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In vivo dynamic metabolic imaging of obstructive cholestasis in mice

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Li FC, Liu Y, Huang GT, Chiou LL, Liang JH, Sun TL, Dong CY, Lee HS. In vivo dynamic metabolic imaging of obstructive cholestasis in mice. *Am J Physiol Gastrointest Liver Physiol* 296: G1091–G1097, 2009. First published February 26, 2009; doi:10.1152/ajpgi.90681.2008.—We tried to image obstructive cholestasis by using a newly developed imaging system to measure the alterations of hepatobiliary function in living mice with their bile ducts ligated. A hepatic imaging window was installed on the upper abdomen soon after the mice underwent ligation of the common bile duct. On the next day, the mice received intravenous injection of rhodamine B isothiocyanate-dextran and carboxyfluorescein diacetate. The later would be transformed into fluorogenic carboxyfluorescein (detected at ~500–550 nm) by hepatocytes and then excreted into bile canaliculi. The images were acquired by multiphoton microscopy. The fluorescence intensities at ~500–550 nm within hepatocytes or sinusoids were measured in time series. In mice with bile duct ligation, bile canaliculi failed to appear during the whole observation period over 100 min following carboxyfluorescein diacetate injection, whereas the fluorescence was retained much longer within sinusoids. Furthermore, the fluorescence intensities in sinusoids were persistently higher than in hepatocytes during the course. Bile duct ligation impedes hepatocytes to excrete carboxyfluorescein into bile canaliculi. The kinetics of fluorescence intensities in hepatocytes and sinusoids indicated there is an active machinery operating backflow of this fluorogenic bile solute from hepatocytes into sinusoids in the liver with obstructive cholestasis.

multiphoton microscopy; hepatocyte transporter; fluorescence imaging; common bile duct ligation

OBSTRUCTIVE CHOLESTASIS is caused by a structural or mechanical obstruction of bile flow at the extrahepatic bile duct. The common etiologies include choledocholithiasis and malignancies such as cholangiocarcinoma and pancreatic or periampullary cancer. It is a serious surgical illness that may lead to jaundice and even death unless the obstructive lesion is removed or the bile duct is bypassed (4). The pathophysiology of obstructive cholestasis is best characterized in animals studied by common bile duct ligation (CBDL). Ligation of the common bile duct would impair bile formation and excretion into intestine. This impairment results in elevated levels in blood of several bile solutes, including cholesterol, bile acids, and bilirubin, that are normally excreted into bile (8).

Bile excretion is part of hepatobiliary function, which is a major function of the liver to remove various organic anionic compounds from circulation. These anions are represented by bilirubin, bile acids, and a variety of xenobiotics. Sinusoidal

uptake and canalicular export of these substrates involve two respective sets of transporter systems. The former set on the basolateral (sinusoidal) membrane of hepatocytes includes sodium-taurocholic acid cotransporting polypeptide (NTCP) for taking up bile acids and organic-anion transporting polypeptides (OATPs) for most amphipathic organic compounds. The latter set on the apical (canalicular) membrane of hepatocytes consists of an array of ATP-binding cassette transporters, each with differential substrate: for example, multidrug-resistance-1 P-glycoprotein for mediating canalicular excretion of bulky lipophilic cations (e.g., various drugs), bile salt export pump for monovalent bile acids, and multidrug resistance protein 2 (MRP2) for divalent bile acids, and a wide range of amphipathic anionic substrates including bilirubin diglucuronide (7, 13). Obstructive cholestasis would induce marked changes in the expression of these transporters in the liver. After CBDL in the rat model, it has been shown that Ntcp, Oatp1, and Mrp2 were downregulated (5, 6, 12). Downregulation of Ntcp and Oatp1 may protect hepatocytes from further taking up organic anionic substrates, whereas downregulation of Mrp2 renders hepatocytes to accumulate potential toxic substrates that are normally excreted into bile canaliculi through MRP2. In contrast, Mrp3 and Mrp4 were upregulated in the liver after CBDL (2, 3, 11). Because of the overlapping substrate specificities between MRP2 and MRP3 and also the immunolocalization of MRP3 at basolateral membrane of hepatocytes after CBDL, it has been postulated that MRP3 may function as a basolateral pump to clear anionic substances into sinusoids as compensatory machinery for impaired MRP2 in CBDL (3). In an Mrp4^{−/−} mouse model, CBDL induced greater liver injury and less elevated level of serum bile acid than in wild-type mice (10). The later data suggested that MRP4 might protect liver tissue from damage presumably induced by accumulation of bile acids in hepatocytes after CBDL. This “overflow” pumping machinery has never been proven, probably because of several technical difficulties. First, although cell culture system is a convenient method to look at the canalicular transport of fluorogenic organic anions, bile duct ligation could not be well simulated in this simple cell culture system. Second, the ex vivo liver perfusion method may theoretically be used to examine the liver after CBDL, but the signal information in the sinusoids will be lost owing to a substitution of normal portal circulation by artificial perfusates.

We have successfully developed a technique capable of examining hepatobiliary excretory function in living mice by using multiphoton microscopy through a newly designed hepatic imaging window (9). A nonfluorogenic substance carboxyfluorescein diacetate (CFDA) was used as the substrate. After being taken up by cells, CFDA was hydrolyzed by esterase into fluorogenic carboxyfluorescein (CF), which emit-

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ted fluorescence at 517 nm (1). In this report, we measured the changes of the kinetics of CF fluorescence intensities within hepatocytes and sinusoids, respectively, in mice after CBDL.

MATERIALS AND METHODS

Animal experimental procedures. C57BL/6 mice beyond 5 wk of age were obtained from Laboratory Animal Center, National Taiwan University College of Medicine. CBDL surgery was performed on mice 1 day before their multiphoton microscopic imaging experiments. They were anesthetized by intraperitoneal injection of 2-2-2 tribromoethanol (0.35 mg/g body weight), and laparotomy was performed to ligate both cystic duct and common bile duct. Ligation of cystic duct was to avoid overdilation of gall bladder, which would push the underlying liver surface away from the central light path of the hepatic imaging window on the next day's imaging experiments. Following CBDL, the mice were installed with hepatic imaging window on the upper abdomen as previously described (9). They recovered awaiting imaging experiments the next day. The hepatic imaging window used in the present study is modified from previous one (9), and hence the second generation window. This new window consists of only one doughnut-shaped titanium ring (Fig. 1A). At the center of the window, a 10-mm round coverglass was glued on the window, leaving the underneath liver surface forced protruded to lightly touch the coverglass (arrowheads in Fig. 1B). This liver surface area served for image acquisition through multiphoton microscopy. A major advantage of the second generation window is allowing the usage of oil immersion objectives and hence allowing the acquisition of high-magnification and high-resolution images. Figure 1C shows a diagram illustrating the microscopic observation on the liver surface through the hepatic window. Figure 1D shows serial photos of fixing the window and mouse on the microscopic stage. The control mice were those in which the imaging window was installed but CBDL surgery was not performed. Five mice each were included in the control and CBDL groups. Four in each group were used for mul-

tiphoton microscopic experiments, and the fifth in each group was used for liver RNA extraction.

Multiphoton microscopy. The settings of the multiphoton microscope were as previously described (9). A titanium-sapphire laser with 780-nm output (Tsunami, Spectra-Physics, Mountain View, CA) pumped by a diode-pumped solid-state laser (Millennia X, Spectra-Physics) was used for excitation. The laser beam was scanned by an x-y mirror scanning system (model 6220, Cambridge Technology, Lexington, MA) and guided toward the modified inverted microscope (TE 2000, Nikon, Tokyo, Japan). Then the laser beam was focused via an objective (SFluor $\times 40$ oil immersion, numerical aperture 1.3, Nikon) into the sample in vivo. Finally, the emission was collected in the epi-illuminated or backscattering geometry and then separated into four simultaneous detection channels by serial dichroic mirrors (435DCXR, 495DCXR, 555DCLP, Chroma Technology, Rockingham, VT) and additional band-pass filters (HQ390/20, HQ460/50, HQ525/50, HQ590/80, Chroma Technology). For fluorescein injection, an indwelling catheter (PE-10) was inserted into right jugular vein and stretched out the body from posterior neck right before imaging experiments. After the mouse was fixed on the stage, rhodamine B isothiocyanate-dextran 7000 (10 mg/mouse, Sigma, Saint Louis, MO) was injected intravenously for labeling vessels. This labeling would persist for longer than 5 h (personal observations) and thus the vascular patterns were always used as landmarks. To image hepatobiliary function, 50 μ g of CFDA (Sigma) in 100 μ l of normal saline was intravenously injected. The depth of imaging area is ~ 30 μ m under liver capsule. Fluorescence images were acquired on the fixed microscopic areas serially at 1-min intervals. Zero time was prior to CFDA injection. The fluorescence signals at ~ 500 – 550 nm and ~ 550 – 630 nm were recorded. They were then pseudocolored into green and red, respectively. The earlier signal would detect CF and the later one would detect rhodamine. Image J software (National Institute of Health, Bethesda, MD) and MetaMorph (Universal Imaging, Downingtown, PA) were used for image processing. The procedures of animal experiments were approved by the Institutional

Fig. 1. A: diagrams illustrating the structure of the second generation hepatic imaging window. The diameters and thickness are shown. B: a close view of a hepatic window installed on the upper abdomen of a mouse. White arrowheads indicate underneath liver lightly attaching the under surface of the coverglass at its central part. This area served for intravital microscopic examination of the liver. C: a diagram showing the spatial relationship between liver, hepatic window, and microscopic objective during microscopic examination. D: serial photos showing how to fix the mouse with its installed hepatic window on the microscopic stage. A set of parallel recesses on the hepatic window was fitted into the U-shaped groove of a square plate. The other set of parallel recesses was then fixed by a smaller plate inserted into the groove. The square plate with the mouse was then turned over and screwed on a round plate, which has a central hole of 8 cm in diameter. The round plate was then put on a microscopic stage that has been trimmed to hold the round plate well.

Animal Care and Use Committee, National Taiwan University College of Medicine.

Dynamics of CF distribution in the hepatocyte and sinusoid compartments. To trace the dynamics of CF fluorescence ($\sim 500\text{--}550\text{ nm}$) within both hepatocyte and sinusoid compartments, we arbitrarily circled five small areas, respectively representing each compartment in each image series. For circling, the hepatocyte nuclei, bile canaliculi, and the supposed stellate cells (asterisks and yellow and white arrows, respectively, in Fig. 2, A and B) were kept away to avoid underestimating or overestimating, respectively, fluorescence intensities in either compartment (Fig. 3A). The fluorescence intensity at $\sim 500\text{--}550\text{ nm}$ per pixel in each circled area was measured and averaged. Before starting the experiment of injecting CFDA, we searched and picked an optimal microscopic area based on the landmarks of sinusoid images at $\sim 550\text{--}630\text{ nm}$, regardless of the depth from liver surface. Because of scattering effect, the fluorescence intensities get dimmer when the distance from the surface for image acquiring goes deeper. Uncontrollable variation between animals in the depth from liver surface for imaging would make the detected fluorescence intensity incomparable. The absolute intensities were not reliable to be compared between animals. Nevertheless, they were comparable between different compartments in the same images and hence the comparisons between hepatocyte and sinusoid compartments were expressed as ratio of intensities in hepatocyte to sinusoid fluorescence at each time point in individual animals and then averaged in each group (as in Fig. 3C). To compare the trends of intensity changes in either compartment between two groups, we have no choice but to normalize the data within each group before plotting and comparing (as in Fig. 3, D and E).

Total RNA extraction and RT-PCR. Total RNA of the liver was extracted by using Rezol reagent (Protech Technology, Taipei, Taiwan) with homogenization. The lysates were extracted with chloroform, and total RNA was precipitated with isopropanol. One microgram of total RNA was reverse transcribed by use of random hexamers and reverse transcriptase. The first-strand cDNA product was subjected to PCR using oligonucleotide primer pairs (Table 1). The PCR protocol was an initial denaturation at 95°C for 3 min and followed by 30 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 45 s. PCR products were analyzed by electrophoresis in 2% agarose gels containing ethidium bromide and visualized with a charged-coupled device camera.

Statistics. The data of normalized fluorescence intensities in hepatocytes and sinusoids and the ratio of intensities in hepatocyte to sinusoid fluorescence (RHS) were presented as means. A linear mixed model was implemented. A model including group, time, and an interaction term consisting of group and time was established to examine the effects of group and time; meanwhile, by the significance of the interaction term, we confirmed whether the effect of time was similar for both groups or not. All statistics were performed by SAS software, and the PROC MIXED procedure was applied (version 9.1.3, SAS, Cary, NC). A P value less than 0.05 was considered statistically significant.

RESULTS

A time series of images from a representative control mouse is shown in Fig. 2A and in Supplementary Movie S1. The uptake of CFDA and hydrolysis into CF occurred within 1 min after CFDA injection (Fig. 2A2) as the green fluorescence in hepatocytes began to appear at 1 min (green arrows in Fig. 2, A2 to A4). The sinusoidal veins containing rhodamine dextran were constantly shown red (red arrows). Blood cells flowing in sinusoids could be clearly seen as dark dots or lines. The green CF began to be translocated into bile canaliculi beginning at 5 min (yellow arrows in Fig. 2, A3 to A5) and nearly completely disappeared from any compartment of the liver after 50 min

(Fig. 2A6). In contrast, the green fluorescence was retained in hepatocytes for as long as 92 min, and bile canaliculi did not appear at any time point in a representative CBDL mouse (Fig. 2B and Supplementary Movie S2). More interestingly, the green fluorescence in the sinusoidal veins was more intense than in the hepatocytes. This is more obviously shown in the later stage as shown in Fig. 2B6; in that image only the original $\sim 500\text{--}550\text{ nm}$ signal was displayed and pseudocolored green. Higher signals were found in the sinusoidal veins (red arrow) than in hepatocytes (green arrow). There were scattered bright fluorescent spots with wide fluorescence spectrum around the sinusoids (white arrows in Fig. 2, A1 and B1). These were supposed to be stellate cells whose cytoplasm contains vitamin A, which would be strongly autofluorescent.

The average intensities per pixel of five arbitrarily selected areas of hepatocyte and sinusoid compartments at each time point of four control and four CBDL mice was plotted in Fig. 3B. The results were in consistence with the findings based on images shown in Fig. 2. In normal control mice, the green fluorescence was nearly completely cleared from both hepatocytes and sinusoids within $\sim 50\text{--}60\text{ min}$. In CBDL mice, by contrast, retention of the green fluorescence, especially in sinusoid compartment, persisted within the whole observation period of 100 min. More interestingly, in CBDL mice the green fluorescence was more intense in sinusoidal veins than in hepatocytes at most time points. The intensities in sinusoids frequently oscillated much more than those in hepatocytes, probably because of variable intermittent appearance of blood cells within the circled areas.

Interactions of fluorescence intensities between hepatocyte and sinusoid compartments in control and CBDL mice can be best illustrated in Fig. 3C showing the trend of RHS in both groups. The RHS was persistently higher in control than in CBDL mice. In control mice, the RHS quickly reached a maximum between ~ 3 and 3.5 within 2 min and then declined to and stabilized at ~ 1 after 60 min. These data indicated the intensities in the hepatocyte were always higher than those in the sinusoid compartment at any time point. The ratios that declined to around 1 after 60 min might represent background signals within both compartments. In contrast, the RHS never reached 1 in CBDL mice and declined to be less than 0.5 after around 30–40 min. These indicated persistently higher intensities in sinusoids than in hepatocytes during the whole observation periods. Statistically, the difference in RHS between control and CBDL group was significant ($P < 0.001$). Also, the descent of RHS was significantly correlated to increasing time ($P < 0.001$). Furthermore, the RHS of CBDL group declined more slowly ($P < 0.05$) compared with the control group.

The time trend of normalized fluorescence intensities in hepatocytes and sinusoids is shown in Fig. 3, D and E, respectively. For normalization, the mean intensities of five selected areas in each compartment of one image series were normalized to give a value of 1 to the maximum intensities. Then the means of the normalized values of the four mice in each group were normalized again to give a value of 1 to the maximum. The last values were plotted in Fig. 3, D and E. For the control group, the average of normalized fluorescence intensities rose from 0.40 in the beginning to 0.95 at 5 min and then started to decline. The average value was 0.15 at 50 min and 0.07 at the end. For the CBDL group, the means were 0.19 at baseline, 0.97 at 9 min, 0.34 at 50 min, and 0.21 at the end.

A **B**

Fig. 3. Kinetics of carboxyfluorescein (CF) intensities in the livers. **A:** example showing arbitrarily circled areas in hepatocytes and sinusoids, respectively. Only fluorescence signals at $\sim 500\text{--}550\text{ nm}$ that would be measured are displayed in the image. Black circles are for hepatocytes, white circles for sinusoids. Scale bar indicates $20\text{ }\mu\text{m}$. **B:** time courses of the green fluorescence intensities in sinusoids and in hepatocytes of control (top 4) and CBDL (bottom 4) mice. Each value represents mean of 5 circled areas. The zero time indicates measurement before injection of CFDA. **C:** time trend of the ratio of intensities in hepatocyte to sinusoid fluorescence. **D:** time trend of normalized fluorescence intensities in hepatocytes. **E:** time trend of normalized fluorescence intensities in sinusoids. Data in **C**, **D**, and **E** are presented as means.

Overall, the fluorescence intensities in hepatocyte area statistically declined ($P < 0.001$) with time; besides, the normalized fluorescence intensity for the CBDL group was statistically higher ($P < 0.001$) than that of control group. However, no interaction between group and time existed. The downward trend in both groups was not statistically significant ($P = 0.2059$). The time trend of normalized fluorescence intensities in the sinusoids is shown in Fig. 3E. It shows that CBDL group had statistically higher ($P < 0.001$) intensities compared with the control group. The averages of normalized intensities were 0.45 for the control and 0.49 for the CBDL group in the beginning, reached peaks of 0.84 and 0.87 at 6 and 11 min, respectively, dropped to 0.35 and 0.66 at 50 min, and then ended up with 0.16 for the control and 0.61 for the CBDL group. The normalized fluorescence intensities significantly declined ($P < 0.001$) as time increased for both groups.

Notably, the interaction between group and time was statistically significant ($P < 0.001$); that is, the decrease of normalized fluorescence intensities in the CBDL group was significantly slower ($P < 0.001$) than that in the control group.

Because hepatocyte membrane transporters play key roles in the uptake and excretion of bile solutes, we then examined the expression of Oatp1, Oatp2, Mrp2, Mrp3, and Mrp4 in the liver of a CBDL mouse. The results clearly showed downregulation of Oatp1 and Mrp2 but upregulation of Mrp4 in the liver of the CBDL mouse (Fig. 4).

DISCUSSION

Taking advantage of our newly developed imaging system in observing hepatobiliary function in living mice, we recorded in the present study the dynamics of a fluorogenic bile solute in two

Fig. 2. Time series of multiphoton images from 1 representative normal mouse (**A**) and 1 common bile duct ligation (CBDL) mouse (**B**). The numbers in minutes indicate time after intravenous injection of CFDA. The sinusoids were marked by intravenous injection of rhodamine dextran (detected at $550\text{--}630\text{ nm}$) and shown red. White arrows, stellate cells; red arrows, sinusoids; green arrows, hepatocytes; yellow arrows, bile canaliculi; asterisks, nuclei of hepatocytes. The dark shadows in sinusoids were circulating blood cells. Bile canaliculi never appeared in CBDL mice. Each scale bar indicates $20\text{ }\mu\text{m}$.

Table 1. Sequences of RT-PCR primers

Gene	Primers	Size, bp
Oatp1	Forward: 5'-TGTGTAAGAAAGGCCCTGAGT-3' Reverse: 5'-TCTCGACCTTAGGACTTCTGC-3'	489
Oatp2	Forward: 5'-TCATACATGTCAGCTTGCCTC-3' Reverse: 5'-TTCCTTGGCAGTGAGCTTC-3'	567
Mrp2	Forward: 5'-GAGGATGAATCTCGACCCCTT-3' Reverse: 5'-TCTGTGCCCTATGACACAGT-3'	544
Mrp3	Forward: 5'-CAGGACCTGAAGTGGTAAAGC-3' Reverse: 5'-CACAAGTCAAAGTAGCGCCT-3'	307
Mrp4	Forward: 5'-CCGCTACACATTCAGTGATT-3' Reverse: 5'-CCCGTTTGATTCCAGCTT-3'	378
β-actin	Forward: 5'-GCCTTCCTTCTTGGGTATGG-3' Reverse: 5'-ACGCAGCTCAGTAACAGTCC-3'	361

microscopic liver compartments, i.e., hepatocytes and sinusoidal veins. In previous studies involving obstructive cholestasis induced by CBDL, kinetic changes of organic anions in these two microscopic compartments have never been demonstrated because of technical inaccessibility.

Our second generation liver window capable of using high power oil immersion microscopic objective enabled us to get optic biopsy images of high magnification and high resolution. In this study we showed again a quick processing of CFDA in the livers of normal mice as reported previously (9). The hepatocyte nuclei, bile canaliculi, and blood cells in the sinusoids could be clearly demonstrated (Fig. 2A). The fixation of the mice on the microscopic stage was steady enough to get stable images during the experimental periods. All these characteristics of this system enable us to describe and compare the dynamic changes of CF in two microscopic liver compartments: the hepatocyte and sinusoid.

In CBDL mice, our present data clearly showed a failure of CF excretion into bile canaliculi (Fig. 2B) and also a delayed clearance of this anion out of the liver (Figs. 2B and 3, D and E). In control mice, the intensities were always higher in hepatocytes compared with sinusoids, especially in the initial periods (Fig. 3, B and C). This may be explained by a relative tendency for hepatocytes in normal mice to keep the anion within for further processing and excretion into bile canaliculi. In contrast, the CF intensities in sinusoids were persistently higher than those in hepatocytes in CBDL mice (Figs. 2B and 3C). It should be noted that the intensities in the sinusoids might be underestimated because nonfluorogenic blood cells might appear time to time in the circled areas. Despite this fact, there still showed higher intensities in the sinusoids of CBDL mice. Provided the fluorescence intensities were positively correlated with CF concentrations, our findings open a question of how this disequilibrium of the solute movement between these two compartments resulted. To address this question, we examined the expression of membrane transporter genes by RT-PCR. Consistent with previously reported results in rats, our data showed a downregulation of Oatp1, Mrp2, and to a less extent Oatp2 in the liver of the CBDL mouse. We did not see obvious change in the expression of Mrp3, but Mrp4 was markedly upregulated (Fig. 4). These findings of gene expression may lead to a hypothesis that, in CBDL-induced obstructive cholestasis, mechanical impedance of bile flow with consequence of downregulation of canalicular excretion transporter Mrp2, hepatocytes would develop two mechanisms for

reducing toxic bile solutes inside. The first is reducing uptake of bile solutes from sinusoids by reducing inward transporters Oatp1 and Oatp2; the second is increasing outward transporter Mrp4. The net results will be keeping more solutes in the sinusoids and hence in the circulation. As CFDA become fluorogenic CF after hydrolysis in hepatocytes (1), the higher signals within the sinusoids in the livers of CBDL mice more importantly resulted from the second mechanism, the overflow machinery.

Though retarded, the CF fluorescence did gradually decline in both hepatocytes and sinusoid veins in CBDL mice (Fig. 3, B, D, and E), suggesting that alternative but less efficient routes may be responsible for the compensating excretion of this organic anion in obstructive cholestasis. Cells other than hepatocytes may also metabolize CFDA and excrete its metabolite CF (14). Renal compensation is most likely the salvage excretion pathway (10).

One thing to note in the mouse experiments is the necessity of simultaneous ligation of cystic duct in addition to ligation of CBD; otherwise marked distention of gallbladder would occur the next day when only CBD ligation was performed. The distended gallbladder would push away the liver surface on the coverglass for microscopic imaging. Furthermore, mice died soon after CBDL, usually before 72 h. This prohibited us from studying the effects of CBDL after longer periods of time. But nonetheless our present data support previous biochemistry and molecular works hypothesizing an overflow pumping machinery after CBDL (2, 3, 10).

Fig. 4. RT-PCR analysis of the expression of Oatp1, 2 and Mrp2, 3, 4 in the liver of a normal and a CBDL mouse. In the liver of the CBDL mouse, the expression of Oatp1 and Mrp2 was obviously downregulated, whereas Mrp4 was upregulated. Oatp2 and Mrp3 were not significantly changed. Loading of PCR products of β-actin served as internal control.

We proved by this report that our newly developed technique is capable of providing new insights into elucidating alterations of hepatobiliary function in obstructive cholestasis.

GRANTS

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