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Characteristics of Human Hepatocellular Carcinoma Cell Lines (Hep-KANO) Derived from a Non-hepatitic, Non-cirrhotic Hepatitis B Virus Carrier

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We have established two cell lines of hepatocellular carcinoma [Hep-KANO, clone 1 (CL-1) and clone 2 (CL-2)] from tissue obtained at autopsy of a hepatitis B virus (HBV) carrier without histological signs of hepatitis or liver cirrhosis. These cell lines differed considerably from each other in morphology, proliferation pattern, α-fetoprotein secretion, albumin synthesis, cytokine secretion, modal chromosome number and transplantability to nude mice. Histologic examinations also revealed differences between them. Amplification of N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras*, c-*erb*-B and c-*erb*-B-2 and rearrangement of p53 were not found in either of the cell lines. However, CL-1 and CL-2 showed an identical HBV-DNA integration pattern. A 4-fold amplification of c-*myc* was observed in CL-1, but not in CL-2. Hep-KANO cell lines, CL-1 and CL-2 may be useful in clarifying the question of whether hepatocarcinogenesis is directly caused by HBV infection.

Key words: Human hepatocellular carcinoma cell line (Hep-KANO) — HBV-DNA integration — HBV hepatocarcinogenesis — Amplification of c-*myc*

The hepatitis B virus (HBV) and hepatitis C virus (HCV) are etiologically linked to hepatocellular carcinoma (HCC).[1] Generally, it is assumed that there is a multistep process by which chronic hepatitis develops into liver cirrhosis and then into HCC through genetic alterations occurring during liver regeneration.[2,3] However, we have encountered some HBV carriers who developed HCC without any histologic sign of hepatitis or liver cirrhosis. We therefore considered that HBV itself or its integration to the host DNA might directly cause cell transformation. Several studies on the molecular biology of HBV have indicated that it has oncogenic potential.[4,5] However, no consensus exists on the transforming activity or carcinogenic mechanism of HBV. To elucidate this, it is important to study the correlation between the cellular integration of HBV-DNA and abnormalities in DNA, including oncogenes and tumor suppressor genes.[6] Such investigations could be performed by using cells of HBV carriers in whom HCC developed without the prior complication of hepatitis or liver cirrhosis. To our knowledge, however, there have been few reports of the establishment of such model cell lines.[7–11] Furthermore, in those cases, the presence or absence of hepatitis or liver cirrhosis in noncancerous liver tissue of HCC was not clearly described. We have established two cell lines, differing from each other in cytobiologic characteristics, from tumor tissue obtained at autopsy of a patient who was an HBV carrier with HCC, but with no history of chronic hepatitis or liver cirrhosis. The cytobiologic characteristics of these cell lines, the integration pattern of HBV-DNA, and the abnormalities of oncogenes and tumor suppressor genes (amplification of various oncogenes, rearrangement of p53) were studied.

MATERIALS AND METHODS

Case report The tumor tissue was obtained at the autopsy of a 42-year-old man with HCC (multiple nodular type, trabecular and solid type, Edmondson II–III, α-fetoprotein (AFP) 990,000 ng/ml, protein induced by vitamin K absence II (PIVKA-II) 40 Au/ml). The serum virus markers were HBsAg(+), HBsAb(−), HBeAg(+), HBeAb(−) and HBcAb(−). Immunostaining of the noncancerous liver tissue was positive for intracellular HBsAg and HBcAg. Histologically, neither chronic hepatitis nor liver cirrhosis was evident in noncancerous parts of the liver (Fig. 1).

Primary culture The primary culture was started on the day of autopsy. DM-170 (Kyokuto Pharmaceutical Co., Ltd.) supplemented with 10% fetal calf serum (FCS) (GIBCO) was used as the medium. The tumor tissue was washed with cold medium, minced into small pieces with a scalpel, and incubated in medium containing 1,000 U/ml of Dispase (Godo Shusei), a neutral bacterial protease, at 37°C for 30 min. After centrifugation at 1,000 rpm for 5 min, the supernatant, containing the Dispase, was removed. Medium was added to the pellet to prepare a cell suspension. The cells were rinsed several times with Ca^{2+},Mg^{2+}-free phosphate-buffered saline (PBS)(−),

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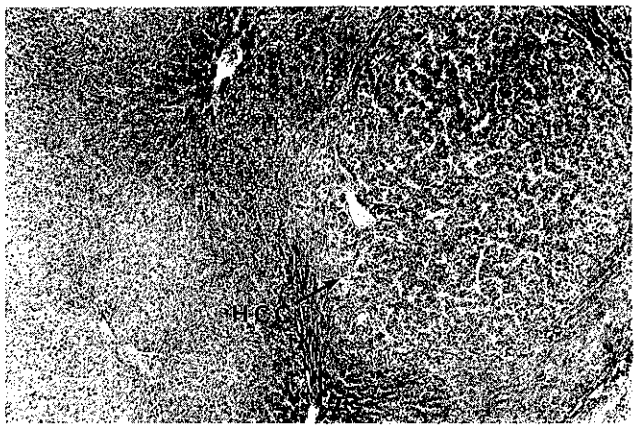

Fig. 1. Histology of cancerous and noncancerous liver tissue by light microscopy. HE staining. HCC; hepatocellular carcinoma.

then plated in 25 cm^2 culture flasks (Falcon) and incubated at 37°C under 5% CO_2.

Subculture After culturing for 9 months, two epithelioid cell lines differing from each other in proliferation pattern were identified in different culture flasks. Cells of one line (clone 1) were relatively oblong in shape and tended to pile up as they reached confluency. Cells of the other line (clone 2) were relatively large and round, forming a regular paving stone-like arrangement at confluency. Even after reaching confluency, these cells did not pile up. About 9 months after the start of primary culture, the first subculture of these cell lines was made. For subculturing, the cultured cells were washed several times with PBS(−), then incubated with 200 U/ml Trypsilin (Mochida Pharmaceutical Co., Ltd.) in PBS(−) for 5 min. The cells were then detached from the culture flask with a rubber policeman. After inactivating the Trypsilin by adding 0.2 ml of FCS, the cell suspension was centrifuged at 1,000 rpm for 5 min to obtain the cell pellet. New medium was added to the cell pellets, and the cells were subcultured at a splitting ratio of 1:3. The medium was renewed twice a week. The passage number has exceeded 50 passages, with one passage every three weeks with a splitting ratio of 1 to 3 over 4 years. The cell lines were named Hep-KANO clone 1 (CL-1) and clone 2 (CL-2).

Light microscopic study The cultured cells were observed under a phase-contrast microscope. Moreover, the cells were cultured on cover slips each placed in a flat-type, long culture tube. The cover glasses were removed, fixed with 10% formaldehyde solution, and stained with hematoxylin-eosin (HE) for light microscopy.

Electron microscopic study Confluent culture flasks were rinsed three times with Tyrode solution, and then fixed with 1% glutaraldehyde in Sorenson's phosphate buffer (SPB) (pH 7.2) at 4°C for 5 min. The cells were scraped off with a rubber policeman and centrifuged at 1,000 rpm for 5 min. The cell pellets were fixed at 4°C for 50 min, washed 8 times with SPB, post-fixed with osmium tetroxide, and embedded in Epon. Thin sections were cut by an ultramicrotome, mounted on a coated grid, stained with uranyl acetate and lead citrate, and observed by electron microscopy.

Growth curve Cultured cells were dispensed into round, short culture tubes at an initial count of 10^4–10^5 cells per tube, and incubated for 7 days, according to the simplified replicate culture method.[12] The cells were counted at fixed intervals to determine the growth curve.

Chromosomal analysis When proliferating cells reached 80% confluency, the medium was renewed. Ninety minutes before the completion of culture, ethidium bromide was added to the medium at a final concentration of 0.75 μg/ml. Sixty minutes before completion, colcemid was added at a final concentration of 0.2 μg/ml. After removal of this medium, the cells were treated with Trypsilin (200 U/ml) in PBS(−) for 5 min, and then treated with 0.2 ml of FCS to inactivate the Trypsilin. The cells were scraped off with a rubber policeman and centrifuged at 1,000 rpm for 7 min. Then 10 ml of a hypotonic solution (KCl 0.075 M:1% sodium citrate = 4:1) was added to the cell pellets, which were kept in a 37°C water bath for 20 min. They were fixed (methanol: acetate = 3:1) and suspended in a small amount of the fixing solution. A drop of suspension was then placed on a slide glass which had been immersed in 50% ethanol, immediately dried over an alcohol lamp and stained with Giemsa for microscopy. For identification of chromosomes, G-banding was employed.[13] Karyotyping was performed according to the International System for Human Cytogenetic Nomenclature.[14]

Transplantation into nude mice Approximately 10^7 cultured cells in 0.2 ml of PBS(−) were subcutaneously inoculated into the dorsal side of Balb/c nu/nu female mice, 4 weeks of age. After 1 week, the tumor formed was extirpated and stained with HE for histologic study and comparison with the original tumor tissue.

Secretory substances in the culture supernatant The culture medium was renewed at confluency. After 72 h of incubation, the concentations of human albumin, AFP, PIVKA-II, carcinoembryonic antigen (CEA), ferritin, and cytokines (interleukin-1(IL-1)β, IL-6, interferon (IFN)-γ) as well as HBsAg and HBeAg in the culture supernatant were determined by radioimmunoassay or enzyme-linked immunosorbent assay.

Integration of HBV-DNA in Hep-KANO (CL-1, CL-2) The integration patterns of HBV-DNA into the cell lines and original HCC tissue were analyzed by Southern blotting. DNA was extracted from 10^7 cells of each cell line, and 100 mg of tumor tissues, then purified by the

phenol-chloroform method. After digestion by restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII) (Boehringer Mannheim Biochemicals), samples were electrophoresed in a 0.8% agarose gel, and transferred to a Hybond-N membrane (Amersham). Hybridization with a ^{32}P-labeled whole HBV-DNA of adw type (3.2 kb) was performed following the protocol of the manufacturer. As a negative control, DNA from human placenta was used, and as a positive control, that of PLC/RPF/5 cells was used.

Amplification of oncogenes and rearrangement of p53 Amplification of oncogenes (c-*myc*, L-*myc*, N-*myc*, K-*ras*, H-*ras*, N-*ras*, c-*erbB*, c-*erbB*-2) and rearrangement of p53 were studied by Southern blotting. Purified DNA extracted from the cells was digested by restriction enzymes and hybridized, after agarose gel electrophoresis and membrane transfer, with ^{32}P-labeled c-DNA probes derived from human cells (c-*myc*, L-*myc*, K-*ras*, H-*ras*, N-*ras*, p53: ONCOR Co., Ltd.), (c-*erbB*, c-*erbB*-2: Amersham Japan Laboratories), followed by autoradiography. As a negative control, DNA from placenta was used; as positive controls, DNAs of HL-60 (human promyelocytic leukemia), IMR-32 (human neuroblastoma), A-431 (human epidermoid carcinoma) and SK-BR-3 (human breast adenocarcinoma) were used for c-*myc*, N-*myc*, c-*erb*-B and c-*erb*-B-2 cells, respectively.

RESULTS

Morphologic features CL-1 cells were polygonal but slightly oblong, showing a fibroblast-like morphology (Figs. 2 and 3). Upon reaching confluency, they piled up. CL-2 showed a relatively round, polygonal morphology (Figs. 2 and 3). Upon reaching confluency, it exhibited a paving stone-like arrangement and did not show any tendency to pile up, even during long-term culture.

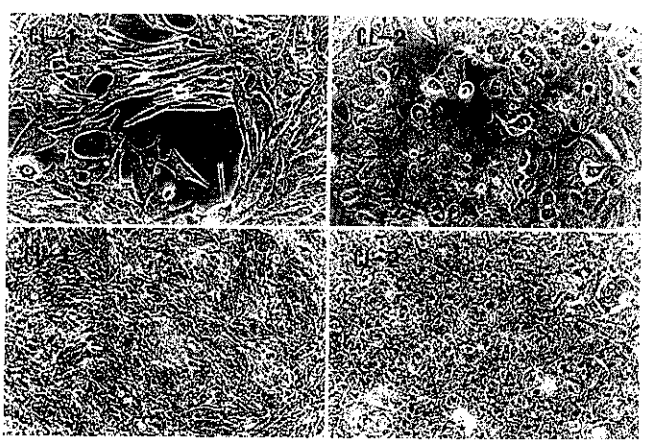

Fig. 2. Morphologic features of cultured Hep-KANO cells (CL-1, 2) by phase-contrast microscopy. Original magnification: upper panels ×50, lower ×25.

Ultrastructurally, microvilli and tight junctions were present in both cell lines. Within the cells, the nucleus was constricted, with mitochondria and lysosomes abundant in the cytoplasm. No characteristic ultrastructural differences were found between the two cell lines (Fig. 4).

Growth curve The doubling time, calculated from the growth curve of each cell line, was 45 h for CL-1 and 36 h for CL-2 (Fig. 5).

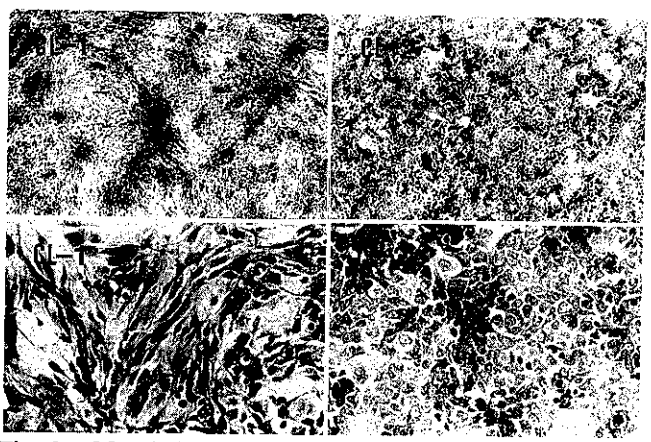

Fig. 3. Morphologic features of cultured Hep-KANO cells (CL-1, 2) by light microscopy. HE. Original magnification: upper panels ×25, lower ×125.

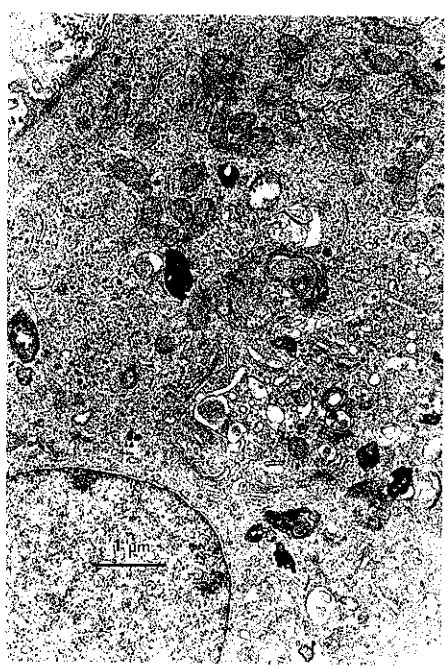

Fig. 4. Electron micrograph of cultured Hep-KANO cells (CL-1).

Chromosomal analysis The karyotypes were large and metacentric, which is characteristic of human cells. CL-1 showed a modal chromosome number of 35, while the modal chromosome number of CL-2 was 65 (Fig. 6).

Transplantation to nude mice CL-1 demonstrated transplantability to nude mice: 1 week after inoculation, small tuberous tumors were formed, with a proliferation of solid-type cells, resembling those in the original tissue (Fig. 7). But the tumor did not kill the animals. On the other hand, the transplantability of CL-2 to nude mice was low. After 1 week, the cells were mainly necrotic. Histologically, they were trabecular. As both solid and trabecular tumor tissue were present in the original hepatoma tissue, we considered that the two cell lines had been derived from these two morphologically different cell populations.

Secreta in culture supernatants Human albumin was secreted more abundantly by CL-2 than by CL-1. AFP was secreted only by CL-1. Neither cell line secreted PIVKA-II. There were differences in the amounts of IL-6 secreted between the two cell lines. HBsAg and HBeAg were not detectable in either medium (Table I).

Integration of HBV-DNA in Hep-KANO (CL-1, CL-2) Figs. 8 and 9 show the integration patterns of HBV-DNA in Hep-KANO cells (CL-1, CL-2) and the original HCC tissue (superior and lateral portions) as determined by Southern blotting after digestion with restriction enzymes. Because a band was detected in the molecular mass region higher than 23.1 kb in the lane of DNA not digested with the enzyme, integration of HBV-DNA into the cells is evident. The degree of integration of HBV-DNA into both Hep-KANO cell lines was about 5 times that of PLC/RPF/5 cells, according to densitometric analysis. On the basis of the band pattern after *Hin*dIII digestion, it was suggested that integration had occurred at 3 or 4 sites in Hep-KANO (CL-1, CL-2), and at 5 sites in PLC/RPF/5 cells used as the positive control, as *Hin*dIII restriction sites were not found on the HBV genome. However, upon treatment with *Eco*RI with one restriction site on the HBV genome, a large number of bands was detected. It was thus considered that the integration pattern was more complicated. Both CL-1 and CL-2 cells exhibited almost identical integration patterns. Moreover, these patterns were very similar to that of the original HCC tissues. Accordingly, it can be concluded that these two cell lines were derived from the HCC tissue. However, when their integration patterns were compared to that of the original tissues, it was seen that they lacked several bands. This is considered to be a result of changes associated with the establishment of the two cell lines. It was estimated that the number of HBV

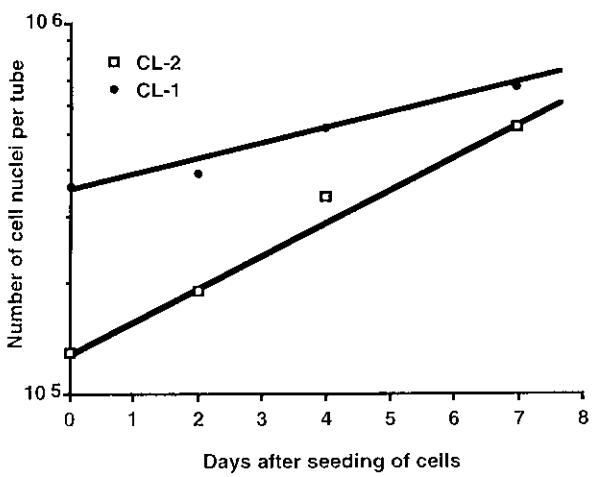

Fig. 5. Growth curve of cultured Hep-KANO cells (CL-1, 2). Each point represents the mean value from three culture tubes.

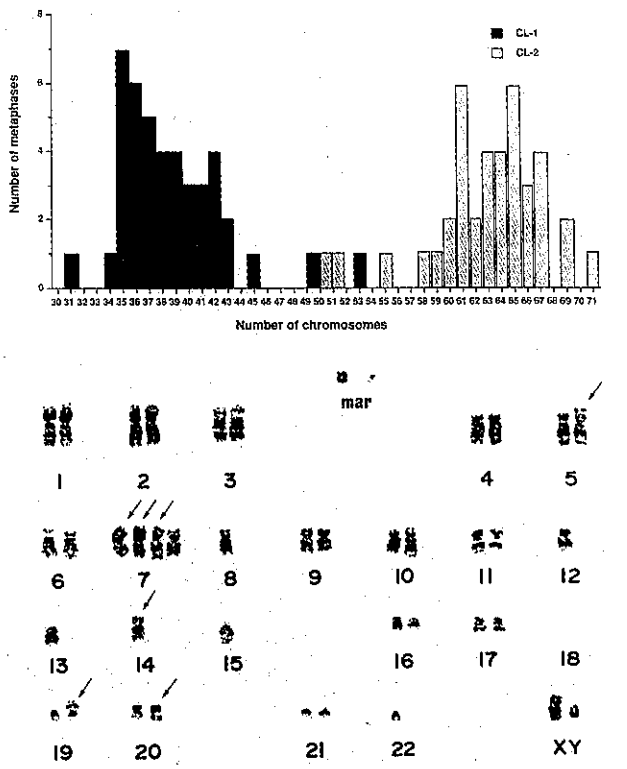

Fig. 6. Chromosomal analysis of Hep-KANO cells (CL-1, 2). The upper figure shows the distribution of chromosome number of Hep-KANO cells. The lower figure shows a typical karyotype of Hep-KANO cells (CL-1). Karyotype; 42,XY, -5,-7,-8,-12,-13,-14,-14,-15,-18,-18,-19,-20,-22, +der(5)t(5;14)(p11;p11), +der(?)t(7;?)(q11;?), +der(?)t(7;?)(q11;?), +der(7)t(7;?)(q11;?), +der(14)t(14;?)(p11;?), +der(19)t(19;?)(q11;?), +der(20)t(20;?)(q12;?), marl, +mar2 (der; derivative, t; translocation, mar; marker).

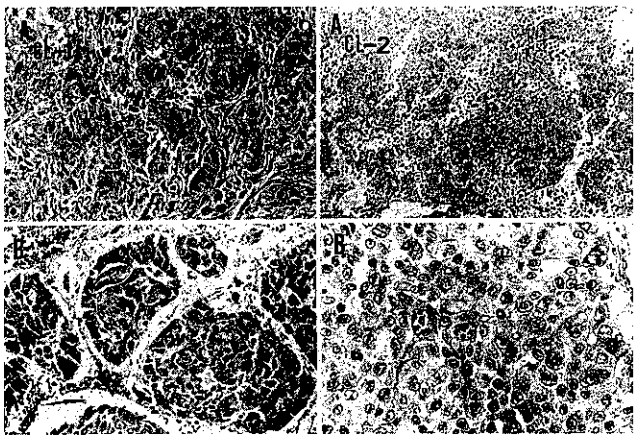

Fig. 7. A. Histology of tumor grown in nude mice after transplantation of Hep-KANO cells. HE. Original magnification: left panel ×125, right ×25. B. Histology of the original HCC tumor tissue. HE. Original magnification: left panel ×125, right ×250.

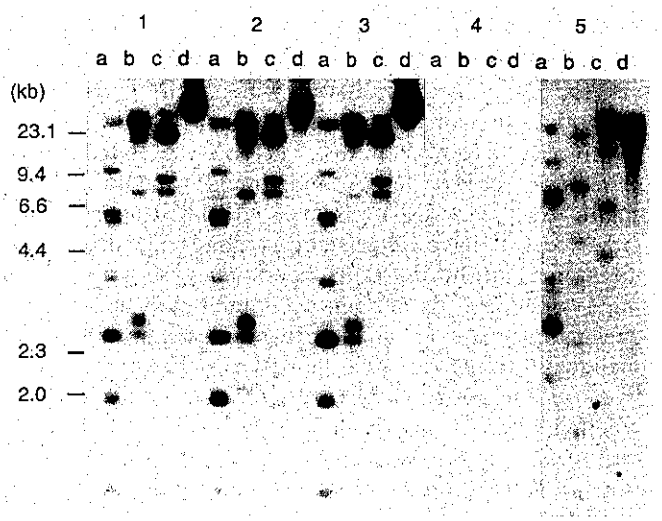

Fig. 8. Integration of HBV-DNA in Hep-KANO cells (CL-1, 2) 1. CL-1 after 36 passages. 2. CL-1 after 9 passages. 3. CL-2 after 42 passages. 4. Human placenta (negative control). 5. PLC/RPF/5 (positive control). a. Digested with *Eco*RI. b. Digested with *Bam*HI. c. Digested with *Hin*dIII. d. Not digested.

copies might have been as much as 30 copies/cell (at least 4 copies/cell) on the basis of the results of *Hin*dIII treatment and dot blot hybridization comparing sample DNA with the HBV-DNA diluted with human placental DNA (data not shown).

Amplification of various oncogenes and rearrangement of p53 Fourfold c-*myc* gene amplification was observed in

Table I. Secretions in Supernatant from Cultured Hep-KANO Cells (CL-1, 2) (Confluent, 72 h)

	Hep-KANO	
	Clone 1	Clone 2
Albumin (ng/ml)	500	53400
AFP (ng/ml)	170	<1.0
PIVKA-II (AU/ml)	<0.06	<0.06
CEA (ng/ml)	<0.5	<0.5
Ferritin (ng/ml)	1600	100
IL-6 (pg/ml)	892	7.3
HBs Ag	(−)	(−)

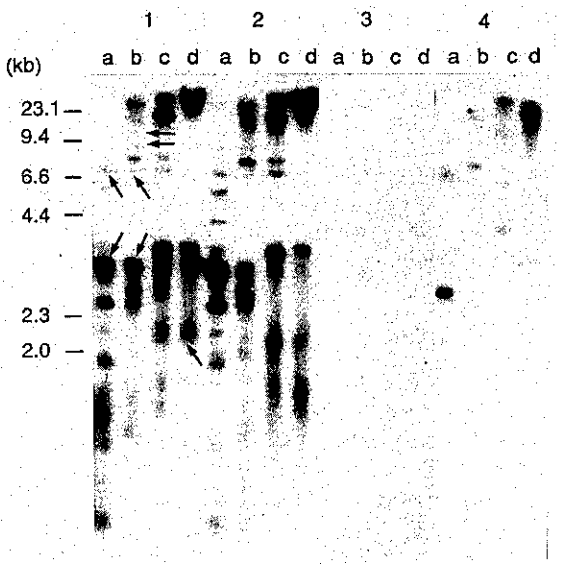

Fig. 9. Integration of HBV-DNA in original HCC tissue of Hep-KANO cells (CL-1, 2) 1. Original HCC tissue (superior portion). 2. Original HCC tissue (lateral portion). 3. Human placenta (negative control). 4. PLC/RPF/5 (positive control). a. Digested with *Eco*RI. b. Digested with *Bam*HI. c. Digested with *Hin*dIII. d. Not digested. Black arrows show deleted bands in Hep-KANO cells.

CL-1, but not in CL-2 by densitometric analysis (Fig. 10). No amplification of other oncogenes (N-*myc*, L-*myc*, H-*ras*, K-*ras*, N-*ras*, c-*erbB*, c-*erbB*-2) was found in either of the two cell lines, nor was rearrangement of the p53 gene observed (Table II).

DISCUSSION

We established two cell lines, namely Hep-KANO CL-1 and CL-2, from a human hepatocellular carcinoma of an HBV carrier who had no history of overt hepatitis or cirrhosis. Although the two cell lines originated from the same tumor, they came to differ cytobiologically *in vitro*.

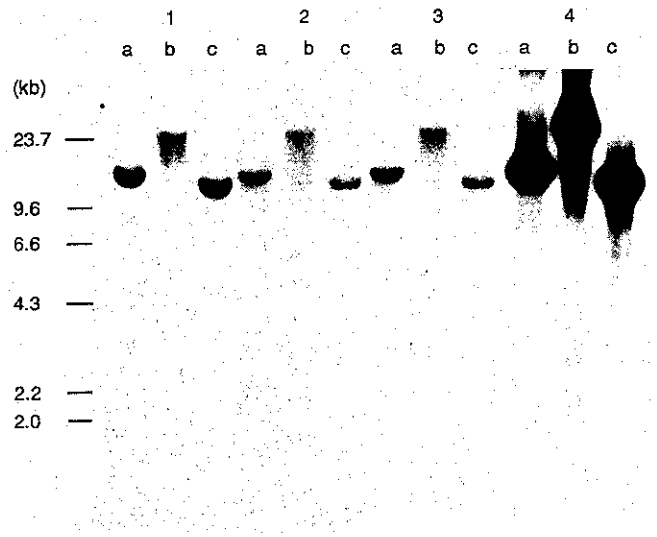

Fig. 10. Amplification of c-*myc* in Hep-KANO cells (CL-1, 2) 1. CL-1. 2. CL-2. 3. Human placenta (negative control). 4. HL-60 (positive control). a. Digested with *Eco*RI. b. Digested with *Bam*HI. c. Digested with *Hin*dIII.

As far as we know, the establishment of a human HCC cell line from a patient with a cancer which arose without prior complications of hepatitis and cirrhosis is without precedent. Most established cell lines were derived from carcinomas arising in livers with prior chronic hepatitis caused by HBV, which progressed to cirrhosis.[7-11] The new cell lines could be useful as models for understanding the mechanism of hepatocarcinogenesis caused by HBV infection and integration without long processes of repeated cell death and regeneration.

Considerable differences were found in the morphology, doubling time, and secretion of albumin, AFP and cytokines between the two cell lines. However, there was no significant difference in the HBV-DNA integration pattern. Among the oncogenes examined, c-*myc* was amplified in one of the lines. If the integration of HBV-DNA directly induces abnormalities in proliferation and differentiation, including abnormalities in oncogenes, the process of carcinogenesis as a result of integration of HBV-DNA might change the cellular character, including chromosomes, accounting for the production of two different cell clones. The changes might have occurred *in vivo* rather than *in vitro* after the initiation of culture, because CL-1 and CL-2 were propagated and established in separate culture flasks from the start of culture. A transformed cell clone *in vivo* would change in character due to interaction with host systems, so that the tumor formed would consist of a cytobiologically heterogenous cell mass.[15]

Table II. Abnormalities of Oncogenes and Rearrangement of p53 in Hep-KANO Cells (CL-1, 2)

Oncogene	Hep-KANO	
	Clone 1	Clone 2
Amplification of		
c-*myc*	(+)	(−)
L-*myc*	(−)	(−)
N-*myc*	(−)	(−)
K-*ras*	(−)	(−)
N-*ras*	(−)	(−)
H-*ras*	(−)	(−)
erbB	(−)	(−)
erbB-2	(−)	(−)
Rearrangement of		
p53	(−)	(−)

Many theories have been put forward on the basis of fragmentary experimental results to explain the relation between the integration of HBV-DNA into hepatocytes and carcinogenesis. One suggestion, based on experiments using transgenic mice, was that the HBV-X gene is possibly a protooncogene, which, together with a hybrid gene of cellular and HBV-X derived fragments, might exert a *trans*-activator function, resulting in carcinogenesis.[4, 16-19] Another possibility is that the 3′-truncated preS2/S region of integrated HBV generates a transactivating function, resulting in HBV-associated oncogenesis,[20, 21] or HBV-DNA might integrate adjacent to the cyclin A gene and hst-I to cause cell transformation.[22, 23] Other reports have suggested carcinogenesis through indirect mechanisms based on experiments using transgenic mice, wherein HBs antigen accumulates in hepatocytes causing liver injury, which in turn induces mutation and malignant transformation in the secondary process of liver regeneration.[2] Other questions to consider are whether HBV is directly or indirectly involved in multistep hepatocarcinogenesis through structural or functional abnormalities of oncogenes and tumor suppressor genes, and if so, where and how it is involved.[6, 24] In the present study, in view of the origin of the cell lines, it appeared that HBV was directly implicated in hepatocarcinogenesis. Therefore, these cell lines we have established may provide useful models to clarify the mechanism of HBV-induced carcinogenesis. We plan to study at the molecular level the quantitative and qualitative abnormalities of various oncogenes and tumor suppressor genes in these cell lines, along with analysis of the integration of HBV-DNA.

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