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The Pattern of Differentially Expressed Genes in Biliary Atresia

Biliary atresia is a progressive obliterative cholangiopathy, but the etiology of this disorder remains uncertain. Identifying genes specifically expressed in biliary atresia and analyzing the pattern of expression may lead to a better understanding of the pathogenesis. Liver tissues were taken from a recipient with biliary atresia and a normal donor during liver transplantation. Total RNA was extracted from each sample and reversely transcribed to cDNA. Then radiolabeled cDNA probe pools were made by random primed DNA labeling method and used for screening of differentially expressed genes by hybridizing with expressed sequence tags (EST) dot blot panel. Northern blot hybridization was done to confirm that these genes are also differentially expressed in other liver tissues. Among 1,730 EST clones, 26 cDNA clones were significantly overexpressed in biliary cirrhosis, while 2 clones were significantly decreased in biliary atresia. By Northern blot hybridization, the results of tissue inhibitor of metalloproteinase (TIMP)-1 and IGFBP-2 were well correlated with differential EST screening (DES). This study identified the pattern of differentially expressed genes in the biliary cirrhosis due to biliary atresia using DES technique.

Key Words : *Genes; Biliary Atresia; Liver Cirrhosis; Expressed Sequence Tags*

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INTRODUCTION

Biliary atresia is a neonatal obstructive cholangiopathy characterized by progressive obliteration or discontinuity of the extrahepatic bile duct. The obstruction of bile flow results in worsening cholestasis, hepatic fibrosis, and cirrhosis, which lead to portal hypertension and a decline in hepatic synthetic function. Biliary atresia is the leading indication for liver transplantation in children (1, 2).

In fact, little is known about the etiology of this disorder, so it would be very meaningful to identify genes that are specifically expressed in liver tissue caused by biliary atresia and analyze the pattern of expression of those genes. This would lead to a better understanding of the pathogenesis as well as developing methods of early detection.

Recently, several studies have been done to find out specific gene expression. Malizia et al. suggested that transforming growth factor (TGF) β1 and platelet-derived growth factor (PDGF) are involved in human liver inflammation and fibrosis, so that the expression of growth factor mRNAs in proliferating ductular cells may indicate a role for these cells in liver fibrogenesis and may help explain the pathophysiology of conditions such as biliary atresia progressing to fibrosis despite the absence of marked inflammation (3, 4).

The pattern of expression of genes may be more informative than analysis of a few individual genes. Therefore, we studied the gene expression difference in biliary atresia liver tissues compared to normal liver tissues, which were obtained mainly from living related liver transplantation, to understand the pathogenesis and to analyze the pattern of gene expression in biliary atresia by using a dot blot panel of 1,730 different expressed sequence tags (ESTs) clones which were isolated from a human hair dermal papilla cell cDNA library in Kyungpook National University (KNU).

MATERIALS AND METHODS

Human cDNA clone

The cDNA library was constructed with mRNA from human dermal papilla cells. Inserts (>400 bp) were unidirectionally cloned into the lambda Uni-Zap phage vector to generate 10^7-10^8 recombinants. Bacterial transformants were obtained by cotransfection with a helper Sequena phage. Colonies were picked at random and minilysate DNA was prepared by alkaline lysis using REAL preps (Qiagen, Hilden, Germany). cDNA was sequenced from 5′ end of the insert using a Sequenase DNA sequencing kit. Sequences were compared with GenBank database.

cDNA array preparation

A total of 1,730 cDNAs were arrayed in nylon membranes using 96-well format dot blotter (BioRad, Hercules, CA, U.S.A.). Two identical blots were produced for each set of cDNA samples. Briefly, the membranes were cut to the size of the dot blot manifold and soaked in 0.4 M Tris (pH 7.5) for 5 min. The membrane was placed into the manifold and clamped. The plasmid DNAs were denatured for 10 min at room temperature in 0.25 N NaOH/0.5 M NaCl. The DNAs were diluted in 0.1 ×SSC/0.125 N NaOH so that 200 ng DNA was fixed per dot. The DNA samples were loaded into the manifold and suction was applied. The membranes were removed from the manifold, neutralized by rinsing in 0.5 M NaCl/0.5 M Tris (ph 7.5) air dried, and then fixed by UV crosslinking at 240 mJ using a Stratalinker (Stratagene, La Jolla, CA, U.S.A.).

Preparation of RNA from biliary atresia and normal liver tissues

Liver parenchymal tissues from 5 biliary atresia patients and 3 normal controls were put into liquid nitrogen immediately after resection to avoid RNA degradation during living related liver transplantation and liver operation. Total RNA was extracted from one hundred milligrams of pulverized tissues by a modified acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi, 1987) using TRIzol (Life Technologies, Bethesda, U.S.A.).

Preparation of probe and hybridization

The cDNAs to be used as probes were synthesized from total RNAs prepared from biliary atresia and normal liver tissues, using an oligo (dT) primer and Superscript II reverse transcriptase. The cDNA was radiolabeled with ^{32}P-dCTP by random priming. Equal counts of the probes were heat-denatured and used to probe the DNA dot blots in Express-Hyb Hybridization Solution.

Films were scanned with Epson GT-8500 scanner and density of each dot was measured using public domain NIH Image program. Density was calculated by integration of the values of pixels of each dot. The ratio of density of each dot between biliary atresia and normal control was calculated and the ratio over 1.5 was arbitrarily counted as significantly increased, just for screening (Table 1).

Northern blotting

Northern blotting was done to confirm those clones differentially expressed in the differential EST screening. Five infants (4 recipients of liver transplantation around 1 yr of age and 1 from Kasai portoenterostomy operation around 10 weeks of age) with extrahepatic biliary atresia and 3 controls (2 normal donor livers and 1 normal portion of liver in a hepatocellular carcinoma patient) were studied. 10 μg of total RNA prepared from normal and biliary atresia liver tissues were used.

RESULTS

Among the total of 1,730 EST clones, 26 cDNA clones were significantly overexpressed in biliary cirrhosis due to biliary atresia. They were sorted by the classification of cDNA library in KNU (http://hair.knu.ac.kr/ebtoc.htm) and revealed homology to genes encoding (Table 1):

1) Cell division/DNA synthesis related: bcl-w. 2) Cell signaling and communication related: laminin binding protein (BP), hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), thymosinβ-4, 10, TGF-β. 3) Gene/protein expression

Table 1. Differential profile of differentially expressed genes identified in DES

Transcript identity	Intensity		Ratio (BA/Normal)
	Normal	BA	
Bcl-w	16.7	28.8	1.7
Laminin BP (binding protein)	0.7	7.7	11
HRS (HGF-regulated tyrosine kinase substrate)	0.5	3.5	6.9
Thymosin β-4	0.6	4.1	6.5
Thymosin β-10	0.5	2.5	5.4
TGF-β	0.6	2.1	3.3
TIMP (tissue inhibitor of metalloproteinase)-1	1.8	3.9	2.2
SRP (signal recognition particle) 4	2	7.1	3.6
SRP9	1.7	5.6	3.3
SNAP (soluble NSF attachment proteins) 45	1.2	5.6	4.5
Alu RNA BP	0.8	6.9	8.5
supt5h (human homologue of the yeast transcription factor, SPT5)	1.9	4.5	2.3
eIF-2α kinase	4.5	12.1	2.7
HSP (heat shock protein) 27	2.1	3.7	1.8
Lysyl oxidase	10.8	22.2	2.1
MLC (myosin light chain) kinase	12	37.2	3.1
Aldolase A	29.4	57.3	1.9
γ-glutamylcystein synthetase	17.3	45	2.6
Collagen type Iα1	1.5	10.1	6.8
Collagen type Iα2	1.1	9.4	8.7
Collagen type III	3	8.5	2.9
Fibronectin	1.5	7.3	4.9
Osteonectin	3.3	5.8	1.8
IGFBP (insulin-like growth factor binding protein)-2	1.7	6.1	3.6
IGFBP (insulin-like growth factor binding protein)-3	5.7	8.3	1.5
Pentaxin 3	1.9	4.5	2.4
Gastrula zinc finger protein*	14.5	2.2	6.6^{-1}
A novel gene: K0059*	8.5	1.4	6.1^{-1}

*: genes of which the expression was decreased.

related: tissue inhibitor of metalloproteinase (TIMP)-1, signal recognition particle (SRP)4, SRP9, soluble NSF attachment proteins (SNAP)45, Alu RNA BP, supt5h (human homologue of the yeast transcription factor, SPT5), eukaryotic initiation factor (eIF)-2α kinase. 4) Cell/organism defence and homeostasis related: heat shock protein (HSP)27. 5) Metabolism related: lysyl oxidase, myosin light chain (MLC) kinase, aldolase A, γ-glutamylcysteine synthetase. 6) Cell structure and motility related: collagen type Iα 1, 2, collagen type III, fibronectin, osteonectin. 7) unclassified: insulin-like growth factor binding protein (IGFBP)-2, 3, pentaxin 3.

The expressions of 2 clones were significantly decreased in biliary atresia, which were gastrula zinc finger protein and one novel gene (Fig. 2).

Northern blot hybridization confirmed that TIMP-1 and IGFBP-2 were significantly overexpressed in biliary cirrhosis due to biliary atresia (Fig. 3).

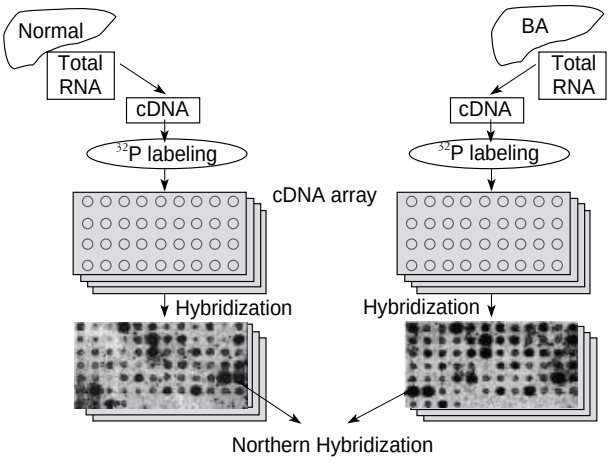

Fig. 1. Flow diagram of the differential expressed sequence tags screening (DES) technique (EST: Expressed Sequence Tags, BA: biliary atresia).

DISCUSSION

Proposed mechanisms of the pathogenesis of biliary atresia include occult viral infection, toxin, defect in morphogenesis, disorder of immunologic/inflammatory system, and defect in fetal/perinatal circulation. Therefore, biliary atresia is suggested to be a heterogeneous disorder and a common phenotype of several different disorders (2).

It is important to find genes specifically expressed in liver cells of biliary atresia in order to study biologic properties of biliary cirrhosis due to biliary atresia. Comparing expression

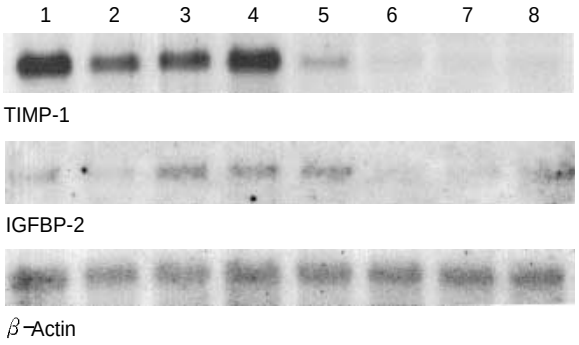

Fig. 3. Northern blot analysis of 2 out of 28 genes of which the expression was shown to be altered in differential EST screening. Each 10 μg of total RNA was electrophoresed on 1% gel containing formaldehyde and blotted onto positively charged nylon membrane. Samples are biliary atresia tissues obatined from liver transplantation recipients (lanes 1 to 4, around 1 yr of age), biliary atresia from wedge biopsy during Kasai operation (lane 5, around 10 weeks of age), normal liver tissues from liver transplantation donors (lanes 6 and 7), and normal portion of hepatocellular carcinoma liver tissue during hepatectomy (lane 8).

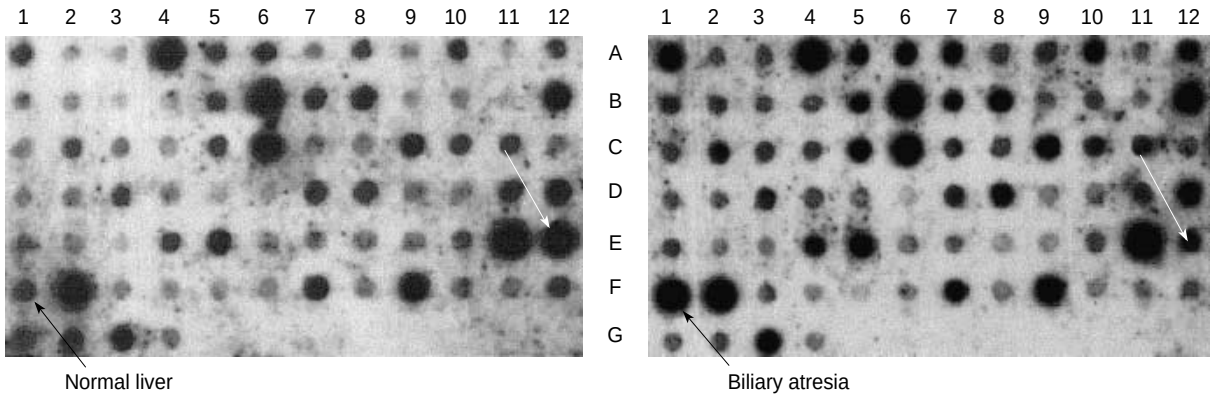

Fig. 2. Results of DES in normal liver and biliary atresia liver tissues. EST dot blot panels were hybridized in ExpressHyb hybridization solution (Clontech, USA) with radioactive cDNA probe pools at the concentration of 2×10^6 cpm/mL at 68℃ for 1 hr. After hybridization, membranes were washed in 2 ×SSC/0.05% SDS at room temperature for 40 min with two changes, followed by washes in 0.1 ×SSC/0.1% SDS for 40 min with one change. Then the membranes were wrapped and exposed to X-ray film at -70℃ for 3 weeks. Arrows indicate differentially expressed genes.

patterns of diseased tissues with counterpart normal tissues enables finding specific gene function. So, it is important to analyze the changes of gene expression in developmental or pathologic tissues or cells, in order to make clear the functions of these genes. Furtheremore, it is mandatory to develop a technique that can analyze a large number of genes simultaneously. Current methods to distinguish mRNA in comparative studies rely largely on subtractive hybridization and differential hybridization (5, 6). However, these techniques are rather difficult to establish, and require large amounts of RNA so that they are inappropriate for weakly expressed genes in tissues.

With the differential EST screening (DES) method like as DNA chip analysis, many genes can be analyzed simultaneously and interrelationships of gene expression and functional or developmental phenomena can be determined. Relatively small amounts of RNA are necessary so that the method is ideally suited for measurement of gene expression in time course experiments, in studies of development, or when the amount of tissue is very limited or the sample size is small. Typically a 10 μg total RNA reaction yields sufficient radioactivity to probe more than 400 genes. The most important advantages of this method are that the identities of the spots on the filter are known in advance and that differential hybridization clones are readily available for use in further characterization.

In this study, we found 28 differently expressed genes in biliary cirrhosis due to biliary atresia by DES using a dot blot panel of 1,730 different EST clones which had been isolated from a human hair stromal cell cDNA library constructed by the Department of Immunology at Kyungpook National University School of Medicine. Twenty six cDNA clones were significantly overexpressed in biliary cirrhosis due to biliary atresia and were classified as genes associated with hepatic fibrosis and genes with unclear roles.

Genes known to be associated with hepatic fibrosis were as follow: TGF-β1 (7-9), TIMP-1 (10, 11), lysyl oxidase, collagen type Iα1, 2, collagen type III (7), laminin, laminin binding protein, fibronectin, osteonectin, HRS, IGFBP-2, -3 (2, 12), MLC kinase, thymosin β-4, -10, and pentaxin 3. Genes with unclear roles in biliary atresia were bcl-w, SRP-4,-9, SNAP 45, Alu RNA binding protein, supt5h, eIF-2α kinase, HSP 27, aldolase, and γ-glutamylcysteine synthetase.

The mechanisms responsible for increased collagen production and hepatic fibrosis in biliary atresia are unknown. During liver injury, hepatic stellate cells (HSCs) proliferate and transform to fibrogenic myofibroblasts. In early injury, collagen types III , V, and fibronectin accumulate, but in chronic injury there is an increasing deposition of collagen types I, IV, undulin, elastin, and laminin. HSC activation is regulated by a wide variety of growth factors and cytokines derived from other liver cells or from HSCs themselves. TGF-β1 upregulates HSC expression of collagens type I, III, and IV, fibronectin, and laminin (7).

By definition, progressive fibrosis occurs when the rate of matrix synthesis exceeds that of matrix degradation. A recent work suggests that the HSCs are also a source of matrix degrading metalloproteinase (MMPs), indicating that, together with other cells, HSCs could participate in matrix remodeling. In the early phases of their cellular activation, HSCs release MMPs with the ability to degrade the normal liver matrix. When HSCs are fully activated, there is a net downregulation of matrix degradation mediated by increased synthesis and extracellular release of tissue inhibitors of metalloproteinase (TIMP)-1 and -2, which are powerful MMP inhibitors. TIMP-1 and -2 may therefore promote progression of hepatic fibrosis through inhibition of matrix degradation. In this 'activated' phenotype the HSCs are the major source of the interstitial collagens, which characterize fibrosis (10, 11, 13, 14).

The liver plays a central role in the IGF-I axis producing the majority of circulating hormones and some of its binding proteins (IGFBPs). Cirrhosis of the liver is characterized by changes in IGF-I, which is known to regulate the action of growth hormone, and IGFBPs associated with liver fibrosis and regeneration (12). The IGFs are present in the circulation and throughout the extracellular space almost entirely bound to members of a family of high-affinity IGF-binding proteins, six of which have been identified as IGFBP-1 to -6 that are essential to coordinate and regulate the biological functions of the IGFs (4, 15). Holt et al. concluded that hepatic gene expression of IGF-I are reduced in pediatric liver disease. IGFBP-2 mRNA expression was increased, but there were no changes in hepatic gene expression of IGFBP-1 and -3 between biliary atresia and normal liver (4). In the study of Ross et al., IGFBP-2 and IGFBP-3 tended to be more highly expressed in cirrhotic liver and IGFBP-1 was more highly expressed in normal liver. Although they studied the concentration of serum IGF-BP as a prognostic factor of hepatoportoenterostomy, the results were not consistent (12).

We think that DES is a useful method for analyzing gene expression patterns simultaneously, because we could ascertain many genes involved in the process of hepatic fibrosis. The results of TIMP-1 and IGFBP-2 were well correlated between DES and northern blot analysis.

Among overexpressed genes in biliary atresia, genes not known to be associated with hepatic fibrosis were bcl-w, HSP 27, SRP-4, -9, SNAP 45 and eIF-2α kinase. These genes are known to be increased when there is cell destruction, production or increase of cellular metabolism. Since the association of hepatic fibrosis and biliary atresia with these genes is not clear yet, we think that further studies about the function of these genes in biliary atresia should be conducted. Two underexpressed genes in biliary atresia are similar genes of gastrular zinc finger protein identified in *Caenorhabditis elegans* and one unknown novel gene. Since there may be a possibility that important regulatory factors are structurally or functionally affected at a certain time point of their development,

we think that genes expressed in normal liver tissues but not in pathologic liver tissues may have more important meanings in identifying the cause of biliary atresia.

Though we have used resected liver tissues instead of purifying biliary epithelial cells from the removed livers in this experiment, we believe that using a monoclonal antibody (HEA125) for isolating biliary epithelial cells is required in order to find genes specifically expressed in biliary atresia (16).

Furthermore, we can use human liver specific cDNA chip to get more information about change in gene expression in biliary atresia and we may be able to predict the severity of disease in patients in whom portoenterostomy was not done or unsuccessful and to determine the optimal time for liver transplantation.

This study identified 28 differentially expressed genes in the biliary cirrhosis from progressive biliary atresia using DES technique. Searching for the genes involved in hepatic fibrosis itself may not be so helpful in finding the etiology of biliary atresia. However, it may be helpful in researching the prevention of hepatic fibrosis, early diagnosis of biliary atresia, and predicting prognosis after hepatoportoenterostomy. We believe this study can lead to a better understanding of the pathogenesis of biliary atresia and hepatic fibrosis and contribute to the development of an early diagnostic kit of biliary atresia and discovery of therapeutic and preventive agents against liver cirrhosis.

REFERENCES

1. Schreiber RA, Kleinman RE. *Biliary atresia. J Pediatr Gastroenterol Nutr 2002; 35: S11-6.*
2. Balistreri WF, Grand R, Hoofnagle JH, Suchy FJ, Ryckman FC, Perlmutter DH, Sokol RJ. *Biliary atresia: current concepts and research directions. Summary of a symposium. Hepatology 1996; 23: 1682-92.*
3. Malizia G, Brunt EM, Peters MG, Rizzo A, Broekelmann TJ, McDonald JA. *Growth factor and procollagen type I gene expression in human liver disease. Gastroenterology 1995; 108: 145-56.*
4. Holt RI, Crossey PA, Jones JS, Baker AJ, Portmann B, Miell JP. *Hepatic growth hormone receptor, insulin-like growth factor I, and insulin-like growth factor-binding protein messenger RNA expression in pediatric liver disease. Hepatology 1997; 26: 1600-6.*
5. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. *Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature 1984; 308: 149-53.*
6. Mason IJ, Taylor A, Williams JG, Sage H, Hogan BL. *Evidence from molecular cloning that SPARC, a major product of mouse embryo parietal endoderm, is related to an endothelial cell 'culture shock' glycoprotein of Mr 43,000. EMBO J 1986; 5: 1465-72.*
7. Benyon RC, Arthur MJ. *Mechanisms of hepatic fibrosis. J Pediatr Gastroenterol Nutr 1998; 27: 75-85.*
8. Milani S, Schuppan D, Herbst H, Surrenti C. *Expression of transforming growth factor β1 in normal and fibrotic human liver. In: Gressner AM, Ramadori G, editors, Molecular and cell biology of liver fibrogenesis. Marburg: Kluwer Academic Publisher, Proceedings of the International Falk Symposium, 1992; Jan 254-61.*
9. Bedossa P, Paradis V. *Transforming growth factor-β (TGF-β): a key-role in liver fibrogenesis. J Hepatol 1995; 22: 37-42.*
10. Arthur MJ, Mann DA, Iredale JP. *Tissue inhibitors of metalloproteinases, hepatic stellate cells and liver fibrosis. J Gastroenterol Hepatol 1998; 13: S33-8.*
11. Iredale JP. *Tissue inhibitors of metalloproteinases in liver fibrosis. Int J Biochem Cell Biol 1997; 29: 43-54.*
12. Ross RJ, Chew SL, D'Souza Li L, Yateman M, Rodriguez-Arnao J, Gimson A, Holly J, Camacho-Hubner C. *Expression of IGF-I and IGF-binding protein genes in cirrhotic liver. J Endocrinol 1996; 149: 209-16.*
13. Olaso E, Friedman SL. *Molecular regulation of hepatic fibrogenesis. J Hepatol 1998; 29: 836-47.*
14. Friedman SL. *The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. N Engl J Med 1993; 328: 1828-35.*
15. Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M. *Analysis of serum insulin-like growth factor binding proteins using western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem 1986; 154: 138-43.*
16. Joplin R, Strain AJ, Neuberger JM. *Immuno-isolation and culture of biliary epithelial cells from normal human liver. In Vitro Cell Dev Biol 1989; 25: 1189-92.*