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

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**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[1-3]. Previously, a variety of studies have described the differences in gene expression which distinguished tumor from nontumor[4-6,9]. 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°C 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 RNaseasy 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’-CTAAATTAGTTCGAGCCGGCCGGCGGT-3’

Ad2: 5’-GTGAGCGTGAAGACGACAGAAGGCGGTTG-GCGGAGGCCGGG-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 molecules. 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 adaptor 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. Valencia, CA, USA) and cloned into pT7Blue(R) T vector (Novagen, Madison, WI, USA). Positive clones were selected by blue-white phenotype. Recombinant DNAs were isolated from minipreps of individual clones, and digested by Rsr 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 digoxigenin/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 | % homology |
|-------|----------------|------------|
| Tumor |                |            |
| T8    | Human chromosome 1 (UT751, L1637). | 54% in 280bp overlap |
| T18   | Unknown protein, uterine endometrium (x7723) | 60% in 151bp overlap |
| T19   | Ribosomal protein L35A (x103475) | 88% in 91bp overlap |
| T22   | Human T cell receptor beta chain (L166059) | 61% in 97 bp overlap |
| T6    | None | |
| T7    | None | |
| T11   | None | |
| T24   | None | |
| T25   | None | |
| Nontumor |            |            |
| 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 (L, 02321) | 75% in 248 bp overlap |
| N13   | Beta-2 glycoprotein 1 from HepG2 (S80305) | 79% in 159bp overlap |
| N8    | None | |

aThe 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.

bProbes 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-activating 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[31], and may also stimulate the production of IGF-1[32], 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 non-tumor 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[33,34], its inhibition of proteosome function[35,36], its ribo/deoxy APTase[37], or AMP kinase activation[38], and/or its ability to alter signal transduction pathways[39]. 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.

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REFERENCES

1 Jiang RL, Lu QS, Luo KX. Cloning and expression of core gene cDNA of Chinese hepatitis C virus in cosmid pTM3. World J Gastroenterol, 2000;6:220-222
2 Zhang SZ, Liang JJ, Qi ZT, Hu YP. Cloning of the non-structural gene 3 of hepatitis C virus and its inducible expression in cultured cells. World J Gastroenterol, 1999;5:125-127
3 Yu MC, Gu CH. Mutation of hepatitis B virus and its association with liver diseases. Shijie Huaren Xiaohua Zazhi, 1999;7:978-979
4 Zhou XP, Wang HY, Yang GS, Chen ZJ, Li BA, Wu MC. Cloning and expression of MRX7 gene in human HCC tissue. World J Gastroenterol, 2000;6:87-60
5 Assy N, Gong YW, Zhang M, Minuk GY. Appearance of an inhibitory cell nuclear antigen in rat and human serum during variable degrees of hepatic regenerative activity. World J Gastroenterol, 1999;5:103-106
6 Begum NA, Mori M, Matsumata K, Sugimachi K, Barnard GF. Differential display and integrin alpha 6 messenger RNA overexpression in hepatocellular carcinoma. Hepatology, 1995;22:1447-1455
7 Darabi AS, Gross M, Watabe M, Malafa M, Watabe K. Differential gene expression in experimental hepatocellular carcinoma induced by woodchuck hepatitis B virus. Cancer Lett, 1995;95:153-159
8 Inui Y, Higashiyama S, Kawata S, Tamura S, Miyagawa JI, Taniguchi N, Matsuzawa Y. Expression of heparin-binding epidermal growth factor in human hepatocellular carcinoma. Gastroenterology, 1994;107:1799-1804
9 Wu GS, Kar S, Carr BI. Identification of a human hepatocellular carcinoma-associated tumor suppressor gene by differential display polymerase chain reaction. Life Sci, 1995;57:1077-1085
10 Yamashita N, Ishibashi H, Hayashida K, Kudo J, Takenaka K, Itoh K, Nisho Y. High frequency of the MAGE-1 gene expression in hepatocellular carcinoma. Hepatology, 1996;24:1437-1440
11 Ueki T, Fujimoto J, Suzuki T, Yamamoto H, Okamoto E. Expression of hepatocyte growth factor c-met proto-oncogene in hepatocellular carcinoma. Hepatology, 1997;25:862-866
12 Diatchenko L, Lau YF, Campbell AJ. Suppression subtractive hybridization: a method for generating differentially regulated or tissue specific cDNA probes and libraries. Proc Natl Acad Sci USA, 1996;93:6025-6030
13 Liu J, Lian ZR, Pan JP, Hu JL, Zhu MH, Fan DM, Feitelson MA. Identification of differentially expressed genes in HBxAg transfected HepG2 cells by suppression subtractive hybridization. Zhonghua Xiaohua Zazhi, 2000;20:11-15
14 Liu J, Lian ZR, Pan JP, Hu JL, Zhu MH, Fan DM, Feitelson MA. Function of one novel gene identified by SSH PCR differentially expressed in HBx transfected HepG2 cells. Zhonghua Yixue Zazhi, 2000;80:1-5
15 Tang ZY. Advances in clinical research of hepatocellular carcinoma in China. World J Gastroenterol, 1998;4(Suppl 2):4-7
16 Wu GY, Wu CH. Gene therapy and liver diseases. World J Gastroenterol, 1999;5:1:218-119
17 Roberts LR, LaRussio NF. Potential roles of tumor suppressor genes and microsatellite instability in hepatocellular carcinogenesis in southern African blacks. World J Gastroenterol, 2000;6:37-41
18 Schmid R. Prospect of genotoxicology and hepatology in the next century. World J Gastroenterol, 1999;5:185-190
19 Yip D, Findlay MA, Beyer M, Tattersall MH. Hepatocellular carcinoma in central Sydney: a 10-year review of patients seen in a medical oncology department. World J Gastroenterol, 1999;5:135-140
20 Lau GKK. Immunological approaches to the breakdown of hepatitis B viral persistence. World J Gastroenterol, 1998;4(Suppl 2):32
21 Bian JC, Shen FM, Shen L, Wang TR, Wang XH, Chen GC, Wang JB. Susceptibility to hepatocellular carcinoma associated with null genotypes of GSTM1 and GSTT1. World J Gastroenterol, 2000;6:228-230
22 Martins C, Kedda MA, Kew MC. Characterization of six tumor suppressor genes and microsatellite instability in hepatocellular carcinoma in southern African blacks. World J Gastroenterol, 1999;5:470-476
23 Wei HS, Li DG, Lu HM. Hepatic cell apoptosis and fas gene. Shijie Huaren Xiaohua Zazhi, 1999;7:531-532
24 Ning XY, Yang DH. Research and progress in vivo gene therapy for primary liver cancer. Shijie Huaren Xiaohua Zazhi, 2000;8:899-903
25 Assy N, Minuk GY. A comparison between previous and present histologic assessments of chronic hepatitis C viral infections in humans. World J Gastroenterol, 1999;5:107-110
26 He P, Tang ZY, Ye SL, Liu BB. Relationship between expression of α-fetoprotein messenger RNA and some clinical parameters of human hepatocellular carcinoma. World J Gastroenterol, 1999;5:111-115
27 Sun HC, Li XM, Xue Q, Chen J, Gao DM, Tang ZY. Study of angiogenesis induced by metastatic and non-metastatic liver cancer by nearmicropocket model in nude mice. World J Gastroenterol, 1999;5:116-118
28 Luo YQ, Wu MC, Cong WM. Gene expression of hepatocyte growth factor and its receptor in HCC and nontumorous liver tissues. World J Gastroenterol, 1999;5:122-127
29 Wang YJ, Li MD, Wang YM, Nie QH, Chen GZ. Experimental study of bioartificial liver with cultured human-liver cells. World J Gastroenterol, 1999;5:135-137
30 Yang JM, Han DW, Liang QC, Zhao JL, Hsiao SY, Ma XH, Zhao YC. Effects of endotoxin on expression of ras, p53 and bcl-2 oncogene in hepatocarcinogenesis induced by thioacetamide in rats. China Natl J New Gastroenterol, 1997;5:213-217
31 Yuan SL, Huang RM, Wang XJ, Song Y, Huang GQ. Reversing effect of Tansin on the malignant phenotypes of human hepatocarcinoma cell line. World J Gastroenterol, 1998;4:317-319
32 Zhao GQ, Xue L, Xu HY, Tang XM, Hu RD, Dong J. In situ hybridization assay of androgen receptor gene in hepatocarcinogenesis. World J Gastroenterol, 1998;4:503-505
33 Ma ZY, Fan QS, Zhang DF. The effect of acupuncture on the IL2-IFN-NK immunoregulatory system of mice with HAC
graffing hepatocarcinoma. World J Gastroenterol, 2000;6(Suppl 3):32
34 Sun HC, Li XM, Xue Q, Chen J, Gao DM, Tang ZY. Study of angiogenesis induced by metastatic and non-metastatic liver cancer by corneal micro-pocket model in nude mice. World J Gastroenterol, 2000;6:116-118
35 Li WJ, Gao QX, Zhou GM, Wei ZQ. Micronuclei and cell survival in human liver cancer cells irradiated by 25MeV/u 4Ar++. World J Gastroenterol, 1999;5:365-368
36 Wu ZQ, Fan JQ, Zhou J, Tang ZY. The value of postoperative hepatic regional chemotherapy in prevention of recurrence after radical resection of primary liver cancer. World J Gastroenterol, 2000;6:131-133
37 Yuan JH, Zhang RP, Zhang RG, Guo LX, Wang XW, Luo D, Xie Y, Xie H. Growth-inhibiting effects of taxol on human liver cancer in vitro and in nude mice. World J Gastroenterol, 2000;6:210-215
38 Liu LX, Jiang HC, Zhu AL, Zhou J, Wang XQ, Wu M. Gene expression profiles in liver cancer and normal liver tissues. World J Gastroenterol, 2000;6(Suppl 3):85
39 Lin NF, Tang J, Mohamed Ismael HS. Study on environmental etiology of high incidence areas of liver cancer in China. World J Gastroenterol, 1999;5:757-761
40 Xu HY, Yang YL, Guan XL, Song G, Jiang AM, Shi LJ. Expression of regulating apoptosis gene and apoptosis index in primary liver cancer. World J Gastroenterol, 2000;6:721-724
41 Gu GW, Zhou YQ. Traditional Chinese Medicine in prevention of liver cancer. Shijie Huaren Xiaohua Zazhi, 1999;7:80-81
42 Meng QZ, Yu EX, Song MZ. Inhibition of telomerase activity of human liver cancer cell SMMC-7721 by chemotherapeutic drugs. Shijie Huaren Xiaohua Zazhi, 1999;7:252-254
43 Wu YD, Song XQ, Zhou DN, Xu HX, Gan QY, Li ZG, Liao P. Experimental and clinical study on targeting treatment of liver cancer using radionuclide-anti-AFP antibody-MMC double bomb. Shijie Huaren Xiaohua Zazhi, 1999;7:387-390
44 Fu JM, Yu XF, Shao YF. Telomerase and primary liver cancer. Shijie Huaren Xiaohua Zazhi, 2000;8:461-463
45 Mao H, Yuan AL, Zhao MF, Lai ZS, Zhang YL, Zhou DY. Effect of p38MAPK?signal pathway on ultrastructural change of liver cancer cells induced by VEGF. Shijie Huaren Xiaohua Zazhi, 2000;8:536-538
46 Cui J, Yang DH. CSF-1 receptor/c-fms and liver cancer. Shijie Huaren Xiaohua Zazhi, 2000;8:696-697
47 Okuda K. Hepatocellular carcinoma: recent progress. Hepatology, 1992;15:948-963
48 Matsubara K, Tokino T. Integration of hepatitis B virus DNA in hepatocellular carcinoma. Proc Natl Acad Sci USA, 1990;87:2970-2974
49 Okuda K. Hepatocellular carcinoma: recent progress. World J Gastroenterol, 2000;8:696-697
50 Caselmann WH, Meyer M, Kekule AS, Lauer U, Weiss L, Luber B, Hofschneider H. Novel aspects of regulating apoptosis gene and apoptosis index in primary liver cancer. World J Gastroenterol, 2000;6:721-724
51 Feitelson MA, Duan LX. Hepatitis B virus X antigen in the serum of patients with primary hepatocellular carcinoma. Oncogene, 1999;8:1109-1117
52 Benn J, Schneider RJ. Hepatitis B virus HBx protein downregulates cell cycle checkpoint controls. Proc Natl Acad Sci USA, 1995;92:11215-11219
53 Benn J, Schneider RJ. Hepatitis B virus HBX protein deregulates cell cycle checkpoint controls. Proc Natl Acad Sci USA, 1995;92:11215-11219
54 Feitelson MA, Zhu M, Duan XL, London WT. Hepatitis B X antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene, 1999;8:1109-1117
55 Benn J, Schneider RJ. Hepatitis B virus HBX protein downregulates cell cycle checkpoint controls. Proc Natl Acad Sci USA, 1995;92:11215-11219
56 Feitelson MA, Zhu M, Duan XL, London WT. Hepatitis B X antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene, 1999;8:1109-1117
57 Benn J, Schneider RJ. Hepatitis B virus HBX protein regulates cell cycle checkpoint controls. Proc Natl Acad Sci USA, 1995;92:11215-11219
58 Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc Natl Acad Sci USA, 1994;91:2230-2234
59 Trum R, Antunovic J, Greenblatt J, Prives C, Cromlish JA. Direct interaction of Hepatitis B virus HBX protein with p35 response element-directed transactivation. J Virol, 1995;69:1851-1859
60 Henkler F, Koshy R. Hepatitis B virus transcriptional activators: mechanism and possible role in oncogenesis. J Virol, 1995;70:3375-3385