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Induction of Hepatoma in Mice by Direct Deep Black-Extra (DDB-EX) and Occurrence of Serum AFP

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Summary (Report I)

Hepatic tumor was observed in about 80% of ICR(SLC) mice which were given 0.3% solution of Direct Deep Black-Extra (DDB-EX) as drinking water. One characteristic of this chemically induced hepatoma is its high degree of resemblance to human hepatoma especially in histological appearance. The tumor showed a histology of hepatocellular carcinoma; however, cholangiocellular carcinoma and cirrhosis were not observed. Serum AFP was detected in about 90% of tumor-bearing mice by either single radial immunodiffusion (SRID) method or latex fixation (LF) method. A correlation between the size of the tumor and AFP level was observed in the group in which the AFP was detectable by SRID method. The larger the occupancy of the tumor in proportion to the entire liver, the higher the serum AFP level was found to be. These tumors had a histological appearance of poor differentiation. However, there were many cases in which depite the presence of a large tumor, serum AFP could not be detected by SRID method. These tumors showed a histological appearance of moderate or well differentiation. Besides hepatic tumor, soft tissue tumor was observed in about 30% of the mice with histology of squamous cell carcinoma originating in the mammary gland.

Introduction

Since the first report of experimental cancer of the liver by Yoshida in 1932[10], many kinds of chemically induced hepatoma have been reported[4,7]. One of the most common and convenient models for studying primary cancer of the liver is DAB hepatoma in rats. It has been used for many years and has contributed greatly to the study of liver cancer. But this chemically induced hepatoma is considerably different from human hepatoma in its morphology.

While benzidine, one of the aromatic amines, has long been recognized in relation to genesis of cancer of the urinary bladder, chiefly in the field of occupational disease[9], it has also recently come to be regarded as one of the carcinogenic agents on other organs such as the liver, the

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stomach or the colon. MIYAKAWA and YOSHIDA[5] reported carcinogenic effects of oral administration of five kinds of benzidine dye [Direct Deep Black-Extra (DDB-EX), Direct Dark Green-B (DDG-B), Direct Bordeaux-BK (DB-BK), Congo Red (CR) and o-Tolidine (o-T)] in mice (Fig. 4). They observed induction of primary cancer of the liver in mice given DDB-EX and DDG-B.

The present study describes the details of one method of induction of DDB-EX hepatoma and pathological findings in relation to serum alphafetoprotein (AFP) concentrations.

Materials and methods

Animals and experimental groups

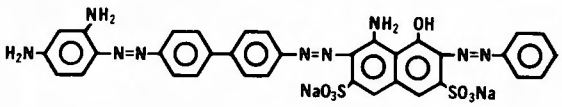
One hundred and twenty ICR (SLC) mice (Shizuoka Laboratory Animals Co., Shizuoka, Japan), four weeks old, weighing between 25 and 30 g were used.

The mice were housed in aluminum cages, two mice to a cage, under air conditioning at 24°C, and maintained on a commercial stock diet (CLEA Japan Ind., Japan).

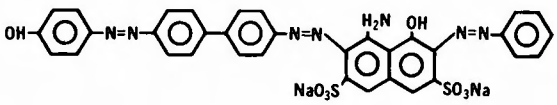
DDB-EX (Fig. 1) was obtained from Kyoei Chemicals, Ltd., Kyoto, and was given ad libitum as drinking water in 0.3% solution to the animals. The experimental groups were as follows:

1) Group I consisted of 20 mice given water without DDB-EX for 60 weeks.

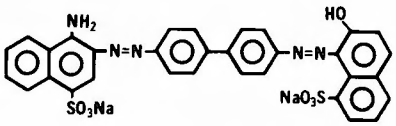
Direct Deep Black EX



Direct Dark Green B



Direct Bordeaux BK



Congo Red

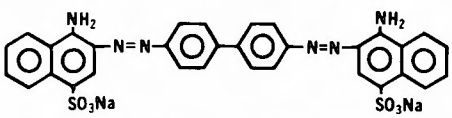


Fig. 1. Structural formulas of four kinds of benzidine dye reported to be carcinogenic.

2) Group II consisted of 40 mice given 0.3% DDB-EX solution. Every two weeks from the 16th week, two animals were randomly selected and exsanguinated through the exposed abdominal aorta.

3) Group III consisted of 60 mice given 0.3% DDB-EX solution. They were sacrificed between the 55th and 60th week. Blood Samples were obtained through the abdominal aorta and stored at −21°C After complete autopsy including careful macroscopic examination, organs were fixed in 10% formaldehyde solution or in 95% cold ethyl alcohol. The tissues were embedded in paraffin, sectioned and stained routinely with hematoxylin and eosin.

Preparation of mouse AFP and its specific antibody

Alphafetoprotein (AFP) was purified from 10 ml of pooled serum of mice with DDB-EX hepatoma by using preparative acrylamide gel electrophoresis as described by JOVIN, et al[3]. Protein content of the elution buffer was recorded by ultraviolet photometer (Fig. 2). A crude antigen containing mouse AFP was obtained in two successive groups (Nos. 3 and 4 on the chart). Samples in serial fraction tubes were gathered, condensed with a diaflomembrane and stored at −21°C Mouse AFP obtained from Dr. H. WATABE (Dept. of Int. Biochemistry, School of Medicine, Hokkaido University).

Antiserum was prepared in the rabbit by subcutaneous injections of crude antigen with single doses of 4 mg protein content at weekly intervals for four weeks. The crude antigen was emulsified with Freund's complete adjuvant. Two weeks after the last injection, rabbit serum was collected from the ear vein. Absorption of the antiserum was carried out with normal adult mouse serum. An appropriate mixing ratio of antiserum to normal mouse serum was determined

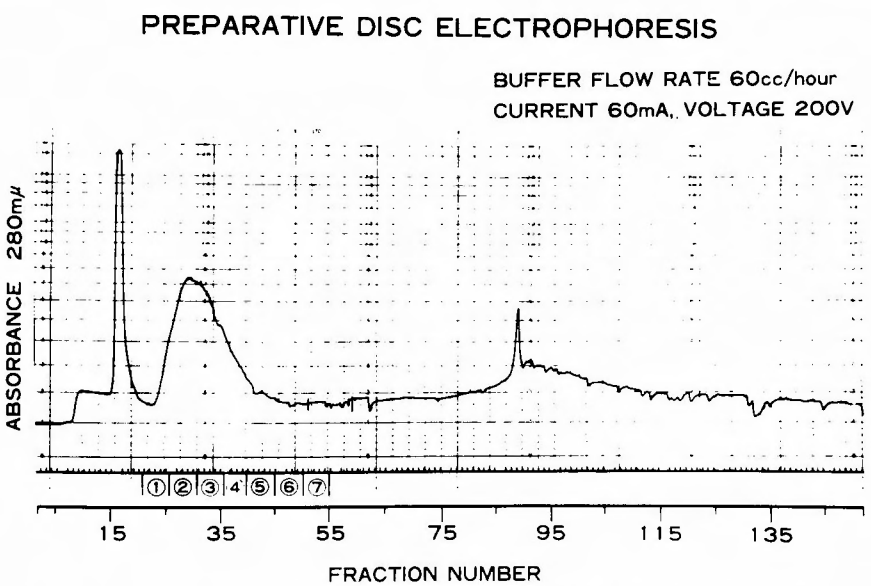


Fig. 2. Protein content of the elution buffer as recorded by ultraviolet photometer. AFP was obtained from Groups 3 and 4.

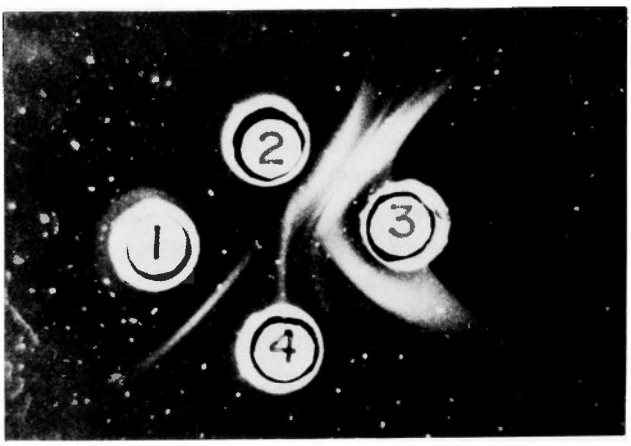


Fig. 3. Results of agar-gel precipitation method. Well 1 contains the absorbed pure antibody; Well 2, normal mouse serum; Well 3, crude antibody; and Well 4, mouse serum containing AFP. A clear single line is apparent between Wells 1 and 4 indicating that the absorbed antiserum is monospecific for the mouse AF.

according to the agar-gel precipitation method. Antigen-antibody mixtures were incubated at 37°C for two hours, placed in a cold room for four days, and centrifuged at 15,000 g for one hour. The absorbed antiserum was demonstrated to be monospecific for the mouse AFP (Fig. 3).

Detection and quantification of mouse AFP

Agar-gel precipitation method and latex fixation (LF) method were used for detection and quantification of mouse serum AFP. Micro-Ouchterlony double diffusion method and Mancini's single radial immunodiffusion (SRID) method were used as agar-gel precipitation methods. It was observed that the lowest value determined by SRID method was approximately 10 μg/ml. Latex fixation method was carried out according to the modification described by SINGER and PLOTZ. Relative sensitivities of LF and SRID methods were compared using the original AFP-containing material. By the LF method as little as 250 ng/ml of serum AFP could be detected, indicating that the LF method has 100 times the sensitivity of the SRID method.

Results

Time of appearance of tumor and serum AFP level (Table 1)

Hepatic tumor was first observed in a mouse sacrificed at the 20th week after administration of 0.3% DDB-EX solution. The tumor was white, solitary, 5×4×5 mm in size and located in the right lobe. Serum AFP was negative not only by the SRID method but also by the LF method. During the period between the 22nd–30th week, hepatic tumors were observed in two out of ten mice. Serum AFP was detected only by LF method in these two tumor-bearing mice. During the 32nd–40th week, hepatic tumors were observed in four out of ten mice. Serum AFP was detected in three out of four, two by SRID and one by LF method. During the last period between the 42nd–50th week, nine out of ten mice had hepatic tumor, the serum AFP of which

Table 1. Time of appearance of tumor and concomitant serum AFP level.

Duration of Administration (Animal)	16～20 week (6)	22～30 (10)	32～40 (10)	42～50 (10)
Hepatic Tumor	1	2	3	7
LF	0	2	0	1
SRID	0	0	2	5
Hepatic & Mammary Tumor	0	0	1	2
LF	0	0	1	1
SRID	0	0	0	1
Mammary Tumor	0	0	0	0
LF	0	0	0	0
SRID	0	0	0	0
Total	1	2	4	9

was detected in five by SRID method and in two by LF method.

Mammary tumor was first observed in a mouse at the 38th week simultaneously with hepatic tumor. Serum AFP was detected by the LF method. No tumor was observed in the mice of the control group at the time of sacrifice.

Incidence of hepatic tumor and mammary tumor and quantification of serum AFP in mice sacrificed during 55th–60th week (Table 2)

Fifty-nine of 60 mice in Group III survived more than 55 weeks. Hepatic tumor was observed in 46 of 59 (77.9%) and mammary tumor in 20 of 59 (33.8%). Nine of the mice had both kinds of tumors. No tumor was observed anywhere in the two remaining mice. Serum AFP was detected in 36 mice (78.3%) by the SRID (30 with hepatoma alone and six with both tumors) and in five (10.8%) by the LF method (four with hepatoma and one with both tumors). No serum AFP was detected by either method in the eleven mice with mammary tumor alone and the two mice with no tumor.

Table 2. Incidence of tumor in Group III mice and the number of mice showing AFP-positive serum.

	Number	AFP(+) SRID	AFP(+) LF
Tumor Bearing Animal	57	36	5
Hepatic Tumor	37	30	4
Heaptic & Mammary Tumor	9	6	1
Mammary Tumor	11	0	0
Non-Bearing Animal	2	0	0
Total	59	36	5

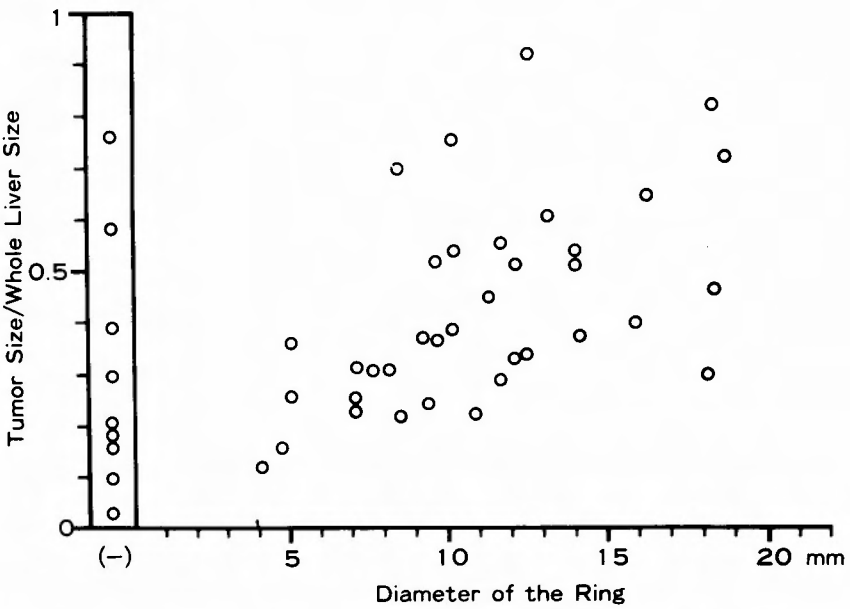


Fig. 4. The serum AFP concentration is represented on the horizontal axis by the diameter of the precipitate ring.

Relationship between the size of hepatic tumor and serum AFP concentration in nine mice sacrificed during 55th–60th week (Figure 4)

Results obtained from a comparison of the size of the hepatic tumor with serum AFP concentration are shown in Figure 4. The ratio of the size of the tumor to that of the entire liver is represented on the vertical axis. The serum AFP concentration was converted to the diameter of the precipitate ring in the agar gel of the SRID method, which is represented on the horizontal axis. As shown in Figure 4, a correlation between size and AFP level was observed in the group in which the AFP was detectable by SRID method. These tumors had a histological appearance of poor differentiation. However there were many cases in which despite the presence of a large tumor serum AFP could not be detected by SRID method. These tumors showed a histological appearance of moderate or well differentiation.

Pathology

1) Hepatic tumor (Figures 5, 6 and 7)

Most of the hepatic tumors were solid, but slightly soft. Many of them were solitary tumors, but some had multiple nodular lesions. Liver tissue around the tumor showed normal appearance with no fibrosis or cirrhosis. Clear demarcation was observed between the tumor and surrounding normal tissue, however no capsule could be found. A more common change, observed in many of the cases, was a bulging adenomatous zone, within which the liver cell changed atypically with rather eosinophilic cytoplasm. Trabecular growth was remarkable, the majority of the cancer cells being well differentiated (Fig. 6); however,

Fig. 5. Macroscopic observation. Solitary grayish-white tumor. Surrounding tissue shows no fibrosis or cirrhosis.

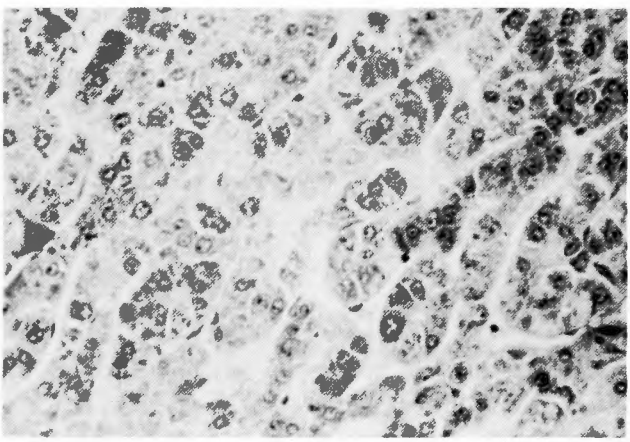


Fig. 6. Histological section showing trabecular growth with fairly well differentiated cancer cells.

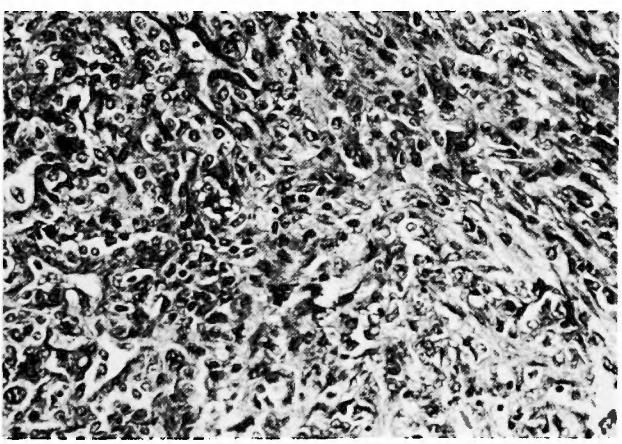


Fig. 7. Histological section showing poorly differentiated cancer cells with more hyperchromatic nucleus and granular acidophilic cytoplasma.

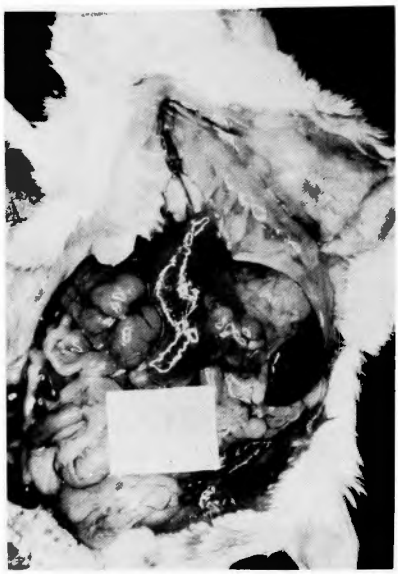


Fig. 8. Macroscopic observation. A solitary tumor can be observed in the subcutaneous tissue of the breast (lower right corner of photo).

moderate and poorly differentiated cells were also present (Fig. 7). No cancerous changes were observed in cholangiogenic cells. Fibrosis was present in some cases, but cirrhosis was not prominent.

2) Mammary tumor (Figures 8 and 9)

A solitary, solid and slightly hard mass was observed in the subcutaneous soft tissue of the shoulder or breast, well demarcated from the surrounding tissue. This tumor was observed only in female animals and first noticed at the 32nd week. At the end of this experimental course, the tumor was observed in 20 out of 59 mice. The tumors were mainly occupied by

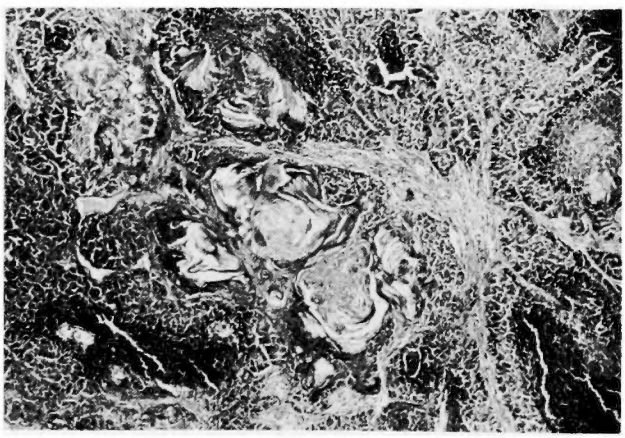


Fig. 9. Microscopic observation. The tumor is mainly occupied by squamous cell carcinoma forming cancer pearl.

adenocarcinoma bulging medullary, the remainder by squamous cell carcinoma forming cancer pearl. These findings confirmed that the origin of this tumor was the mammary gland. These adenoacanthomatous findings were observed in all animals bearing subcutaneous tumor.

Discussion

Benzidine, 2-naphtylamine and 4-quinodiphenyl, known as the aromatic amines, are well documented as powerful carcinogens in the human urinary bladder[5,6,9]. Experimentally, SPITZ et al.[9] produced neoplastic changes including hepatocellular carcinoma, squamous cell carcinoma of the sebaceous glands and adenocarcinoma of the colon in rats injected with both pure and technical grade benzidine. But the incidence of hepatoma in rats induced by technical benzidine, pure benzidine and benzidine sulfate was reported to be very low, 0.65 to 3.9%. OSANAI reported an experiment on the induction of hepatic tumor in ICR mice using several kinds of aromatic compounds, such as benzidine, 3,3'-dichlorobenzidine, dianisidine, 3,3'-dihydroxybenzidine, 1-naphtylamine, 2-naphtylamine and 3,4,4'-biphenylethertriamine. Hepatic tumor was observed in almost 100% of animals given benzidine and dianisidine for six months. He concluded that aromatic compounds would be a potent carcinogen especially in ICR mice.

YOSHIDA and MIYAKAWA[11] carried out an experiment of induction of hepatoma in C3H mice by benzidine dyes. They used 0.1% solution of each of the following: Direct Deep Black-Extra (DDB-EX), Direct Bordeaux-BK (DB-BK), Congo Red (CR) and o-Tolidine (o-T). Hepatic tumor was observed at the 40th week only in the animals given DDB-EX and DDG-B. The incidence of occurrence of the tumor was 33.3%. Using isolated intestines of mice and rats, YOSHIDA[5] et al. showed that DDB-EX was disintegrated by the intestinal bacteria to benzidine and its derivatives. They emphasized that benzidine might play an important role in induction of hepatoma by aromatic compounds.

In the present experiment, ICR (SLC) mouse rather than C3H mouse was used as the experimental animal because of the lower incidence of spontaneous occurrence of hepatoma or mammary cancer. No case of spontaneous occurrence of hepatoma or mammary cancer was observed in the control group. Although mice given 0.3% DDB-EX solution had higher incidence of hepatic tumor at the 40th and the 60th week than those given 0.1% solution, the times of initial occurrence of hepatic tumor under both conditions were almost the same. SPITZ et al.[9] reported that the earliest occurrence of hepatoma induced by benzidine was at 245 days, most cases occurring after three hundred days from the beginning of the experiment.

Gross appearance of DDB-EX hepatoma was grayish-white, solid and solitary with no accompanying fibrosis or cirrhosis. The tumor was well demarcated from the surrounding tissue. Histology showed the tumor to be mainly occupied with fairly well differentiated hepatocytes with cellular atypia. However, cancerous change of the cholangiogenic cell was not observed. These findings closely resemble those of certain types of human hepatoma, e.g. hepatoblastoma.

Generally, in histological findings of DAB-hepatoma, mixed appearance of hepatocellular and cholangiocellular carcinoma is characteristic. In addition, a moderate degree of cirrhosis

is often observed. DDB-EX hepatoma, however, is characteristic for its simple histological aspect without cholangiogenic carcinoma or cirrhosis. These interesting differences are considered to be partly caused by the method of administration of the carcinogenic reagent. In our experiment, DDB-EX was administered in a low concentration for a long period. Although the DAB hepatoma can be induced within two or three months, DDB-EX needs at least five months (the average time is ten months) to induce hepatoma.

AFP was first detected in the serum of a mouse with DDB-EX hepatoma at the 22nd week by LF method; thereafter the number of AFP-positive cases gradually increased in proportion to the duration of DDB-EX administration. In more than 90% of animals sacrificed during the 40th and 50th weeks hepatomas were observed, and also concomitant elevation of serum AFP was revealed in 66.7% by SRID method and in 89% by both SRID and LF method. In human hepatocellular carcinoma, serum AFP can be detected in 70–75% of the patients by agar-gel precipitation method and in 90% by radioimmunoassay[2]. The positivity rates of serum AFP in DDB-EX hepatoma have a high correspondence to those of human hepatoma.

There was a tendency that the larger the size of the tumor, the higher the serum AFP titer was found to be. It is considered that the tumor size has a positive correlation with the titer of the serum AFP. However, there were some exceptions in that even some lesions occupying a large space showed negative AFP by both SRID method and LF method.

The incidence of mammary cancer was as high as 33.9%. In eleven cases with mammary cancer alone no serum AFP could be detected, suggesting the lack of production of AFP in mammary cancer.

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和文抄録

Direct Deep Black-Extra (DDB-EX) による マウス肝癌の発生とその血中 AFP 値

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飲料水として，0.3% Direct. Deep Black-EX を与えた ICR 系マウスの約80%に肝腫瘍の発現が認められた．この実験肝癌は組織学的には，肝細胞由来の腫瘍であり，胆管上皮細胞由来の組織や，肝硬変は認められず，ヒト肝癌によく似た像を示す．血清 AFP は担癌マウスの90%に検出された．未分化な腫瘍組織をもつものでは肝全体に占める割合が大きくなる程，血清 AFP 値が高くなることが見られた．しかし分化度の中等度あるいは高度なものでは AFP 値が陰性である症例も認められ，相関性は見られなかった．約30%のマウスに皮下腫瘍が認められたが，乳線組織を origin とする Squamous cell carcinoma であった．