Altered Expression of Genes Involved in Hepatic Morphogenesis and Fibrogenesis Are Identified by cDNA Microarray Analysis in Biliary Atresia

Limin Chen,[1] Andrew Goryachev,[2] Jin Sun,[1] Peter Kim,[3] Hui Zhang,[4] M. James Phillips,[5] Pascale Macgregor,[6] Sylvie Lebel,[4] Aled M. Edwards,[1] Qiongfang Cao,[7] and Katryn N. Furuya[7]

Biliary atresia (BA) is characterized by a progressive, sclerosing, inflammatory process that leads to cirrhosis in infancy. Although it is the most common indication for liver transplantation in early childhood, little is known about its etiopathogenesis. To elucidate factors involved in this process, we performed comprehensive genome-wide gene expression analysis using complementary DNA (cDNA) microarrays. We compared messenger RNA expression levels of approximately 18,000 human genes from normal, diseased control, and end-stage BA livers. Reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analysis were performed to confirm changes in gene expression. Cluster and principal component analysis showed that all BA samples clustered together, forming a distinct group well separated from normal and diseased controls. We further identified 35 genes and ESTs whose expression differentiated BA from normal and diseased controls. Most of these genes are known to be associated with cell signaling, transcription regulation, hepatic development, morphogenesis, and fibrogenesis. In conclusion, this study serves to delineate processes that are involved in the pathogenesis of BA. (HEPATOLOGY 2003;38:567-576.)

Biliary atresia (BA) is a progressive, sclerosing, inflammatory process that leads to the complete obliteration of the bile ducts at any point from the porta hepatis to the duodenum with replacement by fibrous remnants[1] and results in biliary cirrhosis. BA is the most common cause of liver transplantation in children.[2] It can be separated into 2 clinical classes: (1) the embryonic or fetal type associated with congenital anomalies such as polysplenia and cardiac malformations and (2) the progressive postnatal type, which accounts for 65% to 90% of cases.[3] In an effort to ameliorate this fatal condition, Kasai and Suzuki introduced the hepatoportoenterostomy to reestablish bile flow in these patients.[4] Successful restoration of bile flow occurs in 60% to 94% of infants, although 75% of these children will progress to end-stage liver disease requiring transplantation.[5]

The cause and pathogenesis of BA remains unknown. Studies of racial, gender, viral, and environmental contributions to the development of BA have proven inconclusive.[6,7] There have been case reports of a familial incidence of BA,[8] but discordance[9] in monozygotic twins indicates that environmental factors may play a role.

Genetic factors may contribute to the development of BA through abnormalities in hepatic morphogenesis.[10] It has also been proposed that BA may result from an inherent defect in the epithelial-mesenchymal signaling pathways that would then lead to improper formation of the bile ducts at the porta hepatis during the first trimester.[11]

In an effort to identify possible pathogenetic mechanisms involved in BA, we performed complementary DNA (cDNA) microarray analysis on RNA isolated from normal livers and from livers of patients with BA and diseased controls to determine whether gene expression

Abbreviations: BA, biliary atresia; cDNA, complementary DNA; IGFBP, insulin-like growth factor binding protein; XBP, X-box binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; ECM, extracellular matrix; MMP, matrix metalloproteinase; TGF, transforming growth factor; TIMP, tissue inhibitors of metalloproteinase.

From the [1]Banting and Best Department of Medical Research, Faculty of Medicine, Divisions of [3]General Surgery, [4]Pediatric Gastroenterology, and [5]Pediatric Laboratory Medicine, The Hospital for Sick Children, and the [6]Microarray Center, University Health Network, University of Toronto, Toronto, Ontario, Canada; [2]Genedata AG, Basel, Switzerland; and [7]Division of Pediatric Gastroenterology, Department of Pediatrics, University of North Carolina, Chapel Hill, NC.

Received December 19, 2002; accepted June 11, 2003.

Supported in part by a University Research Council Research grant (University of North Carolina) and by an operating grant from the National Cancer Institute of Canada (to K.N.F.) and the Medical Research Council of Canada (to K.N.F.).

L.C. and A.G. contributed equally to this work.

Address reprint requests to: Katryn N. Furuya, M.D., Division of Pediatric Gastroenterology, 5th floor, 200 Mason Farm Rd., CB 7220, University of North Carolina, Chapel Hill, NC 27599-7220. E-mail: kfuruya@med.unc.edu; fax: 919-966-8641.

0270-9139/03/3803-0007$30.00/0

doi:10.1053/jhep.2003.50363

profiles differed between these groups and to ascertain which pathways are implicated in the etiopathogenesis of BA.

Materials and Methods

Human Liver Tissue. All liver tissues from normal liver and from patients with BA were obtained via hepatectomy and unused donor segments at the time of liver transplantation at The Hospital for Sick Children. One sample was obtained at the time of Kasai portoenterostomy. Four diseased controls were obtained from The Hospital for Sick Children at the time of resection or transplantation (TPN97, HBV42, TYR95, and TYR101). Three diseased control samples were kindly provided by Dr. Elizabeth Rand (Children's Hospital of Philadelphia; WD77, SC81, and SC84), and 2 were obtained from the Cooperative Human Tissue Network (TPN28 and TPN30). Collection of tissue for RNA isolation was approved by the human ethics boards of The Hospital for Sick Children and the Toronto Hospital. Abnormal liver tissue samples were obtained within 15 to 20 minutes of recipient hepatectomy or resection and transported on ice to the laboratory. Normal liver preserved in University of Wisconsin solution was obtained from the unused portion after the donor liver had been cut down on the back table. Approximately 1 to 5 g of liver tissue was immediately homogenized in a guanidinium thiocyanate/phenol solution in preparation for RNA isolation to prevent degradation of RNAs from ribonucleases.[12] After spectrophotometric measurement to determine RNA concentration and purity, 1 μg of RNA was electrophoresed on a 1.2% formamide-agarose gel to determine RNA integrity before microarray analysis. Histologic analysis (hematoxylin-eosin and trichrome stains) was performed by a single pathologist (M.J.P.) on all samples derived from The Hospital for Sick Children and the grade of cirrhosis determined.[13]

cDNA Microarrays. Human 19k microarrays comprising about 19,000 human EST clones were obtained from the Microarray Center (http://www.microarrays.ca/support/glists.html) (OCI Microarray Center and University Health Network, Toronto, Ontario, Canada). These arrays were made by printing the EST clones on CMT-GAPS aminosaline-coated glass slides (Corning, NY) with a 32-pin contact arrayer (SDDC II; Engineering Services, Inc., Toronto, Ontario, Canada). The clones were arrayed on 2 slides, each bearing 9,500 clones spotted in duplicate.

Microarray Experiments. Ten micrograms of total RNA was reverse transcribed as described previously.[14] Total RNA was primed with AncT primer (T20VN; Sigma Genosys, Oakville, Ontario, Canada), and deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada), deoxycytidine triphosphate (Amersham Pharmacia), Cy3–deoxycytidine triphosphate, or Cy5–deoxycytidine triphosphate (NEN, Perkin Elmer LAS, Woodbride, Ontario, Canada) was added. After adding 20 U of RNasin (Promega, Madison, WI), the reaction mixture was heated at 65°C for 5 minutes and then at 42°C for 5 minutes. SuperScript reverse transcriptase was added and the mixture incubated at 42°C for 2 hours. After stopping the reaction and degradation of the RNA template, the mixture was neutralized with 0.5 mol/L acetic acid. The labeled cDNA was precipitated with isopropanol, washed in 70% ethanol, and resuspended in ribonuclease-free water. To eliminate labeling biases, a switch labeling strategy was used. Two pairs of slides were hybridized for each pair of samples: one pair in which the normal pooled liver RNA (pooled from 4 different normal pediatric liver RNAs) was labeled with Cy3 and the RNA from diseased liver was labeled with Cy5, and one pair in which the normal liver RNA was labeled with Cy5 and the diseased liver RNA was labeled with Cy3. The microarray profiles from each of the 4 normal pediatric liver samples were similar (data not shown).

For hybridization, purified Cy3- and Cy5-labeled cDNAs were added to DIG Easy hybridization buffer (Roche Diagnostics Canada, Laval, Quebec, Canada). Yeast transfer RNA (Sigma-Aldrich, Oakville, Ontario, Canada) and single-stranded salmon sperm DNA (Sigma-Aldrich) were added to the hybridization mixture, and the solution was heated at 65°C for 2 minutes before adding to a pair of slides (part 1 and part 2) that were placed face to face before overnight hybridization.

Before scanning, the arrays were washed 3 times in 0.1× standard saline citrate (15 mmol/L NaCl, 1.5 mmol/L sodium citrate)/0.1% sodium dodecyl sulfate at 50°C for 15 minutes, rinsed in 0.1× standard saline citrate 3 times at room temperature for 5 minutes, and dried by centrifugation at 500 rpm for 6 minutes.

The arrays were read with a laser scanner (GenePix 4000; Axon Instruments, Union City, CA), and the images obtained were quantified with the GenePix Pro analysis software (Axon Instruments).

Data Analysis. The raw fluorescent intensities obtained from the quantification of microarray images were processed, and the expression matrix (one expression ratio per clone per tissue sample) was computed. Final expression ratios were obtained by averaging ratios over duplicate spots in both dye-switched experiments performed with each tissue sample as previously described.[15]

Data analysis was performed with Expressionist data mining software (GeneData AG, Basel, Switzerland),

which provides a selection of well-established methods for pattern discovery and classification such as hierarchical and k-means clustering, support vector machines, and principal component analysis.

The analysis started with the full set of 18,968 nonunique clones spotted on the microarray. All samples subjected to microarray analysis were partitioned into 3 groups: BA (n = 11), normal controls (n = 6), and diseased controls (n = 11). Only those clones with expression ratios that were computed with confidence in more than 50% of experiments for each sample group were retained for the following analysis. Further reduction of the clone set was achieved by discarding noninformative clones with expression that remained in the ratio interval (0.5, 2.0) for all samples. A 4,482-clone set was used as the base for all analyses aimed at the identification of groups of genes with specific expression patterns.

Thus, to identify genes discriminating between 2 groups (*e.g.*, BA and normal tissue), we applied the following chain of statistical methods. Each step resulted in a reduction of the number of candidate clones. Genes showing low variability in the 2 groups (variance <0.1) were discarded. Then, according to the results of the *t* test between the 2 groups, only potentially discriminating genes with a *P* value less than .1 were retained. Additional refinement was achieved by requesting that the ratio between mean expression levels in these groups was greater than 1.5 (n-fold regulation test with n = 1.5). The resulting set of clones was partitioned by k-means clustering and further reduced by discarding clusters showing no biologically interesting behavior. The clustering and discarding procedures were repeated recursively until we had a set of clones that statistically significantly discriminated between the 2 groups.

Verification by Reverse-Transcription Polymerase Chain Reaction. Five micrograms of total RNA was reverse transcribed using MMLV reverse transcriptase (Life Technologies, Burlington, Canada) and random primers in a 20 μL reaction volume at 42°C for 1 hour. Oligonucleotide primers (University of North Carolina) for insulin-like growth factor binding protein 7 (IGFBP-7, IGFBP-rP1)[16] and X-box binding protein-1 (XBP-1)[17] were selected based on their NCBI sequences (forward IGFBP-rP1 primer, 5′-AGCTGTGAGGTCATCGGAAT-3′; reverse IGFBP-rP1 primer, 5′-AGGTGGGTTCAGGTGTTCAG-3′; forward XBP-1, 5′-TCAGCCCCTCAGAGAATGAT-3′; reverse XBP-1, 5′-TCAAGGAAAAGGGCAACAGT-3′). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize for sample variation in total RNA amounts and for reaction efficiency. The following GAPDH-specific primer oligonucleotides were used (The Hospital for Sick Children): forward GAPDH primer, 5′-TGCTGGCGCT-GAGTACGTCGTGGAGTCCAC-3′; reverse GAPDH primer, 5′-GCAGTTGGTGGTGCAGGAGGCATT-GCTGAT-3′. After an initial denaturing step of 94°C for 10 minutes, the following polymerase chain reaction (PCR) parameters were used: denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, extension at 72°C for 1 minute for 35 cycles, and a final extension at 72°C for 10 minutes. Each set of reactions included a water-only control and a negative control containing RNA to rule out genomic contamination. The 246–base pair GAPDH, the 300–base pair IGFBP-rP1, and the 305–base pair XBP-1 products were visualized on a 2% agarose gel, and the products were visualized by ethidium bromide staining. The gel image was digitalized and the band intensities quantified. For semiquantitation, appropriate dilutions for each cDNA were determined to ensure that the resulting PCR products were derived only from the exponential phase of amplification. The ratio of IGFBP-rP1 and XBP-1 to GAPDH was calculated using the slopes of the linear phase for each sample.

Results

Explanted liver tissue was obtained from 10 patients with the progressive postnatal form of BA at liver transplantation. Seven of 10 patients underwent transplantation for end-stage liver failure and 3 because of complications of portal hypertension. Ethically, it was difficult to obtain liver tissue from small infants earlier in the natural history of this disease; however, the 11th sample (BA47E) corresponds to the same patient as BA47 but was acquired at the time of Kasai portoenterostomy (0.39 years of age). No patients with the embryonic form of BA were included in this study. RNA was also isolated from 6 normal livers obtained from unused donor segments. In an attempt to control for genes with transcription that would be altered as a result of biliary cirrhosis and end-stage liver disease, we isolated RNA from the liver of 3 age-matched pediatric patients who had undergone transplantation for biliary cirrhosis secondary to total parenteral nutrition. RNA was isolated from explanted liver tissue obtained from patients with other types of end-stage liver disease tyrosinemia (n = 2), Wilson's disease (n = 1), primary sclerosing cholangitis (n = 2), hepatitis C cirrhosis (n = 2), and hepatitis B cirrhosis (n = 1). Clinical and biochemical characteristics for each liver sample (where available) are shown in Table 1. All BA livers showed advanced stage 4 cirrhosis. In the 4 control cases for which histology was available, all had advanced stage 4 cirrhosis; the patient with total parenteral–associated cirrhosis also had biliary hypoplasia, the total parenteral nutrition–treated cirrhotic patient also had biliary hypoplasia, 2 patients with tyrosinemia had extensive dys-

Table 1. Clinical and Biochemical Characteristics of Liver Samples

Liver No.	Age at OLT or Resection (y)	Sex (M/F)	AST (U/L)	ALT (U/L)	Total Bilirubin (μmol/L)	Direct Bilirubin (μmol/L)	Alkaline Phosphatase (U/L)	GGT (U/L)	Albumin (g/L)	INR	Kasai Done	Reason for OLT	Medications
BA													
BA47	0.8	F	417	204	105	74	1,465	672	26	1.5	Yes	FK/LF	Novospirozine, cisapride, rifampin
BA104	3.7	M	112	61	33	22	688	196	34	1.2	Yes	PH	ADEK, UDCA, vitamin D, vitamin K
BA103	0.9	M	335	190	142	113	367	68	29	1.0	Yes	FK/LF	Domperidone, ranitidine, septra, novospirozine, albumin, furosemide
BA85	1.4	F	147	101	149	129	1,364	490	31	1.8	Yes	FK/LF	Rifampin, ADEK, vitamin K, ranitidine
BA34	1	F	195	92	100	77	738	222	26	1.8	Yes	FK/LF	Novospirozine, vitamin K, rifampin, cisapride, ADEK, KCl, septra
BA89	2.2	F	199	157	120	106	656	581	32	1.2	Yes	FK/LF	Spironolactone, rifampin, ADEK, ranitidine, vitamin D, iron, vitamin K
BA59	1	F	229	225	254	234	442	83	21	2.0	Yes	FK/LF	Aldactazide, ADEK, septra
BA05	0.5	F	417	204	480	407	310	136	24	3.6	No	LF	ADEK, aldactazide, atarax
BA94	2.5	M	115	106	39	32	531	358	33	1.0	Yes	PH	ADEK, UDCA, iron, septra, rifampin
BA01	8.6	F	134	76	42	41	578	113	33	1.2	Yes	PH	Omeprazole, salazopyrine, iron, mesalamine
Controls													
HBV42	15.5	M	34	36	8	0	246	23	36	1.0		RS HCC	None
TYR101	0.6	F	59	37	2	0	313	31	31	1.0		LF	NTBC, novospirozine, vitamin K
TYR95	5.7	M	53	40	5	0	293	71	43	1.0		Risk HCC	NTBC
TPN97	0.6	M	364	121	114	92	424	79	26	1.6		LF	Ampicillin, gentamicin, ADEK, metronidazole
TPN28	0.8	M			CHTN							LF	
TPN30	7	M			CHTN							LF	
SC81	13	F			CHOP							LF	
SC84	13	F			CHOP							LF	
HCV56	Adult	F			TGH							RS HCC	
HCV87	Adult	F			TGH							RS HCC	
WD77	17	F			CHOP							LF	
Normal													
NOR06	11	M	55	11	5	ND	84	ND	ND	1.4			DDAVP, neosynephrine
NOR20	8	F	ND	ND	ND	ND	ND	ND	ND	ND			Dopamine
NOR22	14	M	ND	ND	ND	ND	ND	ND	ND	ND			DDAVP, dopamine
NOR58	47	F	ND	ND	ND	ND	ND	ND	ND	ND			ND
NOR86	14	M	ND	ND	ND	ND	ND	ND	ND	ND			ND
NOR96	39	F	139	109	10	4	34	5	23	1.1			ND

Abbreviations: OLT, orthotopic liver transplantation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; INR, international normalized ratio; FK/LF, failed Kasai portoenterostomy/liver failure; PH, complications of portal hypertension; RS HCC, resection for hepatocellular carcinoma; CHTN, tissue obtained from Cooperative Human Tissue Network; CHOP, tissue obtained from Children's Hospital of Philadelphia; TGH, tissue obtained from Toronto General Hospital; ND, not documented; UDCA, ursodeoxycholic acid; ADEK, vitamins A, D, E, and K; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; DDAVP, desmopressin acetate.

plasia and adenomas, and the patient with hepatitis B also had a hepatocellular carcinoma.

BA Samples Can Be Confidently Separated From Normal and Diseased Liver Tissues. Hierarchical clustering of liver samples was performed on the basis of the filtered set of 4,482 expression profiles (Fig. 1A). The degree of correlation between the samples can be inferred from the horizontal position of samples on the figure, so that the most highly correlated samples are the furthest right on the plot. Analysis clearly showed that all BA samples clustered together and formed a distinct group well separated from controls. Surprisingly, we found that TPN97 clustered together with the BA group. Interestingly, histologic examination of the TPN97 liver (from both an earlier liver biopsy and from the explanted liver) showed the presence of primary biliary hypoplasia in addition to total parenteral nutrition–related changes. The other 2 total parenteral nutrition samples were readily distinguishable from the rest of the diseased controls. The remaining diseased controls formed a cluster separate from BA and tended to cluster in accordance with the specific disease.

Principal component analysis often provides more intuitive information on the presence of a group and their interrelationship than does hierarchical clustering. In multidimensional expression space, principal component analysis finds directions of largest variability in the data set. These directions correspond to characteristic expression profiles resulting from the generalization of common trends in the profiles of all genes. Individual experiments

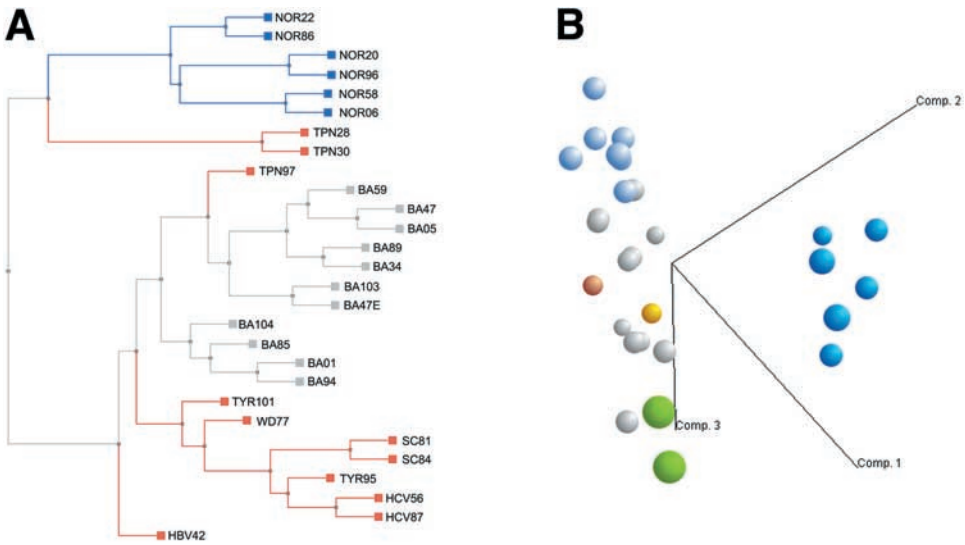

Fig. 1. (A) Hierarchical clustering of all liver samples based on the correlation of the expression profiles. Color coding represents grouping of samples: **gray**, BA; **blue**, normal controls; **orange**, diseased controls. (B) Principal component analysis of liver tissue samples. The size of the balls represents the 3-dimensional perspective in the space of principal components. Color legend: **gray**, BA; **blue**, normal liver; **yellow**, TPN97; **green**, TPN28 and TPN30; **brown**, HBV42; **steel blue**, other diseased controls.

are seen as points in this space, and the presence of clusters can be determined visually (Fig. 1B). All diseased controls lay approximately in one plane in the 3-dimensional space, and all normal controls were found in a tight cluster positioned significantly off that plane. The remarkable difference between expression profiles of normal and diseased tissues dwarfed the differences between the disease expression profiles, suggesting a common pathology among different liver diseases.

Search for Molecular Markers Suggests Novel Genes Involved in the Pathogenesis of BA. We identified genes with transcription that was affected specifically in BA compared with normal and diseased controls. To find all candidate molecular markers of a particular sample group, it was essential to ensure that the expression profiles within this group were homogeneous. Therefore, we applied data mining procedures to the previously described groups of BA samples separately and subsequently integrated the results.

Clones differentiating normal liver and BA were identified first. In total, 169 clones were found. Of 169 genes, we eliminated genes with expression in diseased controls that was similar to that in BA samples. As a result, we arrived at a set of 39 clones representing 35 different genes and EST clusters. Only 5 genes were down-regulated in BA compared with normal liver (Fig. 2); the remainder showed various degrees of up-regulation.

Most of the BA markers belonged to 3 major functional classes: (1) morphogenesis, fibrogenesis, and tissue remodeling; (2) transcription regulation; and (3) cell signaling (Table 2). The largest functional group among BA markers is composed of genes involved in fibrogenesis, morphogenesis, and tissue remodeling. These genes are either structural components or modifiers of extracellular matrix (ECM). The first subgroup of genes with increased expression compared with both normal and diseased controls in our study is represented by several collagens, including types I, III, IV, and VI, and the proteoglycan lumican. Excessive deposition of these proteins has been commonly implicated in the development of hepatic fibrosis.[18]

The second morphogenetic/fibrogenic subgroup consisted of genes with products that are involved in reorganization and destruction of the ECM, such as SPARC (osteonectin) and the matrix metalloproteinases (MMP). We found that SPARC was increased at least 2-fold in BA liver compared with normal liver. SPARC is known to bind to a number of ECM components, including several collagens. We also showed that the MMP-7 was up-regulated in BA samples compared with both normal and diseased controls, whereas MMP-2, a major collagen-degrading enzyme with an important role in hepatic fibrogenesis,[19] was down-regulated. The role of MMP-7 in the liver has yet to be determined.

Third, we found that IGFBP-7, which is now known as IGFBP-rP1, was strongly up-regulated in BA livers compared with normal and diseased controls. IGFBP-rP1 is a representative of a large family of IGFBPs that have profound regulatory activities and is believed to play an essential biological role in senescence, tumor suppression, and vascular pathology.[20] IGFBP-rP1 has not yet been implicated in the pathogenesis of BA.

We also noted that several of the genes identified as BA markers are known to be involved in cell signaling and transcription regulation. For example, citron, a Rho-reacting serine-threonine protein kinase that mediates Rho-Rac interaction in the Ras signaling pathway,[21,22] was up-regulated in BA samples compared with normal and diseased controls, as was SCOP (SCN circadian oscillatory protein), which is a poorly understood protein that

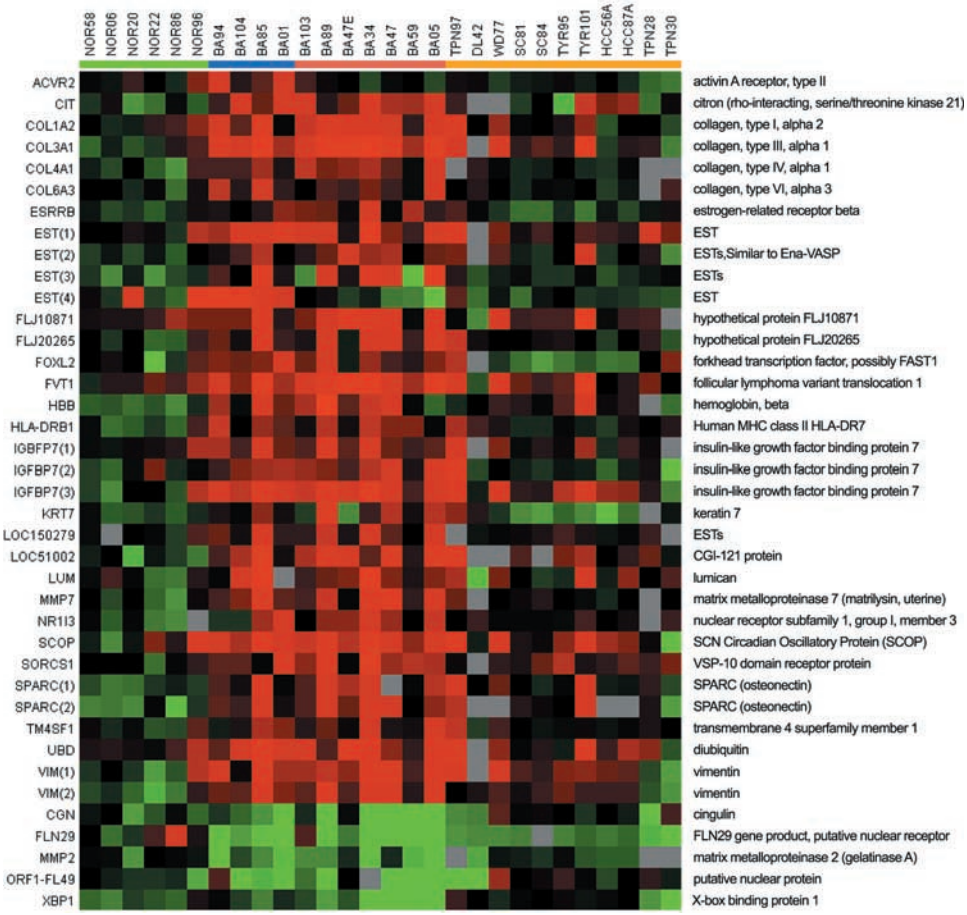

Fig. 2. Molecular markers of BA. cDNA microarray analysis. Different clones belonging to the same gene are denoted as [gene name](number). The color in each block indicates the mean expression for each indicated gene (rows) for each individual liver sample (columns). **Red**, high expression; **green**, low expression. NOR, normal liver; TPN, biliary cirrhosis secondary to total parenteral nutrition; HBV, hepatitis B cirrhosis; WD, Wilson's disease; SC, sclerosing cholangitis; TYR, tyrosinemia; HCV, hepatitis C cirrhosis.

may be involved in Ras signaling.[23] Activin receptor type II was also up-regulated in BA livers, which may indicate changes in the activin signaling pathway leading to alterations in hepatocyte cell growth and apoptosis.[24] A number of other genes listed in the BA marker group are nuclear receptors or transcription factors and include XBP-1, orphan estrogen-related receptor β, and a putative nuclear protein, FLN29, all of which were down-regulated in BA samples compared with controls. Conversely, other genes involved in transcription regulation such as the forkhead transcription factor FAST1 were up-regulated in BA.

Last, there was increased expression of a few genes involved in the immune response, including major histocompatibility complex class II HLA-DR7.

Reverse-Transcription PCR Verification of Microarray Results. To verify some of the gene changers observed by microarray analysis, we compared the expression of IGFBP-rP1 and XBP-1 in 3 different samples by semiquantitative PCR (Fig. 3A). After normalization to GAPDH, we found that IGFBP-rP1 was up-regulated in the 2 BA livers examined whereas XBP-1 was down-regulated compared with normal liver (Fig. 3B).

Discussion

The etiopathogenesis of BA has not yet been delineated. The most widely mentioned hypotheses (*i.e.*, infectious, congenital, toxic, and immune-mediated[3]) are often considered as independent mechanisms leading to BA. Our results suggest that the pathogenesis of this disease is orchestrated by a complex interplay of inflammatory, fibrogenic, and morphogenetic factors but acknowledge the fact that our study is limited by the examination of end-stage livers removed at the time of liver transplantation.

It is known that the inflammatory response is prominent in livers from patients with BA. Our results confirmed earlier observations of major histocompatibility complex class II molecule up-regulation.[25] However, no association has been found between BA and HLA haplotypes.[26] Changes in the expression of some cell adhesion molecules, such as intercellular adhesion molecule 1, which were originally believed to be potential indicators of the immune injury, have now been shown to be part of a normal generic reaction of endothelial cells to proinflammatory cytokines.[27]

Table 2. Molecular Markers of Biliary Atresia

Genbank Accession No.	Description	Fold Change Over Normal Liver	Fold Change Over Diseased Controls	Functional Group
W69802	Activin A receptor, type II	1.11	1.36	Cell signaling
R24294	Citron (rho-interacting, serine/threonine kinase 21)	2.37	1.71	Cell signaling
R24563	VPS-10 domain receptor protein	2.01	1.43	Cell signaling
R38028	Transmembrane 4 superfamily member 1	1.51	1.35	Cell signaling
N92746	SCN circadian oscillatory protein (SCOP)	2.15	1.69	Cell signaling ?
R33392	Keratin 7	1.74	1.82	Cytoskeleton
T40856	Vimentin	2.48	1.61	Cytoskeleton
T50388	Vimentin	2.71	1.82	Cytoskeleton
H28306	EST, similar to Ena-VASP like protein (*H. sapiens*)	1.77	1.37	Cytoskeleton ?
H50622	Human MHC class II HLA-DR7–associated glycoprotein	1.78	1.38	Immune response
N33920	Diubiquitin	1.87	1.54	Immune response
AA011508	Cingulin	−1.67	−1.82	Morphogenesis, fibrogenesis
N30461	Collagen, type I	2.00	1.90	Morphogenesis, fibrogenesis
N32802	Collagen, type III	4.29	2.56	Morphogenesis, fibrogenesis
AA043301	Collagen, type IV	1.87	1.65	Morphogenesis, fibrogenesis
R34259	Collagen, type VI	1.60	1.48	Morphogenesis, fibrogenesis
H49910	IGFBP-7	1.54	1.36	Morphogenesis, growth, development
AA151485	IGFBP-7	1.84	1.83	Morphogenesis, growth, development
W38478	IGFBP-7	2.77	1.58	Morphogenesis, growth, development
H50566	Lumican	2.11	1.72	Morphogenesis
W44529	MMP-2	−1.62	−1.38	Hepatogenesis, ECM remodeling
W52564	MMP-7 (matrilysin)	2.46	1.93	Hepatogenesis, ECM remodeling
H98692	Secreted protein, acidic, cysteine-rich (SPARC, osteonectin)	2.21	1.40	Morphogenesis, fibrogenesis
R12744	Secreted protein, acidic, cysteine-rich (osteonectin)	2.51	1.38	Morphogenesis, fibrogenesis
H19027	Estrogen-related receptor beta	1.65	1.64	Transcription regulation
R45593	FLN29 gene product, putative nuclear receptor	−2.92	−1.49	Transcription regulation
AA156761	Forkhead transcription factor FAST1	1.72	1.87	Transcription regulation
H48870	Nuclear receptor subfamily 1, group I, member 3	1.92	1.32	Transcription regulation
H71793	XBP-1	−1.29	−1.48	Transcription regulation
T48696	Putative nuclear protein	−1.71	−1.90	Transcription regulation?
W02072	CGI-121 protein	2.93	1.46	Tumor suppression?
T93834	Hypothetical protein FLJ10871	1.67	1.50	
T92246	Hypothetical protein FLJ20265	2.04	1.69	
T87876	Follicular lymphoma variant translocation 1	2.54	1.99	
H12314	Hemoglobin, beta	2.63	1.57	
R35456	EST	1.53	1.40	
T79361	EST	−1.02	1.67	
N75215	ESTs	2.33	1.90	
AA044058_1	EST	2.13	1.57	

Various cytokines as well as growth factors released in response to inflammation target biliary epithelial cells and induce remodeling of the epithelial lining of bile ducts. It has been suggested that a defect in remodeling of the biliary epithelium may be responsible for the observed obliteration and subsequent loss of bile ducts in BA.[28] We found that at least one third of our BA markers were directly related to ECM remodeling, hepatic morphogenesis,[29] and cell adhesion, but many of these molecules also play important roles in fibrogenesis.[30] We found that the collagens that are up-regulated in fibrogenesis were increased in expression in patients with BA, with the greatest increase in expression found in type III collagen. Although it is known that the up-regulation of collagen is due in part to overexpression of profibrogenic transforming growth factor (TGF)-β,[31] we did not detect changes in TGF-β expression, which may have been a result of relatively low levels of messenger RNA expression of this cytokine.

Interestingly, we found that MMP-2, a major enzyme with collagenase activity involved in ECM remodeling, was down-regulated in BA livers. This is in contrast to previous findings, where it has been shown that elevated levels of serum and hepatic messenger RNA MMP-2 occur in BA.[32,33] MMP-2 is a gelatinase that can degrade interstitial collagen (I, II, III, X), type IV and type V collagen, and the noncollagenous proteins elastin, fibronectin, and laminin. Up-regulation of MMP-2 may result in regression of fibrosis in the liver,[30] whereas down-regulation of this enzyme that we observed in BA livers may promote a more rapid accumulation of interstitial collagens and increased scarring. In contrast to

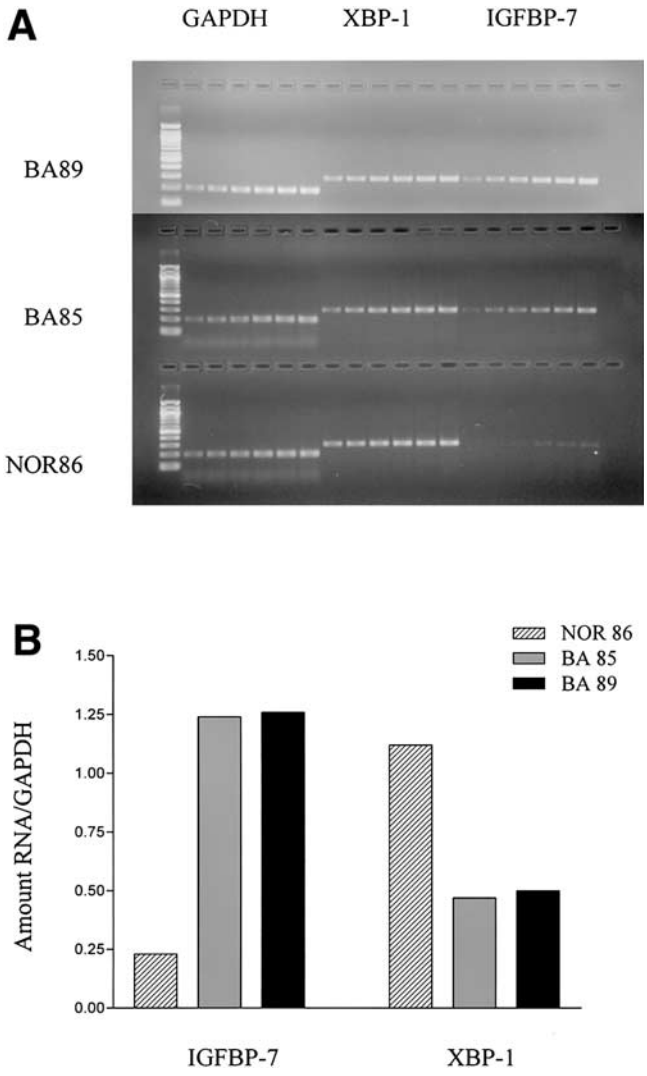

Fig. 3. Reverse-transcription PCR verification of the BA specific genes. (A) Semiquantitative reverse-transcription PCR for IGFBP-7 (IGFBP-rP1), XBP-1, and GAPDH for BA (BA 85, 89) compared with normal liver (NOR86). (B) Semiquantitative analysis of gels shown in A.

MMP-2, we found that MMP-7 or matrilysin was up-regulated in BA livers, similar to findings in other fibrotic liver diseases. Lichtinghagen et al. showed that MMP-7 was elevated in livers with hepatitis C–induced cirrhosis.[34] These proteins also play a role in hepatic morphogenesis not only through regulation of matrix production but also through generation of biologically active fragments such as endostatin, which may be involved in the regulation of early liver development.[35] The tissue inhibitors of metalloproteinases (TIMPs) are the major regulators of extracellular MMP activity and hence affect the balance between matrix synthesis and degradation. It has been reported that increased expression of TIMP-1 and TIMP-2[32,33] has been found in livers of patients with BA and in bile duct–ligated rats[36]; however, we did not observe any change in expression of these genes.

Other genes that were up-regulated in BA livers that are involved in ECM remodeling include SPARC or osteonectin and lumican. SPARC has previously been shown to be up-regulated in chronic hepatitis,[37] alcoholic liver disease, and primary biliary cirrhosis.[38]

Thus, in our study, many of the genes that showed increased expression in BA livers play important roles in matrix remodeling and fibrogenesis. Although it is possible that these genes may have been abnormally expressed during morphogenesis and development, it is more likely that their overexpression is related to hepatic fibrogenesis.

However, the possibility that abnormalities in the morphogenetic/remodeling pathway exist in BA is supported by the association of this disease with laterality defects. Approximately 20% to 30% of patients with BA have associated congenital defects, including situs inversus, polysplenia or asplenia, heart malformations, or intestinal malrotation, many of which are related to abnormalities in left-right asymmetry patterning. Therefore, it is possible that only some elements of the lateralization regulatory pathway are involved in biliary morphogenesis. Embryonic development of left-right asymmetry has been associated with signaling activity of TGF-β superfamily members, such as lefty, nodal, activin, and bone morphogenetic protein.[39] Activin A receptor type II was shown to be the earliest asymmetrically expressed protein in the chick embryo,[40] and the activin signaling pathway has been shown to be involved in the regulation of lateral asymmetry.[41] We found that activin A receptor type II expression was up-regulated in BA livers. Given that activin A induces apoptosis, we speculate that elevated expression of activin A receptor type II in BA livers may reflect attempts to limit cell growth and may contribute to the cachexia and wasting observed in patients with BA with end-stage liver disease, as has been observed in a mouse model associated with elevated activin levels.[24] We also found that expression of the forkhead activin signal transducer FAST1 was increased in BA livers, again implicating a role for the activin signaling pathway in the pathogenesis of BA. FAST1[42,43] is believed to form a DNA-binding complex with SMAD proteins to mediate transcriptional responses of the TGF-β superfamily.[44] Of interest, other TGF-β superfamily members have also been implicated in hepatogenesis. Dichmann et al. showed that misexpression of BMP6 leads to absent bile ducts with fusion of the liver to the duodenum.[45] As of yet, the expression of these proteins has not been examined in BA.

In addition to the aforementioned genes involved in hepatogenesis and hepatic fibrogenesis, we also observed that IGFBP-rP1 expression was increased in BA livers. Although it has been shown that IGFBP-rP1 is expressed

at the messenger RNA and protein level in normal human liver,[20,46] its physiologic role is still largely undetermined. However, it has been shown that it plays a role in skeletal myogenesis, regulation of epithelial cell growth, and tumor suppression[20]; thus, it is possible that IGFBP-rP1 may play a role in hepatic morphogenesis. Like many of the other gene changers observed in our study, the role of IGFBP-rP1 has not been examined in the pathogenesis of liver disease.

We also found that there were changes in gene expression in transcription factors that are involved in hepatic morphogenesis. We observed that XPB-1 expression was down-regulated in BA livers, which may suggest a role for abnormal morphogenesis in BA. Reimold et al. showed that XBP-1–deficient mice displayed hypoplastic fetal livers with diminished hepatocyte growth rates and increased apoptosis.[47]

Thus, not unexpectedly, we found that many of the genes with altered expression in BA were related to hepatic fibrogenesis. However, it is possible that these genes may also play a role in hepatic morphogenesis. Further studies examining pathways involved in hepatic development, morphogenesis, and fibrogenesis will result in clarification of their contributions to the pathogenesis of BA.

Acknowledgment: The authors thank Dr. Alan Hemming (University of Florida) for his contributions and Dr. Elizabeth Rand (Children's Hospital of Philadelphia) for providing access to liver tissue.

References

1. Alagille D. Extrahepatic biliary atresia. HEPATOLOGY 1995;4:7S-10S.
2. Ryckman FC, Alonso MH, Bucuvalas JC, Balistreri WF. Biliary atresia—surgical management and treatment options as they relate to outcome. Liver Transplant Surg 1998;4(suppl):S24-S33.
3. Bates MD, Bucuvalas JC, Alonso MH, Ryckman FC. Biliary atresia: pathogenesis and treatment. Semin Liver Dis 1998;18:281-293.
4. Kasai M, Suzuki S. A new operation for "noncorrectable" biliary atresia: hepatic portoenterostomy. Shujitsu 1959;13:733-739.
5. Otte J-B, de Ville de Goyet J, Reding R, Hausleithner V, Sokal E, Chardot C, Debande B. Sequential treatment of biliary atresia with Kasai portoenterostomy and liver transplantation: a review. HEPATOLOGY 1994;20:41S-48S.
6. Chardot C, Carton M, Spire-Bendelac N, Le Pommelet C, Golmard JLAB. Epidemiology of biliary atresia in France: a national study 1986-96. J Hepatol 1999;31:1006-1013.
7. Balistreri WF, Grand RJ, Hoofnagle JH, Suchy FJ, Ryckman FC, Perlmutter DH, Sokol RJ. Biliary atresia: current concepts and research directions. Summary of a symposium. HEPATOLOGY 1996;23:1682-1692.
8. Ando K, Miyano T, Fujimoto T, Ohya T, Lane G, Tawa T, Tokita A, et al. Sibling occurrence of biliary atresia and biliary dilatation. J Pediatr Surg 1996;31:1302-1304.
9. Hyams JS, Glaser JH, Leichtner AM, Morecki R. Discordance for biliary atresia in two sets of monozygotic twins. J Pediatr 1985;107:420-422.
10. Chandra RS. Biliary atresia and other structural anomalies in the congenital polysplenia syndrome. J Pediatr 1974;85:649-655.
11. Tan CEL, Driver M, Howard ER, Moscoso GJ. Extrahepatic biliary atresia: a first-trimester event? Clues from light microscopy and immunohistochemistry. J Pediatr Surg 1994;29:808-814.
12. Xie W, Rothblum LI. Rapid, small-scale RNA isolation from tissue culture cells. Biotechniques 1991;11:325-327.
13. Ludwig J, Batts K. Practical Liver Biopsy Interpretation, Diagnostic Algorithms. 2nd ed. Chicago: American Association of Clinical Pathology, Joshua Weikersheimer, 1998:292.
14. Furuya KN, Bradley G, Sun D, Schuetz EG, Schuetz JD. Identification of a new P-glycoprotein-like ATP-binding cassette transporter gene that is overexpressed during hepatocarcinogenesis. Cancer Res 1997;57:3708-3716.
15. Goryachev AB, Macgregor PF, Edwards AM. Unfolding of microarray data. J Comput Biol 2001;8:443-461.
16. Yamauchi T, Umeda F, Masakado M, Isaji M, Mizushima S, Nawata H. Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 1994;303:591-598.
17. Liou HC, Boothby MR, Finn PW, Davidon R, Nabavi N, Zeleznik-Le NJ, Ting JP, et al. A new member of the leucine zipper class of proteins that binds to the HLA DR alpha promoter. Science 1990;247:1581-1584.
18. Neubauer K, Saile B, Ramadori G. Liver fibrosis and altered matrix synthesis. Can J Gastroenterol 2001;15:187-193.
19. Olaso E, Friedman SL. Molecular regulation of hepatic fibrogenesis. J Hepatol 1998;29:836-847.
20. Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 1999;20:761-787.
21. Di Cunto F, Calautti E, Hsiao J, Ong L, Topley G, Turco E, Dotto GP. Citron Rho-interacting kinase, a novel tissue-specific Ser/Thr kinase encompassing the Rho-Rac-binding protein citron. J Biol Chem 1998;273:29706-29711.
22. Eda M, Yonemura S, Kato T, Watanabe N, Ishizaki T, Madaule P, Narumiya S. Rho-dependent transfer of citron-kinase to the cleavage furrow of dividing cells. J Cell Sci 2001;114:3273-3284.
23. Shimizu K, Okada M, Takano A, Nagai K. SCOP, a novel gene product expressed in a circadian manner in rat suprachiasmatic nucleus. FEBS Lett 1999;458:363-369.
24. Chen W, Woodruff TK, Mayo KE. Activin A-induced HepG2 liver cell apoptosis: involvement of activin receptors and Smad proteins. Endocrinology 2000;141:1263-1272.
25. Sokol RJ, Mack C. Etiopathogenesis of biliary atresia. Semin Liver Dis 2001;21:517-524.
26. Donaldson PT, Clare M, Constantini PK, Hadzic N, Mieli-Vergani G, Kelly HE, Kelly D. HLA and cytokine gene polymorphisms in biliary atresia. Liver 2002;22:213-219.
27. Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2002;2:787-795.
28. Desmet VJ. Congenital disease of intrahepatic bile ducts: variations on the theme "ductal plate malformation." HEPATOLOGY 1992;16:1069-1083.
29. Amenta PS, Harrison D. Expression and potential role of the extracellular matrix in hepatic ontogenesis: a review. Microsc Res Tech 1997;39:372-386.
30. Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis 2001;21:351-372.
31. Lamireau T, Le Bail B, Boussarie L, Fabre M, Vergnes P, Bernard O, Gautier F, et al. Expression of collagens type I and IV, osteonectin and transforming growth factor beta-1 (TGFβ1) in biliary atresia and paucity of intrahepatic bile ducts during infancy. J Hepatol 1999;31:248-255.
32. Kobayashi H, Li ZX, Yamataka A, Lane GJ, Miyano T. Clinical evaluation of serum levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases as predictors of progressive fibrosis in postoperative biliary atresia patients. J Pediatr Surg 2002;37:1030-1033.
33. Benyon RC, Iredale JP, Goddard S, Winwood PJ, Arthur MJP. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. Gastroenterology 1996;110:821-831.
34. Lichtinghagen R, Michels D, Haberkorn CI, Arndt B, Bahr M, Flemming P, Manns MP, et al. Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibropro-

liferative process in the liver during chronic hepatitis C. J Hepatol 2001; 34:239-247.
35. Quondamatteo F, Knittel T, Mehde M, Ramadori G, Herken R. Matrix metalloproteinases in early human liver development. Histochem Cell Biol 1999;112:277-282.
36. Iredale JP, Benyon RC, Arthur MJP, Ferris WF, Alcolado R, Winwood PJ, Clark N, et al. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. HEPATOLOGY 1996;24:176-184.
37. Nakatani K, Seki S, Kawada N, Kitada T, Yamada T, Sakaguchi H, Kadoya H, et al. Expression of SPARC by activated hepatic stellate cells and its correlation with the stages of fibrogenesis in human chronic hepatitis. Virchows Arch 2002;441:466-474.
38. Frizell E, Liu S, Abraham A, Ozaki I, Eghbali M, Sage EH, Zern MA. Expression of SPARC in normal and fibrotic livers. HEPATOLOGY 1995; 21:847-854.
39. Mercola M, Levin M. Left-right asymmetry determination in vertebrates. Annu Rev Cell Dev Biol 2001;17:779-805.
40. Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C. A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell 1995; 82:803-814.
41. Oh SP, Li E. The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymmetry in the mouse. Genes Dev 1997;11:1812-1826.
42. Fuhrer D. Lessons from studies of complex genetic disorders: identification of FOXL2—a novel transcription factor on the wing to fertility. Eur J Endocrinol 2002;146:15-18.
43. Prueitt RL, Zinn AR. A fork in the road to fertility. Nat Genet 2001;27: 132-134.
44. Zhou S, Zawel L, Lengauer C, Kinzler KW, Vogelstein B. Characterization of human FAST-1, a TGF beta and activin signal transducer. Mol Cell 1998;2:121-127.
45. Dichmann DS, Miller CP, Jensen J, Heller RS, Serup P. Expression and misexpression of members of the FGF and TGFβ families of growth factors in the developing mouse pancreas. Dev Dyn 2003;226:663-674.
46. Degeorges A, Wang F, Frierson HF, Seth A, Sikes RA. Distribution of IGFBP-rP1 in normal human tissues. J Histochem Cytochem 2000;48: 747-754.
47. Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS, Zhang J, Horton HF, et al. An essential role in liver development for transcription factor XBP-1. Gene Dev 2000;14:152-157.