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Evidence of Sex Difference in DNA Synthesis in Hepatocellular Carcinoma

Kazuo Tarao,[1,4] Akio Shimizu,[2] Masaoki Harada,[2] Tetsuo Ono,[2] Yukifusa Kuni,[1] Shinichi Ohkawa,[1] Yoshihiko Ito,[1] Setsuo Tamai,[1] Kazuto Iimori,[1] Yukio Sugimasa,[1] Shoji Takemiya,[1] Takashi Okamoto,[1] Tohru Inoue[3] and Masayoshi Kanisawa[3]

[1]Department of Gastroenterology and [2]Clinical Research Institute, Kanagawa Cancer Center, 54-2, Nakao-cho, Asahi-ku, Yokohama 241 and the [3]Department of Pathology, Yokohama City University, 3-9, Fukuura, Kanazawa-ku, Yokohama 236

To clarify sex differences in DNA synthesis in hepatocellular carcinoma (HCC), bromodeoxyuridine labeling indices (BrdU LI) of HCC cells included in tumor biopsy specimens from 12 consecutive male patients and from 5 consecutive female patients all with liver cirrhosis and HCC were examined using an *in vitro* labeling technique. The mean BrdU LI ± SE of HCC from male patients (7.7 ± 0.8%) was significantly ($P < 0.05$) higher than that from female patients (4.4 ± 1.0%). While 7 of the 12 male HCC patients belonged to the high DNA synthesis group (BrdU LI ≧ 7.0%), none of the 5 female HCC patients showed high DNA synthesis ($P < 0.05$). We conclude that DNA synthesis in HCC was higher in males than in females.

Key words: Sex difference — DNA synthesis — Hepatocellular carcinoma

It is generally accepted that the incidence of hepatocellular carcinoma (HCC) is higher in males than in females,[1] especially in patients with liver cirrhosis (LC).[2] The reason for this is not known, but higher incidences of alcohol abuse,[3] and of chronic viral hepatitis in males are possible causes.[4,5]

Nagasue *et al.*[6,7] demonstrated that the rate of detection of the androgen receptor in HCC cells was higher in males than in females, and that the five-year survival rate in male HCC patients was significantly lower than that in female patients, and proposed that HCC may be an androgen-dependent tumor[8] and that the presence of androgens would accelerate the growth of HCC. However, they did not examine whether these sex differences also existed in the DNA synthesis of HCC cells. We, therefore, studied the DNA synthesis of HCC cells from male and female LC and HCC patients, using the bromodeoxyuridine (BrdU, a thymidine analogue)-anti-BrdU *in vitro* method.[9]

PATIENTS AND METHODS

The patients studied consisted of 19 consecutive patients with LC and HCC (12 men, 5 women) who had undergone hepatectomy on account of HCC, and from whom appropriate specimens of HCC cells had been obtained through tumor biopsy by a Tru-cut needle during hepatectomy. They were inpatients at Kanagawa Cancer Center Hospital (Yokohama) between June 15, 1986 and September 30, 1989. Their ages ranged from 52 to 80 years with a mean of 62.4 ± 7.5 (SD) years for male patients, and from 49 to 75 years with a mean of 61.0 ± 10.2 years for female patients. There was no significant difference in ages between male and female patients. The diagnosis of LC with HCC was made on the basis of histological findings in all cases. The liver cirrhosis was viral in origin, and thus the type of cirrhosis was post-hepatitic cirrhosis in all the patients except one. No drug which would influence the results was administered before biopsy, nor was transcatheter arterial embolization or arterial infusion of anticancer drugs carried out.

Serum α-fetoprotein levels (radioimmunoassay [RIA] method, < 15.0 ng/ml) and HBsAg (enzyme immunoassay [EIA] method) were also measured in all the patients. Clinical features on admission in all the patients are shown in Table I.

The BrdU was purchased from Takeda Chemical Industries Ltd. (Osaka), mixed powder of RPMI 1640 medium from Nissui Pharmaceutical Co. (Tokyo), monoclonal antibody against BrdU[9] from Becton Dickinson Immunocytometry Systems (Mountain View, CA), and antisera and other necessary agents for the avidin-biotin-peroxidase complex (ABC) method[10] from Vector Laboratory (Burlingame, NJ).

Liver biopsy specimens (1.0–1.5 cm long) from the tumor were obtained by using a Tru-cut needle (Travenol Laboratories, Deerfield, IL) during surgery. The specimens were immediately incubated for 45 min in 0.1% BrdU solution in RPMI 1640 at 37°C in a water-bath shaker under a pressure of 3 atmospheres in a mixture of 95% oxygen (O_2) and 5% carbon dioxide (CO_2). They were fixed in 10% phosphate-buffered formalin for about 1 day, embedded in paraffin, and cut into 4-μm sections. After deparaffinization, they were treated

[4] To whom correspondence should be addressed.

with 4 *N* hydrochloric acid (HCl) for 30 min at 37°C to denature the DNA. Immunohistochemical detection of BrdU was performed by the routine ABC method,[10] using the anti-BrdU monoclonal antibody[9] as the first antibody. Neighboring sections were stained with hematoxylin-eosin (H-E) and Azan-Mallory for histological examination.

The sections were examined under a light microscope. The histological diagnoses were based mainly on sections stained with H-E. The degree of cancer cell differentiation was assessed according to Edmondson and Steiner[11]; in this classification, Grade I is extremely well differentiated and Grade IV is poorly differentiated. For measuring the BrdU LI, over 1000 cells were counted in randomly selected high-power fields and the BrdU-positive nuclei were recorded in each field. The LI was defined as the ratio of BrdU-labeled cells to total cells scored and expressed as a percentage.

The number of daughter nodules was determined from specimens of hepatectomized liver including HCC, after angiography and CT scanning (post lipiodolization) data had been obtained for reference. Also, the portal invasions of HCC were examined in specimens of hepatectomized liver.

For statistical analysis, Student's *t* test (Welch's method) and the chi-square test were used.

RESULTS

BrdU-positive nuclei were stained brown and could be clearly distinguished from other nuclei under the light microscope (Fig. 1). The BrdU LI for all patients is shown in Table I. The mean ± SE BrdU LI of HCC cells in male patients (7.7 ± 0.8%) was significantly ($P < 0.05$) higher than that in female patients (4.4 ± 1.0%). While 7 of the 12 (58%) male HCC patients belonged to the high DNA synthesis group (BrdU LI ≥ 7.0%), none of the 5 female HCC patients showed high DNA synthesis (Table II, $P < 0.05$). Many daughter nodules (≥2) were detected in 4 of the 7 patients with high DNA synthesis, but they were not detected in the patients with low DNA synthesis (BrdU LI < 7.0%) ($P < 0.05$, Table III). All patients with many daughter nodules were male.

As to the incidence of portal vein invasion, there was no significant difference between patients with high and low DNA synthesis (Table IV). Although the incidence of portal vein invasion in male patients (4 of 12) was higher than that in female patients (none of 5), this difference was not significant.

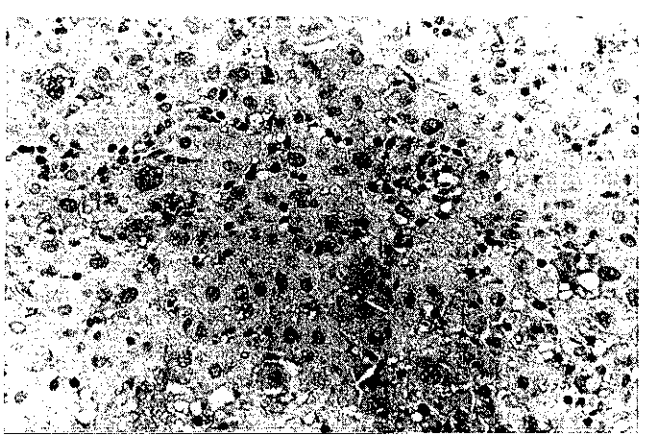

Fig. 1. Representative microphotograph of HCC cells from male patients with LC and HCC. Many nuclei of HCC cells are positively stained by BrdU (BrdU immunostain, ×230)

DISCUSSION

Recently, Nagasue *et al.*[6,7] found that the presence of the androgen receptor (AR) in HCC cells was significantly higher in male patients (74%) than in female ones (37%), whereas about the same ratio (37, 40%) was found for the estrogen receptor (ER).[7,12] In addition, they demonstrated that the AR concentration on HCC cells was higher in male patients than in female ones.[6,7] They also demonstrated the active uptake of testosterone by ARs of HCC in humans,[8] and suggested that HCC may be an androgen-dependent tumor and that a higher incidence of HCC in male patients than female ones,[1] which has often been explained by the fact that alcoholism,[3] chronic liver disease, and, in particular, chronic hepatitis B virus infection[4,5] are more prevalent among males than among females, could be partially explained by this hypothesis.[8]

In support of this hypothesis, Morris and Firminger[13] found that, in a hepatic carcinogenesis model in rats given 2-diacetylaminofluorene, HCC occurs more frequently in intact males and castrated females treated with testosterone than in intact females, castrated males, castrated females, or castrated males treated with diethylstilbestrol. The results of Bannister *et al.*[14] and Ostrowski *et al.*,[15] demonstrating the relationship between the increased hepatic AR concentrations and diethylnitrosamine-induced hepatocarcinogenesis in rats, seem to support the validity of the classical study of Morris and Ferminger,[13] showing that HCC occurs more frequently under androgenic conditions.

We found that DNA synthesis in HCC cells was significantly higher in males than in females. In addition, when we divided the patients with LC and HCC into high and low DNA synthesis groups, 7 of the 12 male patients exhibited high DNA synthesis, in contrast to none of the females.

Table I. Bromodeoxyuridine Labeling Index (%) in HCC Cells from Male and Female Patients with Liver Cirrhosis and Hepatocellular Carcinoma

Case	Age/sex	Type of cirrhosis	HBsAg	ICG (15′)	α-Feto-protein (ng/ml)	Child's classification	Histologic types of tumor	Edmondson and Steiner's classification	Labeling index (% labeled) in HCC cells
Male									
1	57/M	Posthepatitic	−	31.4	3350	A	Trabecular	I-II	7.2
2	56/M	Posthepatitic	+	10.9	5	A	Trabecular	II	4.2
3	69/M	Posthepatitic	−	18.6	870	A	Trabecular	II	4.8
4	66/M	Posthepatitic	−	26.5	3	A	Trabecular	II	5.2
5	63/M	Alcoholic	−	45.5	3147	B	Trabecular, pseudoglandular	II	5.9
6	62/M	Posthepatitic	−	31.4	8	A	Trabecular, pseudoglandular	II	6.4
7	65/M	Posthepatitic	−	18.4	216	A	Solid	II	7.1
8	54/M	Posthepatitic	−	14.7	1740	A	Solid, pseudoglandular	II	8.8
9	52/M	Posthepatitic	−	38.8	28	A	Trabecular	III	7.9
10	80/M	Posthepatitic	−	28.0	16	A	Trabecular	III	10.2
11	63/M	Posthepatitic	−	8.4	4	B	Trabecular, solid	III	11.9
12	62/M	Posthepatitic	−	8.0	12	A	Trabecular, pseudoglandular	III	12.7
Mean±SE	62.4±2.3			23.4±3.7	783±381				7.7±0.8
Female									
1	75/F	Posthepatitic	−	47.8	19	B	Trabecular	I	4.3
2	49/F	Posthepatitic	+	13.2	3990	A	Trabecular	I-II	2.6
3	54/F	Posthepatitic	−	49.5	12790	A	Trabecular, pseudoglandular	II	6.2
4	66/F	Posthepatitic	−	55.5	210	A	Trabecular	II	6.6
5	61/F	Posthepatitic	−	10.7	435	A	Trabecular, solid	II-III	2.5
Mean±SE	61.0±5.1			35.3±10.8	3489±2726				4.4±1.0

Table II. Incidence of Low DNA Synthetic HCC and High DNA Synthetic HCC in Male and Female Patients

	Low DNA synthetic patients (BrdU LI <7.0%)	High DNA synthetic patients (BrdU LI ≧7.0%)
Male patients (n=12)	5 (42%)	7 (58%)
Female patients (n=5)	5 (100%)	0 (0%)

$P<0.05$.

Table III. The Relationship between BrdU LI (%) and the Number of Daughter Nodules in Patients with HCC and LC

Number of daughter nodules	BrdU LI (%)	
	below 7.0%	above 7.0%
nil	7 (5)/10 (70.0%)	1 (0)/7 (14.3%)
1	3 (0)/10 (30.0%)	2 (0)/7 (28.6%)
2	0 (0)/10 (0%)	0 (0)/7 (0%)
3	0 (0)/10 (0%)	4 (0)/7 (57.1%)

$P<0.05$. Parentheses show the numbers of female patients.

Here, we must consider the possible reasons why the DNA synthesis in HCC cells from male patients is higher than that from female ones again. Although human hepatocyte growth factor (hHGF) is the most potent hepatotropic factor, there is generally no difference between male and female patients in serum hHGF concentration.[16] It has been speculated that testosterones might enhance the growth and invasiveness of human HCC, but it is not known whether the testosterone acts directly on HCC cells through AR or indirectly through other factors.

Table IV. The Relationship between BrdU LI (%) and Portal Vein Invasion

Portal vein invasion	BrdU LI (%)	
	below 7.0%	above 7.0%
(−)	9 (5)	4 (0)
(+)	1 (0)	3 (0)

Not significant. Parentheses show the numbers of female patients.

With regard to the sex differences in EGF, it was demonstrated that salivary gland EGF levels in mature male mice were 10–17 times greater than that in mature female mice,[17-19] and that EGF concentration in female salivary glands increases to very high levels upon testosterone treatment, whereas castrated males have levels comparable to untreated females.[17] These findings indicate that androgenic stimulation of the submaxillary gland is essential for EGF synthesis. As to the effects of EGF on the liver, EGF was implicated as a hepatotrophic factor during liver regeneration,[20,21] and found to stimulate DNA synthesis in normal liver.[20] It was also demonstrated that EGF plays a role in the proliferation of rat hepatoma cells.[22] In humans, there have been several studies demonstrating the existence of EGF receptors in HCC[23-25] and there is a strong possibility that EGF stimulates the growth of human HCC.

It is generally accepted that the more the DNA synthesis of the tumor increases, the greater is the biological malignancy. We found that the incidence of many daughter nodules was significantly higher in patients with high DNA synthesis than in those with low DNA synthesis. Although the incidence of many daughter nodules was markedly higher in males than in females, this difference was not significant ($P=0.075$) possibly because of the low number of patients examined. Considering that BrdU LIs in all female patients were below 7.0%, it is possible that the incidence of many daughter nodules in female patients is lower than that in male patients, and it would be expected that HCC in male patients is thus more biologically malignant than that in female ones. In accordance with this concept, Nagasue *et al.*[26] found that the 5-year survival rate for female patients with HCC (52%) was significantly higher than that in male patients with HCC (19%) in 137 patients who underwent radical hepatic resection. They also noted that the recurrence rate was significantly higher in patients with AR-positive HCC than in those with AR-negative HCC.[26]

Finally, it was found that the DNA synthesis of HCC cells in male patients was significantly higher than those of female patients, and that the biological malignancy of HCC as indicated by the numbers of daughter nodules may be influenced by the DNA synthesis of HCC. Androgen might be a possible cause of this phenomenon, but further investigation is needed to clarify the mechanism of the action of androgen.

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