REVIEW

Recent Advances in the Study of Hepatocellular Carcinoma

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Abstract. Among the known causes for the occurrence of hepatocellular carcinoma (HCC), chemical carcinogens, chronic alcoholic intake and hepatitis B virus (HBV), especially in Asia, has been emphasized. HBV has been industriously studied and many queries about the relationship between HBV infection and hepatocarcinogenesis have been clarified. Recent discovery of hepatitis C virus (HCV) revealed that there may be the participation of this virus in hepatocarcinogenesis. However, a precise mechanism in such a viral infection has not been known. Host immunological defence mechanisms including the role of cytokines should be also taken into consideration. Cellular gene abnormalities have been noted in the late period of cancer cell progression. The technical development in the clinically available diagnostic procedures have enabled us to detect early phase of HCC. Some new concepts in the pathological diagnosis of precancerous lesions of HCC and also early HCC have been reported recently. We gave an outline of the recent advances and references in the study of HCC. (Keio J Med 41 (4): 195–204, December 1992)

Key words: hepatitis B virus, hepatitis C virus, immunological defence mechanism, early detection

Introduction

It has been regarded that major hepatocarcinogens are some chemical agents,^{1–3} chronic alcoholic intake⁴ and hepatitis B virus (HBV). Recent studies have suggested that hepatitis C virus (HCV) can likewise be regarded as one of carcinogen. For several years, we have been studying the host's immunological abnormalities as noted in chronic liver diseases; a significant decrease in host defense mechanisms could be considered as one important factor for the progression of liver cirrhosis (LC) into hepatocellular carcinoma (HCC). There may be other unknown causes for the occurrence of HCC which remains to be explored.

Hepatitis B Virus (HBV)

It is well known from epidemiological studies,^{5–7} that there is a correlation between the occurrence of HCC and HBV infection. Since such a significant finding has been discovered, the study of HBV has been emphasized and a lot of important discoveries have been made regarding this special area of cancer research.^{8,9} HBV has been very well investigated.¹⁰ Its DNA sequences have

been completely studied.¹¹ The proteins produced by this virus are encoded in specific DNA regions (Fig 1); eg, hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), and DNA polymerase (DNA-p) which are some of the proteins produced by this virus, are encoded in their respective regions in the HBV DNA loci. Moreover, special attention should be focused on the fact that although oncoviruses, which are usually retroviruses, have their oncogenes included in their genomes, this phenomenon does not hold true for HBV genome, then another interesting feature of HBV which remains to be explained. HBV has interesting replication strategies.¹² As shown in Fig 2, the HBV DNA is transcribed into RNA and translated into specific proteins. On the other hand, during replication of this virus, HBV DNA is reversely transcribed^{13,14} into minus strand DNA and the plus strand DNA reproduced and finally the replication is completed. This complicated way of replication brings about an important change in the hepatocytes infected with HBV; that is, HBV DNA integration. Our results¹⁵ showed that HBV DNA was integrated in all of the 8 HCC tissues from HBsAg-positive patients examined by Southern blot analysis. Okubo *et al*¹⁶ demonstrated that HBV DNA integration

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Fig 1 The schematized construction of the hepatitis B virus. The relationship between the genomic structure and viral antigens is explained.

occurred only 2 or 3 days after the viral infection to human primary hepatocyte culture. However, the DNA binding pattern was so different from each other. This finding was supportive of the claim that there is no specific HBV DNA integration pattern in the development of HCC.¹⁷ It is important that the integration is associated with insertion and deletion formation and is often associated with host chromosomal rearrangements.^{18,19} Moreover, some of the integrated viral genomes exert transactivation of the host genes.^{20,21} Even these results suggested that HBV is an oncogenic virus.

Turning on the possibility of HCC as being multicentric,²² DNA from various lesions from a HCC patient were analyzed by Southern blotting. Interestingly enough, some of HBV DNA integration patterns were found to be different, supporting then the claim of multicentricity of HCC.²³ We also experienced a case whose HBV DNA integration patterns in tissue DNA were different between a main tumor and a metastasized lymph node.¹⁵ There is a great possibility that after a solitary HCC has been surgically removed, tumors in other regions have

Fig 2 Replication strategies of the HBV.

a great propensity to develop, eg, postoperatively, if indeed another tumor develops, we cannot definitely say if it is a metastasis or a primary tumor in itself.

Many reports have been focused and accumulated about HBV X-protein. Recent studies have shown that X-protein¹⁰ (hbx) which is drawn from X gene of HBV DNA promotes hepatocarcinogenesis. It has been demonstrated that this protein has a transactivating activity²⁴ of enhancing the genes in hepatocyte DNA including HBV genome itself, the SV40 promotor and enhancer, long terminal repeat (LTR) of human immunodeficiency virus (HIV), Rous sarcoma virus (RSV), human T cell leukemia virus I (HTLV-I), β -interferon gene, *c-fos*, *c-myc* and RNA-polymerase II and III promoters.²⁵⁻²⁹ Each gene is specifically activated according to the type of cells. However, hbx can not directly combine with DNA itself. It is most possible that hbx transactivates various genes by the interaction through some hbx responsive element binding factors.³⁰⁻³⁴ There are reports that hbx has a protein kinase activity³⁵ or a serine protease inhibitor activity.³⁶ Kim *et al* have revealed that the transgenic mice transfected with a part of HBV DNA including X gene, in which hbx was excessively produced in the liver, soon (8-10 months later) developed HCC.³⁷ However, there are reports which disclaim the direct correlation between HBV DNA integration and hepatocarcinogenesis^{38,39} even in transgenic mice.⁴⁰ Chisari *et al*⁴¹⁻⁴³ made the transgenic mice in which large envelope polypeptides of HBV was produced excessively. In these mice, hepatic necrosis, inflammation and regeneration were observed in the liver and at last HCC appeared after about 1 or 2 years. This model is compatible to the development of human HCC which always accompanied with chronic liver diseases. In chronic liver diseases, there continues inflammation and the recurrence of necrosis and regeneration. Some minor stimulations like oxidants^{44,45} may result in the occurrence of HCC through this recurrence. Thus, it is strongly suggestive that HBV is a hepatocarcinogenic virus. Many reports have been

accumulating which are interesting enough to understand HBV biology.^{17,46–49}

Hepatitis C Virus (HCV)

Since the discovery of a part of hepatitis C virus (HCV) genome,⁵⁰ the development of the study in HCV biology is so rapidly performed. On the other hand, the direct demonstration of its hepatocarcinogenicity has not been reported yet. We established a human hepatoma cell line HCC-T from a nonA nonB (NANB) hepatoma patient⁵¹ and it was demonstrated that he had been infected with HCV though there was no HCV RNA nor DNA in this cell line detected by RT-PCR technique. It is obvious that in patients afflicted with HCC, positive rate for their serum HCV antibodies are high.^{52–61} Although positivity of serum HCV antibody in children below 15 years old was reported to be very low in 1990⁶² and this led us to speculate that there is no vertical transmission of HCV, Thaler *et al*⁶³ and Kuroki *et al*⁶⁴ reported the possibility of the vertical transmission in 1991. This should stimulate further inquiry or studies⁶⁵ to clarify the relationship between HCV and hepatocarcinogenesis as studied in HBV. Now it has been demonstrated that HCV genome (HCV-J) derived from a Japanese NANB hepatitis patient is composed with apparently different nucleotide sequence from that reported by Chiron group (HCV-US) by using RT-PCR method.^{66–68} Other types of HCV (K2, group II) whose nucleotide sequence showing less identical with that of HCV-US were also reported.^{69,70} Kato *et al*⁷¹ determined the entire nucleotide sequence of HCV-J genome. Takamizawa *et al*⁷² determined that of one type (HCV-BK). US-type HCV genome was also analyzed and it became to be possible to postulate genomic products. HCV genome contains a long open reading frame encoding a protein of 3010 amino acid residues. The genomic analyses and comparative study with the pestivirus have shown that HCV proteins encoded by the extreme 5' region are (1) capsid protein (p22); (2) envelop protein (gp 35, gp 70); followed by non-structural proteins (NS1-5) which may be necessary for viral replication. The sequence diversity has been found among the virus isolates with about 10% in nucleotide sequence and 5% in amino acid sequence and two hypervariable regions in the envelope region.^{73,74} Now, HCV genotype was categorized into 4 groups.⁷⁵ The genomes isolated from USA (HCV-H, HCT 18, 23, 27; Th; ECI, 10), Germany (GM2), France (HCVE1) and England are homologous to type I. The genomes isolated from Japan, Taiwan (HCV-T3) and China are homologous to type II. HC-J5, HC-J6 and HCV-K2a are categorized into type III. HC-J6, J7; HCV-K2b, clone A are categorized into type IV. The conserved region of the HCV genome in all isolates is found at the extreme 5' end, capsid region and

NS4 region of the genome. These 3 regions are most useful regions for diagnosis of the HCV genome or the detection of antibodies.

As we mentioned before, there are possibility of the HCV as a carcinogen. The first possibility is the existence of oncogenic gene in the HCV genome. The second is the integration of the HCV genome into the host genome as observed in the HBV biology. The third is that gene products like hbx activate the cellular gene transcription. As HCV is considered to belong to a new genus distinct from the *Flaviviridae*, which is categorized into RNA virus not retrovirus, the first and second possibilities can be neglected. The third possibility should be further studied. But we believe necrosis and regeneration of the hepatocytes in chronic liver diseases may be more important cause of the hepatocarcinogenesis.

Host Immunological Defence Status

We have demonstrated that the host's immunological defense status in patients with LC decreases compared with that of healthy control.⁷⁶ NK activity decreases in patients with LC.^{77,78} It has been noted that many HCC developed in patients with LC,⁷⁹ leading us to conclude that NK activity is one of the factors which can explain the development of HCC in LC. Similar aspects have been reported by others.^{80,81} We examined the relationship between NK activity and the resistance against the graft challenge of a human HCC cell line in nude mice.⁸² Those studies suggested that nonspecific cytotoxic cells play crucial roles in the resistance against tumor cell challenge and that the total level of cytotoxic activity of these cells at the time of tumor cell challenge is a key factor which determines tumor development. We further examined the role of neutrophils in the early phase of tumor elimination.⁸³ It is suggested that the oxidative stress plays an important role in neutrophil-mediated cytotoxicity. Shimizu *et al*⁸⁴ analyzed tumor infiltrating lymphocyte (TIL) from primary and metastatic hepatic tumor and reported that most TIL were activated T lymphocytes. TIL from primary tumor had a stronger activity to kill auto-tumor cells than those from metastatic tumor. Among the sinusoidal cells, especially Kupffer cell function should be also taken into consideration in the elimination of metastatic tumor cells.⁸⁵ Recent our study demonstrated that nitric oxide is a key factor in Kupffer cell-mediated cytotoxicity (paper in submitted).

Transforming Growth Factor- β (TGF- β)

TGF- β has been studied in the field of regulation of hepatic fibrogenesis by Kupffer cells.^{86,87} Besides that, Ito *et al*⁸⁸ reported increased level of TGF- β mRNA transcription in resected HCC tissues. Although TGF- β 1 is known to inhibit the proliferation of hepatocytes,

the proliferation of HCC cells can not be inhibited, suggesting that HCC cells lose the sensitivity against TGF- β 1.⁸⁹

Oncogenes and Chromosome Abnormalities

We transfected the cellular DNA of a human hepatoma cell line HCC-M⁹⁰ into a murine fibroblast cell line NIH/3T3 and found that both HBV DNA with a human repetitive sequences integrated into NIH/3T3 cells.⁹¹ This suggested that transformation occurred as the result of the transfer of proto-oncogene which might be closely associated with HBV genome. However, it was then demonstrated that there was no specific proto-oncogene expressions nor *ras* gene mutations in 3 human hepatoma cell lines including HCC-M.¹⁷ Tada *et al*⁹² also reported that there is a scarce correlation between *ras* mutation and hepatocarcinogenesis. On the other hand, Ogata *et al*⁹³ and Richards *et al*⁹⁴ reported that *ras* mutation was important event in hepatocarcinogenesis in HCC tissues and a cell line. Many reports suggested that no sepecific oncogene expression is responsible to hepatocarcinogenesis. Tumor suppressor gene was also focused about HCC development in recent years.^{93,95-100} One of such genes, p53 is known to be located in chromosome 17p and to code one of nuclear phospholiration protein which negatively regulate a cell cycle. It was reported that a half of HCC cases have a mutation of this gene in China¹⁰¹ and south Africa.¹⁰² while they might be related to chemically occurred HCC with aflatoxin. Abnormalities in chromosome 1,¹⁰³ and deletion of chromosomes 4,^{95-97,104} 5,⁹⁷ 8,^{96,97} 10,⁹⁷ 11,^{96,97} 13,^{96,97} 16,^{96,97,100,104} and 17^{96,97} have been reported. However, these chromosomal changes are suggested to be the events which occur at the late progressive period of cancer cells. There remains unclear what the initial event of malignant transformation in the hepatocyte is.

Early Detection of HCC

Cellular changes occur in the hepatocyte and after the clonal proliferation of the abnormal hepatocytes resulting into a hepatic malignant tumor, which should be clinically detected. It is said that it takes about 20 years from the occurrence of a cancer cell until when we can detect a tumor clinically. A careful detection of hepatic malignant tumors must be done particularly in patients with LC as well as with the HBsAg-positive carriers, since these are conditions which are thought to be highly predisposed to the occurrence of HCC. As it is well known, these clinically available examinations are ultrasonography,¹⁰⁵ CT-scan,¹⁰⁶ MRI,^{107,108} tumor markers¹⁰⁹⁻¹¹⁵ and liver chemistries. The development of the techniques for a needle biopsy under ultrasonography and the relative pathological study between biopsy samples and surgically

resected tissues enabled us to characterize pathological features of early small hepatic tumors.¹¹⁶⁻¹²⁰

After initial detection of the hepatic tumor, a hepatic angiography is recommended to make sure whether a tumor is definitely malignant or not. Moreover, it was only until a few years ago, when angiography has been selected as the best procedure to further define hepatic tumors. However, recently a small hepatic tumor which is under one or two cm in diameter can always be detected by ultrasonography. Such a small tumor cannot easily be detected by CT-scan nor angiography, since most of the time it results into a negative study.

Pathological examinations of these small tumors revealed that the preexisting liver structure was preserved. All of these cases were hypercellular lesions and it has been suggested by some pathologists that these small hepatic tumors can be classified into three categories, namely; adenomatous hyperplasia, which lacked structural atypia; atypical adenomatous hyperplasia with nodules exhibiting focal abnormalities; and finally, the very differentiated HCC with little cellular atypia. From these examinations,¹²¹⁻¹²³ it has been thought that there is a multistep development of HCC. One of them is adenomatous hyperplasia which might be a precancerous lesion of HCC¹²⁴.

Figure 3 shows a series of ultrasonograms and CT-scan photoes, all belonging to one patient of ours, who had been examined during a year and a half, that is, from the 13th of February, 1990 up to 1st of August, 1991 (dates: 13/2/90, 10/9/90, 14/1/90, 5/3/91, 1/8/91). The lower photographs are CT-scan photos taken during the first and last date. From here, we could see that we could not distinguish the characteristics of the tumor just by the CT-scan with ordinal use. However, these could be notably distinguished by the ultrasonograms. The median photograph shows the presence of a low density area or hypoechoic region which can detected in the high density area or high echoic region. And then, the low echoic region spreads diffusely into the high echoic area. Figure 4 shows the schematic representation of such a phenomenon. That is, a small hypoechoic region is first detected, which then develops into the hyperechoic area, gradually diffusing or spreading out till it occupies the whole region. This superimposed low-high echoic area, gradually grows larger and larger. It is interesting to note here, that when biopsy samples are taken from these regions, that sample taken from the hyper-echoic region will show fatty changes.¹²⁵ Meanwhile, in the hypo-hyperechoic area, adenomatous hyperplasia can be discovered. However, in some cases, early stages of HCC, and likewise, its undifferentiated stage, may even be seen.

A possible explanation could account for such a phenomenon; that is, during the development of the

Fig 3 The development of early HCC detected by the ultrasonography. A series of photographs in one patient from February 13th, 1990 to August 1st, 1991.

Fig 4 The schematic changes in the photographs of ultrasonography during the development of early HCC.

hypo-echoic area, vascularization may occur in the tumor itself and nutrition is being given, promoting the further growth of this tumor.¹²⁶ Thus, before this

vascularization period, hepatic angiography of this tumor may result into a negative study.^{127–129} During pre-vascularization period, the tumor may be nourished via the blood supply coming from the portal vein. Thereafter, the tumor's post-vascularization nutrition may come from the hepatic artery. During such stage, the HCC cells undergo a significant change from an early stage to an undifferentiated state.

A summary of the recent diagnostic procedures about HCC is shown on Figure 5. The CT during arterial portography (CTAP, portal CT-scan),¹³⁰ in which perfusion of the contrast medium from the superior mesenteric artery is done, is useful in detecting early HCC.^{131–133} By using this procedure, we can distinguish significantly the tumor into which nutrition is being supplied through the portal circulation from through the hepatic artery. Lipiodol computed tomography is also recommended to evaluate small HCC.¹³⁴

It is well known that mature hepatocytes produce albumin, while the fetal counterparts produce α -fetoprotein (AFP). Since some hepatoma cells can produce AFP,

	adenomatous hyperplasia (AH)	early cancer	cancer
Ultra Sound	hyper (fat+) → iso -hypo	iso -hypo	hypo
CT scan			
simple	iso - low	iso - low	low
dynamic early phase	iso - low	slight high - iso	high
late phase	iso - low	low - iso	low
enhance	iso - low	iso - low	low
angiography	hypo	iso - slightly hyper	hyper
MRI			
T1-enhance	iso - high (low)	iso - high (low)	low
T2-enhance	iso - high	iso - high	high
portal CT	enhance	perfusion defect	defect
nutrition

Fig 5 The summary of the recent available diagnostic procedures to detect early HCC.

the process of malignant transformation suggests to be frequently associated with a change in hepatocytes towards the immature state. This corresponds to a certain developmental stage of the fetus. Therefore, it is possible to say that hepatocarcinogenesis is a gradual process of transformation into the undifferentiated or immature state of the hepatocyte.¹³⁵ The similar discussion has already been carried out in leukemic cells.

In summary, we have discussed the important issues with regards to hepatocarcinogenesis. Among them, HBV is the most significant factor which continues to be the main focus of investigations currently conducted. Its amazing DNA replication strategies, as well as integration into the host's DNA have indeed made the HBV DNA study more challenging than ever to any investigator. Chronic inflammation of the liver may play some important role in the occurrence of malignant transformation in the hepatocyte. Although the direct demonstration of HCV as a hepatocarcinogenesis has not been reported, recent interferon therapy against chronic hepatitis B and C might achieve in future the decrease in number of HCC occurrence.¹³⁶ Many investigators, as well as our group, have been continuously involved in conducting studies which will unfold to us more of the mysteries behind HCC, and many clinicians have been trying to detect the HCC earlier and treat the patients better.

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