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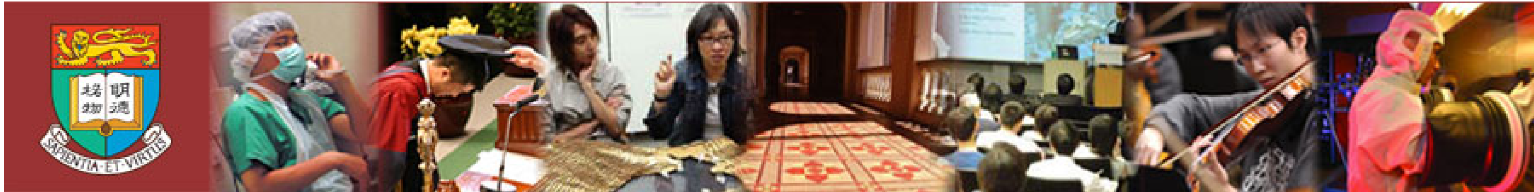

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• LIVER CANCER •

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

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Abstract

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

METHODS: The ^{33}P 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 showed by RT-PCR analysis.

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.

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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 hepatitic B virus and hepatitic C virus and the subsequent multistage pathogenesis of HCC have been extensively studied. In addition, tumor suppressor genes, such as Rb and p53[2], 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[3-5], drug discovery and development[6]. 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.* 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[8]. 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[10]. Xu *et al* identified that 156 genes were down-regulated and up-regulated in >50% of cancer samples of HCC[12]. So much work has focused on HCC expression profile, however the data are still far less.

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, HuH7and the line of normal liver cells L02 were obtained from the Cell Bank of the Chinese Academy of Sciences

Table 1 Primer sequences and PCR conditions used for synthesis of amplicons applied as probes in Northern blot and RT-PCR

Genes name	Primer (5'-3')	PCR fragment size (bp)	Annealing temperature℃	Number of cycles
Pir51	F gtggaagatgatgttggtggtg	527	58	32
	R aaggcggagactctgattgg			
RbAp48	F gaactgcctttctttcaatc	826	58	30
	R atggctcagacacctacctc			
Beclin 1	F cttaccacagcccaggcgaaac	814	58	30
	R gccagagcatggagcagcaa			
Aldolase b	F gccactctcaacctcaatgc	423	55	32
	R tctccttcccaacctaccac			
β-actin	F tgacggggtcacccacactgtgcc	666	60	25
	R cttagaagcattgcggtggacgatg			

(Shanghai, P.R. China). HepG2, HuH7, Bel-7404 and Bel-7402 cells were cultured in Dulbecco' s Modified Eagle Medium (DMED; Life Technologies Inc., Grand Island NY, U.S.A.) supplemented with 10% fetal calf serum (FCS; Life Technologies Inc.) and L02 and SMMC-7721 cells were cultured in RPMI medium 1640 (Life Technologies Inc.) plus 10% FCS.

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 1ml 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℃, 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 manufacture' 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 12×22 cm nylon membrane in a double-spotted pattern at a density of approximately 16 800 spots and 27 controls. ^{33}P-labeled cDNA probe was generated by reverse transcription of 1 μg of each analyzed polyA^+ RNA sample in the presence of α-^{33}P dCTP and the percent label incorporation was not less than 40%. Each cDNA probe was then hybridized to a microarray. Overnight incubation was followed by stringent washing as recommended by the manual. Membranes were exposed to phosphor screen overnight. The data were analyzed by Incyte Genomics.

Northern blot and semi-quantitative RT-PCR

All probes were obtained by amplification of gene fragments by PCR under conditions listed in Table 1. For conventional Northern blot analysis, 15 μg total RNA was fractionated by electrophoresis through 1% agarose gel containing formaldehyde and blotted in 20×SSC onto Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Ltd. Buckinghamshire, UK) by capillary force overnight. The blotted RNA was immobilized by incubating at 80℃ for 1 h. Hybridization was performed at 68℃ in ExpressHyb hybridization buffer (CLONTECH Inc., California, USA) for 3 h. Membranes were washed once in 2×SSC, 0.1% SDS at room temperature, twice in 0.1×SSC, 0.1% SDS at 68℃ for 20 min and exposed to X-ray film (Eastman Kodak Co., Rochester MA) at -80℃ for 1-6 d. Three μg total RNA was reverse transcribed in 20 μl reaction mixture with Superscrip™ II (Life Technologies Inc., NY., USA). The PCR cycle number at the linear phase of amplification was chosen to compare differential gene expression among different genes.

RESULTS

Global gene expression analysis of HCC by high density cDNA microarray

The ^{33}P labeled cDNAs were synthesized by RT of message RNA from one liver cancerous tissue and adjacent normal liver tissue from the same patient and used to hybridize to Life Grid 1.0 cDNA microarray blot. General expression profile of 8400 genes was obtained (Figure 1A). We first performed a global analysis of gene expression of 8400 genes and compared the gene expression profiles for normal liver and liver tumor tissues. To eliminate data with low reliability, genes whose expression was regarded as absent in both normal liver and liver tumor by software analysis were excluded, and genes for which two spots were greater than 2.5 fold different and one of the spots was not 2 fold above background were also excluded. There were 6542 remaining genes in normal liver and liver tumor tissues. Fold changes in gene expression between normal liver and liver tumor are shown in Figure 1B. 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 and adjacent normal liver 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.

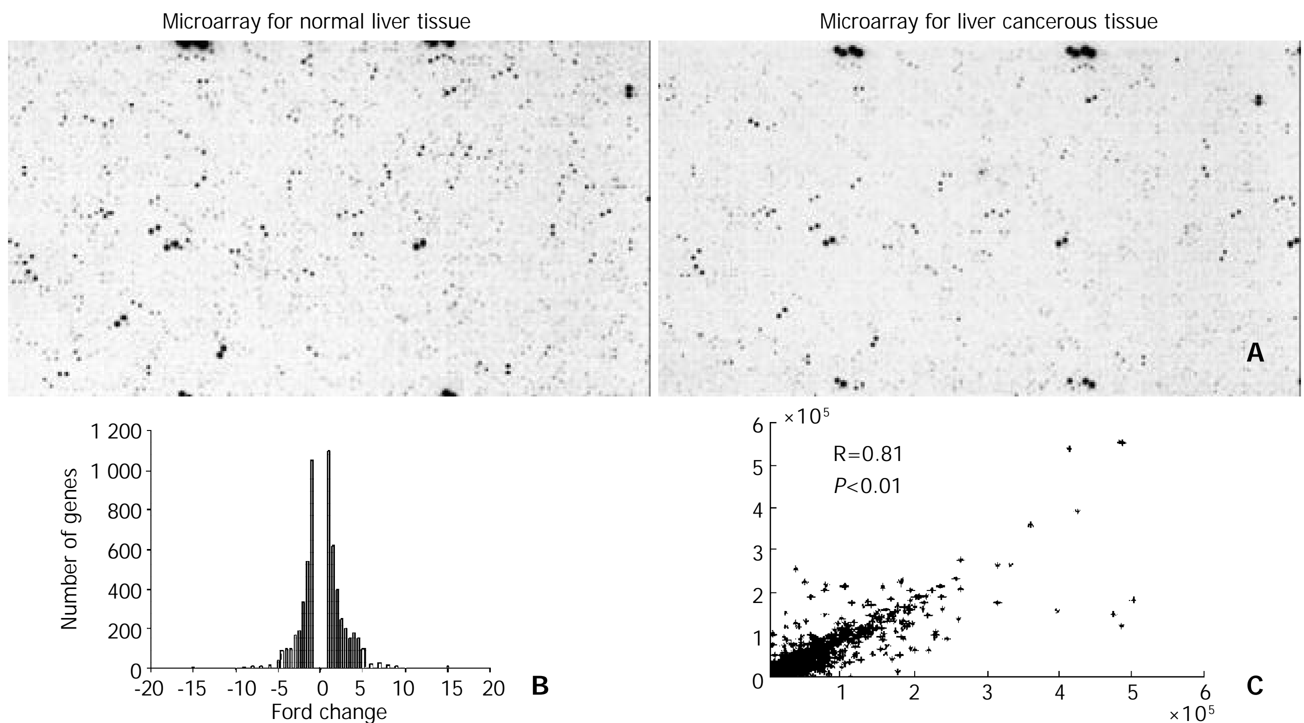

Figure 1 Parallel analysis of gene expression in paired human liver tumor sample (A). Histogram analysis of fold change in differentially expressed genes in cDNA microarray (B). Scatterplot of two cDNA microarray analyses of normal liver and liver tumor samples. Each point stands for a gene with the 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).

Table 2 Classification of number of known functioning genes differentially expressed (>4 folds) in HCC

Gene functions	Number of down-regulated genes in HCC	Number of up-regulated genes in HCC
Cell division	17	14
Cell, organism defense	39	24
Metabolic enzymes, transporters ion channels	17	9
Nuclear proteins (transcription factors, DNA processing enzymes)	21	20
Cell structure, extracellular matrix	10	6
Cell signalling, communication	37	26
EST	41	53
Other genes	84	102

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).

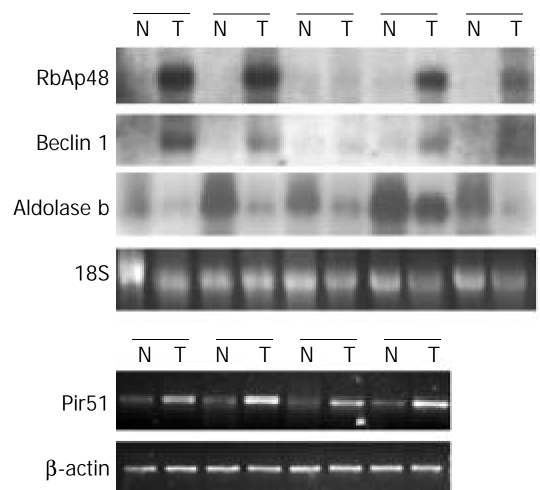

Figure 2 Northern blots of RbAp48, Beclin 1 and aldolase b genes in paired liver tumor (T) and normal liver tissues (N). RT-PCR analysis of Pir51 in the same paired HCC samples. 18S rRNA shown as a loading control.

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.

Table 3 Genes showing differential expression levels in liver cancerous tissue and adjacent normal liver tissue

Gene Name	GenBank access number	Density in filter for tumor liver	Density in filter for normal liver	Fold change by microarray	Fold change by Northern blot
Beclin 1	AF077301	6 971	0	99	99
RbAp48	X74262	14 029.9	0	99	99
aldolase b	XM_005563	2 765	26 589	-9.65	-4.7
Pir51	NM_006479	8 654.61	323.2	26.8	5.7

GenBank access number, density, fold change verified by cDNA microarray and Northern blot were described.

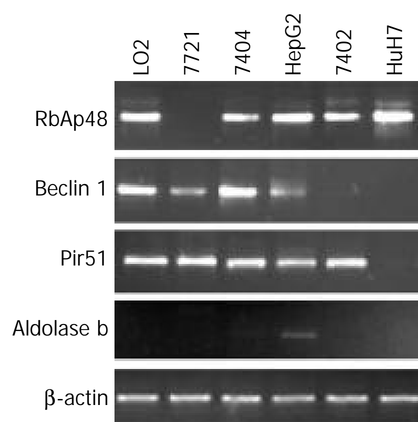

Figure 3 RT-PCR analysis of RbAp48, Beclin 1, Pir51 and aldolase b expression in one hepato (L02) and 5 hepatoma cell lines (SMMC-7721, Bel-7404, HepG2, Bel-7402, HuH7). β-actin was shown as an internal control.

DISCUSSION

In this study, we used cDNA microarray to identify the genes 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[12]. 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[13], and also a mammalian autophagy gene that could inhibit tumorigenesis and could be expressed at decreased levels in human breast carcinoma[14]. It has been considered as a tumor suppressor gene in breast cancer[14,15]. However, our results showed that the expression of Beclin 1 mRNA was increased in liver tumor tissues and HCC cell lines and could not be detected in normal liver tissues. 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[16]. 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[17]. RbAp48 was found to be one of the three subunits of chromatin assembly factor 1[18,19] and components of histone deacetylase complexes[20,21]. RbAp48 could also interact with a complex of CREB binding protein and phosphorylated CREB[22]. RbAp48 was physically associated in the presence of Rb and HDAC1, suggesting that RbAp48 could be involved in transcription repression of E2F responsive genes[23]. Binding of RbAp48 correlated with the ability of Rb to block cell proliferation[17,24]. Furthermore, the *C. elegans* homologues of both Rb and RbAp48 were recently cloned as two proteins that belong to the same Ras-inhibitory way[25]. However, the mechanisms by which RbAp48 gene product regulated tumor growth are largely unknown. RbAp48 mRNA was also shown to increase in HCC tissues. Although the biological significance of this finding needs further study, our results showed that altered expression of RbAp48 gene might be related to HCC development.

Aldolase B, fructose-biphosphate Aldolase, an important enzyme for glucose and fructose metabolism, is a liver-specific gene. Some hepatic genes, for instance, albumin, ornitin transcarbamylase and transthyretin, were down-regulated in carcinogenesis of hepatocytes[26,27]. This down-regulation was mainly attributed to the diminishing of some of liver-enriched transcription factors (LETF). Kovalenko *et al.*[28] reported AlF-C1 enriched in fetal and regenerating livers, and down-regulated the aldolase B gene promoter in rat hepatoma cells. In our results, aldolase B was down-regulated in HCC samples and HCC cell lines, which may reflect the function change of liver cancer from metabolism to proliferation.

Pir51 protein could strongly interact with human Rad51 recombinase[29]. Eukaryotic Rad51 protein has been reported to play a central role in homologous recombination by carrying out the pairing of homologous DNA molecules and initiating the strand exchange reaction[30,31] and mammalian Rad51 was essential for cell proliferation. Rad51 activity is regulated by its associated proteins. Pir51 protein could bind to both single and double stranded DNA and was capable of aggregating DNA[29]. However, functional significance of biochemical properties of pir51 is unclear.

In conclusion, our data demonstrate that profiling of HCC samples can help reveal genes that are commonly expressed in HCC. Information from these studies may be useful in the development of therapeutic drugs for liver cancer.

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