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REVIEW

Comprehensive and innovative techniques for laparoscopic choledocholithotomy: A surgical guide to successfully accomplish this advanced manipulation

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Author contributions: Hori T performed this laparoscopic surgery, reviewed previous papers in this field, and wrote this article; all illustrations and schemas were originally drawn by Hori T.

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Abstract

Surgeries for benign diseases of the extrahepatic bile duct (EHBD) are classified as lithotomy (*i.e.*, choledocholithotomy) or diversion (*i.e.*, choledochojejunostomy). Because of technical challenges, laparoscopic approaches for these surgeries have not gained worldwide popularity. The right upper quadrant of the abdomen is advantageous for laparoscopic procedures, and laparoscopic choledochojejunostomy is safe and feasible. Herein, we summarize tips and pitfalls in the actual procedures of choledocholithotomy. Laparoscopic choledocholithotomy with primary closure of the transductal incision and transcystic C-tube drainage has excellent clinical outcomes; however, emergent biliary drainage without endoscopic sphincterotomy and preoperative removal of anesthetic risk factors are required. Elastic suture should never be ligated directly on the cystic duct. Interrupted suture placement is the first choice for hemostasis near the EHBD. To prevent progressive laceration of the EHBD, full-layer interrupted sutures are placed at the upper and lower edges of the transductal incision. Cholangioscopy has only two-way operation; using dedicated forceps to atraumatically grasp the cholangioscope is important for smart maneuvering. The duration of intraoperative stone clearance accounts for most of the operative time. Moreover, dedicated forceps are an important instrument for atraumatic grasping of the cholangioscope. Damage to the cholangioscope requires expensive repair. Laparoscopic approach for choledocholithotomy involves technical difficulties. I hope this document with the visual explanation and literature review will be informative for skillful surgeons.

Key words: Laparoscopic surgery; Choledocholithotomy; Bile duct; Laparoscopy; General surgery

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Core tip: The right upper quadrant of the abdomen is advantageous for laparoscopic procedures. Laparoscopic choledocholithotomy is safe and feasible, although this laparoscopic approach involves technical difficulties. Endoscopic sphincterotomy destroys the physiological function of Oddi's sphincter. Laparoscopic choledocholithotomy has excellent clinical outcomes; however, emergent biliary drainage and removal of anesthetic risk factors are required preoperatively. Cholangiographic removal of stones strongly affects operative time. Cholangioscopy has only two-way operation; using dedicated forceps to atraumatically grasp the cholangioscope is important for smart maneuvering.

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INTRODUCTION

Laparoscopic surgery has been adopted in various fields[1-8]. Laparoscopic surgery has substantial advantages over open surgery, including less blood loss, less pain, lower morbidity rates, shorter time to a postoperative diet, shorter hospital stay, earlier social reintegration and modest cost savings[1,4,9-13]. Laparoscopic surgeries that do not require advanced techniques such as anastomotic reconstruction or lymphoid dissection (*e.g.*, appendectomy, cholecystectomy, distal pancreatectomy and rectopexy)[3,5-7] have rapid learning curves[11]. Hence, laparoscopic surgeries are widely used worldwide for benign diseases[5,7].

Unfortunately, laparoscopic hepatobiliary and pancreatic (HBP) surgery has developed slowly because of technical challenges and a protracted learning curve[9,14], with the exception of laparoscopic cholecystectomy[15,16]. Acute cholangitis (choledocholithiasis) is itself a benign disease, but associated cholangiovenous reflux and subsequent sepsis can easily result in a life-threatening situation[17-19]. Surgical treatments for benign diseases of the extrahepatic bile duct (EHBD) are classified according to their therapeutic purpose as lithotomy (*i.e.*, choledocholithotomy) or diversion (*i.e.*, choledochojejunostomy)[20,21]. General surgeons do not perform these surgeries laparoscopically because they require advanced skills and anatomical precision[22-26], although a laparoscopic approach is safe and feasible for choledocholithotomy and choledochojejunostomy[22,25-31].

The basic skills required for open surgeries are clearly different from those used in laparoscopic procedures[7,8,14,32-34]. Notably, experience alone is not enough to ensure successful performance of laparoscopic surgeries[7,8,14,32-24]. We herein focus on laparoscopic approaches for choledocholithotomy, summarizing tips and pitfalls of this advanced surgery, on the basis of a review of important studies and our own experience. Also, important previous documents in this field are carefully reviewed.

ANATOMICAL RECOGNITION OF THE BILIARY SYSTEM

The anatomy of the biliary system is shown in Figure 1A. The common hepatic duct (CHD), common bile duct (CBD) and intra-pancreatic bile duct compose the EHBD. The cystic duct contains Heister's valves (spiral folds). The gallbladder infundibulum and cystic duct meet to form the infundibulum–cystic duct junction. The CHD, cystic duct and CBD together constitute the biliary confluence. Biliary drainage is physiologically regulated by Oddi's sphincter.

ACUTE OBSTRUCTIVE SUPPURATIVE CHOLANGITIS

Charcot first documented acute cholangitis in 1877[35]; Charcot's triad (upper abdominal pain, fever and jaundice) was proposed for diagnosis of acute

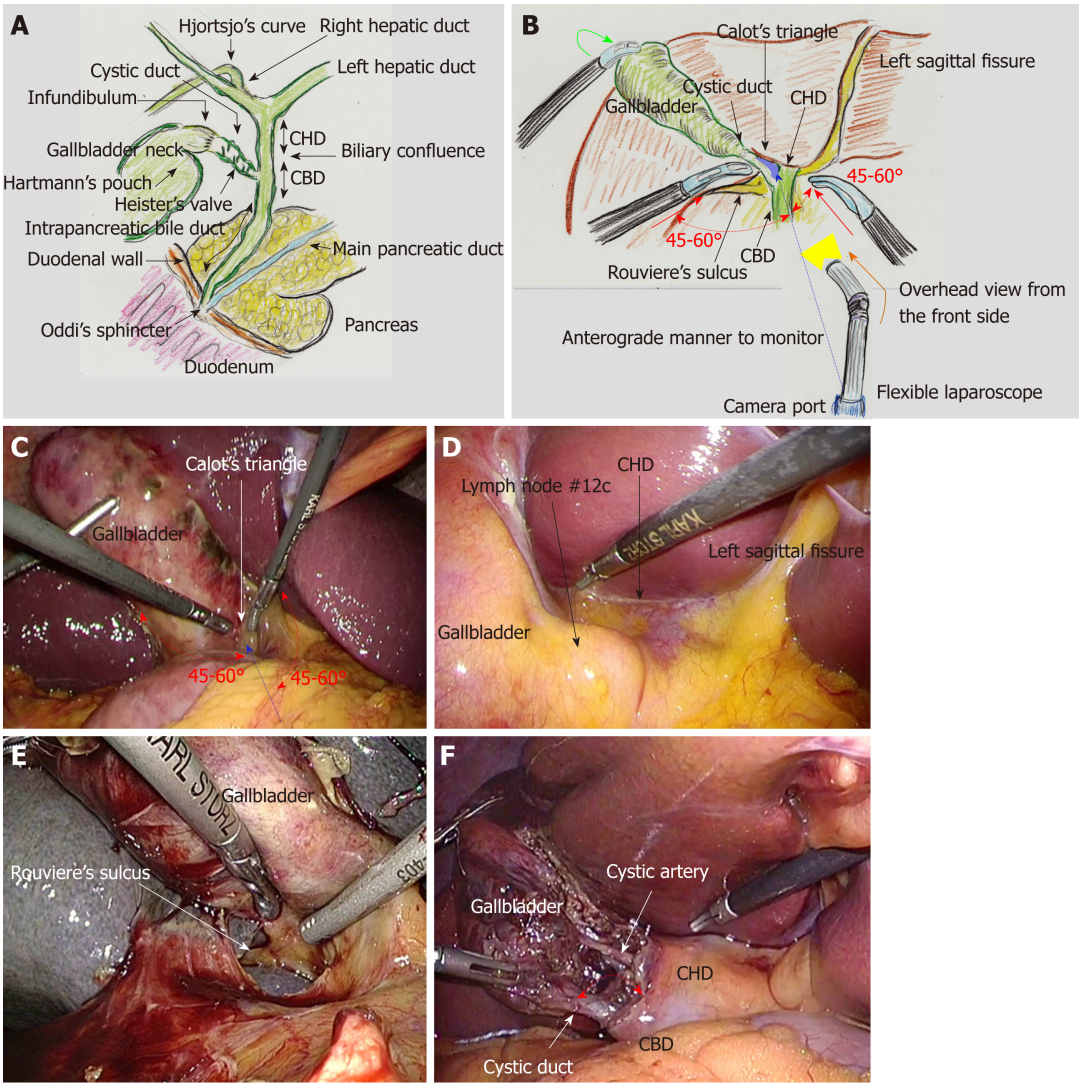

Figure 1 Biliary system and actual surgical procedures of laparoscopic choledocholithotomy. A: The common hepatic duct (CHD), common bile duct (CBD) and intra-pancreatic bile duct compose the extrahepatic bile duct. Biliary drainage is regulated by Oddi's sphincter. Recognition of Hjortsjo's curve on cholangiography is useful for detecting the posterior branch from the right hepatic duct; B and C: The gallbladder fundus is superiorly and cranially lifted (green arrow). The target site is Calot's triangle (blue shaded area). The two forceps of the main surgeon (red arrow) form appropriate angles (approximately 45°-60°) (red dotted arrow) to the axis from the camera port to Calot's triangle (blue dotted arrow). A flexible laparoscope provides an overhead view from the upper anterior side (orange arrow), anterograde to the visual monitor; D: The bottom plateau of the U-shaped line from the left sagittal fissure to the gallbladder, which necessarily involves the CHD; E: Rouviere's sulcus always involves the right hepatic duct; F: The whiter color change of the cystic duct is recognized, and a wider angle is created between the cystic duct and CHD (red arrow). CHD: Common hepatic duct; CBD: Common bile duct.

cholangitis[36,37]. Biliary stagnation caused by obstruction [*e.g.*, stone, tumor, liver failure or dysfunction of Oddi's sphincter (DOS)] or bacterial infection with increased biliary pressure easily result in cholangiovenous reflux and subsequent sepsis[17,18]. Reynolds and Dargan classified a clinical syndrome characterized by Charcot's triad, lethargy (or mental confusion) and shock state as acute obstructive cholangitis in 1959[38]. Thereafter, these five symptoms were called Reynold's pentad[39]. Reynolds and Dargan suggested that the only way to treat this severe cholangitis was emergent surgery and biliary drainage[38]. Longmire first defined severe cholangitis fulfilling Reynold's pentad as acute obstructive suppurative cholangitis (AOSC) in 1971[40]. The high mortality rate of AOSC (8%-71%) has been do-cumented[41-44].

AOSC commonly occurs in elderly patients[42,44]; emergent biliary drainage is required in these patients[19]. Interventional endoscopists may choose endoscopic sphincterotomy (EST). EST destroys the physiological function of Oddi's sphincter, even in elderly patients with subtle DOS. This situation raises a simple question. Is EST the first choice for emergent biliary drainage, even in younger patients? EST alone is not required as initial treatment[45], and we should not forget that EST destroys the physiological function of Oddi's sphincter. Recovery of the physiological function of Oddi's sphincter is impossible after EST. To preserve physiological function, elective surgery has advantages over emergent EST; transpapillary biliary

drainage without EST may be performed initially as emergent therapy[45].

ACUTE CHOLANGITIS AND BILE DUCT STONES

Initial management of acute cholangitis with bile duct stones has been documented[19,45]; biliary drainage should be performed as soon as possible in these patients[19]. The clinical indications and therapeutic techniques of biliary drainage for acute cholangitis have been clearly established[45]. Endoscopic transpapillary biliary drainage, whether *via* nasobiliary drainage or biliary stenting, should be selected as first-line therapy[45]. EST is not routinely recommended for biliary drainage alone because of concerns about bleeding[45].

DOS and anatomical abnormalities (*e.g.*, periampullary diverticulum) result in acute biliary infection, and subsequently cause primary bile duct stones[46]. The etiology of bile duct stones should be recognized preoperatively. In patients with concomitant bile duct stones, stone removal can be performed *via* an endoscopic approach (*e.g.*, EST, papillary dilation and balloon enteroscopy-assisted and/or ultrasonography-guided methods)[45] or with surgical treatment[47-49]. Laparoscopic choledocholithotomy is considered a safe and feasible therapeutic option[22,26-28,31]. As described above, AOSC frequently occurs in elderly patients[42,44]; laparoscopic surgery should be chosen even in this population.

In patients whose condition is stable before surgery, both emergent and elective laparoscopic surgeries are safe and feasible[50]. Perioperative analgesic agents are important; however, some analgesic agents (*e.g.*, opioids or morphine) cause drug-induced DOS[51,52]. Effective biliary drainage should be achieved preoperatively to avoid sepsis[19,45], as should compete removal of risk factors for general anesthesia (*e.g.*, unstable hemodynamic state, obstructive jaundice, sepsis and analgesic contraindications)[53-55].

BILE DUCT STONES AND ASSOCIATED CHOLANGITIS AFTER ABDOMINAL SURGERY

Acute cholangitis and bile duct stones are critical problems after abdominal surgery, especially in elderly patients[28,56,57]. Gastrectomy surgically alters the biliary system, because the inevitable dissection of lymph nodes and nerves results in physiological disorders (*e.g.*, DOS, reduced bile secretion, atonic gallbladder and paralytic bowels)[7,58]. Hence, repeated cholangitis and bile duct stones easily occur after gastrectomy[57,59].

Severe adhesions and dense tissue are often intractable during reoperative surgery. Moreover, the location of important ducts and vessels may easily be shifted after gastrectomy because of digestive anastomosis and postoperative adhesions, especially in Billroth I reconstruction[7]. However, a laparoscopic approach is advantageous, even for re-operative choledocholithotomy[28,59], and is a safe, effective and feasible treatment even in elderly patients after complicated abdominal operations[56]. Laparoscopic choledochoduodenostomy (not choledochojejunostomy) may be chosen as an alternative treatment, if gastrojejunostomy has been performed in the Roux-en-Y fashion[57,60]. Laparoscopic cholecystectomy should be the first choice for gallbladder stones and cholecystitis in patients with a history of abdominal surgery[7,8,61], although cholecystectomy after EST for biliary duct stones does not reduce the incidence of recurrent cholangitis[62].

HISTORY OF CHOLEDOCHOLITHOTOMY AND BILIARY DRAINAGE

Abbe first performed bile duct drainage after choledocholithotomy in 1892[63,64]; Deaver reported use of a modified T-tube drain in 1904[63,64]. Kehr propounded the usefulness of T-tube drainage in 1909[63-67]; thereafter, transductal T-tube drainage after choledocholithotomy became commonly used worldwide. The material of the T-tube is important, because low reaction in either the EHBD or the peritoneal cavity results in a lack of tissue tract formation around the tube due to material inertness[63,64]. Various vulcanized rubber products can be produced from natural rubber and sulfur. The degree of vulcanization influences the hardness and irritant nature of the resulting rubber[64]. Red rubber is the most irritant and silicone rubber is the least[63,64,68]. Latex rubber tubes are preferred for long-term drainage, because they create a good

tissue tract as a result of tissue reaction against the material irritant[63,64]. Silicone rubber T-tubes often fail to elicit tissue tract formation[63,64,68]. Red or latex rubber should be chosen as T-tube material[63,64,68].

Acute cholangitis can be managed with transpapillary biliary drainage, EST, transductal drainage (T-tube) or transcystic drainage (C-tube)[22]. Transductal T-tube drainage has higher rates of stone clearance and biliary leakage than other treatment options[22]. EST has a higher rate of procedural morbidities and serious consequences may occur[22]. Transcystic C-tube drainage is an accessible technique that simplifies surgical procedures and has a lower complication rate than other treatments[22]. The choice of choledocholithotomy *via* conventional open surgery with transductal T-tube drainage versus laparoscopic primary closure with transcystic C-tube drainage remains controversial[69]. Currently, laparoscopic choledocholithotomy with primary closure and transcystic C-tube drainage is superior to conventional open surgery with transductal T-tube drainage[69], which has inspired HBP surgeons to end the use of transductal T-tube drainage[47,49,70].

INTENTIONAL PRESERVATION OF PHYSIOLOGICAL FUNCTION OF ODDI'S SPINCTER

EST destroys the physiological function of Oddi's sphincter, and moreover, recovery of the physiological function of Oddi's sphincter is impossible after EST. Even though emergent EST is easier than elective laparoscopic surgery, transpapillary biliary drainage without EST is the initial treatment for acute cholangitis[45]. Completion of EST results in destruction of the physiological function of Oddi's sphincter. Ill-considered use of EST should be avoided[20], though many physicians may consider that the arguments against EST is really limited. To preserve sphincter function, emergent EST should be performed only in special situations (*e.g.*, elderly patients with critical comorbidities, severe disease, prolonged jaundice, or severe DOS resulting from previous surgeries[20,71]. Paradoxically, EST may be permissive in patients who already have DOS. For example, elder or postoperative patients may have severe DOS[7,57-59].

RECURRENT STONES AND ASSOCIATED CHOLANGITIS

Recurrent stones (not remnant stones) in the bile duct after choledocholithotomy or EST is another critical matter[20,57,72]. Early stone recurrence after surgical or endoscopic treatment is a dreaded occurrence for physicians[20,57]. Though laparoscopic choledocholithotomy provides safe and feasible treatment for recurrent stones and associated cholangitis[28,30,56,59].

As described above, DOS, reduced bile secretion and paralytic bowels raise concerns about recurrent stones and associated cholangitis; elective laparoscopic cholecystectomy after complete removal of bile duct stones does not reduce the recurrence rate of repeat cholangitis[62]. The etiology of bile duct stones should be carefully evaluated and therapeutic strategies should be chosen according to definitive or suggested etiology. Though the arguments against endoscopic managements of bile duct stones may be really limited and laparoscopic choledocholithotomy is a safe and feasible treatment even for recurrent stones[28,30,56,59], laparoscopic choledochojejunostomy may be a possible therapeutic option to provide biliary diversion according to physiological disorders[20,30].

PREOPERATIVE EVALUATION OF BILE DUCT STONES

Stone clearance should be achieved without any remnant stones or debris[73]. Specialized instruments, including endoscope and forceps, are crucial for successful surgical stone removal[74,75]. Clearance of some stones (*e.g.*, impacted, multiple or intrahepatic stones) involves technical challenges[76]. Operative time is greatly affected by the duration of stone removal[73,74]; detailed preoperative imaging studies shorten operative time[77]. Removal of impacted and/or large stones is especially difficult[27,73], although even an impacted stone will float after preoperative biliary drainage[76]. Preoperative evaluation with endoscopic retrograde cholangiography or cholangiography *via* drainage tube is strongly recommended[78]. The presence of a duodenal parapapillary diverticulum, which causes DOS and contraindicates EST, should also be ruled out preoperatively[79].

TECHNICAL DIFFICULTY OF LAPAROSCOPIC SURGERIES OF THE EHBD

Gallbladder stones with acute cholecystitis is an indication for surgery[7,8,80]; inflammatory severity may be an important risk factor in these cases[81,82]. Extrinsic compression of the EHBD, including Mirizzi syndrome and hidden cystic duct, make laparoscopic cholecystectomy technically difficult[81,83]. However, the concept of the critical view of safety (*i.e.*, positive identification of the cystic duct and artery) has been established in 1995[34]. Compliance with this protocol makes laparoscopic cholecystectomy safe, even in severe cholecystitis[7,8]. The right upper quadrant provides a suitable location for the surgical field in laparoscopic procedures[7,30,84], which is one reason laparoscopic cholecystectomy has spread worldwide[15,16]. Reliable stability during laparoscopic cholecystectomy is indispensable for successful laparoscopic choledocholithotomy[7,8,32,34,81].

However, technical challenges have prevented laparoscopic surgeries for the EHBD (*e.g.*, choledocholithotomy and choledochojejunostomy) from gaining worldwide popularity[22,23]. These advanced surgeries should be mastered by skillful HBP surgeons[24,25]. Although laparoscopic choledocholithotomy and choledochojejunostomy[22,25-31] are safe and feasible, technical challenges have prevented the worldwide dissemination of these advanced HBP surgeries[22-26].

ACTUAL SURGICAL PROCEDURES OF LAPAROSCOPIC CHOLEDOCHOLITHOTOMY

The patient is placed in a supine position. Carbon dioxide pneumo-peritoneum at 10 to 12 mmHg is achieved through an umbilical port. Transductal incision is accompanied by bile outflow, and cholangioscopy requires continuous saline flow for intraluminal observation. A saline irrigator and suction tip (StrykeFlow, Stryker Co., Kalamazoo, MI, United States) are used. Frequent, continuous suction is needed during laparoscopic choledocholithotomy, though suction causes collapse of pneumoperitoneum. Pneumoperitoneum stability is very important to maintain the surgical field during laparoscopic surgery[85]. Therefore, we employ an automatically maintained pneumoperitoneum system (AirSeal Intelligent Flow System, Conmed Co., Utica, NY, United States). A flexible laparoscope with an adequate light source (Endoeye Flex, Olympus, Tokyo, Japan) is required; laparoscopic procedures are performed under various angled views.

A total of four working ports are placed in the upper abdomen. An assistant surgeon retracts the gall bladder fundus ventrally. The target site is Calot's triangle; both forceps of the main surgeon make adequate angles (approximately 45°-60°) relative to the axis from the camera port to Calot's triangle (Figure 1B and C). An excessively narrow or wide angle complicates laparoscopic procedures, including fully intracorporeal suture[7]. Moreover, a flexible laparoscope provides an overhead view from the upper anterior aspect, anterograde to the visual monitor (Figure 1B and C). Hence, the right upper quadrant is highly suitable for set-up of surgical procedures[7,30,84]. Surgeons should not hesitate to place additional ports if needed, because stab incisions are minimally invasive[7].

The liver is held cranially with a snake retractor located below the xiphoid process. The hepatoduodenal ligament is well stretched[7]. The hepatorenal fossa is widely dilated, and a working space is obtained. The bottom plateau of the U-shaped line from the left sagittal fissure to the gallbladder, which necessarily involves the CHD (Figure 1D), and Rouviere's sulcus, which always involves the right hepatic duct (Figure 1E), are routinely confirmed.

The whiter color change at the junction of the infundibulum and cystic duct is recognized[7] (Figure 1F). The angle between the cystic duct and CHD is widened to prevent a tenting injury resulting from a parallel junction of these biliary ducts[7,32,34,81,86] (Figure 1F). The critical view of safety is established on both the anterior (Figure 2A) and posterior aspects (Figure 2B)[33,87].

Pneumoperitoneum pressure caused by infiltration of carbon dioxide gas helps to create a dissectible layer. To avoid unexpected injuries, the dissectable layer should be traced as close to the gallbladder as possible[7]. Tissue dissection and membrane cutting should be extended from the visualized side of the correct layer, not from the unseen side[7]. The gallbladder is then removed from the liver bed.

The cystic duct is straightened and a semicircular incision is made on the cystic duct near the gallbladder (Figure 2C). Full cutting with removal of the gallbladder disturbs further procedures. Heister's valves are carefully destroyed before transcystic placement of a C-tube into the EHBD (Figure 2D) and removal of stones from the

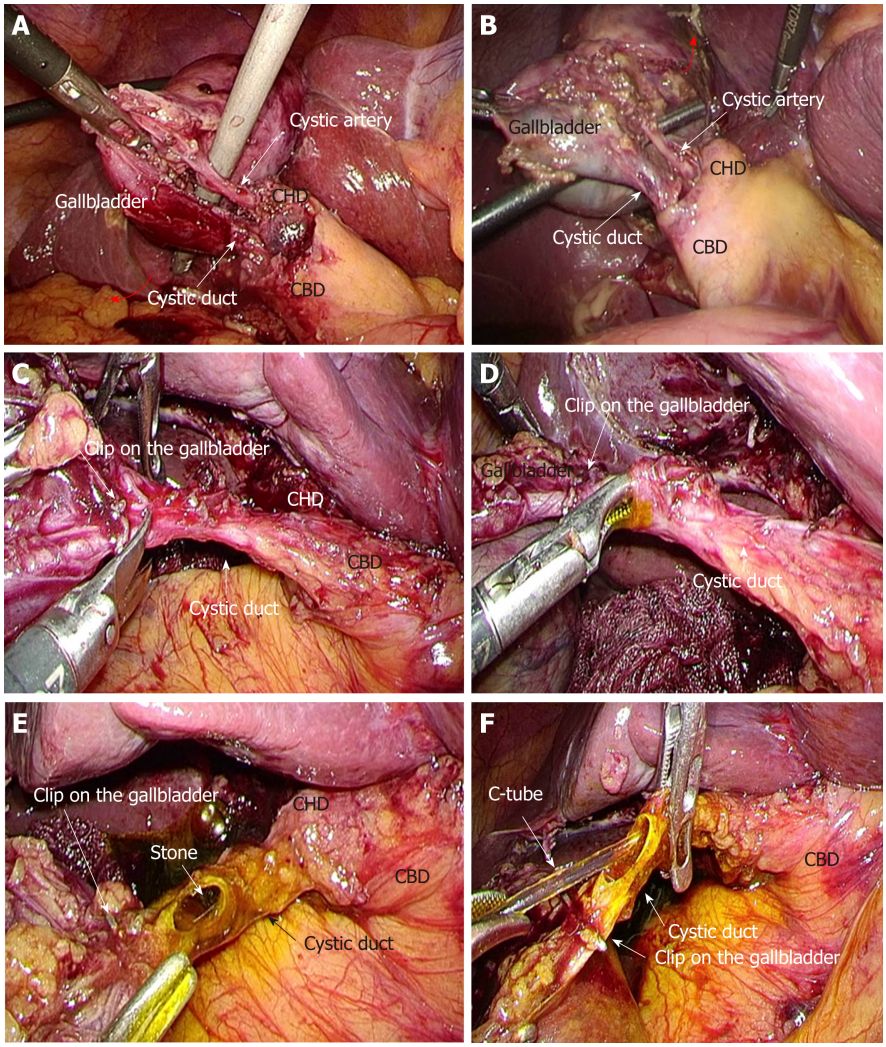

Figure 2 Actual surgical procedures of laparoscopic choledocholithotomy. A and B: Critical view of safety is established in the anterior (A, arrow) and posterior (B, arrow) aspects; C: A semi-circular incision is made in the cystic duct; D: Heister's valves are carefully destroyed; E: Stones in the cystic duct are removed; F: The golden-brown bile flows from the extrahepatic bile duct. The C-tube is cannulated. CHD: Common hepatic duct; CBD: Common bile duct.

cystic duct (Figure 2E). Careless stabbing procedures to destroy Heister's valves can easily result in severe injury to the posterior walls of the biliary confluence or EHBD. The golden brown bile flows from the EHBD, after which the C-tube is cannulated (Figure 2F). The C-tube can be used as a cholangiographic tube for intraoperative cholangiography. Elastic suture is not ligated directly to avoid overtightening of the C-tube; insufficient drainage will trigger postoperative complications. Optimal transcystic fixation of the C-tube is completed with elastic suture and clips[7] (Figure 3A-C); the second clip prevents slippage of the first clip[7] (Figure 3D).

During laparoscopic cholecystectomy, the CHD, biliary confluence and CBD should be recognized, but direct exposure of these structures should be avoided[7,8]. However, these biliary structures should be intentionally explored during laparoscopic choledocholithotomy (Figure 3E). Although blunt dissection is carefully completed to explore the wall of the EHBD, intentional dissection of the autonomic nerves for biliary malignancies is not required for benign biliary diseases[88,89]. Feeding and drainage vessels surrounding the EHBD should be preserved to prevent ductal necrosis and postoperative biliary leakage (Figure 3F)[7]. The EHBD is opened with sharp dissection (Figure 4A); energy devices should not be used, to avoid even mild thermal damage. Intra-corporeal suture placement and subsequent ligation at the bleeding point are the first choice for hemostasis near the bile duct wall (Figure 4B). If oozing is intractable at the anterior wall of the EHBD, a button-shaped electrode with suction used in conjunction with a soft-coagulation system (VIO, Erbe, Tübingen, Germany) is an effective tool for safe hemostasis. After opening of the EHBD, the inner cavity of the EHBD is sufficiently flushed to raise biliary stones (Figure 4C).

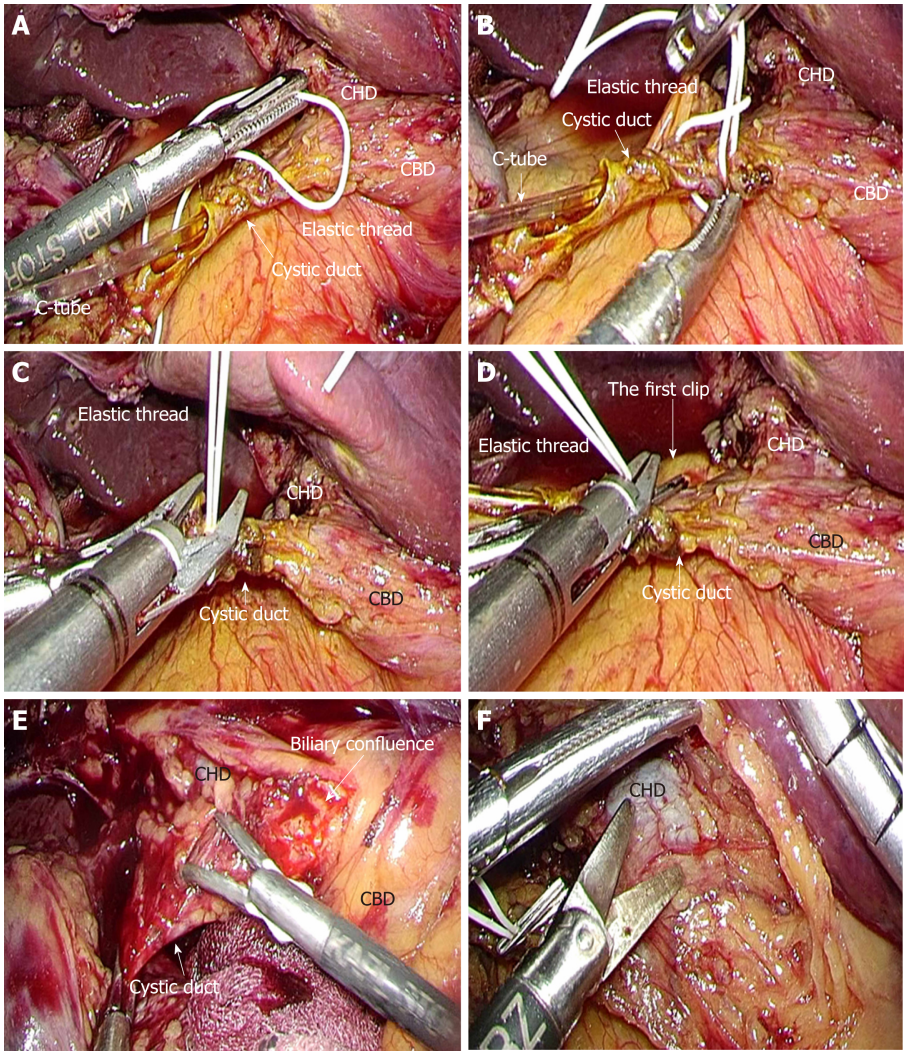

Figure 3 Actual surgical procedures of laparoscopic choledocholithotomy. A-C: Optimal transcystic fixation of C-tube is completed with elastic suture and clips. The elastic suture is never ligated directly; D: The second clip prevents any slippage of the first clip; E: The common hepatic duct, biliary confluence and common bile duct are intentionally explored; F: Feeding and drainage vessels surrounding the extrahepatic bile duct should be preserved. CHD: Common hepatic duct; CBD: Common bile duct.

Thereafter, all stones are completely removed[90] (Figure 4D).

After transductal incision along the long axis, interrupted absorbable monofilament sutures (PDS II, 4-0, violet, SH-1, Ethicon Inc., Bridgewater, NJ, United States) are placed through all layers at the upper and lower edges of the incision to prevent progressive laceration resulting from subsequent procedures, including cholangioscope maneuvers (Figure 4E and F). Thereafter, extracorporeal sutures are placed bilaterally with absorbable monofilament suture (PDS II, 4-0, violet, SH-1, 90 cm, Ethicon Inc.) as fixation sutures to open the transductal orifice (Figure 5A and B). These fixation sutures are adequately set through the abdominal wall at different points from the laparoscopic trocars with a trocar site closure device (Endo Close; Medtronic, Dublin, Ireland) (Figure 5C).

Spilled stones and/or infected bile should be completely removed[90]. Intraoperative cholangioscopy through the laparoscopic trocar is essential for laparoscopic choledocholithotomy. Dedicated elastic forceps to adequately grasp the cholangioscope without damaging the special coating and to allow maneuvering of the cholangioscope for stone removal (A66070A, 10 mm, Olympus or CLICKline BERCI, K33531 PG, 10 mm; Karl Storz Endoskope, Tuttlingen, Germany) is a key tool for successful laparoscopic choledocholithotomy (Figure 5D). Direct grasping with conventional laparoscopic forceps results in irreparable damage to the endoscope surface and should be avoided. Intraluminal findings should be carefully observed *via* the cholangioscope. Bifurcation of the bilateral hepatic ducts at the CHD side (Figure 5E) and the characteristic findings (so-called 'actinia') of the end of the intrapancreatic bile duct at the CBD side (Figure 5F) should be confirmed.

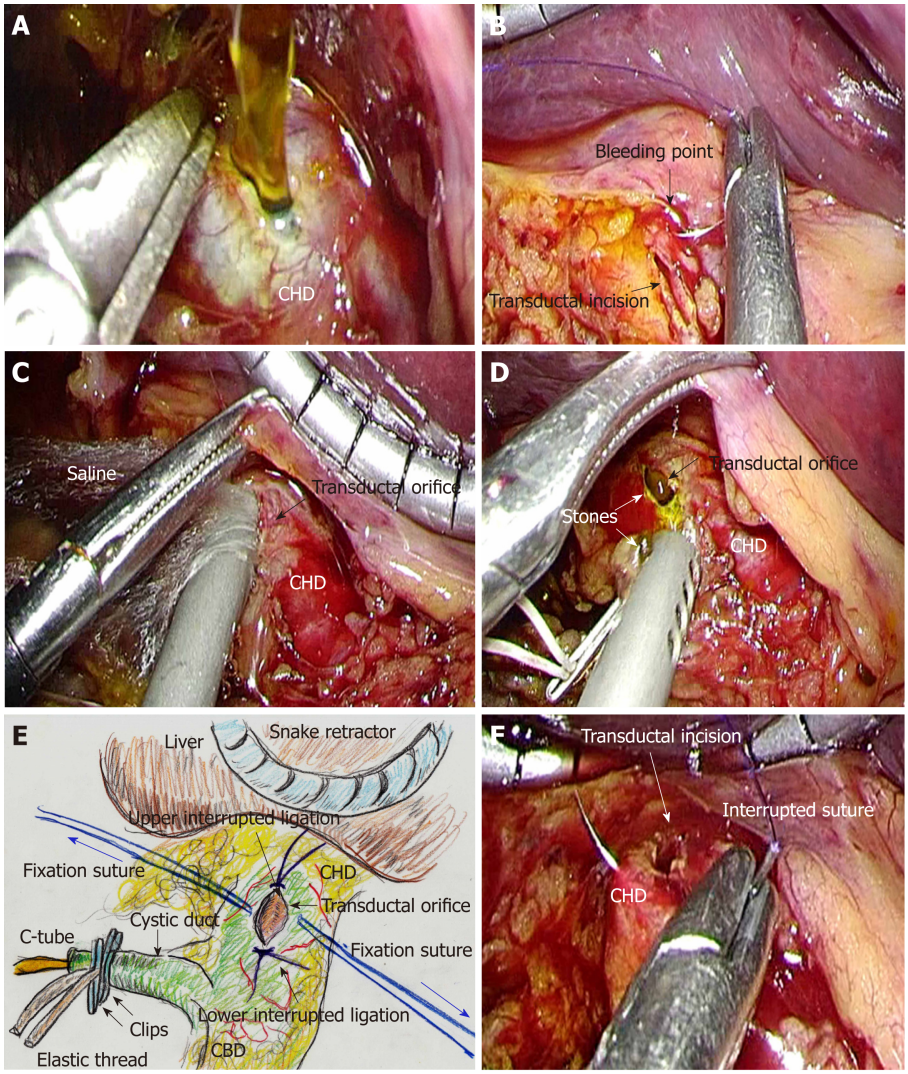

Figure 4 Actual surgical procedures of laparoscopic choledocholithotomy. A: The extrahepatic bile duct (EHBD) is opened with sharp dissection; B: Intracorporeal suture placement and subsequent ligation are the first choice for hemostasis. No energy devices should be used; C: The cavity of the EHBD is sufficiently flushed. Frequent continuous suction is needed during laparoscopic choledocholithotomy. An automatically maintained pneumoperitoneum system is used to preserve an adequate surgical field; D: All stones are removed; E: Interrupted sutures are placed and subsequently ligated at the upper and lower edges of the transductal orifice to prevent progressive laceration resulting from cholangioscopic maneuvers. Thereafter, fixation sutures (blue arrows) are bilaterally placed to open the transductal orifice. These fixation sutures are adequately set through the abdominal wall at different points from the laparoscopic trocars; F: Interrupted sutures and subsequent ligation are placed at the upper and lower edges of the transductal incision, to prevent progressive laceration due to cholangioscopic maneuvers. CHD: Common hepatic duct; CBD: Common bile duct; EHBD: Extrahepatic bile duct.

The diameter of the EHBD is generally > 10 mm[31]; the method of primary closure of the transductal incision is chosen according to the EHBD diameter[91-93]. Generally, both the transductal incision closure and subsequent primary closure are performed in the same direction along the long axis (Figure 6A). In cases of EHBD diameter less than 7 to 8 mm, primary closure is performed in the direction of the short axis to avoid postoperative stenosis (Figure 6B). The transductal incision is primarily closed with intra-corporeal ligation, with primary full-layer interrupted sutures of absorbable monofilament suture (PDS II, 5-0, violet, RB-1; Ethicon, Inc.).

Finally, real-time intraoperative cholangiography *via* C-tube is performed with contrast agent and atoxic dye (indigo carmine or indocyanine green). Remnant stones, biliary leakage and passage obstruction are carefully checked. Biliary passages, especially through the primary-closed portion and Oddi's sphincter, are evaluated during surgery. Intraperitoneal lavage and drain placement are usually performed; total bilirubin level in the drain discharge is monitored after surgery.

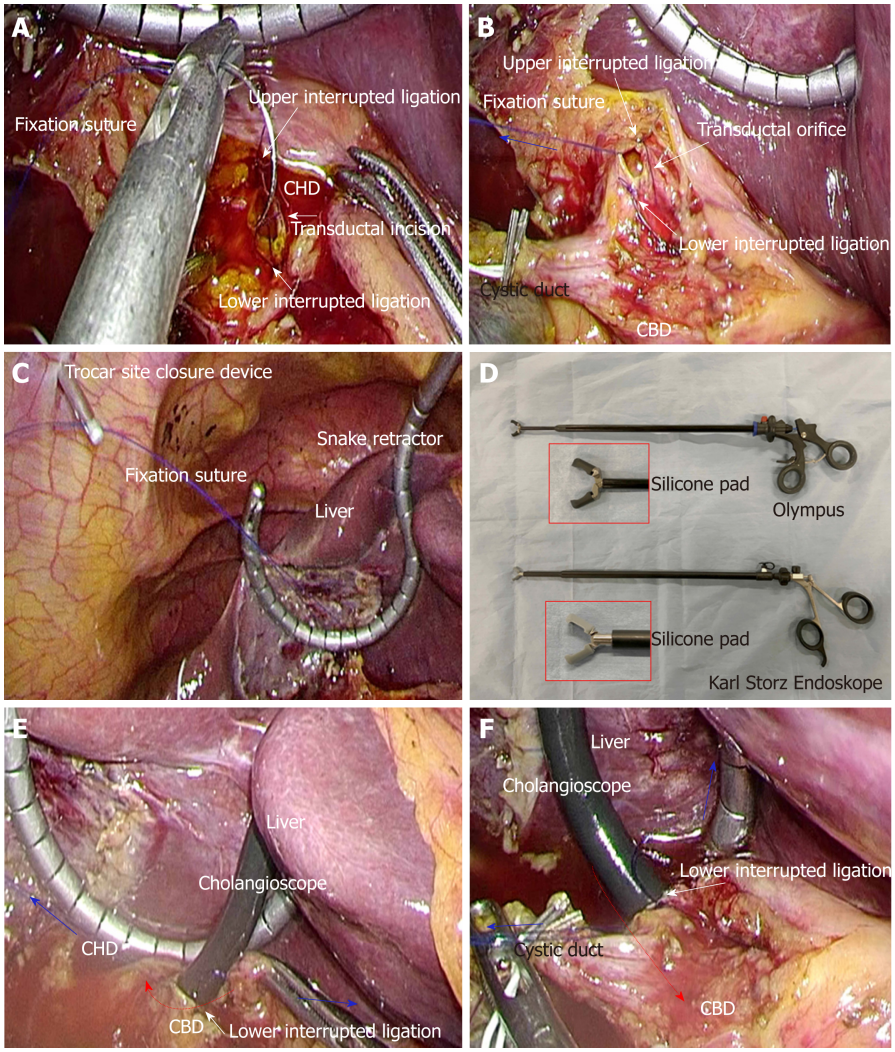

Figure 5 Actual surgical procedures of laparoscopic choledocholithotomy. A and B: Fixation sutures (blue arrow) are bilaterally placed to open the transductal orifice; C: Fixation sutures are adequately set through the abdominal wall at different points from the laparoscopic trocars with a trocar site closure device. The liver is held cranially with a snake retractor to stretch the hepatoduodenal ligament; D: A dedicated elastic forceps is important for successful laparoscopic choledocholithotomy. The tip of the forceps contains a silicone pad to avoid damaging the cholangioscope. Olympus (A66070A; Tokyo, Japan) and Karl Storz Endoskope (K33531 PG; Tuttlingen, Germany) provide made-to-order forceps, respectively; E and F: The bifurcation of hepatic ducts on the common hepatic duct side (E) and characteristic findings of the end of the intra-pancreatic bile duct on the common bile duct side (F) should be confirmed. Interrupted ligations at the upper and lower edges of the transductal incision prevent progressive laceration during cholangioscope maneuvers (red arrows). Fixation sutures (blue arrows) are removed. CHD: Common hepatic duct; CBD: Common bile duct.

AVOIDANCE OF A MISIDENTIFICATION OF THE EHBD AND CYSTIC DUCT DURING LAPAROSCOPIC CHOLEDOCHOLITHOTOMY

In a patient who has a history of laparotomy, severe adhesions and dense tissue are often intractable (Figure 6C and D). Moreover, the location of important ducts and vessels may be shifted. Intraoperative recognition of a "second cystic duct" or an "accessory duct" strongly indicates misidentification of the cystic and CHD[32]. Intraoperative cholangiography is the recommended solution to detect this misidentification[32]. Identification of Hjortsjo's curve is a helpful way to detect the branches from the right hepatic duct[7,70]. Although there is no evidence for routine cholangiography[34], laparoscopic surgeons should not hesitate to perform intraoperative cholangiography when indicated[94-98].

IMPORTANCE OF THE SPECIALIZED FORCEPS TO

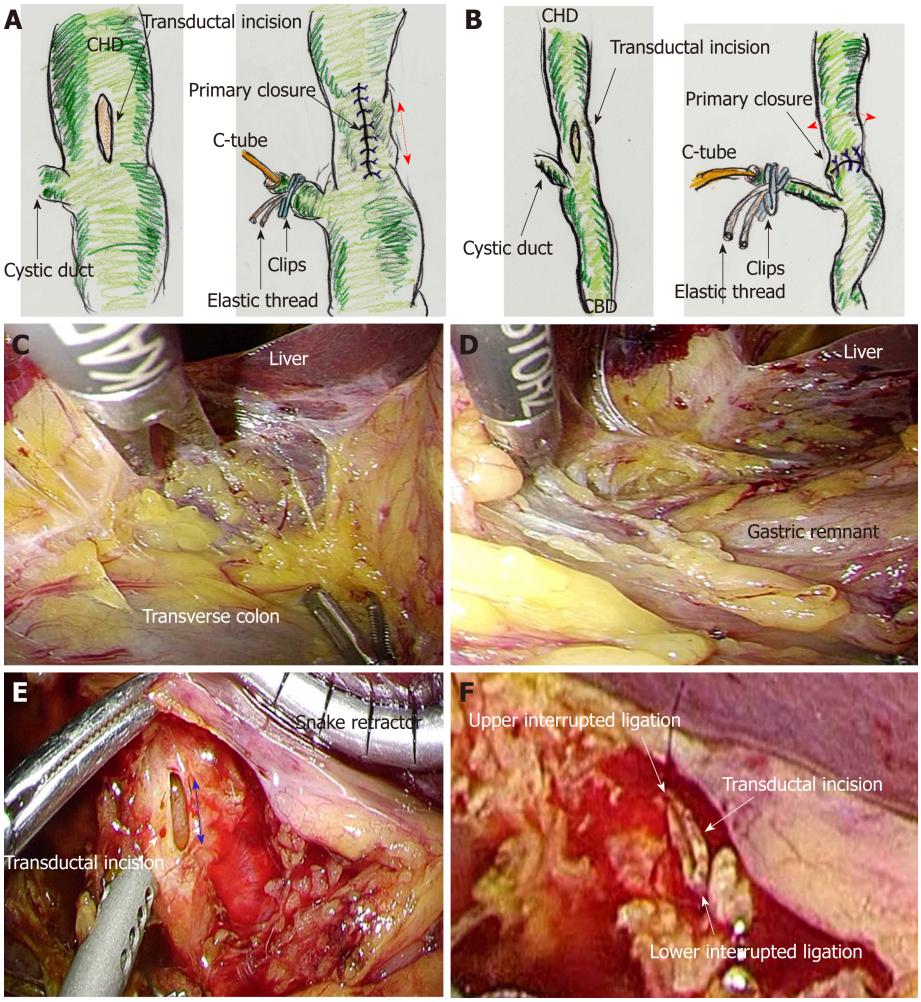

Figure 6 Laparoscopic choledocholithotomy. A: Generally, both the transductal incision and subsequent primary closure are made in the same direction along the long axis; B: In ducts with diameter smaller than 7-8 mm, primary closure is performed in the direction of the short axis to avoid postoperative stenosis; C and D: Though severe adhesions and dense tissue are often intractable during reoperative surgery, a laparoscopic approach is safe and feasible for choledocholithotomy; E and F: A transductal incision (blue arrow) is made along the long axis, and full-layer interrupted sutures are placed at the upper and lower edges of the transductal incision to avoid severe laceration of the extrahepatic bile duct during cholangioscope maneuvers. CHD: Common hepatic duct; CBD: Common bile duct.

ADEQUATELY OPERATE THE CHOLANGIOSCOPE DURING LAPAROSCOPIC CHOLEDOCHOLITHOTOMY

Intraoperative cholangioscopy is an important procedure for observing intraluminal findings and removing stones. Frustrating procedures should be avoided. The duration of intraoperative stone clearance accounts for most of the operative time. Cholangioscopy has only two-way operation. Hence, a dedicated forceps to atraumatically grasp the cholangioscope is a key tool for smart cholangioscope maneuvers and successful laparoscopic choledocholithotomy. Conventional laparoscopic forceps cause severe damage to the surface coating of the cholangioscope. Even mild damage requires very expensive repair, which may reach nearly 10000 USD. Olympus (Product standard number: A66070A) and Karl Storz Endoskope (Product standard number: K33531 PG) provide made-to-order forceps, respectively. Actual forceps we use are shown in Figure 5D. The transductal incision is made along the long axis (Figure 6E). Thereafter, full-layer interrupted sutures should be placed at the upper and lower edges of the transductal incision (Figure 6F), because cholangioscopic maneuvers can easily cause severe laceration of the EHBD along its long axis.

UNEXPECTED THERMAL DAMAGE AROUND THE BILE

DUCT

Cautery-induced injury results in necrotizing loss of ductal and/or perivascular tissues[32]. Cautery, laparoscopic coagulation shears and stronger devices may cause thermal necrosis of adjacent structures[32,99], and may subsequently cause delayed thermal injury[100]. This spread of thermal damage results in biliary complications after surgery[7]. Developed vessels on the surface of the EHBD should be carefully protected from thermal damage[7]. Minimized interrupted sutures and ligation are the first choice to achieve hemostasis near the biliary wall (Figure 4B). If safe hemostasis is not possible with additional suture, a button-shaped electrode with suction and a soft-coagulation system (VIO, Erbe) may be permissible for hemostasis near the EHBD.

CLINICAL MANAGEMENT AFTER LAPAROSCOPIC CHOLEDOCHOLITHOTOMY

An initial cholangiography is performed on postoperative day 4, with contrast agent adjustment according to the purpose of cholangiography. For intraoperative cholangiography, a full-concentration contrast agent with atoxic dye is used to detect even subtle leakage and stenosis. Half-concentration contrast agent without dye is used for postoperative cholangiography, because full concentration may hide small stones. The C-tube is thereafter removed based on the cholangiographic findings and need for ongoing biliary drainage[7]. Management of C-tube drainage is simple; transcystic drainage is easily replaced with transpapillary biliary drainage, using a so-called "rendezvous technique" that uses endoscopic cannulation *via* the guidewire through the C-tube[101-103]. In contrast, transductal T-tube drainage ineluctably requires drain placement for a period of at least 3 wk[65,66]; long-term drainage results in electrolyte abnormalities, disordered digestion, diarrhea and dehydration. Intake of autogenous bile (*i.e.*, drained bile) is difficult even when bile is cut with cola or snowball syrup[104,105]. Moreover, the discharge with T-tube placement is burdensome.

DISCUSSION

Laparoscopic HBP surgery for benign diseases has several advantages, including excellent magnified visualization and an adequate surgical field located on the right anterior side of the body[7,30,84]. Laparoscopic surgeons should be proficient in a variety of techniques and devices[6,14]; laparoscopic choledocholithotomy requires skillful manipulation of the forceps[30]. This advanced surgery is feasible in the clinical setting[7,30,84]. Even when endoscopic transpapillary biliary drainage is emergently required in patients with acute cholangitis[19,45], subsequent laparoscopic HBP surgery for benign diseases of the EHBD have an excellent clinical course and acceptable outcomes[22,26-28,31].

Laparoscopic choledocholithotomy with transcystic C-tube drainage results in an excellent rate of stone clearance, less bile leakage, less blood loss, acceptable mortality and morbidity rates, shortened hospital stay, and earlier social reintegration than conventional open surgery with transductal T-tube drainage[22,27,69,106-109]. However, in a laparoscopic approach, operative time was prolonged and cost becomes more expensive[110,111]. Overall, we should never forget that laparoscopic choledocholithotomy with transcystic C-tube drainage is the first choice for biliary stones in the EHBD.

Severe acute cholangitis and AOSC easily result in sepsis[17,18]; the elderly population is a target of AOSC[42,44]. Emergent biliary drainage is critical for patients with life-threatening conditions[19]. To preserve the physiological function of Oddi's sphincter, ill-considered use of EST should be avoided[20]. HBP surgeons should make an effort to end conventional open surgery with transductal T-tube drainage[47,49,70]. The right upper quadrant of the abdomen provides adequate space for laparoscopic HBP surgery[7,30,84]. One-stage laparoscopic choledocholithotomy has excellent clinical outcomes[22,26-28] and cost-effectiveness[112,113], even though emergent biliary drainage to prevent sepsis[19,45] and complete removal of risk factors for general anesthesia[53-55] are required.

Robotic surgery offers a promising frontier in the field of HBP surgery[114-116]; however, laparoscopic approaches are safe and feasible for benign biliary diseases of the EHBD[22,25-30,84]. During recent decades, laparoscopic surgery has been well developed, especially in the field of HBP surgery. Biliary stone in the EHBD is a common disease, and laparoscopic choledocholithotomy is a routine surgery and is

not so skill-demanded in many centers nowadays. However, especially in Japan, laparoscopic choledocholithotomy is not a routine surgery in spite of a cover of medical insurance[117,118], and many physicians condone an ill-considered use of EST for biliary stones[20]. Here, actual procedures of laparoscopic choledocholithotomy are described in detail, and also important documents for this advanced surgery are summarized in Table 1. We hope that our article with visual explanation and literature review will be informative for skillful HBP surgeons.

CONCLUSION

Laparoscopic choledocholithotomy is not rocket science. Safe laparoscopic HBP surgery is the recommended approach for benign diseases of the EHBD. I hope that this article results in benefits for these patients.

Table 1 Important documents for laparoscopic choledocholithotomy

Reference number	Remarks
[7,8,14,32-24]	Experience alone is not enough to ensure successful performance of laparoscopic surgeries.
[7,30,84]	The right upper quadrant provides a suitable location for the surgical field in laparoscopic procedures.
[22,23]	Technical challenges have prevented laparoscopic surgeries for the EHBD (*e.g.*, choledocholithotomy and choledochojejunostomy) from gaining worldwide popularity.
[26-28,30,31,56,59]	Laparoscopic choledocholithotomy provides safe and feasible treatment for recurrent stones and associated cholangitis.
[22,26-28,110,111]	For acute cholangitis and bile duct stone, one-stage laparoscopic choledocholithotomy has excellent clinical outcomes and cost-effectiveness.
[19,45]	For patients with acute cholangitis, biliary drainage should be performed as soon as possible.
	Risk factors for general anesthesia should be completely removed by preoperative biliary drainage.
[45]	Transpapillary biliary drainage without EST (*i.e.*, nasobiliary drainage or biliary stenting) should be performed initially as an emergent therapy for acute cholangitis.
	For patients with acute cholangitis, EST is not routinely recommended for biliary drainage alone.
[20]	Ill-considered use of EST should be avoided.
[28,56-59]	Acute cholangitis and bile duct stones are critical problems in a patient after abdominal surgery.
[28,59]	Laparoscopic approach is advantageous even for reoperative choledocholithotomy in a patient with a past history of laparotomy.
[62]	Cholecystectomy after EST for biliary duct stones does not reduce the incidence of recurrent cholangitis.
[22]	Transcystic C-tube drainage has a lower complication rate than transductal T-tube drainage or EST.
[69]	Previously, choledocholithotomy *via* conventional open surgery with transductal T-tube drainage versus laparoscopic primary closure with transcystic C-tube drainage remains controversial.
[22,27,69,106-109]	Currently, laparoscopic choledocholithotomy with primary closure and transcystic C-tube drainage is superior to conventional open surgery with transductal T-tube drainage.
[47,49,70]	Currently, HBP surgeons intend to end the use of transductal T-tube drainage.
[73,74]	Operative time is greatly affected by the duration of stone removal.
[77,78]	Detailed preoperative investigation is important for successful laparoscopic choledocholithotomy with a shortened operative time.
[91-93]	The method of primary closure of the transductal incision is chosen according to the EHBD diameter.
[32]	Cautery-induced injury results in necrotizing loss of ductal and/or perivascular tissues.
	Anatomical misidentificaion should be avoided.

EHBD: Extrahepatic bile duct; EST: Endoscopic sphincterotomy; HBP: Hepatobiliary and pancreatic.

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MINIREVIEWS

Hepatocellular carcinoma surveillance: An evidence-based approach

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Abstract

Hepatocellular carcinoma (HCC) makes up 75%-85% of all primary liver cancers and is the fourth most common cause of cancer related death worldwide. Chronic liver disease is the most significant risk factor for HCC with 80%-90% of new cases occurring in the background of cirrhosis. Studies have shown that early diagnosis of HCC through surveillance programs improve prognosis and availability of curative therapies. All patients with cirrhosis and high-risk hepatitis B patients are at risk for HCC and should undergo surveillance. The recommended surveillance modality is abdominal ultrasound (US) given that it is cost effective and noninvasive with good sensitivity. However, US is limited in obese patients and those with non-alcoholic fatty liver disease (NAFLD). With the current obesity epidemic and rise in the prevalence of NAFLD, abdominal computed tomography or magnetic resonance imaging may be indicated as the primary screening modality in these patients. The addition of alpha-fetoprotein to a surveillance regimen is thought to improve the sensitivity of HCC detection. Further investigation of serum biomarkers is needed. Semiannual screening is the suggested surveillance interval. Surveillance for HCC is underutilized and low adherence disproportionately affects certain demographics such as non-Caucasian race and low socioeconomic status.

Key words: Liver cancer; Hepatocellular carcinoma; Surveillance

Core tip: Hepatocellular carcinoma (HCC) is a leading cause of cancer related death and 80%-90% of new cases occur in patients with cirrhosis. Surveillance programs have been

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developed on the basis that earlier detection of disease provides more curative treatment options and a better prognosis. This comprehensive review focuses on current literature regarding the utility of HCC surveillance, high-risk populations, surveillance modalities and adherence and recall.

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INTRODUCTION

Primary liver cancer is projected to be the sixth most commonly diagnosed and fourth most common cause of cancer death worldwide in 2018 with hepatocellular carcinoma (HCC) making up 75%-85% of all primary liver cancers[1]. HCC has a higher incidence in developing countries with > 80% of HCC cases occurring in either sub-Saharan Africa or Eastern Asia. HCC is three times more prevalent among men than women. The mean age at diagnosis varies among geographical location depending on the local burden of disease. While the incidence of HCC is decreasing in some Chinese and Japanese populations due to vaccination and treatment programs for viral hepatitis, HCC cases are increasing in the United States. In fact, HCC is the fastest growing cause of cancer-related deaths in the United States, with a decrease in the mean age at diagnosis[2-4]. Chronic liver disease of any etiology remains the most significant risk factor, with 80% to 90% of new HCC cases occurring in this population[4-6]. Given the international burden of disease, surveillance programs have been developed for earlier detection and mortality reduction. Current guidelines recommend enrollment in surveillance programs for adults with cirrhosis and high-risk patients without cirrhosis using ultrasound (US) with or without alpha-fetoprotein (AFP) at six-month intervals. These guidelines are largely unanimous among major international societies including the American Association for the Study of Liver Disease (AASLD), the European Association for the Study of the Liver (EASL) and the Asian Pacific Association for the Study of the Liver (APASL)[7-9]. Our objective is to summarize the current literature regarding utility of HCC surveillance, high-risk populations, surveillance modalities and adherence and recall.

LITERATURE SEARCH

A comprehensive literature search was performed using PubMed and Google Scholar for research papers regarding HCC surveillance and related literature was analyzed to prepare this review article. We did not restrict the search to a certain period of time. Articles written in English and published in peer-reviewed journal were included.

HCC SURVEILLANCE

Optimal screening tests are designed to detect an asymptomatic or subclinical disease and must meet several criteria including high sensitivity, cost effectiveness and availability. Diseases suitable for screening include those that are of high burden in selected populations with a proven treatment strategy and outcomes that improve with early detection[10]. When screening tests are used at regular intervals in at-risk populations, this is considered surveillance[11].

A randomized clinical trial (RCT) is the optimal way to measure the effectiveness of cancer surveillance programs but unfortunately there is limited RCT data available to address whether HCC surveillance programs reduce disease-related mortality. A key study that is often cited was performed by Zhang *et al*[12] in China and included 18816 patients with current or previous evidence of hepatitis B infection. The selected patients were randomly assigned to surveillance group (n = 9373) or control group (n = 9443). Surveillance in this study consisted of measurement of serum AFP levels and US imaging every 6 mo. Study adherence was poor (60%) but showed a significant

reduction (37%) in mortality related to HCC in the surveillance group. While this landmark study is the basis for many of the surveillance recommendations, it is not without its criticisms and limitations. Limitations of this study include lack of outcome data other than death and lack of information regarding all-cause mortality. The authors also failed to account for clustering which could produce misleading statistical significance. Additionally, the study population included only HBV patients. With these points in mind, some have argued that there is limited ability to extrapolate this data and its conclusions to Western countries[13]. However, any attempts to affirm these conclusions with an RCT in North America or Europe is largely impractical due to ethical concerns in randomizing patients at risk for HCC to a no surveillance group and the sheer difficulty of enrolling patients who are informed of the potential risks and benefits of HCC surveillance[14].

Although the RCT data is of limited quality and unable to be replicated, this does not disprove the effectiveness of HCC surveillance and there is observational data available to support a survival benefit from HCC surveillance. A 2014 meta-analysis of forty-seven cohort and case-controlled studies looked at the effect of HCC surveillance on early tumor stage detection, receipt of curative therapy and overall survival in patients with cirrhosis. Of the 15158 patients analyzed, 6284 (41.4 %) had HCC detected by surveillance while 8874 (58.6%) had HCC detected incidentally or due to presence of symptoms. Rates of HCC detected by surveillance were higher among studies in the United States (51%) and Europe (45%). Of the studies that included data on tumor stage and curative treatment, HCC surveillance was associated with improved early stage detection, curative treatment rates and prolonged survival. The pooled 3-year survival rate was 50.8% among patients undergoing surveillance compared to 27.9% among those without surveillance. Overall the data is encouraging, however, limitations include short duration of follow up and failure to adjust for liver function or lead-time bias. This data suggests that given the association of HCC surveillance with significant improvements in early tumor detection, these patients are more likely to receive curative treatment and thus overall survival benefit providing evidence to support regular HCC surveillance guidelines[15].

Given the poor 1-year and 3-year survival rates in patients with HCC (36% and 17%, respectively), early detection may provide curative treatment options including surgical resection, transplantation and percutaneous ablation. Finding late stage or advanced HCC removes these options and leaves only palliation[16-18].

HCC surveillance has also been shown to be cost effective. Both Lin *et al*[19] and Arguedas *et al*[20] found that HCC screening using either biannual AFP and annual abdominal US or triple phase computed tomography (CT) were cost effective compared to no surveillance, with cost effectiveness ratio less than $50000 quality-adjusted life year. This is comparable to other frequently used screening strategies including colonoscopy and mammography[19,20].

HIGH RISK POPULATIONS

The AASLD recommends offering surveillance when the risk of HCC is at least 1.5% per year and the incidence is greater than 0.2% per year, which includes patients with cirrhosis and some non-cirrhotic hepatitis B carriers[7]. The risk for HCC in chronic liver disease differs based on the underlying etiology of disease. Chronic hepatitis C virus (HCV) infection is associated with a 15- to 20-fold higher risk of HCC compared to those without HCV and patients with HCV related cirrhosis have a 3.5% annual rate of HCC development[4]. While HCC can develop in HCV infected patients in the absence of cirrhosis, the odds decrease significantly when elastography shows a lack of advanced fibrosis (< 10 kPa)[21]. Currently, HCC surveillance is not recommend in patients with chronic hepatitis C without cirrhosis[7]. Eradication of HCV with sustained viral response (SVR) has been shown to decrease the risk for HCC. Morgan *et al*[22] previously showed that in the interferon era, eradication of HCV with SVR resulted in a reduced risk for HCC (relative risk = 0.24).

The landscape of HCV treatment has evolved with the availability of effective direct antiviral agents (DAAs). As opposed to IFN-based therapies, DAAs are better tolerated in patients with advanced liver disease and can provide SVR rates > 95%[23-25]. Despite the utility of DAAs, there has been a debate regarding increased incidence of HCC (recurrence or *de novo*) in contrast to IFN-based treatment. There are conflicting results from various retrospective studies looking at DAA therapy and HCC. An initial small cohort study by Reig *et al*[25] suggested an increase in rates of HCC following DAA therapy, however a large meta-analysis subsequently found no difference in HCC occurrence in patients following SVR from DAA *vs* IFN-based

treatment[26]. Kwong *et al*[27] recently showed that although the incidence of *de novo* HCC in patients with HCV cirrhosis has increased in the DAA era, these changes may be explained by changes in the rates of liver transplantation among HCV patients and wait list mortality. Increasing age and severity of liver disease likely contributes to a higher incidence of HCC in transplant candidates as well[23,27]. Current guidelines continue to recommend HCC surveillance in patients with cirrhosis even after eradication of HCV with DAA therapy[7].

Patients with chronic hepatitis B virus (HBV) represent a unique population who require HCC surveillance outside of the setting of cirrhosis. Specific recommendations for surveillance in patients with chronic hepatitis B without cirrhosis include Asian and black males > age 40, Asian females > age 50, African/North African blacks with hepatitis B > age 20, patients with hepatitis D co-infection, and patients with a first-degree relative with HCC[7,28]. High levels of HBV DNA are associated with a higher risk of developing HCC and worse prognosis in those with HCC[29]. It is thought that active HBV viral proliferation promotes carcinogenesis through both direct and indirect mechanisms and therefore antiviral treatment can lower the risk for HCC occurrence in these patients[30]. A previous study showed that patients with advanced fibrosis or cirrhosis who received lamivudine had a significantly lower risk (3.9%) of developing HCC compared to placebo (7.4%)[30]. Despite the reduced risk, these patients still require routine monitoring for HCC occurrence. Alanine aminotransferase (ALT) is a marker of liver injury and can be used in conjunction with other host factors such as age and duration of infection to identify high-risk HBV carriers[28,29]. Other important risk factors include environmental exposures such as alcohol, cigarettes and the mycotoxin aflatoxin[31] as well as a family history of HCC[32].

Heavy alcohol use and subsequent alcohol related liver disease has also been associated with the development of HCC. The incidence of HCC in patients with alcohol related cirrhosis (Child-Pugh A or B) has been previously reported to be 2.5%[33]. A previous review found that alcohol use greater than 80 g/d for more than 10 years led to a 5-fold increase in risk for development of HCC[34]. A synergistic effect can occur between alcohol use and other risk factors for HCC, most prominently viral hepatitis. It has been suggested that screening patients younger than age 55 with platelet counts > 125000 mm^3 may not be cost effective[34], however current guidelines still recommend surveillance for all patients with cirrhosis[7].

Non-alcoholic fatty liver disease (NAFLD) and its complications are of increasing clinical significance, particularly in Western nations due to a rising burden of metabolic syndrome[35]. A prior retrospective analysis has shown that cumulative incidence of HCC is slightly lower in NAFLD related cirrhosis compared to HCV cirrhosis (2.6% *vs* 4%)[36], however surveillance is still recommended in this population. A very low incidence of HCC has been described in patients with NAFLD without cirrhosis, however incidence rates do not meet surveillance criteria at this time[37,38]. Continued investigation of these relationships is of utmost importance given the increasing prevalence and incidence of NAFLD[7].

Less common etiologies of cirrhosis that can also increase the risk for HCC include hereditary hemochromatosis (HH), primary biliary cholangitis (PBC), autoimmune hepatitis (AIH) and alpha 1-antitrypsin deficiency (A1AT). Patients with HH have been shown to have a 20-fold higher risk of HCC without an increased risk for non-hepatic malignancies in a large Swedish based population cohort study[39]. While the incidence of HCC in HH patients with cirrhosis is unknown, the AASLD endorses a surveillance benefit in these patients[7]. HCC also occurs with increased frequency in patients with cirrhosis secondary to PBC[40]. The risk of HCC in these patients has been shown to be similar to the risk of HCC in patients with HCV cirrhosis[41], and therefore the AASLD recommends routine surveillance[7]. Although there are no formal recommendations regarding surveillance in patients with cirrhosis secondary to AIH, several studies note an annual incidence rate > 1.5% and therefore it is reasonable to include these patients in standard surveillance protocols[42]. The incidence of HCC in patients with cirrhosis secondary to A1AT deficiency has been previously shown to be 0.88%/year in one small retrospective study[43], however guidelines still recommend biannual surveillance at this time[7]. Additional studies would be helpful in these less common causes of cirrhosis to more accurately determine annual incidence and suitability for surveillance programs.

As mentioned, the mortality benefit in HCC surveillance lies in the advantages of early detection so that curative therapies, including liver transplantation, can be considered. As such, patients with Childs Pugh C cirrhosis who are not eligible for HCC treatments and are not candidates for liver transplantation should not be offered surveillance programs. Liver transplant candidates should continue to undergo surveillance up until the time of transplantation[7].

SURVEILLANCE METHODS

The AASLD recommends surveillance using US with or without AFP every 6 mo. We will look at the evidence behind the surveillance methods including imaging techniques and biomarkers as well as the time intervals when they should be performed.

Imaging techniques

US is an inexpensive and noninvasive surveillance method without any risk or radiation exposure for the patient. US detection of HCC in a cirrhotic liver is limited by several factors including hepatic features such as abnormal liver texture, patient characteristics such as obesity and technical limitations such as quality of US and experience of ultrasonographer[44]. A meta-analysis looking at the performance characteristics of surveillance US to detect early HCC found a sensitivity of 94% for detecting HCC lesions at any stage and sensitivity of 63% for early stage tumors. Adding AFP measurement to the US regimen did not provide a statistically significant increase in sensitivity. Performing the surveillance every 6 mo as opposed to annually increases the sensitivity to 70% for detecting early stage HCC[45]. An additional study looking strictly at patients with Child-Pugh classes A and B cirrhosis found that by combining AFP to US the sensitivity increased from 32% to 65% for detecting early stage HCC[46]. Given the variation in reported sensitivity of US, one study looked at predictors of surveillance failure and found that one in five USs for HCC surveillance are classified as inadequate. This study showed that US quality is diminished in obese patients and those with cirrhosis from alcohol or NAFLD. It is thought that this deficiency is related to altered US visualization from the presence of subcutaneous fatty tissue in addition to hepatic steatosis. Consequently, this leads to under-recognition of small or early stage HCC nodules[47,48]. Pocha *et al*[49] randomized 163 patients with cirrhosis to receive either biannual US or annual triphasic CT to compare performance and costs. Biannual US was found to be more sensitive (71.4%) when compared to CT (66.7%). Overall costs were less for biannual US. In addition to lacking cost-effectiveness, CT has risks of radiation exposure and renal injury that must be kept in mind when considering imaging modalities[50]. Magnetic resonance imaging (MRI) is the most sensitive imaging modality for HCC but its use is limited by high cost and low accessibility. A recent prospective study of 407 South Korean patients compared surveillance with MRI to US and found that MRI with liver specific contrast had a higher detection rate and a lower false-positive rate. MRI was significantly more sensitive in detecting very early stage HCC meaning a single lesion < 2 cm with a sensitivity of 84.8% compared to 27.3% detected by US. This study may not be generalizable to other populations as the majority of patients (74.4%) had HBV related cirrhosis and the average body mass index (BMI) was low (24.3). Given the obesity epidemic in the United States, with a prevalence of obesity (BMI > 30) in adults greater than 30%, the sensitivity of ultrasound may be reduced in this population [51,52]. While the AASLD practice guidelines acknowledge limited US reliability in patients with truncal obesity or marked parenchymal heterogeneity, CT or MRI is not recommended as the primary imaging technique for HCC surveillance. CT or MRI may be utilized in select patients with inadequate US visualization or at high risk for inadequate US[7].

Serum biomarkers

Novel biomarkers are being introduced as simple blood tests with growing applications for cancer screening in patients carrying a diagnosis of cirrhosis, including early detection, prognostication, and surveillance. Biomarkers in development are variable in approach, including biochemical metabolites, proteins, and RNA. Perhaps the most promising biomarker in cirrhosis screening is AFP, which has gained favor as a supplement to US screening [53]. It has gained popularity as an affordable and accessible screening test and received a 'conditional' recommendation to be used in conjunction with semiannual US according to AASLD guidelines[7].

As mentioned, screening US may be limited among select populations secondary to body habitus, obesity, and early HCC disease[15]. In such cases, biomarkers may supplement US in the detection of early disease. And while the combined performance characteristics of AFP in conjunction with US are not yet known in full, it is believed that AFP does improve the sensitivity of interval screening[7]. In a retrospective analysis of all etiologies of cirrhosis, the performance characteristics for serum values above 20 ng/mL approach 70% sensitivity and 90% specificity[54]. When AFP is implemented alongside US screening, one analysis found an improvement in curative treatment rates and improved 3-year overall survival rates when compared to groups that did not receive routine HCC screening[15].

Some of the largest criticisms of biomarkers, and specifically AFP, appear to be

drawn from its inconsistent performance characteristics across different etiologies of chronic liver disease. Among patients with chronic HCV, AFP levels may be falsely elevated due to acute inflammation and therefore the upper limit of normal may need to be adjusted in this population. This is in contrast to patients with non-HCV related cirrhosis, in which AFP levels greater than 11 ng/L have more accurate performance characteristics[54]. As a result, there is the possibility of confusion among clinicians wishing to screen for HCC, as multiple thresholds may be needed, depending on the sub-population.

European guidelines continue to recommend against the use of AFP despite estimated improvement of 6% to 8% detection rate, as it is met with a larger number of false positives[8]. But the future of biomarker screening is promising, with numerous other molecules under research and development: osteopontin, Midkine, AFP-L3, DCP, GPC3, and alpha-1-fucosidase. Predictive models, such as the GALAD, have also been validated as a tool to address the heterogeneity in biology among cirrhosis etiologies[53]. As alternative biomarkers progress through development, the landscape of HCC screening will assuredly change alongside it.

Surveillance intervals

There is evidence to support the suggested six-month surveillance interval. A study by Santi *et al* compared patients with semiannual surveillance to annual surveillance. The semiannual surveillance group was associated with increased detection rate of early stage HCC tumors leading to higher chance of curative therapies and overall better prognosis[55]. In the aforementioned meta-analysis by Singal *et al*[45] surveillance US every 6 mo significantly improve the sensitivity for detection of early stage HCC when compared to annual exams. More frequent imaging (every 3 mo) has not been shown to improve survival or increase detection of small HCC lesions and is therefore not recommended at this time [56].

ADHERENCE AND RECALL

Adherence

Proper screening for HCC is a continuum of services, extending from initial patient screening, diagnosis, treatment and ultimately surveillance. As one may expect, there are numerous chances for failure in the delivery of cancer screening care. Patient adherence seems to be a major barrier in colorectal cancer screening, where nearly 40% of patients were found to have missed their first colonoscopy or flexible sigmoidoscopy appointment[57]. But in the case of HCC, one analysis suggested that only 3% of patients missed screening once ordered by a provider. The most significant barrier identified in this same retrospective cohort was the lack of surveillance orders from a provider, which neared 40% missed opportunities on behalf of the healthcare system[58]. Among referring providers, there seems to be a measurable difference in frequency of screening between primary care physician (PCP) settings and subspecialty gastroenterology clinics. The most prominent barriers perceived by PCPs are related to falling out-of-date with regards to the newest HCC screening guidelines, ineffective communication with at-risk patients and prioritizing other issues in clinic[59]. Of course, referring patients to be screened for HCC is more nuanced than a simple referral, and requires recognizing at-risk patients, establishing a diagnosis of cirrhosis, and then actively counseling the patient. A meta-analysis of 9 studies looked utilization rates and factors that affect them for HCC surveillance. Pooled utilization rates for HCC surveillance were 18.4%. Utilization rates were better in patients followed by subspecialists (51.7%) compared to primary care physicians (16.9%). This study also found other demographics associated with lower surveillance rates including non-Caucasian race and low socioeconomic status[60]. Studies have found that quality improvement measures can be used to increase the rate of HCC surveillance. By enrolling cirrhotic patients into a chronic disease management program that incorporates automatic reminders for surveillance, surveillance rates increased from 73% to 90% over 3 years[61]. Including reminders for HCC surveillance along with screening for other known complications of cirrhosis such as varices or ascites could be helpful as well. Overall, data on patient adherence suggests an opportunity for improvement available on the part of providers as well as systems based approach.

Recall

Surveillance programs need a reliable recall strategy for abnormal findings on US imaging. Lesions less than 1 cm can be followed with repeat US (with or without AFP) in 3-6 mo. Further management of abnormal surveillance imaging including lesions >

1 cm can be managed according to the Liver Imaging Reporting and Data System (LI-RADS). Diagnostic liver biopsy may be needed for indeterminate lesions that fall into LI-RADS 4 (probably HCC) or LI-RADS M categories (malignancy but not definitely HCC)[7].

CONCLUSION

To summarize, there is sufficient evidence to support the importance and survival benefit of HCC surveillance (Table 1). Early identification through surveillance provides more curative treatment options. Surveillance programs are indicated for all cirrhotic patients and high-risk HBV patients without cirrhosis. Surveillance for obese and NAFLD patients is of increasing interest as this population continues to rise in the United States. Semiannual US (with or without AFP) is the recommended imaging modality for surveillance but clinicians must consider alternative imaging if the US is limited. Surveillance rates are low and disproportionately affect certain populations. Clinicians must recognize the importance of adherence to surveillance and continue to explore options to improve surveillance rates through systems based approaches and awareness.

Table 1 Patients at the highest risk for hepatocellular carcinoma

Population group	Threshold incidence for efficacy of surveillance (> 0.25 LYG; % per year)	Incidence of HCC (% per year)
High risk of HCC for whom surveillance benefit is indicated		
Asian male hepatitis B carriers over age 40	0.2	0.4%-0.6% per year
Asian female hepatitis B carriers over age 50	0.2	0.3%-0.6% per year
Hepatitis B carrier with family history of HCC	0.2	Increased
African and/or North American blacks with hepatitis B	0.2	HCC occurs at a younger age
Hepatitis B carriers with cirrhosis	0.2-1.5	3%-8% per year
Hepatitis C cirrhosis	1.5	3%-5% per year
Stage 4 PBC	1.5	3%-5% per year
Genetic hemochromatosis and cirrhosis	1.5	Probably > 1.5% per year
Alpha-1 antitrypsin deficiency and cirrhosis	1.5	Probably > 1.5% per year
Cirrhosis secondary to other etiologies	1.5	Unknown
High risk of HCC for whom surveillance benefit is uncertain		
Male hepatitis B carriers younger than 40	0.2	< 0.2% per year
Female hepatitis B carriers younger than 50	0.2	< 0.2% per year
Hepatitis C and stage 3 fibrosis	1.5	< 1.5% per year
NAFLD without cirrhosis	1.5	< 1.5% per year

Adapted with permission from AASLD guidelines on management of HCC[7] and HCC Surveillance[62]. LYG: Life-years gained; AASLD: American Association for the Study of Liver Disease; HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease.

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MINIREVIEWS

Cellular therapy: A promising tool in the future of colorectal surgery

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Abstract

Cellular therapy may be the solution of challenging problems in colorectal surgery such as impaired healing leading to anastomotic leakage and metastatic colorectal cancer (CRC). This review aimed to illustrate the role of cellular therapy in promotion of wound healing and management of metastatic CRC. An organized literature search for the role of cellular therapy in promotion of wound healing and management of metastatic CRC was conducted. Electronic databases including PubMed/Medline, Scopus, and Embase were queried for the search process. Two types of cellular therapy have been recognized, the mesenchymal stem cells (MSCs) and bone marrow-mononuclear cells (BM-MNCs) therapy. These cells have been shown to accelerate and promote healing of various tissue injuries in animal and human studies. In addition, experimental studies have reported that MSCs may help suppress the progression of colon cancer in rat models. This article reviews the possible mechanisms of action and clinical utility of MSCs and BM-MNCs in promotion of healing and suppression of tumor growth in light of the published literature. Cellular therapy has a potentially important role in colorectal surgery, particularly in the promotion of wound healing and management of metastatic CRC. Future directions of cellular therapy in colorectal surgery were explored which may help stimulate futures studies on the role of cellular therapy in colorectal surgery.

Key words: Cellular therapy; Future; Colorectal surgery; Stem cells

Core tip: Cellular therapy may be the solution of challenging problems in colorectal surgery such as impaired healing leading to anastomotic leakage and metastatic colorectal cancer. Two types of cellular therapy have been recognized, the mesenchymal stem cells (MSCs) and bone marrow-mononuclear cells therapy. These cells have been shown to accelerate and promote healing of various tissue injuries in animal and human studies. In addition, experimental studies have reported that MSCs may help suppress the progression of colon cancer in rat models.

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INTRODUCTION

Colorectal surgery entails several technical aspects and various postoperative morbidities that may compromise the outcome of patients. Among these morbidities, improper healing and spread of colorectal cancer (CRC) are considered to be the most challenging problems.

Improper or delayed wound healing after reconstruction is considered a major challenge for many surgeons in the daily practice. Impaired wound healing can result in reconstruction failure which may lead to serious consequences in colorectal surgery such as anastomotic leakage (AL) and persistent fecal incontinence after failing anal sphincter repair.

It is worthy to remember that even when optimal surgical strategies and techniques are followed, failure of surgical reconstruction still occurs in a distressing rate as the case with AL after colorectal anastomosis which ranges between 1.5% and 15.9%[1,2]. It became apparent that, in addition to optimizing the surgical technique, alternative strategies may be necessary for further improvement in the healing process in order to decrease the rate of reconstruction failure.

Another serious problem is the loco-regional recurrence and distant metastasis of CRC occurring after an apparently curative surgery. Approximately 20%-25% of patients with CRC have synchronous liver metastases and another 25%-50% will develop liver metastases after apparently curative intent surgery[3]. Although the current chemotherapy has been reported to cause regression of metastatic CRC[4] with subsequent improvement in the overall survival after curative intent surgery[5], the management of patients with systemic disease is mostly palliative and metastatic cancer remains generally incurable and a major cause of cancer-related mortality. Most colorectal metastases affect the liver and only 10%-20% of them are resectable with a five-year survival rate of 30%-40%[6].

Even when radical colorectal resection is conducted, functional impairments are frequently encountered postoperatively with remarkable impact on patients' quality of life. Radical excision of rectal cancer usually results in poor bowel function, particularly low anterior resection syndrome (LARS) which affects up to 80% of patients after low anterior resection[7]. This encouraged many surgeons to adopt the "wait and watch" policy after complete clinical response of rectal cancer to neo-adjuvant chemo-radiotherapy. Organ preservation strategies have been increasingly used for rectal cancer in the modern surgical practice.

It has been suggested that the solution of the problems aforementioned may not be in more refinement or improvement in surgical technique or chemotherapy, but in biological interventions including cellular and immunological therapies. Cellular therapy is a promising modality that may become the future of colorectal surgery. This article highlights the role of cellular therapy in promotion of healing and suppression of progression of CRC which may shed light on the potentials and future directions of this innovative therapy.

ROLR OF CELLULAR THERAPY IN TISSUE HEALING

In order to comprehend the rationale of using cellular therapy in promotion of healing, the basic concepts of cellular response to injury should be emphasized. It has been demonstrated that tissue injury stimulates the mobilization of progenitor cells from the bone marrow to the site of injury to regenerate the stroma. These progenitor cells include the fibrocytes which then differentiate into fibroblasts and deposit collagen and extracellular matrix proteins[8,9]; and the endothelial progenitor cells which form new blood vessels[10]. The end result of migration of these mononuclear cells from the bone marrow to the site of injury is the formation of granulation tissue which subsequently matures into fibrous tissue.

It has been shown that mesenchymal stem cells (MSCs) also are mobilized from the bone marrow to the site of injury to promote healing[11] without integration in the tissues[12]. Therefore, the bone marrow mononuclear cells (BM-MNCs) and MSCs are

being mobilized in response to tissue trauma from the bone marrow to the site of injury to contribute to promotion of healing as illustrated in Figure 1. Since both have the same origin and pathway homing at the site of injury, the MSCs and BM-MNCs may belong to the same differentiation line, may have common cellular features and functions, and may have similar therapeutic efficacy[13].

The use of cellular therapy in promotion of wound healing is mainly based on the stem cell paradigm in which stem cells injected into the injured tissues will differentiate into parenchymal cells resulting in better healing and tissue regeneration. However, tissue regeneration secondary to differentiation of injected stem cells was not proved to occur in experimental studies. Another possible mechanism was postulated that stem cells may improve wound healing by secreting different healing promoting mediators, instead of differentiation into parenchymal cells[14].

On the other hand, an alternative concept, the stroma paradigm, was suggested. In this paradigm, the progenitor cells are first mobilized from the bone marrow to the site of injury to contribute to regeneration of the stroma, then the local stem cells start infiltrating the preformed stroma to regenerate the parenchyma.

In light of this paradigm, it would be logical that impaired stroma regeneration can prevent its infiltration by local parenchymal stem cells, resulting in healing by fibrosis rather than by regeneration. Many experimental studies supported the stroma paradigm against the stem cell paradigm. Three experimental studies examined the effect of local injection of stem cells on healing of injured anal sphincters[14-16] and concluded that the injected stem cells do not differentiate into skeletal muscles, yet they accelerate a normal regenerative mechanism that begins by regeneration of the stroma which is then infiltrated by muscle fibers from the nearby muscles[17].

Based on previous arguments; the use of MSCs may be not ideal in promoting healing and the BM-MNCs may be a more suitable alternative. This fraction of bone marrow contains the cells responsible for stroma regeneration ready to act, the fibrocytes and endothelial progenitor cells, in contrast to the MSCs which are supposed to be less differentiated[18,19].

Experimental and clinical studies have shown that both MSCs and BM-MNCs are equally effective in promotion of healing. Mazzanti *et al*[20] showed that local injection of MSCs and BM-MNCs have the same therapeutic efficacy in promotion of healing of injured anal sphincter muscles in rats. Other investigators have also reported that MSCs and BM-MNCs are equally effective in inducing regenerative changes in animal models of myocardial infarction and osteoarthritis[13,21].

Being equally effective with the MSCs, the BM-MNCs have the advantages of being less costly, easier to prepare, and not requiring weeks of in-vitro culture rendering them more suitable for clinical use[22]. The preparation of BM-MNCs takes approximately one hour after withdrawal of bone marrow. Orthopedic surgeons[22-24] have used bone marrow aspirate concentrate (BMAC) which is composed mainly of BM-MNCs[25] instead of *ex-vivo* cultivated stem cells in the treatment of bone defects, bone healing disorders, and osteonecrosis with promising results. Our group has also used BMAC to augment healing of repaired external anal sphincter in humans with promising results[26].

ROLE OF CELLULAR THERAPY IN TREATMENT OF METASTAIC CRC

Many studies demonstrated that MSCs home into various tumors as breast cancer, prostate cancer[27] and colon cancer[28]. It has been assumed that tumors tend to behave biologically as a wound that never heals, releasing several inflammatory mediators that recruit MSCs[29].

The effect of MSCs on tumor growth is controversial as some studies reported that MSCs can either enhance[30,31] or inhibit tumor growth[32,33]. Waterman *et al*[34] documented that MSCs can be primed by stimulation of toll like receptor 3 or 4 (TLR3 or TLR4) into immunosuppressive or proinflammatory MSCs, respectively. While the non-primed and immunosuppressive MSCs tend to enhance tumor growth, the proinflammatory MSCs tend to inhibit it. This concept may shed light on the controversial role and dual action of MSCs in tumor biology.

The key in using MSCs in inhibition of tumor growth lays in shifting the polarization of these cells from the immunosuppressive phenotype, which helps formation of tumor stroma (pro-tumor), to the proinflammatory phenotype which stimulates the immune system to destroy the tumor (anti-tumor). One of the strategies used for shifting polarization of MSCs to the proinflammatory phenotype is local injection of bacteria into the tumor.

Coley[35] treated patients with inoperable soft tissue sarcomas by local injection of

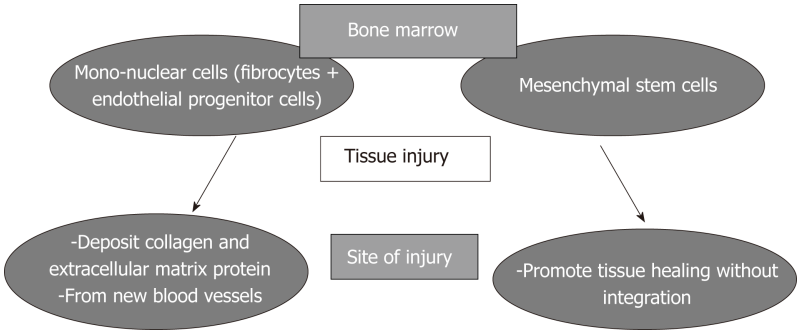

Figure 1 Mobilization of mono-nuclear cells and mesenchymal stem cells from the bone marrow to the site of tissue injury.

heat killed bacteria "Coley's toxin" with long term disease free survival of about 50% which is considered extraordinary. Although Coley's toxin is not used now in clinical practice, intra-vesical Bacillus Calmette Guerin (BCG) is considered the standard of care in patients with superficial bladder cancer[36]. In general, the antitumor effect of BCG on superficial bladder cancer is due to activation of the patient's immune response against the tumor[37] as evidenced by infiltration of the bladder wall by immune cells after BCG therapy[38]. To be effective, BCG therapy requires a competent host immune system[39]. We speculate that these bacterial products may prime MSCs that infiltrate the tumor to become proinflammatory, resulting to tumor regression. Although certain evidence is still lacking, combining MSCs with bacteria may help priming the MSCs to become proinflammatory which makes them a strong weapon against cancer.

Former experimental studies have documented the inhibitory effect of MSCs therapy on the progression of CRC. Francois *et al*[40] showed that intravenous injection of MSCs attenuated both initiation and progression of CRC in an immunocompetent rat model of colon cancer. In line with the previous study, Tang *et al*[41] showed that intravenous MSCs helped suppress the development of colon cancer in a colitis rat model. El-Khadragy *el al*[42] also showed that intra-rectal injection of non-manipulated bone marrow cells suppressed the progression of colon cancer in a rat model.

Similar to MSCs, fibrocytes seem also to either promote or suppress tumor growth through differentiation into different phenotypes. Fibrocytes that express $CD34^+$ were suggested to help inhibition of tumor growth in different cancers[43]. On the other hand, loss of $CD34^+$ on fibrocytes in tumor stroma with increased α-smooth muscle $actin^+$ are associated with increased invasive behavior of different tumors[44,45]. This may be explained by loss of the antigen presenting function of fibrocytes that lack $CD34^+$ expression, eventually leading to impaired immune response to malignant cells[40]. This concept of polarization of fibrocytes and the effect of this polarization on tumor biology is so similar to that of MSCs which may suggest common origin and functions of both cell types.

Although fibrocyte-based cellular therapies were not used yet to treat tumors even experimentally, the biologic similarity between fibrocytes and MSCs as aforementioned makes us postulate that local injection of BM-MNCs may have similar effects on tumor growth as MSCs. Perhaps the addition of a bacterial product such as BCG to either MSCs or BM-MSCs may help polarize stem cells or fibrocytes to the tumor suppressing phenotype, however, thorough and extensive research on this hypothesis is needed to ascertain its validity.

CONCLUSION

In conclusion, cellular therapy may be the future solution for difficult surgical problems such as impaired healing and tumors. Cells can be locally injected at sites of reconstruction to augment healing as to prevent AL. The use of MSCs and potentially BM-MNCs may help suppress the progression of metastatic CRC without the morbidity, mortality and limitations of major surgery. Further animal studies are highly required to prove the validity of these concepts.

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ORIGINAL ARTICLE

Basic Study

Characterization of hepatitis B virus X gene quasispecies complexity in mono-infection and hepatitis delta virus superinfection

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Abstract

BACKGROUND

Hepatitis delta virus (HDV) seems to strongly suppress hepatitis B virus (HBV) replication, although little is known about the mechanism of this interaction. Both these viruses show a dynamic distribution of mutants, resulting in viral quasispecies. Next-generation sequencing is a viable approach for analyzing the composition of these mutant spectra. As the regulatory hepatitis B X protein (HBx) is essential for HBV replication, determination of HBV X gene (*HBX*) quasispecies complexity in HBV/HDV infection compared to HBV mono-infection may provide information on the interactions between these two viruses.

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AIM

To compare HBV quasispecies complexity in the *HBX* 5′ region between chronic hepatitis delta (CHD) and chronic HBV mono-infected patients.

METHODS

Twenty-four untreated patients were included: 7/24 (29.2%) with HBeAg-negative chronic HBV infection (CI, previously termed inactive carriers), 8/24 (33.3%) with HBeAg-negative chronic hepatitis B (CHB) and 9/24 (37.5%) with CHD. A serum sample from each patient was first tested for HBV DNA levels. The *HBX* 5′ region [nucleotides (nt) 1255-1611] was then PCR-amplified for subsequent next-generation sequencing (MiSeq, Illumina, United States). HBV quasispecies complexity in the region analyzed was evaluated using incidence-based indices (number of haplotypes and number of mutations), abundance-based indices (Hill numbers of order 1 and 2), and functional indices (mutation frequency and nucleotide diversity). We also evaluated the pattern of nucleotide changes to investigate which of them could be the cause of the quasispecies complexity.

RESULTS

CHB patients showed higher median HBV-DNA levels [5.4 logIU/mL, interquartile range (IQR) 3.5-7.9] than CHD (3.4 logIU/mL, IQR 3-7.6) (P = n.s.) or CI (3.2 logIU/mL, IQR 2.3-3.5) ($P < 0.01$) patients. The incidence and abundance indices indicated that HBV quasispecies complexity was significantly greater in CI than CHB. A similar trend was observed in CHD patients, although only Hill numbers of order 2 showed statistically significant differences (CHB 2.81, IQR 1.11-4.57 *vs* CHD 8.87, 6.56-11.18, $P = 0.038$). There were no significant differences in the functional indices, but CI and CHD patients also showed a trend towards greater complexity than CHB. No differences were found for any HBV quasispecies complexity indices between CHD and CI patients. G-to-A and C-to-T nucleotide changes, characteristic of APOBEC3G, were higher in CHD and CI than in CHB in genotype A haplotypes, but not in genotype D. The proportion of nt G-to-A *vs* A-to-G changes and C-to-T *vs* T-to-C changes in genotype A and D haplotypes in CHD patients showed no significant differences. In CHB and CI the results of these comparisons were dependent on HBV genotype.

CONCLUSION

The lower-replication CHD and CI groups show a trend to higher quasispecies complexity than the higher-replication CHB group. The mechanisms associated with this greater complexity require elucidation.

Key words: Hepatitis B virus; Hepatitis delta virus; Hepatitis B X gene; Next-generation sequencing; Viral quasispecies; Hepatitis B virus-hepatitis delta virus interaction

Core tip: Hepatitis B virus (HBV) replication is lower in the presence of hepatitis delta virus (HDV), but little is known about the mechanism of this interaction. HBV X gene quasispecies study in HBV/HDV infection could provide data regarding this interaction. With use of next-generation sequencing, we analyzed HBV quasispecies complexity and found a trend to greater complexity in chronic HDV and chronic HBV infection (CI, previously termed inactive carriers) than in chronic hepatitis B. This suggests that HDV may drive the HBV quasispecies to acquire a situation of diversity similar to that of CI patients.

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INTRODUCTION

An estimated 257 million people worldwide are chronically infected with hepatitis B virus (HBV), and 15 to 20 million of them are also infected with hepatitis delta virus (HDV)[1]. HDV is a defective RNA virus that requires the helper function of HBV surface antigen (HBsAg) to achieve transmission[2]. HDV infection can occur as an acute coinfection (simultaneous HBV/HDV infection) or as a superinfection in individuals already chronically infected with HBV[3]. Acute HBV/HDV coinfection is usually self-limited and shows a course similar to that of acute HBV mono-infection, with clearance rates of both agents greater than 95% in immunocompetent adults. Nonetheless, it can also cause severe acute hepatitis with a high-risk of developing a fulminate course[4]. In superinfections, HDV progresses to chronicity in > 80% of cases[4]. Chronic HDV infection leads to more severe liver disease than chronic HBV mono-infection. In fact, it is the most severe form of viral hepatitis in humans, with accelerated progression of fibrosis, a slightly increased risk of hepatocellular carcinoma (HCC), and early decompensation in the setting of established cirrhosis[2].

HBV is an enveloped DNA virus consisting of a 3.2-kb partially double-stranded genome that replicates via an RNA intermediate[5] and encodes 7 proteins: PreCore, core, pol, X (HBx), and the three envelope proteins, L, M, and S. The HDV genome is comprised of a 1.7-kb single-stranded circular RNA of negative polarity, the smallest among known mammalian viruses[6]. As HDV does not encode an RNA-dependent RNA polymerase, its replication relies on the host cell's RNA polymerase II (RNA pol II)[7]. The HDV genome contains a single functional open reading frame (ORF) encoding hepatitis delta antigen (HDAg). Through the action of RNA-specific adenosine deaminase 1 (ADAR 1) editing during replication, HDV manages to produce two HDAg isoforms from this single ORF: The small (S-HDAg) and large (L-HDAg) delta antigens. As compared to S-HDAg, L-HDAg contains 19 additional amino acids at the C terminus[7]. Remarkably, the two HDAg isoforms have different functions: S-HDAg binds to HDV RNA and promotes HDV replication, whereas L-HDAg inhibits replication and is known to be involved in HDV packaging by direct binding to HBsAg[8]. As the two viruses use the same envelope proteins, HDV and HBV share common attachment and entry steps. Direct contact between HBsAg and HDAg for HDV virion envelopment is considered the main interaction[9], but the two viruses must also interact with each other at different stages of their replication cycles. For example, previous studies have shown that HDV can strongly suppress HBV replication and become the predominant virus in HBV/HDV infection[10,11]. However, other patterns of predominance are also seen in HBV/HDV infection, related to the fluctuating patterns of HBV and HDV replication over time[12]. Currently, little is known about the specific mechanisms of this interaction. It has been suggested that the suppressive effect of HDV on HBV replication may be mediated by the interaction of HDAg with HBV enhancers[13], the L-HDAg-RNA pol II interaction[14], or by the antiviral activity of interferon-inducible MxA protein activated by L-HDAg[13].

As is the case of other RNA and DNA viruses that replicate by low-fidelity polymerases, HDV and HBV both exhibit high mutation rates, and their populations show a dynamic distribution of mutants. This characteristic results in a complex swarm of sequences that are highly similar, but not identical, known as a viral quasispecies[15,16]. Next-generation sequencing (NGS), also referred to as massive or deep sequencing, is an ideal approach to analyze the composition and complexity of the mutant spectra within a viral quasispecies[17,18]. This information can explain or predict the response of the virus to specific environmental changes. The HBV quasispecies in chronic mono-infection and the HDV quasispecies have been analyzed individually by NGS[19-23]. However, there are no studies comparing the quasispecies in HBV mono-infection and HBV/HDV infection using this technique. Characterization of the HBV population in each of these situations could provide valuable information on the interference between HBV and HDV. The pleiotropic, trans-activating HBx protein has an important role in regulating the HBV life cycle, host-virus interactions, and HBV-related HCC[24], and HDV/HBV interactions at this level may impair HBV replication/transcription. Therefore, the hepatitis B X gene (*HBX*) could be an interesting target to study potential interactions between these two viruses.

The aim of this study was to evaluate and compare the complexity of the HBV quasispecies in serum samples from HBV mono-infected patients in the chronic infection phase (CI, previously termed inactive carrier), patients with chronic hepatitis B (CHB) mono-infection, and patients with chronic hepatitis delta (CHD) superinfection, using a high-throughput NGS-based approach. We focused our study on the 5′ end of the *HBX* coding region and its upstream non-coding region [nucleotides (nt) 1255-1611], which was also examined in a previous study by our group in patients in different stages of chronic HBV infection, including CHB and

CI[23].

MATERIALS AND METHODS

Patients and samples

Twenty-four patients with chronic HBV infection in two different clinical stages including some with HDV superinfection were recruited from the outpatient clinics of Vall d'Hebron University Hospital (Barcelona, Spain). According to the guidelines of the European Association for the Study of the Liver[25], patients were classified into three groups: Hepatitis B e-antigen (HBeAg)-negative CI (7 patients, 29.2%), HBeAg-negative CHB (8 patients, 33.3%), and CHD (9 patients, 37.5%). The study was approved by the Ethics Committee of Vall d'Hebron Research Institute and all patients provided written informed consent for participation.

One serum sample from each patient with HBV DNA ≥ 2.5 logIU/mL (sensitivity limit of the PCR to amplify the target region) was selected for the study. Exclusion criteria were positive testing for hepatitis C virus (HCV) or human immunodeficiency virus (HIV) antibodies, current antiviral therapy, or liver transplantation in the 2 years before the sample was obtained.

Serological and virological determinations

HBV serological markers (HBsAg and HBeAg) and anti-HCV antibodies were tested using commercial electrochemiluminescent immunoassays on a COBAS 8000 instrument (Roche Diagnostics, Rotkreuz, Switzerland). Anti-HDV antibodies were tested using the HDV Ab kit (Dia.Pro Diagnostics Bioprobes, Sesto San Giovanni, Italy), and anti-HIV antibodies with the Liaison XL murex HIV Ab/Ag kit (DiaSorin, Saluggia, Italy). HBV-DNA was quantified by real-time PCR with a detection limit of 10 IU/mL (COBAS 6800, Roche Diagnostics, Mannheim, Germany). HDV-RNA was quantified by an in-house method[26] using the HDV RNA international standard of the World Health Organization (1st World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Techniques-based assays; PEI code number: 7657/12), with a quantification limit of 100 IU/mL.

Amplification of HBV and HDV regions of interest by next-generation sequencing

The region of the *HBX* gene (nt 1255 to 1611) selected for HBV sequencing is included in the 5′ end of all *HBX* transcripts. It encompasses a non-coding upstream region (nt 1255-1373) and the 5′ end of the *HBX* coding region (nt 1374-1611)[23]. This latter sequence encodes the N-terminal HBx domain (HBx amino acids 1-50), which acts as negative regulator of HBx transactivation and has an essential role in multiple functions of the protein. The region selected for HDV genotyping covered a 360-bp fragment of the HDV genome, from nt positions 910 to 1270. These genome regions have both been used to respectively determine HBV and HDV genotypes in previous studies[22,23].

For each sample, total viral nucleic acid was extracted from 200 μL of serum with the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Molecular amplification of HBV-DNA was performed using a 3 PCR protocol. The first-round PCR was performed using external primers (forward 5′-TGTATTCCCATCCCATCATC, and reverse 5′-AGWAGCTCCAAATTCTTTATAAGG, which cover the region from nt 599 to 1936) with the following protocol: 95 ºC for 5 min followed by 35 cycles of 95 ºC for 20 s, 53 ºC for 20 s, and 72 ºC for 15 s, and finally 72 ºC for 3 min. The second- and third-round PCRs were performed as previously described by our group[23]. By adding another PCR to the amplification protocol described in that previous study, we were able to increase the sensitivity limit from 3.5 logIU/mL to 2.5 logIU/mL, thereby allowing sequencing of samples from patients with low HBV replicative activity (especially CI and CHD samples). Amplification of the region selected for HDV genotyping was performed by RT-nested PCR as previously described[21,22]. After amplification of the HBV and HDV regions of interest, the final products were flanked by universal M13 sequences at both ends. In the last PCR amplification [multiplex identifier (MID) PCR], a specific pair of primers was used, consisting of an M13 universal primer and a MID or barcode sequence. Each individual patient sample required a different MID. The PCR products of this amplification, also known as amplicons, were visualized as single bands on 1.5% agarose electrophoresis gel, stained with SybrSafe DNA Gel Stain (Invitrogen, United States) with 1 × TAE running buffer. PCR products from the gel were subsequently purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicon quality was analyzed using the Agilent 2200 TapeStation System with the D1000 ScreenTape kit (Agilent Technologies,

Waldbronn, Germany). Purified DNA from each sample was quantified by fluorescence using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, United States), adjusted to the same concentration, and pooled. The pools, one for HBV (24 amplicons) and another for HDV (9 amplicons), were NGS-sequenced on the MiSeq Platfrom (Illumina, San Diego, United States) following the manufacturer's protocol.

Data treatment

PCR artefacts and sequencing errors can occur when using NGS. Thus, the sequences obtained (referred to as reads: Sequences obtained by NGS that do not always cover the full amplicon length) require bio-informatic processing to minimize the scoring of these errors. To this end, we developed a haplotype-centric data analysis pipeline to exclude full reads that did not meet minimum quality requirements, essentially consisting of the following steps:

Quality control of fastq files: Inspect profiles for per-site quality, read length, and general quality-related instrument parameters.

Overlapping paired reads: In paired-end experiments, use FLASh[27] to impose a minimum of 20 overlapped base pairs (bp) with a maximum of 10% mismatches (yield 60%-80% for 450 to 500-bp amplicons).

Discarding reads: Reads are discarded if more than 5% of bases are below a Phred score[28] of 30, corresponding to an estimated accuracy of 99.9% (yield 75%-85%).

Demultiplexing reads: Demultiplexing is done by identifying oligonucleotide sequences at both ends within windows of expected positions in the reads (yield 70%-85%). First, the individual MIDs (10 oligonucleotide sequences) are used to distinguish between samples from different patients/origins. Only one mismatch is allowed. Second, specific primers (20 to 30-bp oligos) are used to distinguish between different regions in the genome or different genomes, and between the two strands. Up to three mismatches are allowed. Finally, MIDs and primers are trimmed and a fasta file is obtained for each combination of MID, primer, and strand in the run, where reads are collapsed to haplotypes (unique sequences covering the full amplicon observed on the clean set of sequences) with the corresponding frequencies.

Aligning haplotypes: In each fasta file haplotypes are aligned to the wild-type reference sequence or the master sequence (most abundant haplotype in the file) and quality filter (yield > 90%). This quality filter consists in discarding haplotypes that do not cover the full amplicon and those that have more than two indeterminations, three gaps, or more than 99 differences with respect to the reference. Finally, the accepted indeterminations and gaps are repaired as per the reference sequence.

Intersecting haplotypes: For this step, haplotypes with abundance not below 0.1% in both strands are selected (yield 50%-60%), whereas those unique to one strand are discarded. The coverage of haplotypes passing the filter is taken as the sum of reads in both strands.

Final result: All haplotypes with abundances not below 0.25% are kept. The final haplotypes are called consensus haplotypes, and these are the basis for the downstream analysis in this study (final overall yield 15%-25%).

Genotyping

HBV and HDV genotypes were determined by NGS and phylogenetic analysis of the amplified genome regions of both viruses. NGS allows detection of mixtures of viral genotypes in patient samples, which could have an impact on HBV quasispecies complexity. The nt haplotypes aligned at 0.25% obtained by NGS were genotyped by distance-based discriminant analysis (DB rule)[29,30]. For this analysis, we used reference sequences of the HBV and HDV regions analyzed extracted from the full-length genomes representative of HBV genotypes A to H and HDV genotypes 1 to 8 obtained from GenBank (Supplementary Figures 1 and 2). This analysis takes into account the inter- and intra-class variability of each genotype. Genetic distances were computed according to the Kimura-80 model[31]. UPGMA trees were designed to visualize the genetic distances between sequences.

Quasispecies complexity measures

Quasispecies complexity was analyzed in HBV sequences obtained by NGS (5' *HBX* gene, nt 1255-1611). Multiple alignment displays the entities (haplotypes, polymorphic sites, and mutations) present in the viral quasispecies. In this study, six parameters were used to describe quasispecies complexity: Number of haplotypes

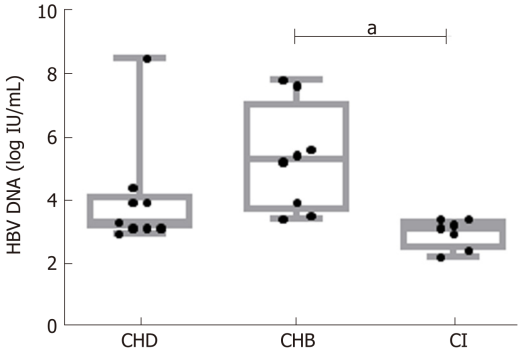

Figure 1 Comparison between hepatitis B virus-DNA serum levels in the three groups. $^{a}P < 0.01$. CHD: Chronic hepatitis delta; CHB: Chronic hepatitis B; CI: Hepatitis B virus chronic infection; HBV: Hepatitis B virus.

(nHpl) and number of mutations (nMuts) as incidence-based indices; Hill numbers of order 1 and 2 (q = 1, the exponential of Shannon's entropy and q = 2, the inverse of Simpson's index) as abundance-based indices; and the mutation frequency (Mf) and nucleotide diversity (Pi) as functional indices[18,32]. The complexity parameters are defined in Supplementary Materials.

Analysis of nucleotide substitutions

The innate immune system is suggested to contribute to HBV genetic variability through the cytidine deaminase APOBEC (apolipoprotein B mRNA editing enzyme) family[33], such as APOBEC3G (A3G) which promotes G–to-A, and in some cases, C-to-T hypermutation of HBV genomes[34,35]. To infer whether this nt substitution pattern could be associated with HBV quasispecies complexity in the groups analyzed, we used a point mutation approach to detect bias towards a specific nt change. This approach consists in assessing the nt changes in the sequence of haplotypes relative to the reference sequence of the same genotype (consensus sequence of all GenBank patterns of the same genotype used for HBV genotyping), taking into account only one nucleotide change per position, regardless of the number of haplotypes where it appears.

In addition, we compared the proportion of positions with a G-to-A nt change in any haplotype (relative to the genotype reference sequence) with the proportion of positions with an A-to-G nt change (G→A/G *vs* A→G/A) between CHB, CI and CHD. The same comparison was done for C-to-T changes: (C→T/C *vs* T→C/T).

Statistical analysis

Statistics were carried out using GraphPad Prism version 7.0 (GraphPad software, La Jolla, United States). All parameters are expressed as the median value and interquartile range (IQR). For qualitative variables the chi-square test was performed. The Kruskal Wallis and Dunn test (post hoc) were used for multiple comparisons of independent samples. To compare the proportions of nt changes, a 2-sample test for equality of proportions with continuity correction was performed. The Mann-Whitney test was used for the two-group comparisons (HBeAg+ *vs* HBeAg- in CHD, and cirrhotic *vs* non-cirrhotic patients). *P* values < 0.05 were considered significant. The bioinformatics and biostatistics methods used in this study were reviewed by Dr. Josep Gregori from the Liver Disease-Viral Hepatitis Laboratory of Vall d'Hebron Hospital (Barcelona, Spain), CIBERehd research group, and Roche Diagnostics SL.

RESULTS

Patient characteristics

Clinical, virological, and serological parameters were obtained from the 24 patients. Baseline characteristics are summarized in Table 1. Median (IQR) age was 37 (30-45 years), 58% were men, 71% were Caucasians, and the remainder were of Sub-Saharan origin. Regarding laboratory characteristics, 79% were negative for HBeAg and median (IQR) alanine aminotransferase levels were 43 IU/L (31.75-118.5). HBV DNA levels were lower in the CI [3.2 logIU/mL (2.3-3.5)] and CHD [3.4 logIU/mL (3-7.6)] groups than in CHB [5.4 logIU/mL (3.5-7.9)] (*P* < 0.01 and n.s., respectively) (Figure 1). Liver histology was available in all cases: 2/9 patients with CHD (1 of whom additionally had HCC), and 3/8 patients with CHB had liver cirrhosis.

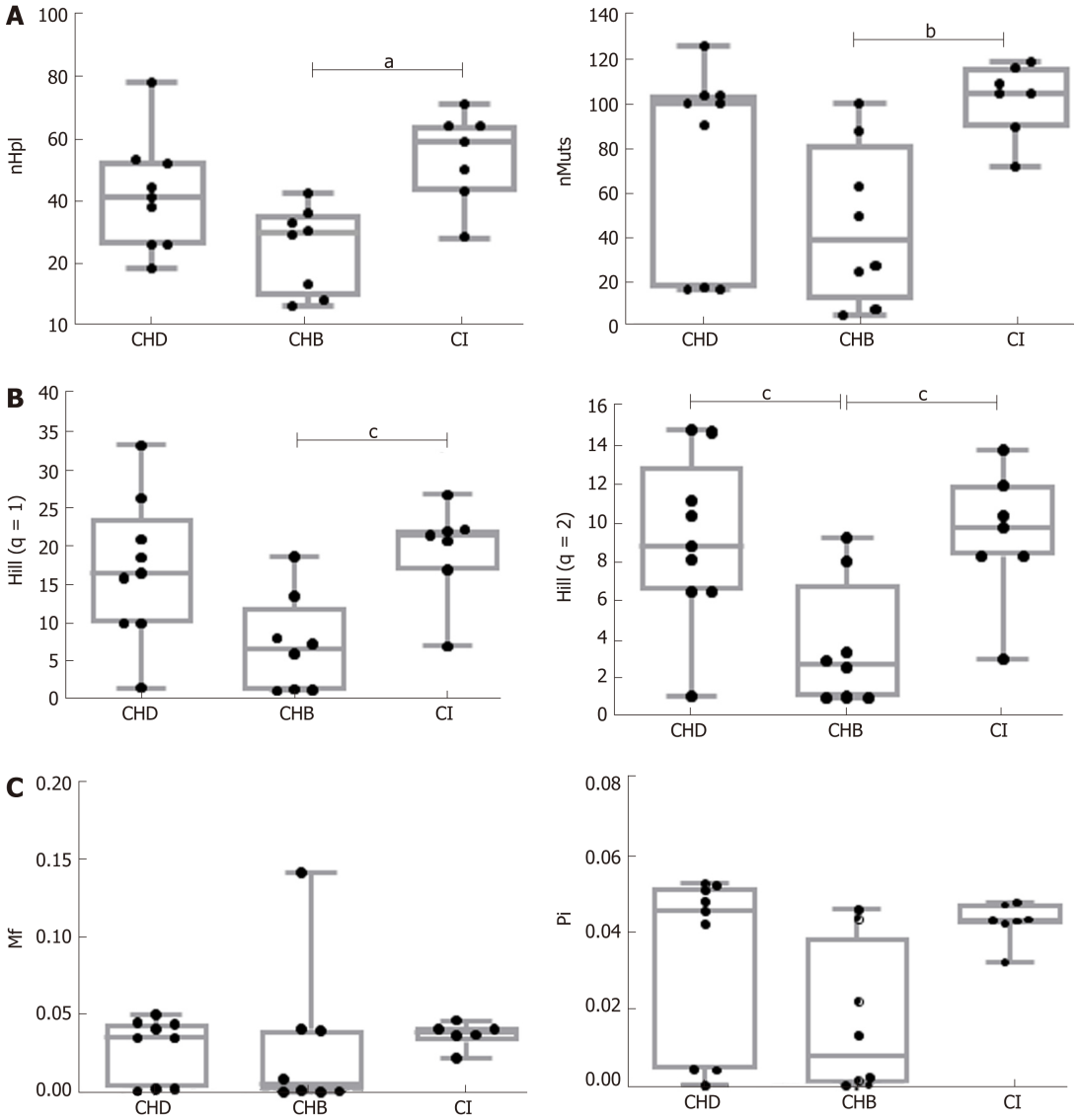

Figure 2 Comparison of the indices of hepatitis B virus quasispecies complexity between the three groups. A: Incidence-based indices: Number of haplotypes (nHpl), number of mutations (nMuts) ($^{a}P < 0.05$, $^{b}P < 0.01$); B: Abundance-based indices: Hill numbers (Hill q = 1 and Hill q = 2) ($^{c}P < 0.05$); C: Functional-based indices: Mutation frequency (Mf) and nucleotide diversity (Pi). One outlier value in the CI group was eliminated in the representation of Mf results, but this value was included in the statistical comparisons. nHpl: Number of haplotypes; nMuts: Number of mutations; Mf: Mutation frequency; Pi: Nucleotide diversity; CHD: Chronic hepatitis delta; CHB: Chronic hepatitis B; CI: Hepatitis B virus chronic infection.

Analysis of NGS sequences obtained and genotyping results

After applying the quality filters, 791036 sequences from the HBV target region were obtained from the 24 serum samples, yielding a median (IQR) of 26459 (17649-44852) sequences per patient. Regarding HDV, 287541 sequences were obtained from the 9 samples, yielding a median (IQR) of 17609 (14475-22677) sequences per patient. HBV genotyping showed that in the region analyzed (nt 1255 to 1611), 15/24 patients (62.5%) had a complex mixture of genotypic variants, mainly A/D/C. None of the patients included showed genotype B, G, or H haplotypes. With regard to the HDV region analyzed (nt 910 to 1270), 7/9 (78%) CHD patients were classified as genotype HDV-1, whereas 2/9 (11%) were HDV-2 and HDV-6, respectively.

Characterization of HBV quasispecies complexity

To analyze HBV quasispecies complexity data from samples of notably different coverage, samples were made comparable by down-sampling and fringe trimming to a common coverage of 6000 reads, retaining haplotypes at a frequency above 0.2% with 95%CI. Quasispecies complexity in the HBV target region was evaluated using six parameters. Of note, all CHB and CI patients were HBeAg-negative, whereas 5/9 CHD patients were HBeAg-positive. The complexity of the viral population in the preCore/Core region of the viral genome has been reported to differ between HBeAg-positive and -negative HBV mono-infected patients[36]. To determine whether HBeAg

Table 1 Baseline characteristics according to the clinical stage of hepatitis B virus or hepatitis B virus/hepatitis delta virus infection

	Total, *n* = 24	Chronic HBV infection, *n* = 7	Chronic hepatitis B, *n* = 8	Chronic hepatitis delta, *n* = 9	*P* value
Age, yr, median (IQR)	36.5 (30.75-48)	40 (33.5-60.5)	35.5 (32.75-42)	38 (30-41)	ns
Male, *n* (%)	14 (58)	4 (57)	4 (50)	6 (67)	ns
Ethnic group, *n* (%)					
Caucasian	17 (71)	5 (71)	5 (62)	7 (78)	ns
Sub-Saharan	7 (29)	2 (29)	3 (38)	2 (22)	
ALT, IU/L, median (IQR)	43 (31.75-118.5)	27 (16-30.5)	60 (44.25-118.5)	80 (39-138)	0.0007
HBeAg-, *n* (%)	19 (79)	7 (100)	8 (100)	4 (44)	0.005
HBsAg, logIU/mL, median (IQR)	3.8 (3.5-4.1)	3.6 (2.6-3.9)	3.6 (3.4-3.7)	4.1 (3.8-4.5)	0.0152
HBV DNA, logIU/mL, median (IQR)	3.5 (3.2-5.4)	3.2 (2.8-3.4)	5.4 (3.9-6.2)	3.4 (3.2-4)	0.0038
HDV RNA, logIU/mL, median (IQR)				5.5 (4.3-5.5)	-

IQR: Interquartile range; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e-antigen; HBsAg: Hepatitis B virus surface antigen; HBV: Hepatitis B virus; HDV: Hepatitis delta virus.

status had an effect on quasispecies complexity in the *HBX* 5′ region in CHD patients, the six parameters used to assess this factor were compared between HBeAg-negative and –positive CHD patients, but no statistically significant differences were found (Table 2). Thus, the patients' HBeAg status was not considered to be a potential interfering factor. In addition, we compared these complexity indices between 5 patients with liver cirrhosis, including 2 CHD and 3 CHB, and 19 patients without progression to severity. No significant differences were found (Table 3).

Comparison between HBV mono-infection (CHB and CI) *vs*. CHD (HBeAg-negative and HBeAg-positive) in two-group tests showed no significant differences in the incidence, abundance, or functional indices related to quasispecies complexity. However, significant differences were found between CHB and CI in incidence and abundance-that is, median nHpl (IQR), CHB 31 (12-75, 34.75) *vs* CI 60 (47.5-65) (P = 0.011), nMuts, 39 (20.75-69.25) *vs* 105 (97.5-112.5) (P < 0.01), and Hill numbers of order 1, 6.70 (1.37-9.43) *vs* 21.54 (18.99-22.14) (P = 0.012), and 2, 2.81 (1.11-4.57) *vs* 9.86 (8.37-11.22) (P = 0.027)-with the CI quasispecies showing greater complexity than that of CHB (Figure 2). No statistically significant differences were observed for the functional indices (Mf and Pi), which are sensitive to the number of differences between the different haplotypes, although the results showed a trend towards greater complexity in CI and CHD than in CHB patients (Figure 2C). Regarding the effect of HDV on the HBV quasispecies, the most interesting finding was that the HBV viral populations in CHD and CI showed a similar trend, with greater complexity (higher incidence, abundance and functional values) than that of CHB patients, although only Hill numbers of order 2 showed a statistically significant difference, CHB 2.81 (1.11-4.57) *vs* CHD 8.87 (6.56-11.18) (P = 0.038) (Figure 2). There were no significant differences in any HBV complexity indices between CHD and CI patients.

Types of nucleotide changes in HBV sequences

All types of nt changes were computed in the three patient groups. As the patterns of nt changes varied between the different genotypes (data not shown), we only took into account genotype A (Figure 3A) and D (Figure 3B) haplotypes, which were the most abundant in our samples [genotype A 511/1065 haplotypes (47.98%) and genotype D 264/1065 haplotypes (24.79%)], for the comparisons. In this analysis we found that the pattern of nt changes in genotype A haplotypes (Figure 3A) differed from those in genotype D (Figure 3B). The nt changes G-to-A and C-to-T, which are characteristic of the modifications introduced by the A3G enzyme, were higher in CHD and CI than in CHB patients in genotype A haplotypes (Figure 3A), but not in genotype D (Figure 3B). We then compared the proportion of G-to-A *vs* A-to-G nt changes and C-to-T *vs* T-to-C in genotype A and D haplotypes by group to investigate bias in the nt change pattern, which could be associated with the effect of A3G. There were no significant differences in these changes in either genotype A or genotype D in CHD patients. The results in CHB and CI were dependent on HBV genotype. In genotype A haplotypes, the proportion of C-to-T nt changes was higher than T-to-C in the CI group (0.22 *vs* 0.07 respectively, P < 0.01), whereas in genotype D the proportion of G-to-A nt changes was higher than A-to-G in the CHB group (0.20 *vs*

Table 2 Comparison of hepatitis B virus quasispecies complexity indices between hepatitis B e-antigen-negative and hepatitis B e-antigen-positive chronic hepatitis D patients

	CHD HBeAg-negative (*n* = 4)	CHD HBeAg-positive (*n* = 5)	*P* value
nHpl, median (IQR)	42 (36-47)	42 (27-54)	1.0
nMuts, median (IQR)	97.5 (72.5-104)	100 (18-100)	1.0
Mf, median (IQR)	0.039 (0.027-0.045)	0.035 (0.002-0.041)	0.4127
Pi, median (IQR)	0.047 (0.033-0.052)	0.046 (0.004-0.049)	0.5556
Hill number (q = 1), median (IQR)	17.29 (14.46-20.49)	16.62 (10.06-20.96)	1.0
Hill number (q = 2), median (IQR)	10.03 (8.29-12.11)	8.15 (6.56-10.42)	0.5556

IQR: Interquartile range; CHD: Chronic hepatitis D patients; nHpl: Number of haplotypes; nMuts: Number of mutations; Mf: Mutation frequency; Pi: Nucleotide diversity.

0.04 respectively, $P = 0.025$).

DISCUSSION

Clinical and experimental data support the existence of interference between HDV and HBV. Although several hypothesis have been raised to define the interactions between these viruses[37], the molecular mechanisms through which HDV affects HBV remain elusive. In the clinical setting, most HBV/HDV co-infected patients show a pattern of HDV dominance, with a significant decrease in HBV-DNA viral load when compared to that of mono-infected patients[12,38,39]. In accordance with these data, our results showed lower HBV-DNA levels in HDV-infected patients, with values similar to those in the chronic HBV infection group.

The viral population analyses carried out found no significant differences in the complexity of the HBV quasispecies between the total of patients with HBV mono-infection and patients with CHD. These results would seem to suggest that HBV quasispecies complexity is unaffected by the presence of HDV. However, when the clinical phases of the disease were taken into account, HBV quasispecies complexity was found to be greater (significant in incidence and abundance-based indices and non-significant in functional indices) in CI than in CHB patients. Surprisingly, the viral population was more complex in the group with lower replication. This may indicate that the higher incidence of mutations in the 5′ of *HBX*, distributed in different haplotypes in CI patients could cause HBV replication to be closer to the quasispecies error threshold, that is, the point beyond which the mutation rate is so high that the genetic information carried by the replicating genome is lost[40]. In this sense, it should be taken into account that HBx is essential for HBV replication. For this reason, it seems logical to associate *HBX* variability with HBx functionality, which would affect HBV replication and result in the low HBV replication levels observed in the CI stage of HBV infection.

As to the interaction between HDV and HBV, it seems that CHD drives the HBV quasispecies to a situation similar to that observed in CI patients: Lower replication level and higher *HBX* quasispecies complexity than CHB patients. Two hypotheses could explain the mechanism by which HDV enhances HBV quasispecies complexity. The first is activation of the host innate immune response under the effect of HDV stimulation[39,41,42]. A3G activity, which provides broad innate immunity[34,43], could therefore be responsible for the hyper-mutation of HBV genomes. To investigate this possibility, we analyzed nucleotide changes in the CHB, CI, and CHD groups to determine whether there was some bias in favor of those produced by A3G. Although HDV activates the immune system, we did not find a hyper-mutation pattern associated with A3G. Nonetheless, this hypothesis should be more extensively analyzed in further, more specific studies, and it could be extended to other innate immunity enzymes.

The second hypothesis postulates a possible interaction between HDAg and RNA pol II, which could affect the replicative capacity and functionality of this enzyme. As Yamaguchi *et al*[14] reported, HDAg not only increases the elongation rate, it also reduces transcriptional fidelity by interacting with and loosening the RNA pol II clamp. This would increase the error rate, introduce a larger number of mutations, and give rise to a more complex quasispecies. This mechanism has been suggested to explain the extremely high mutation rate that occurs in HDV replication[22,44]. In addition to HDV, this loss of fidelity would also affect HBV transcription. It is

Table 3 Comparison of hepatitis B virus quasispecies complexity indices between patients with and without liver cirrhosis

	Cirrhotic (*n* = 5)	Non cirrhotic (*n* = 19)	*P* value
nHpl, median (IQR)	34 (28.5-44)	42 (27-60)	0.5456
nMuts, median (IQR)	28 (21-95.5)	100 (50-105)	0.2130
Mf, median (IQR)	0.035 (0.0019-0.0905)	0.03715 (0.0025-0.0440)	0.8311
Pi, median (IQR)	0.0223 (0.00347-0.0445)	0.0434 (0.0044-0.0481)	0.3937
Hill number (q = 1), median (IQR)	10.06 (6.7-18.69)	16.62 (7.02-22.02)	0.4994
Hill number (q = 2), median (IQR)	6.56 (2.81-10.23)	8.37 (3.05-10.42)	0.7223

IQR: Interquartile range; nHpl: Number of haplotypes; nMuts: Number of mutations; Mf: Mutation frequency; Pi: Nucleotide diversity.

important to keep in mind that HBV cccDNA is the template for transcription of all viral mRNA including pregenomic RNA, essential for progeny production, and that pregenomic RNA transcription from both cccDNA and integrated HBV DNA is mediated by the host RNA pol II[45,46]. This effect of HDV on RNA pol II could also affect cellular mRNAs, thereby worsening cell homeostasis, and this could be linked to the poorer prognosis of HDV hepatitis when compared to the other viral hepatitis. To test this possibility, NGS studies investigating the complexity of cellular mRNAs in CHD are needed.

Thus, hyper-mutation due to the innate immune system and loss of RNA pol II fidelity by the effect of HDV could have an impact on HBV DNA synthesis, which, along with the error rate of the HBV polymerase itself, could give rise to a situation similar to that seen in CI patients, driving the HBV quasispecies closer to its error threshold. However, we would expect to find significant differences between CHD and CHB in other complexity indices in addition to Hill numbers (q = 2) in this scenario. Hence, larger samples taken at different time points and other regions of the genome should be analyzed in future studies to confirm these results.

In this line, 2 recent studies[47,48] investigated HBV sequence variation in the viral genome surface (S) ORF (encoding HBsAg) in large patient cohorts. The authors compared consensus sequences obtained from HBV/HDV infected *vs* HBV mono-infected patients. Both studies concluded that HDV can exert selective pressure over some positions of the S ORF, constraining HBV evolution. In the light of these findings, it would be interesting to assess the effect of HDV on the HBV quasispecies in the S ORF and compare it with the effect in other regions of the viral genome, such as that analyzed in the present study. These efforts illustrate the relevance of studies investigating the HBV quasispecies in HDV superinfected or coinfected patients to deepen current knowledge on the interference between HDV and HBV. In addition, cellular and animal models of HBV/HDV infection[42,49] enable *in vitro* and *in vivo* functional studies to test whether the presence of HDV has an effect on HBV replication and genetic diversity.

Of note, the patients included in this study had to have HBV replication at high enough levels for amplification by our PCR protocol. This obliged us to include HBeAg-positive CHD patients (5/9), whose numbers are limited in HBV/HDV infection. We believed it was necessary to determine whether HBeAg status may have had an effect on quasispecies complexity, as we found a more complex viral population in the preCore/Core region of the HBV genome in HBeAg-negative than HBeAg-positive CHB patients in a previous analysis[36]. However, the present study, focusing on the 5′ region of *HBX* (nt 1255-1611), showed that HBV quasispecies complexity was similar in HBeAg-positive and -negative CHD. These differences may be related to the regions studied: the 5′ region of *HBX* does not have a direct relationship with HBeAg status; hence, it would not have a significant influence on HBV quasispecies complexity in this region. Thus, as HBeAg status did not seem to significantly affect HBV quasispecies complexity in the 5′ *HBX* region, we were able to compare all CHD patients (both HBeAg-positive and HBeAg-negative) with CHB and CI patients. Another factor that could be related to HBV quasispecies complexity is liver disease progression to cirrhosis or HCC, which had occurred in 5 patients (both CHB and CHD) included in this study. Comparison of quasispecies complexity between these patients and the 19 who did not progress to severity showed no statistically significant differences. Nonetheless, although HBeAg status and more severe disease stage did not affect HBV quasispecies complexity in our sample, analysis of larger patient groups is needed to define the actual role of these virological

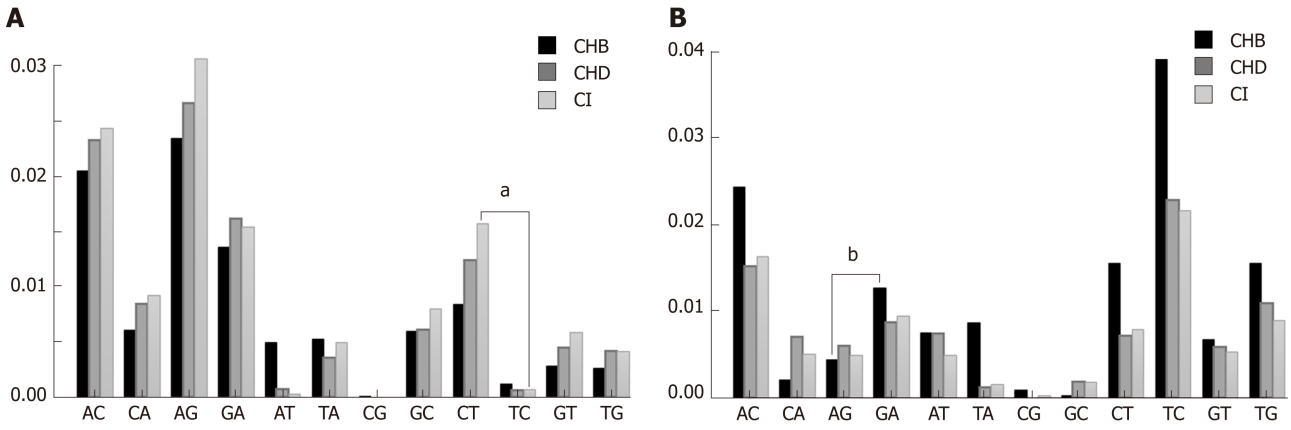

Figure 3 Nucleotide change patterns. A: Genotype A substitution rates by group; B: Genotype D substitution rates by group. [a]*P* < 0.05, [b]*P* < 0.01. CHD: Chronic hepatitis delta; CHB: Chronic hepatitis B; CI: Hepatitis B virus chronic infection.

and clinical factors.

In summary, this study provides the first data on the influence of HDV on HBV genetic diversity in the *HBX* gene, obtained using NGS. Our results showed that in HBV stages with lower replication (CHD and CI), the HBV quasispecies in the 5'end of *HBX* exhibited a trend toward higher complexity than in CHB. This was mainly evident in terms of incidence and abundance, that is, a higher incidence of mutations, distributed in different haplotypes. The mechanisms associated with this greater complexity are unknown, but two hypotheses could explain them: involvement of the innate immune response or HDAg interaction with RNA pol II, which should be explored in greater depth.

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ARTICLE HIGHLIGHTS

Research background

Hepatitis delta virus (HDV) causes the most severe form of chronic viral hepatitis in persons simultaneously infected with hepatitis B virus (HBV). In longitudinal clinical studies, HDV infection has been associated with a considerable temporary or permanent reduction in HBV viral load, whereas HBV surface antigen levels are usually high. Thus, beyond the interaction with HBV envelope proteins, there are other mechanisms by which HDV inhibits HBV-DNA replication.

Research motivation

To date, little information has emerged on the interaction between HDV and HBV. In this study, we investigated whether HDV can affect the complexity of the HBV quasispecies, and proposed possible mechanisms by which it may do so, to further characterize the interaction between these two viruses.

Research objectives

Considering the essential role of the HBV X protein (HBx) on viral replication, the aim of this study was to analyze the 5' end of the hepatitis B X gene (*HBX*) coding region and its upstream non-coding region (nt 1255-1611) by next-generation sequencing (NGS) to evaluate HBV quasispecies complexity between chronic hepatitis delta (CHD)-infected patients and chronic HBV mono-infected patients [HBV chronic infection (CI) and chronic hepatitis B (CHB)].

Research methods

The *HBX* 5' end region, nucleotide (nt) 1255-1611, was PCR-amplified for subsequent NGS (MiSeq, Illumina, United States) in 7 CI, 8 CHB, and 9 CHD patients. HBV quasispecies complexity in the region analyzed was evaluated using incidence-based indices [number of haplotypes (nHpl) and number of mutations (nMuts)], abundance-based indices (Hill numbers of order, q = 1 and q = 2) and functional indices [mutation frequency (Mf) and nt diversity (Pi)]. The pattern of nt changes was evaluated to investigate the cause of HBV quasispecies complexity.

Research results

HBV quasispecies complexity was significantly higher in the CI group than in CHB for abundance (Hill numbers q = 1 and q = 2) and incidence (nHpl and nMuts). In CHD, the HBV quasispecies showed a trend towards higher complexity similar to that of CI patients. No significant differences were observed in Mf or Pi between the groups, although CI and CHD showed a trend towards greater quasispecies complexity than CHB patients. The proportion of G-to-A vs. A-to-G and C-to-T vs. T-to-C nt changes in genotype A and D haplotypes by group did not provide conclusive evidence of a hyper-mutation pattern associated with the innate immune system enzyme APOBEC3G.

Research conclusions

The HBV quasispecies showed a trend to higher complexity in groups with lower viral replication (CHD and CI) than in the higher-replicating CHB patients. This could indicate that HDV has an effect on the 5′ *HBX* sequence, increasing HBV quasispecies complexity. Two different mechanisms are proposed to explain how HDV can change the HBV quasispecies: hypermutation by activation of the innate system through HDV stimulation or loss of RNA pol II fidelity due to its interaction with hepatitis delta antigen. Further studies are needed to determine the clinical impact of the increased HBV quasispecies complexity in CHD patients, which may be of help to devise new therapy strategies.

Research perspectives

CHD drives the HBV quasispecies to a situation similar to that found in HBV CI: Lower replication level and higher *HBX* quasispecies complexity than in CHB patients. Further studies are needed to characterize the mechanisms by which HDV acts on the HBV quasispecies, which may include the innate immune system or RNA pol II fidelity.

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ORIGINAL ARTICLE

Basic Study

Plasma microRNAs as potential new biomarkers for early detection of early gastric cancer

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Abstract

BACKGROUND

Early gastric cancer (EGC), compared with advanced gastric cancer (AGC), has a higher 5-year survival rate. However, due to the lack of typical symptoms and the difficulty in diagnosing EGC, no effective biomarkers exist for the detection of EGC, and gastroscopy is the only detection method.

AIM

To provide new biomarkers with high specificity and sensitivity through analyzed the differentially expressed microRNAs (miRNAs) in EGC and AGC and compared them with those in benign gastritis (BG).

METHODS

Gansu Province, No. zdsyskfkt-201704; and the Foundation of The First Hospital of Lanzhou University, No. ldyyyn2015-16.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the First Hospital of Lanzhou University. Informed consent was obtained from all individual participants included in the study.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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We examined the differentially expressed miRNAs in the plasma of 30 patients with EGC, AGC, and BG by miRNA chip analysis. Then, we analyzed and selected the significantly different miRNAs using bioinformatics. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) confirmed the relative transcription level of these miRNAs in another 122 patients, including patients with EGC, AGC, *Helicobacter pylori* (*H. pylori*)-negative gastritis (Control-1), and *H. pylori*-positive atrophic gastritis (Control-2). To establish a diagnostic model for the detection of plasma miRNA in EGC, we chose miRNAs that can be used to determine EGC and AGC from Control-1 and Control-2 and miRNAs in EGC from all other groups.

RESULTS

Among the expression profiles of the miRNA chips in the three groups in the discovery set, of 117 aberrantly expressed miRNAs, 30 confirmed target prediction, whereas 14 were included as potential miRNAs. The RT-qPCR results showed that 14 potential miRNAs expression profiles in the two groups exhibited no differences in terms of *H. pylori*-negative gastritis (Control-1) and *H. pylori*-positive atrophic gastritis (Control-2). Hence, these two groups were incorporated into the Control group. A combination of four types of miRNAs, miR-7641, miR-425-5p, miR-1180-3p and miR-122-5p, were used to effectively distinguish the Cancer group (EGC + AGC) from the Control group [area under the curve (AUC) = 0.799, 95% confidence interval (CI): 0.691-0.908, $P < 0.001$]. Additionally, miR-425-5p, miR-24-3p, miR-1180-3p and miR-122-5p were utilized to distinguish EGC from the Control group (AUC = 0.829, 95%CI: 0.657-1.000, $P = 0.001$). Moreover, the miR-24-3p expression level in EGC was lower than that in the AGC (AUC = 0.782, 95%CI: 0.571-0.993, $P = 0.029$), and the miR-4632-5p expression level in EGC was significantly higher than that in AGC (AUC = 0.791, 95%CI: 0.574-1.000, $P = 0.024$).

CONCLUSION

The differentially expressed circulatory plasma miR-425-5p, miR-1180-3p, miR-122-5p, miR-24-3p and miR-4632-5p can be regarded as a new potential biomarker panel for the diagnosis of EGC. The prediction and early diagnosis of EGC can be considerably facilitated by combining gastroscopy with the use of these miRNA biomarkers, thereby optimizing the strategy for effective detection of EGC. Nevertheless, larger-scale human experiments are still required to confirm our findings.

Key words: Biomarker; MicroRNA; Plasma; Early gastric cancer

Core tip: Early gastric cancer (EGC) has no typical symptoms and difficulty to diagnosis. We filtrated the differentially expressed microRNAs (miRNAs) in the plasma of EGC, advanced gastric cancer and benign gastritis by miRNA chip analysis. Then, reverse transcription quantitative real-time polymerase chain reaction confirmed the relative transcription level of target miRNAs. The 5 plasma miRNAs can be used as new potential biomarkers for the diagnosis of EGC.

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INTRODUCTION

Gastric cancer (GC) is one of the most common upper digestive tract cancers, the fourth most commonly diagnosed cancer, and the second leading cause of cancer-

related deaths worldwide[1,2]. China is well known to have a high incidence of GC. This incidence and mortality are the second and third highest, respectively, among all cancers, and the incidence and mortality rates are more than two-fold higher than world averages. Moreover, recent reports showed that the incidence rate of GC in Gansu Province, China, is significantly higher than the national rate, which is 55.25/100000, and the mortality rate is 36.94/100000[3], This province ranks first in terms of mortality caused by malignant tumors, accounting for 28.74% of all malignant tumor mortalities, far higher than the national average (30.00/100000, 21.48/100000, 12.80%)[4]. The prognosis of GC is related to the clinical progress and the early diagnosis, and its treatment is of critical significance to the prognosis. In early GC (EGC), the gastric mucosal lesion and the development of invasion cancer do not reach the submucosa, but their spread is limited to the mucous layer. This type of cancer is called intramucosal carcinoma. Most EGC can be treated with radical surgery with gastroscopy. The gastroscopy treatment using endoscopic submucosal dissection (ESD) can completely remove the gastric mucosal lesion, eliminating the pain of laparotomy and organ removal. The application of this approach can increase the survival rate to 90% within five years, whereas the survival rate from advanced GC (AGC) is less than 20%. However, the diagnosis of EGC is difficult due to the absence of typical symptoms. Furthermore, no clinically effective biomarkers have been established for EGC detection. Using simple methods to detect high-risk groups of GC and improve the diagnostic rate of EGC are some of the crucial strategies for prevention of GC. Currently, no reliable method or effective biomarkers for EGC that have high sensitivity and specificity exist for detection of high-risk groups of GC. The early detection and diagnosis of EGC at the subclinical stage would substantially improve the prognosis of GC patients.

It is estimated that approximately two-thirds of the genes in the human body are manipulated by specific microRNA (miRNA) or groups of miRNAs, and more than 60 percent of genes encoding human protein were manipulated by miRNAs. These small miRNAs always target one mRNA or multiple mRNAs and degrade or block the translation of mRNAs by base pairing RNA-induced silencing complex with the target gene mRNA. Additionally, they adjust the expression of genes at the translation level. Previous findings indicate that the results of the testing of the miRNA level in tissues, cells, and body fluids can be used as biomarkers for early diagnosis, treatment, and prognosis of tumors[5]. miRNAs can circulate in the blood in stable extracellular forms. The tests for circulating miRNAs can be employed to establish various disease statuses. Therefore, circulating miRNAs should be considered potential blood-based biomarkers, which are new tools for early diagnosis of cancer[6].

The purpose of this research was to identify the differentially expressed miRNAs in the plasma of patients with EGC, AGC, and benign gastritis (BG) through miRNAs chips and to determine their transcription levels at different stages of the disease through reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). Finally, new specific biomarkers for detection of EGC were identified.

MATERIALS AND METHODS

Patients

The protocol for this study was approved by the Ethics Committee of the First Hospital of Lanzhou University (Lanzhou, Gansu, China; ethic number: LDYYLL2018-60), and all patients signed informed consent forms. The present study is part of the project of "The Early Cancer Screening Program of the Upper Gastrointestinal Tract of Gansu Province" conducted from July 2016 to December 2017. The patients diagnosed with EGC, AGC, BG, *Helicobacter pylori* (*H. pylori*)-negative gastritis (Control-1), and *H. pylori*-positive atrophic gastritis (Control-2) received a gastroscopy examination and treatment using the OLYMPUS EVIS 290 electronic endoscope system. The biopsy specimen was analyzed and diagnosed by pathologists at the First Hospital of Lanzhou University using the Vienna classification. *H. pylori* infection was detected by ^{14}C-urea breath test. Blood specimens were analyzed in the Key Laboratory of Biotherapy and Regenerative Medicine of Gansu Province. Patients who were diagnosed for the first time were included, whereas those with other types of previous malignant tumors were excluded (Figure 1).

Obtaining plasma and extracting total RNA

Samples of 5 mL of blood were collected into EDTA anticoagulant tubes after an overnight fast. After in vitro placement from 30 min to 2 h, each specimen was centrifuged at 3000 r/min for 10 min, followed by transfer of plasma into an EP tube

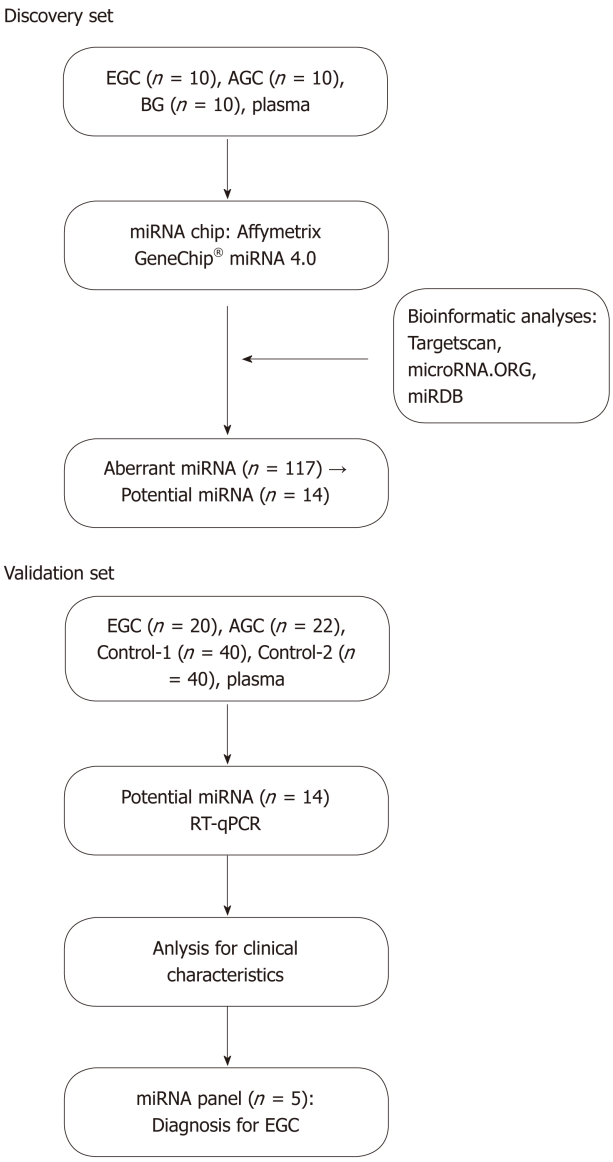


Figure 1 Overview of the study design. EGC: Early gastric cancer; AGC: Advanced gastric cancer; BG: Benign gastritis; Control-1: *Helicobacter pylori*-negative gastritis; Control-2: *Helicobacter pylori*-positive atrophic gastritis; RT-qPCR: Reverse transcription quantitative real-time polymerase chain reaction.

in a liquid nitrogen canister and then transport into a -80 °C refrigerator. We extracted total RNA using TRIzol, and the total RNA was analyzed by NanoDrop 2000 and Agilent Bioanalyzer 2100.

miRNA chip expression profile

The Affymetrix GeneChip® miRNA 4.0 system (Affymetrix, CA, United States) was used following the manufacturer's protocol. FlashTagTM Biotin HSR Labeling Kit was utilized for Poly(A) biotin labeling and hybridization. Next, a GeneChip Hybridization Wash and Stain Kit was used to dye the array and pictures, and original data were obtained by scanning.

Bioinformatic analysis

Differentially expressed miRNA were selected at logFC > 2. The microRNA target prediction was performed with three databases, TargetScan, microRNA.ORG, and miRDB, and the common target genes were obtained. We conducted pathway analysis, followed by enrichment analysis in accordance with the gene information from KEGG and BIOCARTA pathways and showing the results in order of *P*-value.

miRNA RT-qPCR verification

Total RNA was extracted from 200 μL plasma using TRIzol, and another 3 referential plasma samples were used for comparison. Synthetic short sequences of reference

spike-in 1 (ID3EAL Spike-in control for Isolation, MiRXES, Singapore) RNA were put into lysis buffer. This process was considered quality control for the whole experiment, including extracting RNA, reverse transcription, pre-amplification, and the real-time fluorescent PCR. A target miRNAs sequence was obtained from miRBase 21 to design SYBR Green reverse transcription primers, while reverse transcription and miRNA pre-amplification were performed as described above. SYBR Green real-time fluorescent PCR provided the relative transcription and two technical duplications of every miRNAs in every specimen.

Statistical analysis

The Ct attenuation value of each type of miRNA in each of the samples was corrected by the internal reference spike-in 1 and three housekeeping genes, miR-454-3p, miR-423-3p, and miR-191-5p, which are stably expressed in humans. The normalized Ct values of the miRNAs are expressed as the mean ± standard deviation. The Wilcoxon rank sum test was used for comparison between the two groups. Receiver-operating characteristics (ROC) curves were used to evaluate the diagnostic value of miRNAs, and logistic regression was used to calculate the weighting coefficients of miRNAs. $P < 0.05$ was considered to be statistically significant. The software uses SPSS 20.0.

RESULTS

Clinical characteristics of the patients

Experiments were conducted with the miRNA chip expression profiles of the plasma of 30 patients with EGC, AGC, and BG. The validation set was based on the plasma RT-qPCR test results of 20 EGC patients, 22 AGC patients, 40 *H. pylori*-negative gastritis (Control-1) patients, and 40 *H. pylori*-positive atrophic gastritis (Control-2) patients (Table 1).

Bioinformatics analysis of miRNA chip expression profiles

The logFC value of the chip expression profiles of the miRNAs from patients with EGC, AGC, and BG had to be larger than 2 (logFC > 2). Differential expression was found in 117 miRNAs of the three groups of miRNA chips. The miRNAs with the biggest differential multiple were screened to identify the target gene predictions in the three databases: TargetScan, microRNA.ORG, and miRDB. A total of 30 miRNAs passed the target gene predictions. Based on previous findings reported in the literature, 14 potential miRNAs were screened.

RT-qPCR verification results of the potential miRNAs

RT-qPCR tests were run for identification of 14 potential miRNAs, 3 housekeeping genes (miR-454-3p, miR-423-3p, and miR-191-5p), and a spike-in 1 of the internal references using the plasma of 20 EGC, 22 AGC, 40 *H. pylori*-negative gastritis (Control-1), and 40 *H. pylori*-positive atrophic gastritis (Control-2) patients. Reverse transcription and advanced amplification of 18 miRNAs mentioned above and the relative expression of miRNAs was read by real-time fluorescence PCR. The results indicated that no obvious differences were available between Control-1 and Control-2 in the relative expression of the 14 miRNAs with differential expression. The P-values were larger than 0.05 on the average. Thus, they were classified as the Control group.

Four miRNAs distinguished the GC group (EGC + AGC) from the Control group, whose *P*-values, sensitivity, and specificity were as follows, respectively: miR-7641 ($P = 0.006$, 76.2%, 60.0%), miR-425-5p ($P = 0.021$, 66.7%, 65.0%), miR-1180-3p ($P = 0.001$, 81.0%, 60.0%) and miR-122-5p ($P = 0.026$, 71.4%, 60.0%). The equation was risk score factor (RSF) = 1.569 × miR-7641 + 1.312 × miR-425-5p + 1.852 × miR-1180-3p + 1.322 × miR-122-5p (Figure 2), and the area under the curve (AUC) was 0.799 [95% confidence interval (CI): 0.691-0.908, $P < 0.001$]. Additionally, four miRNAs distinguished EGC from the Control group, whose *P*-values, sensitivity, and specificity were as follows, respectively: miR-425-5p ($P = 0.012$, 80.0%, 67.5%), miR-24-3p ($P = 0.031$, 70.0%, 80.0%), miR-1180-3p ($P = 0.007$, 80.0%, 65.0%), miR-122-5p ($P = 0.021$, 80.0%, 60.0%). The equation was RSF = 2.117 × miR-425-5p + 2.234 × miR-24-3p + 2.005 × miR-1180-3p + 1.792 × miR-122-5p (Figure 3), and the value of AUC was 0.829 (95%CI: 0.657-1.000, $P = 0.001$). In addition, miR-24-3p and miR-4632-5p were used to distinguish EGC and AGC ($P = 0.029$, 70.0%, 90.0% and $P = 0.024$, 81.8%, 70.0%) (Figure 4).

The relative expressions of miR-7641, miR-425-5p, miR-1180-3p and miR-122-5p in the GC group (EGC + AGC) were lower than those in the Control ($P < 0.05$), with miR-7641 (AUC = 0.714, 95%CI: 0.563-0.865, $P = 0.006$, sensitivity 76.2%, specificity 60.0%), miR-425-5p (AUC = 0.681, 95%CI: 0.534-0.828, $P = 0.021$, sensitivity 66.7%, specificity 65.0%), miR-1180-3p (AUC = 0.767, 95%CI: 0.635-0.899, $P = 0.001$, sensitivity 81.0%, specificity 60.0%) and miR-122-5p (AUC = 0.675, 95%CI: 0.515-0.835,

Table 1 Characteristics of the subjects included in this study

Characteristics	Discovery set (*n* = 30)			Validation set (*n* = 122)			
	EGC	AGC	BG	EGC	AGC	Control-1	Control-2
Number	10	10	10	20	22	40	40
Age (yr) (Mean ± SD)	53.4 ± 2.6	57.0 ± 7.5	51.4 ± 10.0	63.3 ± 10.1	55.6 ± 13.9	50.2 ± 10.3	56.1 ± 11.3
Gender							
Male	6	6	5	9	12	20	22
Female	4	4	5	11	10	20	18
H. pylori						-	+
Pathology							
HGIN	4			13			
Intramucosal cancer	6			7			
TNM							
HGIN	4			13			
T1a	6			7			
…							
T4N2M0		2			2		
T4N3M0		4			14		
T4N3M1		4			6		

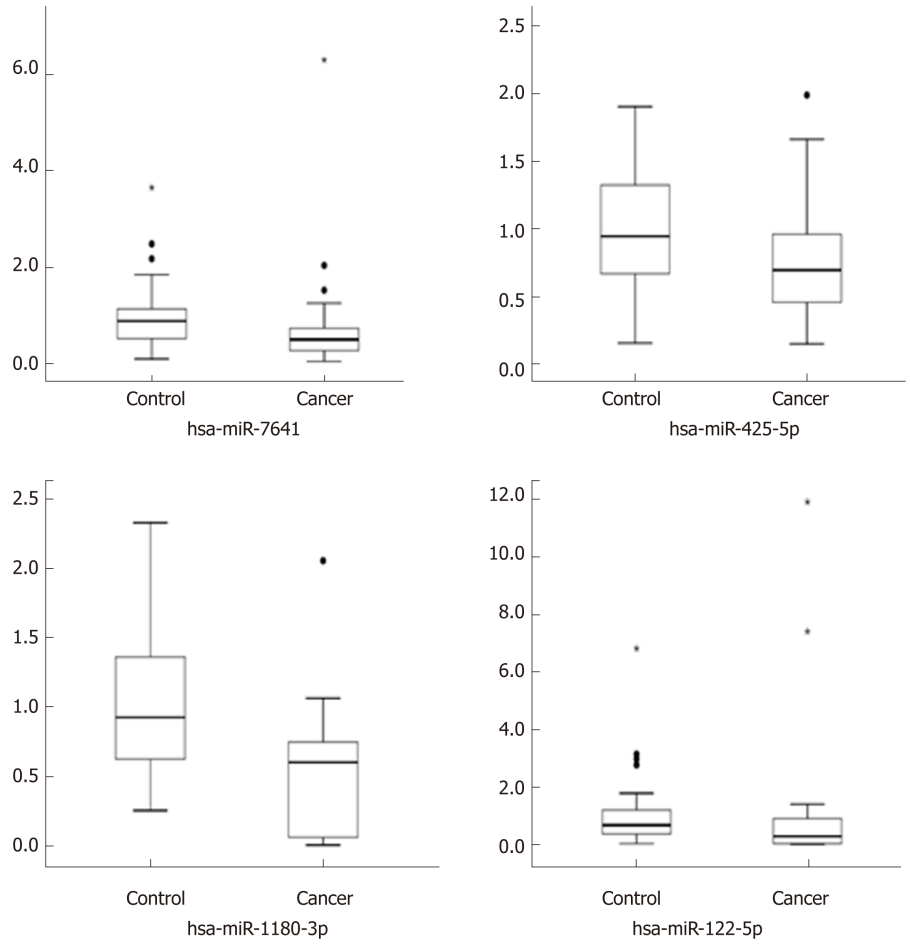
EGC: Early gastric cancer; AGC: Advanced gastric cancer; BG: Benign gastritis; Control-1: *Helicobacter pylori*-negative gastritis; Control-2: *Helicobacter pylori*-positive atrophic gastritis; *H. pylori*: *Helicobacter pylori*; HGIN: High Grade Intraepithelial Neoplasia.

P = 0.026, sensitivity 71.4%, specificity 60.0%), The AUC was significantly increased after combining the four miRNAs (AUC = 0.799, 95%CI: 0.691-0.908, P < 0.001), and the equation was RSF = 1.569 × miR-7641 + 1.312 × miR-425-5p + 1.852 × miR-1180-3p + 1.322 × miR-122-5p. The relative expression levels of miR-425-5p, miR-24-3p, miR-1180-3p and miR-122-5p in the EGC were lower than those in the Control Group, and combining the miRNAs significantly improved the diagnostic value (AUC = 0.829, 95%CI: 0.657-1.000, P = 0.001). The equation was RSF = 2.117 × miR-425-5p + 2.234 × miR-24-3p + 2.005 × miR-1180-3p + 1.792 × miR-122-5p. The expression of miR-24-3p in the EGC group was significantly lower than that in the AGC group (AUC = 0.782, 95%CI: 0.571-0.993, P = 0.029). The expression of miR-4632-5p in the EGC group was significantly higher than that in the AGC (AUC = 0.791, 95%CI: 0.574-1.000, P = 0.024).

DISCUSSION

According to clinical data for Singapore, just one patient is diagnosed with GC out of every 170 patients who are subjected to gastroscopy. Due to its large population and unbalanced development of the medical career, China provides no universal screening programs or early cancer screening activities in medical institutions at the primary level. Liquid biopsy is used to identify related markers from blood and various body fluid samples instead of invasive tests or biopsy[7]. The risk of gastric mucosa lesion can be found through a test of small quantities of a few bodily fluids, such as 2 mL of venous blood. No classical symptoms are evident in most EGC patients. Thus, high-risk patients can be detected through liquid biopsy as early as possible and then subjected to meticulous gastroscopy. Meticulous examinations such as magnifying endoscopy and dye gastroscopy should be used. In addition to comprehensive pathological examination, the diagnosis rate of EGC will be improved. Therefore, the use of liquid biopsy in EGC screening is minimally invasive, safe, economical, and convenient. In addition, it is suitable for the screening of a wide range of people, which makes it valuable for the improvement of EGC diagnosis and treatment.

In a previous study, Li *et al*[8] used a miRNA microarray chip analysis with GC patients and discovered the simultaneous presence of upregulated and downregulated miRNA expression profiles. In addition, Tsai *et al*[9] reported that miR-196a/b was upregulated in both the plasma and tissue of GC patients. Moreover, Fang *et al*[10] found that some carcinogenesis-related miRNAs (miR-10b, miR-21, miR-223, and miR-338) and tumor suppressor miRNAs (miR-30a-5p, miR-126, and let-7a)



AUC of 4 miRNAs in validation phase

miRNAs	AUC	95%CI	*P* value	Cut-off	Sensitivity	Specificity
hsa-miR-7641	0.714	0.563 - 0.865	0.006	20.246	76.2%	60.0%
hsa-miR-425-5p	0.681	0.534 - 0.828	0.021	16.674	66.7%	65.0%
hsa-miR-1180-3p	0.767	0.635 - 0.899	0.001	22.020	81.0%	60.0%
hsa-miR-122-5p	0.675	0.515 - 0.835	0.026	16.661	71.4%	60.0%

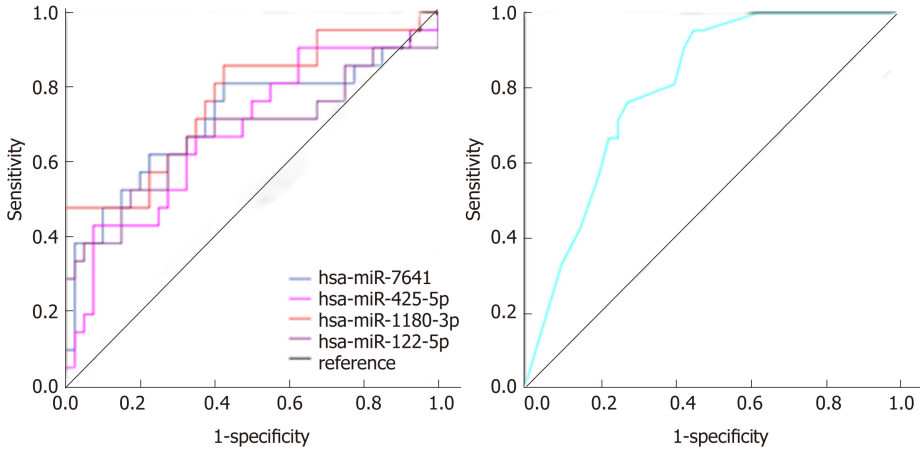
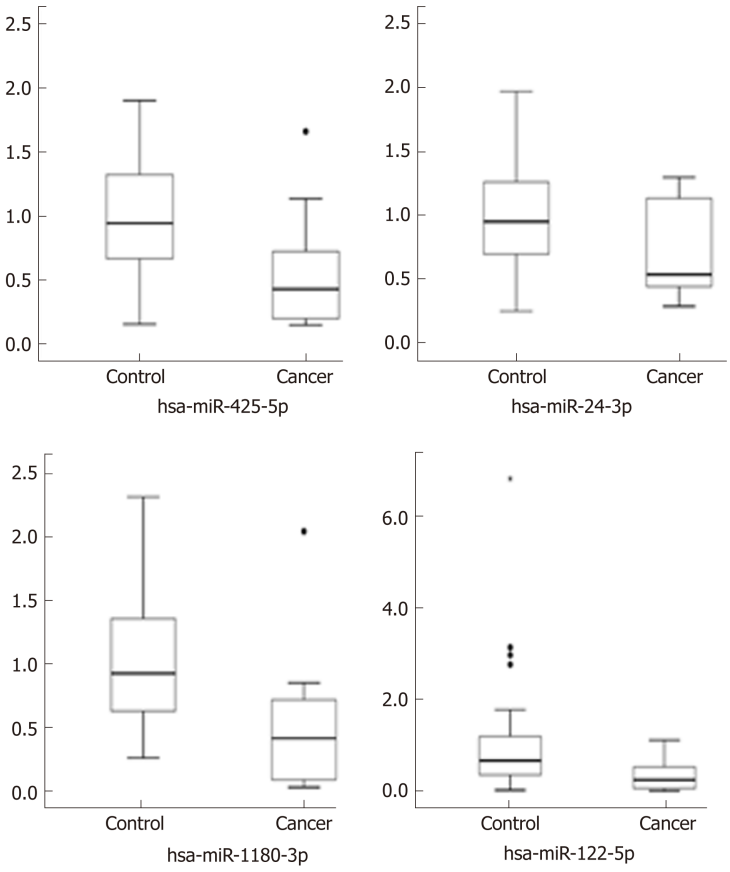


Figure 2 Expression of the four identified microRNAs between the control (Control-1 + Control-2) and cancer (early gastric cancer + advanced gastric cancer) groups. To assess the diagnostic utility of miR-7641, miR-425-5p, miR-1180-3p and miR-122-5p, a linear combination of the risk score of the four miRNAs weighted by the regression coefficient was used to calculate a risk score factor (RSF) for the four-microRNA panel for each subject. The RSF was calculated as follows: RSF = 1.569 × miR-7641 + 1.312 × miR-425-5p + 1.852 × miR-1180-3p + 1.322 × miR-122-5p. Area under curve = 0.799, 95% confidence interval: 0.691-0.908, $P < 0.001$. AUC: Area under curve; CI: Confidence interval; miRNA: MicroRNA.

can be used as prognosis markers in GC patients. The findings from Zhou *et al*[11] show that the differential expression of plasma miRNAs can be utilized as diagnostic markers of GC. Furthermore, plasma miR-106b, miR-20a, and miR-221 can be employed as new types of noninvasive markers for the early diagnosis of GC, whereas miR-21 can be used as a maker of early (stage I) and advanced (stage IV) GC.



AUC, sensitivity and specificity (Control, EGC)

miRNA	AUC	95%CI	*P* value	Cut-off	Sensitivity	Specificity
hsa-miR-425-5p	0.760	0.567 - 0.953	0.012	16.706	80.0%	67.5%
hsa-miR-24-3p	0.722	0.536 - 0.909	0.031	21.292	70.0%	80.0%
hsa-miR-1180-3p	0.777	0.589 - 0.966	0.007	22.109	80.0%	65.0%
hsa-miR-122-5p	0.738	0.552 - 0.923	0.021	16.661	80.0%	60.0%

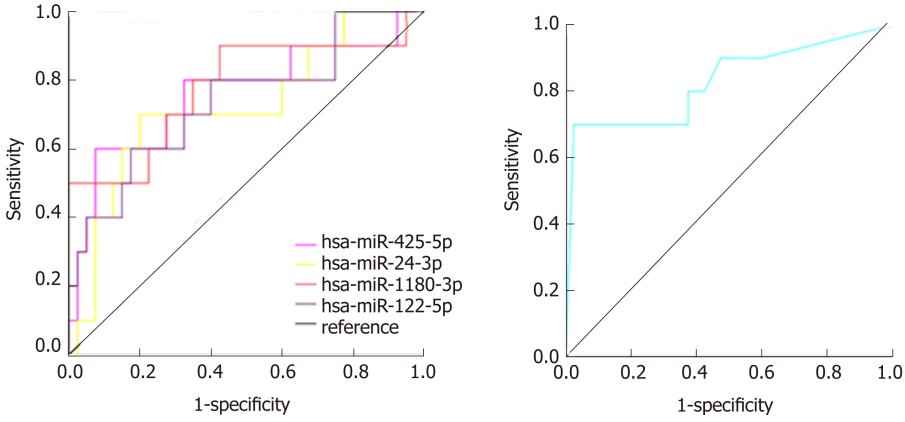


Figure 3 Expression of the four identified microRNAs between the control (Control-1 + Control-2) and early gastric cancer groups. To assess the diagnostic utility of miR-425-5p, miR-24-3p, miR-1180-3p and miR-122-5p, a linear combination of the risk score of the four microRNAs (miRNAs) weighted by the regression coefficient was used to calculate a risk score factor (RSF) for the four-miRNA panel for each subject. The RSF was calculated as follows: RSF = 2.117 × miR-425-5p + 2.234 × miR-24-3p + 2.005 × miR-1180-3p + 1.792 × miR-122-5p. Area under curve = 0.829, 95% confidence interval: 0.657-1.000, *P* = 0.001. AUC: Area under curve; CI: Confidence interval; miRNA: MicroRNA; EGC: Early gastric cancer.

Jiang detected high expression of miR-421 in EGC and suggested that it could serve as a diagnostic marker[12]. Additionally, Fehmida *et al*[13] discovered that miR-200c-3p was considerably downregulated in EGC tissue, which can be used for early diagnosis of the disease. Racial differences exist in the expression of GC-related miRNAs. It is noteworthy that Li discovered that the level of miRNA-199a-3p in the plasma of EGC

miRNA	AUC	95%CI	*P* value	Cut-off	Sensitivity	Specificity
hsa-miR-24-3p	0.782	0.571 - 0.993	0.029	21.293	70.0%	90.0%
hsa-miR-4632-5p	0.791	0.574 - 1.000	0.024	31.179	81.8%	70.0%

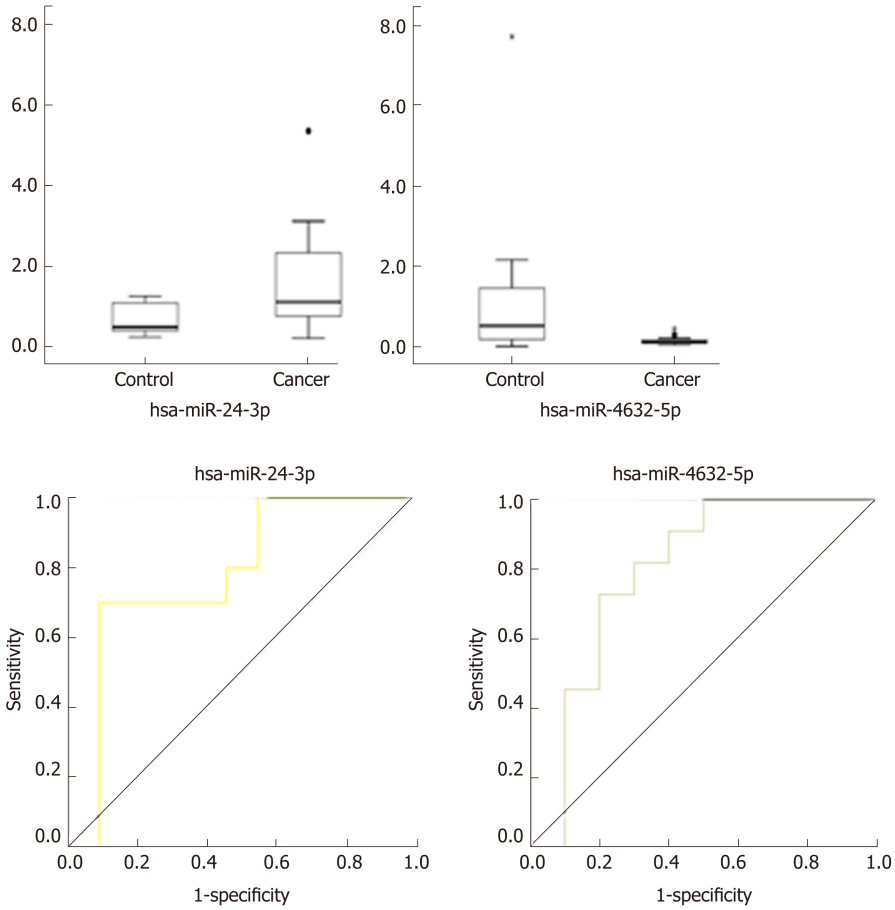


Figure 4 Expression of the two microRNAs between the advanced and early gastric cancer groups. The expression of miR-24-3p in the early gastric cancer (EGC) group was significantly lower than that in the advanced gastric cancer (AGC) [area under curve (AUC) = 0.782, 95% confidence interval (CI): 0.571-0.993, *P* = 0.029] group. The expression of miR-4632-5p in the EGC group was significantly higher than that in the AGC (AUC = 0.791, 95%CI: 0.574-1.000, *P* = 0.024) group. AUC: Area under curve; CI: Confidence interval; miRNA: MicroRNA.

patients changed with the treatment, but its expression level was not related to depth of tumor infiltration[14]. In many studies, the use of combinations of circulating miRNAs can lead to high diagnostic accuracy, which is indicated by an area under the ROC curve larger than 0.8. Although, limited research has been conducted, similar diagnostic accuracy was reported using long noncoding RNAs or proteins of extracellular vesicles[15].

At present, a number of studies have been carried out on the use of miRNAs in GC patients related to the occurrence, development, diagnosis, treatment, and prognosis of the disease. Therefore, miRNAs can be used as early diagnostic markers of GC. However, substantial discrepancies exist among the findings of those studies, and there is no consistent conclusion. Relatively few studies have been conducted on the application of miRNAs in the diagnosis and screening of EGC. In the present investigation, we used "The Early Cancer Screening Program of the Upper Gastrointestinal Tract of Gansu Province" biobank. The differences in the expression profiles of the plasma miRNAs in EGC, AGC, and BG patients were established through miRNA chip analysis, and 14 potential miRNAs were screened through bioinformatics analysis. Since *H. pylori* infection is a well-known GC carcinogen, and atrophic gastritis is an exceedingly common precancerous disease, EGC screening should eliminate the negative influence of test sensitivity and specificity to which precancerous diseases such as *H. pylori* infection, atrophic gastritis, and precancerous lesion might contribute to. Thus, *H. pylori*-negative gastritis (Control-1) and *H. pylori*-positive atrophic gastritis (Control-2) groups should be available in the validation set to replace the BG group in the discovery set to eliminate the influence that

precancerous diseases such as *H. pylori* infection and atrophic gastritis might have on miRNA metabolism.

Initially, this study found no statistically significant difference among the relative expression levels of 14 potential miRNAs from the groups of *H. pylori*-negative gastritis and *H. pylori*-positive atrophic gastritis patients. Hence, these patients were combined and classified as a Control group. The combination of miR-7641, miR-425-5p, miR-1180-3p and miR-122-5p distinguished the Cancer group, which included EGC and AGC, from the Control group, whose AUC was 0.799 (95%CI: 0.691-0.908, $P < 0.001$). On the other hand, the combination of miR-425-5p, miR-24-3p, miR-1180-3p and miR-122-5p distinguished the EGC group from the Control group. The AUC was 0.829 (95%CI: 0.657-1.000, $P = 0.001$). Additionally, miR-24-3p and miR-4632-5p can be used to distinguish EGC from AGC. Of note, screening strategies can be employed during the statistical analysis of the relative expression and pathological results of RT-qPCR testing of target miRNAs. First, miR-7641, miR-425-5p, miR-1180-3p and miR-122-5p should be used to distinguish EGC and AGC from the Control group. In addition, EGC can be compared to and distinguished from the Control group using miR-425-5p, miR-24-3p, miR-1180-3p and miR-122-5p. Finally, miR-425-5p, miR-1180-3p and miR-122-5p are shared potential miRNAs, whereas miR-24-3p and miR-4632-5p can distinguish EGC from AGC. Thus, a joint testing mode that contains miR-425-5p, miR-1180-3p, miR-122-5p, miR-24-3p and miR-4632-5p can be established and used for predictive EGC diagnosis.

No relevant research is present in the existing literature on the value of miRNA as a biomarker for screening and diagnosis of EGC. At present, the correlation between hsa-miR-1180-3p and hsa-miR-4632-5p and GC, especially EGC, has not been reported. miR-425-5p is upregulated in human GC and promotes its invasion and metastasis *in vitro* and *in vivo*, which may serve as a predictor of poor prognosis[16,17]. Earlier studies found that miR-425-5p passed the mechanism of ubiquitinating enzyme (CYLD) as an oncogene and promoted the progression of GC, whereas competitive endogenous RNA (ceRNA) targeting miR-425-5p inhibited the development of GC by p53[18,19]. Moreover, Xu *et al*[20] discovered that the expression of miR-122-5p was downregulated in GC tissues and cells, whereas the overexpression of miR-122-5p inhibited the migration and invasion of GC cells and the occurrence of lung metastases by downregulating DUSP4. These findings indicate that miR-122-5p inhibits GC cell proliferation and induces apoptosis by targeting MYC[21]. miR-24-3p targets and negatively regulates Prdx-6, which significantly inhibits the growth, migration, and invasion of GC cell lines and promotes apoptosis. Of note, *H. pylori* infection may decrease the expression of miR-24-3p[22].

In the present investigation, we found that the abnormal expression levels of plasma miR-425-5p, miR-1180-3p, miR-122-5p, miR-24-3p, and miR-4632-5p can be used as a new combination of specific biomarkers for predictive diagnosis of EGC. In addition, we established a predictive diagnosis mode for EGC circulating plasma miRNAs markers, which can be used as a new specific combination of biomarkers for predictive diagnosis of EGC. In such a way, more EGC patients will be diagnosed after combining the application of this mode with gastroscopy, and the strategies for EGC screening will be considerably improved. It has a working mode, in which liquid biopsy can be used to identify high-risk groups and shrink the screening scope, while using gastroscopy can be implemented for diagnosis of EGC patients. Moreover, the screening efficiency should be improved, which requires verification of our findings in a larger sample size of people. This replication will be our objective in the next screening project of "The Early Cancer Screening Program of the Upper Gastrointestinal Tract of Gansu Province".

In summary, there are increasing demand on early diagnosis of GC, especially the EGC for the more higher survival rate than AGC after radical treatment. The stability of miRNAs makes it suitable biomarker in liquid biopsy, and previous and this study showed the miRNAs pannel maybe have better sensitivity and specificity. We first identified that five miRNAs can be used in early detection of EGC, the pannel will useful to screening high risk population, nevertheless, larger-scale human experiments are still required to confirm our findings.

ARTICLE HIGHLIGHTS

Research background

The early gastric cancer means the gastric mucosal lesion and the development of invasion cancer do not reach the submucosa, but their spread is limited to the mucous layer. Compared with advanced gastric cancer, it performs better in prognosis. Yet due to the lack of typical symptoms and biomarkers, the early gastric cancer is hard to be diagnosed and the golden standard is gastroscope. However, due to the patient's acceptance with certain risks and the

difficulty in gastroscopic diagnosis, the detection rate of early gastric cancer in China is still low. Some existed research suggests that microRNA (miRNA) in the peripheral blood can be used as biomarkers in the diagnosis and prognosis of gastric cancer especially for advanced gastric cancer, but few shows whether miRNA can be used as the biomarker for predictive diagnosis of early gastric cancer.

Research motivation

MiRNA is relatively stable in the circulation system, and the detection technique is simple and easy to popularize. The several combinations of miRNAs can improve the accuracy of diagnosis which found in kinds of cancers. Through the predictive diagnosis model of the combination of several miRNAs, screening out high-risk or suspect patients and then being confirmed by gastroscopy combined with biopsy, will improve the accuracy and efficiency of gastroscopy, and promote the detection of early gastric cancer and early diagnosis of advanced gastric cancer, thereby improving the overall treatment effect and prognosis of gastric cancer.

Research objectives

The focus of the study is whether miRNA in peripheral blood can be used as sensitive and specific biomarkers for predictive diagnosis of early gastric cancer. First of all, it is to be studied that whether there is one or there are several miRNAs used to diagnose gastric cancer and non-cancer. Furthermore, it is to be known that whether such miRNAs can be used to suggest the occurrence of early gastric cancer. If there are indeed such miRNAs, it needs to be known that whether it can be applied in the screening of early gastric cancer and the sensitivity and specificity as well as influence factors. All these are possibly applied to clinical practice.

Research methods

First, in the discovery set, miRNA array was applied to detect the differential expressions of plasma miRNA in the early and advanced gastric cancer patients as well as the control group. Then through the bioinformatics, miRNAs possibly related to disease staging were screened out. In the validation set, in order to rule out the effects of *Helicobacter pylori* (*H. pylori*) infection, atrophic gastritis and other diseases on miRNA, the control group was divided into *H. pylori* infection with atrophic gastritis and *H. pylori*-negative superficial gastritis. Then RT-qPCR was used to verify target miRNAs selected in the last stage and miRNAs or combinations that may be used for predictive diagnosis of early gastric cancer are selected.

Research results

Fourteen target miRNAs were screened from the miRNA array by bioinformatics and they show differential expressions in early and advanced gastric cancer and control group. Subsequent reverse transcription quantitative real-time polymerase chain reaction verification suggested that five miRNAs combinations might be used for predictive diagnosis of early and advanced gastric cancer while not being affected by diseases such as *H. pylori* infection and atrophic gastritis.

Research conclusions

In this article, we found that miRNAs in early and advanced gastric cancer as well as control group show differential expressions. Through further confirmatory experiment, it is found that combinations of several miRNAs may suggest the occurrence of early and advanced gastric cancer. Gastroscopy combined with biopsy can be used to further confirm the diagnosis and then this combination of miRNAs may be regarded as the biomarker of predictive diagnosis of early gastric cancer.

Research perspectives

Currently, there is a lack of effective tumor biomarker in the diagnosis of gastric cancer, which is related to the heterogeneity of tumors. Although gastroscopy and biopsy are the golden standards for the diagnosis of gastric cancer, they are currently difficult to spread in China due to the large population. This study hopes to find high-risk patients with early gastric cancer in the population through simple and economical liquid biopsy of new miRNA biomarkers. Then gastroscope and pathological examination can be used to confirm the diagnosis and treatment of early gastric cancer, and early diagnosis and early treatment to advanced gastric cancer, so as to improve the overall prognosis and curative effect of gastric cancer. Certainly, the new combinations of these miRNAs biomarkers need to be further validated in a larger sample population.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comparison of Hemospray® and Endoclot™ for the treatment of gastrointestinal bleeding

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Author contributions: Rath T designed the study; Vitali F, Naegel A, Atreya R, Neufert C and Rath T participated in the acquisition, analysis, and interpretation of the data, and drafted the initial manuscript; Vitali F wrote the article; Siebler J and Neurath MF revised the article critically for important intellectual content.

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Informed consent statement: As retrospective cohort study informed patients' consent was waived from the ethic committee

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Abstract

BACKGROUND

Gastrointestinal (GI) bleeding is a common indication for endoscopy. For refractory cases, hemostatic powders (HP) represent "touch-free" agents.

AIM

To analyze short term (ST-within 72 h-) and long-term (LT-within 30 d-) success for achieving hemostasis with HP and to directly compare the two agents Hemospray (HS) and Endoclot (EC).

METHODS

HP was applied in 154 consecutive patients (mean age 67 years) with GI bleeding. Patients were followed up for 1 mo (mean follow-up: 3.2 mo).

RESULTS

Majority of applications were in upper GI tract (89%) with following bleeding sources: peptic ulcer disease (35%), esophageal varices (7%), tumor bleeding (11.7%), reflux esophagitis (8.7%), diffuse bleeding and erosions (15.3%). Overall ST success was achieved in 125 patients (81%) and LT success in 81 patients (67%). Re-bleeding occurred in 27% of all patients. In 72 patients (47%), HP was applied as a salvage hemostatic therapy, here ST and LT success were 81% and 64%, with re-bleeding in 32%. As a primary hemostatic therapy, ST and LT success were 82% and 69%, with re-bleeding occurring in 22%. HS was more frequently applied for upper GI bleeding ($P = 0.04$)

CONCLUSION

Both HP allow for effective hemostasis with no differences in ST, LT success and re-bleeding.

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Core tip: Hemostatic powders represent "touch-free" hemostatic agents for the treatment of gastrointestinal bleeding. Within this study, we analyzed the hemostatic efficacy of hemostatic powders as first line or salvage therapy in several clinical scenarios in a large cohort of prospectively included patients. As shown in our report, both hemostatic powders allow for excellent short term bleeding control while at the same time, long term efficacy over a period of 4 wk is maintained in a considerable amount of patients. No differences were observed between Hemospray and Endoclot in their hemostatic efficacy.

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INTRODUCTION

Gastrointestinal (GI) bleeding represents a major challenge for the GI endoscopist both in term of frequency, which is estimated to be 150/100000 for upper and 33/100000 for lower GI bleeding with a mortality ranging between 2 and 10%[1-3], and in terms of technical efforts to reach a stable hemostasis. As the administration of direct oral anticoagulants[4] and the use of assistant devices in terminal cardiomyopathy[5] is increasing, sufficient and effective treatment of GI bleeding is mandatory while at the same time can be clinically challenging. In the last years, endoscopists increasingly face emergency bleeding in a clinical scenario in which coagulation parameters cannot always be corrected to normal range. Further, with increasing development of advanced endoscopic therapeutic procedures, iatrogenic bleeding after endoscopic resections represents another emerging problem[6]. Conventional treatment approaches achieve hemostasis in more than 90% of cases[7], however, depending on the bleeding site and source can be technically challenging, and might not be optimal for diffuse oozing bleeding as frequently observed in patients with impaired coagulation or cancer bleeding.

Hemostatic powders (HP) act as "touch-free" agents that can be easily administrated for the treatment of GI bleeding, which are generally safe and well tolerated[8-11]. Hemospray (HS, TC-325, Cook Medical, Bloomington, Indiana, United States) is an inert mineral based compound, which, in contact with blood, absorbs water and acts cohesively and adhesively, thereby forming a covering mechanical tamponade. By fluid absorption, HS enhances clot formation by deforming and packing erythrocytes, concentrates activated platelets with clotting factors and interacts with the fibrin matrix[12] and within 24 to 72 h, the adherent coat sloughs off into the GI lumen[10]. With his local hemostatic proprieties, first studies suggest that HS is equally effective in both patients with and without systemic antithrombotic therapy[9].

Endoclot (EC, Micro-Tech Europe, Düsseldorf, Germany) is a starch-derived agent composed of absorbable hemostatic polysaccharides. Similar to HS, in contact with blood, EC initiates a dehydration process leading to a concentration of clotting factors, platelet and erythrocytes thereby accelerating the physiological clotting cascade and the formation of a mechanical shell of gelled matrix which adheres to the bleeding tissue[13]. Although data on the efficacy of EC are still limited, first clinical evidences suggest that both HS and EC allow for effective bleeding control[8,11,14-20]. Further, no direct comparison of the efficacy of these two HP is available to date. Against this background we set off: (1) To analyze short and long term hemostatic effectiveness of HP; and (2) to compare the efficacy between the agents HS and EC in achieving hemostasis in a large cohort of patients treated for emergency GI bleeding in our center.

MATERIALS AND METHODS

Patients and methods

Prospective data collection was performed including patients who were treated with HS and EC for endoscopic hemostasis during emergency endoscopy between September 2013 and September 2017 in our university hospital. After application of HP patients were followed-up for at least one mo. After completion of follow-up (FU) of all patients data analysis was performed. The study was approved by the local institutional review board and the ethics committee of the Friedrich-Alexander University Erlangen Nuereмberg (approval at 31 January 2018) and our study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Indications for treatment with HP were: refractory GI bleeding (e.g. due to difficult anatomical location or diffuse oozing bleeding without definite source); application of HP as salvage therapy after failure of other endoscopic methods; application of HP as prophylactic means to avoid delayed bleeding in lesions with high re-bleeding risk, application of HP as primary treatment means usage of HP as monotherapy. Primary endpoints were short term (ST, hemostasis for 72 h) and long term (LT, hemostasis for a period of 30 d) success in achieving hemostasis with HP as a primary or salvage therapy.

Re-bleeding rate (RBR) was defined as the number of the patients who showed recurrent bleeding among the patients who underwent FU. Recurrent bleeding was defined if one of these criteria had been met: (1) Hematemesis or melena; (2) a drop in hemoglobin > 2 mg/dL or transfusion of 4 or more blood packs; or (3) hemodynamic instability as previously described[10,21]. Complete Rockall Score was utilized to stratify high-risk patients. Secondary endpoint was the direct comparison of hemostatic efficiency between EC and HS.

Statistical analyses

Descriptive statistics consisted of the mean, median, SD and range. The $\chi 2$ analysis was used for discrete variables. The Fisher exact probability test was used for the 2 × 2 contingency tables, where suitable. A two-sided $P < 0.05$ was considered to be significant. The statistics were processed using the SPSS statistical program (SPSS Inc, Chicago, Ill, United States).

RESULTS

Clinical characteristics of the patient cohort

HP was applied a total of 239 times in 154 patients with a mean age of 67 years. The majority of the patients were male (n = 101, 66%). One child treated with HS was also included in the analysis. Clinical FU for at least one month was performed in 134 patients (87%) with a mean FU of 3.2 SD 5.5 mo (range 1-29). No patient was lost during FU; however, in 20 patients FU was not completed as they died from other causes than GI bleeding within 30 d after the first HP application. The mean complete Rockall score[22] in the total patient cohort was 7.1 with 61 (40%) patients exhibiting a Rockall score > 7 and 27 patients (18%) with a Rockall score > 8.

Therapeutic anticoagulation was present in 45 patients (29%). Of these, 17 (11%) received heparin, low molecular weight heparin or argatroban in therapeutic dosages while 17 patients (11%) and 11 patients (7%) were taking vitamin K antagonist and direct oral anticoagulants, respectively. Antiplatelet drugs were administered in 34 (22%), 8 patients received dual antiplatelet therapy (5.2%). Among co-morbidities, 20 patients had localized (13%) and 21 patients metastasized cancer (14%) while 6 patients suffered from malignant lymphoproliferative disease (4%). 40 patients (26%) suffered from liver cirrhosis and 74 patients (48%) exhibited renal insufficiency, of which 35 patients (23%) had terminal kidney failure requiring hemodialysis. 13 patients had coronary heart disease (8%). 53 patients (35%) presented with hemorrhagic shock at the time of application of HP. Vasopressors were administered in 65 patients (42%). Clinical characteristics of the total patient cohort are summarized in Table 1.

Overall Efficacy of HP in the management of GI bleeding

In patient cohort, HP exhibited an overall ST and LT success for achieving hemostasis of 82% and 69% with a RBR of 21% when applied as primary therapy. As salvage therapy, overall ST success, LT success and RBR rate were 83%, 68% and 29%, respectively. In the cohort, no significant difference was observed for achieving hemostasis between HS and EC under primary or salvage therapy (Table 1). Due to refractory bleeding a total of 20 patients treated with HP had to undergo surgery or

Table 1 Clinical characteristics of the total patient cohort treated with hemostatic powders for gastrointestinal bleeding *n* (%)

	HS and EC *n* = 154	Hemospray *n* = 111	Endoclot *n* = 32	*P* value
Sex (M)	101 (65.6)	76 (68.5)	17 (53.1)	ns
Age, yr				
mean ± SD	66.6 ± 14	67 ± 13.8	67.4 ± 15.1	ns
range	11-93	29-93	11-89	
Rockall risk score				
median ± SD	7.1 ± 1.7	7.1 ± 1.7	7.1 ± 1.8	ns
range	2-10	2-10	2-10	
Comorbidities				
Coagulopathy	48 (31.2)	36 (32.4)	6 (18.8)	ns
Renal insufficiency	74 (48.1)	53 (47.7)	15 (46.9)	ns
Hemodialysis	35 (22.7)	26 (23.4)	5 (15.6)	ns
Liver cirrhosis	40 (26)	32 (28.9)	5 (15.6)	ns
Bleeding locale				
upper GI bleeding	137 (89)	102 (91.8)	25 (78.1)	0.04
lower GI bleeding	17 (11)	8 (8)	7 (21)	ns
Application as				
Primary therapy	82 (53.2)	64 (57.7)	14 (43.8)	ns
Salvage therapy	72 (46.8)	47 (42)	18 (56)	ns
Multiple applications of HP	42 (27.3)	27 (24.3)	5 (15.6)	ns
Definite hemostatic therapies after HP failure				
Coiling	13 (8.4)	11 (9.9)	1 (3.1)	ns
Surgery	9 (5.8)	7 (6.3)	1 (3.1)	ns
Short term success (total)	125 (81.2)	92 (82.9)	26 (81.2)	ns
Primary therapy	67/82 (81.7)	53/64 (82.8)	11/14 (78.6)	
Salvage therapy	58/72 (80.6)	39/47 (82.9)	15/18 (83.3)	
Long term success	81/121 (66.9)	59 (69.4)	18 (66.7)	ns
Primary therapy	45/65 (69.2)	35/49 (71.4)	8/13 (61.5)	
Salvage therapy	36/56 (64.3)	24/36 (66.7)	10/14 (71.4)	
Re-bleeding rate	41 (26.6)	27 (24.3)	8 (25)	ns
Primary therapy	18/82 (21.9)	13/64 (20.3)	3/14 (21.4)	
Salvage therapy	23/72 (31.9)	14/47 (29.8)	5/18 (27.8)	

HS: Hemospray; EC: Endoclot; HP: Hemostatic powders; HS and EC: Including patients who received both Hemospray and Endoclot at different time points; GI: Gastrointestinal.

interventional radiology for bleeding control after failure of HPs.

Efficacy of HP in the management of upper GI bleeding

The majority of patients exhibited upper GI bleeding (*n* = 137, 89%). Of these, 91 patients (66%) presented with Forrest Ib bleeding while 15 patients (11%) exhibited a Forrest Ia bleeding source. Further, 4 patients (3%) had Forrest III lesions. Clinical characteristics of the patients with upper GI bleeding are shown in Table 2.

Overall, ST success of HP within the upper GI tract was achieved in 113 patients (82.5%) with LT success maintained in 71 patients (66%) and an overall RBR of 25%. HP as salvage therapy was applied in 65 patients (47%) with upper GI bleeding. The ST and LT success of HP as primary and salvage therapy is shown in Table 2. Within the upper GI Tract, bleeding was derived from the following sources: peptic ulcer disease (*n* = 49, gastric ulcer: *n* = 12; duodenal ulcer: *n* = 37), malignant tumor (*n* = 15), esophagogastric varices (*n* = 13), reflux esophagitis (*n* = 12), angiodysplasias or angioectasias (*n* = 8), Mallory Weiss lesions (*n* = 5) and diffuse oozing bleeding and erosions (*n* = 21). We then performed subgroup analyses on the efficacy of HP according to the bleeding location (Table 3). In peptic ulcer disease (Figures 1 and 2), HP achieved hemostasis with a ST and LT success of 80% and 57% and a RBR of 34%. When applied as a primary therapy in peptic ulcer disease, ST and LT success and RBR were 79%, 67% and 21%, respectively; when applied as a salvage therapy ST and LT were comparable (81% and 67%); however RBR was considerably higher under

Table 2 Clinical characteristics of the patients with upper gastrointestinal bleeding and efficacy of hemostatic powders in the treatment of upper gastrointestinal bleeding *n* (%)

	HS and EC (*n* = 137)	Hemospray (*n* = 102)	Endoclot (*n* = 25)	*P* value
Sex (M)	86 (62.8)	68 (66.7)	11 (44)	0.04
Age, yr				
mean ± SD	66.4 ± 14.2	66.4 ± 14.0	67.9 ± 16.5	ns
range	(11-93)	29-93	11-89	
Rockall risk score				ns
median ± SD	7.1 ±1.7	7.1 ± 1.7	7.1 ± 1.8	
range	2-10	2 -10	2 - 10	
Comorbidities				
Coagulopathy	45 (32.8)	34 (33)	5 (2)	
Renal insufficiency	68 (49.6)	59 (49)	12 (48)	
Hemodialysis	32 (23.4)	24 (23)	12 (48)	
Liver cirrhosis	38 (27.7)	30 (29.4)	5 (20)	
Therapeutic anticoagulation	35 (25.5)	28 (27.5)	6 (24)	
Dual antiplatet therapy	7 (5.1)	5 (5)	2 (8)	
Vitamin K antagonists	14 (10.2)	11 (11)	3 (12)	
DOAC	8 (5.8)	7 (7)	1 (4)	
Antiaggregation therapy	29 (21.2)	21 (20.6)	7 (28)	
Application as				ns
Primary Therapy	72 (52.6)	59 (58)	10 (40)	
Salvage Therapy	65 (47.4)	43 (42)	15 (60)	
Multiple Applications of				ns
HS	37 (27)	24 (23)	3 (0.12)	
Definite hemostatic therapies after HP failure				ns
Coiling	13 (9.5)	11 (11)	1 (4)	
Surgery	8 (5.8)	7 (6.9)	0	
Short term success (total)	113/137 (82.5)	68/102 (66.6)	21/25 (84)	ns
Primary therapy	60/72 (83.3)	50/59 (84.7)	8/10 (80)	
Salvage therapy	53/65 (81.5)	36/43 (83.7)	13/15 (86.6)	
Long term success	71/108 (65.7)	53/78 (67.9)	15/22 (68.2)	ns
Primary therapy	39/57 (68.4)	32/45 (71)	6/10 (60)	
Salvage therapy	32/51 (62.7)	21/33(63.6)	9/12 (75)	
Re-bleeding rate	34/137 (24.8)	24/102 (23.5)	4/25 (16)	ns
Primary therapy	15/72 (20.8)	11/59 (18.6)	2/10 (20)	
Salvage therapy	19/65 (29.2)	13/43 (30.2)	2/15 (13)	

DOAC: Direct acting oral anticoagulant; HS: Hemospray; EC: Endoclot; HP: Hemostatic powders; HS and EC: Including patients who received both Hemospray and Endoclot at different time points.

salvage therapy (46%). A total of 15 patients suffered from diffuse cancer bleeding, here ST and LT success were 81% and 85%, re-bleeding occurring in only 1 patient.

For variceal bleeding, overall ST success was achieved in 91%. In oesophageal bleeding HP was used as salvage therapy in 8 patients. LT success was achieved in 3/4 (75%) patients. Re-bleeding was present in 2/7 (28.5%). In 3 patients with fundic varices bleeding, 1 LT success was achieved after applying HP as salvage therapy (33.3%). HP as a primary therapy in fundic varices bleeding is in our experience not suitable to achieve a stable hemostasis alone. In patients under therapeutic anticoagulation ST and LT success of HP were 81% and 58%, re-bleeding in 33% of patients. Regardless of whether they were applied as primary or salvage therapy or in which bleeding location, no significant differences for achieving ST or LT hemostasis and recurrence of bleeding were detected between HS and EC.

Efficacy of HP in the management of lower GI bleeding

HP was applied in 17 patients with lower GI bleeding (Table 4). Among these, 9 patients were treated with HS, 7 patients with EC while in 1 patient with lower GI bleeding, both HS and EC were applied. Overall ST and LT success was 71% (12/17)

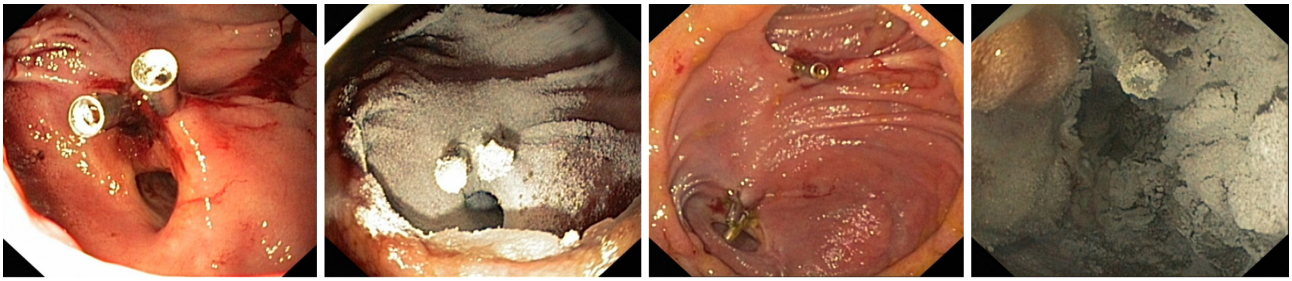

Figure 1 Before (left side) and after application of Hemospray (right side) within the upper gastrointestinal tract (duodenal ulcer).

and 59% (9/13), respectively with a RBR of 41%. Clinical characteristics of patients with lower GI bleeding are shown in Table 4.

DISCUSSION

Herein we report on our experience in the treatment of GI bleeding both with HS and EC in a single tertiary university care center. To the best of our knowledge our work is the first to directly compare two different HP for the treatment of GI bleeding in the upper and lower GI tract. Our study confirms the findings of other investigators where an excellent immediate control of the bleeding source was achieved with HP[8,11,18,19,21]. HP exhibited an overall short-term success of 82% in our study. With this, ST success was higher in our cohort compared to a previous report on a smaller cohort by Chen and colleagues[8], although this study analyzed of success rates of HS only.

According to the literature, hemostatic success of HP within 7 to 8 d range between 51% and 87.5%[14,15,19]. Within this study, we performed FU for at least one month in the vast majority of patients (87%) and long-term success dropped to 67% in our study. Hence, these data are consistent with results from GRAPHE registry in which LT success rates of 66% were reported[19]. A graphic illustration (Figure 3) of the mean incidence of re-bleeding after application of HP according our and past studies[8-11,14-16,18,19,22] shows that RBR increase over time after HP application across studies and with this, although allowing for excellent immediate bleeding control, HP appears to be not suitable as a definitive long-term hemostasis tool in patients with a high-risk profile of bleeding recurrence. On the other hand, the benefit of HP is the high immediate hemostasis rate and that can be administered more than once without risk of "overdosing" or induction of bleeding due to mechanical irritation.

When performing subgroup analyses according to bleeding etiology, overall ST and LT success in peptic ulcer disease was 81% and 68% with a RBR of 19%. With this, our results are consistent to those reported in the literature[11,13,14,17,18,23], with immediate hemostasis ranging between 78 and 96% and RBR between 10.5 and 38%. However, when analyzing peptic ulcer disease with Forrest Ia bleeding in our study, ST and LT success were only 67% and 33% respectively. Together with results from other studies that have reported a re-bleeding risk of Forrest Ia lesions under HP between 67% and 73%[10,11,13,14,17,18,24], our data show that HP are not effective as a first-line therapy in Forrest Ia peptic ulcer bleeding. Nevertheless, HP but might still be useful in this scenario as a bridging or rescue strategy until an alternative therapy as another endoscopic procedure, a radiological embolization or surgical therapy can be performed.

HP have also been reported to be effective as rescue therapy for variceal bleeding when band ligation fails[25] and also in gastric varices and gastric bleeding derived from portal hypertension[23]. Within our study, we observed an overall ST success of 85% and LT success of 56%. It is important to note that in the majority of applications for variceal bleeding, the bleeding was serious with 70% of patients presenting with hemorrhagic shock. Against this background, the overall ST success can be regarded as high, and thus HP might represent a promising addition to arsenal of the endoscopist for severe and refractory variceal bleeding.

Due to their touch-free application and large coverage, HP are also well suited for the treatment of tumor bleeding. As shown in our study, HP provide immediate hemostatic efficacy of 95%, a short-term success of 83% and a long-term success 87% in patients with diffuse tumor bleeding. With this, our results are comparable to previous studies, in which immediate efficacy of HP and RBR ranged between 93%-100% and 20%-32%, respectively[10,14,18,21,26]. Since tumor bleeding is frequently diffuse

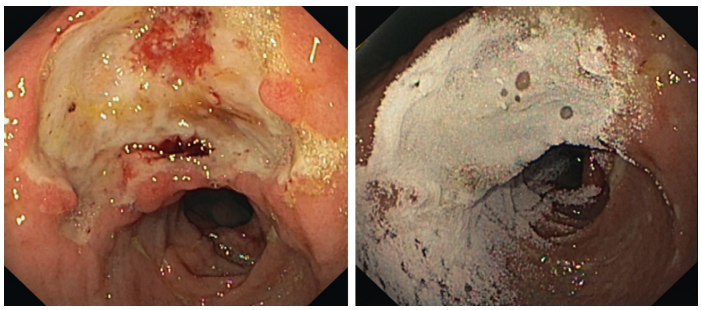

Figure 2 Before (left side) and after application (right side) of Endoclot within the upper gastrointestinal tract (duodenal ulcer).

and exhibits a large bleeding area, high RBR ranging up to 49% have been reported with conventional hemostatic approaches[24,27]. Together, with data from the largest multicenter retrospective study, in which an immediate hemostasis of HP for tumor bleeding was achieved in almost 98% of patients, our data show that HP are allow for effective control of tumor bleeding.

To date, no direct comparison between HS and EC is available. When looking across studies rates for achieving primary hemostasis in the upper GI tract with EC and HS have been reported to range between 82%-100%[13,16] and 85%-98%[8,9,11,14,15,18,19,21], respectively. Our study is the first to directly compare the efficacy of HS and EC and no significant different in their hemostatic efficacy and RBR were observed between these two agents. Nevertheless some technical differences between the two HP should be noted: first HS is sprayed at high pressure with a propellant CO_2 cartridge. Such feature might be an advantage in cases of high pressure bleeding or scenarios where a large surface needs to be covered. On the other hand, high-pressure application can potentially cause further tissue injury to the point of perforation especially in friable or inflamed mucosa. Indeed, in two of the patients treated with HS (1.3%), perforation occurred as major adverse events after application of HS in the current study. Occurrence of intestinal perforation after HS application have been reported in other series as well[15,18], therefore some caution of using HS might be necessary. In contrast, with EC the pressure of spraying is much lower, allowing a more sectorial area of targeting, making EC more suitable for localized bleeding lesions like a peptic ulcers or a surface after resection. On the other hand, the area that can be covered with EC might be lower with EC as compared to HS and also high pressure bleeding might be less controlled. However, more systematic studies are clearly needed to investigate on these aspects.

For lower GI bleeding ST and LT success of HP were 75% and 56.3% with a RBR of 37.5%. Data on the role of HP for lower GI bleeding are relatively scarce to date and long-term FU data are completely lacking. In the largest series of low GI bleeding treated with EC, hemostasis was achieved in 83% of the cases with a RBR of 11%[16]. Although limited by the number of patients included in the study, our results do support the concept that HP represent valuable therapeutic options for lower GI bleeding when conventional hemostatic approaches fail.

Limitations of the current study also need to be addressed. Although our study included a large number of patients, its setting in a single high volume university centre might have led to a certain bias in terms of patients characteristics. As shown by the clinical data, a large percentage exhibited a variety of severe co-morbidities and therefore most likely do not represent an average cohort. Further, we did not utilize a randomized study protocol and the decision to apply HS or EC was at the discretion of the endoscopist and therefore subjective.

In conclusion, our study demonstrates that both HPs HS and EC allow for bleeding control with high short-term efficacy when used as primary or salvage therapy. Further, both EC and HS exhibit high efficacy for achieving hemostasis in impaired coagulation status or friable tissues. With these properties, HPs represent powerful and effective additions to the armentarium of the endoscopist for treatment of GI bleeding.

Table 3 Etiology of upper gastrointestinal bleeding and success in bleeding management (short term, long term, re-bleeding rate)

	HS and EC (*n* = 137)	Hemospray (*n* = 102)	Endoclot (*n* = 25)
Reflux esophagitis, *n*	17	16	1
Overall ST, LT, RBR (%)	92, 60, 0	100, 33, 0	100, 0, 0
Primary ST, LT, RR (%)	100, 100, 0	100, 100, 0	0
Salvage ST, LT, RR (%)	100, 100, 0	100, 100, 0	100, 0, 0
OG variceal disease, *n*	13	11	2
Overall ST, LT, RBR (%)	85, 56, 38	91, 50, 45	100, 100, 0
Primary ST, LT, RBR (%)	75, 25, 75	66, 66, 100	100, 100, 0
Salvage ST, LT, RBR (%)	100, 80, 22	100, 80, 25	100, 0, 0
Peptic ulcer disease, *n*	49	34	12
Overall ST, LT, RBR (%)	80, 57, 34	80, 59, 29	84, 50, 31
Primary ST, LT, RBR (%)	79, 67, 21	81, 71, 18	75, 50, 25
Salvage ST, LT, RBR (%)	81, 67, 46	78, 40, 50	90, 62,3 0
Angiodysplasia, -ectasia, *n*	8	6	1
Overall ST, LT, RBR (%)	75, 85, 0	66, 80, 0	100, 100,0
Primary ST, LT, RBR (%)	75, 100, 0	75, 100, 0	0
Salvage ST, LT, RBR (%)	75, 75, 0	50, 50, 0	100, 100, 0
Diffuse bleeding and erosions, *n*	22	16	4
Overall ST, LT, RBR (%)	77, 72, 36	87, 84, 25	66, 66, 33
Primary ST, LT, RBR (%)	78, 67, 33	100, 100, 0	75, 50, 25
Salvage ST, LT, RBR (%)	66, 70, 58	71, 66, 57	100, 50, 50
Cancer bleeding, *n*	15	12	1
Overall ST, LT, RBR (%)	81, 85, 10	85, 92, 10	100, 100, 0
Primary ST, LT, RBR (%)	100, 100, 0	100, 100, 0	100, 100, 0
Salvage ST, LT, RRB (%)	67, 50, 0	67, 75, 17	0
Other bleeding sources, *n*	13	7	4
Overall ST, LT, RBR (%)	70, 70, 40	75, 58, 58	86, 75, 28
Primary ST, LT, RBR (%)	62, 60, 50	50, 43, 62	80, 67, 20
Salvage ST, LT, RBR (%)	77, 69, 36	100, 100, 0	100, 75, 30

Other bleeding sources: Mallory Weiss lesions, aortoduodenal fistula, posttraumatic, bleeding after surgery, anastomosis bleeding. ST: Short term success; LT: Long term success; RBR: Re-bleeding rate; OG: Oesophageal and gastric; HS and EC: Including patients who received both Hemospray and Endoclot at different time points.

Table 4 Clinical characteristics of the patients treated with Hemospray and Endoclot for lower gastrointestinal bleeding *n* (%)

	HS and EC (*n* = 17)	Hemospray (*n* = 9)	Endoclot (*n* = 7)	*P* value
Sex (M)	15	8	6	ns
Age, yr				0.007
mean ± SD	67.8 ± 12.2	72.9 ± 9.2	65.6 ± 9.2	
range	37-81	51-81	37-76	
Application as				ns
Primary therapy	10 (59)	5 (55)	4 (57.1)	
Salvage therapy	7 (41.2)	4 (44)	3 (56)	
Definite therapy after HP failure				ns
Coiling	0	0	0	
Surgery	1 (5.9)	0	1 (14)	
Comorbidities				
Coagulopathy	3 (17.6)	2 (22)	1 (14)	
Renal insufficiency	6 (35.3)	3 (33)	3 (43)	
Hemodialysis	3 (17.6)	2 (22)	1 (14)	
Liver cirrhosis	2 (11.8)	2 (22)	0	
Therapeutic anticoagulation	10 (59)	3 (33)	6 (86)	

Dual antiplatet therapy	1 (5.9)	0	1 (43)	
Vitamin K Antagonists	3 (17.6)	0	3 (43)	
DOAC	3 (17.6)	0	2 (29)	
Antiaggregation therapy	5 (29.4)	2 (22)	3 (43)	
Short term success	12 (79.6)	6 (67)	5 (71)	ns
Primary therapy	7 (70)	3/5 (60)	3/4 (75)	
Salvage therapy	5 (71.4)	3/4 (75)	2/3 (67)	
Long term success	10 (76.9)	6/7 (86)	3/5 (75)	ns
Primary therapy	6 (75)	3/4(75)	2/3 (67)	
Salvage therapy	4 (57.1)	3/3 (100)	1/2 (50)	
Re-bleeding rate	7 (41.2)	3 (33)	4 (57)	ns
Primary therapy	3 (30)	2/5 (40)	1/4 (25)	
Salvage therapy	4 (57.1)	1/4 (25)	3/3 (100)	

DOAC: Direct acting oral anticoagulant; HS: Hemospray; EC: Endoclot; HP: Hemostatic powders; HS and EC: Including patients who received both Hemospray and Endoclot at different time points.

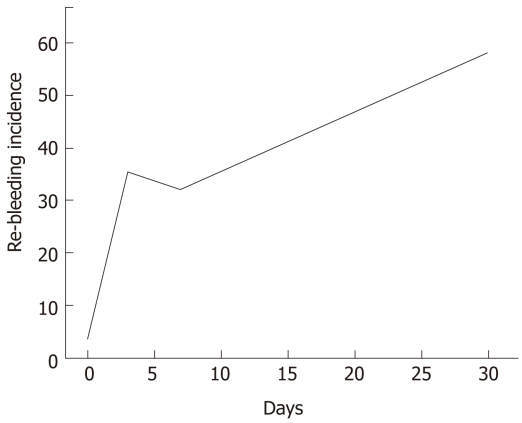

Figure 3 Incidence (%) of re-bleeding after application of hemostatic powder according our data and past studies[8-11,14-16,18,19,21,26].

ARTICLE HIGHLIGHTS

Research background

Gastrointestinal (GI) bleeding frequently leads to hospital admission and is associated with relevant morbidity and mortality, particularly in the elderly. Due to the increasing administration of direct oral anticoagulants in the last years and the emerging role of antiplatelet agents, sufficient and effective treatment of GI bleeding is mandatory while at the same time can be clinically challenging. In the last years, endoscopists increasingly face emergency bleeding in a clinical scenario in which coagulation parameters cannot always be corrected to normal range. Further, with increasing development of advanced endoscopic therapeutic procedures, iatrogenic bleeding after endoscopic resections represents another emerging problem. For refractory cases, hemostatic powders (HP) represent "touch-free" agents.

Research motivation

Although data on the efficacy of Endoclot (EC) are still limited, first clinical evidences suggest that both Hemospray (HS) and EC allow for effective bleeding control. Further, no direct comparison of the efficacy of these two HP is available to date.

Research objectives

Against this background we set off: (1) To analyze the short and long term success in achieving hemostasis with HP; and (2) to directly compare the two agents HS and EC in their efficacy for achieving hemostasis in a large cohort of patients treated for emergency GI bleeding in our center.

Research methods

Data were prospectively collected on patients who were treated with HS and EC for endoscopic hemostasis during emergency endoscopy between September 2013 and September 2017 in our center. Patients were followed-up for at least one month after index endoscopy and data analysis was performed after follow-up was completed

Research results

HP was applied in 154 consecutive patients (mean age 67 years) with GI bleeding in our center. Patients were followed up for at least 1 month (mean follow up: 3.2 mo). The majority of HP applications were in the upper GI tract (89%) with the following bleeding sources: Peptic ulcer disease (35%), esophageal varices (7%), tumor bleeding (11.7%), reflux esophagitis (8.7%), diffuse oozing bleeding and erosions (15.3%). Overall short term (ST) success with HP was achieved in 125 patients (81%) and long term (LT) success in 81 patients (67%). Re-bleeding occurred in 27% of all patients treated with HP. In 72 patients (47%), HP was applied as a salvage hemostatic therapy, here ST and LT success were 81% and 64%, respectively, with re-bleeding in 32% of patients. As a primary hemostatic therapy, ST and LT success were 82% and 69%, respectively, with re-bleeding occurring in 22%. Subgroup analysis showed a ST and LT efficacy for cancer bleeding of 83% and 87%, for peptic ulcer disease of 81% and 56% and in patients under therapeutic anticoagulation of 80% and 60.5%. There was no statistical difference in the ST or LT efficacy between EC and HS for the various indications; however, HS was more frequently applied for upper GI bleeding ($P = 0.04$)

Research conclusions

Within this study, we retrospectively analyzed the hemostatic efficacy of HPs HS and EC as first line or salvage therapy in several clinical scenarios in a large cohort of prospectively included patients. As shown in our report, both HPs allow for excellent ST bleeding control when applied as primary or salvage therapy. At the same time, LT efficacy over a period of 4 weeks is maintained in a considerable amount of patients.

Research perspectives

Both EC and HS exhibit high efficacy for achieving hemostasis in impaired coagulation status or friable tissues. With these properties, HPs represent powerful and effective additions to the armentarium of the endoscopist for treatment of GI bleeding.

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ORIGINAL ARTICLE

Retrospective Study

Performance of tacrolimus in hospitalized patients with steroid-refractory acute severe ulcerative colitis

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Institutional review board statement: The study was reviewed and approved by the Heidelberg University Institutional Review Board.

Informed consent statement: The requirement for informed consent was waived due to the retrospective nature of the study.

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Abstract

BACKGROUND

Acute severe ulcerative colitis unresponsive to systemic steroid treatment is a life-threatening medical condition requiring hospitalization and often colectomy. Despite the increasing choice of medical therapy options for ulcerative colitis, the condition remains a great challenge in the field of inflammatory bowel diseases (IBD). The performance of the calcineurin inhibitor tacrolimus in this clinical setting is insufficiently elucidated.

AIM

To evaluate the short and long-term outcomes of tacrolimus therapy in adult inpatients with steroid-refractory acute severe ulcerative colitis.

METHODS

We conducted a retrospective monocentric study enrolling 22 patients at a tertiary care center for the treatment of IBD. All patients who were admitted to one of the wards of the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital with acute severe ulcerative colitis between 2007 and 2018, and who received oral or intravenous tacrolimus for steroid-refractory disease were included. Baseline characteristics and data on the disease courses were retrieved from entirely computerized patient charts. The primary study endpoint was clinical response to tacrolimus therapy, resulting in discharge from the hospital. Secondary study endpoints were colectomy rate and time to colectomy, achievement of clinical remission under tacrolimus therapy, and the occurrence of side effects.

RESULTS

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In the majority of the 22 included patients (68.2%), tacrolimus therapy was initiated intravenously and subsequently converted to oral administration. The treatment duration was 128 ± 28.5 d (mean ± SEM), and the patients were followed up for 705 ± 110 d after treatment initiation. Among all patients, 86.4% were discharged from the hospital under continued oral tacrolimus therapy. In 36.4% of the patients, the administration of tacrolimus resulted in clinical remission at some point during the treatment. Thirty-two percent of the patients underwent colectomy between 5 and 194 d after the initiation of tacrolimus treatment (mean: 97.4 ± 20.8 d). Colectomy-free survival rates at 1, 3, 6 and 12 mo after the initiation of tacrolimus therapy were 90.9%, 86.4%, 77.3% and 68.2%, respectively. The safety profile of tacrolimus was overall favorable. Only two patients discontinued the treatment due to side effects.

CONCLUSION
The short-term outcome of tacrolimus in steroid-refractory acute severe ulcerative colitis was beneficial, and side effects were rare. In all, tacrolimus therapy appears to be a viable option for short-term treatment of steroid-refractory acute severe ulcerative colitis besides ciclosporin and anti-tumor necrosis factor α treatment.

Key words: Acute severe ulcerative colitis; Steroid-refractory; Tacrolimus; Rescue therapy; Calcineurin inhibitor; Inflammatory bowel disease; Hospitalized

Core tip: Steroid-refractory acute severe ulcerative colitis requires hospitalization and is frequently a risky tightrope walk between surgery and medical treatment. Whereas sufficient data has been provided over time to justify ciclosporin and infliximab as salvage therapies in this clinical scenario, guideline recommendations are still more reluctant towards tacrolimus due to the relative lack of data. However, tacrolimus may have advantages over ciclosporin especially due to its different toxicity profile. Our study provides more insight in the potential of tacrolimus in the strictly defined situation of steroid-refractory acute severe ulcerative colitis in hospitalized patients.

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INTRODUCTION

The global incidence of ulcerative colitis is increasing[1]. Ten to 15% of the patients with ulcerative colitis suffer from an episode of fulminant colitis during the course of their disease[2]. Intravenous corticosteroids remain the first-line therapy for such severe attacks[3]. However, approximately 30% of the patients with acute severe ulcerative colitis respond insufficiently to corticosteroid treatment, which necessitates some type of rescue therapy[4,5]. Conventional salvage therapies to avoid colectomy comprise antibodies against tumor necrosis factor α (TNFα)—typically infliximab and calcineurin inhibitors, *i.e.*, ciclosporin or tacrolimus, both drug classes yielding comparable results[3]. Ciclosporin and tacrolimus are efficient immuno-suppressants widely used in clinical routine to prevent allograft rejection after organ transplantation[6]. Ciclosporin was the first calcineurin inhibitor to be successfully tested in the clinical setting of steroid-refractory acute severe ulcerative colitis[7]. Tacrolimus is a calcineurin inhibitor with a more potent inhibitory effect on activated T cells in comparison with ciclosporin, as tacrolimus influences both ciclosporinsensitive and ciclosporininsensitive T-cell activation pathways[6,8]. Ciclosporin and tacrolimus also display different toxicity profiles[9].

Regarding the treatment of acute severe ulcerative colitis, less is known about tacrolimus therapy than on ciclosporin therapy. To date, two randomized controlled

trials (RCTs) have been published on tacrolimus therapy in steroid-refractory ulcerative colitis: In one, two different serum trough concentrations of tacrolimus were compared to each other (5-10 ng/mL *vs* 10-15 ng/mL)[10]. That trial revealed a dose-dependent effect of tacrolimus; however, it was underpowered for the detection of a significant difference between the two subgroups. Another Japanese trial published by the same group examined oral tacrolimus in the management of hospitalized patients with steroid-refractory ulcerative colitis and demonstrated a clinical response rate of 50% in the tacrolimus group *vs* 13.3% in the placebo group after only two weeks of treatment, while the rate of clinical remission was 9.4% *vs* 0%[11]. Furthermore, several small and heterogeneous retrospective studies have dealt with the use of tacrolimus in severe steroid-resistant ulcerative colitis. For example, in a recently published open-label trial including 100 patients with moderate-to-severe ulcerative colitis, tacrolimus was compared to anti-TNFα treatment. Efficacy and safety data were similar in both groups[12].

This is the basis on which national and international guidelines recommend both ciclosporin or tacrolimus in steroid-refractory acute severe ulcerative colitis, even though the recommendation for ciclosporin is stronger than the one for tacrolimus due to the larger quantity of available data[3,13]. The aim of the present study is to extend the knowledge on the suitability of tacrolimus in steroid-refractory acute severe ulcerative, only considering critically ill patients on the verge of colectomy.

MATERIALS AND METHODS

Study design

This is a retrospective single-center observational study performed at the University Hospital Heidelberg, a tertiary care center in Southwest Germany treating a large number of patients with IBD. The study embraces a time span of 12 years (January 2007 to December 2018). The cut-off time point for data acquisition was 31 December 2018. The study protocol was reviewed and approved by the institutional Ethics Committee (Alte Glockengießerei 11/1, 69115 Heidelberg, Germany; protocol number: S-006/2019). It was performed in accordance with the ethical principles of the 1975 Declaration of Helsinki, as revised in 2000. The requirement for informed consent was waived due to the retrospective nature of the study.

Inclusion and exclusion criteria

The following inclusion criteria were defined: (1) Ascertained diagnosis of ulcerative colitis according to ECCO criteria[3]; (2) endoscopic disease extent of at least Montreal E2 (left-sided colitis)[14]; (3) age of at least 18 years at the time of the initiation of tacrolimus therapy; (4) inpatient treatment at the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital between January 2007 and October 2018; (5) presentation with an acute severe flare of ulcerative colitis according to Truelove and Witts criteria[15]; (6) no or insufficient response to intravenous prednisone or prednisolone according to national guideline recommendations[13]; (7) treatment of the flare with tacrolimus (oral or intravenous application). Exclusion criteria were: (1) Patients who were already scheduled for colectomy at start of tacrolimus therapy; (2) patients in whom the diagnosis of ulcerative colitis was changed to Crohn's colitis in the follow-up after the initiation of tacrolimus therapy; (3) patients with untreated intestinal infections, including *Clostridium difficile* (*C. difficile*), *Campylobacter spp*., and *Cytomegalovirus*.

Definitions

Acute severe ulcerative colitis at admission to the hospital was defined according to the Truelove and Witts criteria[15]. The Truelove and Witts[15] criteria include a stool frequency of ≥ 6 per day, and at least one of the following: Pulse rate > 90 bpm, temperature > 37.8 °C, hemoglobin concentration < 10.5 g/dL, and erythrocyte sedimentation rate (ESR) > 30 mm/h. Steroid-refractoriness was defined as no sufficient clinical response to intravenous treatment with prednisone or prednisolone at a daily dose of 1 mg/kg body weight according to guideline recommendations[3,13].

Clinical response was defined as a significant decrease of stool frequency, rectal bleeding, and plasma C reactive protein (CRP) concentration, as well as an amelioration of general well-being as documented in the patient chart, resulting in the possibility to discharge the patient from the hospital to continue the therapy on an outpatient basis. Clinical remission was considered if a Partial Mayo Score of 0 or 1 was documented in the electronic patient chart by the treating physician[16]. Disease extent was categorized according to the Montreal classification based on all available endoscopy reports[14]. Loss to follow-up was considered when the last contact to the

patient (counted from the cut-off time point for data acquisition) was more than two years ago.

Treatment algorithm

Patients with a severe flare of ulcerative colitis were first treated with intravenous corticosteroids according to guideline recommendations[3,13], in case that had not already been performed at a different inpatient facility prior to the referral to our department. Intestinal infections were excluded by sigmoidoscopy and biopsies for *Cytomegalovirus* PCR or immunohistochemistry, and stool cultures for *Salmonella, Campylobacter, Yersinia* and *Shigella spp*. as well as an assay for *C. difficile* toxin. Antibiotics, mainly ciprofloxacin and metronidazole, were applied at the discretion of the treating physician, even without proof of infection, *e.g.*, if translocation of intestinal bacteria was suspected. Intravenous nutritional support was administered in malnourished patients. Intravenous fluid and electrolyte replacement as well as blood transfusions were performed as required.

Steroid-refractoriness was considered if no sufficient clinical response occurred under intravenous treatment with prednisone or prednisolone at a daily dose of 1 mg/kg for at least three d according to guideline recommendations[3,13]. In patients with steroid-refractory disease, the treating physicians' team (always including a senior consultant in gastroenterology with experience in IBD therapy) decided on the basis of disease severity, comorbidities, patient age, prior medications, and patients' wishes which rescue therapy was most appropriate. In most cases, a visceral surgeon was involved in the decision-making process. Tacrolimus has been used as the standard first-line rescue medication of the department in patients with steroid-refractory acute severe ulcerative colitis over the last two decades. It was administered every 12 h, and dosage was adjusted to blood trough levels of 10-15 ng/mL. Intravenous tacrolimus was consistently applied over six hours twice per day *via* a rate-controlled syringe pump, and tacrolimus trough levels were determined shortly before the morning application. The first trough level measurement was performed one or two days after treatment initiation; thereafter, tacrolimus trough concentrations were measured on a daily basis during the hospital stay. After at least four weeks of tacrolimus treatment, the target trough level was decreased to 5-10 ng/mL at the discretion of the treating physician. Where intravenous tacrolimus treatment resulted in improvement of colitis symptoms and the medication was tolerated by the patient, the treatment was continued orally at the discretion of the attending physician. Patients with distinct amelioration of disease activity according to clinical symptoms—including stool frequency, occurrence of bloody stools, abdominal cramps, and fever—were released to outpatient treatment. After the decision to initiate tacrolimus therapy was made, steroid therapy was completely discontinued or tapered off depending on the total duration of steroid treatment. The decision on the introduction of a second immunosuppressive agent during the hospital stay to maintain remission was individualized mainly according to prior therapies and the risk of opportunistic infections.

Study end points

The primary study end point was clinical response to tacrolimus salvage therapy, as defined above. Secondary endpoints were clinical response under tacrolimus therapy, colectomy rate, time to colectomy, and the occurrence of side effects.

Data collection

Names of suitable patients were retrieved from electronically available lists of inpatients of all wards of the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital who were admitted between January 2007 and October 2018. All data were available as entirely electronic patient records in the Hospital Information System. The patient records were monitored until the cut-off time point for data collection on 31 December 2018, or to loss to follow-up. The following data were collected in an Excel spread sheet: Patient age, disease duration at admission to the hospital, disease extent according to the Montreal classification[14], medications for ulcerative colitis at admission and discharge from the hospital, prior nonresponse to biological therapy, endoscopic findings, laboratory findings, number of bowel movements per day, presence and amount of blood in stool, body temperature, necessity of blood transfusions, performance of colectomy and time span between initiation of tacrolimus therapy and colectomy, duration of hospital stay, duration of steroid therapy until start of tacrolimus treatment, total duration of tacrolimus therapy, results of stool cultures and rectal biopsies for *Cytomegalovirus* PCR, suspected side effects of tacrolimus, doses and blood trough levels of tacrolimus during the hospital stay, concomitant medications administered in the ward, subsequent ulcerative colitis therapies, reasons for discontinuation of tacrolimus

therapy, and disease course after discharge from the hospital.

Statistical analysis

This is a descriptive study. Categorical variables are presented as frequencies and percentages. For numerical variables, means ± standard errors of the mean (SEM) were calculated. A Kaplan-Meier survival plot was applied to illustrate cumulative colectomy-free survival. Statistical analyses were performed with Microsoft Excel 2010 and IBM SPSS Statistics 25 (IBM corporation, Armonk, New York, United States).

RESULTS

Patient characteristics

The present study included 22 patients (13 females) who were treated for acute severe ulcerative colitis refractory to steroid treatment in one of the wards of the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital between 2007 and 2018. Figure 1 illustrates in a flowchart how many patients had to be excluded and for which reasons. The demographic characteristics and disease-specific baseline data of the included patients are presented in detail in Table 1. The mean age at first diagnosis of ulcerative colitis was 25.5 ± 5.6 years, and the disease duration at hospitalization was 6.2 ± 1.3 years. Disease extent according to the Montreal classification[14] was mostly extensive colitis (E3). None of the patients had been treated with tacrolimus prior to their hospitalization. Prior failure to anti-TNFα therapy (but not during the hospital stay of interest) had occurred in five patients (22.7%). At admission to the hospital, the patients' mean plasma CRP concentration was 87.5 ± 14.3 mg/L (normal: < 5 mg/L), the body temperature 38.0 ± 0.2 °C, the heart rate 97.2 ± 2.9 bpm, and the number of bowel movements 13.5 ± 1.4 per 24 h.

Follow-up and loss to follow-up

The average time span between the first dose of tacrolimus and the last follow-up visit was 705 ± 110 d (range: 63-1870 d). In total, six patients (27.3%) were lost to follow-up at the cut-off time point for data acquisition. The time to loss to follow-up ranged from 65 to 1557 d (mean: 107 ± 225 d). It was shorter than one year in only one patient.

Data obtained during hospitalization

The average duration of hospitalization was 22.8 ± 4.9 d. Further characteristics of the study cohort during the hospital stay may be viewed in Table 2. Notably, all but one of the patients received empirical systemic antibiotic treatment at admission, mostly intravenous ciprofloxacin plus metronidazole, for suspected septic complications without positive blood cultures. At that, parenteral nutritional support was administered in nine patients (40.9%), and ten patients received at least one blood transfusion during their hospital stay (45.5%).

Tacrolimus dosing and treatment duration

In 15 of the 22 included patients (68.2%), tacrolimus therapy was initiated intravenously, while seven patients (31.8%) received oral tacrolimus from the start. The initial dose for intravenous tacrolimus was 1.4 ± 0.4 mg/24 h, corresponding to 26 ± 3 μg/kg body weight, while dosage was 5.3 ± 2.2 mg/24 h for oral treatment initiation, corresponding to 95 ± 31 μg/kg body weight. Overall, the target trough concentration of 10-15 ng/ml was reached after 3.1 ± 0.8 d. The time until achievement of target trough concentration was longer for the oral than for the intravenous treatment scheme (4.2 ± 1.2 *vs* 3.1 ± 0.4 d, n = 21, as one patient discontinued the therapy due to side effects on day 2). The mean oral tacrolimus dose per 24 h at the time of discharge from the hospital (n = 19) was 10.2 ± 1.1 mg (equaling 186 ± 23 μg/kg body weight). The mean duration of intravenous tacrolimus treatment was 4.0 ± 0.9 d. The total duration of tacrolimus treatment during hospitalization was 15.9 ± 3.4 d. The mean total duration of tacrolimus therapy (duration of inpatient treatment plus duration of outpatient treatment) was 128 ± 28.5 d.

Concomitant colitis-specific medications

At hospital admission, 15 patients (68.2%) were already undergoing oral systemic steroid therapy with prednisone or prednisolone, one patient was on infliximab, and one patient on azathioprine. In all, 12 patients (54.5%) received oral mesalamine and three patients (13.6%) oral budesonide to treat IBD. As part of the therapeutic concept of using tacrolimus as a bridge to a less toxic maintenance therapy, five patients (22.7%) were started on vedolizumab while hospitalized, while thiopurine therapy was introduced in five patients (22.7%). None of the patients was administered a TNFα antibody during inpatient treatment.

Table 1 Demographic and disease-specific baseline characteristics of the 22 included patients

Characteristic	*n* = 22
Gender, *n* (m/f)	9/13
Age at admission (yr, mean ± SEM)	33.2 ± 7.1 (range: 18-66)
Age at first diagnosis (yr, mean ± SEM)	25.5 ± 5.6 (*n* = 21, uk in 1) (range: 14-58)
Disease duration at admission (yr, mean ± SEM)	6.2 ± 1.3 (*n* = 21, uk in 1) (range: 0-19)
Disease extent according to Montreal classification at admission, *n* (E2:E3)	4:18
Previous anti-TNFα therapy failure, *n* (%)	5/22 (22.7)
Previous thiopurine therapy, *n* (%)	9/22 (40.9)
Systemic steroid therapy at admission, *n* (%)	15/22 (68.2)
Oral mesalamine at admission, *n* (%)	15/22 (68.2)
Anti-TNFα therapy at admission, *n* (%)	1/22 (4.5) (third infliximab infusion had been applied 23 d prior to admission)
Thiopurine therapy at admission, *n* (%)	1/22 (4.5) (on azathioprine for 32 mo prior to admission)
Body mass index (BMI) at admission (kg/m^2, mean ± SEM)	20.3 ± 4.3 (range: 12.1-26.8)
Body temperature at admission (°C, mean ± SEM)	38.0 ± 0.2 (range: 36.6-39.6)
Heart rate at admission (beats per minute, mean ± SEM)	97.2 ± 2.9 (range: 80-135)
Number of bowel movements per 24 h at admission (mean ± SEM)	13.5 ± 1.4 (range: 7-30)
Presence of bloody stools at admission, *n* (%)	22/22 (100)
Plasma CRP concentration at hospital admission (mg/L, mean ± SEM)	87.5 ± 14.3 (range: 2.0-310.4)
WBC count at admission (/nL, mean ± SEM)	12.6 ± 1.0 (range: 4.4-22.8)
Platelet count at admission (/nL, mean ± SEM)	453 ± 29 (232-724)
Blood hemoglobin concentration at admission (g/dL, mean ± SEM)	10.8 ± 0.3 (7.7-14.5)
Endoscopic Mayo score at admission, *n* (Mayo 2:Mayo 3) (sigmoidoscopy)	7:15

n: Number; m: Male; f: Female; SEM: Standard error of the mean; TNF: Tumor necrosis factor; CRP: C-reactive protein; uk: Unknown; WBC: White blood cell.

Short-term efficacy of tacrolimus

All but three patients (86.4%) were discharged from the hospital under continued oral tacrolimus treatment. Distinct primary treatment failure of tacrolimus despite achievement of target trough levels was observed in two patients (9.1%), resulting in their direct transfer to the surgery department for subtotal colectomy. In one patient, tacrolimus was discontinued after two days due to severe vomiting. Clear clinical response to tacrolimus indicated by a reduction of stool frequency and a reduction or disappearance of blood in stool was documented in 18 patients (81.8%). One patient was discharged from the hospital on her own urgent wish even though distinct clinical response to tacrolimus had not occurred. In that patient, the therapy was changed to adalimumab after discharge from the hospital, and she achieved clinical remission under that therapy. Six patients (27.3%) achieved complete clinical remission at some point during their tacrolimus therapy which was attributable to the calcineurin inhibitor and not to any concomitant medication.

Directly prior to the first administration of tacrolimus, the mean plasma CRP concentration was 87.5 ± 12.2 mg/L, and it decreased to 24.3 ± 10.5 mg/L at discharge from the hospital (*n* = 20, the two patients who were transferred to the surgery department were excluded). It was 51.5 ± 11.4 mg/L at day 5 of tacrolimus therapy and 42.9 ± 11.8 mg/L at day 7 of tacrolimus therapy. The occurrence of blood in stool was documented in 100% of the patients at admission to the hospital, while blood in stool was documented in 11/20 (55%) patients at discharge from the hospital. The mean stool frequency was 13.5 ± 1.4 at admission (*n* = 22) and decreased to 5.4 ± 0.6 at discharge (*n* = 20). The mean body temperature at admission was 38.0 ± 0.2 °C at admission, decreasing to 36.9 ± 0.1 °C at discharge from the hospital.

Reasons for discontinuation of tacrolimus therapy

The most prevalent event (36% of cases) resulting in discontinuation of tacrolimus therapy in this study was medium-term treatment failure after discharge from the hospital, including inadequate response and secondary treatment failure. In 27% of the patients, tacrolimus was stopped after initial response and after introduction of an overlapping immunosuppressive therapy with azathioprine or vedolizumab in order to find out whether the immunosuppressant intended for maintenance therapy was

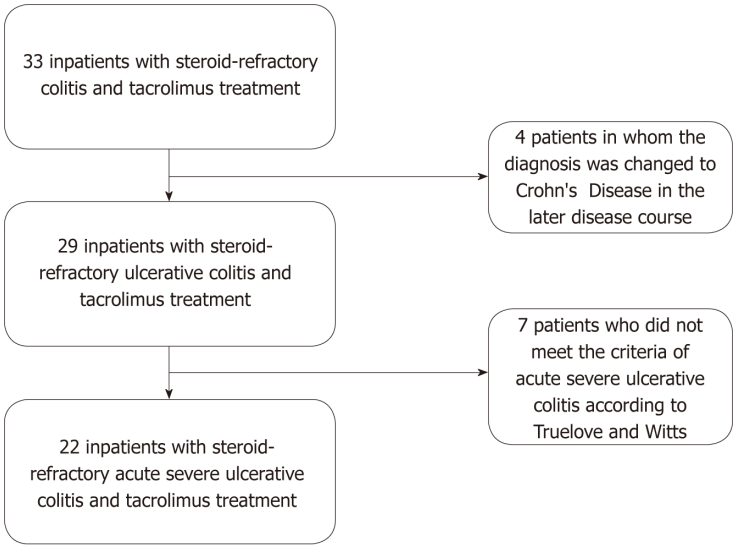

Figure 1 Flow diagram for patient inclusion and exclusion. Finally, 22 patients who met the inclusion criteria were enrolled in the study.

successful as monotherapy. The whole spectrum of reasons for tacrolimus discontinuation is presented in detail in Figure 2.

Colectomy-free survival

Seven among the 22 included patients (31.8%) underwent colectomy for treatment-refractory ulcerative colitis during the follow-up after the initiation of rescue therapy with tacrolimus. The mean time span from the initiation of tacrolimus therapy to surgical intervention was 97.4 ± 20.8 d (range: 5-194 d) (Table 3 and Figure 3). Two patients (9.1%) underwent colectomy within one month of the initiation of tacrolimus therapy, three (13.6%) within three mo, five (22.7%) within six mo, and seven (31.8%) within 12 mo. Vice versa, colectomy-free survival rates at 1, 3, 6 and 12 mo were 90.9%, 86.4%, 77.3% and 68.2%, respectively.

Long-term outcomes including other immunosuppressive medications

At the time of their last follow-up visits at the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital, only three patients were on continued tacrolimus therapy. Two of them were in clinical remission at that time point. In total (independent of whether tacrolimus therapy was ongoing), the outcome of all included 22 patients at their respective last follow-up visits was as follows: 8/22 patients (36.4%) were in clinical remission, and 7/22 patients had undergone colectomy (31.8%); ongoing disease activity was documented in 7/22 patients (31.8%).

Among the eight patients with documented clinical remission at their last follow-up visits, one was under therapy with tacrolimus and oral mesalamine, one under a combination therapy with tacrolimus and vedolizumab (induction with vedolizumab not yet finished), four under infliximab monotherapy, one under adalimumab monotherapy, and one under azathioprine monotherapy.

Among the seven patients with documented disease activity at their last follow-up visits, one was under therapy with tacrolimus and vedolizumab, one under therapy with systemic steroids and vedolizumab, two under systemic steroid treatment alone, one under adalimumab monotherapy, one under azathioprine monotherapy, and one under mesalamine monotherapy.

Outcome of tacrolimus/thiopurine or tacrolimus/vedolizumab combination

Among the five patients in whom vedolizumab therapy was initiated after having achieved clinical response to tacrolimus as a maintenance concept during the hospital stay, three had to discontinue their vedolizumab therapy due to lack of response after the induction had been completed, and all three patients underwent colectomy. Two of these three patients had also failed on anti-TNFα treatment before. One of the patients on tacrolimus and vedolizumab combination therapy had not undergone at least 10 wk of vedolizumab at the cut-off time point for data acquisition, so that the effect of vedolizumab could not be assessed in this patient. In none of the five patients, the combination of tacrolimus and vedolizumab resulted in serious infections. In our study, five patients received additional thiopurine therapy after treatment initiation with tacrolimus: one of them underwent colectomy, three stopped

Table 2 Clinical data obtained during the hospital stay

Variable	*n* = 22
Systemic antibiotic treatment during hospital stay, *n* (%)	21/22 (95.5)
Duration of IV steroid therapy prior to start of tacrolimus therapy (mean ± SEM)	6.7 ± 0.7
Use of parenteral nutrition during hospital stay, *n* (%)	9/22 (40.9)
Blood transfusion during hospital stay, *n* (%)	10/22 (45.5)
Oral mesalamine therapy during hospital stay, *n* (%)	17/22 (77.3)
Stay in intermediate care unit during part of the hospitalization, *n* (%)	4/22 (18.2)
Duration of hospital stay, d (mean ± SEM)	22.8 ± 4.9
Addition of a second immunosuppressive as a maintenance therapy during hospital stay, *n* (%)	10/22 (45.5)
Anti-integrin (vedolizumab)	5/22 (22.7)
Thiopurine (azathioprine or 6-mercaptopurine)	5/22 (22.7)

SEM: Standard error of the mean; IV: Intravenous.

azathioprine therapy for treatment failure and changed to a different regimen, and one discontinued 6-mercaptopurine therapy because of side effects.

Safety of tacrolimus therapy

Only adverse events that were suspected to be caused by tacrolimus were considered. In none of the cases was tacrolimus treatment discontinued because of an infectious complication. No patients died during the follow-up period. The suspected side effects of tacrolimus in our study cohort are listed in detail in Table 4. The most frequently documented side effect was tremor of the limbs, especially of the hands. It was dose-dependent and provoked therapy interruption in none of the cases. It was completely reversible after tacrolimus had been stopped for other reasons. Two patients discontinued tacrolimus therapy because of intolerable suspected side effects. One male ended treatment because of severe nausea and vomiting after a treatment duration of only two days; in that patient, ciclosporin was subsequently tried and discontinued for the same reason. The other had to stop her intake of tacrolimus for anemia and leukopenia after 50 d, when she presented as an outpatient for a follow-up of her disease course. That patient was on treatment with 6-mercaptopurine at the same time, so the side effect cannot be definitely attributed to tacrolimus. We also analyzed glomerular filtration rates determined directly prior to the start of tacrolimus therapy and at discharge from the hospital: they were 114.9 ± 26.4 mL/min *vs* 111.7 ± 25.6 mL/min, arguing against a short-term detrimental effect of tacrolimus on renal function at the high doses that were administered.

DISCUSSION

We performed a retrospective analysis to explore both the short and long-term outcomes of tacrolimus rescue therapy in hospitalized patients with steroid-refractory acute severe ulcerative colitis. Non-response to steroid treatment in ulcerative colitis represents a negative selection concerning other classes of immunosuppressive medications. The key finding of our study is that in this critically ill group of patients, tacrolimus had a very beneficial short-term effect and was able to prevent direct referral to the surgery department for colectomy in the vast majority of cases. However, in the long term, outcome results became more disappointing, as can be best derived from a cumulative colectomy rate of 31.8% at a mean of 97.4 d after the initiation of tacrolimus therapy.

Data on the long-term outcome of tacrolimus in steroid-refractory acute severe ulcerative colitis are overall scarce. Cohort studies on the performance of tacrolimus in the treatment of ulcerative colitis have been published by several other authors, starting in 1998 by Fellermann *et al*[17], who presented a case series of six patients with ulcerative colitis and five with Crohn's disease or indeterminate colitis. The largest published patient series covered 156 patients from five treatment facilities suffering from moderate to severe courses of steroid-refractory ulcerative colitis[18]. In the majority of these studies, tacrolimus was administered orally from the beginning, and study populations were rather non-homogeneous. Also, many of the studies - including the only two RCTs on this subject - originated from Japan, so the number of published data in North America and Europe is limited. Our rationale for adding

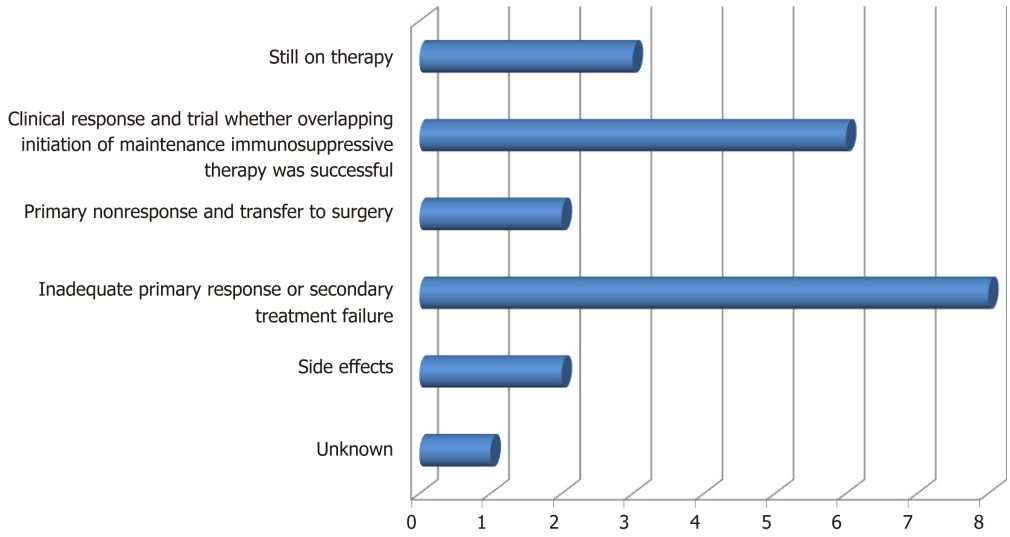

Figure 2 Detailed information on the reasons for which tacrolimus treatment was discontinued.

another study to the body of research on this subject was that published trials on the use of tacrolimus in ulcerative colitis for the most part do not focus on the distinct situation of acute severe ulcerative colitis in the hospital ward setting. However, it is exactly that scenario where calcineurin inhibitors with their advantage of short elimination half-life may have ongoing importance in the treatment algorithm of ulcerative colitis, even if its long-term use is not recommended[13]; treatment options must therefore take into consideration which other - possibly more slowly acting medication - may supplement tacrolimus after its successful initiation[3,13].

The question may be raised why we used tacrolimus as the standard medical salvage therapy in steroid-refractory acute severe ulcerative colitis. At our department, tacrolimus is preferred over ciclosporin in patients who underwent liver transplantation. This choice is made for the following reasons: Liver-transplant patients treated with tacrolimus were less likely to experience acute rejection than those receiving ciclosporin[19]; mortality and graft loss at one year were significantly reduced in tacrolimus-treated liver-transplant recipients[20]; and finally, conversion from ciclosporin to tacrolimus has been shown to improve the cardiovascular risk profile in patients after liver transplantation[21]. Owing to our experience with tacrolimus, which is based on the relatively large number of liver-transplant patients followed up at our department, the administration of tacrolimus to patients with steroid-refractory acute severe ulcerative colitis has become our standard approach over the last one to two decades. The most important reason for the preference of tacrolimus over anti-TNFα was its shorter elimination half-time. Thus, ciclosporin and infliximab were used much less frequently than tacrolimus in steroid-refractory acute severe ulcerative colitis.

The two most prominent features characterizing the present study are the strict inclusion criteria, ensuring a very homogeneous study population, and the long follow-up time with the maximal time span being 5.1 years. According to ECCO guidelines[3], patients with bloody diarrhea ≥ 6/day and any signs of systemic toxicity (pulse > 90/min, temperature > 37.8 °C, hemoglobin < 10.5 g/dL, ESR > 30 mm/h, or CRP > 30 mg/L) have severe colitis and should be admitted to a hospital for intensive treatment. Our study cohort consists exclusively of patients with considerable disease activity, all meeting the criteria by Truelove and Witts[15] for the definition of acute severe ulcerative colitis, necessitating in-ward treatment. The severity of disease in our cohort is illustrated by the large percentages of patients receiving systemic antibiotic treatment, intravenous nutrition support, and blood transfusions, and by the fact that nearly 20% of the patients needed transient intermediate care treatment during their hospitalization. Of note, this is a selection of critically ill patients in whom perpetuating medical therapy may be life-threatening, and timely performed colectomy may be the better alternative.

Despite the severity of disease, our study revealed very good short-term outcomes of tacrolimus therapy in steroid-refractory acute severe ulcerative colitis: As many as 86.4% of the patients were discharged from the hospital with ongoing oral tacrolimus therapy. Overall clinical response was documented in 81.1% of the patients (one patient was released with only slight amelioration of her symptoms on her own urgent request). Clinical remission under tacrolimus therapy not attributed to any

Table 3 Characteristics and outcome of tacrolimus therapy

Variable	*n* = 22
Intravenous initiation of tacrolimus treatment, *n* (%)	15/22 (68.2)
Duration of intravenous tacrolimus therapy (d, mean ± SEM)	4.0 ± 0.9 (range: 2-13)
Duration of tacrolimus therapy until discharge from the hospital or transfer to surgery (d, mean ± SEM)	15.9 ± 3.4 (*n* = 20)
Initial dose of intravenous tacrolimus (mg/24 h, mean ± SEM)	1.4 ± 0.4 (*n* = 15)
Initial dose of intravenous tacrolimus per body weight (μg/kg/24 h, mean ± SEM)	26 ± 3 (*n* = 15)
Initial dose of oral tacrolimus (mg/24 h, mean ± SEM)	5.3 ± 2.2 (*n* = 7)
Initial dose of oral tacrolimus per body weight (μg/kg/24 h, mean ± SEM)	95 ± 31 (*n* = 7)
Time to achievement of target tacrolimus trough level after intravenous treatment initiation (d, mean ± SEM)	3.1 ± 0.4 (*n* = 14)
Time to achievement of target tacrolimus trough level after oral treatment initiation (d, mean ± SEM)	4.2 ± 1.2 (*n* = 7)
Total duration of tacrolimus therapy to end of therapy or last follow-up (d, mean ± SEM)	128 ± 28.5 (range: 2-266)
Patients discharged from the hospital under continued tacrolimus therapy, *n* (%)	19/22 (86.4)
Clinical response to tacrolimus therapy, including remission, *n* (%)	18/22 (81.8)
Clinical remission under tacrolimus therapy, *n* (%)	8/22 (36.4)
Colectomy during follow-up, *n* (%)	7/22 (31.8)
Direct transmission to the surgery department after primary failure of tacrolimus therapy, *n* (%)	2/22 (9.1)
Time from start of tacrolimus therapy to colectomy (d, mean ± SEM)	97.4 ± 20.8 (range: 5-194)

SEM: Standard error of the mean.

other medication occurred in 36.4% of patients. Two patients were already on thiopurine or anti-TNFα (infliximab) therapy, respectively, when they were admitted to the hospital. As they had been on their therapies for 9 wk (infliximab) and 32 mo (azathioprine) when they were admitted to the hospital with acute severe ulcerative colitis, we do not think that their prior therapies interfered with our results of response to tacrolimus therapy.

A meaningful outcome parameter which is routinely used in many studies dealing with the treatment of acute severe ulcerative colitis is the cumulative colectomy-free survival over time after medical treatment initiation. That is why we explored cumulative colectomy-free survival rates at 1, 3, 6 and 12 mo after the first administration of tacrolimus; our data shows 90.9%, 86.4%, 77.3% and 68.2% survival rates, respectively. Of critical note, however, is that the rate of colectomy in our study may have been underestimated due to the loss to follow-up of some patients. However, only one patient was lost to follow-up within one year of the initiation of tacrolimus therapy. A recent meta-analysis on tacrolimus treatment of steroid-refractory acute severe ulcerative colitis revealed colectomy-free survival rates of 86%, 84%, 78% and 69% at 1, 3, 6 and 12 mo[22]. Thus, the colectomy-free survival rates were fairly similar to those we identified in our relatively small study. A recent European prospective randomized controlled multi-center study compared colectomy-free survival rates of patients treated with ciclosporin or infliximab for steroid-refractory acute severe ulcerative colitis[23]. The authors found colectomy-free survival rates after one year of 70.9% for patients initially treated with ciclosporin and of 69.1% for patients initially treated with infliximab. Both treatments thus showed similar efficacy. The one-year colectomy-free survival rate of 68.2% identified for tacrolimus treatment in our study is in the same range and argues against the inferiority of tacrolimus to ciclosporin and infliximab for this indication. It is of note that the risk of colectomy appears to be highest within the first year after initiation of medical salvage therapy, independent of other therapies which may have been introduced subsequently or additionally during the span of the year.

No systematically obtained results have been published on the question of how tacrolimus should best be administered in steroid-refractory acute severe ulcerative colitis. In nearly all published studies on tacrolimus in acute severe ulcerative colitis, tacrolimus was administered orally from the beginning[24]. A potential advantage of initial intravenous treatment is that the target trough level and thus efficacy may be achieved more rapidly than by using oral tacrolimus, keeping in mind that acute severe ulcerative colitis is a highly time-sensitive situation with impending emergency colectomy. Food intake is known to reduce serum levels of tacrolimus due to its low absorption rate[11,25]. In our study, the time until achievement of the target tacrolimus trough level was indeed one day shorter in the intravenously treated subgroup compared to the orally treated subgroup. As far as it can be assessed in the

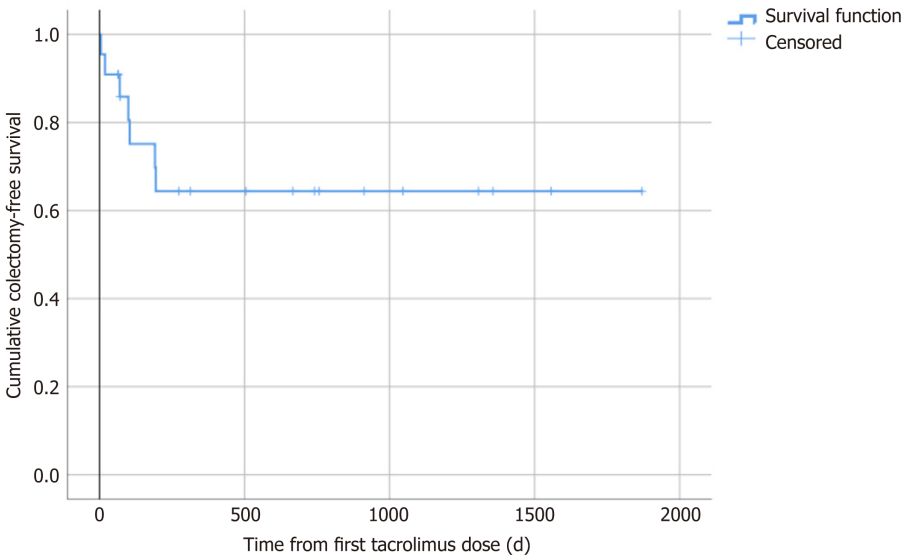

Figure 3 Kaplan-Meier plot of colectomy-free survival after initiation of tacrolimus therapy. Vertical lines in the curve ("censored") demonstrate the duration of follow-up and do not necessarily indicate loss to follow-up.

relatively small subgroups, the prevalence and intensity of side effects did not differ between the intravenous and the oral administration route, so intravenous treatment should be considered, especially if the patient tends to suffer from nausea and vomiting, which may both be further provoked by the oral intake of more medications. Yamamoto *et al*[12] started oral tacrolimus therapy at a dose of 0.1 mg/kg/day and reached the aspired tacrolimus trough concentration of 10-15 ng/mL on day 5. In comparison, we used similar initiation doses and reached the target concentration on day 4. On one hand, it may be favorable to start the therapy at a higher dose, then quickly reduce it later on if the targeted range has been surpassed. On the other hand, the small therapeutic index of tacrolimus may result in severe side effects and possibly premature treatment discontinuation using such an approach.

As soon as patients with steroid-refractory acute severe ulcerative colitis respond to a medical rescue therapy with a calcineurin inhibitor or TNFα antibody, the question remains: how to maintain the response or, ideally–remission, as tacrolimus is not recommended as a long-term maintenance therapy in ulcerative colitis due to its expected long-term toxicity[13]. Indeed, we can unfortunately not add more data on long-term side effects of tacrolimus in the cohort of young people suffering from ulcerative colitis, as according to our standard operating procedure, tacrolimus was only used as a bridging therapy. However, these data may differ from data obtained in patients after organ transplantation who are usually older than the patients described in our study and who are often treated with more than one immunosuppressant concurrently. As for the choice of an additional immunosuppressant for maintenance therapy following successful treatment initiation with tacrolimus, there have been two common options during our study phase: The use of a thiopurine like azathioprine or 6-mercaptopurine, or, more recently, the anti-integrin antibody vedolizumab. This choice has to be made on an individual base taking into consideration patient age, prior therapies, concomitant diseases, potential intolerances and access to outpatient intravenous therapies. Data have been published on both of these two options. For example, Schmidt *et al*[18] conducted a multi-center study examining the role of purine analogues in the long-term outcome of steroid-refractory ulcerative colitis after tacrolimus treatment. In that study, colectomy was performed in 29% (45/156) of patients after a median of 0.5 years from initiation of tacrolimus treatment. One percent of the patients on tacrolimus plus a purine analogue had to discontinue therapy due to adverse events, while 14% of the patients on tacrolimus monotherapy discontinued treatment due to side effects. Among the five patients who were started on azathioprine or 6-mercaptopurine shortly after the initiation of tacrolimus therapy in our study, the concept proved to be successful in none, but no serious infections were documented. The combination of a calcineurin inhibitor and vedolizumab for remission induction and maintenance therapy in steroid-refractory severe ulcerative colitis was addressed in a recent study from France[26]. After a median follow-up period of 11 mo, 11 patients (28%) had undergone colectomy. At 12 mo, 68% of the patients survived without colectomy and 44% survived without vedolizumab discontinuation. Analyzing our small subgroup of five patients

Table 4 Documented suspected side effects of tacrolimus during intravenous and oral treatment

Suspected side effect	*n* = 22
None, *n* (%)	10/22 (45.5)
Treatment discontinuation due to side effects, *n* (%)	2/22 (9.1) (1 due to severe vomiting, 1 due to anemia and leukopenia)
Nausea ± vomiting, *n* (%)	3/22 (13.6)
Stomach pain, *n* (%)	1/22 (4.5)
Headache, *n* (%)	1/22 (4.5)
Tremor, *n* (%)	4/22 (18.2)
Paresthesias, *n* (%)	1/22 (4.5)
Photosensitivity, *n* (%)	1/22 (4.5)
Itching rash, *n* (%)	1/22 (4.5)
Joint or back pain, *n* (%)	1/22 (4.5)
Muscle pain or cramps, *n* (%)	2/22 (9.1)
Temperature intolerance, *n* (%)	3/22 (13.6)
Anemia, leukopenia, *n* (%)	1/22 (4.5)
Loss of hair, *n* (%)	1/22 (4.5)

receiving vedolizumab after tacrolimus initiation, three had to undergo surgery for refractoriness to the anti-integrin antibody, and in two, the final outcome was not clear when they visited the outpatient clinic for the last time.

The adverse events which occurred under the therapy with tacrolimus were mostly mild or moderate. Only two patients stopped the therapy due to adverse events, neither of those a life-threatening situation. These results largely conform to those of other studies on tacrolimus in ulcerative colitis[22]. However, as according to our standard operating procedure–tacrolimus was perceived as a bridging therapy to a different immunosuppressive medication with fewer expected long-term side effects, our study results do not allow for an assessment of long-term toxicity of tacrolimus.

There are several limitations to this study. The main drawbacks are its being restricted to a single treatment center and its retrospective, uncontrolled design. Due to the relatively small number of patients, this study was underpowered to perform regression analyses and thus to identify risk factors for primary treatment failure of tacrolimus in steroid-refractory acute severe ulcerative colitis. Also, we have not treated a sufficient number of patients with infliximab or ciclosporin during the time span of the study, so that a controlled comparison between different treatment groups could not be incorporated in the study. Even though the follow-up rate of this study is satisfactory, considering that the study spans over 12 years, some patients were lost to follow-up, which may have influenced our results, especially those of colectomy rates. Documentation of short-term outcomes was overall very thorough, as the patients were treated in the hospital ward. However, disease scores were not calculated on a routine basis, so they could not be incorporated in the study. Laboratory markers in the blood were determined every day due to the severity of disease and impending colectomy, but stool markers such as lactoferrin and calprotectin were not regularly determined, especially in the first half of the study period, as those measurements had not entered clinical routine at that time. Also, endoscopies were only performed before the start of therapy and not repeated to assess the short-term efficacy of tacrolimus. Due to the retrospective character of the study, the term of "clinical response" was not clearly defined by quantitative parameters or cut-off values and depended much on the assessment by the treating physicians. This is why we chose to also incorporate the possibility to discharge the patient from the hospital into the definition of "clinical response", as this is a relatively "hard" clinical endpoint in the "real world".

Nearly all of the patients were on systemic antibiotic treatment, which was usually started directly upon hospital admission. These interventions were not performed as part of a standard operating procedure for the treatment of acute severe ulcerative colitis, as the use of antibiotics for ulcerative colitis itself is contrversial[27]. Treatment decisions were made at the discretion of the attending physicians' team and reflect the concern of septic complications in this critically ill patient group. However, there are data from studies demonstrating some effects of antibiotics on disease activity in acute severe ulcerative colitis, and these effects may have interfered with our efficacy data of tacrolimus[28]. This potential confounder was probably minor in our study, as: (1) Antibiotic treatment was started before tacrolimus therapy – together with steroid

therapy – and did not obviate the need of tacrolimus use; (2) all but one of the included patients received antibiotics, which ensures homogeneity of the study population, and (3) plasma CRP concentrations were similar at admission and on the day prior to the start of tacrolimus therapy, by which the argument could be made against any significant therapeutic effects of not only the steroids, but also the antibiotics in our cohort.

In conclusions, in a retrospective analysis including 22 inpatients suffering from steroid-refractory acute severe colitis, we found that the vast majority of patients could be discharged from the hospital after introduction of intravenous or oral tacrolimus therapy, while only two patients had to undergo surgery after primary failure of tacrolimus treatment. We conclude that the short-term efficacy of tacrolimus in this situation is very good. However, long-term evaluations revealed that in spite of initial response to tacrolimus therapy, the cumulative colectomy rate after one year for inpatients in the described clinical scenario was as high as 31.8%. It remains to be elucidated whether novel therapeutic options with a potential of rapid efficacy are able to effect the relatively high short- to medium-term colectomy rates observed after hospitalization of ulcerative colitis patients for acute steroid-refractory flares, and how these novel treatment options compare to either calcineurin inhibitors or TNFα antagonists as rescue medications. For future research projects, a direct prospective comparison of ciclosporin and tacrolimus as has already been performed in transplantation medicine would also be interesting in the setting of steroid-refractory acute severe ulcerative colitis.

ARTICLE HIGHLIGHTS

Research background

Steroid-refractory acute severe ulcerative colitis is a life-threatening medical condition requiring hospitalization and frequently emergency colectomy. Although there is a steadily growing choice of medications for ulcerative colitis, the treatment of steroid-refractory acute severe ulcerative colitis continues to be very challenging. Calcineurin inhibitors - mainly ciclosporin and tumor necrosis factor α (TNFα) antagonists have been shown to be viable therapeutic options to avoid colectomy in this scenario.

Research motivation

In contrast to that of ciclosporin, the performance of the calcineurin inhibitor tacrolimus in the clinical setting of steroid-refractory ulcerative colitis is insufficiently elucidated, but nonetheless recommended in national and international treatment guidelines for ulcerative colitis.

Research objectives

The objective of our study was to extend the current knowledge on the use of tacrolimus in steroid-refractory ulcerative colitis by assessing the short- and long-term outcomes of tacrolimus in adult inpatients suffering from steroid-refractory acute severe ulcerative colitis.

Research methods

We conducted a retrospective monocentric study enrolling 22 patients at a tertiary care center for the treatment of inflammatory bowel diseases. All patients who were admitted to one of the wards of the Department of Gastroenterology and Hepatology of the Heidelberg University Hospital with acute severe ulcerative colitis between 2007 and 2018 and who received oral or intravenous tacrolimus for steroid-refractory disease were included. Baseline characteristics and data on the disease courses were obtained from entirely computerized patient charts. The key study endpoints were clinical response to tacrolimus therapy, colectomy rate, time to colectomy and the occurrence of side effects.

Research results

Our study revealed that intravenous or oral tacrolimus, as in previous studies by other authors ciclosporin and infliximab, was able to prevent emergency colectomy in the majority of adult inpatients with steroid-refractory acute severe ulcerative colitis. At the same time, the safety profile of high-dose tacrolimus in this setting was acceptable. However, colectomy rates due to therapy-refractory disease courses over the year following tacrolimus rescue therapy reached nearly one-third of the patients. These results are also comparable to those of other studies dealing with the use of ciclosporin or infliximab in steroid-refractory acute severe ulcerative colitis.

Research conclusions

In all, tacrolimus appears to be a viable option for short-term treatment of steroid-refractory acute severe ulcerative colitis besides ciclosporin and anti-TNFα treatment.

Research perspectives

Even though not recommended for long-term maintenance therapy in ulcerative colitis, tacrolimus is a valuable tool for the short-term treatment of steroid-refractory severe ulcerative colitis, where rapid induction of symptom relief is warranted to gain time for the introduction of

other, more slowly acting substances, with more favorable long-term toxicity profiles. Prospective trials are required to define its role among other medications, and to examine the safety of an overlapping combined use with these medications.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy and complications of argon plasma coagulation for hemorrhagic chronic radiation proctitis

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Abstract

BACKGROUND

Chronic radiation proctitis (CRP) is a complication which occurs in 1%-5% of patients who undergo radiotherapy for pelvic malignancies. Although a wide range of therapeutic modalities are available, there is no literature to date showing any particularly appropriate therapeutic modality for each disease stage. Argon plasma coagulation (APC) is currently recommended as the first-choice treatment for hemorrhagic CRP, however, its indication based on long-term follow-up is still unclear. On the hypothesis that the long-term efficacy and safety of APC are not fully understood, we reviewed APC treatment for patients with hemorrhagic CRP from a single center.

AIM

To assess the long-term efficacy and safety of APC for hemorrhagic CRP.

METHODS

This is a retrospective study of consecutive patients treated with APC for hemorrhagic CRP from January 2013 to October 2017. Demographics, clinical variables, and typical endoscopic features were recorded independently. Success was defined as either cessation of bleeding or only occasional traces of bloody

nature. Patients agreed to undergo treatment by written consent.

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stools with no further treatments for at least 12 mo after the last APC treatment. We performed univariate and multivariate analyses to identify factors associated with success and risk factors for fistulas.

RESULTS

Forty-five patients with a median follow-up period of 24 mo (range: 12-67 mo) were enrolled. Fifteen (33.3%) patients required blood transfusion before APC. Successful treatment with APC was achieved in 31 (68.9%) patients. The mean number of APC sessions was 1.3 (1-3). Multivariate analysis showed that APC failure was independently associated with telangiectasias present on more than 50% of the surface area [odds ratio (OR) = 6.53, 95% confidence interval (CI): 1.09-39.19, $P = 0.04$] and ulcerated area greater than 1 cm^2 (OR = 8.15, 95%CI: 1.63-40.88, $P = 0.01$). Six (13.3%) patients had severe complications involving rectal fistulation. The only factor significantly associated with severe complications was ulcerated area greater than 1 cm^2 ($P = 0.035$).

CONCLUSION

The long-term efficacy of APC for hemorrhagic CRP is uncertain in patients with telangiectasias present on > 50% of the surface area and ulceration > 1 cm^2.

Key words: Argon plasma coagulation; Chronic radiation proctitis; Radiation proctopathy; Efficacy; Safety

Core tip: Argon plasma coagulation (APC) is currently recommended as the first-choice treatment for hemorrhagic chronic radiation proctitis, however, its indication based on long-term follow-up is still unclear. The purpose of this study was to review APC's long-term efficacy and safety. Forty-five patients with a median follow-up period of 24 mo were enrolled. Successful treatment was achieved in 31 (68.9%) patients. APC failure was independently associated with telangiectasias present on > 50% of the surface area and ulceration > 1 cm^2. Six (13.3%) patients experienced severe complications involving rectal fistulation. The only factor significantly associated with severe complications was ulceration > 1 cm^2.

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INTRODUCTION

Chronic radiation proctitis (CRP) is a complication that occurs in 1%-5% of patients who undergo radiotherapy for pelvic malignancies[1]. Hemorrhagic CRP is a syndrome characterized by rectal bleeding, tenesmus, mucus discharge, and fecal incontinence[1,2]. It persists beyond three months after the completion of radiotherapy or begins three months after the initiation of radiotherapy[3]. The underlying pathological mechanisms of CRP are endarteritis obliterans and submucosal fibrosis, which lead to ischemia[4]. Telangiectasias, typical endoscopic findings of hemorrhagic CRP, are considered to be a compensatory mechanism for ischemia[5]. However, these superficial vascular lesions may bleed occasionally or even cause severe rectal bleeding requiring transfusion[6].

Current treatment modalities for hemorrhagic CRP include three main categories: medical, interventional, and surgical. Medical treatments mainly include formalin application[7,8] and sucralfate retention enemas[9,10]. Interventional treatments mainly include endoscopic argon plasma coagulation (APC)[11,12] and hyperbaric oxygen therapy[13,14]. Nonsurgical therapy is preferable to surgical treatment, as the latter may cause high morbidity or mortality[15]. Surgery should be reserved for refractory bleeding or cases complicated by fistulas, abscesses, or strictures. Although a wide range of therapeutic modalities are available, there is no literature to date showing

any particularly appropriate therapeutic modality for each disease stage. APC is currently recommended as the first-choice treatment for hemorrhagic CRP, due to its coagulation depth control, easy accessibility, relatively high effectiveness, and low cost[16,17]. However, the indication of APC for hemorrhagic CRP is still unclear. The purpose of our study was to review the long-term efficacy and safety of APC for hemorrhagic CRP, and to evaluate the prognostic and risk factors.

MATERIALS AND METHODS

Patients

We retrospectively reviewed consecutive patients with hemorrhagic CRP treated with APC between January 2013 and October 2017 at the Sixth Affiliated Hospital of Sun Yat-sen University. The indications for APC were persistent rectal bleeding despite several treatment attempts with various topical agents including sucralfate, almagate, corticosteroids, and 5-aminosalicylic acid enemas. The exclusion criteria for the present study included the following: (1) patients had received treatments other than medical therapy prior to APC, such as APC, formalin irrigation, fecal diversion, and proctectomy; (2) primary tumor residue/relapse or large bowel cancer occurring during follow-up; and (3) patients had causes of rectal bleeding other than CRP. Patients agreed to undergo treatment by written consent. Informed consent for this study was waived due to its retrospective nature, and the study was approved by the Institutional Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University.

APC technique

Patients maintained a clear fluid diet for 24 h before the APC procedure and underwent standard bowel preparation with 2-L polyethylene glycol. Preparation with enemas was performed in a few patients: 8.9% (4/45) for the first APC procedure and 29.4% (5/17) for the latter procedures. Patients received sedation with individual doses of midazolam, pethidine, or propofol if required. A total large bowel evaluation was not essential for patients who had received a complete evaluation previously or who could not tolerate a complete colonoscopy. A front-firing APC probe with a diameter of 2.3 mm was inserted through the working channel of a therapeutic colonoscope (PCF-Q260J, Olympus). If blood or other contaminating material was present, a volume of water was used to rinse the mucosal surface of the affected colorectum to prepare the surface for APC application. An argon flow of 1.0-3.0 L/min at a power of 40-60 W was applied to the lesions in 1-2 s pulses by the endoscopist, while an argon flow of 1.8 L/min at a power of 50 W was routinely adopted. The endoscopist aimed to ablate all the visible telangiectasia that might require multiple endoscopic procedures. During the APC procedure, adequate endoscopic aspiration was required to avoid overdistension with argon gas. Patients received a low or no residue diet for at least one week after the APC procedure. Repeat procedures, if necessary, were often performed at intervals of 3-4 wk.

Definition and follow-up

Follow-up was scheduled through outpatient clinic or by telephone at 6 and 12 mo after the procedure and thereafter at 12-mo intervals. Patients were advised to contact our departments in the event of recurrence of hemorrhage or anemia. Medical records were reviewed retrospectively. Endoscopic severity of CRP was derived from the highly-detailed endoscopic images in combination with the description on the endoscopic reports. CRP was endoscopically characterized according to the system advocated by Zinicola *et al*[18]. In addition, ulceration was an important feature according to the Vienna grading system[19]. Four factors were recorded independently: telangiectasia distribution, the surface area involved, the presence of fresh blood and ulceration (Figure 1).

Success was defined either as cessation of bleeding or only occasional traces of bloody stools with no further treatment for at least 12 mo after the last APC treatment[20].

Statistical analysis

Categorical variables were compared using the $\chi 2$ test or Fisher's exact test. A two-sided P-value < 0.05 was considered significant. We performed univariate and multivariate analyses to identify factors associated with success and risk factors for fistulas. Multivariate models were developed using the enter stepwise method with a removal cutoff of $P = 0.10$. All statistical analyses were performed with SPSS version 20 (SPSS, Inc., Chicago, IL, United States).

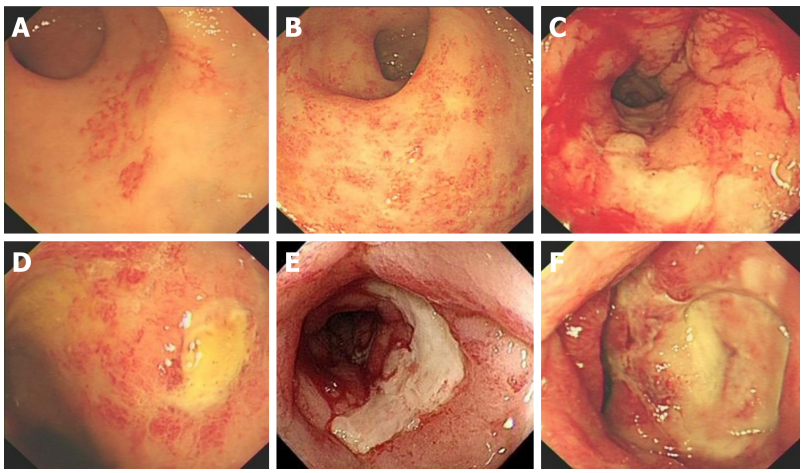

Figure 1 Endoscopic characteristics of hemorrhagic chronic radiation proctitis. A: Telangiectasias present on less than 50% of the surface area; B: Telangiectasias present on more than 50% of the surface area; C: Presence of fresh blood; D: Ulceration < 1 cm^2; E and F: Ulceration > 1 cm^2.

RESULTS

Demographics

Between January 2013 and October 2017, 52 consecutive patients had not received treatments other than medical therapy before undergoing APC treatment for hemorrhagic CRP. After exclusion, a cohort of 45 patients were enrolled in this study. Reasons for exclusion were: (1) the patient was lost to follow-up (n = 6), and (2) the patient had a tumor relapse (n = 1). Approximately 88.9% of patients were treated with radiotherapy for gynecological malignancies, including cervical (n = 39) and vaginal (n = 1) cancers. The remaining five patients had prostate cancer. The median age at the time of the first APC treatment was 60 years (range: 43-88 years). The median duration between cessation of radiotherapy and onset of radiation proctitis was 8 mo (range: 0-78 mo). The median follow-up time from the most recent APC procedure was 24 mo (range: 12-67 mo). The median hemoglobin level at the time of first APC treatment was 10.1 g/dL (2.8-13.1 g/dL), and 15 (33.3%) patients required blood transfusion.

Efficacy of APC

At the time of first APC treatment, 14 (31.1%) patients had extensive telangiectasia distributed more than 10 cm from the anal verge, 26 (57.8%) had more than 50% of the surface area covered by telangiectasias, 29 (64.4%) had fresh blood in the lumen, and 12 (26.7%) had ulceration greater than 1 cm^2 (Table 1).

Successful treatment with APC was achieved in 31 (68.9%) patients. The mean number of APC sessions was 1.3 (1-3). Bleeding was not successfully controlled by APC treatment in the remaining 14 (31.1%) patients. The univariate analysis of clinical and endoscopic variables showed statistically significant associations between APC failure and telangiectasias present on more than 50% of the surface area (P = 0.011) and ulceration greater than 1 cm^2 (P = 0.006). Further multivariate analysis showed that APC failure was independently associated with telangiectasias present on more than 50% of the surface area [odds ratio (OR) = 6.53, 95% confidence interval (CI): 1.09-39.19, P = 0.04] and ulceration greater than 1 cm^2 (OR = 8.15, 95%CI: 1.63-40.88, P = 0.01) .

For all 14 patients for whom APC was unsuccessful, bleeding was successfully controlled after fecal diversion.

Complications

Six (13.3%) patients had severe complications involving rectal fistula formation (including rectovaginal fistula and rectourethral fistula) at 1, 1, 2, 4, 9, and 9 mo after the first treatment session. They were treated with surgical interventions, including fecal diversion (n = 5) and restorative resection with pull-through coloanal anastomosis (n = 1). In the univariate analysis, the only factor significantly associated with severe complications was ulceration greater than 1 cm^2 (P = 0.035) (Table 2).

Table 1 Univariate analysis of factors associated with argon plasma coagulation treatment failure for hemorrhagic chronic radiation proctitis.

Variable	No. of failure/total patients	*P*-value
Distribution of telangiectasias		
Distal rectum (within 10 cm from anal verge)	7/31	0.136[1]
Entire rectum +/− sigmoid (more than 10 cm from anal verge)	7/14	
Surface area covered by telangiectasias		
Less than 50%	2/19	0.011[2]
More than 50%	12/26	
Presence of fresh blood		
No	3/16	0.320[1]
Yes	11/29	
Ulceration		
< 1 cm^2	6/33	0.006[1]
> 1 cm^2	8/12	
Gender		
Female	14/40	0.305[3]
Male	0/5	
Hypertensive		
No	11/39	0.356[3]
Yes	3/6	
Diabetic		
No	11/40	0.166[3]
Yes	3/5	
Abdominal surgery		
No	11/33	0.865[1]
Yes	3/12	
Acute radiation injury		
No	14/39	0.156[3]
Yes	0/6	
Requiring blood transfusions		
No	7/30	0.210[1]
Yes	7/15	
Number of APC		
1	11/33	0.865[1]
≥ 2	3/12	
Age, yr		
< 60	8/22	0.457[2]
≥ 60	6/23	
BMI at the first treatment of APC, kg/m^2		
< 21	7/21	0.763[2]
≥ 21	7/24	
Time from the end of radiotherapy to bleeding, mo		
< 8	6/17	0.637[2]
≥ 8	8/28	
Time from the end of radiotherapy to the first time of APC, mo		
< 14	8/23	0.586[2]
≥ 14	6/22	

[1]Data were calculated using continuity correction;
[2]Data were calculated using the $\chi 2$ test;
[3]Data were calculated using the Fisher exact test.
APC: Argon plasma coagulation; CRP: Chronic radiation proctitis; BMI: Body mass index.

DISCUSSION

Table 2 Univariate analysis of factors associated with argon plasma coagulation treatment complications for hemorrhagic chronic radiation proctitis

Variable	No. of complication/total patients	*P*-value
Distribution of telangiectasias		
Distal rectum (within 10 cm from anal verge)	4/31	1.000[2]
Entire rectum +/− sigmoid (more than 10 cm from anal verge)	2/14	
Surface area covered by telangiectasias		
Less than 50%	2/19	1.000[2]
More than 50%	4/26	
Presence of fresh blood		
No	1/16	0.399[2]
Yes	5/29	
Ulceration		
< 1 cm^2	2/33	0.035[2]
> 1 cm^2	4/12	
Gender		
Female	5/40	0.529[2]
Male	1/5	
Hypertensive		
No	4/39	0.367[1]
Yes	2/6	
Diabetic		
No	4/40	0.125[2]
Yes	2/5	
Abdominal surgery		
No	6/33	0.171[2]
Yes	0/12	
Acute radiation injury		
No	6/39	0.699[1]
Yes	0/6	
Requiring blood transfusions		
No	3/30	0.384[2]
Yes	3/15	
Number of APC		
1	5/33	1.000[2]
≥ 2	1/12	
Age, yr		
< 60	1/22	0.187[2]
≥ 60	5/23	
BMI at the first treatment of APC, kg/m^2		
< 21	3/21	1.000[2]
≥ 21	3/24	
Time from the end of radiotherapy to bleeding, mo		
< 8	2/17	1.000[2]
≥ 8	4/28	
Time from the end of radiotherapy to the first time of APC, mo		
< 14	4/23	0.665[2]
≥ 14	2/22	

[1]Data were calculated using continuity correction;
[2]Data were calculated using the Fisher exact test.
APC: Argon plasma coagulation; CRP: Chronic radiation proctitis; BMI: Body mass index.

Radiation therapy is widely used for pelvic cancer. Although radiation techniques have made substantial advances in delivering more targeted radiation to tumors, as

many as 5% of patients treated with radiotherapy for pelvic cancer will suffer from hemorrhagic CRP. Several treatment modalities, including APC, are strongly recommended by the American Society of Colon and Rectal Surgeons (ASCRS); however, none of them are based on high-quality evidence[17]. APC has been widely reported as an effective and safe modality for the treatment of hemorrhagic CRP; however, reported effectivity and complication rates vary across studies, with effectivity rates from 50% to 100% and complication rates from 0% to 63.6%[21]. Few studies have investigated the prognostic factors and risk factors of APC for the treatment of hemorrhagic CRP. Based on the strict definition of success, which states that the follow-up time should be at least 12 mo from the last treatment of APC, our study confirmed that APC was an effective modality with the complete control of bleeding in 68.9% of all patients. The independent prognostic factors were both endoscopic features prior to APC, including telangiectasias present on more than 50% of surface area and ulceration greater than 1 cm^2. Our study also showed that APC was not a risk-free procedure, with 13.3% of patients developing rectal fistulas. The only risk factor identified was ulceration greater than 1 cm^2.

The efficacy of APC in our study seemed to be less than that observed in some prior studies[22-24]. Two main reasons may contribute to this. First, we defined treatment success on the basis of the long-term effect (at least 12 mo of follow-up from the last treatment of APC). A study in Japan analyzed 64 patients who developed hemorrhagic CRP, with a median follow-up period of 35 mo (range: 12-69 mo) to assess treatment efficacy[25]. In this study, 12 patients received APC therapy, and 5 of them (42%) successfully had their bleeding stopped. Second, 24 (53.3%) patients in our study were categorized as having severe radiation proctitis prior to APC therapy according to the endoscopic severity of hemorrhagic CRP developed by Zinicola *et al*[18]. Half of the patients were treated successfully by APC. The results of this study were consistent with Zinicola *et al*[18], in which only one of the three patients with severe radiation proctitis was treated successfully by APC. The authors concluded that success was not certain for patients with severe hemorrhagic CRP.

Whether endoscopic severity has predictive value in the treatment of hemorrhagic CRP with APC remains controversial. In a series of 50 patients, Swan *et al*[26] found that the endoscopic grade did not predict the likelihood of treatment success. A study by Siow *et al*[22] also suggested that endoscopic grading was not a predictor of the number of APC sessions needed to achieve hemostasis. However, Zinicola *et al*[18] suggested that endoscopic severity could predict the success of APC. The endoscopic score they developed was based on the telangiectasia distribution, surface area covered by telangiectasias, and presence of fresh blood. APC failed in 2 of 14 patients, both of whom had more than 50% of the surface area covered by telangiectasias and fresh blood in the rectum. The authors considered these two factors to be significant in predicting the treatment success of APC. Karamanolis *et al*[20] also confirmed a statistically significant correlation between endoscopic severity and the treatment success of APC. They used a modified 2-grade scale instead of the 3 grades proposed by Zinicola *et al*[18] to assess the endoscopic severity. The modified scale included telangiectasia distribution and surface area covered by telangiectasias. Our study also confirmed that telangiectasias present on more than 50% of surface area was an independent prognostic factor for treatment failure of APC. We did not find a significant correlation between the presence of fresh blood and the treatment failure of APC. Some authors argued that luminal blood might result in an unclear view and prevent adequate telangiectasia ablation, which could reduce the efficacy of APC. During the APC procedure, we used a water pump (OFP-2; Olympus) to rinse away blood or other contaminating material, which might have minimized the adverse impact of the blood. According to the Vienna grading system, ulceration represents a severity feature of radiation proctitis. Goldner *et al*[27] thought that patients who had received high doses at a certain volume could develop histopathological changes such as ulcers in addition to congested mucosa or telangiectasia. Our study found that ulceration greater than 1 cm^2 was another independent prognostic factor. The explanation for this is still unclear. One possible explanation is that ulceration greater than 1 cm^2 indicates more severe disease; another is that the endososcopist might restrict the application of APC in terms of argon flow, power, and time in the case of a large ulceration.

Some gastroenterologists consider APC to be a safe and "risk-free" treatment modality. The major complications reported are mucus discharge, rectal pain, and rectal ulcerations, which are most likely self-limiting and rarely require intervention[24]. Severe complications, including fistulation and stricture formation, were reported to affect approximately 3% of patients in several studies[22]. However, Andreyev *et al* suggested that APC should be used with caution in patients with CRP. They warned that the severe complication rate of APC could reach as high as 26% when used in patients with severe ischemia in the rectum[28]. Weiner *et al*[29] reported

the long-term results of 35 patients who received APC treatment for hemorrhagic CRP, with a median follow-up time of 56 mo (range: 3-112 mo). In their study, two (5.7%) patients developed fistulation, with one mortality. In our study, six (13.3%) patients developed severe complications involving rectal fistulation; however, all of them survived. We further identified ulceration greater than 1 cm^2 as the only risk factor for severe complications. APC is a noncontact thermal coagulation technique, in which the thermal energy is delivered to the superficial blood vessels by ionized gas. The depth of coagulation is limited to approximately 0.5-3 mm; thus, the risk of perforation is generally considered to be low. Rectums with ulcers developing after pelvic radiation could be considered as having fragile, ischemic, and poor healing tissue. This may partly explain why patients with large ulceration have a higher risk of developing fistulation.

There is no consensus on the optimal APC settings for hemorrhagic CRP. A recent systematic review summarized different APC settings in 32 trials, with the electric power ranging from 25 W to 80 W (median 50 W), and the argon flow ranging from 0.6 L/min to 3.0 L/min (median 1.5 L/min)[21]. In one study, the optimal APC settings were determined by using a swine rectum at an argon flow of 1.2 L/min and a power of 40 W with application to the lesions in 2-s pulse. Sato *et al*[30] concluded that this setting was sufficient to ablate telangiectasia but did not damage the muscle layer. However, the review showed that there was no difference in the corresponding complications rates (0%-63.6% *vs* 0%-58.1%) between two electric power settings (50-80 W *vs* 30-50 W, respectively). In addition, four studies using a current of 60 W and an argon flow of > 1.5 L/min reported complications rates of 0%, 0%, 13.3%, and 35.7%. Peng *et al*[21] noted that APC settings seemed to have no correlation with complication rates. In our study, an argon flow of 1.0-3.0 L/min at a power of 40-60 W with application to the lesions in 1-2 -s pulses was determined by the endoscopist, while an argon flow of 1.8 L/min at a power of 50 W was routinely adopted. This is consistent with the guidelines advocated by ASCRS.

The present study was limited by its retrospective design and a relatively small number of cases. Further large-cohort prospective studies are therefore required to confirm our findings.

In conclusion, the long-term efficacy of APC for hemorrhagic CRP is uncertain in patients with telangiectasias present on more than 50% of the surface area and ulcerated area greater than 1 cm^2. Ulcerated area greater than 1 cm^2 is also a risk factor for severe complications.

ARTICLE HIGHLIGHTS

Research background

Radiotherapy is widely used in the treatment of pelvic malignancies. Hemorrhagic chronic radiation proctitis (CRP) is one of the most concerning complication that occurs in 1%-5% of patients who received pelvic radiotherapy for cancer. Current treatment modalities for hemorrhagic CRP include three main categories: medical, interventional, and surgical. Although a wide range of therapeutic modalities are available, there is no literature to date showing any particularly appropriate therapeutic modality for each disease stage.

Research motivation

Argon plasma coagulation (APC) is currently recommended as the first-choice treatment for hemorrhagic CRP, due to its coagulation depth control, easy accessibility, relatively high effectiveness, and low cost. However, its indication based on long-term follow-up is still unclear.

Research objectives

This study aimed to review the long-term efficacy and safety of APC for hemorrhagic CRP, and to evaluate the prognostic and risk factors.

Research methods

We retrospectively analyzed demographics, clinical and endoscopic characteristics, and long-term outcomes of consecutive patients who had received APC treatment for hemorrhagic CRP from January 2013 to October 2017. Success was defined as either cessation of bleeding or only occasional traces of bloody stools with no further treatments for at least 12 mo after the last APC treatment.

Research results

This study enrolled 45 patients with a median 24-mo follow-up period (range: 12-67 mo), 33.3% of whom required blood transfusion before APC. The success rate was 68.9%, with the mean number of APC sessions being 1.3 (1-3). This study showed that telangiectasias present on more than 50% of the surface area [odds ratio (OR) = 6.53, 95% confidence interval (CI): 1.09-39.19, P = 0.04] and ulcerated area greater than 1 cm^2 (OR = 8.15, 95%CI: 1.63-40.88, P = 0.01) were poor prognostic indicators for APC treatment of hemorrhagic CRP. Six (13.3%) patients had severe

complications involving rectal fistulation. The only risk factor for severe complications was ulcerated area greater than 1 cm^2 (P = 0.035). Further large-cohort prospective studies are required to confirm our findings.

Research conclusions

Endoscopic severity could predict the success of APC. The long-term efficacy of APC for hemorrhagic CRP is uncertain in patients with telangiectasias present on more than 50% of the surface area and ulcerated area greater than 1 cm^2. APC is not a "risk-free" treatment modality. Ulcerated area greater than 1 cm^2 is also a risk factor for severe complications.

Research perspectives

Although APC is currently recommended as the first-choice treatment for hemorrhagic CRP, its long-term efficacy and safety are still not well understood. Our study showed that endoscopic characteristics could predict the success and severe complications of APC. Prospective, multicenter, large-scale studies involving different APC settings ought to be conducted in the follow-up research.

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META-ANALYSIS

Systematic review with meta-analysis on transplantation for alcohol-related liver disease: Very low evidence of improved outcomes

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Abstract

BACKGROUND

Alcohol-related liver disease (ALD) is a leading cause of liver failure and indication for liver transplantation that arises in the setting of alcohol use disorder (AUD). Previous reviews of transplantation for ALD are limited in scope of outcomes and type of ALD studied. A comprehensive systematic review could improve use of transplantation in ALD and improve future research. We hypothesize that while transplanting ALD may improve mortality and relapse, findings will be limited by pre-specified causes of heterogeneity - assessment and treatment of AUD, definition of ALD, spectrum of ALD studied, assessment and rates of relapse, and study quality and bias.

AIM

To optimize liver transplantation for ALD, understanding existing research to guide future research, we conducted a systematic review with meta-analysis.

METHODS

We conducted a systematic review, comparing liver transplant to no-transplant in patients with ALD, with a primary outcome of both short- and long-term mortality and relapse. We performed a comprehensive search of MEDLINE, EMBASE, Web of Science, and The Cochrane Library databases for peer-reviewed journal articles comparing use of liver transplant in ALD to no-transplant. Two reviewers independently conducted screening, full text review, and data

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extraction according to the PRISMA guidelines. We report the quality of the evidence according to the GRADE criteria.

RESULTS

We analyzed data from 10 studies. Of 1332 participants, 34.2% (456/1332) had undergone liver transplantation, while 65.8% (876/1332) had not. While random effects meta-analysis suggested transplant in comparison to no-transplant had an association of reduced mortality that did not reach statistical significance, relative risk (RR) = 0.51 (0.25-1.05), but not relapse risk, RR = 0.52 (0.18-1.53), significant heterogeneity limited these findings. When restricted to prospective data, transplant compared to no-transplant significantly reduced mortality, RR = 0.25 (0.13-0.46, $P < 0.01$), and relapse, RR = 0.25 (0.14-0.45, $P < 0.01$), with insignificant heterogeneity but persistent small-study effects. The overall quality of the evidence was Very Low. Heterogeneity analysis suggested that AUD assessment and treatment was often not reported while ALD, relapse assessment and rate, and data collection were institutionally rather than standardly defined.

CONCLUSION

Systematic review of liver transplantation for ALD suggests reduced mortality and relapse in heterogeneous, institution-specific populations with inherent bias. To understand efficacy of transplanting ALD, our research approach must change.

Key words: Alcohol-related hepatitis; Alcohol-related cirrhosis; Alcohol use disorder; Liver transplantation; Standardization

Core tip: Our findings suggest the dearth of well-published literature on transplantation in alcohol-related liver disease (ALD) and the urgent need for rigorous standardization in studying ALD. Such standardization would enable global scale assessment on the efficacy of transplanting ALD. Standardization should include addressing the presence and treatment of alcohol use disorder, the clinical definition of ALD, reporting the spectrum of the population studied (acute, chronic, acute on chronic, hepatocellular carcinoma in the setting of ALD), data collection, and definition and detection of relapse.

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INTRODUCTION

Alcohol-related liver disease (ALD) is a leading cause of liver failure in the United States that arises in the setting of alcohol use disorder (AUD)[1-3]. Recent studies report a rising prevalence on the transplant waiting list and among privately insured persons[1,2]. Additionally, population studies suggest rising ALD death rates, and most recently, ALD was found to have the greatest risk for death among gastrointestinal diseases with a rate of 6.8 per 100000[4-6]. Treatment options for ALD are limited and at the minimum involve treating the underlying AUD, possibly in combination with liver transplantation. Given the increasing burden of disease and mortality in the setting of a profound shortage of donor organs, it is imperative to understand our current use of transplantation in the ALD population in order to optimize care and future research.

ALD occurs on a pathological spectrum and assessment of transplant use within ALD is limited. ALD ranges from asymptomatic steatosis to symptomatic cirrhosis and its complications[7], and this process can be categorized into acute alcohol-related hepatitis (AH), severe alcohol-related hepatitis (SAH), chronic ALD, acute-on-chronic ALD, and hepatocellular carcinoma due to ALD[7]. While a prior published systematic review with meta-analysis investigated alcohol relapse as primary outcome and 6-mo

mortality as a secondary outcome, this review has significant limitations that restrict clinical applicability[8]. The limitations include the narrow inclusion criteria of observational studies focused in AH, the inclusion of studies with a lack of comparator (potentially biasing the results in favor transplantation), the failure to extract or comment on AUD, the focus on short-term 6-mo mortality outcome, ambiguity if the PRISMA guidelines were followed, failure to report the overall quality of the evidence using GRADE (which is different than bias assessment), and limiting assessment of heterogeneity to removal of studies without "stringent criteria for selecting candidates for liver transplantation"[8]. Moreover, the review did not include data extraction of factors associated with abstinence, which were reported in a prior systematic review published by McCallum *et al*[9] - social stability, no nuclear family history of alcohol disease, older age, no prior rehabilitation treatment failure, no co-existing psychiatric problem[8,9]. Exploration of heterogeneity causes and use of pre-specified control for heterogeneity is necessary to accurately study clinical efficacy of liver transplantation.

Overall, a more comprehensive systematic review of the literature, thoroughly assessing both long-term outcomes and pre-specified causes of heterogeneity to identify best practices, was needed to improve care, establish future research priorities, in particular related to the use of transplant, in the context of underlying AUD and in the broader ALD population, and inform clinical practice guidance documents. We hypothesized that the literature would be limited by a lack of standardization of terminology, and that the use of transplantation in the ALD population would also be highly variable. To assess this, we systematically reviewed the use of transplantation in all forms of ALD, including all studies comparing transplant to no-transplant and investigating short- and long-term outcomes. All ALD populations were included without restriction. Placement of the cohort on the ALD disease spectrum, assessment of underlying AUD and treatment, definition of ALD and relapse, assessment of relapse, reporting of data associated with abstinence, and study quality and bias were collected. By systematically reviewing the published literature, in particular the definitions used to assess the ALD population and outcomes, we aimed to fully characterize any heterogeneity, with the goal of assessing and combating bias to optimize and standardize care when considering patients with ALD for transplant, allowing best use of a limited resource.

MATERIALS AND METHODS

This study was constructed using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines[10]. Accordingly, a protocol was registered in PROSPERO, an international prospective register of systematic reviews (Registration #: CRD42017016195; URL: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=16195), and the PRISMA checklist was submitted with our manuscript.

Data sources and searches

Electronic searches: A comprehensive literature search identifying studies investigating transplant compared to no-transplant for ALD was conducted. The initial search was performed on February 3, 2017 *via* Ovid MEDLINE® and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, and Daily and Versions®. Follow-up searches via Ovid EMBASE (1974 to present); Web of Science (Core Collection); and The Cochrane Library (Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials (CENTRAL), Cochrane Methodology Register, Technology Assessments (HTA)) were run on February 22, 2017. Search terms included all subject headings and/or keywords associated with "Alcoholic Liver Disease", "Liver Transplantation", "Survival Rate", "Mortality", "Treatment Outcome", "Prognosis", "Recurrence", "Recidivism", "Alcohol Drinking", "Patient Compliance", "Temperance", "Alcohol Abstinence", and "Alcohol Cessation". There were no language, publication date, or article-type restrictions implemented. These searches were re-run on February 28, 2018 to capture potentially relevant articles published after our initial search. The full OVID Medline Search strategy is available in the supporting information.

Study selection

Types of studies: Randomized controlled trials, observational, and case-control studies investigating adults with ALD comparing those with and without transplant.

Types of participants: Adults aged ≥ 18 years with ALD.

Types of interventions: Use of liver transplant (intervention) in comparison to no-transplant (control).

Types of outcome measures: Primary outcomes assessed short-term (≤ 6 mo) and long-term (> 6 mo) mortality and rate of alcohol relapse. Secondary outcomes included adverse events such as graft dysfunction and/or failure, bacterial infection (ascites, pulmonary, urinary, bacteremia, other), hepatorenal syndrome, gastrointestinal bleeding, and mechanical ventilation.

Data extraction and quality assessment

Selection of studies: After excluding duplicates, two researchers (Londono C and Gold S) independently screened titles and abstracts. An independent, third investigator (NTS) resolved any conflicts. All articles were reviewed against pre-defined inclusion criteria. Employing the same process, articles underwent full-text review, and those meeting inclusion criteria moved on to data extraction.

Data extraction and management: Two investigators (Londono C and Gold S) independently extracted data using a standardized form separating the cohorts into transplant and no-transplant. Patients on the waitlist at time of data reporting were excluded from the analysis in order to not bias the results. Extracted data included trial design and methodology (assessment and treatment of AUD, definition of the diagnosis of ALD, required period of abstinence prior to transplant, definition of relapse, recognition of the presence of AH or SAH), patient demographics (age, sex, ethnicity, Child-Turcotte-Pugh (CTP) score, model for end-stage liver disease (MELD) score, history of prior alcohol-related decompensating events, medical management (pentoxifylline or steroids), Maddrey's discriminant function, labs on presentation (bilirubin, prothrombin time), Lille score at 7 d, length of abstinence in months (pre-transplant where applicable), time to transplant listing, loss to follow-up, and length of follow-up), and primary and secondary outcomes. Where data was missing or unclear, the manuscript corresponding authors were contacted for further information.

Risk of bias and quality assessment: Two investigators (Londono C and Gold S) independently assessed trial risk for bias using the Newcastle-Ottawa Scale for cohort and case-control studies and the Cochrane Risk of Bias tool for randomized controlled trials[11,12]. The Newcastle Ottawa Scale assesses for bias using a star system across 3 categories - selection, comparability, and exposure[11]. The Cochrane Risk of Bias tool assesses for bias, categorizing the risk as high, low, or unclear across the following components: selection (randomization, allocation concealment), performance (blinding of participants and personnel), detection (blinding of outcome assessment), attrition (incomplete outcome data), reporting (selective reporting), and other (funding, *etc*)[12]. Studies lacking the maximum stars available across categories using the Newcastle Ottawa Scale or with low or unclear risk of bias according to the Cochrane Risk of Bias tool were considered to be at risk for bias. A third investigator (NTS) resolved disagreements. The overall quality of the evidence was evaluated using the GRADE system[13], downgrading based on study design, study limitations, inconsistency of results, indirectness of evidence, imprecision, and reporting bias.

Data synthesis and analysis

Statistical analysis: Studies meeting the inclusion criteria were tabulated. Using random effects meta-analysis method of DerSimonian and Laird, statistically and clinically appropriate studies were combined to calculate a summary relative risk (RR) and 95% confidence interval (CI). Pre-specified subgroup analyses were undertaken to evaluate whether the estimated effect was modified by study design. Using the statistical software Stata, version 14 (StataCorp LP, College Station, TX, United States), data analysis was conducted.

Assessment of heterogeneity: To assess heterogeneity, the *I2* statistic and the chi-squared test were computed[14]. Pre-specified explanations for heterogeneity included: study design, definition of ALD, definition and assessment of relapse, and inclusion criteria.

Assessment of publication bias: Funnel plots and Egger's regression were used to evaluate for publication bias[15-17].

RESULTS

Description of studies

Included studies: Of the 4010 articles screened, 125 underwent full text review and 10 studies (6 prospective, 4 retrospective) met inclusion criteria (Figure 1). These 10 studies included a total of 1332 participants, of which 34.2% (456/1332) underwent liver transplantation and 65.8% (876/1332) did not. Table 1 shows details of the included studies and their transplant and no-transplant groups baseline characteristics, with weighted averages where applicable reported in Table 2. Included studies were performed between 1993 to 2017, with high representation of a French population, 40% (4/10), with later studies more likely to report findings restricted to SAH populations[18,19]. The majority of both transplant and no-transplant populations were men, 80.1% and 64.1%, respectively and had mean weighted ages of 46.7 and 49.6. Data reporting CTP and MELD broken down into transplant and no-transplant cohorts was limited (Tables 1 and 2); the study populations appeared to be CTP class B/C with lower MELD scores in studies not restricted to SAH populations[20,21] in comparison to those restricted to patients with SAH[18,19]. Most studies did not report ethnicity, history of prior alcohol-related decompensating events, use of medications, Maddrey's discriminant function, labs on presentation, or Lille score (data not shown).

Gaps in reporting: Supplementary Table 1 shows individual trial details, highlighting gaps in reporting regarding assessment and treatment of underlying AUD, diagnosis of ALD, requirement of a period of abstinence prior to transplant, details of relapse (definition and assessment), and accounting for or restricting to the presence of AH and or SAH. Two of the studies did not report how ALD was diagnosed[22,23], and the remaining 8 studies used a range of diagnostic criteria - some studies included mixed liver etiology (alcohol and other causes of liver disease)[24]; others defined ALD by alcohol consumption ranging from 50 g/d for both men and women[25], greater than 3 units per day for men or 2 units per day for women[26], or greater than 80 g/d for men or 60 g/d for women[21]; and the most recent studies did not specifically quantify the amount[18,19,27]. The majority of studies did not require pathological diagnosis, specify a required period of abstinence, or differentiate where patients were classified on the spectrum of ALD. Relapse was rarely defined, mostly assessed for by interviews at non-standardized intervals, and only three studies accounted for quantity of alcohol consumed among those with relapse[18,19,26].

Risk of bias in the included studies: Supplementary Table 2 shows the study quality and risk of bias assessment. The randomized control trial[23], the only abstract included in the analysis, had an unclear risk of bias across the different categories. Four of the later studies appeared at substantial risk of bias - the two case-control studies had a higher risk of selection and outcome bias[18,19] while the other two were at risk of comparability bias[21,27]. Two earlier studies were additionally at risk of outcome bias[24,25]. Of the three studies that appeared to have low risk of bias[20,22,26], only one was prospectively conducted[26].

Effects of liver transplantation

Mortality: Random effects meta-analysis suggested a trend toward transplantation reducing mortality risk in comparison to no-transplant (RR = 0.51; 95%CI: 0.25-1.05; P = 0.07), but heterogeneity ($I2$ = 86.7%; P < 0.01) was significant (Figure 2A). This heterogeneity was no longer significant ($I2$ = 4.1%; P = 0.35) and a statistically significant reduction in mortality (RR = 0.25; 95%CI: 0.13-0.46; P < 0.01) was observed when restricting the study population to prospectively collected data (Figure 2B)[18,19,24]. When restricting to studies reporting early mortality, the remaining two studies included prospectively collected data in steroid non-responsive SAH populations[18,19], suggesting significantly reduced 6-mo mortality (RR = 0.30; 95%CI: 0.15-0.58; P < 0.01) with insignificant heterogeneity ($I2$ = 0.0%; P = 0.35). All six studies with mortality data reported presence of AH patients, with three specifying presence of SAH[18,19,25], of which two studied only steroid non-responsive SAH[18,19]. The lack of details of the AH patients within the other studies prevented further meta-analyses. Graphical evidence of publication bias was observed when including all studies (Supplementary Figure 1A) and when restricted to prospectively collected studies (Supplementary Figure 1B), with suggestion of the presence of small-study effects, P = 0.14 and P = 0.07, respectively.

Relapse: Relapse risk in both the transplant and no-transplant cohorts was reported in 4 of the studies[20,22-24], and random effects meta-analysis was not significantly different (RR = 0.52; 95%CI: 0.18-1.53; P = 0.24) with significant heterogeneity ($I2$ = 82.3; P < 0.01) (Figure 3A). None of these studies differentiated between short or long-term relapse. When restricting the analysis to prospectively collected data[23,24], relapse risk significantly decreased for transplant patients (RR = 0.25; 95%CI: 0.14-0.45; P < 0.01) with insignificant heterogeneity ($I2$ = 0.0%; P = 0.61) (Figure 3B). Funnel plot

Table 1 Details of included studies and cohort baseline characteristics

Study	Place	Study design	Transplant					No-transplant				
			n (%)	Age[1] (SD or range)	Male sex *n* (%)	CTP[1] (SD or range)	MELD (SD or range)	*n* (%)	Age[1] (SD or range)	Male sex *n* (%)	CTP[1] (SD or range)	MELD (SD or range)
Gish *et al*[24]	United States	P, C	29 (62)	47[2]	30 (64)[2]	12 (9-15)[2]		14 (30)	47[2]	30 (64)[2]	13 (9-15)[2]	
Anand *et al*[22]	United Kingdom	R, C	39 (28)	48 (37-69)	34 (87)	11[3]		94 (69)	51 (30-71)[3]	67 (74)	11[3]	
Poynard *et al*[25]	France	R, CC	169 (50)	47 (39-55)	NS	9 (5-15)[3]		169 (50)	47 (39-54)	NS	9 (5-15)[3]	
Veldt *et al*[26]	France	P, C	2 (3)	59 (37-82)[2]	47 (64)[2]	11 (10-15)[2]		72 (97)	59 (37-82)[2]	47 (64)[2]	11 (10-15)[2]	
DiMartino *et al*[23]	France	P, RCT	60 (50)	50[2]	92 (77)[2]	8.2[2]		60 (50)	50[2]	92 (77)[2]	8.2[2]	
Immordino *et al*[20]	Germany	R, C	110 (45)	53 (30-68)	95 (86)	10 (6-13)	14 (4-35)	113 (46)	50 (33-64)	64 (57)	6.5 (5-13)	12 (6-40)
Alvarez *et al*[21]	Spain	P, C	5 (3)	< 65	NS	> 8	14 (13-15)[2]	156 (95)	56 (54-58)[2]	135 (82)[2]	9 (8-9)[2]	14 (13-15)[2]
Mathurin *et al*[18]	France	P, CC	26 (27)	47 (35-61)	15 (58)	NS	30 (22-47)	69 (73)	52 (47-54)	41 (59)	NS	NS
Im *et al*[19]	United States	P, CC	9 (10)	41 (30-60)	5 (55)	NS	39 (27-42)	79 (84)	48 (26-68)[2]	54 (57)[2]	NS	31 (16-52)[2]
Onishi *et al*[27]	Japan	R,	7 (7)	44 (28-51)	4 (57)	10.1(2)[2]	NS[4]	50 (75)	52 (31-69)	37 (74)	10.1 (2)[2]	NS[4]

[1]Median values used if both mean and median were reported;
[2]Value is for the entire study population (both transplant and no-transplant);
[3]Calculated as the weighted average;
[4]Mean model for end-stage liver disease reported as 1.9 for the entire study population was omitted given concern for publication error.
P: Prospective; R: Retrospective; C: Cohort; CC: Case-control; RCT: Randomized controlled trial; CTP: Child-Turcotte-Pugh score; MELD: Model for end-stage liver disease; NS: Not specified.

suggested publication bias when including all studies (Supplementary Figure 2A) and when restricted to prospectively collected data (Supplementary Figure 2B). Of the 4 studies, 3 included a mixed population of AH that did not specify SAH and lacked details to allow additional meta-analyses (Supplementary Table 1)[20,22,24].

Adverse events: Four studies specified the occurrence of graft dysfunction and re-transplantation[18,19,25,27]. Of these four, the study with the longest follow-up observed graft dysfunction in two of their 31 transplant patients with relapse (6%), of which one (50%) underwent re-transplant, the other which died (Supplementary Table 3)[25]. Other adverse events including bacterial infection (ascites, pulmonary, urinary, bacteremia, other), hepatorenal syndrome, gastrointestinal bleeding, and mechanical ventilation were only reported in two studies (data not shown).

Quality of the evidence: Using the GRADE system, the quality of the evidence across studies was classified as Very Low for use of transplantation in ALD and relapse risk, with downgrading for study design, risk of bias, inconsistency, imprecision, and publication bias.

DISCUSSION

ALD is a leading indication for liver transplantation[28,29], with increasing prevalence[1] and incidence[30] on the liver transplant waiting list, but our understanding of the utility and application of transplant in this population compared to no-transplant is limited with no prior comprehensive systematic review. Random effects meta-analysis of the currently reported literature supports that transplantation for ALD reduced mortality, but not relapse risk, and significant heterogeneity limited these findings. The overall quality of evidence for both outcomes by GRADE criteria was very low. When analyzing prospectively collected data, transplant in comparison to no-transplant significantly reduced mortality and relapse with insignificant

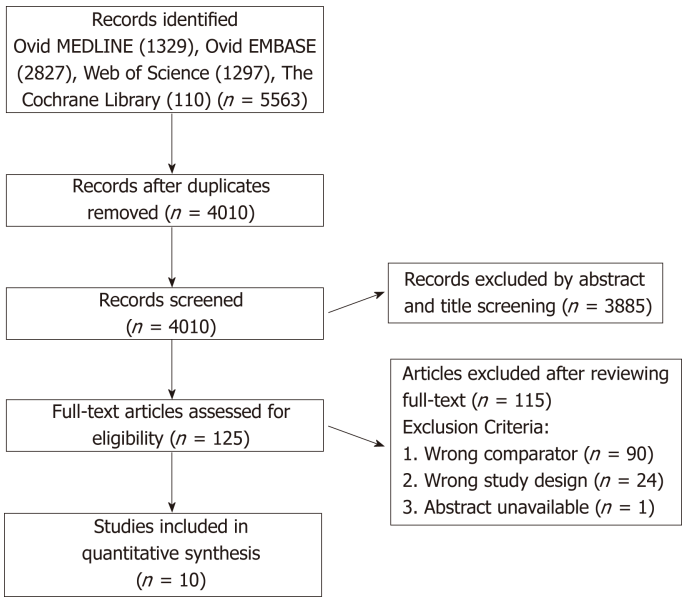


Figure 1 The flow diagram shows search results, studies screened, excluded, and reasons for exclusion or inclusion.

heterogeneity, but the suggestion of small-study effects driving these results persisted. Similar to our findings, significant heterogeneity observed in a prior systematic review focused on transplanted AH patients[8] corrected with restricting analysis to studies with strict candidate selection criteria and SAH. The prior systematic review failed to explore the causes for heterogeneity beyond this corrective maneuver, but in order to improve our study of ALD so that future studies report clear findings that allow larger scale compilation of data, a detailed understanding of prior causes of heterogeneity to allow successful future standardization is necessary.

None of the studies explicitly comment on the severity or treatment of AUD present in their study cohorts, which would impact outcomes of interest, in particular relapse. AUD when diagnosed according to the Diagnostic and Statistical Manual (DSM) 5th edition put forth by the American Psychiatric Association is defined by meeting two of eleven possible criteria over a 12-mo period and further categorized into mild (presence of 2-3 symptoms), moderate (presence of 4-5 symptoms) or severe (presence of 6 or more symptoms) AUD[31]. Characterization of underlying AUD is imperative as prior research suggests that patients with less severe AUD are less likely to relapse[32]. Additionally, effective treatments for AUD include medications and behavioral therapies, and a recent systematic review found that interventions increase abstinence[33]. Overall, this failure of studies to provide detailed information pertaining to underlying AUD severity and treatment likely contributed to heterogeneity, reducing the reproducibility of prior publications.

Furthermore, all of the studies included defined ALD inconsistently. The majority of published research studies rely on clinical history rather than pathology, even when transplant makes explant liver pathology easily available. Additionally, studies use varying alcohol consumption cut-offs, vague terminology such as "alcoholism" without elaboration, and include mixed disease processes (alcohol in combination with another cause of liver disease, *e.g.*, hepatitis C). Similar to findings reported by McCallum *et al*[9], we report that this persistently vague definition of ALD inevitably leads to greater heterogeneity and prevents comparability among studies. Studies also fail to consistently characterize the clinical spectrum of ALD studied lie - acute, chronic, acute on chronic, hepatocellular carcinoma due to ALD - though this spectrum is commonly used in clinical practice[7]. This reduces the clinical applicability and external validity of the reported literature. The current literature investigating the use of transplantation in ALD defines ALD in a manner that not only introduces selection bias into the patients studied to date, but also may affect the study outcomes and raises the question of the presence of country and or transplant center bias in access to transplantation for patients with ALD.

In addition to heterogeneous disease definitions, the current reporting of pre-transplant abstinence and relapse rates in ALD allows significant under-reporting and variability[7,9]. Pre-transplant abstinence in the transplant population was not reported in the majority of studies (Table 3), and though detailed breakdown of relapse appears more clinically useful[34], only 4 of the studies defined relapse, varying from

Table 2 Weighted average of baseline characteristics

	n (%)	Male *n* (%)	Age	CTP	MELD
Transplant	456 (34.2)	153 (80.1)	46.7	9.8	18.4
No-transplant	876 (65.8)	183 (64.1)	49.6	8.7	15.2[1]

[1]Used data from 26 matched controls of Mathurin *et al*[18] trial to calculate weighted average as data for all 69 of the entire cohort was not available. CTP: Child-Turcotte-Pugh score; MELD: Model for end-stage liver disease.

"any consumption" to "slips." Additionally, though patient self-report, interviewing, and biochemical tests of blood, urine, or hair all present with limitations in terms of sensitivity, specificity, cost, and feasibility for monitoring for relapse[35], the reported studies likely suffered from underreporting; the majority of studies used "short" or "random" intervals and relied only on interviews to detect relapse. A standardized protocol for defining and detecting relapse pre- and post-transplant in general practice and in clinical trials is needed.

There appears to be a critical need for standardized data collection tools to capture underlying AUD severity and treatment, spectrum of ALD studied, patient demographics (*e.g.*, race, ethnicity, socioeconomics) and adverse events that may influence mortality and relapse outcomes. While the National Institute on Alcohol Abuse and Alcoholism (NIAAA) made recommendations to help with standardization of the study of AH[36], the focus on AH and lack of incorporation of underlying AUD definitions and diagnoses limit the recommendations. This suggests the need for newer, more comprehensive recommendations, which were recently put forth by Shen *et al*[37], proposing a standardized flow chart approach to patients with ALD and a comprehensive data collection tool.

Limitations and strengths

Not only does the heterogeneity due to lack of standardization limit our ability to fully assess transplantation in ALD, but this systematic review also suggests that the published data is of poor and limited quality - small studies with suggestion of small study effects in analysis, mostly observational or case-control cohort study design with only a single randomized controlled trial in abstract form, under-representation of many countries, and lack of long-term follow-up. Once definitions and data collection are standardized, within the spectrum of AUD and ALD, future multi-center prospective consortia and preferably controlled randomized clinical trials with long-term follow-up should be organized to capture and optimize the use of transplantation in the ALD population.

Our systematic review explores the extensiveness of study heterogeneity, even affecting the definition of ALD and lack of accounting for AUD. Additionally the majority of studies failed to report our pre-specified outcome of adverse events and lacked long-term follow-up to capture graft dysfunction or failure. Similarly, the definition of relapse, monitoring of it, and presence of social support or underlying demographics that might influence it were inconsistently reported. Despite the limitations, our analyses do suggest a short-term mortality benefit for transplantation in at least a subgroup of the ALD population, patients with steroid non-responsive SAH. Overall, the review highlights the need for more detailed studies in the ALD population, particularly the non-SAH.

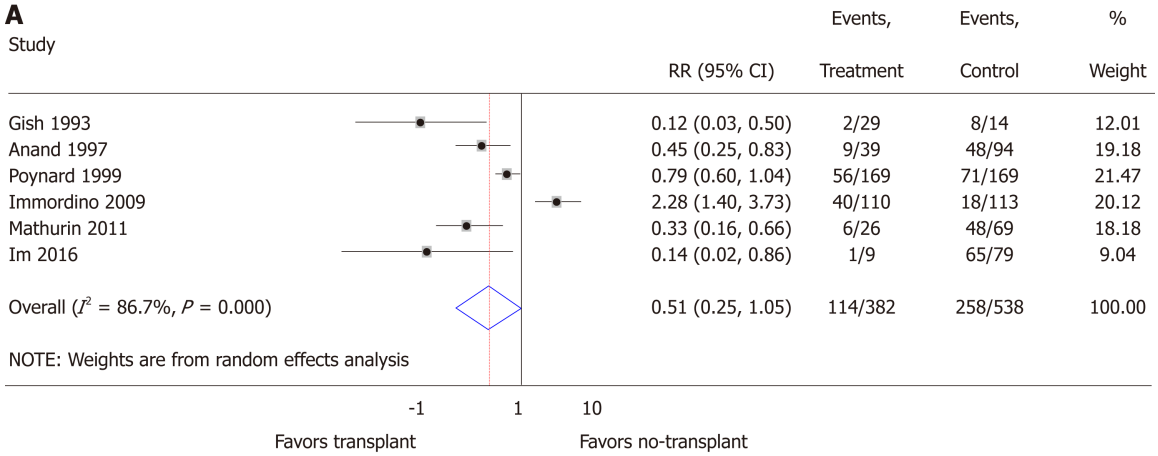
Implications for clinical practice and research

Overall prior literature to date has focused on requirements of pre-transplant abstinence prior to listing and transplanting AH patients, but our systematic review suggests that this focus may be premature. Our findings suggest the urgent need for rigorous standardization in studying ALD, including the presence and treatment of AUD, the clinical definition of ALD, reporting the spectrum of the ALD population studied, data collection, and definition and detection of relapse. Only with such standardization can the needed international, large-scale, randomized controlled trials with long-term follow-up be conducted in a clinically useful manner.

Table 3 Outcomes of included studies

Study	Transplant					No-transplant				
	Abstinence length (SD or range)[1]	Relapse *n* (%)	Mortality *n* (%)	Loss to follow-up *n* (%)	Follow-up length (SD or range)[1]	Abstinence length (SD or range)[1]	Relapse *n* (%)	Mortality *n* (%)	Loss to follow-up *n* (%)	Follow-up length (SD or range)[1]
Gish *et al*[24]	21	6 (21)	2 (7)	0 (0)	24 (12-41)[2]	5	13 (93)	8 (57)	5 (36)	24 (12-41)[2]
Anand *et al*[22]	18 (6-130)	5 (13)	9 (23)	0 (0)	25 (7-63)	11 (2-86)[3]	27 (37)	48 (51)	11 (12)	24 (6-90)
Poynard *et al*[25]	≥ 6[4]	31 (18)	56 (33)	0 (0)	28 (21-37)	NS	NS	71 (42)	NS	NS
Veldt *et al*[26]	≥ 6	NS	NS	NS	NS	NS	NS	NS	NS	NS
DiMartino *et al*[23]	NS	4 (6)	NS	NS	42[2]	NS	13 (21)	NS	NS	42[2]
Immordino *et al*[20]	NS	13 (12)[5]	40 (36)	NS	120	NS	5 (6)	18 (18)	14 (12)	120
Alvarez *et al*[21]	NS	NS	116 (70)[2]	9 (5)[2]	54 (19-96)[2]	NS	NS	116 (70)[2]	9 (5)[2]	54 (19-96)[2]
Mathurin *et al*[18]	NS	3 (12)	6 (23)	NS	24	NS	NS	48 (70)	NS	24
Im *et al*[19]	NS	2 (22)	1 (11)	NS	25 (6-39)	NS	NS	65 (76)[6]	NS	NS
Onishi *et al*[27]	21.2 (17.4)[7]	1 (14)	NS	NS	61.2	8.8 (13.6)	NS	NS	NS	NS

[1]Median values used if both mean and median were reported and length was reported in months;
[2]Value is for the entire study population (both transplant and no-transplant);
[3]Calculated as the weighted average;
[4]All of the patients were abstinent for greater than 6 mo prior to transplant with the exception of one patient;
[5]Only 1 patient of the 8 with acute alcohol-related hepatitis relapsed;
[6]Includes 6 patients on the transplant waiting list;
[7]Includes transplant and waitlist patients. SD: Standard deviation; NS: Not specified.



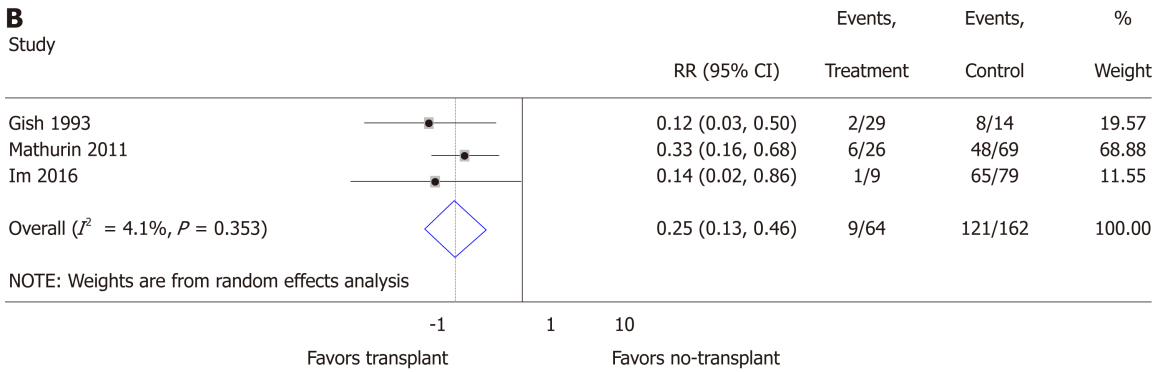


Figure 2 Meta-analysis of overall mortality in patients with alcohol-related liver disease transplant *vs* no-transplant including all studies (A) and only prospective trials (B). CI: Confidence interval.

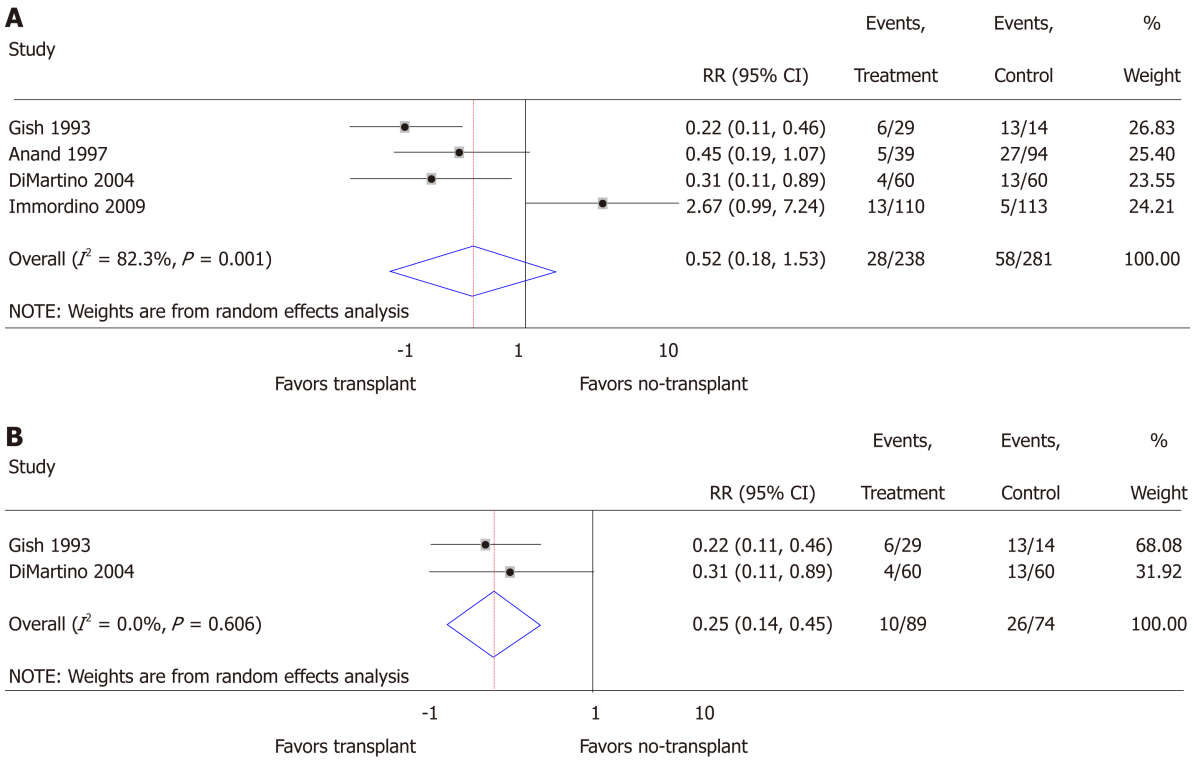


Figure 3 Meta-analysis of relapse in patients with alcohol-related liver disease transplant *vs* no-transplant including all studies (A) and only prospective trials (B). CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Alcohol-related liver disease (ALD) is a leading cause of liver failure and indication for liver transplantation, thus optimizing use of liver transplantation in this patient population is imperative. Systematically reviewing the literature, comparing transplanting ALD to not transplanting ALD is necessary to understand how to optimize use of liver transplantation in ALD and to direct future research.

Research motivation

Systematically reviewing the existing literature on the use of liver transplant compared to no-transplant in patients with ALD could help guide clinical care and future directions of research.

Research objectives

To help inform optimal use of liver transplantation in ALD and understand limitations of existing research to guide future research, we conducted a comprehensive systematic review.

Research methods

We systematically reviewed the existing literature for studies comparing liver transplant to no-transplant with a primary outcome of both short- and long-term mortality and relapse. Pre-specified causes of heterogeneity included assessment and treatment of alcohol use disorder (AUD), definition of ALD, spectrum of ALD studied, assessment and rates of relapse, and study quality and bias.

Research results

We analyzed data from 10 studies including 1332 participants. While meta-analysis comparing liver transplant to no-transplant suggested improved mortality, relapse was found to be insignificant and both meta-analyses were limited by significant heterogeneity. Outcomes and heterogeneity improved with restriction to prospectively collected data; liver transplant in comparison to no-transplant had significantly reduced mortality and relapse with insignificant heterogeneity, though results remained limited by small-study effects. Overall, the quality of the evidence was very low.

Research conclusions

Current systematic review with meta-analysis comparing liver transplant to no-transplant suggests a mortality and relapse benefit in heterogeneous, institution-specific populations with inherent bias.

Research perspectives

To understand efficacy of liver transplantation for ALD on a global scale, formal recognition of the dearth of well-published literature on transplantation in this population is necessary, and there is an urgent need to standardize our approach to studying ALD. Such standardization should include assessment of the presence and treatment of AUD, the clinical definition of ALD, reporting the spectrum of the ALD population studied, data collection, and definition and detection of relapse.

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