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Genes encoding Pir51, Beclin 1, RbAp48 and aldolase b are up or down-regulated in human primary hepatocellular carcinoma

Hai Song, Shuang-Luo Xia, Cheng Liao, Yi-Liang Li, Yi-Fei Wang, Tsai-Ping Li, Mu-Jun Zhao

AIM: To reveal new tumor markers and target genes from differentially expressed genes of primary tumor samples using cDNA microarray.

METHODS: The 32P labeled cDNAs were synthesized by reverse transcription of message RNA from the liver cancerous tissue and adjacent non-cancerous liver tissue from the same patient and used to hybridize to LifeGrid 1.0 cDNA microarray blot containing 8400 known and unique human cDNA gene targets, and an expression profile of genes was produced in one paired human liver tumor tissue. After a global analysis of gene expression of 8400 genes, we selected some genes to confirm the differential expression using Northern blot and RT-PCR.

RESULTS: Parallel analysis of the hybridized signals enabled us to get an expression profile of genes in which about 500 genes were differentially expressed in the paired liver tumor tissues. We identified 4 genes, the expression of three (Beclin 1, RbAp48 and Pir51) were increased and one (aldolase b) was decreased in liver tumor tissues. In addition, the expression of these genes in 6 hepatoma cell lines was also decreased in liver tumor tissues. In addition, the expression of these genes in 6 hepatoma cell lines was also decreased in liver tumor tissues. By a global analysis of gene expression profile using cDNA microarray, we found that 10 genes were up-regulated and 9 genes were down-regulated in >50% of HCC samples.

CONCLUSION: cDNA microarray permits a high throughput identification of changes in gene expression. The genes encoding Beclin 1, RbAp48, Pir51 and aldolase b are first reported that may be related with hepatocarcinoma.

INTRODUCTION
Hepatocellular carcinoma (HCC), an aggressive malignancy with poor prognosis and one of the most common tumors in human beings, has become a leading cause of cancer-related death in adults from Asia and sub-Saharan-Africa[1]. The multiple pathogenic factors, including food contamination with aflatoxin B1 and infection with hepatitis B virus and hepatitis C virus and the subsequent multistage pathogenesis of HCC have been extensively studied. In addition, tumor suppressor genes, such as Rb and p53[2,3], may play a significant role in hepatocarcinogenesis. However it is not clear how these disorders result in HCC. Recent advances in molecular genetics have identified various genetic abnormalities in tumors. However, little is known about the genetic alterations responsible for specific phenotypes of HCC.

With the advent of cDNA microarray technology, genome-wide expression of hundreds of genes can be simultaneously analyzed, facilitating differential expression monitoring of a large number of activated or suppressed genes under various biological conditions, including carcinogenesis[4-6], drug discovery and development[7]. With cDNA microarrays, it is now possible to perform a large-scale expression survey to identify candidate target genes[7]. Efforts to classify human HCC based on gene expression profile using cDNA microarray have been successfully processed in recent year[8-12]. Shirota et al. [10] found that 10 genes were up-regulated and 9 genes were down-regulated in >50% HCC and identified the changes of 22 genes associated with the degree of differentiation of HCC[11]. Kawai et al. showed that AFP-producing hepatoma cell lines shared a distinct expression profile of genes in various categories compared with those of AFP-negative hepatoma cell lines and non-hepatotic cancer cell lines[12]. Xu et al. identified that 156 genes were down-regulated and up-regulated in >50% of cancer samples of HCC[10].

In this study, we used cDNA microarray representing 8400 cDNA clusters to analyze HCC specific expression profile. The aims were to identify complex alterations of genes expression responsible for the development of HCC and to identify differentially expressed genes and differentially expressed novel genes of potentially biological or medical importance for HCC. In this report, we showed that 523 genes were differentially expressed over 4 folds in the microarray analysis. We confirmed 4 genes which were consistently up or down-regulated in >50% of HCC samples.

MATERIALS AND METHODS

Tumor materials and cell lines
All samples were obtained from Eastern Hepatobiliary Surgery Hospital and Zhongshan Hospital (Shanghai, China). All patients were diagnosed as HCC. Tissue specimens were quickly frozen shortly after surgical resection and stored in liquid nitrogen. Tissue for cDNA microarray hybridization was obtained from a 47-year-old male patient with primary hepatocellular carcinoma stage III, HBV positive.

The HCC cell lines HepG2, SMMC-7721, Bel-7404, Bel-7402, HuH7 and the line of normal liver cells L02 were obtained from the Cell Bank of the Chinese Academy of Sciences.
RNA preparation and poly A' mRNA preparation
Total RNA was extracted with TRIZOL reagent (Life Technologies, Inc., N.Y., USA). Tissue samples were homogenized in 1 ml of TRIZOL reagent per 50-100 mg of tissue and incubated for 5 min at room temperature, then 200 μl chloroform was added and mixed vigorously and incubated at room temperature for 2-3 min. After centrifugation at 1 2000 rpm for 15 min at 4°C, the aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol, and incubated at room temperature for 10 min. Total RNA was collected and washed in 75% ethanol. Total RNA was run on a denaturing formaldehyde agarose gel to check quality. Poly A' RNA was isolated using the oligotex mRNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two hundred micrograms of total RNA was routinely used for mRNA isolation.

cDNA microarray hybridization
Gene expression was analyzed using the Human Life Grid 1.0 array (Incyte Genomics Inc. California, USA). Approximately 8 400 human PCR products chosen from Incyte Genomics's library of proprietary clones were girded onto a 12 x 22 cm nylon membrane in a double-spotted pattern at a density of about 33 spots per cm2. About 92% genes had no significant expression change. The result reflected the reliability of the gene expression profile. The scatter plot of intensity of all genes on arrays of liver tumor was statistically examined to evaluate the accuracy of experiment (Figure 1C). A high correlation was observed. This result suggested a high reliability of the experiment for analysis of differentially displayed genes by cDNA microarray analysis of this sample.

Characterization of expression profile of HCC
Among the 6542 genes expressed in paired liver tumor tissue, 256 genes were up-regulated and 267 genes were down-regulated, which were greater or less than 4 fold in liver tumor tissue. Known functioning genes differentially expressed (>4 fold) in HCC were classified into six functional categories with respect to selected functional properties of their products. The six categories were included in cell division; cell, organism defense; metabolic enzymes, transporters ion channels; nuclear proteins; cell structure, extracellular matrix; cell signaling, communication. The numbers of classified genes are shown in Table 2. The group of 'other genes' summarized individual genes that could not be included in any of the above categories.
Table 2 Classification of number of known functioning genes differentially expressed (>4 folds) in HCC

<table>
<thead>
<tr>
<th>Gene functions</th>
<th>Number of down-regulated genes in HCC</th>
<th>Number of up-regulated genes in HCC</th>
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<tr>
<td>Cell division</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Cell, organism defense</td>
<td>39</td>
<td>24</td>
</tr>
<tr>
<td>Metabolic enzymes, transporters ion channels</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Nuclear proteins (transcription factors, DNA processing enzymes)</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Cell structure, extracellular matrix</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Cell signalling, communication</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>EST</td>
<td>41</td>
<td>53</td>
</tr>
<tr>
<td>Other genes</td>
<td>84</td>
<td>102</td>
</tr>
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Verification of differentially expressed genes in cDNA microarray

To verify the data, we then performed Northern blot analysis and RT-PCR analysis. Only genes with expression levels that were altered by >4 fold between normal and tumor tissues were selected. These analyses were carried out with a total 10 paired HCC samples. We identified 4 genes that were differential expression in >50% paired samples. Three genes were up-regulated and one was down-regulated. Northern blot analysis of Beclin 1 and RbAp48 mRNA showed a significantly increased expression level in 50% paired tumor samples (Figure 2), which could not be detected in normal liver tissues. Northern blot analysis of aldolase b mRNA showed a significantly decreased expression level in 70% paired tumor samples. Northern blotting signal of Pir51 was difficult to obtain, the semi-quantitative RT-PCR carried out in the linear detection range was used to estimate the relative amount of mRNA. Pir51 mRNA was up-regulated by RT-PCR analysis in 60% paired tumor samples (Figure 2).

Figure 1 Parallel analysis of gene expression in paired human liver tumor sample (A). Histogram analysis of fold change in differentially expressed genes in cDNA microarray (B). Scatterplot of two cDNA microarray analyses of normal liver and liver tumor samples. Each point stands for a gene with the X coordinate value as the gene expression level in the normal liver microarray and the Y coordinate value as the gene expression level in the liver tumor microarray. A R of 0.81 was produced and suggested high reliability of the experiments (C).

Figure 2 Northern blots of RbAp48, Beclin 1 and aldolase b genes in paired liver tumor (T) and normal liver tissues (N). RT-PCR analysis of Pir51 in the same paired HCC samples. 185 rRNA shown as a loading control.

In contrast we investigated the expression of Beclin 1, RbAp48, Pir51 and aldolase b in one hepato (L02) and 5 hepatoma cells lines by RT-PCR analysis. RbAp48 was detected in L02, Bel-7404, HepG2, Bel-7402 and HuH7, was undetectable in SMMC-7721. Beclin 1 was detected in L02, SMMC-7721, Bel-7404, and HepG2, was weak or undetectable in Bel-7402 and HuH7. Pir51 was expressed in all of hepatoma cell lines detected excluding HuH7. The data also showed that only HepG2 cells, most of cells had not, had a weak expression of aldolase b, (Figure 3). These results from cells coincided with that from tissues (Figure 2). The data of 4 genes obtained from microarray and Northern bolt or RT-PCR are summarized in Table 3. The fold changes of the 4 genes by Northern blot analysis were significantly consistent with cDNA microarray.
was found to be one of the three subunits of chromatin.

RbAp48 was isolated as an Rb binding protein. Information to further determine its new biological role.

Enhanced expression of Beclin 1 in HCC provided us important clues of Beclin 1 might be induced by the infection of virus. The patients were infected with HBV or HCV, increased expression in host defense against Sindbis virus infection in vivo showed that the expression of Beclin 1 mRNA was increased detected in normal liver tissues. Beclin 1 has an unknown function in HCC. Overexpression of Beclin 1 in neurons could inhibit Sindbis virus replication, reduce central nervous system (CNS) apoptosis, and provide protection against fatal Sindbis virus infection, which might play a role in host defense against Sindbis virus infection. 80% of HCC patients were infected with HBV or HCV, increased expression of Beclin 1 might be induced by the infection of virus. The enhanced expression of Beclin 1 in HCC provided us important information to further determine its new biological role.

RbAp48 was isolated as an Rb binding protein. RbAp48 was found to be one of the three subunits of chromatin RbAp48 was recently cloned as two proteins that may play roles in hepatocarcinogenesis. We demonstrated up- or down-regulated genes in liver cancerous tissues commonly found in patients, and compared them with those in adjacent normal tissues. Then we selected several differentially displayed genes for further verification by Northern blots or RT-PCR. Four genes (Beclin 1, RbAp48, Pir51 and aldolase b) were confirmed to have a differential expression pattern in normal and cancerous liver tissues. The low percentage appeared to be the heterogeneity of tumors. The genes identified through this approach are potential candidates for factors implicated in carcinogenesis, and are useful in both cancer diagnosis and HCC therapy.

Beclin 1 has been found to be a novel Bcl-2-interacting cellular protein which was mono-allelically deleted in 40-75% of sporadic human breast cancers and ovarian cancers, and also a mammalian autophagy gene that could inhibit tumorigenesis and could be expressed at decreased levels in human breast carcinoma. It has been considered as a tumor suppressor gene in breast cancer and HCC therapy. Beclin 1 has an unknown function in HCC. Overexpression of Beclin 1 in neurons in vivo could inhibit Sindbis virus replication, reduce central nervous system (CNS) apoptosis, and provide protection against fatal Sindbis virus infection, which might play a role in host defense against Sindbis virus infection. 80% of HCC patients were infected with HBV or HCV, increased expression of Beclin 1 might be induced by the infection of virus. The enhanced expression of Beclin 1 in HCC provided us important information to further determine its new biological role.

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