Jpn. J. Cancer Res. 83, 871–877, August 1992

New Hepatocellular Carcinoma Cell Line SUHC-1 Established from a Patient with Hepatitis C Virus RNA in Serum

Kaname Yoshizawa,[1] Kendo Kiyosawa,[1,4] Seiichi Usuda,[1] Koji Yabu,[1] Yoshiyuki Nakatsuji,[1] Shuhei Yamada,[1] Kiyoshi Furuta,[1] Eiji Tanaka,[1] Takeshi Sodeyama,[1] Seiichi Furuta,[1] Shuichi Kaneko[2] and Paul K. Nakane[3]

[1]*Second Department of Internal Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390,* [2]*First Department of Internal Medicine, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920 and* [3]*Third Department of Anatomy, Nagasaki University School of Medicine, 12-4 Sakamoto-cho, Nagasaki 852*

A new human hepatocellular carcinoma cell (HCC) line, designated SUHC-1, was derived from a Japanese patient with hepatocellular carcinoma having antibody to hepatitis C virus (HCV) and HCV-RNA in his serum, and established in tissue culture. This cell line exhibited typical epithelial cell morphology in culture as observed by phase-contrast and electron microscopy. The SUHC-1 cells produced albumin and α_2-macroglobulin. Chromosomal analysis showed several rearrangements at short and long arms of chromosome 1, 17 and 20 (1p−, 1q−, i(1q), i(17q) and 20q+) with a modal number of 91. HCV-RNA was not detected in the supernatant of SUHC-1 cells by nested polymerase chain reaction assay or in the SUHC-1 cells by the *in situ* hybridization method. We concluded that complete HCV does not exist in the SUHC-1 cell line. The SUHC-1 cell line is the first line of HCC to have been derived from a patient with persistent HCV infection, and may provide a suitable model for studies of hepatocarcinogenesis related to HCV.

Key words: Hepatocellular carcinoma cell line — Hepatitis C virus — HCV-RNA

The incidence of hepatocellular carcinoma (HCC)[5] has been rising in Japan. The majority of cases are caused by the hepatitis B virus (HBV) and the hepatitis C virus (HCV).[1-3] It has been reported that HBV-DNA was integrated into the chromosomes of HCC cells derived from patients with HBV-related chronic liver disease.[4-6] HBV is considered to play an important role in the development of HCC.[7-9] HCV and the development of HCC were found to be closely related in an epidemiologic study,[2] though the oncogenicity of HCV is not known. Several HCC cell lines have been established, in some of which HBV-DNA is integrated,[10-13] while in others it is not.[14, 15] Some of the HCC cell lines in which HBV-DNA is not integrated may be derived from patients with non-A, non-B hepatitis virus-related chronic liver disease. However, to our knowledge, there is no report of the establishment of an HCC cell line derived from a patient in whom the underlying liver disease is definitively associated with HCV infection. For the study of hepatic carcinogenesis, it would be useful to establish an HCC cell line related to HCV. We have established a new HCC cell line, designated SUHC-1, from a male Japanese with anti-HCV antibody and HCV-RNA. We also investigated the existence of the HCV genome in the cell using the nested polymerase chain reaction (PCR) assay and the *in situ* hybridization method.

MATERIALS AND METHODS

Patient The patient was an 81-year-old Japanese man admitted to Shinshu University Hospital with a diagnosis of HCC. He had received blood transfusions on three occasions at the age of 26, 49 and 67 years. His serum was positive for anti-HCV antibody and HCV-RNA. Anti-HBs antibody was positive and anti-HBc antibody was weakly positive, although the serum HBs antigen and HBV-DNA were negative. These data pointed to a past infection with HBV. Tests were negative for serum alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and CA 19-9. Prior to the operation, chemotherapy or transcatheter arterial embolization for HCC was not done. At surgery, the anterior-superior (S8) and posterior-superior (S7) segments of the right lobe of the liver were found to be involved in a neoplastic process. Subsegmentectomy of S8 and partial resection of S7 were performed in order to remove a 3×3×2 cm encapsulated tumor.

Tissue culture The hepatoma specimen was minced with forceps and scissors, dispersed using 1000 pU/ml Dispase (Sanko Junyaku, Tokyo) for one hour and washed. Cells were cultivated in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-

[4] To whom correspondence should be addressed.

[5] Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; PCR, polymerase chain reaction; AFP, alpha-fetoprotein; CEA, carcinoembryonic antigen.

inactivated fetal calf serum (FCS) (GIBCO, Grand Island, NY), 100 IU/ml penicillin and 100 μg/ml streptomycin and 2 m*M* glutamine in 6-well culture plates (Falcon, Lincoln Park, NJ) at 37°C with 5% CO_2 in air. An epithelial cell colony grew among the fibroblasts in about 2 months and gradually became dominant. The cells were removed with a cell scraper (Falcon) and transferred to a 25 cm^2 culture flask (Falcon). The cultured cells have been maintained for 24 months and for more than 40 passages.

Morphological studies Part of the hepatoma tissue was fixed in 10% buffered formalin and embedded in paraffin. Histological specimens were stained with hematoxylin and eosin. Cultured cells, seeded in a petri dish (Falcon), were observed by phase-contrast microscopy. For electron microscopic observation, the hepatoma tissue and SUHC-1 cells grown on a LAB-TEC chamber (Nunc Inc., Naperville, IL) were prefixed with 2.5% glutaraldehyde in phosphate buffer (PB), and postfixed with 1% osmium tetroxide in PB. They were routinely dehydrated in a series of ethanol and embedded in Luveak-812 (Nakarai, Tokyo). Ultrathin sections were cut with an ultramicrotome and doubly stained with uranyl acetate and lead citrate. The sections were observed in a Hitachi HS-9 electron microscope.

Growth kinetics Cultured cells were trypsinized and 2×10^5 viable cells were seeded in each 60 mm petri dish. Cells were harvested every day with trypsin-EDTA (GIBCO) treatment and the number of viable cells was counted by trypan blue dye exclusion using a hemocytometer.

Transplantation to nude mouse SUHC-1 cells at the 20th passage were inoculated subcutaneously in 4-week-old athymic nude mice, BALB/c *nu/nu* (Nihon Clea Ltd., Osaka), at concentrations ranging from 1×10^6 to 2×10^7 per mouse.

Chromosome analysis SUHC-1 cells at the 20th passage were incubated with 0.1 μg/ml colcemid (GIBCO) for 2 h, then lysed with 0.075 *M* KCl for 15 min and fixed with methanol/acetic acid (3:1). Chromosomal preparations were examined by Giemsa staining and G-band techniques.[16]

Plasma protein and tumor markers The 10-fold concentrated supernatant from the 5-day cell cultures were tested for albumin, ceruloplasmin, haptoglobin and α_1-antitrypsin by using a laser nephrometer (Behring, Marburg, Germany). We examined AFP (latex photometric immunoassay; Dia-Iatron, Tokyo), CEA (Dainabot CEA RIA kit; Dainabot Co. Ltd., Tokyo) and CA 19-9 (CA 19-9 RIA kit; Centocor, PA) in 10-fold concentrated spent medium of SUHC-1 cells. To detect the production of albumin, α_2-macroglobulin and AFP of SUHC-1 cells, the indirect immunoperoxidase method was used, as previously described.[17] SUHC-1 cells were seeded in LAB-TEC chamber slides (Nunc) and cultured for 3 days. As the primary antibodies, we used goat anti-human albumin (Sigma, St. Louis, MO), goat anti-human α_2-macroglobulin (BioMakor, Rehovot, Israel) and rabbit anti-AFP (Dako, Carpintera, CA).

HBV markers HBsAg, anti-HBs antibody, and anti-HBc antibody were examined by means of enzyme-linked immunosorbent assays (ELISA) (Behring). Serum HBV-DNA was assayed by dot blot hybridization. The intracellular HBV-DNA of SUHC-1 cells was examined by the Southern blot technique[18] using *Eco* RI-digested cell DNA and a ^{32}P-labeled HBV-DNA probe. DNA of PLC/PRF/5 was used as a positive control.

HCV markers A first-generation anti-HCV antibody (HCV antibody ELISA Test System, Ortho Diagnostic Systems, Raritan, NJ) raised against the polypeptide C100-3 synthesized by the recombinant yeast clone of HCV[19] was tested in enzyme immunoassay (EIA). A second-generation anti-HCV antibody (Ortho) was also tested. HCV-RNA in serum and the supernatant of SUHC-1 cells was examined by the nested PCR assay using HCV-specific PCR primer, which consisted of a sequence of 5′ noncoding region in HCV strains.[20] The sequences of the outer sense and antisense primers were 5′ACTCCACCATAGATCACTCC3′ (nucleotides 7–26) and 5′AACACTACTCGGCTAGCAGT3′ (229–248), respectively, and those of the inner sense and antisense primers were 5′TTCACGCAGAAAGCGTCTAG3′ (46–65) and 5′GTTGATCCAAGAAAGGACCC3′ (171–190), respectively. RNA from the serum and spent medium of the SUHC-1 cells was extracted by the acid guanidinium-phenol-chloroform (AGPC) method described by Chomczymski and Sacchi.[21] *In situ* hybridization was performed as previously described.[22] Oligo-DNAs complementary to the region of HCV-RNA corresponding to the sequences of the prototype HCV (−36 to 8) (European Patent Application #88310922.5) with 5′-TTATTA- and -ATTATTATT-3′ sequences at the 5′ and 3′ end were synthesized by the phosphoramidite method on a 391 PCR mate DNA synthesizer (Applied Biosystems Japan, Tokyo). T-T dimerization of DNAs was done by irradiation (10000 J/m) with ultraviolet light (253.7). SUHC-1 cells cultured on a LAB-TEC chamber were fixed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS), dried in air and baked at 45°C for 4 h. Rehydrated slides were treated with 0.3% H_2O_2 in methanol for 10 min in order to block endogenous peroxidase activity. Prior to hybridization, the specimens were treated with 0.2 *N* HCl for 10 min to remove basic proteins such as histone and ribonuclease. The specimens were digested with proteinase K (1 μg/ml) dissolved in PBS for 15 min at 37°C. After washing with PBS, the specimens were fixed in 4% paraformaldehyde in PBS for 5 min and washed, and the excess

aldehyde was quenched twice by immersion in 2 mg/ml glycine in PBS for 15 min each. T-T dimerized probe DNAs were mixed with deionized formamide and the other constituents, boiled for 10 min, and quickly chilled in an ice-water bath. The final hybridization mixture was as follows: 10 m*M* Tris-HCl (pH 7.3), 1 m*M* EDTA, 0.6 *M* NaCl, 1×Denhardt's solution, 40% (v/v) deionized formamide, 250 μg/ml yeast tRNA, 125 μg/ml sonicated salmon sperm DNA and T-T dimerized probe DNA (3 μg/ml). A 25 μl aliquot of the mixture was applied to each section and incubated for 15 h at 37°C. The specimens were then washed with 50% formamide in 2× standard saline citrate (SSC) three times for 1 h and once for 3 h at 37°C, and then twice with 2×SSC at room temperature for 30 min each. For immunohistochemical detection of T-T dimerized DNA, the specimens were treated for 1 h with PBS containing 5% (w/v) bovine serum albumin (BSA), 500 μg/ml normal goat IgG, 100 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA and 0.05% NaN_3 in order to block the non-specific binding of antibody to specimens. The specimens were reacted with rabbit anti-T-T dimer IgG dissolved in PBS containing 5% (w/v) BSA, 100 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA, and 0.05% NaN_3 for 15 h at 25°C. After washing with PBS for 1 h, the specimens were reacted for 1 h with horseradish peroxidase (HRP)-conjugated Fab of goat IgG against rabbit IgG dissolved in PBS containing 5% BSA, 100 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA, and then washed with PBS for 1 h. The sites of HRP were visualized histochemically using 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide with nickel and cobalt ions.

RESULTS

Morphology The original liver tumor was composed of epithelial cells arranged in a trabecular pattern (Fig. 1). The noncancerous part of the liver showed typical cirrhosis. The established cell line SUHC-1 showed an epithelioid appearance by phase-contrast microscopy (Fig. 2). In electronmicroscopic examination, the tissue HCC cells have well-developed nucleoli (Fig. 3-a). Mitochondria, smooth-surfaced endoplasmic reticulum (SER), glycogen granules and other cell organelles were seen in the cytoplasm. Bile canaliculi were also seen among the cells. In the SUHC-1 cells, ultrathin sections showed that well-developed nucleoli were close to the indented nuclear membranes (Fig. 3-b). Mitochondria and free ribosomes were well developed in the cytoplasm. Microvilli were also observed at the cell surface. These findings indicated that the SUHC-1 cells had some characters of the HCC cells.

Growth kinetics The doubling time of SUHC-1 cells was approximately 24 h in RPMI 1640 with 10% FCS at the 30th passage.

Chromosome analysis The number of chromosomes was distributed in the range of 88 to 93 and the modal number was 91 (Fig. 4). Analysis by the G-band method showed abnormal rearrangements at chromosomes 1, 17 and 20 (1p−, 1q−, i(1q), i(17q) and 20q+) of all cells analyzed.

Plasma proteins and tumor markers Albumin, ceruloplasmin, haptoglobin and α_1-antitrypsin were not detected in the 10-fold concentrated culture medium. However, albumin and α_2-macroglobulin were detected

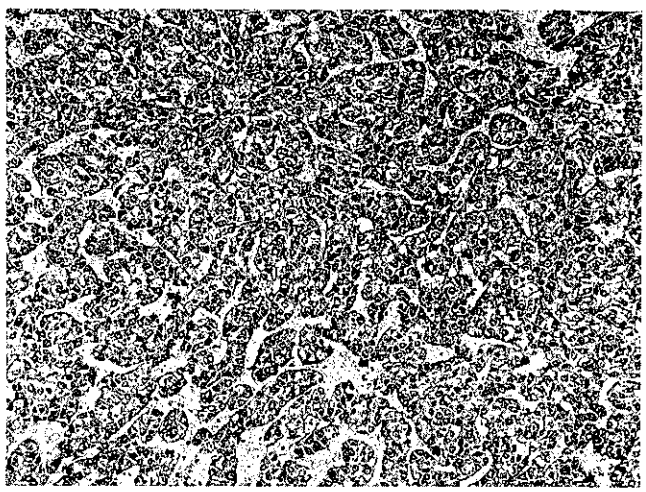

Fig. 1. Morphology of the original HCC tissue. Trabecular-sinusoidal structures are shown. (hematoxylin and eosin; original magnification ×200)

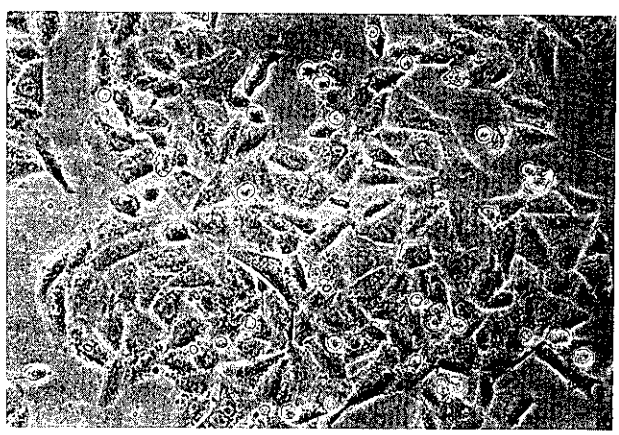

Fig. 2. Phase-contrast micrograph of SUHC-1 cells. They show a polygonal, epithelioid appearance. (×400)

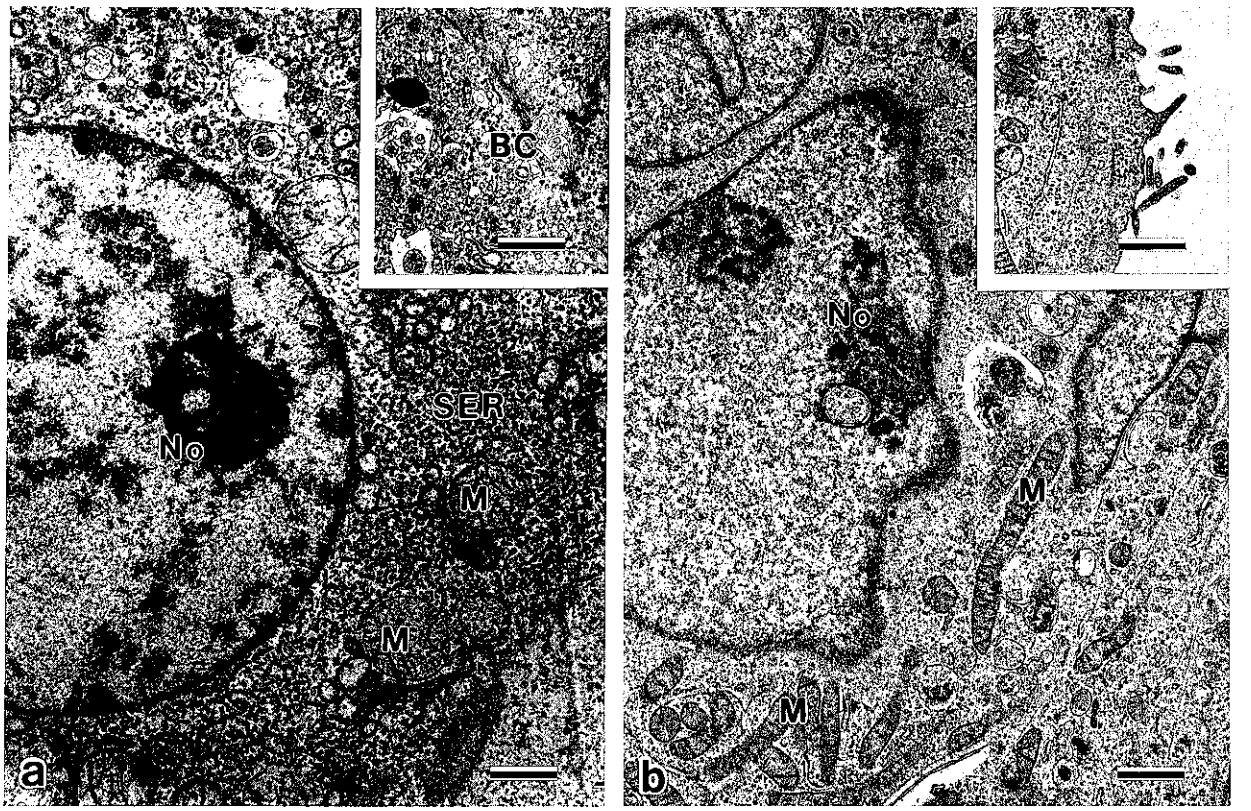

Fig. 3. a. Electronmicrographs of the original HCC tissue. A well-developed nucleolus (No) is seen in the nucleus. Mitochondria (M), smooth-surfaced endoplasmic reticulum (SER), and glycogen granules are observed in the cytoplasm. Inset demonstrates bile canaliculi (BC) among the cells. b. Electronmicrographs of the SUHC-1 cell. Nucleoli (No) are localized close to the indented nuclear membrane. Mitochondria (M) and ribosomes are developed in the cytoplasm. Inset; microvilli are also observed at the cell surface. Bar=1 μm.

by the indirect immunoperoxidase method. AFP, CEA and CA19-9 were not detected in the spent medium of SUHC-1 cells. AFP was not detected by the indirect immunoperoxidase method. AFP was detectable in the supernatant and the cytoplasm of PLC/PRF/5, used as a positive control.

Transplantation to nude mouse An amount of 1×10^6 to 2×10^7 SUHC-1 cells was inoculated subcutaneously in nude mice. Despite two attempts, no tumors were observed after 5 months.

HBV-DNA Southern hybridization assay HBV-DNA was not detected in SUHC-1 cells by Southern blot analysis with total HBV-DNA as a probe. As a positive control, PLC/PRF/5 showed specific HBV-DNA bands (data not shown).

HCV-RNA PCR assay HCV-RNA was not detected in the supernatant of the SUHC-1 cells by the nested PCR assay, even though the patient's serum was positive for HCV-RNA (Fig. 5).

***In situ* hybridization** HCV-RNA was not detected in the SUHC-1 cells using the *in situ* hybridization method (Fig. 6). We previously proved the existence of HCV-RNA in liver cells derived from patients with chronic hepatitis type C by the same method.[22]

DISCUSSION

The SUHC-1 cell line showed an epithelioid appearance on phase-contrast microscopy and had some characters of the HCC cells based on electronmicroscopic findings. Cell morphologic features and production of albumin and α_2-macroglobulin indicated that the origin of SUHC-1 cells was hepatocytes.

The modal chromosome number of the cell line was 91. An abnormal rearrangement at chromosomes 1, 17 and 20 (1p−, 1q−, i(1q), i(17q) and 20q+) was noted in all cells analyzed. Simon *et al.* reported abnormalities of chromosome 1 and loss of heterozygosity on 1p in HCC cell lines

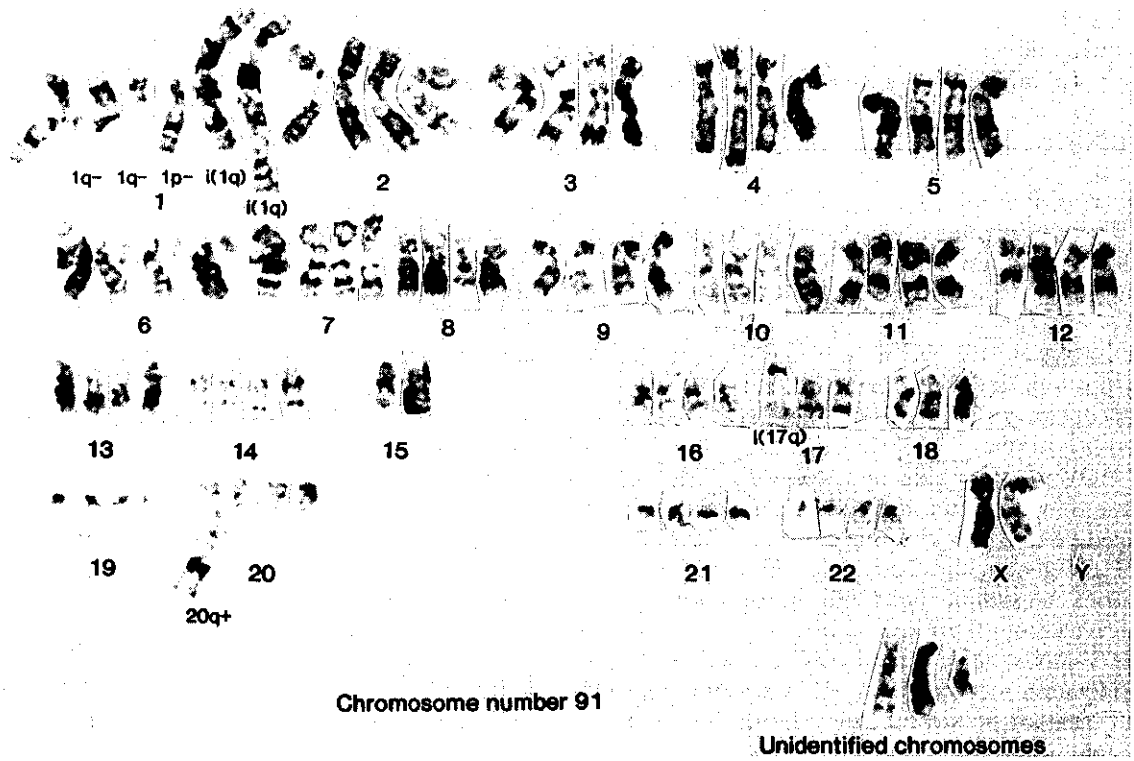

Fig. 4. Karyology of SUHC-1 cell line. A G-banded karyotype with 91 chromosomes is shown. SUHC-1 cells contain a distinctive rearrangement of chromosomes 1, 17 and 20, evident in all cells analyzed. Unidentified chromosomes are arranged at the bottom.

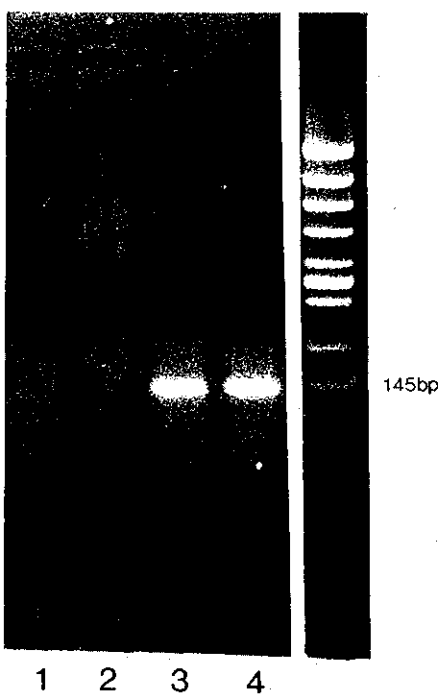

Fig. 5. Detection of HCV-RNA in the patient's serum and the spent medium of the SUHC-1 cells by the nested PCR assay. Lane 1; negative control; lane 2; the supernatant of SUHC-1 cells; lane 3; patient's serum; lane 4; positive control.

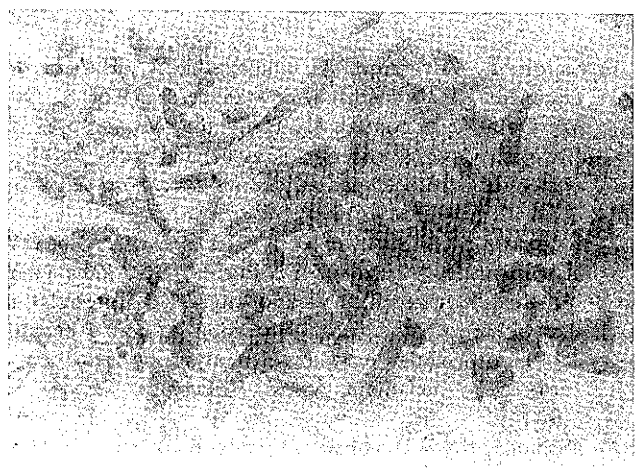

Fig. 6. Examination for HCV-RNA in the SUHC-1 cells by *in situ* hybridization. HCV-RNA is not detectable in the cells.

(HepG2, Hep3B, PLC/PRF/5, Huh-1, HA22T, Hep10 and NCA2) and suggested that chromosome 1 abnormalities characterize HCC and that loss of a gene(s) located on 1p might play a role in the induction of HCC.[23]

HBV-DNA was not integrated in the SUHC-1 cells, although the patient's serum was positive for anti-HBs antibody. Recent studies have revealed that HBx[7] and preS2/S[8] gene products acted as a transcriptional transactivator and HBx gene induced liver cancer in transgenic mice.[9] The role of HCV in the development of HCC is not known. HCV-RNA has reportedly been detected in tumor tissues of patients with anti-HCV antibody-positive HCC.[24] However, such tissues obtained at operation or autopsy may have been contaminated by serum. We should demonstrate the existence of HCV in tumor cells by detecting HCV-RNA in cells cultured in a medium kept free of human serum for a prolonged period. To our knowledge, there is no report that the HCV genome has been detected in an HCC cell line. We tried to examine the HCV genome of the SUHC-1 cells derived from the HCC patient whose serum contained HCV-RNA, as detected by PCR assay and the *in situ* hybridization method. HCV-RNA was not detected in the supernatant of SUHC-1 cells by the nested PCR assay system using HCV-specific primer consisting of sequences of the 5′ noncoding region. HCV-RNA was also not detected in the SUHC-1 cells by the *in situ* hybridization method. We conclude that complete HCV does not exist in SUHC-1 cells. It cannot be excluded that some fragment of HCV gene was integrated in the host DNA by reverse transcriptase. However, Itoh *et al.* found that reverse transcriptase activity was not detectable in human and chimpanzee sera with a high infectivity for non-A, non-B hepatitis.[25]

The established cell line, SUHC-1, is the first example of an HCC cell line derived from a patient with persistent HCV infection, although some of the HCC cell lines previously established elsewhere might possibly have been derived from HCV-infected patients with chronic liver disease, whose sera were not assayed for anti-HCV antibody or HCV-RNA.[14, 15] This cell line will provide a useful *in vitro* system for studies of the molecular biology of HCV and hepatocarcinogenesis, and of the activation of cellular oncogenes and inactivation of tumor suppressor genes.

ACKNOWLEDGMENTS

We thank Mr. Yoshihiro Kato and Mr. Masayuki Nozawa (Daiichi Pure Chemicals Co. Ltd., Tokyo) for providing the oligo-DNA probes. We also thank Dr. Takehiko Koji (Third Department of Anatomy, Nagasaki University School of Medicine, Nagasaki) for valuable advice. This work was supported in part by a Grant-in-Aid for Cancer Research (4-6) from the Ministry of Health and Welfare of Japan.

(Received March 24, 1992/Accepted May 1, 1992)

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