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• BRIEF REPORT •

Cloning of differentially expressed genes in human hepatocellular carcinoma and nontumor liver

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INTRODUCTION

The mechanism of hepatocellular carcinoma (HCC) is still unclear, although some genes have been found to play a role in the transformation of liver cells, and a variety of studies have described differences in gene expression which distinguished tumor from nontumor[1-6]. The new genes, especially the functional genes directly related with tumor are still worth being found.

The purpose of our study is to find the different genes between human liver tumor and normal tissues using suppression subtractive hybridization.

MATERIALS AND METHODS

Patients samples

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.

PCR selected cDNA subtraction, cloning, sequencing and identification of cloned gene fragments

The difference in gene expression between human tumor and nontumor tissues were evaluated by a commercially available subtraction hybridization approach (the PCR selected cDNA subtraction kit from Clontech, Palo Alto, CA, USA) according to the instruction provided by the manufacturer. Briefly, we got total RNA and mRNA from tumor and nontumor tissues using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA), and then both mRNA (2 μg each) were converted into cDNA. We refer to the cDNA from tumor 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 moleclule. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adapters. The driver cDNA had no adaptor. Two hybridization was 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 to reduce any background and to further enrich differentially expressed genes. The cDNA fragments were directly inserted into a T/A cloning vector(Novagen, Medison, WI, USA), and homology analysis was undertaken within GeneBank. On the other hand, we used normal tissues as the tester and tumor as the driver to do PCR select cDNA hybridization. The procedure was as above.

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 differed in tumor compared to normal tissue. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according to 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 19 differentially expressed genes in tumors and nontumors. Among them, 14 cDNA fragments had considerable homology with known genes in GeneBank (Table 1). For example, T2 and T3 had homology with ribosomal protein and elongation factor EF-1α, suggesting that these genes may stimulate cell growth. N1 from normal tissues had homology with interferon gamma gene, suggesting that this gene may be a negative regulator for cell growth. Interestingly, one gene from tumor

and three genes from normal liver tissues had no homology as compared with those in GeneBank, which implied that these may be new genes.

Validation and in vivo expression patterns of these genes

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

Table 1 Differentially expressed genes in human tumor and nontumor liver

Clone	GeneBank search	
	Match	% homology
Tumor		
T1	Retinoblastoma gene (L11910)	75% in 193 bp overlap
T2	Ribosomal protein L7(L16588)	87% in 209 bp overlap
T3	Elongation factor EF-1α (J04617)	85 % in 157 bp overlap
T4	2-oxoglutarate dehydrogenase (D10525)	89% in 258 bp overlap
T5	Proteasome activator HPA28 subunit β (D45348)	93% in 204 bp overlap
T6	Ribosomal protein S2 (X57432)	89% in 195bp overlap
T7	Rab geramylgeranyl transferase-α Subunit(Y08200)	90% in 110 bp overlap
T8	Nuclear-encoded mitochondrial NADH-ubiquitinone reductase	93% in 197 bp overlap
T9	None	
Nontumor		
N1	Interferon gamma gene (L07633)	88% in 308bp overlap
N2	None	
N3	V-fos transformation effector protein	92% in 200bp overlap
N4	Sigma-1 receptor (266537)	75% in 123bp overlap
N5	Glycoprotein gll gene (D00464)- 3'flanking region	62% in 549bp overlap
N6	None	
N7	RABAPTIN-5 protein(X91141)	86% in 110bp overlap
N8	Dishevelled-3 (DUL3) protein	89% in 72bp overlap
N9	None	
N10	None	

DISCUSSION

Hepatocellular carcinoma is one of the major causes of death in the world[7-10]. The mechanism of carcinogenesis is unknown, although it is widely accepted that hepatitis B virus (HBV) and hepatitis C virus (HCV) are closely related to liver cancer, especially hepatitis B virus X antigen[11-14]. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[15-17]. The sites of HBV integration are scattered throughout the host genome[18], 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[19], 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[20-24]. However, only HBxAg transforms a mouse hepatocyte cell line in culture[25,26], and gives rise to liver tumors in at least one strain of transgenic mice[27-29]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[30-34]. The expression of HBxAg is more consistent than that of 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*[35-37], and that it may bind to and alter the function of other transcriptional factors in the cells[38], 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[39], and may also stimulate the production of IGF-11[40], both of which may help sustain the survival and/or growth of tumor cells.

Because the mechanism of HCC induced by HBV still need to be elucidated, cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer. By the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor. 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. It is not known whether these differences are associated with HBxAg associated trans-activation[41,42], its inhibition of protesome function[43] its ribo/deoxy APTase[44], or AMP kinase activation[45], and/or its ability to alter signal transduction pathways[46], because 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 hepatocellular carcinoma (HCC)[47-60]. 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 which have considerable homology with known products from GeneBank, for example, ribasomal protein and elongation factor EF-12, suggesting that the

function of these genes is likely to positively regulate cell growth. Several genes are generated from normal tissues and one has >88% homology with interferon gamma gene, suggesting that these genes may be the negative regulators for cell growth. In addition, one gene from tumor and three genes 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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