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World J Gastroenterol 2001;7(4):575-578
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORT •

Differentially expressed genes in hepatocellular carcinoma induced by woodchuck hepatitis B virus in mice

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Received 2001-04-06 **Accepted** 2001-05-18

Subject headings liver neoplasm/virology; carcinoma, hepatocellular/virology; hepatitis B virus/genetics; genes, viral; gene expression

Cao XY, Liu J, Lian ZR, Clayton M, Hu JL, Zhu MH, Fan DM, Feitelson M. Differentially expressed genes in hepatocellular carcinoma induced by woodchuck hepatitis B virus in mice. *World J Gastroenterol*, 2001;7(4):575-578

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major causes of death in the world. The mechanism of carcinogenesis is unknown, although it is widely accepted that HBV and HCV are closely related to liver cancer[1-5]. Previously, a variety of studies have described the differences in gene expression which distinguished tumor from nontumor[6-11]. Cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer.

Traditionally, several methods were used to clone the new genes, which means to compare two population of mRNA and obtain clones of genes that expressed in one population but not in the other. Although these methods have been successful in some cases, they require many rounds of hybridization and are not well suited for the identification of rare messages. The suppression subtractive hybridization is a latest method employed in the gene cloning, which is a unique method based on selective amplification of differentially expressed sequences and overcomes technical limitation of traditional subtraction methods[12-14]. Hence, the purpose of our study is to find the differentially expressed genes in liver tumor and nontumor tissues induced by woodchuck hepatitis B virus using suppression subtractive hybridization.

MATERIALS AND METHODS

Patient samples

The tumor and nontumor tissues induced by woodchuck hepatitis B virus were obtained from Department of Pathology & Cell Biology, Thomas Jefferson University, Philadelphia, USA. The other HCC and surrounding nontumor liver tissues used for analysis were obtained from the patients who had undergone surgery for the removal of their tumors in Xijing Hospital. Fresh frozen blocks and -80℃ snap frozen paired liver and tumor samples from individual patients were collected, and were then made available for RNA extraction and *in situ* hybridization.

Total RNA and mRNA extraction

Total RNA and mRNA were extracted separately from tumor and nontumor tissues by using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA) and the quality of extraction was determined by assaying 18S and 28S rRNA with agarose gel electrophoresis and ethidium bromide staining.

RT-PCR and adaptor ligation

The reverse transcriptase PCR was started with 2μg poly-(A) + RNA isolated from tumor and nontumor tissues. Two adaptors were ligated to the fraction of *Rsa* I digested cDNA generated by RT-PCR. The sequence of two adaptors is as follows:

Ad1: 5'-CTAATACGACTCAC-TATAGGGCTCGAGCGGCC-GCCCGGGCAGGT-3'
Ad2: 5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGG-TGCGGAGGGCGGT-3'

cDNA subtraction and suppression PCR

The cDNA from tumor was referred to as tester, and the reference cDNA from nontumor as driver. The tester and driver cDNA were digested with *Rsa* I to obtain shorter, blunt-ended moleclules. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adaptors. The driver cDNA had no adaptor. Two hybridizations were then performed. In the first hybridization, an excess of driver cDNA was added to each sample of tester for equalization and enrichment of differentially expressed gene. During the second hybridization, templates for PCR amplification were generated from differentially expressed sequence. The entire population of molecules was then subjected into PCR to amplify the desired differentially expressed genes. In the first PCR, only differentially expressed genes were amplified exponentially because of using suppression PCR. The second PCR was performed using nested primer which matched the sequence of adaptors to reduce the background and further enrich the differentially expressed genes.

Sequencing and GeneBank search of cloned genes

Following agarose gel electrophoresis, the unique fragments were eluted from the gels (using Qiagen gel extraction kit,

Qiagen, Inc. Valencea, CA, USA) and cloned into pT7Blue (R) T vector (Novagen, Medison, WI, USA). Positive clones were selected by blue-white phenotype. Recombinant DNAs were isolated from minipreps of individual clones, and digested by *Rsa* I to check insert size, and then both strands were individually analysed by sequence analysis in the DNA sequence facility at the Kimmel Cancer Institute of Thomas Jefferson University in USA. The sequences obtained were compared with those in GeneBank using the FASTA command in the GCG software package for homology to known genes.

In situ hybridization (ISH)

The gene fragments obtained from PCR select cDNA subtraction were used as probes for *in situ* hybridization (ISH). ISH was conducted to verify that the subtraction hybridization procedure yielded probes whose expression was different between tumor and normal tissues. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according the instruction provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

RESULTS

PCR selected cDNA subtraction, cloning, sequencing and GeneBank search

PCR select cDNA subtraction generated totally 14 differentially expressed genes in tumors as compared with nontumors. Among them, 8 cDNA fragments from both tumor and nontumors had considerable homology with known genes in GeneBank (Table 1). Five genes from tumor and one gene from normal liver tissues had no homology as compared to those in the GeneBank, which implied that these may be new genes. PCR select cDNA subtraction was also performed with HBV virus X gene transfected HepG2 cells and control HepG2 cells. Ten genes were differentially expressed in HepG2X compared with HepG2 cells(data not shown). Interestingly, three genes cloned from the tumor tissue of woodchuck mouse liver shared considerable homology with sequences independently found to be upregulated in HBV-X [+] cells, suggesting that the different expressions of HBxAg effector can be independently observed in the tumor and nontumor tissues induced by woodchuck hepatitis B virus.

Table 1 Differentially expressed genes in tumor and nontumor liver induced by woodchuck hepatitis B virus

Clone	GeneBank search	
	Match	% homology
Tumor[a]		
T8[b]	Human chromosome 1(UT751,L1637).	54% in 280bp overlap
T18	Unknown protein, uterine endometrium(x7723)	60% in 151bp overlap
T19	Ribosomal protein L35A(x03475)	88% in 91bp overlap
T22	Human T cell receptor beta chain(L166059)	61% in 97 bp overlap
T6	None	
T7[b]	None	
T11[b]	None	
T24	None	
T25	None	
Nontumor[a]		
N7	Human aminopeptidase N(x13276)	93% in 54 bp overlap
N10	Human IFN receptor gene (U10360)	79% in 271 bp overlap
N11	Human glutathione S-transferase(L02321)	75% in 248 bp overlap
N13	Beta-2 glycoprotein 1 from HepG2(S80305)	79% in 159bp overlap
N8	None	

[a]The clones represent fragments of genes whose expression is activated (T6,T7, T11, T18, T19, T22, T24, T25) or suppressed (N7, N8, N10, N11, N13) in HCC compared to nontumor cells.
[b]Probes whose sequences share considerable homology with sequences independently found to be upregulated in HbxAg[+] cells.

Validation and in vivo expression patterns of these genes

The cDNA fragments obtained from subtraction hybridization of tumor and nontumor tissue were then used as probes for *in situ* hybridization.In all cases, the probes from tumor showed transcripts that were preferentially expressed in tumor tissue compared with nontumor tissues. In contrast, the genes from nontumor tissue demonstrated strong hybridization in normal tissues, but little or no signal in tumor tissues.

DISCUSSION

Hepatocellular carcinoma is one of the major causes of the death in the world[15-20]. Although many researchers worked on HCC, the mechanism is still unclear[21-46]. It is widely accepted that HBV is closely associated with HCC, especially HBxAg. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[47-49]. The site of HBV integration is scattered throughout the host genome[50], making it unlikely that HBV brings about hepatocellular transformation by cis acting mechanisms in most cases. With regard to virus sequences, integration commonly occurs within a small region near the end of the virus genome[51], which is consistent with the hypothesis that transformation may be associated with the expression of one or more virus proteins from the integrated templates acting in trans. Integrated fragments of HBV DNA have been shown to make a truncated preS/S and or HBX polypeptides, both of which have trans-activting activities[52-56]. However only HBxAg transforms a mouse hepatocyte cell line in culture[57,58], and gives rise to liver tumors in at least one strain of transgenic mice[59-61]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[62-66]. HBxAg is more consistently expressed than preS in the liver of infected patients. In addition, the findings that HBxAg binds to and inactivates the tumor suppressor p53 both *in vitro* and *in vivo*[67-69], and that it may bind to and alter the function of other transcriptional factors in the cells[70], implied that HBxAg

function is important to the pathogenesis of HCC. There is some evidence that HBxAg naturally trans activates the insulin-like growth factor-1 (IGF-1) receptor[71], and may also stimulate the production of IGF-11[72], both of which may help sustain the survival and/or growth of tumor cells.

Because lots of factors are involved in the development of HCC induced by HBV and the mechanism need to be further elucidated, the new genes, especially the functional genes directly related with tumor are still worth being found in the liver tissues infected by HBV. Using the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor induced by woodchuck hepatitis B virus. The use of these fragments as probes for *in situ* hybridization of tumor and nontumor tissues verified that the PCR-selected cDNA subtraction actually yielded differences in the gene expression that distinguished tumor from nontumor, and that its differential expression may be relevant to the pathogenesis of HCC. Because of hepatitis B virus is closely associated with the development of chronic liver diseases, such as hepatitis and cirrhosis, as well as with the development of HCC, it is not known whether these differences are associated with HBxAg associated trans-activation[73-77], its inhibition of protesome function[60], its ribo/deoxy APTase[78], or AMP kinase activation[79], and/or its ability to alter signal transduction pathways[80]. However, experiments are in progress to firmly address these issues.

The results of this study showed that the up-regulation of multiple genes in tumor had considerable homology with known products from GeneBank, suggesting that the function of these genes is likely to positively regulate cell growth, while several genes generated from normal tissues suggests that these genes may be the negative regulators for cell growth. In addition, five genes from tumor and one gene from normal liver tissues had no homology as compared with entries in GeneBank, which implied that these may be new genes, and that it is very important to clone the full-length genes of these cDNA fragments to do the functional analysis. This kind of experiments are already on the way.

ACKNOWLEDGEMENTS Prof. Bo Rong Pan in Oncology Center, Xijing Hospital has made great contributions to this article.

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Edited by Wu XN and Ma JY