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Clonal state of human hepatocellular carcinoma and non-tumorous hepatocytes

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Summary. We determined the clonal state in specimens of hepatocellular carcinoma (HCC) and non-tumorous hepatocytes from the integration mode of hepatitis B virus (HBV) DNA. The integration mode of HBV DNA in several parts of the tumors and non-tumorous regions of the same liver, as well as in metastatic tumors, was examined using the Southern blot analysis. In 13 of the 14 cases of HCC, the liver tumors, including metastatic tumors in lymph nodes and the lungs, were monoclonal. In one case, a different HCC clone was found in one part of the liver tumor. The integration of HBV DNA was also observed in non-tumorous tissues in 38 of the 78 cases (49%) of chronic hepatitis with and without HCC; in 16 cases of chronic hepatitis in which HBV DNA was integrated, several clones of the hepatocytes that had HBV DNA integrated into their chromosomal DNA and had proliferated clonally were found in non-tumorous tissues. These clones were different from the tumor clone of the same liver. Thus HCCs were usually monoclonal. The development of different tumor clones appeared to be unusual, but the non-tumorous hepatocytes could have proliferated clonally from different multicentric clones before carcinogenesis. The clonal growth of the non-tumorous hepatocytes suggests that the integration of HBV DNA plays an important role in hepatocarcinogenesis.

Introduction

Hepatitis B virus (HBV) infection is etiologically associated with the development of hepatocellular carcinoma (HCC) [10]. Hepatitis B virus DNA has been found to be integrated into the cellular DNA of HCCs [1, 5, 7] and occasionally into that of the non-tumorous tissues of patients with chronic hepatitis [2, 5, 6, 8]. In this work, we analyzed the clonal origin of HCCs and the presence or absence of clonal growth in non-tumorous tissues through studies on the integration of HBV DNA into cellular DNA.

With the Southern blot hybridization analysis of HCC DNA, a restriction fragment containing HBV DNA can be seen as a definite band because of the clonal growth of a tumor cell that has HBV DNA integrated into its chromosomal DNA. On the other hand, bands of integrated HBV DNA are not distinct in non-tumorous tissues without clonal growth because of the different modes of integration in different hepatocytes. Therefore, an analysis of the integration modes of HBV DNA, including the molecular size and the number of restriction fragments of integrated HBV DNA, can be used to determine whether various parts of the HCC of a given case are of clonal origin, and to examine whether there is clonal growth in non-tumorous tissues.

Results

The clonal origin of HCCs

The clonal origin of HCCs was determined in autopsy specimens from 14 cases with HBV surface antigen in sera [3]. Samples of 30–800 mg tumor tissues were obtained from between two and nine parts of each HCC. The DNAs were purified from these samples and digested with *Hin*dIII, *Eco*RI or *Pvu*II. These enzymes do not cleave the HBV DNA of subtype *adr*. The DNA digests were subjected to a Southern blot hybridization analysis [9] using cloned HBV DNA [4] labeled with [α-^{32}P]dCTP as a probe. As shown in Fig. 1, HBV DNA was integrated into the tumor cells in all six samples of case 7, and a single fragment

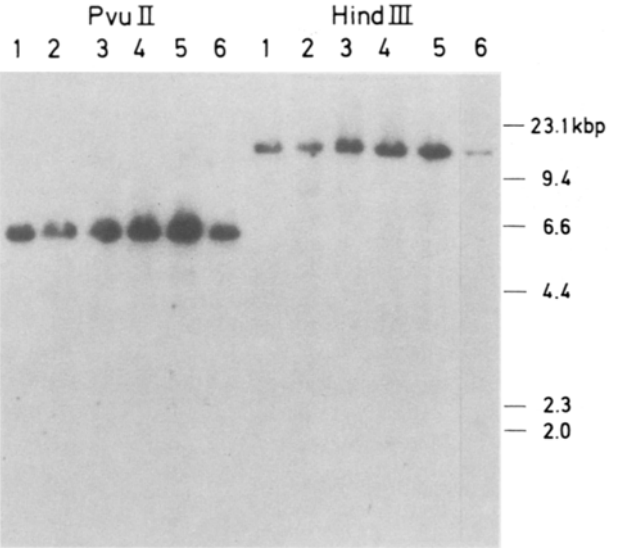

Fig. 1. The Southern blot analysis of cellular DNA from six parts of the HCC in case 7 after digestion with *Pvu*II or *Hin*dIII

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Table 1. Clonal origin of hepatocellular carcinoma

Case	HBsAg/Ab	HBeAg/Ab	No. of liver samples/ metastases	HBV DNA integration[a]	Clonal state
1	+/−	+/−	5/3	1	Same
2	+/−	+/−	4/2	1	Same
3	+/−	−/−	6	2	Same
4	+/−	−/−	7	1	Same
5	+/−	−/−	5	4	Same
6	+/−	−/−	4	2	Same
7	+/−	−/+	6	1	Same
8	+/−	−/+	4	7	Same
9	+/−	−/+	2	2	Same
10	+/−	−/+	3	3	Same
11	+/−	−/+	5/4	1	Same
12	+/−	ND[b]	3	5	Same
13	+/−	ND	7	3	Same
14	+/−	−/+	4	2 + 1	Different

[a] Number of bands determined by Southern blot analysis of *Hind*III fragments of DNA
[b] Not determined

of the same size was obtained from all six DNAs. HBV DNA was therefore integrated into the same site in the cellular DNA in these six tumor samples. The tumor cells growing in separate nodules were of the same clone. As shown in Table 1, in 12 other cases all the tumor cells had HBV DNA integrated into the same site of the cellular DNA. In the advanced stage at least, the scattered HCCs, including the metastatic tumors, were usually of the same clone; that is, they were generated from a single tumor cell.

In case 14, one part of the HCC gave an extra integrated band besides the two bands of the other three HCCs (Fig. 2). This extra band was specific for HBV DNA, since the control probe, pBR322 DNA, did not hybridize with it. Sample 1 contained a new HCC clone.

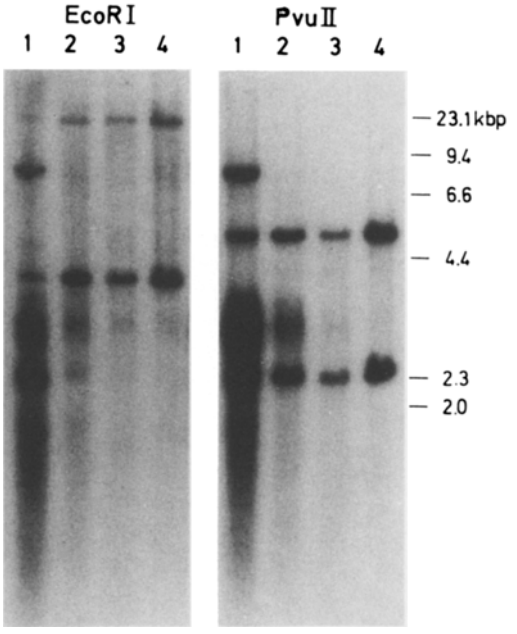

Fig. 2. The Southern blot analysis of cellular DNA from four parts of the HCC in case 14 after digestion with *Eco*RI or *Pvu*II

Table 2. Integration of HBV DNA in non-tumorous liver tissues

Diagnosis	Total	HBV DNA integration	Clonal growth
Chronic hepatitis (CH)	35	14 (40%)	4
Chronic hepatitis (CH) with liver cirrhosis (LC)	25	13 (52%)	4
CH, LC with hepatocellular carcinoma	18	11 (61%)	8
Total	78	38 (49%)	16

The clonal growth of non-tumorous hepatocytes

The integration of HBV DNA into the biopsy and autopsy specimens from liver tissues of 78 cases of chronic hepatitis was examined [11, 12]. As shown in Table 2, HBV DNA was also integrated into non-tumorous hepatocytes in 49% (38/78) of the cases. A Southern blot analysis showed the various sizes of integrated fragments as smears, since the integration sites usually differed with different hepatocytes. In 16 cases, however, a number of definite bands were observed (Fig. 3); that is, several subpopulations of non-tumorous hepatocytes had acquired the ability to grow clonally. This clonal growth was frequently found in non-tumorous tissues from cases of HCC (Table 2). In some cases of HCC, multiple clones were found in different non-tumorous samples of the same liver (Fig. 3), and these clones were different from the tumor clone in the same liver.

Discussion

Through the examination of the mode of integration of HBV DNA, we have demonstrated in this work that HCCs at their advanced stages are usually monoclonal. A clone with a growth advantage may survive, replace other clones, and form a tumor. However, it is possible that some putative monoclonal tumors are biclonal or multiclonal, with one or more subpopulation of tumor cells containing no detectable HBV DNA. In fact, a polyclonal

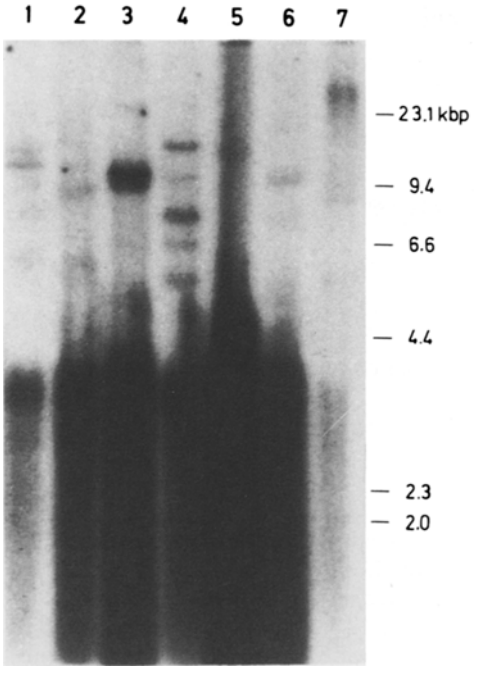

Fig. 3. The Southern blot analysis of cellular DNA from seven parts of non-tumorous tissues in a case of HCC after digestion with *Hin*dIII

state was observed in the HCC of case 14. The 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.

Then what about the polyclonal growth of HCC in the early stage of tumor development? Although nothing is yet known about this, in a stage prior to HCC, such as chronic hepatitis with liver cirrhosis, some populations of non-tumorous hepatocytes begin growing clonally to form multicentric clones each with small-cell populations. In this work, through the examination of the integration of HBV DNA, we detected the clonal growth of non-tumorous hepatocytes, it is therefore possible that other non-tumorous hepatocytes that do not have integrated HBV DNA may also grow clonally. We may have, therefore, underestimated the clonal growth of non-tumorous hepatocytes; in fact, in this work we could not estimate it in the case of hepatocytes that did not have integrated HBV DNA. Interesting problems arising from these results include the question of whether these multicentric clones reflect the histological presence of the regenerative nodules of hepatocytes, and whether HCC is generated from one or more of these multicentric clones.

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