different nodules apart from one another were of the same clone; that is, they were generated from a single tumor cell. In Cases 2 to 5 also, tumor cells spreading through the
liver were all of the same clone and had the same histological features. In Case 2 in particular, 7 distinct integrations of HBV DNA were accurately sustained during expanding growth of tumor cells (Fig. 2).

As shown in Fig. 3A, Case 6 had a nodular type of HCC in the left lobe with liver cirrhosis. There were another separate tumor in the right lobe, tumor emboli in the portal vein, and metastases in peripancreatic and perigastric lymph nodes. Histologically, intrahepatic tumors were mainly poorly differentiated trabecular HCCs with scattered giant tumor cells, whereas the portal emboli appeared to be relatively differentiated HCCs (Fig. 3B). Moreover, a metastasis in a perigastric lymph node showed more pleomorphism with desmoplastic change (Fig. 3B). However, as seen in Fig. 3C, Southern blot analysis of these tumor DNAs showed that HBV DNA was integrated into a single site of cellular DNA which was the same in all the scattered tumors. Thus all the tumor cells, including the metastatic tumor cells, were of the same clone. In Cases 7 to 13 also, all the tumor cells in each case were monoclonal in origin, although the morphologies of the various tumors differed (Fig. 4).

Dissimilar Clone of HCC. In Case 14, the main tumor was in the right lobe, and there were a small number of small separated tumor nodules in the left lobe with liver cirrhosis (Fig. 5A). Histologically all 4 samples presented more or less similar features of poorly differentiated HCC (Fig. 5B). Southern blot analysis, however, showed that HCC from the edge of the main tumor had an extra integrated band besides the 2 bands of the other 3 HCCs (Fig. 5C). None of 3 bands containing an extra band was hybridized with a control probe of pBR322 DNA. Samples 1 and 2 contained nontumorous tissues besides HCC. Smears from 2 to 4 kilobase pairs indicated the presence of free HBV DNA in the nontumorous tissues. Sample 1 contained a new clone of HCC. This new clone had HBV DNA integrated into a single new site of cellular DNA or into a new site as well as one or both of the sites of integration in the other tumor samples. Tumor cells of intrahepatic metastases in Samples 2 to 4 were of monoclonal origin.
DISCUSSION

In this work we investigated whether HCCs were monoclonal. Results showed that, in the advanced stage at least, HCCs, including metatstatic tumors, were usually monoclonal. Growth selection during tumor development, if any, may result in apparent monoclonal origin of HCC; a clone with growth advantage may survive, replace, and occupy the tumor. Even when the morphologies of different HCCs in a single case varied, these tumors were found to be of clonal origin. Thus their histological features may have been regulated by environmental factors, not by genetic information.

Then is there a polyclonal state during tumor development or tumor growth? Case 14 was a case in point. Fig. 5 shows that the edge of the main HCC contained a new clone distinct from that of the intrahepatic metastasis. There are 2 possibilities about the new clone in this case: one is that a tumor cell had HBV DNA integrated into a single new site of chromosomal DNA; the other is that the tumor cell had HBV DNA integrated in 2 or 3 sites, one or 2 of which were the same as those in other clones and one of which was an extra new site of chromosomal DNA. The former possibility would mean that the new tumor clone was generated independently, while the latter would mean that it was generated by rearrangement of chromosomal DNA or by superinfection followed by integration of HBV DNA into an established tumor that already had HBV DNA integrated into 2 sites. In either case, the new clone of tumor cells may have been generated by chance around the main tumor. Then it becomes possible that, of 13 putative monoclonal tumors, some of them may actually be biclonal, with one subpopulation of tumor cells containing no detectable HBV DNA. The chance generation of a new clone of tumor cells suggests the generation of various types of tumor cells with respect to metastatic ability and sensitivity to cancer chemotherapy or embolization therapy. In fact, although no tumor developed immediately after transarterial embolization, HCC was found again 5 yr later. Thus a clone that was resistant...
to embolization therapy may have survived and have had a growth advantage. Further investigations are necessary on polyclonal growth of HCC, especially in the early stage of tumor development.

REFERENCES

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