肝内胆管細胞のheterogeneity(多様性)についてのプロテオーム解析

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平成16～17年度科学研究費補助金(基盤研究(C))　研究成果報告書

平成18年3月

研究代表者　上野　義之

(東北大学・病院・講師)

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はしがき

PBC の標的細胞は胆管上皮細胞であるが、同細胞の解析は遅れており、最新の手法を用いて、その多様性(heterogeneity)や、病的分子の表出を検討することにより、新たな治療法の標的となる分子の探索を行うことを目的とした。

A. 研究方法

平成16年度は主に生体肝移植の摘出肝および正常動物より分離された胆管細胞を用いて、μアレイ、プロテオミクス法といった最新の技術を用いて、胆管細胞特異的に発現される蛋白の探索を行った。人組織からの分離は、手術件数の減少のため、基礎条件設定に難渋したが、それを補うべく動物モデルを用いて、基礎検討を進めた。特に胆管細胞の多様性に寄与する分子に着目し、そのうちの一つとして ephrin が挙げられることを明らかにした。また、μアレイ法によりレトロウイルスのエントリーに関与する蛋白が小葉間胆管特異的に発現されることを見出したため、逆転写酵素阻害剤を用いた二重盲検試験を組織し、実際の PBC 患者に投与した。平成17年度は、AMA 産生を伴う胆管炎動物モデルから胆管細胞を分離し、AMA の産生と関連する蛋白の探索を行った。

B. 研究結果

平成16年度の研究より小型胆管により優位に発現される蛋白の一つとして、EphA5 が見出された。膜蛋白である Eph 受容体 family はリガンドである ephrin 刺激によるチロシンキナーゼの活性化を介する系と、非刺激下で Eph 受容体に結合する RhoGEF 等を介する系による Rho ファミリーの調節を経て、アクチンの構築を制御していると考えられており、神経突起、血管新生、細胞の malignant transformation に関与している。NMC-S、NMC-L 上の EphA member の発現と局在を検討し、細胞運動、形態に与える機能を検討するために、小型、大型肝細胞上の EphA 発現を RT-PCR, direct sequence で確認し、免疫染色、共焦点レーザー顕微鏡で局在を検討した。EphA の機能の検討にはリガンドである ephrinA5-Fc キメラを用い、糸状仮足の形態を観察した。糸状仮足の形成を膜透過性の cyclic AMP analogue である N6, 2'-O-dibuityryladenosine 3': 5'-cyclic (dcAMP)刺激下で検討した。その結果、EphA8 は大型、小型の胆管細胞双方に認められたが、EphA5 は小型胆管細胞に強く発現が認められた。EphrinA5 は糸状仮足を有意に退縮させた($p<0.01$)。EphA5 の発現は主に糸状仮足に局在し、細胞質にも一部認められた。さらに EphA8 は細胞質と葉状仮足に認められた。dcAMP 刺激により、EphA5、EphA8 の membrane 上への translocation が認められ、小型胆管細胞の糸状仮足の伸展が認められたが、大型胆管細胞には認められなかった。さらに、今回新たに開発した胆管細胞の分離法が、純度の高い胆管細胞を得る方法として有望であることを確認した。

また、今回用いた手法を応用して、胆管細胞の多様性や、細胞特性についての共同研究をまとめて発表した。

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研究代表者　　上野　義之　　(東北大学・病院・講師)

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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 regarding secretory, proliferative and apoptotic activities. Distinct expression profiles of two murine cholangiocytes lines, termed small and large were 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 lines. Namely large cholangiocytes are characterized as "transport" and "immune/ inflammatory responses". In contrast to large, 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 to understanding the heterogeneiety of cholangiocytes.

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. 30000-40000 genes expressed in mammalian cells). Therefore, it is essential for future advances to reliably preserve a database containing all of the raw data. The establishment of the 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 the 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 of 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 a 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 20-100μm in diameter and lined by cuboidal epithelium), and septal bile ducts (which are more than 100μm 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 were suspected to play a major role in this type of destruction. Tetramer technology revealed 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 cholangiocytes 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 of regenerating the bile ducts i.e. with normal structures, in contrast to the rapid regeneration of hepatocytes in cases with acute liver injury. The proliferation of cholangiocytes that potentiates 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 is essential to understanding the regulation of bile ducts 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 profiles of cholangiocytes are 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 scale of sample 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 of 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 controversial issue. Another measure feature of cholangiocyte is the 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 minimal level in amount 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.

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Legends of the table

Differences in cDNA expression between small and large cholangiocyte lines

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

GO[b] annotation/ Gene name	Gene accession	GO accession	Small[a]	Large[a]	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 (Uvm)	X06115		32	42	1.29
Cytoskeleton		GO:0005856			
cytoplasmic beta-actin (ACTB)	M12481		455	275	0.60
vimentin (Vim)	X51438		260	73	0.28
Angiogenin		GO:0001525			
matrix metalloproteinase 14 (MMP14); membrane-type matrix metalloproteinase 1 (MTMMP1)	X83536		2585	1339	0.52
Cell death		GO:0006915			
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		382	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	986	3.14
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 10	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					
PDC-E3BP					
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		985	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		306	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	662	1.89
purinergic receptor P2X, ligand-gated ion channel 4	NM_013526		711	2229	3.13
Detection of Stimulus		GO:0051606			
toll-like receptor 2	NM_011905		85	58	0.72
toll-like receptor 5	NM_016928		169	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		166	152	0.92
heparin-binding growth factor 5 (HBGF5); fibroblast growth factor 5 (FGF5)	M30643		356	152	0.43
fibroblast growth factor receptor 3 (FGFR3); heparin-binding growth factor receptor (HBGFR)	M81342		192	486	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; TGFBR1; ESK2	D25540		44	29	0.65
Cell cycle		GO:0007049			
proliferating cell nuclear antigen (PCNA); cyclin	X53068		211	134	0.64
Others					
interferon gamma receptor (IFN-gamma receptor; IFNGR)	M28233		61	30	0.49