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Identification of Novel Cellular Targets in Biliary Tract Cancers Using Global Gene Expression Technology

Donna E. Hansel,* Ayman Rahman,* Manuel Hidalgo,† Paul J. Thuluvath,‡ Keith D. Lillemoe,§ Richard Shulick,§ Ja-Lok Ku,¶ Jae-Gahb Park,¶ Kohje Miyazaki,‖ Raheela Ashfaq,** Ignacio I. Wistuba,†† Ram Varma,‡‡ Lesleyann Hawthorne,‡‡ Joseph Geradts,‡‡ Pedram Argani,* and Anirban Maitra*

From the Departments of Pathology, Oncology,† Gastroenterology,‡ and Surgery,§ The Johns Hopkins University School of Medicine, Baltimore, Maryland; the Cancer Research Institute,¶ Seoul National University College of Medicine, Seoul, Korea; Saga Medical School,‖ Saga, Japan; University of Texas Southwestern Medical School,** Dallas, Texas; Catholic University,†† Santiago, Chile; and Roswell Park Cancer Institute,‡‡ Buffalo, New York*

Biliary tract carcinoma carries a poor prognosis, and difficulties with clinical management in patients with advanced disease are often due to frequent late-stage diagnosis, lack of serum markers, and limited information regarding biliary tumor pathogenesis. RNA-based global analyses of gene expression have led to the identification of a large number of up-regulated genes in several cancer types. We have used the recently developed Affymetrix U133A gene expression microarrays containing nearly 22,000 unique transcripts to obtain global gene expression profiles from normal biliary epithelial scrapings (n = 5), surgically resected biliary carcinomas (n = 11), and biliary cancer cell lines (n = 9). Microarray hybridization data were normalized using dCHIP (http://www.dCHIP.org) to identify differentially up-regulated genes in primary biliary cancers and biliary cancer cell lines and their expression profiles was compared to that of normal epithelial scrapings using the dCHIP software as well as Significance Analysis of Microarrays or SAM (http://www-stat.stanford.edu/~tibs/SAM/). Comparison of the dCHIP and SAM datasets revealed an overlapping list of 282 genes expressed at greater than threefold levels in the cancers compared to normal epithelium (t-test P <0.1 in dCHIP, and median false discovery rate <10 in SAM). Several pathways integral to tumorigenesis were up-regulated in the biliary cancers, including proliferation and cell cycle antigens (eg, *cyclins D2* and *E2*, *cdc2/p34*, and *geminin*), transcription factors (eg, *homeobox B7* and *islet-1*), growth factors and growth factor receptors (eg, *hepatocyte growth factor*, *amphiregulin*, and *insulin-like growth factor 1 receptor*), and enzymes modulating sensitivity to chemotherapeutic agents (eg, *cystathionine β synthase*, *dCMP deaminase*, and *CTP synthase*). In addition, we identified several "pathway" genes that are rapidly emerging as novel therapeutic targets in cancer (eg, *cytosolic phospholipase A2*, an upstream target of the cyclooxygenase pathway, and *ribosomal protein S6 kinase* and *eukaryotic translation initiation factor 4E*, two important downstream mediators of the mitogenic Akt/mTOR signaling pathway). Overexpression of selected up-regulated genes was confirmed in tissue microarrays of biliary cancers by immunohistochemical analysis (n = 4) or *in situ* hybridization (n = 1), and in biliary cancer cell lines by reverse transcriptase PCR (n = 2). The majority of genes identified in the present study has not been previously reported in biliary cancers, and represent novel potential screening and therapeutic targets of this cancer type. ***(Am J Pathol 2003, 163:217–229)***

Biliary tract carcinomas, which include cancers of the gallbladder and intra- and extrahepatic biliary tree, affect 7500 individuals in the United States each year, and nearly 3500 patients die as a direct consequence of this lethal disease.[1] Once established, biliary tract cancers are notoriously challenging to diagnose and treat. At present, only surgical excision of detectable malignancy is associated with improvement in 5-year survival. Distant metastases, extensive regional lymph node metastasis, and vascular encasement or invasion preclude resection.[2] In general, the outcome for patients with advanced biliary tract cancer at any site is dismal, and neither radiation nor conventional chemotherapy significantly improves survival or quality of life. Early diagnosis has a significant impact on prognosis of biliary cancers. For example, patients with lesions confined to the gallbladder mucosa have a 32% 5-year survival rate, while patients with more advanced lesions have only a 10% 5-year survival rate.[3] Thus, urgent efforts are needed for identification of reliable tumor markers that will facilitate the early detection of biliary cancer in at-risk individuals. In

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Address reprint requests to Anirban Maitra, M.D., 720 Rutland Avenue, Ross 632 Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21212. E-mail: amaitra1@jhmi.edu.

addition, we need to identify cancer-specific cellular targets that would form the basis for novel therapeutic approaches for established biliary tract malignancies.

Global expression platforms such as oligonucleotide microarrays are a robust technique for identification of differentially up-regulated cancer-specific genes in tumors. This technology has recently been successfully applied for the identification of novel tumor markers in a large variety of human neoplasms.[4–7] To identify differentially expressed genes in biliary cancers *versus* non-neoplastic biliary epithelium, we applied RNA-based global gene expression profiling using the recently developed Affymetrix U133A expression microarrays to a series of surgically resected biliary cancers, biliary cancer cell lines, and biliary epithelial scrapings. We report the identification of 282 genes, conforming to a variety of potential tumorigenic pathways, expressed threefold or greater in biliary cancers compared to normal biliary epithelium. The majority of these genes has not been previously described in biliary tract carcinoma and may serve as potential therapeutic targets and novel tumor markers for this lethal disease.

Materials and Methods

Non-Neoplastic Epithelial Scrapings, Primary Biliary Cancers, and Cell Lines

Permission for this study was obtained through The Johns Hopkins Joint Committee on Clinical Investigation. Fresh scrapings from five non-neoplastic biliary epithelial samples were collected as previously described[8] from patients undergoing Whipple resection or incidental cholecystectomies for non-biliary disorders at The Johns Hopkins Hospital; this method has been shown to yield highly enriched sheets of epithelial cells without contaminating stromal elements. The epithelial scrapings were collected within 10 minutes of surgical resection and stored at −80°C, and included 3 extrahepatic biliary 2 gallbladder epithelial samples. Eleven cancer samples were collected from patients undergoing surgery for biliary tract cancer, and included 7 gallbladder carcinomas, 2 intrahepatic cholangiocarcinomas, and 2 distal bile duct carcinomas. All tumor samples were collected within 10 minutes of surgical resection, snap-frozen in liquid nitrogen, and stored at −80°C. Hematoxylin and eosin-stained sections from adjacent frozen tissue were prepared before sample harvest to confirm the diagnosis and assess neoplastic cellularity. RNA was extracted from tumor samples containing >50% neoplastic cells on frozen section examination.

The nine human biliary cancer cell lines used for this study included EGI-1 and TFK-1[9,10] (obtained from the German Collection of Microorganisms and Cell Cultures Department, Braunschweig, Germany), HUH28[11] and HUCCT-1[12] (obtained from the Health Science Research Resources Bank, Osaka, Japan), SNU 245, SNU 308, and SNU 1079[13] (obtained from the Korean Cell Line Bank, Seoul, Korea), GB-H3, and GB-D1.[14] Of these, SNU308, GB-H3, and GB-D1 cell lines were derived from gallbladder carcinomas, HuH28, HuCCT-1, and SNU 1079 were derived from intrahepatic cholangiocarcinomas, and EGI-1, TFK-1, and SNU 245 were derived from extrahepatic biliary cancers. All cell lines except EGI-1 were grown in RPMI (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Inc.); EGI-I was grown in Dulbecco's MEM (Life Technologies Inc.) supplemented with 10% FBS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

RNA Extraction and Hybridization

Sample preparation and processing procedure was performed at the Roswell Park Cancer Institute Microarray Core Facility, as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix Inc., Santa Clara, CA). Briefly, frozen tumor tissues were crushed in TRIzol (Invitrogen Inc., Carlsbad, CA) by using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Total RNA was then extracted from the crushed tissue and cleaned using RNeasy columns according to manufacturer's protocol (Qiagen Inc., Valencia, CA). For biliary cancer cell lines and biliary epithelial scrapings, the RNeasy protocol for human cell lines was directly used for extraction of total RNA. The integrity of total RNA was confirmed in each case using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Using 5 to 40 μg of total RNA, double-stranded cDNA was synthesized following SuperScript Choice system (Invitrogen Inc.). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using Phase Lock Gel, phenol/chloroform extraction, and precipitated with ethanol. The cDNA pellet was collected and dissolved in appropriate volume. Using cDNA as template, cRNA was synthesized using a T7 MegaScript In-Vitro Transcription (IVT) kit (Ambion, Austin, TX). Biotinylated-11-CTP and 16-UTP ribonucleotides (Enzo Diagnostics Inc., Farmingdale, NY) were added to the reaction as labeling reagents. IVT reactions were carried out at 37°C for 6 hours and, the labeled cRNA obtained was purified using RNeasy columns (Qiagen Inc.). The cRNA was fragmented in a fragmentation buffer (40 mmol/L Tris-acetate, pH 8.1, 100 mmol/L KOAc, 30 mmol/L MgOAc) for 35 minutes at 94°C. Fragmented cRNA (10 to 11 μg/probe array) was used to hybridize to human U133A GeneChip array at 45°C for 24 hours in a hybridization oven with constant rotation (60 rpm). The chips were washed and stained using Affymetrix fluidics stations. Staining was performed using streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR), followed by the addition of biotinylated antibody to streptavidin (Vector Laboratories, Burlingame, CA), and finally with streptavidin phycoerythrin conjugate. Probe arrays were scanned using fluorometric scanners (Hewlett Packard Gene Array Scanner; Hewlett Packard Corporation, Palo Alto, CA). The scanned images were inspected and analyzed using established quality control measures.

Data Filtering and Analysis

The 25 .CEL files generated by the Affymetrix Microarray Suite (MAS) version 5.0 were converted into .DCP files using dCHIP (www.dCHIP.org), as described previously by Li and Wong.[15] The .DCP files were normalized, and raw gene expression data generated using the dCHIP system of model-based analysis. To evaluate how the 25 samples grouped together according to the similarity of their gene expression profiles, we used hierarchical clustering with the average linkage method, with a subset of 2308 genes demonstrating the largest variation across samples (SD/mean $\geq$1). For hierarchical cluster analysis, data were log-transformed, median-centered, and visualized using the CLUSTER and TREEVIEW programs.[16] For comparison of global gene expression profiles between normal and cancer samples, a two-pronged strategy was used. The first comparison was performed using the dCHIP software itself, wherein the five biliary epithelial scrapings were designated as "baseline" (B), and the 20 biliary cancer specimens designated as "experiment" (E). Genes expressed threefold or higher in the cancers *versus* normal samples were then identified by defining the appropriate filtering criteria in the dCHIP software (mean E/mean B $>$3; mean E $-$ mean B $=$ 100, $P < 0.1$, *t*-test). The second comparison was performed using significance analysis of microarrays or SAM v1.13 (http://www-stat.stanford.edu/~tibs/SAM/),[17,18] which contains a sliding scale for false discovery rate (FDR) of significantly up-regulated genes. The output criteria selected for SAM included threefold or greater expression in the biliary cancers as compared to normal tissues, and a significance threshold expected to produce a median FDR of less than 10 genes.

Immunohistochemistry

A biliary cancer tissue microarray was generated from 40 biliary tract cancers (15 gallbladder carcinomas, 10 intrahepatic cholangiocarcinomas, and 15 extrahepatic cholangiocarcinomas), as previously described.[19] Each cancer specimen was represented by four 1.4-mm cores on the tissue microarrays, to obtain adequate representation of neoplastic cells. In addition, non-neoplastic biliary tissues, and additional control tissues from extrabiliary sites were also included on the tissue array. Slides were deparaffinized in fresh xylenes and rehydrated through sequential graded ethanol steps. Antigen retrieval was performed by citrate buffer incubation (18 mmol/L citric acid, 8.2 mmol/L sodium citrate, pH 6.0) using a household vegetable steamer (Black and Decker) for 60 minutes. Slides were incubated for 5 minutes with 3% hydrogen peroxide, washed in TBS/T (20 mmol/L Tris, 140 mmol/L NaCl, 0.1% Tween-20, pH 7.6), and incubated in appropriate antibody dilutions for cdc2(p34) (Zymed, South San Francisco, CA, 1:100), topoisomerase II α [topoIIα] (Neomarkers, Fremont, CA, 1:3200), bone morphogenetic protein receptor 1A [BMPR1A/Activin A receptor/ALK3] (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:500), and geminin (1:500, a generous gift from Dr. Anindya Dutta, Brigham and Woman's Hospital, Boston, MA) for 60 minutes at room temperature. Normal saline was substituted for the primary antibody in control sections. The avidin-biotin-peroxidase complex method from DAKO (Glostrup, Denmark) was used and slides were subsequently counterstained with hematoxylin. Assessment of immunohistochemical labeling in the tissue microarrays was performed by two of the authors (D.E.H. and A.M.). For the three nuclear markers (cdc2, geminin, and topo IIα), scoring was performed as follows: negative, $<$5% nuclear labeling; focal, 5 to 25% nuclear labeling; and diffuse, $>$25% nuclear labeling; for BMPR1A, labeling was scored as negative ($<$5% cytoplasmic and membranous staining), focal (5 to 25% cytoplasmic and membranous staining), and diffuse ($>$25% cytoplasmic and membranous staining).

In Situ *Hybridization*

Non-radioisotopic *in situ* hybridization was performed for the transcription factor *islet-1* (*ISL-1*). Sense and antisense riboprobes were prepared from the corresponding sequence-verified I.M.A.G.E. clone (Invitrogen, Inc.), as previously described,[20] and labeled using Digoxin RNA Labeling mix (Boehringer Mannheim, Germany). Biliary cancer tissue microarrays were rehydrated through ethanol gradients (as described above), incubated for 10 minutes in 1% hydrogen peroxide (Super G Brand), washed in TBS, treated with proteinase K for 30 minutes at 37°C, and incubated overnight in 300 μl of diluted sense or antisense probe at 50°C. The next day, slides were washed in 2X SSC, treated with 250 μl Rnase A/T1 cocktail (Ambion, Austin, TX) for 30 minutes at 37°C, washed in 2X SSC with 50% formamide (American Bioanalytical, Natick, MA), washed in 0.8X SSC, blocked, incubated with rabbit HRP-anti-DIG (DAKO), and developed in the dark with biotinyl-tyramide (DAKO). Slides were then counterstained as previously described.[20] The specificity of hybridization was assessed by absence of signal in the sense riboprobe slide; the antisense riboprobe slide was then used for analysis of transcript expression pattern in neoplastic *versus* non-neoplastic biliary cells.

Semi-Quantitative RT-PCR

The differential expression of two genes, homeobox B7 (*HoxB7*) and dickkopf 1 homolog (*Dkk-1*), was validated by reverse transcriptase PCR. Total RNA was prepared from five biliary cancer cell lines (GB-H3, SNU1079, SNU308, SNU245, and HuCCT1), and three normal biliary epithelial scrapings using the RNeasy Mini kit (Qiagen, Inc.). For cDNA preparation, a mix of 4 μg total RNA and Oligo (dT) primers (Invitrogen, Inc.) was incubated at 70°C for 10 minutes to denature RNA and briefly placed on ice. A final buffer mixture of 1X First Strand Buffer, 20 mmol/L DTT, and 2 mmol/L dNTP (Invitrogen) was added, samples were incubated at 42°C for 2 minutes, 200 units of Super Script II RNase H-reverse transcriptase (Invitro-

gen, Inc.) were added, and samples were again incubated at 42°C for 50 minutes. A final incubation at 70°C for 15 minutes was performed. Polymerase chain reaction was performed in a final mixture of 1X Platinum PCR Supermix (Invitrogen, Inc.), 400 nmol/L forward and reverse primer, and 2 μl cDNA. PCR reactions were performed in a Thermo Hybaid MBS 0.2S cycler as follows: 96°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 3 minutes, followed by 72°C for 5 minutes, then 4°C. PCR products were visualized by 1% agarose gel electrophoresis, followed by eithidium bromide staining. The primer sequences were as follows: *HoxB7* foward primer 5′-AGCCTCAAGTTCGGTTTTCG-3′, *Hox B7* reverse primer 5′-GCGTCAGGTAGCGATTGTAG-3′, *Dkk-1* forward primer 5′-ACCAGACCATTGACAACTAC-3′, *Dkk-1* reverse primer 5′-GTGTCTAGCACAACACAATC-3′. Standardization was performed using GAPDH; forward primer and reverse primers were used as previously described.[4]

Results

Identification of Differentially Up-Regulated Genes in Biliary Cancers

RNA samples extracted from neoplastic and non-neoplastic biliary tissues and cell lines were hybridized to the Affymetrix U133A expression microarray containing ~22,000 unique transcripts, and normalized gene expression data were generated using the dCHIP software. Hierarchical clustering was performed using a subset of 2308 genes with the greatest variation between 25 samples. Based on gene expression profiling, two major clusters were identified, the first containing 9 of 9 cell lines and 4 of 11 resected tumors, and the second containing 5 of 5 normal biliary epithelial scrapings and 7 of 11 primary tumors (Figure 1A). As expected, all five normal biliary scrapings clustered with remarkable identity and separately from the nine biliary cancer cell lines on the dendrogram; the clustering of a subset of primary biliary cancers near the biliary epithelial samples was not unexpected given the presence of residual stroma and non-neoplastic epithelium in many of the resected tumor samples. Although there were examples of site-of-origin-specific clustering (for example, three gallbladder carcinoma lines on the same branch of the dendrogram), in general, we found intermingling on the dendrogram between cancers from different sites in the biliary tree (Figure 1A). Thus, while most cancer specimens, especially cell lines, clustered separately from normal epithelium, there was no unequivocal site-of-origin clustering. A pictorial representation of the principal component analysis of biliary cancers and normal biliary epithelium is illustrated in Figure 1B, with red representing relative overexpression and green representing relative underexpression (TREEVIEW software).

To achieve a high level of stringency for identification of differentially up-regulated genes in biliary cancers, a two-pronged strategy was used. First, the dCHIP normalized hybridization data from biliary cancers and normal epithelium was compared using the dCHIP software itself, which yielded 512 Affymetrix fragments expressed at threefold or greater intensity in the cancers *versus* normal samples ($P < 0.1$, *t*-test). Second, the dCHIP normalized hybrdidization data were exported into, and analyzed by SAM, which yielded 373 Affymetrix fragments expressed at threefold or greater intensity in the cancers, using a threshold median discovery rate (FDR) of <10 genes. The merging of the two datasets from dCHIP and SAM analysis yielded 347 overlapping Affymetrix fragments. After purging this set of 347 Affymetrix fragments for duplicate fragments from the same gene, unnamed hypothetical proteins, and ESTs, we identified 282 unique known genes up-regulated at least threefold or higher in primary resected cancers and biliary cancer cell lines *versus* normal epithelial scrapings. Table 1 lists a representative subset of 50 annotated genes identified in our analysis; the complete list of 282 up-regulated genes is available publicly on the Johns Hopkins gallbladder and bile duct cancer website (http://pathology2.jhu.edu/gbbd/microarray). We then performed a similar series of analyses comparing only the nine biliary cancer cell lines with the five biliary epithelial scrapings. This direct comparison of neoplastic *versus* non-neoplastic epithelium yielded 700 Affymetrix fragments by dCHIP analysis, 717 fragments by SAM analysis, and 638 fragments that were common to both analyses. The 638 fragments were then parsed for duplicates, unnamed hypothetical proteins, and ESTs, identifying 514 known genes that were overexpressed threefold or greater in the cell lines *versus* normal epithelium (data available publicly on our website). The larger number of differentially expressed genes

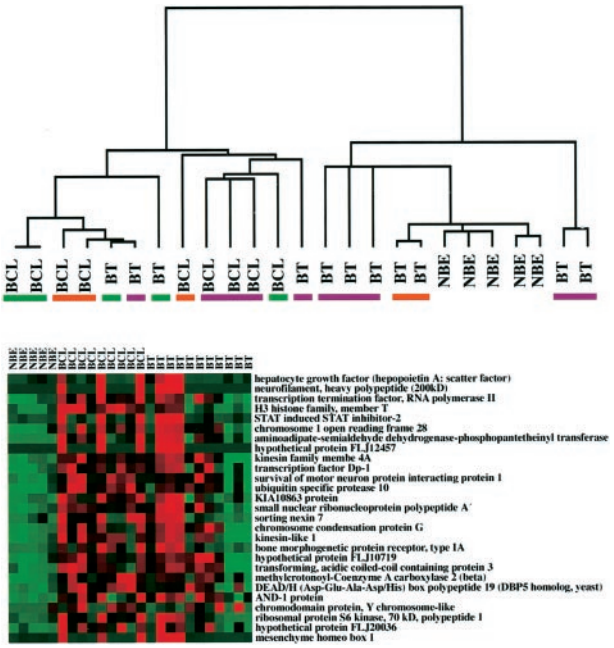


Figure 1. Cluster analysis of biliary cancers. **A:** Cluster analysis of 9 biliary cancer cell lines (BCL), 11 primary biliary cancers (BT), and 5 normal biliary epithelial samples (NBE). The colored **bars** under the tumor samples indicate their location in the biliary tree: purple, gallbladder; green, extrahepatic bile duct; orange, intrahepatic bile duct. **B:** Representative **panel** demonstrating results of principal component analysis (PCA) of biliary cancers and normal biliary epithelium. Red indicated relative overexpression, green indicates relative underexpression (TREEVIEW software).

when only cell lines were compared with normal epithelium possibly reflects either the "dilution" effect caused by non-neoplastic epithelial and stromal elements within primary cancers, or the effects of *in vitro* culture. In passing, it should be mentioned that we also identified 513 genes that were down-regulated threefold or greater in the cancers *versus* normals; however, since the objective of the current study was to identify novel tumor markers, the ensuing discussion will focus on up-regulated genes only.

Multiple classes of differentially up-regulated genes were identified in this study and included proliferation and cell cycle antigens (*cyclins D2* and *E2*, *cdc2/p34*, and *geminin*),[21–23] transcription factors (*homeobox B7* and *islet-1*),[24,25] growth factors and growth factor receptors (*hepatocyte growth factor*, *amphiregulin*, and *insulin-like growth factor 1 receptor*),[26–28] and enzymes modulating sensitivity to chemotherapeutic agents (*cystathionine β synthase*, *dCMP deaminase*, and *CTP synthase*)[29–31] Subsets of genes involved in biologically relevant pathways were also identified, such as genes in the transforming growth factor β pathway (*bone morphogenetic protein receptor 1A* [*BMPR1A*], *mothers against decapentaplegic homolog 5* [*MADH5*], and *TGF-β receptor-associated protein 1* [*TRAP-1*]),[32–34] or genes involved in steroid metabolism, particularly in the estrogen/androgen pathways (*estrogen-related receptor-γ*, *sterol isomerase*, and *steroid-5-α-reductase*).[35–37] Interestingly, we identified several genes that are related to pathways rapidly emerging as novel therapeutic targets in many cancer types. Notable among these were *cytosolic phospholipase A2*, which generates arachidonic acid, the substrate for the cyclooxygenase (COX) enzyme,[38,39] and two important downstream mediators of the tumorigenic *Akt/mammalian target of rapamycin* (*mTOR*) pathway in humans, *ribosomal protein S6 kinase*, and *eukaryotic translation initiation factor 4E*.[40]

A literature search of PubMed (www.ncbi.nlm.nih.gov/PubMed) revealed that several of the genes up-regulated in this study have previously been reported as overexpressed, either singly or through global microarray expression analyses in other cancer types, in principle validating our approach. A second PubMed search using the gene name and either "cholangiocarcinoma," "biliary," "bile duct," or "gallbladder" in the text search revealed that only three up-regulated genes (*hepatocyte growth factor*, *proliferating cell nuclear antigen*, and *cytosolic phospholipase A2*) have been previously reported as specifically overexpressed in biliary cancers.[41–43] Thus, the overwhelming majority of up-regulated genes reported in this study represent novel tumor markers and cellular targets for this cancer type.

Validation of Differentially Up-Regulated Genes in Biliary Cancers

The differential overexpression of a subset of genes ($n = 7$) was validated using a combination immunohistochemistry and *in situ* hybridization in archival biliary cancers and RT-PCR on biliary cancer cell lines. A biliary cancer tissue microarray containing 40 biliary cancers was immunolabeled with antibodies against BMPR1A, geminin, topoIIα, and cdc2/p34 to confirm the overexpression of the corresponding protein product. The results of immunohistochemical analysis are summarized in Table 2.

BMPR1A is a member of the cell surface receptor TGF-β superfamily and is expressed in human skeletal muscle, heart, and placenta under normal conditions.[32] BMPR1A immunolabeling was absent in normal biliary epithelium (<5% labeling) (Figure 2A). In contrast, 91% of cancers demonstrate diffuse or focal cell membrane and cytoplasmic immunolabeling of BMPR1A (Figure 2B). Similarly, 85% of biliary cancers demonstrate diffuse or focal nuclear overexpression of the novel protein geminin,[23] while normal biliary epithelium was negative (Figure 2, C and D). The cell cycle protein cdc2/p34 serves as a promoter of mitosis and is regulated by tyrosine phosphorylation.[44] Normal biliary epithelium lacked nuclear cdc2/p34 expression (Figure 2E), whereas 83% of biliary tract cancers demonstrated diffuse or focal nuclear expression (Figure 2F). Similarly, labeling with topoIIα, which regulates the topology of DNA,[45] was absent in normal biliary tract epithelium (Figure 2G), but was diffusely or focally expressed in 71% of biliary cancers (Figure 2H) (Table 2).

In situ hybridization for the transcription factor islet-1 (*ISL-1*) was performed using the biliary cancer tissue microarray to confirm differential overexpression. *ISL-1* is a downstream target of the Sonic hedgehog pathway, and is essential for development of the endocrine pancreas.[46,47] *In situ* hybridization for *ISL-1* demonstrated neoplastic epithelial expression in 26 of 40 (65%) biliary tract carcinomas, while minimal expression was seen in non-neoplastic biliary epithelium (Figure 3,A and B); sense control yielded no signal in the cancer cells (Figure 3C).

We additionally confirmed the overexpression of two genes, *homeobox B7* (*HoxB7*) and *dickkopf 1* (*Dkk-1*), in five biliary cancer cell lines and three non-neoplastic biliary epithelial scrapings by semi-quantitative RT-PCR. *HoxB7* is a homeobox containing gene normally expressed during development and aberrantly overexpressed in several human cancers,[48] whereas *Dkk-1* is a p53-responsive gene that inhibits canonical *Wnt* signaling pathways.[49] Both transcripts were expressed and up-regulated in the cancer cell lines we examined in comparison to normal biliary epithelium (Figure 4).

Discussion

Biliary tract carcinoma represents the second most common primary cancer of the hepatobiliary system; each year as many as 3500 individuals succumb to this disease in the United States alone, while mortality rates are far higher in some parts of the world such as the Far East, Chile, and parts of Southeast Asia.[2] Known risk factors include primary sclerosing cholangitis, cholelithiasis, chronic parasitic infection, and chemical exposure.[3] Although early preventative steps can be taken to lower the risk of developing biliary cancers, the overall prognosis once cancer develops is poor, with only a 10% 5-year

Table 1. Representative Subset of Differentially Overexpressed Genes in Biliary Cancers

Affymetrix tag number	Gene name	Fold change	*p* Value	Chromosome	Function
205239_at	Amphiregulin (schwanomma-derived growth factor)	5.64793	0.011387	4q13-q21	Autocrine growth factor, mitogen
204832_s_at	Bone morphogenetic protein receptor, type IA	3.92410	0.000314	10q22.3	Integral membrane protein, TGF-β mediator
209642_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	3.37973	0.000275	2q14	Spindle assembly checkpoint regulator
216602_s_at	Calreticulin	3.08473	0.000315	19p13.3-p13.2	Protein folding, calcium storage
206075_s_at	Casein kinase 2, alpha 1 polypeptide	3.41682	0.000074	20p13	Phosphorylation of proteins (eg, p53)
203968_s_at	CDC6 cell division cycle 6 homolog	5.16898	0.000004	17q21.3	DNA replication checkpoint control
203213_at	Cell division cycle 2, G1 to S and G2 to M	3.51015	0.000139	10q21.1	Cell cycle regulator
205394_at	CHK1 checkpoint homolog (*S. pombe*)	3.64472	0.000261	11q24	Cell cycle regulator
202613_at	CTP synthase	4.20409	0.000019	1p34.1	Phospholipid and nucleic acid biosynthesis; multidrug resistance associated
203418_at	Cyclin A2	5.02650	0.000076	4q27	Cell cycle regulator
200953_s_at	Cyclin D2	3.61139	0.005114	12p13	Cell cycle regulator
205034_at	Cyclin E2	4.01328	0.000196	8q21.3	Cell cycle regulator
212816_s_at	Cystathione beta synthase	7.39167	0.00009	21q22.3	Homocysteine metabolism; induces susceptibility to cytosine arabinoside
201571_s_at	dCMP deaminase	3.87652	0.000056	4q35.1	Target enzyme for gemcitabine
202576_s_at	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 19 (DBP5 homolog, yeast)	3.29770	0.000058	16q22.3	mRNA nuclear export
204602_at	Dickkopf homolog 1 (*Xenopus laevis*)	12.2781	0.000046	10q11.2	Wnt signaling antagonist
213789_at	Emopamil binding protein (sterol isomerase)	3.50690	0.024251	Xp11.23-p11.22	Binds tamoxifen, SR31747A
203499_at	EphA2	3.75649	0.001234	1p36	Receptor tyrosine kinase
207981_s_at	Estrogen-related receptor gamma	5.67041	0.006618	1q41	Binds estrogen response elements
201436_at	Eukaryotic translation initiation factor 4E	4.03826	0.000002	4q21-q25	Akt/mTOR signaling pathway
218350_s_at	Geminin	3.90463	0.000120	6p21.32	Cell proliferation antigen
210997_at	Hepatocyte growth factor (hepapoietin A; scatter factor)	7.70259	0.016513	7q21.1	Cell development and growth
203138_at	Histone acetyltransferase 1	4.75385	0.000009	2q31.2-33.1	Histone acetylation and gene expression
204779_s_at	Homeo box B7	7.98564	0.000113	17q21-q22	Gut and lymphoid development
203627_at	Insulin-like growth factor 1 receptor	3.57119	0.000542	15q25-q26	Insulin receptor signaling
206104_at	ISL1 transcription factor, LIM/ homeodomain, (islet-1)	13.3186	0.003433	5q11.1	Insulin enhancer-binding protein
20908_s	Jagged-1	5.41679	0.004756	20p12.1	Notch ligand
216815_at	Laminin receptor 1	3.18114	0.00713	3p21.3	Receptor for cell adhesion molecule laminin
205397_x_at	MAD, mothers against decapentaplegic homolog 5 (Drosophila)	3.50543	0.002022	5q31	TGF-β receptor signaling, transcription
203362_s	MAD2 mitotic arrest deficient-like 1	3.85297	0.000011	4q27	Mitotic checkpoint control
203552_at	Mitogen-activated protein kinase kinase kinase kinase 5	3.08865	0.000236	14q11.2-q21	Ser/Thr protein kinase
219148_at	PDZ-binding kinase; T-cell originated protein kinase	3.39109	0.000268	8p21.1	Mitotic kinase
204186_s_at	Peptidylprolyl isomerase D (cyclophilin D)	7.04929	0.000001	4q31.3	Estrogen receptor complex member
210145_at	Phospholipase A2, group IVA (cytosolic)	3.54762	0.000421	1q25	Catalyzes arachidonic acid release
213226_at	Polymyositis/scleroderma autoantigen 1	5.16640	0.000039	4q28.1	Nucleolar and nucleoplasm protein
205512_s_at	Programmed cell death 8 (apoptosis-inducing factor)	3.43048	0.000079	Xq25-26	Cell death mediator

(*Table continues*)

Table 1. (*continued*)

Affymetrix tag number	Gene name	Fold change	*p* Value	Chromosome	Function
201202_at	Proliferating cell nuclear antigen	4.02376	0.000006	20pter-p12	Cell cycle regulator
209228_x_at	Putative prostate cancer tumor suppressor	5.91911	0.000976	8p22	Transmembrane protein, CpG methylated
209849_s_at	RAD51 homolog C (*S. cerevisiae*)	4.54601	0.000179	17q23.1	DNA repair, DNA recombination; "17q23 amplicon"
204146_at	RAD51-interacting protein	5.17207	0.000000	12p13.2-p13.1	Genetic recombination
217457_s_at	RAP1, GTP-GDP dissociation stimulator 1	3.21201	0.000089	4q21-q25	GTP-GDP dissociation stimulator
215098_at	Retinoid X receptor, beta	3.13613	0.000124	6p21.3	Transcription factor
204171_at	Ribosomal protein S6 kinase, 70kD	3.10282	0.001765	17q23.1	Akt/mTOR pathway; "17q23 amplicon"
211056_s_at	Steroid-5-alpha-reductase, alpha polypeptide 1	5.17492	0.00052	5p15	Androgen metabolism
202817_s_at	Synovial sarcoma translocation, chromosome 18	3.26773	0.000037	18q11.2	Chromosomal translocation
205210_at	TGF beta receptor associated protein 1	5.56609	0.000628	2q12.1	TGF-β receptor binding protein
201291_s_at	Topoisomerase (DNA) II alpha (170kD)	3.90944	0.000021	17q21-q22	DNA maintenance and structure
204147_s_at	Transcription factor Dp-1	4.29968	0.000410	13q34	Cell cycle regulator, transcription
203856_at	Vaccinia related kinase 1	3.19922	0.000418	14q32	Cell cycle regulator
212533_at	WEE1+ homolog (S. pombe)	8.28110	0.000007	11p15.3-p15.1	Cell cycle regulator
217821_s_at	WW domain binding protein 11	3.62317	0.000076	12p13.31	SH3 domain-binding protein

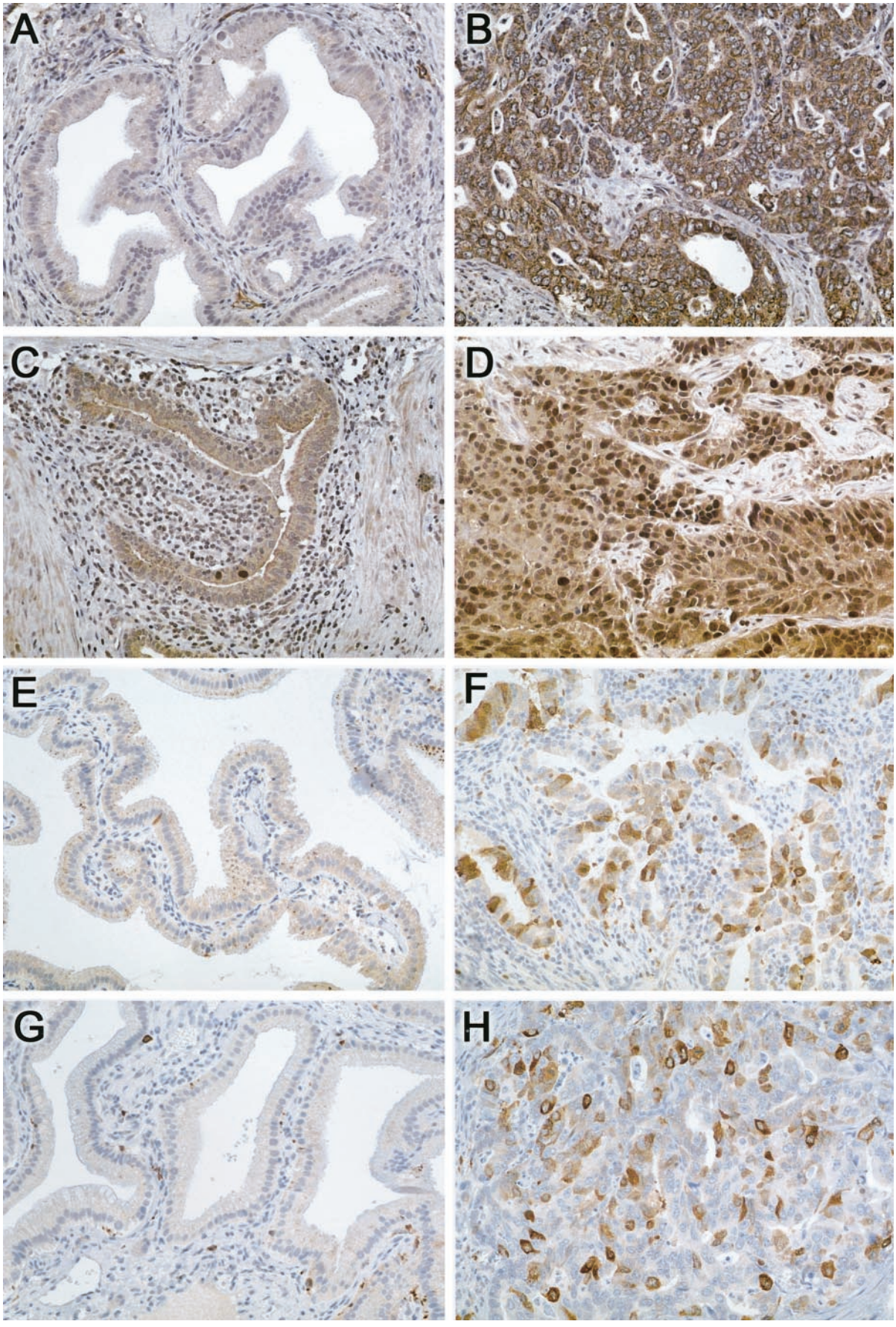
survival rate for advanced disease.[3] New methods for early detection, a better understanding of the biological mechanisms underlying cancer progression, and cancer-targeted treatment modalities are urgently needed to reduce the mortality from this lethal disease.

The availability of global expression platforms such as oligonucleotide and cDNA microarrays has greatly facilitated the identification of novel tumor markers in many cancer types. For example, oligonucleotide microarrays have enabled us to identify nearly 100 novel tumor markers with immediate diagnostic and therapeutic relevance in pancreatic adenocarcinomas.[4] In this study, we identify for the first time the up-regulation of a large number of genes in biliary cancers relative to non-neoplastic biliary epithelium using the recently developed Affymetrix U133A expression microarrays. Of the 282 genes identified as overexpressed threefold or greater in biliary cancers, only three have been reported previously in the specific context of biliary cancers.[41–43] Thus, this study has enormous potential in terms of expanding our knowledge of biliary cancer pathogenesis and identification of novel cellular targets in this cancer type. In addition to using the recently developed Affymetrix U133A microarray that probes for ~22,000 transcripts on a single platform, other methodological strengths of our study include the use of a large number of biliary cancer cell lines representing different sites in the biliary tree, verification of neoplastic cellularity of primary resected cancers using frozen section examination, and the appropriate use of enriched biliary epithelium (as opposed to "full thickness" biliary ductal sections) as controls for comparison of gene expression. The dCHIP and SAM software used for data analysis in this study have emerged as two of the most widely used and robust programs for two-class comparison of microarray expression data.[17,18,50–52] Finally, we have validated the gene expression data for a subset of genes using a second independent technique; all seven genes were confirmed as overexpressed in biliary cancers compared to non-neoplastic biliary epithelium, irrespective of the detection technique (immunohistochemistry, *in situ* hybridization, or RT-PCR). While we only chose to identify, validate, and discuss overexpressed genes in this study, it should be mentioned that we have also identified over 500 genes that are underexpressed threefold or more in the tumors compared to normal samples; although not discussed in the context of this manuscript, these genes should serve as an invaluable resource in future studies for identification of tumor suppressor genes that are the target of genetic or epigenetic inactivation in biliary cancers.

Of the 282 genes identified as differentially overexpressed in biliary cancers compared to normal epithelium, many conform to cellular processes intuitive to tumor cells, such as proliferation, DNA synthesis and RNA processing, and metabolism. A subset of these genes have emerged as candidates for novel molecular therapy

Table 2. Immunohistochemical Analysis of Candidate Overexpressed Genes in Tissue Microarrays of Biliary Cancer

	Diffuse	Focal	Negative	Not evaluable
BMPR1A	23/40 (58%)	13/40 (33%)	3/40 (7%)	1/40 (2%)
Geminin	22/40 (55%)	12/40 (30%)	5/40 (13%)	1/40 (2%)
Topoisomerase II	15/40 (38%)	13/40 (33%)	11/40 (27%)	1/40 (2%)
Cdc2 (p34)	21/40 (53%)	12/40 (30%)	6/40 (15%)	1/40 (2%)


A
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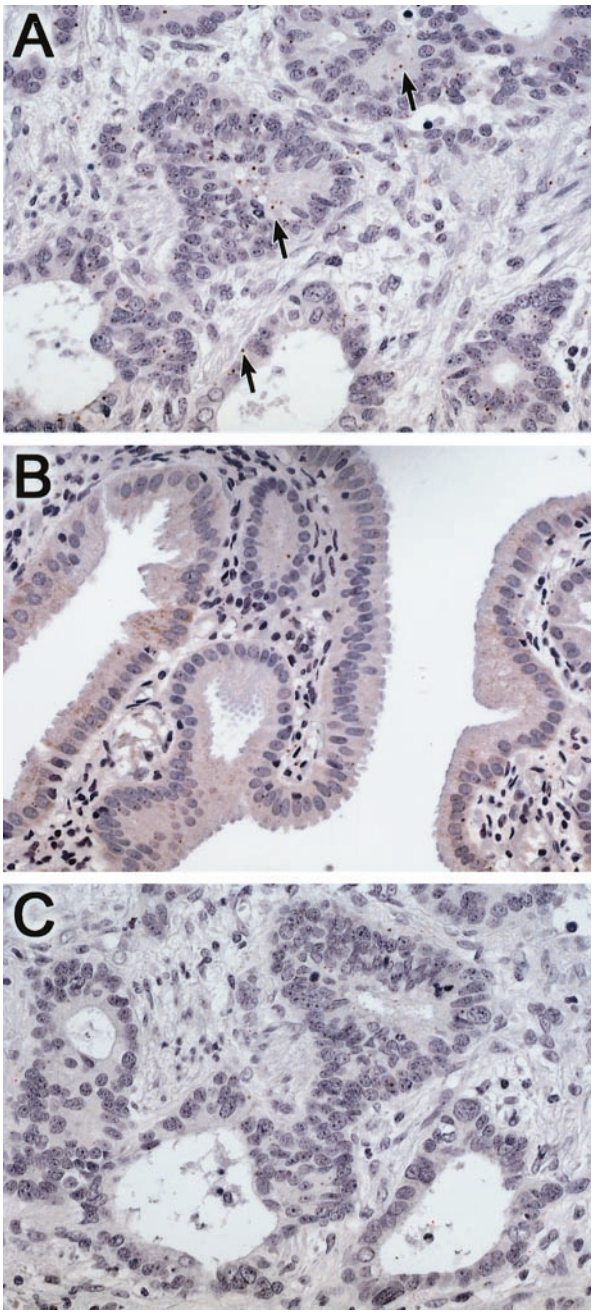

Figure 3. Non-radioisotopic *in situ* hybridization for validation of Islet-1 expression in biliary cancer using a biliary cancer tissue microarray. *Islet-1* transcripts are detected in a biliary adenocarcinoma using the *ISL-1* antisense riboprobe (**arrows**) (**A**), but transcripts are not detectable in normal biliary epithelium using an antisense riboprobe (**B**), or in the serial section of the cancer using the *ISL-1* sense (control) riboprobe (**C**).

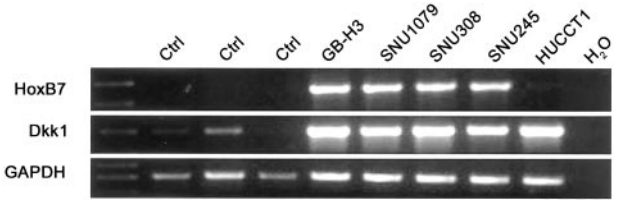

Figure 4. RT-PCR validation of overexpressed genes in biliary cancers. Two genes overexpressed in the Affymetrix analysis, *homeobox B7* (*HoxB7*) and *dickkopf-1 homolog* (*Dkk1*), were selected for RT-PCR in five biliary cancer cell lines (GBH3, SNU1079, SNU308, SNU245, and HuCCT1), and three samples of normal biliary epithelium. *HoxB7* expression is absent in the normal epithelium, but is present in 4 of 5 biliary cancer cell lines; water control is appropriately negative (**top panel**). Dkk-1 expression is present in normal epithelium, but its expression is up-regulated in 5 of 5 cell lines; water control is appropriately negative (**middle panel**). GAPDH is used as an external control (**bottom panel**).

in several cancer models, and thus, their reported overexpression in biliary cancers provides an important basis for future *in vitro* and *in vivo* trials, targeting the corresponding proteins. For example, the cdc2/p34 protein, which we confirmed as overexpressed in our biliary cancer tissue microarrays, is the catalytic subunit of a protein kinase complex, called the M-phase promoting factor that induces entry into mitosis and is universal among eukaryotes.[22] Recently, cdc2/p34 has also been demonstrated to play a role in promoting cell survival, mediated by its site-specific phosphorylation and activation of the anti-apoptotic protein, survivin.[53] Survivin interferes with spontaneous and chemotherapy-induced cell death, and thus loss of survivin activation by pharmacological inhibition of cdc2/p34 has emerged as a novel chemotherapeutic strategy. Topoisomerase IIα, the overexpression of which was also confirmed in our tissue microarrays, is important in mediating susceptibility to drugs that specifically target this enzyme; response to topoisomerase inhibitors is enhanced in breast cancers that overexpress topo II,[54,55] providing an excellent example of how molecular events can direct choice of therapeutic regimens.

Growth factors and growth factor receptors that were up-regulated in biliary cancers included, among others, hepatocyte growth factor (HGF) (7-fold), and the insulin-like growth factor 1 (IGF-1) receptor (3.5-fold). HGF has been previously reported as up-regulated in biliary cancers, often in conjunction with its receptor, c-met.[42] Although c-met was not included in the gene list that compared all cancers *versus* normals, c-met was one of the additional genes that emerged in the comparison of cell lines *versus* normals (http://pathology2.jhu.edu/gbbd/microarray). HGF is identical to scatter factor, and it is a potent mitogen for hepatocytes. Biliary epithelial cells produce little or no HGF, while biliary cancers acquire the ability to secrete this growth factor in conjunction with up-regulation of c-met.[56] In addition to biliary cancers, dysregulation of the HGF/c-met pathway has been reported in hepatocellular, lung, prostate, and breast cancers, glioblastomas, and melanomas, wherein this pathway promotes angiogenesis, cellular motility, growth, and invasion.[26,57,58] HGF may stimulate tumor invasion in an autocrine loop through c-met, via down-regulation of cel-

Figure 2. Immunohistochemical validation of genes overexpressed in biliary cancers using a biliary cancer tissue microarray. Bone morphogenetic protein receptor type 1A (BMPR1A) expression in normal biliary epithelium (**A**) and in adenocarcinoma of the biliary tract (**B**). BMPR1A is absent in normal biliary epithelium, but is diffusely expressed in a cytoplasmic and membranous distribution in the invasive adenocarcinoma. Geminin expression is absent in normal biliary epithelium (**C**) while biliary tract adenocarcinoma is diffusely positive (**D**). Cdc2 (p34) expression is absent in normal biliary epithelium (**E**), while it is diffusely expressed in biliary tract adenocarcinoma (**F**). Topoisomerase II α expression is absent in normal biliary epithelium (**G**) but diffuse nuclear labeling is seen in adenocarcinoma of the biliary tract (**H**).

lular adhesion molecules such as E-cadherin.[59] Besides providing an avenue for therapy by signal antagonism, HGF is also useful as a potential diagnostic tool in biliary cancer patients. Elevated serum HGF levels are found in a variety of cancer types, and may correlate with prognosis.[60–62] Similarly, overexpression of the IGF-1 receptor is commonly found in many cancers, and its level of expression may also correlate with prognosis.[28,63] As a cell surface molecule upstream of many critical signaling pathways, the IGF-1 receptor represents an important target for cancer therapy. Indeed, antagonism of cellular IGF-1 receptor by overexpression of dominant-negative mutant forms or by interference using small interfering RNA (siRNA) results in decreased tumorigenicity in *in vitro* models.[64,65] Thus, the IGF-1 receptor represents another potential target for therapy identified in biliary cancers.

The mammalian target of rapamycin (mTOR) is a member of a recently identified family of protein kinases termed phosphoinositide 3-kinase (*PI3K*)-related kinases, which are involved in many critical regulatory cellular functions pertaining to cell cycle progression, DNA repair, and DNA damage. mTOR functions downstream of the proto-oncogenes *PI3K* and *Akt*, and is itself upstream of two distinct pathways that control translation of specific subsets of mRNA, one involving ribosomal protein p70 synthase kinase ($p70^{s6k}$), and the other eukaryotic initiation factor 4E binding protein-1 (4EBP1).[66] In response to PI3K/Akt signaling, mTOR rapidly phosphorylates both $p70^{s6k}$ and 4EBP1, the latter leading to release of eIF4E, resulting in initiation of translation; these actions of mTOR can be inhibited by the administration of the macrolide rapamycin. Currently, CCI-779, a water soluble analog of rapamycin is undergoing evaluation as an anti-tumor agent in a variety of cancers.[40] It is likely that a subset of biliary cancers will be resistant to inhibition of mTOR. The identification of two pathway genes, $p70^{s6k}$ and eIF4E, that are differentially up-regulated in biliary cancers compared to normal epithelium provide alternative downstream targets for inhibition.

Up-regulation of the pro-inflammatory enzyme cytosolic phospholipase A2 (cPLA2) has been previously reported in biliary cancers.[41] Phospholipase A_2s are a group of enzymes that catalyze the hydrolysis of the sn-2-ester bond of phospholipids, resulting in production of free fatty acid and lysophospholipids, which can then be further metabolized to produce eicosanoids and platelet-activating factor.[67] These lipid molecules are crucial for various cellular responses, such as inflammation, signal transduction, and cell proliferation. cPLA2 is a key enzyme in the liberation of arachidonic acid (AA) from membrane phospholipids for subsequent production of bioactive eicosanoids in activated cells; notably, cPLA2 is itself activated in response to signaling mediated by another differentially overexpressed gene, hepatocyte growth factor.[68] Increasing evidence has suggested a causal relationship between cancer development, including cholangiocarcinoma, and increased expression of eicosanoid-forming enzymes. For example, increased expression of cyclooxygenase (COX) enzymes has been reported in biliary cancers.[69,70] Similar to the anti-proliferative effects of COX inhibition, targeting cPLA2 also results in decreased growth of cholangiocarcinoma cells.[41] Thus, the demonstration of up-regulated cPLA2 in biliary cancers provides another important potential therapeutic target for the control of biliary cancer and, perhaps, chemoprevention in patients with precursor conditions such as primary sclerosing cholangitis.

Several genes involved in steroid metabolism, specifically the estrogen/androgen pathways (*estrogen-related receptor-γ*, *sterol isomerase*, *cyclophilin D*, and *steroid-5-α-reductase*) were also up-regulated in biliary cancers compared to normal epithelium.[35–37] Biliary cancers, especially those arising in the gallbladder, occur more frequently in women,[71] and this has been attributed to the increased incidence of gallstones in women;[2] however, direct or indirect estrogen-related effects may also function to promote carcinogenesis. The expression of sterol isomerase (emopamil-binding protein) correlates with poor prognosis in breast cancers, although the mechanistic aspects remain poorly understood.[72] Notably, SR31747A, a recently described synthetic ligand with anti-proliferative activity in numerous cell lines binds to and inhibits sterol isomerase, and overexpression of the enzyme reverts the anti-proliferative effects of this drug.[73] Sterol isomerase also binds to and is inhibited by the synthetic estrogen homolog 4-hydroxytamoxifen.[74] Additional functional studies are warranted to demonstrate whether inhibition of sterol isomerase can emerge as a therapeutic strategy in biliary cancers. We also identified an up-regulation of the estrogen-related receptor-γ gene in our study. Estrogen-related receptor-γ is a member of an orphan nuclear receptor family that shares significant homology with estrogen receptors and, although this receptor does not recognize naturally occurring estrogens, is capable of binding to the estrogen response element.[75,76] Like sterol isomerase, estrogen-related receptor-γ binds to and is inhibited by tamoxifen.[77]

Cystathionine β synthase (CBS), which is mutated in patients with homocystinuria, is a cytosolic enzyme that also modulates sensitivity to the cytotoxic drug, cytosine arabinoside (ara-C).[29,78] Neoplastic cells with high levels of CBS demonstrate increased sensitivity to ara-C. Therefore, a sevenfold up-regulation of *CBS* transcripts in biliary cancers merits considering ara-C-containing regimens for therapy of biliary cancers. Finally, we also report up-regulation of *dCMP deaminase*, whose product is the target enzyme for the cytotoxic agent 2′,2′-difluorodeoxycytidine (gemcitabine).[30,79] Gemcitabine has emerged as a widely used chemotherapeutic agent for solid gastrointestinal malignancies, including biliary cancers. Fourfold up-regulation of dCMP deaminase in biliary cancers may suggest that, while gemcitabine should be considered as a viable therapeutic option, the corresponding dosages may need to be calibrated accordingly.

In summary, we report for the first time a global gene expression analysis of biliary cancers using Affymetrix oligonucleotide microarrays and a combination of primary resected tumors, biliary cell lines, and non-neoplastic biliary epithelial scrapings. We report 282 unique known genes as being differentially up-regulated three-

fold or greater in the cancers compared to normal epithelium, and validate a subset of genes using an alternative platform. The differentially overexpressed genes are publicly available on our website at http://pathology2.jhu.edu/gbbd/microarray, and represents an enormous resource for the scientific community to study the pathogenesis of this lethal disease and develop appropriate cancer-specific targeted therapies.

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