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Highly incidence of Hepatocellular carcinoma in HBV X transgenic mice

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Chronic infection of hepatitis B virus (HBV) has been regarded as one of the major causative agents of hepatocellular carcinoma (HCC). Several mechanisms of oncogenic role of HBV have been proposed including gene activation by integration of the viral cis-elements, induction of recombination by HBV subgenome, imbalanced accumulation of viral proteins, and transactivation by viral proteins. Recently the X-gene product was found to be a viral transactivator (1). Since then, a variety of genes have been shown to be activated by the X-protein (2-5). Activating a broad spectrum of genes, the X-gene was implicated in carcinogenesis as one of the causative factors (6).

The X-gene is one of the four genes in the HBV genome (7). It encodes a polypeptide of 154 aa, the amino acid sequences of which are well conserved among mammalian hepadna viruses such as woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) (8). It is noteworthy that duck hepatitis B virus (DHBV) lacks the X-gene and no HCC developes in DHBV infection in contrast to frequent occurrence of HCC in WHV and less frequent but significant occurrence in GSHV (8). Most of the primary tumors and tumor-derived cell lines have some or all of the X-region and upstream pre-S/S sequences integrated into the host genome (9). In addition, most of the primary tumors produced X-region transcripts, whereas relatively few made transcripts from other regions of the genome (10,11). Many of these integrated fragments make hepatitis-B-virus-encoded X antigen (HBxAg) capable of trans-activation both *in vitro* (12,13) and *in vivo* (14) although the natural targets of HBxAg transactivation in liver diseases, including HCC, remain to be clearly identified. Altogether, these properties provide a strong basis on which to suspect the HBxAg as the most likely cause of HBV-associated hepatic cell transformation. The evidence that HBxAg contributes to hepatocarcinogenesis was reported using a transgenic mouse system by one research group (15,16,17). Altered foci, adenoma, and carcinoma appeared in their HBx transgenic mice with persistently high levels of HBx expression. However, these observations have not been reproducible in other transgenic mouse systems using the X-gene (18-22). Therefore, the oncogenic role of HBx has been strongly

suspected, and the positive role of HBx in the hepatocarcinogenesis remains controversial.

Result and Discussion

We have created transgenic mice bearing HCC by expressing the X-gene to present a useful model for defining the molecular events that follow the expression of the viral X-gene and are responsible for the development of liver cancer. 279 young mice were born from the 946 embryos transferred, however only 3 transgenic founder mice were determined. Integration rate of the transgene on mice chromosomes was very low level comparing to the results of other authors (24) and many mice were also dead in early stage of the growth before examining expression of the X-gene. It might be caused due to the physiological problem by overexpression of the X-protein in liver of transgenic mice. Despite difficulty in getting the HBx transgenic mice, we luckily got one transgenic founder mouse (HEX-3 line) stably transmitting the transgene to its progenies. The expression level of the X-mRNA in tissues was a little different from that of Kim et al (15). It was reported that HBV X mRNA was highly expressed in the liver and testis and weakly in the kidney (15). However, the expression level in HEX-3 line mice was high in the testis and kidney, but very low in the liver. RT-PCR result showed the presence of the transcript in the heart as well. However, expression of the X-protein by immunohistochemical analysis was positive only in liver tissues of all the transgenic mice examined. We have not examined the reason why post-transcriptional expression did not occurr in kidney, testis, and heart of HBx transgenic mice. One of the possibilities is the lack of some factors necessary for post transcriptional-regulation of the X-protein in these tissues (25). There were mild to moderate pleomorphic nuclei or enlarged nuclei and multiple small vacuolar cytoplasmic changes in all HBx transgenic mice from the age of 6 months to 18 months. In 3 of the 4 HBx transgenic mice which did not have grossly diagnosed hepatocellular carcinomas microscopically diagnosed, small hepatocellular carcinomas were found. Grossly diagnosed hepatocellular carcinomas were found in 5 of the 7 HBx transgenic mice after age of 11 months. Overall incidence of the hepatocellular carcinoma was 8 out of the 10 HBx transgenic mice (80%) and their age ranged from 6 to 18 months. The incidence of hepatic tumor may be comparable to that reported in CD-1 strain (15,16). Susceptibility to hepatocarcinogenesis varies in different mouse strains. Some strains, including the C57BL6J, C57BL10Sn, DBA/2J and Balb/c, have very low spontaneous liver tumor incidences ($<$ 4% of animals develop liver tumors), whereas others, such as the C3H/HeJ, have high incidences (40-50%) (26). As our transgenic mice were derived from F1 hybrid (C57BL/6 $\times$ DBA), the spontaneous incidence of hepatic tumors in these mice is expected to be lower than 4%. Therefore, 80% hepatic tumor incidence in our transgenic mice, generated by the expression of HBV X gene, may be very significant. Koike et al. (16) reported that low levels of HBx expression by Northern blot hybridization were insufficient for initiating tumor formation in mouse liver. E1 heterozygous line with relatively low levels of HBx expression developed Hepatic tumors in only 1 out of

16, which is comparable to the incidence of hepatic tumors reported in normal CD-1 strain (27,28). However, although the mRNA level expressed in the liver of our HBx transgenic mice was very low according to Northern blot analysis (Fig.3), the incidence of hepatic tumors was comparable to that in the H9 transgenic mouse line with high level expression of HBx as reported by Koike et al. (16). Therefore, our result suggests that continued expression of the X-gene as shown in Fig. 4A and 4B, even though at very low level, but at the appropriate sites of liver, may be responsible for the development of hepatic tumors at high incidence.

Production of transgenic mice for the X-gene have been reported by several groups (3,15,19,20,21,22,31,29,30). Kim et al. (15) observed high incidence of HCC in transgenic mice by microinjecting a 1.15kb HBV subtype *adr* DNA fragment, which spans nucleotide positions 707 to 1856 in the viral genome, into single-cell embryos derived from outbred CD-1 mice. In contrast, other HBx transgenic lines generated in different mouse strains developed no obvious hepatic pathology, although they expressed the X-gene in liver cells and the HBx protein could be detected in some cases (3,19,20,21,22,23,30). However, in these cases the identity of the used for generating transgenic mice mouse strain might not have been an absolute criterion for the induction of hepatic tumors in the transgenic mice, because we used the same F1 hybrid mouse (C57BL/6×DBA) as that used by Purfumo et al. (21) and Terradillos et al. (23). One of the critical factors responsible for the difference in the outcomes of our experiments and perfumo et al. (21) could be the source of the X-gene used to generate the transgenic mice. We used the *adr* type of X-gene whereas Perfumo et al. used the *ayw* type; the nucleotide homology between these two gene types is only 39%. It could be that some elements responsible for inducing neoplasia in liver by the HBx might be deleted in the sequence of the X-gene of *ayw* type used in generation of HBx transgenic mice by Perfumo et al. (21).

Another possibility is that transgene integration site in the genome of HBx transgenic mice affected the incidence of hepatic tumor induction.

The HEX-3 transgenic mouse has been stably transmitting its transgenic trait to the progeny through generation until now. We are now trying to expand the HBx transgenic mice stock to present a useful model for defining the molecular events responsible for the development of liver cancer.

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