Original

Hepatocellular Carcinoma with PIVKA-II Production in a Young Laboratory Monkey

Katsuhiko Yoshizawa^{1,3}, Yuji Oishi¹, Koichi Sano¹, Kenjiro Tsubota¹,
Kazuhiko Ikeda², Yoshifumi Fukuhara², Hideto Senzaki³, and Airo Tsubura³

¹Department of Toxicologic Pathology, Toxicology Research Laboratories, and

²Department of Toxicology, Toxicology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Kashima 2–1–6, Yodogawa, Osaka 532–8514, Japan

³Department of Pathology II, Kansai Medical University, Fumizono-cho, Moriguchi, Osaka 570–8506, Japan

Abstract: Hepatocellular carcinoma which occurred in one male laboratory cynomolgus monkey at 5 years of age was investigated extensively by immunological, histopathological and electron-microscopic examinations. The animal exhibited no abnormalities in clinical laboratory tests including blood-chemistry, except for elevation of protein induced by vitamin K absence or antagonist II (PIVKA-II) in serum, which is one of the hepatic tumor markers. In virus antibody tests, the serum was positive for hepatitis A virus. On necropsy, a mass measuring 60 × 60 × 65 mm was seen in the hepatic left lobe. The tumor mass had a lobulated structure with hemorrhagic and necrotic areas, and was demarcated by thin fibrous capsules. Histopathologically, the tumor was composed of hepatocyte-like cells, having irregular trabecular structure with various thickness, lined by vascular endothelial cells. Cellular atypia such as polynucleated cells, mitotic figures, and invasion into the vascular cavity were also observed. Immunohistochemically, the tumor cells showed positive reaction for anti-PIVKA-II, anti-epithelial membrane antigen (EMA), anti-carcinoembryonic antigen (CEA), and anti-cytokeratin 18, as well as for proliferating cell nuclear antigen (PCNA). On electron-microscopic examination, the tumor cells had a number of tight junctions and formations of bile canaliculi between adjacent cells, basically resembling hepatocytes. This is the first case of hepatocellular carcinoma with PIVKA-II production in monkeys. Serological and immunohistochemical analyses for PIVKA-II are, therefore, practicable for diagnose as hepatocellular carcinoma in nonhuman primates. (J Toxicol Pathol 2002; 15: 61–68)

Key words: hepatocellular carcinoma, cynomolgus monkey, PIVKA-II, hepatitis virus, immunohistochemistry

Introduction

Although various spontaneous neoplasms have been reported in nonhuman primates, the incidence of neoplasms is very low^{1–3}. In particular, spontaneous hepatic malignant neoplasms have rarely been described in monkeys; only fourteen tumors have been reported in wild or wild-derived aged monkeys (Table 1). The pathogenesis of spontaneous hepatocellular carcinoma (HCC) is not well-established in nonhuman primates due to its low incidence, though the occurrence is often associated with cirrhosis and hepatitis virus infection in humans^{12,13}. Whereas several chemicals, such as 2-amino-3-methylimidazo [4,5-*f*] quinoline¹⁴, pyridoxine¹⁵, cycasin¹⁶,

aflatoxin B₁^{17,18}, and N-nitrosodiethylamine^{19,20}, can induce hepatic tumor in laboratory primates, only limited information is available from these experimentally-induced liver tumors in monkey. In the safety assessment of new chemicals or drugs, it is important to recognize spontaneous lesions in laboratory monkeys, especially in young monkeys that are routinely used in toxicological studies. In this report, we describe the occurrence of spontaneous hepatocellular carcinoma (HCC) in a young laboratory monkey and discuss the relevance to PIVKA-II production.

Materials and Methods

Animal

The male cynomolgus monkey (*Macaca fascicularis*), born and raised in primate colonies in Vietnam, was purchased from U.S.A. Covance Research Products at 4 years of age. The animal had been checked for one month for medical inspection and had no abnormal findings in the following examinations: parasite detection test, hematology and blood-chemistry, as well as serological tests for simian

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Mailing address: Katsuhiko Yoshizawa, Department of Toxicologic Pathology, Toxicology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Kashima 2–1–6, Yodogawa, Osaka 532–8514, Japan

TEL: 81-6-6390-1167 FAX: 81-6-6304-5716

E-mail: katsuhiko_yoshizawa@po.fujisawa.co.jp.

Table 1. Spontaneous Hepatocellular Tumors of Nonhuman Primates

Hepatic Neoplasm	Species	No. of case	Age (years)	Authors (year)	Reference
Hepatocellular carcinoma	<i>Lemur macaco</i>	1	unknown	O'Gara RW <i>et al.</i> (1972)	2
Hepatocellular carcinoma	<i>Cercocebus atys</i>	1	10	Clark JD <i>et al.</i> (1973)	4
Hepatocellular carcinoma	<i>Tupaia belangeri</i>	2	8	Hofmann W <i>et al.</i> (1981)	5
Hepatocellular carcinoma	<i>Pan troglodytes</i>	2	>7	Tabor E (1989)	6
Hepatocellular carcinoma	<i>Macaca mulatta</i> , <i>Tupaia belangeri</i>	3	unknown	Beniasvili DS <i>et al.</i> (1990)	7
Hepatocellular carcinoma	<i>Pan troglodytes</i>	1	12	Abe K <i>et al.</i> (1993)	8
Hepatocellular carcinoma	<i>Saimiri boliviensis</i>	1	>24	Borda JT <i>et al.</i> (1996)	9
Hepatocellular carcinoma	<i>Saimiri boliviensis</i>	1	>13	Morris TH <i>et al.</i> (1996)	10
Hepatocellular carcinoma	<i>Macaca fascicularis</i>	2	5	Reindel JF <i>et al.</i> (2000)	11
Hepatocellular carcinoma	<i>Macaca fascicularis</i>	1	5	Present case	
Hepatocellular adenoma	<i>Presbytis entellus</i>	1	unknown	O'Gara RW <i>et al.</i> (1972)	2
Hepatocellular adenoma	<i>Presbytis entellus</i> , <i>Cercopithecus aethiops</i> , <i>Papio hamadryas</i> , <i>Macaca multa</i>	11	unknown	Beniasvili DS <i>et al.</i> (1990)	7

Table 2. Virus Titers and Hepatic Tumor Markers

	Present Case	Control Cases*	Method
HAV	Inhibition 97.3 %	Inhibition 90.2 %	RIA
HBV	<×16	<×16	PA
HCV	<0.1	<0.1	RIA (C-100-3)
B-virus	negative	negative	RIA
HSV-1	negative	negative	RIA
SIV	negative	negative	RIA
SRV	negative	negative	RIA
Filovirus	negative	negative	RIA
AFP	<0.5 ng/mL	<0.5 ng/mL	RIA
PIVKA-II	24770 mAU/mL	122 mAU/mL	ECLIA

AFP: α -fetoprotein, PIVKA-II: protein induced by vitamin K absence or antagonist II, HAV: hepatitis A virus, HBV: hepatitis B virus, HCV: hepatitis C virus, HSV-1: herpes simplex virus-1, SIV: simian immunodeficiency virus, SRV: simian retrovirus, *: three male untreated monkeys used in the same toxicity study, RIA: radio-immunoassay, ECLIA: electrochemiluminescence immunoassay, PA: peroxidase assay.

retrovirus, simian immunodeficiency virus, herpes B virus, herpes simplex virus type 1, and simian T-lymphotrophic virus type 1 (Table 2). The animal was housed for about one year individually in a stainless steel cage in an environment of $25 \pm 1^\circ\text{C}$ room temperature, $60 \pm 5\%$ relative humidity, and a 12 hr light-dark cycle. The monkey was given daily 100 g of commercial diets in breeder and PS diets (Oriental Yeast Co., Ltd., Tokyo, Japan) in our laboratory and water freely. The present monkey was one of the monkeys employed in the low-dose group of a 4-week toxicity study of a certain test compound and the age was at 5 years of age. During the study period, animals were routinely observed for clinical signs, weighed, and examined for hematology and blood-chemistry. No abnormalities were detected in the liver of other monkeys except this monkey. Therefore, the study report concluded that the test compound had no hepatotoxicity potential. The monkey was handled in accordance with Fujisawa Pharmaceutical's ethical

guidelines for animal care, handling, and termination.

Serological test

The concentrations of two markers for tumors of hepatocyte origin; vitamin K absence or antagonist II [PIVKA-II, ECLIA method, PIVKA-II IRMA Daiichi kit (Daiichi Radioisotope Labs, Tokyo, Japan)²¹] and α -fetoprotein [AFP, RIA method, Alfa-feto RIA beads kit (Dainabot, Tokyo, Japan)²²], and the antibody titers against hepatitis A [HAV, RIA method, HA antibody RIA kit (Dainabot)²³], B [HBV, PA method, Cellodia anti-HBs PA kit (Fuji Rebio, Tokyo, Japan)²⁴] and C [HCV, RIA method, Ortho-HCV Ab IRMA kit (Ortho-Clinical Diagnostics, Tokyo, Japan)²⁵] viruses were determined serologically (Table 2); it has been reported that PIVKA-II, which is an abnormal prothrombin with a relatively high incidence in HCC patients, is one of the useful tumor markers for human HCC^{21,26–30}. These serological tests were performed at Falco

Table 3. Immunohistochemical Profile of the Tumor Cells

Antibodies	Clone	Source	Dilution	Antigen retrieval	Results
PIVKA-II	MU-3	Eisai	50	microwave	Diffuse, ++
Cytokeratin-18	DC-10	DAKO	20	microwave	Focal, +
CEA	polyclonal	DAKO	150	microwave	Diffuse, +
EMA	E29	Nichirei	prediluted	NT	Focal, +
PCNA	PC-10	Novacastra	100	microwave	Diffuse, ++
Vimentin	V9	DAKO	40	microwave	Negative
AFP	polyclonal	Nichirei	prediluted	NT	Negative
Factor VIII	F8/86	Nichirei	prediluted	trypsin	Negative
Actin	HHF-35	Enzo	50	microwave	Negative
p53	DO-7	Nichirei	prediluted	microwave	Negative

PIVKA-II: protein induced by vitamin K absence or antagonist II, CEA: carcinoembryonic antigen, EMA: epithelial membrane antigen, PCNA: proliferating cell nuclear antigen, AFP: α -fetoprotein, NT: not treated, +: positive, ++: strongly positive.

Fig. 1. Gross view of the liver. A large gray and dark red mass is located in the left lobe (a). The tumor is composed of multilobular structures covered by a thin fibrous capsule accompanied by hemorrhage and necrosis (b).

Biosystems Co., Ltd. (Kyoto, Japan) for blood sample obtained from the present monkey; sera from three male monkeys in the control group of the toxicity study served as the controls.

Pathology

The monkey was sacrificed under deep anesthesia by exsanguination from the cervical aorta and subjected to a complete necropsy including measurement of organ weights. Specimens of major organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for routine histopathological examination. Additional serial sections of the hepatic tumor were stained with periodic acid-Schiff (PAS), azan, phosphotungstic acid hematoxylin (PTAH), silver reticulin, Berlin blue for iron, and Hall's method for bile. Representative sections were stained using immunocytochemical technique^{31–33} for PIVKA-II (MU-3, Eisai, Tokyo, Japan), cytokeratin 18 (CK18; DC-10, DAKO, Glostrup, Denmark), carcinoembryonic antigen (CEA;

polyclonal, DAKO), epithelial membrane antigen (EMA; E29, DAKO), proliferating cell nuclear antigen (PCNA; PC-10, Novacastra, Newcastle upon Tyne), vimentin (V9, DAKO), alfa-fetoprotein (AFP; polyclonal, DAKO), factor VIII-related antigen (F8/86, Nichirei, Tokyo, Japan), alfa-smooth actin (HHF-35, Enzo, USA), and p53 (DO-7, Nichirei) (Table 3). Additionally, some specimens of the hepatic tumor were post-fixed in 2.5% glutaraldehyde and 1% osmium tetroxide solutions, then dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate for electron-microscopic observation.

Results

Clinical laboratory findings

Clinical laboratory tests including hematology and blood-chemistry revealed no abnormalities before and during the study period. The terminal values before sacrifice in the parameters of hepatic damage; glutamic oxalacetic

Fig. 2. Photomicrographs of the hepatic tumor. (a) Solid sheets and trabeculae of neoplastic hepatocytes are partially surrounded by a fibrous capsule (arrows). Dilated vascular spaces (arrow heads) are seen. HE staining, $\times 40$. (b) Trabeculae in various thickness are separated by sinusoids lined by endothelial cells. HE staining, $\times 100$. (c) Polynucleated giant cells with cellular pleomorphism in size and shape are seen (arrows). Focal lymphocytic infiltration is seen in sinusoidal area (arrow heads). HE staining, $\times 100$. (d) Note atypical mitotic figure of hepatic tumor cells. HE staining, $\times 100$.

transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase (ALP), were within the normal ranges (36, 28, and 614 mU/mL respectively).

Serological tests for viral antigen and hepatic tumor markers

Serum PIVKA-II concentration before sacrifice was elevated (24770 mAU/mL) compared with those of control levels (mean: 122, min: 106, max: 139 mAU/mL), whereas the AFP value was similar to control levels (below 0.5 ng/mL) (Table 2). The history of HAV infection was suspected in these animals, since the virus antibody reaction before sacrifice was positive for HAV in this animal (inhibition rate 97.3%) as well as in the control animals (inhibition rate 90.2%), whereas they were negative for HBV and HCV.

Macroscopic findings

The $60 \times 60 \times 65$ mm-large gray and dark red mass with hemorrhagic and necrotic areas was located in the hepatic

left lobe (Fig. 1a, b). At the cut surface, the mass had a multilobular structure covered by a thin fibrous capsule. Emboli to hepatic portal vein were not seen, and grayish nests were detected in the other lobes (Fig. 1b). The gross abnormalities were not detected in any other organs or tissues.

Histopathology

The tumor consisted of solid sheets and trabeculae partially surrounded by a fibrous capsule (Fig. 2a). The trabeculae were of various thickness and separated by prominent sinusoids lined with vascular endothelial cells (Fig. 2b). Tumor cells were larger than adjacent normal hepatocytes and had abundant homogenous or vacuolated eosinophilic cytoplasm with well-demarcated cell borders. Collagen fibers were often detected between tumor cords by azan stain; however, reticulin fibers were rarely found by silver stain. Tumor cells had cellular pleomorphism in size and shape, and there were polynucleated giant cells with

Fig. 3. Immunohistochemical characteristics of hepatic tumor. Most of the tumor cells are diffusely positive for PIVKA II (a). Scattered tumor cells are positive for CK18 (b), CEA (c), and EMA (d). Most tumor cells are highly labeled by PCNA (e). Arrow heads show normal area. Immunohistochemical staining, $\times 100$.

single or multiple nucleoli (Fig. 2c) and atypical mitotic figures (Fig. 2d). Tumor invasions to vascular cavities were infrequently observed and tumor cells rarely possessed PTAH-positive granules, so called pale bodies. Tumor cells forming glandular and ductal structures with bile production were not observed by Hall's stain. Moreover, the existence of bile ducts, Glisson's sheath or cirrhotic changes were seldom detected. Focal lymphocytic infiltration (Fig. 2c), hemosiderin deposition by Berlin blue stain, and calcified parasite in dilated vascular space of tumor necrotic area were observed.

The parenchyma surrounding the neoplasm was compressed and atrophic. In other lobes, no metastases were detected, although focal hepatocellular hypertrophy without compression of parenchyma was seen. In accordance with

grayish nests observed macroscopically, focal glycogen-rich areas were detected by PAS-positive reaction. Additionally, the other organs and tissues were demonstrated to be normal for the animal's age in detailed histopathological examinations.

The results of immunohistochemical stain of the tumor are summarized in Fig. 3 and Table 3. Most neoplastic cells showed strongly positive reaction for PIVKA II (Fig. 3a), while several tumor cells were weakly positive for CK18 (Fig. 3b), CEA (Fig. 3c), and EMA (Fig. 3d). Moreover, high PCNA-labeling indicated high cellular proliferation activity in tumor cells (Fig. 3e). They were negative for vimentin, AFP, factor III, actin, and p53 (Table 3). In the normal area surrounding the neoplasm, some hepatocytes were weakly positive for PIVKA II. A few PCNA-labelled

Fig. 4. Electron micrographs of tumor cells. Tight junction (arrow head) and structures resembling bile canaliculi (arrows) are seen between the carcinoma cells. bar: 6 μ m.

cells in addition to bile duct epithelium were positive for CK18.

Electron-microscopically, tumor cells possessing one or two round nuclei had a high number of swollen mitochondria in the cytoplasm. Golgi apparatus, glycogen granules, and lipid droplets were also present in the cytoplasm. Between the tumor cells, tight junctions and structures resembling bile canaliculi were often observed (Fig. 4).

Discussion

The present case demonstrated typical features of hepatocellular carcinoma; trabecular proliferation with sinusoidal structure and vascular invasion of neoplastic cells. Moreover, electron-microscopic features such as junctional structure and bile canaliculi were suggestive of hepatocellular origin³⁴. Based on the aforementioned findings and anatomical location, we diagnosed the tumor as hepatocellular carcinoma (HCC). Review of the literature (Table 1) revealed only fourteen cases of spontaneous hepatic malignant neoplasia in several strains of wild or wild-derived aged monkeys. Our case may be added to the limited number of reports on spontaneously-occurring hepatic tumors in young nonhuman primates.

The etiology of liver tumors in both human and animals documented in the literature includes cirrhosis, viral hepatitis, parasites, and aflatoxin in diet. In our case, there were no factors which suggested any relationship to the diet, because the same quality of commercial diet has been given to monkeys in breeder and our laboratory. Moreover, there were no cirrhotic changes in the liver. However, the history of HAV and parasitic infections in the liver was apparent by histopathological and immunological analyses in our case. It is well known that several species of healthy monkeys show

detectable antibodies to HAV and HBV³⁵; HAV does not seem to cause any hepatic lesions in nonhuman primates³⁶, while HBV can induce subacute hepatitis but not neoplastic changes³⁶. On the other hand, HCV (non-A, non-B hepatitis virus) induces HCC experimentally in some species of monkeys^{6,37,38}. The present case was not related to HBV and HCV infection as indicated by negative immunological reaction. Concerning the parasitic cause, HCC in a chimpanzee has been reported to be associated with chronic *Schistosoma mansoni* infection⁸. It was not clear whether the parasite infection was related to the occurrence of HCC in this animal, although calcified parasite in tumor necrotic area was observed.

It is generally accepted that PIVKA-II is a useful tumor marker for human HCC^{21,26–30}. Although the exact mechanism of the elevation of PIVKA-II levels in HCC is not clear, abnormal prothrombin appears to be produced in relation to excessive synthesis of prothrombin precursors in human HCC tissue²⁸. Moreover, it is also well recognized that AFP is produced by immature hepatocytes of the fetus or in hepatic tumors². Both AFP and PIVKA-II are generally detected in the serum of advanced HCC patients; however, serum PIVKA-II levels do not correlate with serum AFP levels^{28–30}. In our case, serum AFP level was within the normal range and AFP was not detected in tissues immunohistochemically. This finding agrees to the previous reports; though a progressive increase of serum AFP is seen in several cases of chemically-induced HCC in primates^{14,15,19}, neoplastic cells do not always show a positive reaction for AFP immunohistochemically¹¹. In contrast, usefulness of immunohistochemical analyses for PIVKA-II has been reported in human HCC and ovarian hepatoid carcinoma^{31,32}. Our results suggest PIVKA-II may be a useful HCC marker in monkey HCC, as same as is the case in humans.

Immunohistochemical analyses of chemically-induced monkey HCC have revealed positive reactions for cytokeratin 8, 18, and/or 19²⁰. In addition, spontaneous HCC is reported to be positive for glutathione S-transferase, AFP, and/or CEA¹¹. Both tumor types were positive for CK18 antigen. In the present HCC, neoplastic cells revealed positive reactions for CK18, EMA, and CEA, in line with previous reports in monkeys and humans^{11,12,20,38}, but were negative for p53. p53 is a nuclear protein that normally regulates cell growth and suppresses tumor formation. Mutation of p53 has been documented in 50% of human HCC patients³⁹ and in 20% of chemically-induced HCC monkeys⁴⁰; however, the mutation did not occur in the present case, similarly to some other cases in primates^{40,41}.

In chemically-induced tumors in monkeys, usefulness of PIVKA-II has not been widely recognized, yet, although some information characterized by serological, morphological and immunohistochemical data is available. The results of our young monkey case may serve as a useful reference for researchers who will encounter similar lesions in both veterinary and toxicological research in nonhuman primates. Moreover, the present report provides additional

information on the fact that young monkeys such as 5 years of age have the potential for developing spontaneous hepatocellular carcinoma.

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