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In vivo Visualization of Extrahepatic Bile Ducts in Mice Using Indocyanine Green Staining and Its Application in Portal Vein Ligation of the Left Lateral Lobe

Visualización *in vivo* de Conductos Biliares Extrahepáticos en Ratones Mediante Tinción con Verde de Indocianina y su Aplicación en la Ligadura de la Vena Porta del Lóbulo Lateral Izquierdo

Na Yao[1]; Lin Qian[2]; Chubin Luo[1]; Jiayan Yan[1]; Xinrong Yang[1]; Weizhong Wu[1] & Jian Zhou[1]

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SUMMARY: Rodents are frequently utilized in constructing animal models for various liver-related studies, necessitating a comprehensive understanding of liver lobe structures. However, detailed descriptions of the biliary system in mice have been lacking. In this study, we employed intravenous indocyanine green (ICG) injection combined with common bile duct ligation to visualize extrahepatic bile ducts under natural light. We observed that bile duct branches from each lobe typically drained into the superior common bile duct near the gallbladder. Additionally, we addressed the challenge of ligating the portal vein of the left lateral lobe (LLL) in mice, which had not been successfully achieved in previous rodent models of portal vein ligation (PVL) or associating liver partition and portal vein ligation for staged hepatectomy (ALPPS). By examining the liver vasculature structure under a 25¥ microscope with ICG staining, we categorized the LLL portal vein into four patterns. The majority of mice exhibited distinct portal vein branches for the LLL and left middle lobe (LML), contrasting with rats. Selecting the generally or fully exposed types, skilled microsurgeons could effectively ligate the LLL portal vein using our method. On postoperative day 3 (POD 3), the LLL appeared viable without significant necrosis, and the weight ratio of the future liver remnant (FLR) to body weight, as well as the number of Ki67-positive nuclei, were significantly increased, indicating that LLL portal vein ligation induced liver regeneration.

KEY WORDS: Indocyanine green; Bile duct; Portal vein ligation; Liver regeneration.

INTRODUCTION

Rodents are extensively used in constructing animal models for various liver-related studies, including cholestasis, hepatic fibrosis, liver cancer, and surgical research such as hepatectomy, portal vein embolization (PVL), and ALPPS. The rodent liver, like that of other mammals, is multilobulated, with each lobe named after the portal branches that supply them (Lorente *et al*., 1995). Previous studies have described the liver structure in rats, including lobes, arteries, portal veins, hepatic veins, and bile ducts, highlighting the anatomical variations in bile ducts (Martins & Neuhaus, 2007). However, detailed descriptions of the biliary system in mice have been limited.

Indocyanine green (ICG) is a di-sulfonated heptamethine indocyanine with moderate optical properties, primarily binding to albumin and lipoproteins in plasma (Baker, 1966; Gioux, 2010). After intravenous injection, ICG circulates in the bloodstream, is rapidly extracted by the liver, and is eventually secreted into bile (Mitsuhashi *et al*., 2008). Fluorescent intraoperative cholangiography using ICG has been demonstrated as a safe and valuable procedure for visualizing biliary tract anatomy in real-time (Ishizawa *et al*., 2010). This technique has been applied in various clinical settings, such as liver segmentectomy, laparoscopic cholecystectomy, and liver transplantation (Aoki *et al*., 2008).

Liver resection offers the best survival advantage for primary and secondary liver cancers (Abdalla *et al*., 2006). The concept of resectability depends on both oncological clearance and the adequacy of the future liver

[1] Liver Cancer Institute, Zhongshan Hospital, Fudan University; Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Shanghai, P. R. China.
[2] Fudan University Shanghai Cancer Center & Institutes of Biomedical Sciences; Cancer Institutes; Key Laboratory of Breast Cancer in Shanghai; Shanghai Key Laboratory of Radiation Oncology; The Shanghai Key Laboratory of Medical Epigenetics, Shanghai Medical College, Fudan University, Shanghai, P.R. China.
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remnant (FLR) in terms of function and volume (Liu & Zhu, 2009). Given that maintaining stable liver function is crucial for overall body homeostasis (Michalopoulos & Bhushan, 2021), the primary limitation in performing hepatectomy is the insufficient FLR volume. Portal vein occlusion, achieved by PVL or portal vein embolization (PVE), can increase the FLR, thereby reducing the risk of postoperative hepatic failure after hepatectomy (Covey *et al*., 2008). However, there is often a long interval between portal vein occlusion and partial hepatectomy while waiting for a sufficient FLR. In 2011, ALPPS was introduced, generating rapid liver regeneration and reducing waiting time, as well as mitigating the challenges of adhesions and tumor progression (de Santibañes & Clavien, 2012).

To understand how surgical procedures like PVL, PVE, or ALPPS induce regenerative responses, animal models in rodents have been developed (Vorobioff *et al*., 1983; Iwakiri *et al*., 2002; Trenard *et al*., 2014). The left middle lobe (LML) represents approximately 10% of the total liver mass and is typically considered the FLR in procedures mimicking human insufficient FLR scenarios (such as ALPPS, PVL, or extended hepatectomy) (Dili *et al*., 2019). However, the LML shares a common pedicle with the LLL, and some bile ducts of the LML may drain into the LLL (Martins & Neuhaus, 2007), making them more closely connected. Therefore, instead of preserving only the LML portal vein branch, researchers usually ligate the pedicles of the left portion (LML and LLL) (Martins *et al*., 2008). In 2014, Andrea's team established a mouse ALPPS model involving 60% portal vein ligation (of the caudate lobe (CL), right lobe (RL) and right middle lobe (RML)) and a 30% resection of the LLL (Schlegel *et al*., 2014). In 2018, Alexandra's group developed a rat ALPPS model, ligating approximately 90% of the portal vein (of the CL, RL, RML, and LLL) without prior parenchymal resection (Dili *et al*., 2019). In rats, the bifurcation between the LML and LLL often occurs within the LLL parenchyma, and the LLL has two portal vein pedicles (inferior and superior) and two independent venous drainages (Madrahimov *et al*., 2006), rendering the dissection and ligation of the LLL portal vein branch challenging. Additionally, considering the differences in immune function compared to humans and the lack of transgenic tools and antibodies, it is necessary to develop a 90% portal vein ligation model in mice.

MATERIAL AND METHOD

Animals and preoperative anesthesia: Male C57BL/6N mice, approximately 8 weeks old, were obtained from Charles River (Beijing) and housed in a specific pathogen-free environment under a 12-hour light-dark cycle. They were maintained on a standard pellet diet and tap water *ad libitum.* All animal procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and the National Institutes of Health (NIH publication 86-23, revised 1985). Anesthesia was induced by a mixture of 4% chloral hydrate (0.6 ml/20 g body weight) and 1% pentobarbital sodium (0.6 ml/20 g body weight). The abdomen was opened via a midline incision.

Indocyanine green staining of mouse bile ducts: Ten milligrams of indocyanine green was dissolved in 3 ml of water for injection and mixed with an equal volume of normal saline. The intestines and liver lobes were retracted to expose the inferior vena cava, right renal vein, and hepatic vessels. A 0.1 ml ICG solution was injected through a 30 G needle into the right renal vein, directed towards the inferior vena cava and hepatic vessels. Simultaneously, the common bile duct was ligated proximal to the pancreas using 9/0 monofilament nylon thread (Jinhuan, Shanghai). Five minutes post-injection, ICG gradually appeared in the bile ducts and gallbladder. After ten minutes, the bile ducts and gallbladder turned emerald green.

Observation, typing and ligation the LLL portal vein branch: Using an operating microscope (25× magnification, Leica M400 E), the hepatic hilum and the common pedicle of the LML and LLL were inspected. By gently manipulating the LLL parenchyma near the common pedicle with a thin cotton swab, the portal vein, artery, and bile duct branches were clearly identified in the majority of mice, which was significantly different from rats. Based on the depth of the portal vein pedicles' "growth" into the LLL, the LLL portal vein branch was categorized into four types: hidden type, poorly exposed type, generally exposed type, and fully exposed type. The generally or fully exposed types were selected for LLL portal vein ligation. Under the 25¥ microscope, a monofilament nylon thread (9/0) was carefully guided across the hepatic pedicle around the LLL portal vein, ensuring that no visible vessels were included in the suture loop. The suture was then tied to achieve ligation. The sham group underwent laparotomy and liver lobe manipulation without ligation. Finally, the midline laparotomy was closed in two layers using 5/0 silk. The mice were placed in a recovery cage with a heat pad until they regained consciousness.

Assessment of liver growth and cell proliferation: After 72 hours, mice were humanely euthanized. The liver was exsanguinated and carefully removed, with the non-ligated portions (FLR, liver without LLL) being dissected. Liver growth and the restitution of liver mass were assessed by calculating the ratio of FLR weight to body weight. Cell proliferation was evaluated using immunohistochemical (IHC) staining for the proliferation marker Ki67. For IHC

staining, the left middle lobe was fixed in 4% paraformaldehyde for 24 hours, processed, paraffin-embedded, and sectioned at 3 mm. The number of Ki67-positive nuclei was manually counted in 10 random visual fields (200× magnification).

Statistical analysis: GraphPad Prism software (San Diego, CA, USA) was utilized for generating graphs and conducting statistical analyses. Data are presented as mean ± standard error of the mean (SEM). Comparisons were made using the unpaired two-tailed t-test. Statistical significance was set at p values < 0.05, with the following notations: *p < 0.05, **p < 0.01, and ***p < 0.001.

RESULTS

ICG staining the extrahepatic bile ducts: Despite the mouse liver lobe anatomy being highly similar to that of the rat, the smaller and more delicate structure of the mouse has not been as extensively described as that of the rat liver (Martins *et al*., 2008). Five minutes after intravenous injection of ICG, the extrahepatic bile ducts gradually became visible. Due to the ligation of the common bile duct near the pancreas, the concentration of ICG increased, and the extrahepatic bile system turned emerald green. The LLL is most frequently drained by a single biliary branch (Fig. 1a). Occasionally, an additional branch exists at a distance from its portal vein (Fig. 1b). The RML contains one biliary branch (Fig. 1 c, d), which connects to the common bile duct (Fig. 1c) or the biliary branch of LLL (Fig. 1d). The LML shares a common pedicle with the LLL (Rozga *et al*., 1986) and some bile ducts of the LML may drain into the LLL (Martins & Neuhaus, 2008) as mentioned above. ICG staining revealed that the biliary branch of the LML drains into the biliary branch of the LML (Fig. 1e, f). This may be a single branch on the right side of the portal vein (Fig. 1e) or two branches on the left and right sides of the portal vein (Fig. 1f). The Right Lobe (RL) exhibits a high degree of anatomical variation in its biliary branches (Fig. 1g, h, i). Instead of draining into inferior common bile duct nearby (Fig. 1g), the biliary branch of the RL often drains into the superior common bile duct near the gallbladder (Fig. 1h). The RL consists of superior and inferior lobes, each with a branch that joins together to form a common branch before draining into the common bile duct. These branches often join within the RL (Fig. 1g, h), but sometimes they join outside (Fig. 1i). The biliary branches of the CL also show a high degree of variation (Fig. 1j, k, l). It is rare for the biliary branch of the CL to drain into the nearby common bile duct (Fig. 1j). More frequently, the biliary branch of the CL drains into the superior common bile duct near the gallbladder (Fig. 1k). Occasionally, the biliary branches of the two portions (anterior/superior and posterior/inferior) of the CL drain into different positions. One drains into the superior common bile duct (Fig. 1l, superior) for the anterior portion, and the other drains into the biliary branch of the RL for the posterior portion (Fig. 1l, inferior).

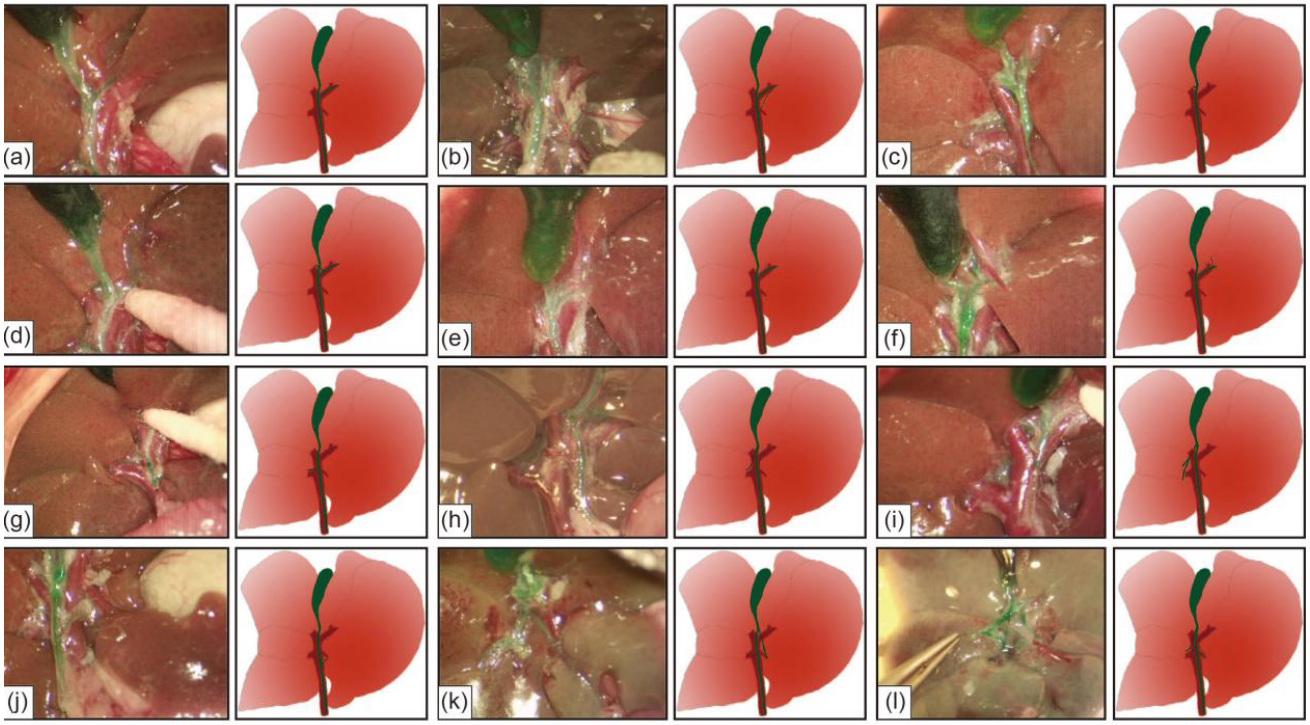

Fig. 1. Mice extrahepatic bile system and schematic diagram. Extrahepatic bile duct branch of LLL (a), (b); RML (c), (d); LML (e), (f); RL (g), (h), (i); CL (j), (k), (l).

Classification of LLL portal vein branches: In our experiments, we encountered a very rare arterial giant variation (Fig. 2a). In this variation, the extrahepatic portal vein is normal, but the portal vein branches of each lobe are exceedingly narrow. The hepatic artery, in contrast, appears broad. The liver appears to be predominantly supplied by the artery rather than the portal vein, as the liver parenchyma is brighter than usual. This rare variation was excluded from further analysis.

Without dissecting the vascular and bile duct branches, we observed that the portal vein branches of the LML and LLL form a common trunk on the visceral surface, as previously reported (Martins *et al*., 2008). Based on the depth of the portal vein pedicles in the LLL, mice can be divided into two major types (more than 200 individuals were counted). In the first type, the portal vein pedicles extend deeply into the LLL parenchyma, and the branch emerging site of the LML portal vein is hidden within the LLL parenchyma (Fig. 2b). In the second type, the portal vein pedicles extend shallowly, and the branch emerging site of the LML portal vein is exposed outside the LLL parenchyma (Fig. 2d, f, h). The degree of exposure varies, leading to further detailed classifications: poorly exposed type (Fig.2c, d), generally exposed type (Fig. 2e, f), and fully exposed type (Fig. 2g, h). In the poorly exposed type, the branch emerging site can be seen on the visceral surface with the help of a cotton swab (Fig. 2d) but is invisible on the diaphragm surface (Fig. 2c). In the generally exposed type, the branch emerging site is clearly visible on the visceral surface (Fig. 2f). In the fully exposed type, the branches of the LLL and LML are distinct on both the visceral and diaphragm surfaces (Fig. 2g, h). Venous collaterals (indicated by black triangles) are always present, bridging over the portal vein branches (Fig. 2c).

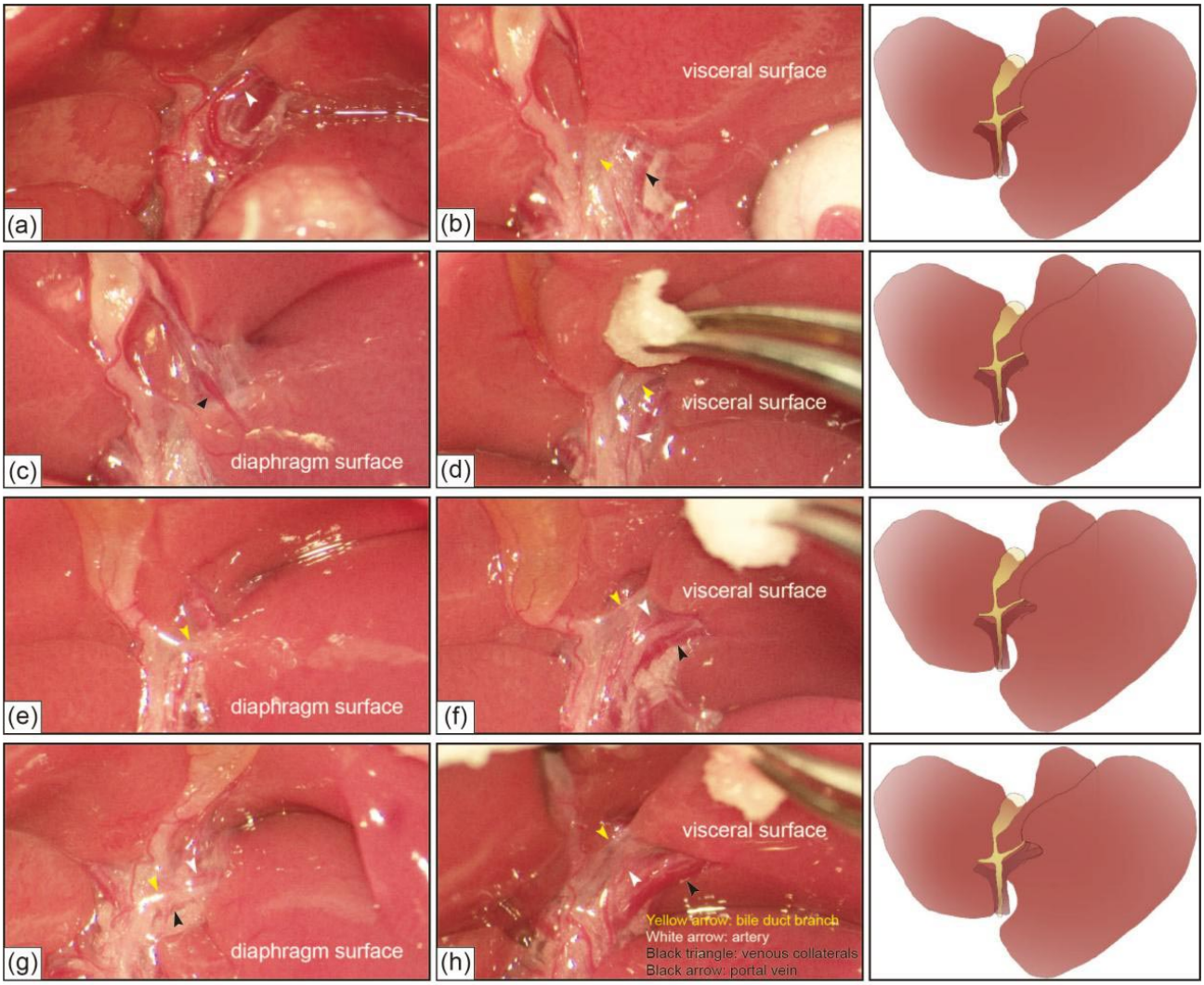

Fig. 2. Different portal vein branches of the LLL. (a) Arterial giant variation. (b) The emerging site of the LLL portal vein branch was hidden in the LLL parenchyma. (c),(d) Poorly exposed type. Venous collaterals overbridge the portal branch. (e), (f) generally exposed type. (g), (h) fully exposed type. Black triangle: venous collaterals. Yellow arrow: bile duct branch. White arrow: artery. Black arrow: portal vein.

Regardless of the type, bile duct branches drain into the common bile duct along the edge. The arteries (indicated by white arrows) are thin and bright red, spreading into the parenchyma together with the bile ducts and portal veins (indicated by black arrows), consistent with previous reports (Martins & Neuhaus, 2007).

Ligation of portal vein branch of LLL and postoperative observation: To perform portal vein branch ligation of the LLL successfully, experimenters should acquire advanced microsurgical skills (Aller *et al*., 2012). The branches of arteries, veins, and bile ducts are extremely small and fragile, and they are closely adherent to each other. Therefore, dissection should be technically avoided. If the portal vein branch of the Left Middle Lobe (LML) is hidden, ligation should not be attempted. In the poorly exposed type, the portal vein branch emerges at a tricky angle, making it difficult to define the boundary of the portal vein. Fortunately, these two types (hidden and poorly exposed) make up only a small proportion of the population (about 1/12), which is suitable for a sham-operated group.

For the generally and fully exposed types, the visceral surface of the LLL was carefully exposed. A monofilament nylon thread (9/0, Jinhuan, Shanghai) was guided across the hepatic pedicle around the LLL portal vein as close as possible, ensuring that no visible tubes such as arteries, bile ducts, nerves, lymphatics, or connective tissues were included in the suture loop (Fig. 3a, b, c). This ensured that partial necrosis of the liver parenchyma was avoided. If venous collaterals were present over the portal branch of the LML, they were also ligated. Under the microscope, the bile duct branch was observed to be far from the point of ligation (Fig. 1a).

There was no perioperative and intraoperative mortality. On POD 3, the LLL showed minimal or no necrosis or sclerosis, and the ML, including the LML, which shares a common pedicle with LLL, appeared to have grown (Fig. 3d, e). The exsanguinated liver was then obtained. Compared to the sham-operated group (Fig. 3f), the number of Ki67-positive nuclei, a marker of cell proliferation, was significantly increased (Fig. 3g, h), and the weight ratio between FLR and body obviously enhanced (Fig. 3i). These findings indicated that ligation of the portal vein of the LLL effectively induced liver regeneration.

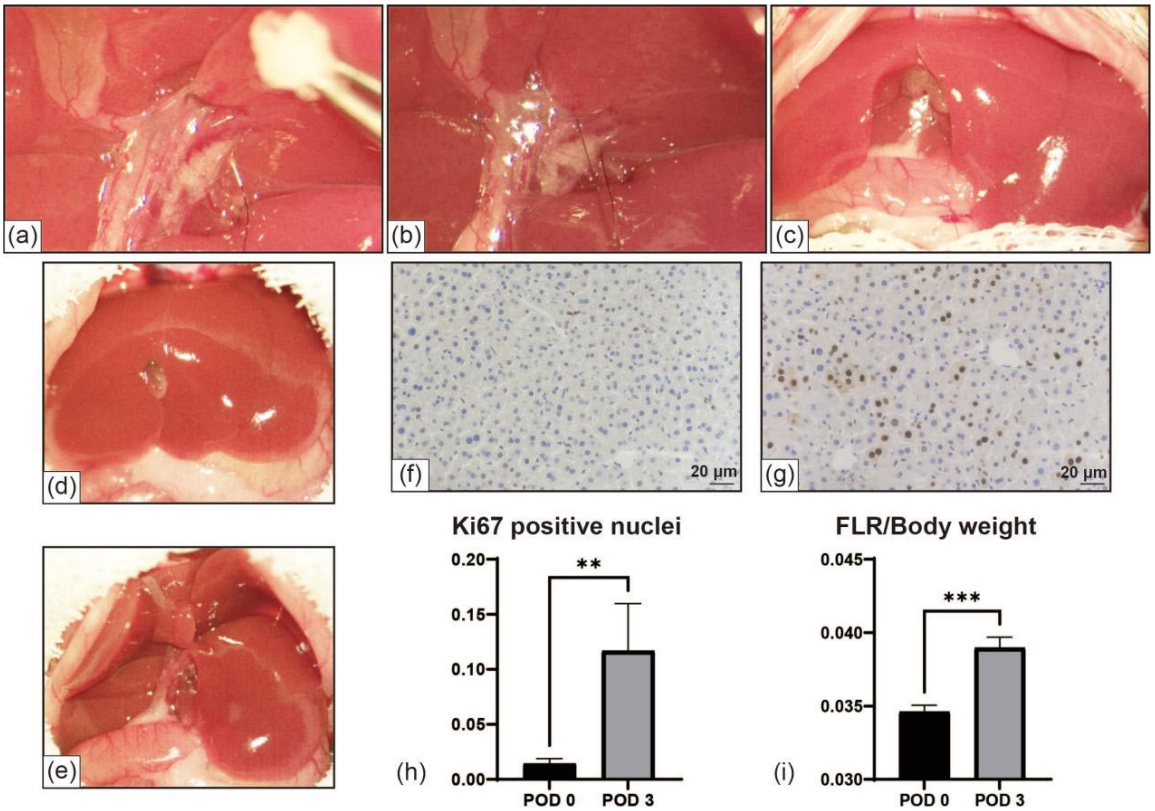

Fig. 3. Ligation of portal vein branch of the LLL and postoperative observation on POD 3. (a),(b),(c) The process of ligation of the portal vein of the LLL. (d), (e) ML and LLL on POD 3. The ML has grown significantly, and the LLL appears healthy. (f), (g), (h) Compared to the sham-operated group (left), the number of Ki67-positive nuclei is increased in the surgery group (right). (i). The weight ratio between the FLR and body is significantly enhanced on POD 3.

DISCUSSION AND CONCLUSION

ICG fluorescent intraoperative cholangiography, combined with a fluorescent imaging system, has been widely used to identify bile or bile leakage during procedures such as liver segmentectomy (Aoki *et al*., 2008), laparoscopic cholecystectomy (Kaneko *et al*., 2012), and liver transplantation (Dai *et al*., 2022). Researchers have previously dissected the biliary tree to observe the bile system (Martins & Neuhaus, 2007) or used dynamic near-infrared fluorescence cholangiography with ICG to detect bile duct injuries in rats (Gao *et al*., 2017). However, until now, there have been no reports on the bile system structure in mice. Our observations revealed that the bile duct branches of each lobe frequently drained into the superior common bile duct near the gallbladder, even in the RL and CL, which are anatomically inferior in location. This finding highlights the high variability in the bile system structure.

During our investigation of the mechanisms underlying liver regeneration induced by PVL and ALPPS, we utilized rodent models, specifically Lewis and C57BL/6N mice. Unlike rats, whose portal vein branches of the LLL and LML are often hidden, mice consistently exhibit visible portal vein branches of the LLL and LML under the microscope. After staining with ICG, the bile ducts appear emerald green. Combined with the bright red arteries and dark red portal veins, this creates a high-contrast visualization of the vascular and biliary structures. Surgeons or technicians proficient in micromanipulation skills can feasibly perform LLL portal vein ligation using our method by selecting appropriate types.

Moderate-depth anesthesia facilitated the operation by reducing diaphragmatic and liver motion. During ligation, it is crucial not to turn over the LLL, as this can disrupt the surgical field. Accurate and complete ligation ensures the preservation of LLL tissue viability and function, thereby promoting regeneration and weight increase in the FLR. How can we confirm that the ligation is complete and accurate? When the needle passes through the liver, minimal or no blood outflow from the tissue indicates that there is no perforation in the vein or main artery. If there is profuse and uncontrollable bleeding, the portal vein has likely been punctured. In such cases, the suture should be removed and re-ligated to avoid further bleeding or partial blood supply to the LLL. If there is minimal bleeding and the blood is bright red, an artery has been punctured. Hemostasis by compression is usually effective. After ligation, the LLL should appear dark red, rather than pale or gray, indicating that the portal vein is within the knot, and the artery is outside. We chose POD 3 as the endpoint because liver regeneration requires an initial period to manifest. Liver edema on POD 2 can interfere with accurate liver weight measurements. Based on our preliminary experiments, regeneration typically ceases around POD 5 when the body achieves normal liver function.

YAO, N.; QIAN, L.; LUO, C.; YAN, J.; YANG, X.; WU, W. & ZHOU, J. Visualización *in vivo* de conductos biliares extrahepáticos en ratones mediante tinción con verde de indocianina y su aplicación en la ligadura de la vena porta del lóbulo lateral izquierdo. *Int. J. Morphol., 43(3)*:1038-1044, 2025.

RESUMEN: Los roedores se utilizan frecuentemente en la construcción de modelos animales para diversos estudios relacionados con el hígado, lo que requiere un conocimiento exhaustivo de las estructuras de los lóbulos hepáticos. Sin embargo, se carece de descripciones detalladas del sistema biliar en ratones. En este estudio, empleamos la inyección intravenosa de verde de indocianina (IVI) combinada con la ligadura del conducto biliar común para visualizar los conductos biliares extrahepáticos con luz natural. Observamos que las ramas de los conductos biliares de cada lóbulo generalmente drenaban hacia el conducto biliar común superior, cerca de la vesícula biliar. Además, realizamos la prueba de ligar la vena porta del lóbulo lateral izquierdo (LLI) en ratones, lo cual no se había logrado con éxito en modelos previos de ligadura de la vena porta (LVP) en roedores ni en la asociación de la partición hepática y la ligadura de la vena porta para la hepatectomía por etapas (ALPPS). Al examinar la estructura vascular hepática con un microscopio de 25x y tinción con ICG, categorizamos la vena porta del LLI en cuatro patrones. La mayoría de los ratones mostraron ramas porta diferenciadas para el LLI y el lóbulo medio izquierdo (LMI), a diferencia de las ratas. Al seleccionar los tipos con exposición general o total, los microcirujanos expertos pudieron ligar eficazmente la vena porta del LLI con nuestro método. Al tercer día postoperatorio (DPO 3), el LLI parecía viable sin necrosis significativa, y la relación peso-peso del remanente hepático futuro (RHF) respecto al peso corporal, así como el número de núcleos Ki67-positivos, aumentaron significativamente, lo que indica que la ligadura de la vena porta del LLI indujo la regeneración hepática.

PALABRAS CLAVE: Verde de indocianina; Conducto biliar; Ligadura de la vena porta; Regeneración hepática.

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Corresponding author:
Jian Zhou
Liver Cancer Institute
Zhongshan Hospital
Fudan University
Key Laboratory of Carcinogenesis and Cancer Invasion
Ministry of Education
NO. 179 Fenglin road
Shanghai 200032
CHINA

E-mail: zhou.jian@zs-hospital.sh.cn