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World J Gastroenterol 2006 June 14; 12(22): 3481-3486
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, *Series Editor*

Bioinformatic approach for understanding the heterogeneity of cholangiocytes

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Supported by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan for the Research on Measures for Intractable Diseases and GRANT-IN-AID FOR SCIENTIFIC RESEARCH C (16590573) from JSPS
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Received: 2006-01-18 Accepted: 2006-02-18

Abstract

It is remarkable that microarray technologies have nearly reached a pinnacle. Establishment of further analysis and management of enormous data derived from microarray technology is currently the highest priority. The heterogeneous functions of cholangiocytes regulate the pathophysiology of the biliary epithelium in relation to secretory, proliferative and apoptotic activities. Distinct expression profiles of two murine cholangiocyte lines, termed small and large have been revealed by microarray analysis. The features of the two cholangiocyte cell lines, categorized partly according to gene ontology, indicate the specific physiological role of each cell line. The large cholangiocytes are characterized as "transport" and "immune/ inflammatory responses". In contrast, small cholangiocytes are associated with properties of limited physiological functional ability and proliferating/migrating potential with specific molecules like Eph receptors, comparable to mesenchymal cells. 'Omic study will be of great help in understanding the heterogeneity of cholangiocytes.

Key words: Cholangiocytes; Heterogeneity

Fukushima K, Ueno Y. Bioinformatic approach for understanding the heterogeneity of cholangiocytes. *World J Gastroenterol* 2006; 12(22): 3481-3486

http://www.wjgnet.com/1007-9327/12/3481.asp

INTRODUCTION

Several research avenues have established novel scientific theories. We initially examined phenotypic manifestations of various forms of induction, analogy and abduction. Subsequent preliminary studies resulted in a firm-working hypothesis. We then tested this hypothesis in order to establish our theoretical groundwork. However, recent advances in the field of bioinformatics have become routine, especially in regards to the initial speculative step in designing a rational approach. Since the initial DNA microarray analysis experiment[1], microarray has developed rapidly and is now well established, with manufacturers meeting market demand[2]. The number of scientific papers with "microarray" as a key word steadily increased on PubMed from 2001 to 2004, reaching a total of 8603 papers. Moreover, an enormous quantity of raw data potentially contained in "microarray" papers is anticipated in the near future, and the estimate is based on the number of the genes analyzed in each paper (e.g. 30 000-40 000 genes expressed in mammalian cells). Therefore, it is essential for future advances to reliably preserve a database containing all the raw data. The establishment of data analysis system as well as data mining and data sharing, is anticipated to be the highest priority in this field. We will focus on such issues pertaining to general microarray experiments as well as the methods and the results of microarray analyses designed to characterize cholangiocytes in this chapter.

MICROARRAY ANALYSIS

We will simply give an overview of the current status, analytic method and data-sharing aspects of microarray analysis, partly in accordance with the description of Knudsen[3]. One of the currently popular microarray systems is the Affymetrix DNA Chip®, which employs *in situ* oligonucleotide synthetic technology as well as mass-production compatible to that of silicon chips. Several microarrays and expression analysis services are commercially available from several companies including Amersham, Clontech (TAKARA), Invitrogen and so on. As in other experiments, the quantity of RNA samples should be standardized, otherwise correction steps will be necessary for the evaluation of the results by

"scaling" generally employing internal controls. Moreover, verification of the linearity of spot intensity-sample quantity curves for all applied spots is also necessary in microarray experiments. The companies supplying ready-made products have overcome these problems and established quality control, making their products popular with researchers applying commercial products to limited numbers of samples. Although array technologies at the bench have nearly reached a zenith during the past several years, there are some in silico systems under development. The parametric *t*-test and non-parametric Wilcoxon's rank-sum/ Mann-Whitney tests are the most popular methods applicable to test the significance of differences between pairs of samples. The normality of each spot intensity for the samples becomes an issue if the *t*-test or ANOVA is used with a routine microarray having thousands of spots. The most conservative statistical method, Bonferroni's correction, which defines the level of significance more strictly according to the number of tested items, might be applicable even to microarray experiments[4]. Even if we employ other less conservative approaches[5], we may need to verify the differential expressions of the specific molecules by other methods in some cases. Several software programs are available, including some that are free like Cluster (http://rana.lbl.gov/EisenSoftware.htm)[6], for analysis of microarray data. Cluster analysis categorizes each gene into distinct clusters according to Euclidian distance based on the variation among the samples at multiple time-points or under the different conditions. Each gene in a cluster shows a similar expression pattern under different conditions, which may indicate a common transcription pathway, RNA degradation process, and/ or similarities of gene function in a cluster. In other words, microarray technology holds promise for elucidating the complex regulatory mechanisms of RNA transcription as well as unknown gene functions. Every gene is currently being systematically defined, allowing organization according to gene ontology (http://www.geneontology.org/). The gene ontology project was originally designed to create a thesaurus-like hierarchy of gene properties, based on molecular function, biological processes and cellular components, in order to integrate databases in a unified way. The combination of raw microarray data and this unified classification will greatly facilitate developing the speculative groundwork for our experiments. This approach differs from the usual methodology based on molecular properties including domain structure, 3D structure, evolution or expression patterns. Therefore, raw microarray data can be a source of new hypotheses, regardless of the original aim of the experiment. This may make it difficult to share raw microarray data. The unified approach, termed MIAME (http://www.mged.org/Workgroups/MIAME/miame.html), for submission of microarray data is described in the guidelines of each paper contributing data to be shared and for peer review. The statement that "MIAME is neither a dogma, nor a legal document-it assumes a cooperative data provider and a fair reviewer" suggests potential difficulty in data sharing, probably stemming from the complexities of ownership and intellectual property rights to data. Microarray data is clearly precious, even in the current era of non-sharing, and should be carefully stored by each lab, organization or government. Such data should be viewed as common property, with potential future benefits to mankind.

FEATURES OF CHOLANGIOCYTES

Bile flows from canalicular spaces, encircled by hepatocytes, through the canals of Hering, interlobular bile ducts (branches of which are 20-100 μm in diameter and lined by cuboidal epithelium), and septal bile ducts (which are more than 100 μm in diameter and lined by a simple tall columnar epithelium) to hilar intrahepatic bile ducts[7]. The cholangiocytes that line the interlobular and bolder bile ducts[7] also contribute to bile secretion[8,9]. The mechanism underlying this phenomenon, described elsewhere in detail, is explained briefly by out-intraluminal vectorial passive transport of water caused by the osmotic gradient which is formed by active transport of several substrates driven by cholangiocytes[10,11]. This physiological function, conserved even in cultured cell lines, characterizes the specialized cholangiocytes. Isolated rat cholangiocytes are classified as large or small, depending on their size. Large cholangiocytes, regarded as representative functional biliary epithelial cells *in vivo*, respond to secretin with increased choleresis[12]. In contrast, little is known about the properties of small cholangiocytes. Cholangiocytes are sometimes altered in a disease-specific manner. For example, primary biliary cirrhosis (PBC), a potentially fatal cholestatic disorder due to ductopenia, is a representative disease characterized by specific destruction of interlobular bile ducts. CD8+ cytotoxic T cells are suspected to play a major role in this type of destruction. Tetramer technology reveals the existence of an E2 subunit of the pyruvate dehydrogenase complex (PDC-E2)$_{159\text{-}167}$-specific to autoreactive cytotoxic T cells in PBC[13]. Hypothetically, there are several specific properties (e.g. adhesion molecules, MHC antigens[14], specific autoantigens, variations in apoptosis and so on) of cholangiocytes lining interlobular bile ducts, which make them vulnerable to attack by cytotoxic lymphocytes. A novel disease etiology was proposed by Gershwin *et al*[15], who demonstrated that IgA class anti-mitochondrial antibodies, transported via transcytosis from the basolateral to the apical surface co-localize with PDC-E2 in cholangiocytes. Moreover, molecules that bind PDC-E2 monoclonal antibody are expressed on the apical membranes of PBC cholangiocytes[15]. These observations indicate the cytotoxicity associated with functional impairment of cholangiocytes to be caused by interaction with IgA-PDC-E2. In this regard, the mechanism of transcytosis may specifically dictate the underlying disease process. Such a hypothesis could be explained by cholangiocyte heterogeneity based on the recognition that large and small cholangiocytes are derived from bile ducts of corresponding diameters[16]. The susceptibility of bile ducts to pathological conditions (e.g. chronic ductopenic rejection[17], GVHD[18], ischemic cholangitis[19], PBC or ductopenia resulting from other pathologies) is due not only to the specific destruction of cholangiocytes, but also to the difficulty in regeneration of the bile ducts i.e. with normal structures, in contrast to the

Table 1 Differences in cDNA expression between small and large cholangiocyte lines

GO[1] annotation/ Gene name	Gene accession	GO accession	Small[2]	Large[3]	Ratio (L/S)
Immune response		GO:0006955			
CD1d1 antigen	NM_007639		107	156	1.46
CD1d2 antigen	NM_007640		80	131	1.64
CD86 antigen; B7-2; CD28 antigen ligand 2 (CD28L2)	L25606		52	17	0.33
Histocompatibility 2, class II antigen E alpha	NM_010381		132	407	3.08
Histocompatibility 2, class II antigen A, beta 1	NM_010379		586	832	1.42
Histocompatibility 2, class II antigen E beta	NM_010382		3579	4481	1.25
Interleukin 2 receptor, beta chain	NM_008368		540	2604	4.83
Cell adhesion		GO:0007155			
Intercellular adhesion molecule 1 (ICAM1)	X52264		39	20	0.51
Vascular cell adhesion molecule 1 (VCAM1)	M84487		17	11	0.65
Cadherin 1 (CDH1); epithelial cadherin (E-cadherin; E-CAD); uvomorulin (UM)	X06115		32	42	1.29
Cytoskeleton		GO:0005856			
Cytoplasmic beta-actin (ACTB)	M12481		455	275	0.60
Vimentin (VIM)	X51438		260	73	0.28
Proteolysis		GO:0006508			
Matrix metalloproteinase 14 (MMP14); membrane-type matrix matalloproteinase 1 (MTMMP1)	X83536		2585	1339	0.52
Cell death		GO:0008219			
Fas antigen ligand (FASL); apoptosis antigen ligand (APTL; APT1LG1); tumor necrosis factor superfamily member 6 (TNFSF6); generalized lymphoproliferative disease protein (GLD)	U06948		37	21	0.57
Fas antigen; fasL receptor; apoptosis antigen 1 (APO1; APT1); CD95 antigen	M83649		18	20	1.07
Fas death domain-associated protein	NM_007829		392	103	0.26
Fas-associating protein with death domain	NM_010175		484	188	0.39
B-cell leukemia/lymphoma protein 2 (BCL2)	M16506		31	18	0.56
Caspase 9	NM_015733		318	996	3.14
Anti-apoptosis					
Insulin-like growth factor 1 receptor alpha subunit (IGFIR-alpha)	U00182	GO:0006916	95	176	1.86
Inflammatory response		GO:0006954			
Tumor necrosis factor	NM_013693		130	275	2.11
Tumor necrosis factor (ligand) superfamily, member 4	NM_009452		98	227	2.32
Tumor necrosis factor (ligand) superfamily, member 7	NM_011617		133	239	1.80
Tumor necrosis factor (ligand) superfamily, member 8	NM_009403		189	514	2.73
Tumor necrosis factor (ligand) superfamily, member 9	NM_009404		278	305	1.10
Tumor necrosis factor (ligand) superfamily, member 19	NM_011615		164	88	0.53
Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A); tumor necrosis factor receptor 1 (TNFR1)	X57796		58	41	0.72
Tumor necrosis factor receptor superfamily member 1B2 (TNFRSF1B2); tumor necrosis factor receptor 2 (TNFR2)	M59378		18	17	0.92
Interleukin 6 receptor alpha subunit (IL6R-alpha; IL6RA)	X51975		57	64	1.13
Cytokine activity		GO:0005125			
Interleukin 6 (IL6)	X06203		36	25	0.70
Oncostatin M (OSM)	D31942		79	509	6.42
Cellular metabolism		GO:0044237			
PDC-E2			NT	NT	
PDC-E3BP			NT	NT	
Transport		GO:0006810			
Solute carrier family 4 (anion exchanger), member 2	NM_009207		80	145	1.82
Solute carrier family 4 (anion exchanger), member 3	NM_009208		52	120	2.31
Solute carrier family 4 (anion exchanger), member 1	NM_011403		995	2146	2.16
Voltage-dependent anion channel 1	NM_011694		111	146	1.32
Voltage-dependent anion channel 2	NM_011695		87	62	0.71
Solute carrier family (organic anion transporter) member 1	NM_013797		76	114	1.50
Aquaporin 1	NM_007472		257	245	0.95
Aquaporin 2	NM_009699		198	276	1.39
Aquaporin 3	NM_016689		124	204	1.64
Aquaporin 5	NM_009701		396	222	0.56
Aquaporin 8	NM_007474		2160	8728	4.04

Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	NM_011387		208	402	1.93
G-protein coupled purinergic receptor P2Y1 (P2RY1)	U22829		346	652	1.89
Purinergic receptor P2X, ligand-gated ion channel 4	NM_011026		711	2229	3.13
Detection of Stimulus		GO0051606			
Toll-like receptor 2	NM_011905		95	68	0.72
Toll-like receptor 5	NM_016928		168	114	0.68
Toll-like receptor 6	NM_011604		255	569	2.23
Cell proliferation		GO:0008283			
FMS-like tyrosine kinase 1 (FLT1); vascular endothelial growth factor receptor 1 (VEGFR1)	L07297		36	23	0.65
Epidermal growth factor (EGF)	J00380		88	116	1.32
Kinase insert domain receptor (KDR); vascular endothelial growth factor receptor 2 (VEGFR2); FLK1	X70842		93	58	0.62
Vascular endothelial growth factor (VEGF); vascular permeability factor (VPF)	M95200		165	152	0.92
Heparin-binding growth factor 5 (HBGF5); fibroblast growth factor 5 (FGF5)	M30643		355	152	0.43
Fibroblast growth factor receptor 3 (FGFR3); heparin-binding growth factor receptor (HBGFR)	M81342		192	485	2.53
Transforming growth factor beta 1 (TGF-beta 1; TGFB1)	M13177		69	105	1.51
Transforming growth factor beta receptor 1 (TGF-beta receptor 1; TGFR1); ESK2	D25540		44	29	0.65
Acetylcholine receptor M3	NM_033269		NT	NT	
Cell cycle		GO:0007049			
Proliferating cell nuclear antigen (PCNA); cyclin	X53068		211	124	0.59
Cell growth					
Estrogen receptor 1 (ESTR1); estrogen receptor alpha (ER-alpha; ESTRA)	M38651	GO:0016049	22	33	1.48
Others					
Nerve growth factor receptor	AF105292	GO:0007411	N.T.	N.T.	
Neurotrophic tyrosine kinase receptor type1	XM283871	GO:0042490	N.T.	N.T.	
Neurotrophic tyrosine kinase receptor type 2	M33385	GO:0042490	93	18	0.20
Neurotrophic tyrosine kinase, receptor, type 3	NM_008746	GO:0042490	2923	3827	1.31
Interferon gamma receptor (IFN-gamma receptor; IFNGR)	M28233	GO:0005615	51	50	0.98

The entire dataset obtained from the Atlas Glass Array mouse (Clontech, Takara Bio Inc., Shiga, Japan) was analyzed by ArrayGauge software (Fuji Photo Film Co., Ltd., Tokyo). [1]gene ontology, [2,3]spot intensities of all samples from small or large cholangiocytes.

rapid regeneration of hepatocytes in cases with acute liver injury. The proliferation of cholangiocytes that potentiate the regeneration of bile ducts occurs in a characteristic manner, *i.e.* secretin, somatostatin or bile duct ligation results in proliferation of large cholangiocytes[20], whereas chemical injury of bile ducts with CCl_4 increases the numbers of small cholangiocytes[21,22]. Therefore, to clarify the mechanisms sustaining cholangiocyte growth it is essential to understand the regulation of bile duct regeneration. We identified an Eph receptor, a membrane bound-type tyrosine kinase, as one of the key molecules for reorganization/proliferation of cholangiocytes, and a subtype of this family, which is expressed mainly in small cholangiocytes[23]. Thus, the heterogeneous expression profile of cholangiocytes is expected to facilitate further study of these cells.

ANALYSIS OF HETEROGENEOUS CHOLANGIOCYTES

For the evaluation of genes that are expressed by small and large cholangiocytes see Table1. Several liver diseases, including non-alcoholic fatty liver disease[24], liver cirrhosis[25], hepatocellular carcinoma[26,27] and cholangiocellular carcinoma[28] have been studied extensively using microarray techniques. Some studies have examined whole liver samples, consisting of various cell types, probably for the purpose of disease classification. Sample size is critical for the detection of subtle changes in the expression of meaningful genes[29], a possible problem in assessing rare diseases. Another problem in the study of biliary diseases like PBC by microarray is that cholangiocytes account for only 3% of the cell population even in the normal liver[30]. Over 75% of the cell population is regarded as necessary to test the significance of differences in expression levels[2]. Therefore, for the purpose of analyzing chlangiocytes, isolation[31] or microdissection[32] is necessary prior to analysis. Our study goals were to characterize the physiological role of biliary epithelia in the mouse and to analyze cholangiocyte heterogeneity. Due to the complexities of the isolation steps, maintaining quality control or reproducibility was possible when obtaining a large sample of RNA from freshly isolated cholangiocytes. Moreover, given the necessity of conducting further functional assays, we immortalized and subcloned the isolated Balb-c mouse cholangiocytes by introducing the SV40 large T antigen[33]. The established large and small cholangiocyte cell lines were evaluated by their morphologies and responsiveness to secretin. We revealed 230 genes (4.74%) showing different expression patterns in the two cells lines, among 4800 genes tested by combining two types of ready-made microarrays[34]. Our large cholangiocyte line was characterized by gene ontology, transport and immune/inflammatory responses, which were apparent, even without the statistical tests presented in the table. The term

"transport" includes movement of anions, water and bile acids, and thus represents the physiological functions of bile ducts. These observations indicate a special feature of large cholangiocytes which presumably play a role in local immune reactions. In contrast, our small cholangiocytes are categorized into a subgroup characterized by rapid cell cycle turnover as well as poor physiological functional ability. In addition to these fundamental properties, our small cholangiocytes are characterized by abundant expressions of actin and vimentin and poor expression of E-cadherin. Together with rich spindle-type cell processes, small cholangiocytes have a feature in common with mesenchymal cells probably originating from epithelial-mesenchymal transition[35]. This remains a controversial issue. Another important feature of cholangiocyte is its capability of the responsiveness to hormones and neuropeptides. Specifically, estrogen receptor[36] and receptor for IGF-1(insulin like growth factor 1)[37], NGF(nerve growth factor)[38] and acetylcholine (M3)[39] have been shown to play a major role in modulating cholangiocyte proliferation. Estrogen receptor expresses at a minimal level in both types of cell lines. In contrast, IGF receptor was preferentially expressed in large cholangiocyte line in our microarray study. The expression of estrogen receptor inducible under pathological conditions like bile duct injury[40] may explain the discrepancy between the experiments results. Predominant expression of IGF receptor in large cholangiocytes may be a marker of differentiated biliary epithelial cells as well as a proliferating effector of maturated cholangiocytes.

CONCLUDING REMARKS

Microarray is a powerful tool for elucidating functional cholangiocyte heterogeneity. Although the evaluation of some crucial biological regulatory processes like protein modification requires methodologies other than microarray, the potential of microarray technology is anticipated to grow with the development of data-analysis theory for the comprehension of complex networks.

ACKNOWLEDGMENTS

We thank Hiroyuki Izu (Center for Functional Analysis of DNA, TAKARA BIO Inc.) for his technical advice on the microarray.

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