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ORIGINAL ARTICLE

Falciform ligament in pancreatoduodenectomy for protection of skeletonized and divided vessels

Nobutsugu Abe · Masanori Sugiyama · Yutaka Suzuki · Osamu Yanagida · Tadahiko Masaki · Toshiyuki Mori · Yutaka Atomi

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Abstract
Background/purpose Pancreatic fistula, which is one of the main causes of late postpancreatectomy hemorrhage (PPH), is a common complication of pancreatoduodenectomy (PD). It may erode the anastomosis site and vascular wall in its vicinity, resulting in pseudoaneurysm formation and/or the rupture of major vessels. To protect the vessels near the area for pancreaticojejunostomy from potential pancreatic fistula, we have adopted a surgical option by which such vessels are separated from the pancreaticojejunostomy using a pedicled falciform ligament. We reviewed 36 patients who underwent PD that included this option.
Methods After the PD was completed (before reconstructions), the pedicled falciform ligament was spread widely on the major vessels exposed during resection, and was fixed to the surrounding retroperitoneal connective tissue. These procedures enabled the complete separation of these vessels from the pancreaticojejunostomy.
Results The mobilization and placement of the falciform ligament in the space between the pancreaticojejunostomy and the major vessels were successfully carried out without any complications. Although ten (28%) patients developed pancreatic fistula and three (8%) developed intraabdominal infection, none of the patients developed late PPH.
Conclusions The present surgical option is technically simple and easy, and may be an effective prophylactic measure against late PPH following PD.

Keywords Pancreatic fistula · Pancreatoduodenectomy · Falciform ligament · Late postpancreatectomy hemorrhage

Introduction

Pancreatic fistula is a common complication of pancreatoduodenectomy (PD) [1]. It exposes skeletonized or divided vessels directly to pancreatic juice and/or intraabdominal infection, creating a condition for vessel erosion and late postpancreatectomy hemorrhage (late PPH) [2, 3]. The protection of these vessels from pancreatic fistula and/or intraabdominal infection is therefore an important surgical option for preventing late PPH. The placement of an omental flap between skeletonized or divided vessels and the pancreaticojejunostomy may be a useful surgical technique for protecting these vessels [3–6]. However, an appropriate omental flap may not always be obtained. We previously reported that the wrapping of the vessels near the pancreatic stump, using a pedicled falciform ligament, was an effective prophylactic measure against late PPH following distal pancreatectomy [7]. Based on this experience, we have recently adopted a surgical option in PD by which skeletonized or divided vessels are separated from the pancreaticojejunostomy using a pedicled falciform ligament. This surgical option is simple and easy, and appears to prevent late PPH following PD. We herein present our procedure, which involves the use of the falciform ligament, and we review the short-term operative outcomes of 36 patients who underwent PD that included this option.

N. Abe (✉) · M. Sugiyama · Y. Suzuki · O. Yanagida · T. Masaki · T. Mori · Y. Atomi
Department of Surgery, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
e-mail: abenbtg@kyorin-u.ac.jp

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Methods and patients

Surgical techniques for use of the falciform ligament

A pedicled falciform ligament was easily and rapidly obtained during a midline abdominal incision [7]. After the incising of the linea alba, the preperitoneal fat is bluntly dissected to the right prior to incising the peritoneum. The falciform ligament is mobilized by dividing it near the umbilicus and incising its anterior peritoneal reflections along the posterior right rectus sheath. An additional length is obtained by continuing the anterior incision cephalad to the undersurface of the diaphragm to the triangular ligament and incising the posterior peritoneal reflections cephalad to the anterior surface of the liver to the triangular ligaments [8]. Note that the pedicled falciform ligament (Fig. 1a) normally reaches the space between the pancreaticojejunostomy and the major vessels exposed during resection. After completion of the PD (before reconstructions), the pedicled falciform ligament is spread widely on the major vessels, such as the common/proper hepatic artery, superior mesenteric vein (SMV), portal vein (PV), splenic vein, and stump of the gastroduodenal artery (Fig. 1b), and fixed with interrupted 3-0 silk sutures to the surrounding retroperitoneal connective tissues (Fig. 1c). This procedure enables the complete separation of these vessels from the pancreaticojejunostomy.

Patients and pancreatoduodenectomy

Between October 2002 and February 2007, 36 patients without prior history of upper abdominal surgery underwent PD employing the placement of a pedicled falciform ligament. The patients were 23 men and 13 women with a mean age of 65 years (range 22–78 years). The indications for surgery included pancreatic ductal cancer (n = 11), intraductal papillary-mucinous tumor of the pancreas (n = 2: adenoma, 1; carcinoma, 1), intraductal papillary-mucinous carcinoma of the pancreas concomitant with intrahepatic bile duct cancer (n = 1), serous cystadenoma of the pancreas (n = 1), solid and pseudopapillary tumor of the pancreas (n = 1), cancer of the papilla of Vater (n = 7), duodenal cancer (n = 2), and bile duct cancer (n = 11). Of the 36 patients, 23 (64%) had normal (soft) pancreas.

Pancreatoduodenectomy included two types: standard PD (n = 17) and pylorus-preserving PD (n = 19). For standard PD, antrectomy was performed. Two patients underwent concomitant resections of other organs: lateral segmentectomy of the liver in one (a patient with intraductal papillary-mucinous carcinoma of the pancreas concomitant with intrahepatic bile duct cancer) and transverse colectomy in one (a patient who showed severe inflammatory adhesion between intraductal papillary-mucinous carcinoma of the pancreas and the transverse mesocolon). PV resection and reconstruction were carried out in four patients with pancreatic ductal cancer. Lymph

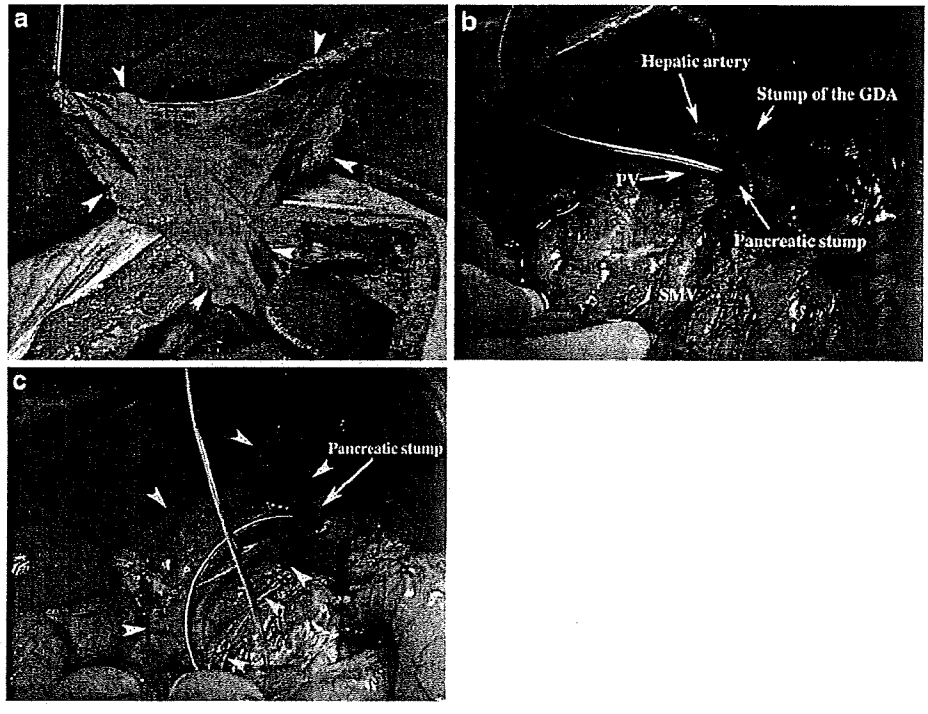

Fig. 1 Protection of skeletonized and divided vessels using falciform ligament. **a** The pedicled falciform ligament (*arrowheads*). **b** Surgical view before the placement of the pedicled falciform ligament. The hepatic artery, stump of the gastroduodenal artery (*GDA*), superior mesenteric vein (*SMV*), and portal vein (*PV*) are exposed. **C** Surgical view after the placement of the pedicled falciform ligament (*arrowheads*). This procedure enables the complete separation of the vessels from the pancreaticojejunostomy

nodes in the hepatoduodenal ligament and around the common hepatic artery were dissected in the patients with pancreatic ductal cancer, bile duct cancer, duodenal cancer, and cancer of the papilla of Vater. The SMV to the PV was skeletonized in all the patients. In the patients with pancreatic ductal cancer, the nerve plexus around the superior mesenteric artery (SMA) was dissected over lengths of approximately 5 cm, up to the origin of the SMA. None of the patients underwent paraaortic lymph node dissection.

The pancreas was transected at the level of the left side of the SMV. During the pancreatic transection, the pancreatic parenchyma was divided using a Harmonic Scalpel (Ethicon Endo-Surgery, Cincinnati, OH, USA) or a surgical scalpel (Harmonic Scalpel, $n = 10$; surgical scalpel, $n = 16$).

Reconstruction was carried out according to a modification of the method described by Child. Pancreaticojejunostomy was performed, using end-to-side anastomosis, in two different ways depending on the diameter of the main pancreatic duct. When the diameter was large (4 mm and more), the pancreatic stump was anastomosed to the jejunum in two layers: the duct-to-mucosa and pancreatic parenchyma-to-jejunal seromuscular layer ($n = 17$). When the diameter was small (3 mm or less), the main pancreatic duct was intubated using a retrograde transjejunal pancreatic tube, with pursestring sutures around the duct and the stab wound in the jejunum, and then the pancreatic parenchyma was anastomosed to the jejunal seromuscular layer ($n = 19$). The common hepatic duct was anastomosed end-to-side to the jejunum 6–8 cm distal to the pancreaticojejunostomy. The duodenum or the stomach was then anastomosed end-to-side to the jejunum. Finally, a Braun jejunostomy was performed near the duodeno- (gastro-) jejunostomy. After completion of the reconstruction, closed suction drains were placed near the biliary and pancreatic anastomoses. The tip of the drain near the pancreatic anastomosis was placed in the compartment between the pancreatic anastomosis and the falciform ligament that was spread widely on the major vessels. No octreotide was administered postoperatively.

Definition of postoperative complications

The definition of pancreatic fistula was a drain output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than three times the serum amylase level [9]. Other complications were categorized and defined as follows: intraabdominal infection (drainage of purulent but amylase-poor fluid); intraabdominal abscess (need for percutaneous or surgical drainage) [6]; delayed gastric emptying (inability to return to a standard diet by the end of the first postoperative week or the need for nasogastric intubation for 10 days or more) [10, 11]; bile leakage (bilious drainage) [12]; intestinal obstruction (need for the insertion of a long tube); and late PPH (intraabdominal hemorrhage 24 h or more after the operation) [13].

Results

The mobilization and placement of the falciform ligament in the space between the pancreaticojejunostomy and the major vessels were successfully carried out without any complications. In none of patients did the pedicled falciform ligament show ischemia at the end of the operation. The mean operating time and blood loss were 445 min (range 258–887 min) and 687 ml (range 110–1,554 ml), respectively. Postoperative complications occurred in 15 of the 36 patients in the series, for an overall postoperative morbidity rate of 42%. There were no postoperative deaths.

Ten patients (28%) developed pancreatic fistula. According to the grading system of pancreatic fistula proposed by the International Pancreatic Fistula Study Group, three were defined as grade B pancreatic fistula and seven as grade A. All ten patients with pancreatic fistula had a soft pancreas. None of the patients with pancreatic fistula required surgical or percutaneous interventions. The pancreatic fistula resolved spontaneously after conservative treatments including pancreatic rest and/or intermittent local irrigation with saline via the drain. Although three patients (8%) developed intraabdominal infection, none of the patients required additional drainage. The intraabdominal infections resolved after conservative treatments including antibiotic administration and local irrigation with saline via the drain. Delayed gastric emptying was observed in two patients (6%), and there was a small intestinal obstruction in one patient (3%). A reoperation (adhesionolysis) was carried out in one patient with intestinal obstruction. Other major complications, including bile leakage, wound abscess, and late PPH did not occur.

Discussion

Late PPH still remains a fatal complication after PD [2]. The incidence of PPH after PD ranges from 1 to 8% [2, 6, 13–17], and the mortality rates associated with PPH are high, ranging from 11 to 58% [13–21]. Late PPH is reportedly most often caused by pancreatic fistula and/or intraabdominal infection [2, 6, 22–24]. These may erode the anastomosis site and vascular wall in its vicinity, resulting in pseudoaneurysm formation and/or the rupture of major vessels such as hepatic arteries, the stump of the gastroduodenal artery, and portal vessels [2]. Of note, pancreatic fistula, which is one of the main causes of late

PPH, is a common complication of PD [1, 9]. Its reported incidence varies from 2 to 28% [9, 12]. Recent advances in medical and surgical care in PD cannot completely eliminate the possibility of pancreatic fistula development [1]. The prevention of late PPH associated with pancreatic fistula is therefore an important step toward improving the short-term outcome after PD.

The falciform ligament is the obliterated umbilical vein (ligamentum teres or round ligament) and its encompassing parietal peritoneum [8]. The pedicled falciform ligament adequately mobilized is therefore a large (15–30 cm) autologous tissue that will usually reach any surgical area in the upper abdomen. It is therefore sufficiently large to cover the major vessels widely. Although its suitability is not widely appreciated, it has been used in several types of abdominal operations, including those for hepatic injury [8] or perforated gastroduodenal ulcer [25], and hiatal herniorrhaphy [26]. Only a few reports have been published on the use of the falciform ligament in pancreatic surgery [7, 27]. We previously showed that the wrapping of the vessels near the pancreatic stump, using a pedicled falciform ligament, was successful for reducing the incidence of late PPH (0%) after distal pancreatectomy [7]. Baker et al. [27]. described the use of the falciform ligament for the protection of the vessels in PD. However, the outcomes of the use of the falciform ligament in PD have not yet been described.

Others [3–6] use an omental flap, whose suitability is widely appreciated, for the same purpose as ours. Indeed, the placement of an omental flap between the major vessels and the pancreaticojejunostomy is successful for reducing the incidence of late PPH (0–1%) after PD [3–6]. However, an adequate omentum is not available in some patients [8]. Because the space between the pancreaticojejunostomy and the major vessels is small, a thick omentum with rich adipose tissue seems to be inappropriate. Complications associated with the use of an omental flap, such as intestinal obstruction, total flap necrosis, and infection, have been reported [6]. Therefore, we consider that the falciform ligament is an excellent alternative to the omentum for the protection of the major vessels.

The presented surgical option, which is the placement of a pedicled falciform ligament between the pancreaticojejunostomy and the major vessels, is a simple and easy technique for the complete separation of the vessels from the pancreaticojejunostomy. It is suggested that the falciform ligament can prevent the diffusion of pancreatic juice with or without bacterial infection and protect the vessels. In the present study, although 28% of the patients developed pancreatic fistula, none of the patients developed late PPH. However, it could not be confirmed whether the present surgical option itself prevented late PPH. Further controlled randomized studies involving large numbers of patients are required to confirm the value of this surgical option.

In conclusion, the present surgical option (the placement of a pedicled falciform ligament between the pancreaticojejunostomy and major vessels) is technically simple and easy, and we consider that it may prevent late PPH caused by pancreatic fistula following PD.

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SURGEON AT WORK

Modified liver hanging maneuver with extraparenchymal isolation of the middle hepatic vein in left hepatectomy

Masanori Sugiyama · Yutaka Suzuki · Nobutsugu Abe · Tadahiko Masaki · Toshiyuki Mori · Yutaka Atomi

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Abstract
Background/purpose The liver hanging maneuver has been employed mostly for right hepatectomy. After the space between the inferior vena cava and the liver parenchyma is bluntly dissected, a tape is passed through the space, with the upper end of the tape between the right hepatic vein and middle hepatic vein (MHV). We devised a modified maneuver with tape repositioning between the MHV and left hepatic vein (LHV) after extraparenchymal isolation of the MHV, in left hepatectomy with the caudate lobe and without the MHV.
Methods The ligamentum venosum is divided. Traction of the cranial stump of the ligament in the cranial and left direction shows an avascular plane between the LHV and MHV, and enables the taping of each MHV and LHV. Finally, the upper end of the hanging tape is repositioned.
Results This maneuver was successfully performed in three patients.
Conclusions The present maneuver is feasible and useful in left hepatectomy.

Keywords Liver hanging maneuver · Ligamentum venosum · Hepatic vein

Introduction

The liver hanging maneuver proposed by Belghiti et al. [1] has been employed mostly for right hepatectomy [2–7]. In the original method, the space between the anterior surface of the inferior vena cava (IVC) and the liver parenchyma is bluntly dissected without liver mobilization. Then a tape is passed through the space, with the upper end of the tape between the right hepatic vein (RHV) and middle hepatic vein (MHV). This tape serves as a guide to the transection plane and as a means of suspending the liver, facilitating the control of bleeding in the deeper parenchyma of the liver and protecting the IVC [1–7].

Only a few articles have described modified hanging maneuvers for left hepatectomy with or without the MHV [7, 8]. For left hepatectomy without the MHV, it is rational to pass a hanging tape just on the left side of the MHV, contrary to the original hanging maneuver. Kim et al. [6] reported a method in which a tape is passed along the anterior surface of the retrohepatic IVC between the MHV and left hepatic vein (LHV) after dividing the liver parenchyma between the MHV and LHV using an ultrasonic dissector. We devised a modified liver hanging maneuver with tape repositioning between the MHV and LHV after extraparenchymal isolation of the MHV, in left hepatectomy with the caudate lobe and without the MHV. This technique obviates the need for liver parenchymal dissection before passing the hanging tape.

Method

After laparotomy, intraoperative ultrasonography is performed, with special attention to confirm the absence of tumor infiltration to the IVC, abnormal short hepatic veins in the space to be dissected, or abnormal branches near the roots of the LHV and MHV [4, 9]. The anterior surface of the suprahepatic IVC is exposed, and the space between the RHV and MHV is dissected 2–3 cm in the caudal direction,

M. Sugiyama (✉) · Y. Suzuki · N. Abe · T. Masaki · T. Mori · Y. Atomi
Department of Surgery,
Kyorin University School of Medicine,
6-20-2 Shinkawa, Mitaka,
Tokyo 181-8611, Japan
e-mail: sgym@kyorin-u.ac.jp

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using a right-angled dissector. The caudal edge of the caudal lobe is lifted from the IVC, and small short hepatic veins are divided up to the level of the inferior right hepatic vein. A long light curved Kelly clamp is inserted behind the caudate lobe, just to the left side of the right inferior hepatic vein, and passed cranially along the anterior surface of the IVC between the 10 and 11 o'clock position, under ultrasonographic guidance [4]. The Kelly clamp is advanced further toward the tip of the operator's left index finger inserted into the previously dissected space between the RHV and MHV. Then, a tape for liver hanging is passed (Fig. 1).

The left triangular and coronary ligaments and the lesser omentum are divided. The anterior surface of the junction of the LHV and MHV is dissected. After the left lobe is lifted up, the ligamentum venosum (Arantius' ligament) is ligated and divided at the cranial one-third. The cranial stump of the ligament is grasped with the Kelly clamp and dissected cranially toward the IVC, almost until the ligament broadens into its attachment (the posterior wall of the LHV or the common trunk of the LHV and MHV). Traction of the ligament in the cranial and left direction puts the walls of the LHV and MHV under tension, showing an avascular plane between the posterior walls of the LHV and MHV and the liver parenchyma. This plane is developed toward the anterior aspect of the dissection. Another Kelly clamp is inserted between the LHV and MHV in the posteroanterior direction, and the LHV is encircled with a tape (Figs. 2, 3). Furthermore, a tunnel is created behind the common trunk of the LHV and MHV. Consequently, extraparenchymal taping of the MHV is easily performed. Finally, the upper end of the hanging tape previously passed between the liver and IVC is repositioned from between the RHV and MHV to between the MHV and LHV, using the tape around the MHV (Fig. 4).

After cholecystectomy, the hepatic hilum is dissected to free the right and left Glisson's pedicles, in the inferior surface of the quadrate lobe. Then, the lower end of the hanging tape is passed between both pedicles. This

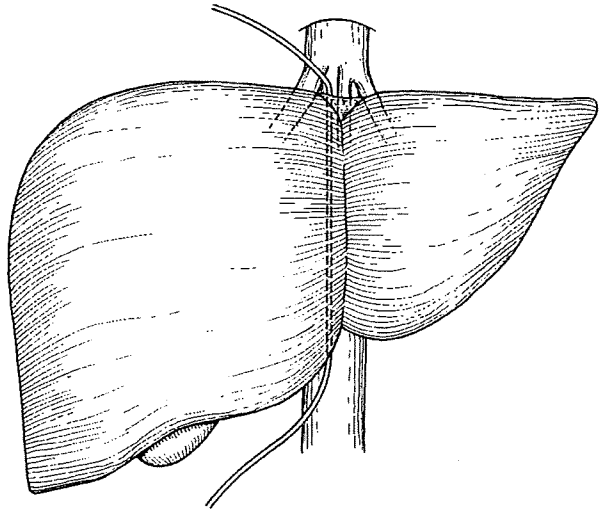

Fig. 1 The hanging tape is passed along the anterior surface of the retrohepatic inferior vena cava (IVC) between the right hepatic vein (RHV) and middle hepatic vein (MHV)

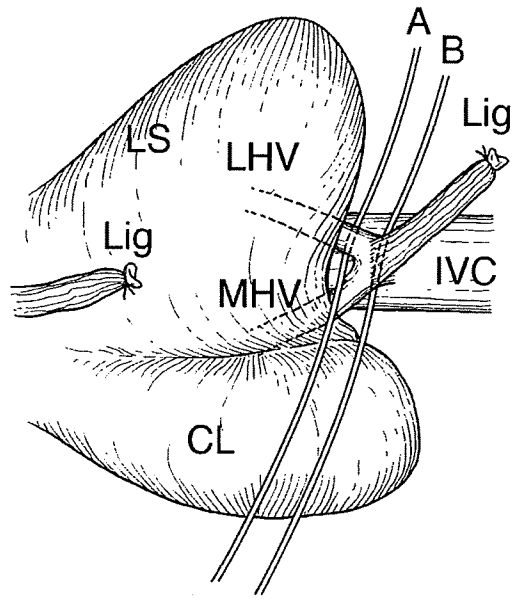

Fig. 2 The ligamentum venosum (*Lig*) is ligated and divided. Traction of the cranial stump of the ligament in the cranial and left direction reveals an avascular plane between the posterior walls of the left hepatic vein (*LHV*) and *MHV* and the liver parenchyma. The LHV is encircled with a tape (*A*). The common trunk of the LHV and MHV is also encircled (*B*). *LS* left lateral segment, *CL* caudate lobe, *IVC* inferior vena cava

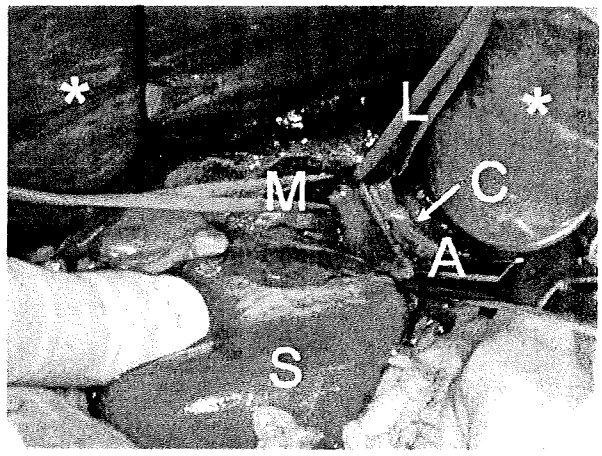

Fig. 3 Intraoperative view of the liver from the *left side*, while the left lobe (*asterisk*) is lifted up. Each MHV (*M*) and LHV (*L*) is encircled. *A* ligamentum venosum (Arantius' ligament), *C* common trunk of the LHV and MHV, *S* spiegel lobe of the caudate lobe

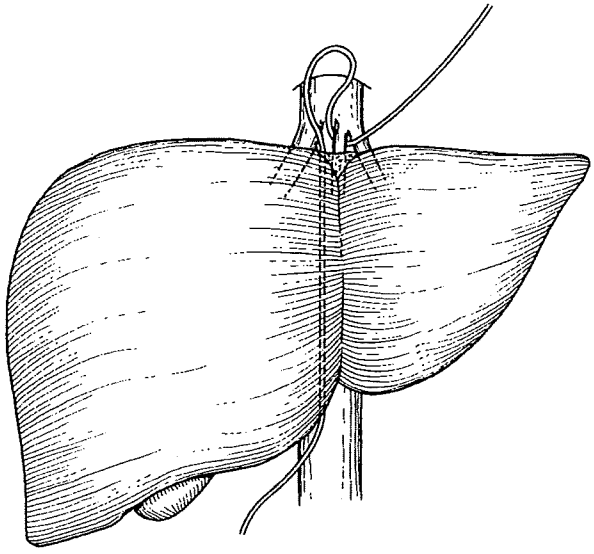

Fig. 4 The upper end of the hanging tape is repositioned between the MHV and LHV, using the tape around the MHV

repositioning of the tape is more effective than previously used positions for suspension of the caudate lobe. Hepatic parenchymal transection is then performed with Pringle's maneuver.

Results

Three patients underwent left hepatectomy with the caudate lobe and without the MHV, by using the present modified liver hanging maneuver. There were two men and one woman, with a mean age of 66 years (range 58–74 years). The diagnoses were: hilar cholangiocarcinoma (n = 2) and colorectal liver metastasis (n = 1). The maneuver was successfully performed. The mean operative time was 260 min (range 176–315 min). The mean intraoperative blood loss was 642 ml (range 320–811 ml). None of the patients required blood transfusion during or after the operation.

Discussion

The present technique is a combination of the original liver hanging maneuver and the extraparenchymal isolation of the MHV. The latter is an application of the maneuver of Majno et al. [9] namely, extraparenchymal isolation of the LHV by dividing and pulling the ligamentum venosum.

Previously, it had been believed that the extraparenchymal isolation of each LHV and MHV was technically difficult. However, Majno et al. [9] proposed the maneuver of extraparenchymal isolation of the LHV. This maneuver is based on the anatomical knowledge that the fibers of the ligamentum venosum insert not on the IVC itself but on the posterior wall of the LHV or the common trunk of the LHV and MHV [9, 10]. The point of this maneuver is traction, not simply division, of the ligamentum venosum. Majno et al. [9] performed this maneuver successfully in 54 (95%) of 57 cases of in-situ liver splitting or hepatic resection. Because the common trunk of the LHV and MHV is easily encircled, the MHV can also be isolated extraparenchymally. An isolated LHV or MHV serves as a goal of hepatic transection. Clamping of these veins can reduce blood loss during hepatectomy via vascular outflow control. We have routinely used Majno's maneuver in left hepatectomy or left lateral sectionectomy. Afterward, we combined this maneuver and the liver hanging maneuver.

The present technique allows liver hanging just on the left side of the MHV, and obviates the need for liver parenchymal dissection before taping. This technique is feasible and useful in left hepatectomy with the caudate lobe and without the MHV. This technique may also be useful in extended right hepatectomy or right trisectionectomy. On the other hand, left hepatectomy without the MHV and without the caudate lobe does not require the present technique. Such hepatectomy is possible if the tape is located along the ligamentum venosum with its upper end between the MHV and LHV.

The extraparenchymal isolation of the MHV can be performed safely in almost all cases, as has been mentioned. However, extraparenchymal isolation of the LHV or MHV is not indicated for cases of tumor near the roots of these veins. Furthermore, potential abnormal venous branches near the roots should be investigated by intraoperative ultrasonography. Incautious dissection between the LHV and MHV may injure these vessels. When a resistance-free dissection route cannot be found, the dissection should be abandoned, and these vessels should be encircled after dividing the liver parenchyma between the MHV and LHV.

In the liver hanging maneuver, the blind dissection between the caudate lobe and IVC carries a potential risk of inadvertent tearing of the short hepatic veins. Strict positioning and gentle advance of a Kelly clamp along the anteromedian surface of the IVC can reduce the risk. Intraoperative ultrasonography is useful for checking for abnormal short hepatic veins and as guidance during the retrohepatic dissection [4]. The liver hanging maneuver is not indicated when the tumor has invaded the retrohepatic IVC.

In conclusion, the present modified liver hanging maneuver is feasible and useful in left hepatectomy with the caudate lobe and without the MHV.

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How I Do It

Pancreatic duct holder for facilitating duct-to-mucosa pancreatojejunostomy after pancreatoduodenectomy

Masanori Sugiyama, M.D.*, Yutaka Suzuki, M.D., Nobutsugu Abe, M.D., Hisayo Ueki, M.D., Tadahiko Masaki, M.D., Toshiyuki Mori, M.D., Yutaka Atomi, M.D.

Department of Surgery, Kyorin University, School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan

KEYWORDS:
Duct-to-mucosa;
Pancreatojejunostomy;
Pancreatic duct holder;
New device

Abstract. Duct-to-mucosa pancreatojejunostomy after pancreatoduodenectomy may be technically difficult, particularly in cases in which the remnant pancreas is soft with a small main pancreatic duct. We devised a pancreatic duct holder for duct-to-mucosa pancreatojejunostomy. The holder has a cone-shaped tip. A one-third circle of the tip is cut away, which makes a slit. As the tip is inserted gently into the pancreatic duct, the duct can be adequately expanded. The holder provides a good surgical field for anastomosis. A slit of the tip allows needle insertion. The holder facilitates stitches of the jejunum also. Twelve patients underwent pancreatoduodenectomy, followed by duct-to-mucosa pancreatojejunostomy using the holder. The holder allowed 8 or more stitches in duct-to-mucosa anastomosis, even in patients with a small pancreatic duct. No patients developed prolonged pancreatic leakage or pancreatic fistula postoperatively. In conclusion, the pancreatic duct holder is a simple and useful tool for facilitating duct-to-mucosa pancreatojejunostomy.

Pancreatoenterostomic leakage is still a common and serious complication after pancreatoduodenectomy, despite recent advances in operative techniques and postoperative care. Various methods have been used to prevent the pancreatic leakage, including duct-to-mucosa anastomosis, pancreatogastrostomy, and pancreatic duct stenting.[1–4] However, duct-to-mucosa pancreatojejunostomy may be technically difficult, particularly in cases in which the remnant pancreas is soft with a small main pancreatic duct, namely high-risk cases for pancreatic leakage or fistula.[5] We devised a pancreatic duct holder for facilitating duct-to-mucosa pancreatojejunostomy.

* Corresponding author: Tel.: +81-422-47-5511; fax: +81-422-47-9926.
E-mail address: sgym@kyorin-u.ac.jp
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Surgical Technique

The pancreatic duct holder or a dilator for suture placement is constructed of metal. The holder consists of a cone-shaped tip (15-mm height, 1-mm diameter at the tip, and 6-mm diameter at the base) and a handle (Fig. 1). At the tip portion, a one-third circle of the cone is cut away, which makes a slit.

The tip of the holder is inserted gently into the pancreatic ductal lumen (Fig. 2). The pancreatic duct can be adequately expanded to 3 to 5 mm with the cone-shaped tip, even in cases with a small pancreatic duct. The holder provides a good surgical field for anastomosis. A slit of the tip allows needle insertion. Furthermore, the holder, which is inserted into a small opening of the jejunum, facilitates stitches of the jejunum also. Surgeons can avoid grasping the pancreatic duct, parenchyma, or the jejunal wall with forceps.

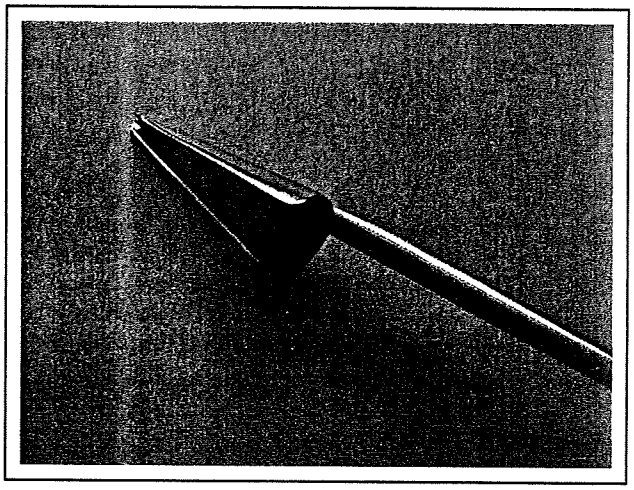

Figure 1 The pancreatic duct holder has a tip in which a one-third circle of a cone is cut away.

After pancreatoduodenectomy, pancreatojejunostomy is conducted in end-to-side anastomosis using 2 layers including duct-to-mucosa and the pancreatic parenchyma-to-the jejunal seromuscular layer[6] (Fig. 3). Duct-to-mucosa anastomosis is performed with the pancreatic duct holder, under visual magnification (2.5×). The pancreatic duct with a little pancreatic parenchyma is anastomosed to the jejunum (the mucosal layer and a small part of the seromuscular layer) using interrupted sutures (5–0 Maxon, monofilament polyglyconate; Davis & Geck, St Louis, MO). A 5F polyvinyl chloride tube (Sumitomo Bakelite, Tokyo, Japan) is inserted through the anastomosis into the pancreatic duct for partial external drainage of pancreatic juice. The opposite end of the pancreatic tube is introduced externally through the jejunal wall and the abdominal wall. Closed drains are placed anteriorly and posteriorly to the pancreatojejunal anastomosis.

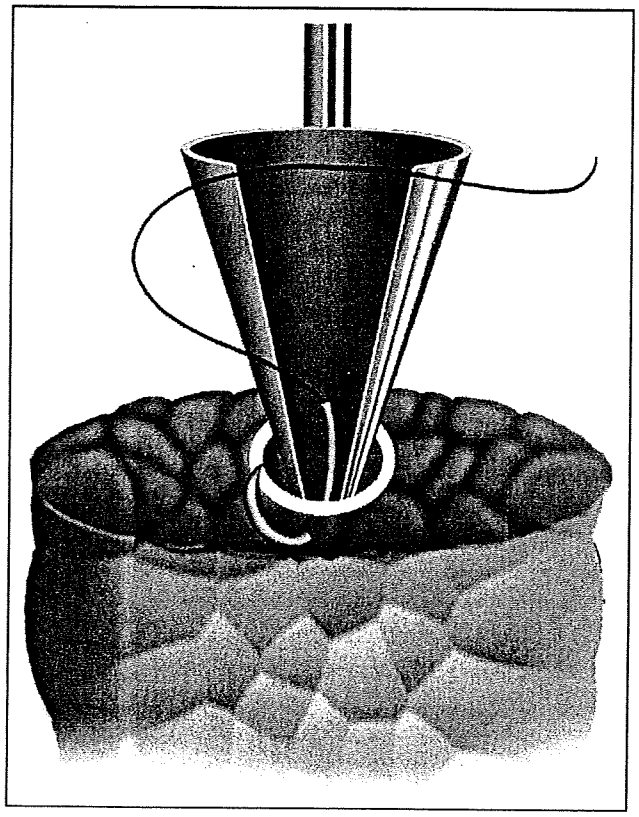

Figure 2 A pancreatic ductal stitch is placed with the pancreatic duct holder in duct-to-mucosa pancreatojejunostomy.

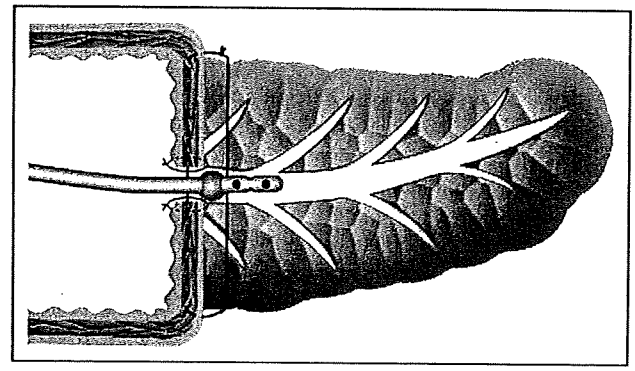

Figure 3 Pancreatojejunostomy is conducted using 2 layers including duct-to-mucosa and the pancreatic parenchyma-to-the jejunal seromuscular layer. For the latter layer, sutures penetrate the pancreatic parenchyma from the anterior surface to the posterior surface and pick up seromuscular layer of the jejunum widely, after Kakita's method.[6] A drainage tube is inserted through the anastomosis into the pancreatic duct.

Results

Between June and December 2007, 12 patients underwent conventional (n = 2) or pylorus-preserving (n = 10) pancreatoduodenectomy followed by duct-to-mucosa pancreatojejunostomy using the pancreatic duct holder. Diagnoses included bile duct carcinoma (n = 6), pancreatic head carcinoma (n = 4), and intraductal papillary mucinous neoplasm (n = 2). At surgery, the diameter of the main pancreatic duct at the cut surface measured ≤2 mm (small duct with soft pancreas) in 7 patients and >2 mm (dilated duct) in the 5 remaining patients. For duct-to-mucosa pancreatojejunostomy, 8 and 12 to 16 stitches were performed in patients with the small and dilated duct, respectively.

Postoperatively, no patients developed pancreatic fistula or prolonged (>7 days) high output (>50 mL/d) of an amylase-rich content (amylase concentration >3 times the upper limit of the normal serum amylase level) from perianastomotic peritoneal drains. The daily discharge from the pancreatic tube was 287 ± 109 mL (mean ± standard deviation).

During the postoperative follow-up period (2–9 months; median, 6 months), no patients had steatorrhea. Postoperatively, none of 10 patients who had been preoperatively nondiabetic became diabetic, but 1 of 2 diabetic patients experienced exacerbation of diabetes. Of 9 patients who

underwent a computed tomography scan or magnetic resonance imaging 3 to 9 months after surgery, none had anastomotic stenosis of pancreatojejunostomy or pancreatic ductal dilatation.

Comments

Duct-to-mucosa pancreatojejunostomy after pancreatoduodenectomy may be technically challenging in cases with a small pancreatic duct. The pancreatic ductal lumen is often collapsed after pancreatic transection. Many surgeons use fine pickup forceps, grasping the pancreatic duct, inserting it into the duct, or trying to open the ductal lumen. However, they usually fail to dilate the ductal lumen in a round shape, and the lumen becomes rather flattened. A stay suture may facilitate the subsequent stitches. Nevertheless, placement of the first stay suture itself is inevitably difficult. Stitches for a small opening of the jejunum may also be difficult because the jejunal wall is somewhat thick and sometimes inverted. Furthermore, forceps manipulation or incorrectly pulled stay sutures may cause a laceration of the pancreatic duct or parenchyma. Such manipulation may also result in inadvertent enlargement of the jejunal opening at the end of anastomotic procedure, despite an initially small incision.

The pancreatic duct holder presented in this article has several advantages. The cone-shaped tip can adequately expand the pancreatic ductal lumen as a round shape and offer excellent visualization for anastomosis. The holder is useful also for jejunal stitches. This device enables precise and fine stitches, allowing 8 or more sutures. The holder gives no damages on the pancreatic duct and parenchyma and the jejunum.

In conclusion, the pancreatic duct holder described here is a simple and useful tool for facilitating duct-to-mucosa pancreatojejunostomy.

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Adverse prognosis of epigenetic inactivation in *RUNX3* gene at 1p36 in human pancreatic cancer

S Nomoto*[,1], T Kinoshita[1], T Mori[1], K Kato[1], H Sugimoto[1], N Kanazumi[1], S Takeda[1] and A Nakao[1]

[1]*Department of Surgery II, Graduate School & Faculty of Medicine, University of Nagoya, Nagoya, Japan*

Alteration in transforming growth factor-β signalling pathway is one of the main causes of pancreatic cancer. The human runt-related transcription factor 3 gene (*RUNX3*) is an important component of this pathway. *RUNX3* locus 1p36 is commonly deleted in a variety of human cancers, including pancreatic cancer. Therefore, we examined genetic and epigenetic alterations of *RUNX3* in human pancreatic cancer. Thirty-two patients with pancreatic cancer were investigated in this study. We examined the methylation status of *RUNX3* promoter region, loss of heterozygosity (LOH) at 1p36, and conducted a mutation analysis. The results were compared with clinicopathological data. Promoter hypermethylation was detected in 20 (62.5%) of 32 pancreatic cancer tissues, confirmed by sequence of bisulphite-treated DNA. Loss of heterozygosity was detected in 11 (34.3%) of 32 pancreatic cancers. In comparison with clinicopathological data, hypermethylation showed a relation with a worse prognosis ($P=0.0143$). Hypermethylation and LOH appear to be common mechanisms for inactivation of *RUNX3* in pancreatic cancer. Therefore, *RUNX3* may be an important tumour suppressor gene related to pancreatic cancer.

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Keywords: *RUNX3*; pancreatic cancer; hypermethylation; loss of heterozygosity

Despite its relatively low incidence of approximately 10 cases/100 000 people, pancreatic cancer is still one of the leading causes of cancer-related death in industrialised countries including Japan. The prognosis remains poor, with an overall 5-year survival rate of less than 5% (Jemal *et al*, 2007). The pathogenesis of pancreatic ductal adenocarcinoma can be described as a step-by-step accumulation of genetic changes, such as K-ras oncogene mutations, p53, p16, and smad4 tumour suppressor gene mutations (Kern *et al*, 2002), in addition to several epigenetic alterations, which together result in self sufficiency of growth signals, insensitivity to antigrowth signals, evasion of apoptosis, angiogenesis, invasion, and metastasis (Ozawa *et al*, 2001). Recently, several reports indicated that every silencing mechanism, such as loss of heterozygosity (LOH) and mutations in a gene, or hypermethylation in its promoter region occurred in a tumour suppressor gene resulting in loss of its function in tumorigenesis (Tokumaru *et al*, 2003).

Transforming growth factor-β (TGF-β) signalling is a well-established tumour suppressor pathway in pancreatic carcinogenesis (Massagu *et al*, 2000). Smad4 is a key transcription factor in the TGF-β1 signalling pathway, and is inactivated in about 50% of pancreatic adenocarcinomas. The human runt-related transcriptional factor 3 (*RUNX3*) gene also plays important roles in the TGF-β signalling pathway. In this pathway, Smad2 and Smad3 activated by TGF-β interact with RUNX3, and induce transcriptional activation of target genes in the nucleus (Ito and Miyazono, 2003; Miyazono *et al*, 2003).

*Correspondence: Dr S Nomoto; E-mail: snomoto@med.nagoya-u.ac.jp
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RUNX3 induced apoptosis in epithelial cells, and the knockout mice of this gene showed hyperplasia in gastric mucosa. In addition, loss of function of RUNX3 caused by DNA hypermethylation, LOH at gene locus, and mutation correlated with the progression of primary gastric cancers (Li *et al*, 2002). RUNX3 might have the important role of TGF-β and Smad proteins in carcinogenesis. Furthermore, *RUNX3* is located on the distal portion of the short arm of human chromosome 1 (1p36), which is commonly deleted in a variety of human cancers, including pancreatic cancer (Nowak *et al*, 2005; Loukopoulos *et al*, 2007). Therefore, the genetic and epigenetic alterations in *RUNX3* may have an important role in pancreatic cancer.

The aim of our present study was to determine whether the *RUNX3* gene alteration might have a role in carcinogenesis in pancreatic cancer. We examined LOH at this gene locus in 1p36 with microdissected DNA, the DNA-methylation status by methylation-specific polymerase chain reaction (MSP) and sequencing, and the mutation of *RUNX3* by reverse transcription-polymerase chain reaction (RT-PCR) single-strand conformation polymorphism (RT-PCR-SSCP) in 32 primary pancreatic cancer tissues and corresponding noncancerous tissues. Then, we correlated these results with the clinicopathological data.

MATERIALS AND METHODS

Patients, sample collection, microdissection, and DNA preparation

Thirty-two primary pancreatic cancer tissues and corresponding noncancerous tissues were collected at Nagoya University Hospital from pancreatic cancer patients during pancreatico-duodenectomy,

distal pancreatectomy, or total pancreatectomy. All tissues were diagnosed histologically as pancreatic cancer. Written informed consent, as required by the institutional review board, was obtained from all patients. Collected samples were stored immediately in liquid nitrogen at −80°C until analysis. Genomic DNA was obtained from these samples by digestion with proteinase K, followed by phenol/chloroform extraction.

Other parts of the specimens were formalin-fixed for 24 h and processed for paraffin embedding. From each tissue block, a series of four 5-μm thick sections were cut. The first section was H&E stained for pathologic evaluation; identification of the tumour epithelia. To avoid normal cell contamination, target epithelial cells from the cancer areas were produced by laser capture microdissection using a Pixcell LCM system (Arcturus Engineering Inc., Mountain View, CA, USA). An average of 200 laser shots (30 μm shot size, 60 ms laser pulse duration, and power of 60 MW) were used for each sample. Microdissected cells were then incubated overnight at 37°C in 50 μl digestion buffer (10 mmol l^{-1} Tris-HCl (pH 8.0), 1 mmol l^{-1} EDTA, 1% Tween 20, 1 mg ml^{-1} proteinase K) and incubated at 95°C for 10 min to inactivate the proteinase K.

Microsatellite analysis

DNAs from primary pancreatic cancer tissues and corresponding noncancerous tissues were analysed for LOH study by amplification of CA repeat sequences using PCR. DNAs of pancreatic cancer epithelia were collected by the microdissection method mentioned above.

Two microsatellite markers, D1S234 and D1S247, were used. D1S234 exists at only 900 Kb on the telomeric side from the RUNX3 locus, and D1S247 is centromeric from the *RUNX3* locus. Polymerase chain reaction amplification was performed containing [α^{32}P]dCTP and 50 ng of genomic DNA. Polymerase chain reaction products were analysed on a 6% polyacrylamide gel and processed by autoradiography. Allelic loss was scored when the band intensity of one allele was decreased significantly (more than 40% reduction) in tumour DNA as compared with that in the normal DNA by using a BASS-2000 image analyzer (Fuji Photo Film Co. Ltd, Tokyo, Japan).

Methylation-specific PCR

DNA from tumour and normal specimens was subjected to bisulphite treatment. Briefly, 2 μg of DNA was denatured by NaOH and modified by sodium bisulphite. DNA samples were then purified using the Wizard purification resin (Promega Corp., Madison, WI, USA), treated again with NaOH, precipitated with ethanol, and resuspended in water. The primer pairs for the unmethylated detecting were in *RUNX3* promoter region near exon 1: S (sense, 5′-GTGGGTGGTTGTTGGGTTAGT-3′) and AS (antisense, 5′-TCCTCAACCACCACTACCACA-3′), which amplify a 138-base pair (bp) product, and those for the methylated detecting were in the same region: S (sense, 5′-CGTCGGGTTAGCGAGGTTTC-3′) and AS (antisense, 5′-GCCGCTACCGCGAAAAACGA-3′), which amplify a 120-bp product. The PCR amplification consisted of 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 15 s, after the initial denaturation step (94°C for 5 min). Each PCR product was loaded directly onto 2% agarose gels, stained with ethidium bromide, and visualised under UV illumination.

Sequence analysis

Genomic bisulphite-treated DNA of primary pancreatic cancer tissues was sequenced. Polymerase chain reaction was performed in methylated cases. The primer pairs for sequence were in RUNX3 promoter region near exon1: S (sense, 5′-GTTTAGGTAGTAGGGATAGTT-3′) and AS (antisense, 5′-CTATTCTCTCCCATCTTACC-3′), which amplify a 388-bp product. The PCR amplification consisted of 36 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, after the initial denaturation step (94°C for 5 min). Polymerase chain reaction products were purified directly using the QIA quick Gel Extraction Kit (QIAGEN, Hilden, Germany). Purified DNA fragments were subcloned into TA cloning vector (Invitrogen™, Carlsbad, CA, USA). Six cloning samples were picked out from one methylated tumour tissue. Each cloning DNA was mixed with 3 μl of specific primer (M13), 4 μl of Cycle Sequence Mix (ABI PRISM Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA). Samples were subjected to the following cycling conditions: 95°C for 30 s; 25 cycles of 95°C for 15 s, 50°C for 15 s, and 60°C for 4 min followed by purification by ethanol precipitation. Sequence analysis was carried out using an Applied Biosystems ABI310, and sequence electropherograms were generated by ABI Sequence Analysis 3.0.

RT-PCR-SSCP

Polymerase chain reaction amplification using random-primed cDNA of 32 primary pancreatic cancer tissues was performed using oligonucleotide primers in the presence of [α^{32}P]dCTP, followed by electrophoretic separation on 6% nondenaturing polyacrylamide gels both in the presence of 5% glycerol at room temperature and in its absence at 4°C. RUNX3 ORF (1248-bp) is divided into four overlapped fragments and each fragment was amplified. The primer pairs used for *RUNX3* mutation were S1 (sense, 5′-GCCGCTGTTATGCGTATTCC-3′) and AS1 (antisense, 5′-CTCAGCGGAGTAGTTCTCGT-3′), amplifying a 370-bp fragment; S2 (sense, 5′-GTGACTGTGATGGCAGGCAA-3′) and AS2 (antisense, 5′-GTTCCGAGGTGCCTTGGATT-3′), amplifying a 398-bp fragment; S3 (sense, 5′-ACAAGCCACTTCAGCAGCCA-3′) and AS3 (antisense, 5-GAGAACTGGTAGGAGCCAGA-3′), amplifying a 368-bp fragment; S4 (sense, 5′-CTACCACCTCTACTACGGGA-3′) and AS4 (antisense, 5′-CCCATCACTGGTCTTGAAGG-3′), amplifying a 326-bp fragment. The PCR amplification consisted of 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, after the initial denaturation step (94°C for 5 min) in F1–R1 and in the presence of 10% dimethylsulphoxide (F2–R2, F3–R3, F4–R4).

Statistical analysis

The correlation between the methylation status of RUNX3 mRNA and clinicopathological data was analysed by Fisher's exact test or χ^2 test for independence. Overall survival rates were calculated using the Kaplan–Meier method, and difference in survival curves was analysed using the log-rank test. Independent prognostic factors were identified by multivariate analysis using the Cox proportional hazards regression model. Data are expressed as mean ± s.d. Statistical significance was considered as $P<0.05$.

RESULTS

Microsatellite analysis of *RUNX3*

We first examined DNA samples obtained by microdissection from the 32 primary pancreatic cancer tissues and corresponding noncancerous tissues for LOH using two microsatellite markers, D1S234 and D1S247, which are close to the RUNX3 locus. D1S234 is telomeric and D1S247 is centromeric to the locus. Allelic imbalance in one or two markers was observed in 11 (34.3%) of the 32 cases (Figure 1). We judged the 11 cases as having an LOH at the locus. The results are summarised in Table 1. No cases evidenced microsatellite instability in this study. Two cases proved noninformative from using the two markers.

Hypermethylation of *RUNX3* promoter region in pancreatic cancer

To investigate whether the gene silencing was due to hypermethylation of *RUNX3*, MSP was performed in the 32 primary pancreatic

cancer tissues and corresponding noncancerous tissues. Promoter hypermethylation was detected in 20 (62.5%) of the 32 primary pancreatic cancer tissues and in only two of the corresponding noncancerous tissues (Figure 1). To confirm the methylation of the RUNX3 promoter region, genomic bisulphite-treated DNA of primary pancreatic cancer tissues, which showed methylation by MSP, were sequenced. Every case showed at least one methylated CpG island of the sequenced fragments. A representative case is shown in Figure 2.

Mutational analysis of *RUNX3* in pancreatic cancer tissues

To investigate the mutation status of this gene, RT-PCR-SSCP analysis was performed. We could not see any aberrant bands (Figure 3). No mutations or polymorphisms were detected in the 32 pancreatic cancer tissues. As we used the bulk frozen samples, normal cells such as fibrosis cells were contaminated in the tumour tissues, making it difficult to identify aberrant bands.

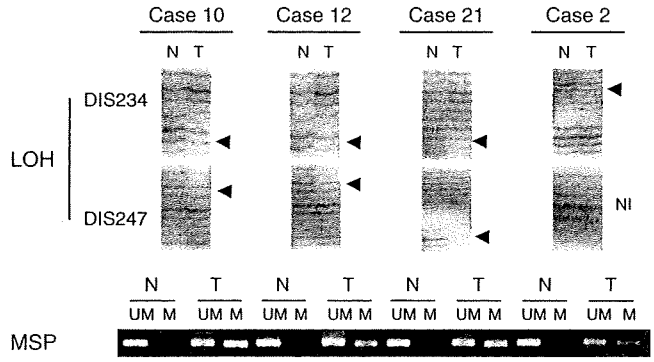

Figure 1 Representative results of LOH and MSP in cases 10, 12, 21, and 2. In the analysis of LOH at RUNX3 locus, cases 10, 12, and 21 showed allelic imbalance at D1S234 as well as at D1S247 (arrowheads). Case 2 showed allelic imbalance at D1S234 (arrowhead), but the D1S247 was not informative (NI). Promoter hypermethylation was observed in the DNA extracted from tumour tissue (T). In noncancerous samples (N), a methylation band was not seen in any lane. All four cases showed both LOH and promoter hypermethylation. These results indicated that biallelic inactivation (LOH at 1p36+ methylation) caused the inactivation of RUNX3 in pancreatic cancer. LOH, loss of heterozygosity; MSP = methylation-specific PCR; RUNX3 = human runt-related transcription factor 3 gene.

Statistical analysis of clinicopathological data and our findings

Subsequently, we analysed the correlation between the clinicopathological data and results of our findings. Table 2 shows the correlation between the clinicopathological data and methylation status. Interestingly, *RUNX3* hypermethylation was significantly correlated with a worse prognosis ($P = 0.0143$) (Figure 4). No other correlation with any clinicopathological parameter was found.

To evaluate the value of *RUNX3* methylation as an independent prognostic determinant, multivariate analysis was performed with prognostic factors that had been found to be significant by univariate analyses. The analysis identified lymph node metastasis, invasion of retroperitoneal tissue, and hypermethylation of *RUNX3* gene as the variables for independently predicting overall survival (Table 3).

DISCUSSION

Transforming growth factor-β plays a key role in regulating the growth and differentiation of many cell types. In TGF-β1-null

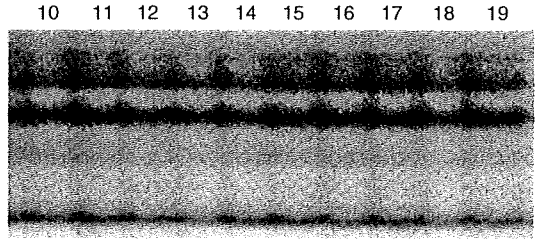

Figure 3 RT-PCR-SSCP analysis of RUNX3 in pancreatic cancer tissues. Representative results (cases 10–19) of RT-PCR-SSCP analysis using F2–R2 primer set. There were no aberrant bands in all cases. RT-PCR-SSCP = RT-PCR single-strand conformation polymorphism; RUNX3 = human runt-related transcription factor 3 gene.

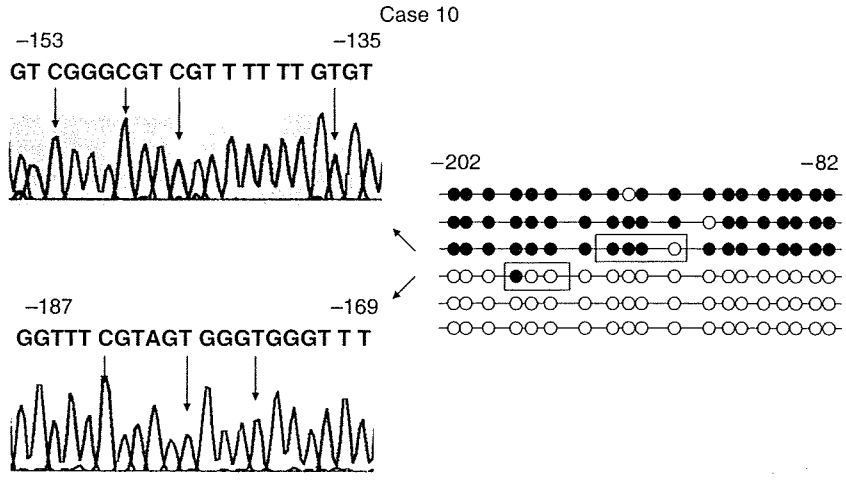

Figure 2 Sequence analysis of bisulphite-treated DNA from tumour sample of case 10 in RUNX3 promoter region. Methylation status of the 19 CpG islands between −82 and −202 from the transcription-initiation site of RUNX3 exon 1 is shown. The fragment was PCR amplified and subcloned into TA cloning vector. Closed circle indicates methylated CpG island, open circle indicates unmethylated CpG island. Each group of six clones showed a different methylation status. Arrows below the sequence indicate CpG islands. The Cs indicate methylated CpG islands. The Ts were converted from C by bisulphite treatment, indicating unmethylated CpG islands. RUNX3 = human runt-related transcription factor 3 gene.

Table 1 Clinicopathological features and results of RUNX3 alterations in pancreatic cancer tissues

					Hypermethylation		LOH[a]	
Case	Gender	Location	Stage[b]	Pathology	N	T	D1S234	D1S247
1	M	Ph	III	Tub. poor	—	—	●	●
2	F	Ph	IVa	Tub. mod	—	—	○	NI
3	M	Ph	IVb	Tub. mod	—	M	●	●
4	F	Ph	III	Tub. mod	—	—	●	NI
5	F	Phb	III	Anap. duc	—	—	NI	●
6	F	Ph	IVa	Tub. well	M	M	●	●
7	M	Ph	III	Tub	—	M	NI	●
8	F	Ph	IVb	Tub	—	M	●	●
9	M	Ph	IVb	Tub. mod	—	M	○	●
10	F	Ph	IVa	Tub. mod	—	M	○	○
11	M	Ph	IVb	Tub. mod	M	M	○	NI
12	M	Pb	III	Tub. poor	—	M	○	○
13	F	Ph	IVa	Tub. mod	—	—	●	●
14	M	Ph	IVa	Tub. mod	—	M	NI	●
15	M	Ph	IVb	Tub. mod	—	—	NI	●
16	M	Ph	IVb	Tub. poor	—	M	●	●
17	M	Ph	IVb	Tub. mod	—	—	●	○
18	M	Ph	IVb	Tub. mod	—	M	○	NI
19	M	Ph	IVb	Undifferentiated	—	M	●	○
20	F	Ph	IVb	Tub. mod	—	—	●	●
21	F	Phbt	IVb	Tub. mod	—	M	●	○
22	M	Ph	III	Acinar cell ca.	—	M	○	○
23	F	Ph	IVb	Tub	—	—	●	●
24	F	Phb	IVa	Tub. mod	—	—	NI	NI
25	F	Ph	IVa	Tub. poor	—	M	NI	●
26	M	Pb	IVa	Tub. well	—	M	NI	NI
27	F	Ph	III	Tub. mod	—	M	●	NI
28	M	Ph	IVa	Tub. mod	—	M	●	NI
29	M	Ph	III	Tub. mod	—	—	●	NI
30	M	Ph	IVa	Tub. well	—	—	●	NI
31	M	Pb	IVa	Tub. poor	—	M	NI	●
32	F	Ph	IVa	Tub. mod	—	M	○	○
					2/32 (6.3%)	20/32 (62.5%)	8/32 (25%) LOH*: 11/32 (34.3%)	7/32 (21.9%)

[a]Anap. duc = anaplastic ductal adenocarcinoma; F = female; LOH = loss of heterozygosity; M = male; M = methylated; mod = moderately differentiated adenocarcinoma; N = normal tissue; NI = not informative; Pb = pancreatic body; Pt = pancreatic tail; poor = poorly differentiated adenocarcinoma; Ph = pancreatic head; T = tumour tissue; tub = tubular adenocarcinoma; well = well-differentiated adenocarcinoma; ; —, unmethylated; open circle = LOH detected; closed circle = retention of heterozygosity; LOH* = cases in which LOH was detected in at least one locus. [b]The stage classification was performed according to the Pancreatic Cancer Study Group of Japan.

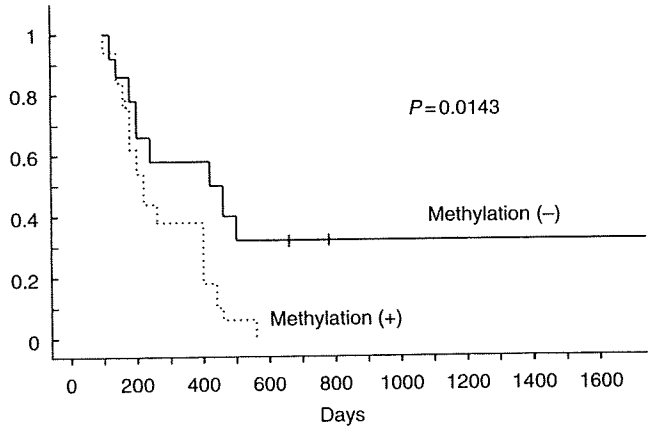

Figure 4 Survival stratified by methylation status in primary pancreatic cancer. RUNX3 hypermethylation was significantly correlated with a worse prognosis ($P = 0.0143$). RUNX3 = human runt-related transcription factor 3 gene.

animals, proliferation of the gastric epithelium is stimulated and hyperplasia occurs (Crawford *et al*, 1998). TGF-β is known to be a potent inhibitor of pancreatic acinar and duct cell proliferation *in vitro* (Bisgaard and Thorgeirsson, 1991; Logsdon *et al*, 1992). RUNX3 is a runt domain transcription factor involved in this signalling pathway. RUNX3 protein binds with the Smad2 and Smad3 proteins. Recently, it has been reported that RUNX3 was one of the tumour suppressor genes in gastric cancer and testicular yolk sac tumour. Runx3-null mice reportedly develop hyperplasia of the gastric mucosa through activation of cellular proliferation and suppression of apoptosis in epithelial cells (Li *et al*, 2002). Interestingly, 1p36, where *RUNX3* exists, is a region commonly deleted in a wide variety of human carcinomas, including pancreatic cancer. To date, there are many reports regarding the TGF-β signalling pathway in pancreatic cancer (e.g. TGF-β receptor II, Smad2 and Smad4), but only a few deal with this gene's alterations in pancreatic cancer (Li *et al*, 2004; Wada *et al*, 2004). Moreover, there are no reports regarding primary pancreatic cancer. Our study further supports a role for RUNX3 in pancreatic cancer.

The 1p36 region is believed to harbour tumour suppressor genes, because previous studies identified frequent allelic imbalance at 1p36 in various types of human cancers (Schwab *et al*, 1996). *RIZ1* and p73 genes are located on 1p36, and LOH was detected at each gene locus in pancreatic cancer (Sakurada *et al*, 2001; Sphyris *et al*, 2004). It is thought that these are one of the tumour suppressor genes in pancreatic cancer, and we think that RUNX3 may also be a candidate.

Previously, Wada *et al* (2004) reported that nine of 12 pancreatic cancer cell lines exhibited no expression of *RUNX3* by both

Table 2 Clinicopathological features and results of RUNX3 hypermethylation in pancreatic cancer tissues

Variable	No. of cases	Hypermethylation +	Hypermethylation −	P^a
Age				
<60	10	5	5	0.325
⩾60	22	15	7	
Gender				
M	18	13	5	0.198
F	14	7	7	
Tumour size				
TS1	5	2	3	>0.9999
⩾TS2	27	18	9	
S				
−	18	11	7	0.854
+	14	9	5	
RP				
−	10	6	4	0.844
+	22	14	8	
CH				
−	13	9	4	0.515
+	19	11	8	
DU				
−	21	13	8	0.923
+	11	7	4	
PV				
−	12	8	4	0.706
+	20	12	8	
A				
−	27	16	11	0.379
+	5	4	1	
PL				
−	27	16	11	0.379
+	5	4	1	
DPM				
−	26	15	11	0.242
+	6	5	1	
N				
0	14	8	6	0.581
1	18	12	6	
Differentiation				
Mod	21	12	9	0.241
Poor	6	5	1	

[a]Analysed by Fisher's exact test or χ^2 test for independence. [b]Tumour size according to the Classification of Pancreatic Carcinoma; A = arterial invasion; CH = choledocal invasion; DPM = dissected peripancreatic tissue margin; DU = duodenal invasion; F = female; PL = peripancreatic nerve plexus invasion; M = male; mod = moderately differentiated adenocarcinoma; N = lymph node metastasis; poor = poorly differentiated adenocarcinoma; pTNM = pathological TNM; PV = portal vein invasion; RP = retroperitoneal invasion; S = serosal invasion. [c]Classified according to the classification of The General Rules for the Clinical and Pathological Study of Primary Pancreatic Cancer. April 2002, Pancreatic Cancer Study Group of Japan.

Table 3 Multivariate analysis of patients with pancreatic cancer

Variable	Odds ratio	95% CI	P
Tumour size (⩾2.0 cm)	1.995	0.639–6.226	0.2342
Lymph node metastasis	2.388	1.026–5.561	0.0435*
Invasion of retroperitoneal tissue (d.p.m.)	5.486	1.409–21.358	0.0141*
Invasion of plexus nerve (PI)	1.759	0.591–5.239	0.3103
Hypermethylation	3.157	1.226–8.130	0.0172*

*Statistical significance. CI = confidence interval.

northern blot analysis and RT-PCR. All of the nine cell lines showed methylation of the promoter CpG island of the gene. Moreover, hemizygous deletion of *RUNX3*, as detected by fluorescence *in situ* hybridisation, was found in most of the cell lines that lacked RUNX3 expression. Our results using primary pancreatic cancer tissue were compatible with their findings.

Li *et al* (2004) reported that RUNX3 expression was low-to-absent in normal pancreatic tissues, but increased in a third of cancer tissues by RT-PCR and immunohistochemistry. RUNX3 expression was present only in islets of the normal pancreas. They

also found that all metastases of pancreatic cancer tissues were devoid of or displayed only very faint RUNX3 expression by immunostaining.

Some groups have advocated islet cells as the cells of origin of pancreatic ductal adenocarcinoma (Pour *et al*, 2003). This would mean that the islet cells in pancreatic tissue are the tissue-specific stem cells in which cancer cells begin from the alteration in the oncogenes or tumour suppressor genes. RUNX3 is expressed in the tissue-specific stem cells, and only in islet cells in normal tissue. When cancer tissue has grown from the tissue-specific stem cells, the cancer cells express the RUNX3 protein. Some cancer tissues do not express RUNX3. In those cancer cells, RUNX3 gene is methylated. In cases with metastatic lesions, more aggressive tumour cells from the original lesion exist, such as RUNX3-methylated cells. Hence, the metastatic pancreatic cancer cells do not express RUNX3 gene.

Thus, it may be hypothesised that there is indeed loss of RUNX3 expression by promoter hypermethylation or LOH in some primary tumours compared with normal islets, and almost a complete loss in metastatic tumours. Our finding that the survival in methylated cases in *RUNX3* gene was significantly worse than that in unmethylated patients is compatible with this hypothesis, although pointing to a tumour suppressor role for RUNX3 in pancreatic cancer.

Nine of 11 LOH detected cases had hypermethylation of the RUNX3 promoter region. These findings imply that silencing of RUNX3 occurred biallelically. Complete silencing of this gene leads to the progression of cancer, and then relates to the worse prognosis.

In conclusion, we have clearly demonstrated for the first time that *RUNX3* is frequently methylated in primary pancreatic cancer tissues, frequent hemizygous deletion occurs at its locus in 1p36, and RUNX3-inactivated cases showed worse survival. We propose that inactivation of RUNX3 plays an important role in alteration of the TGF-β signalling pathway and in the tumorigenesis of pancreatic cancer.

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A gastrin transcript expressed in gastrointestinal cancer cells contains an internal ribosome entry site

AM Grabowska*[,1], CA Berry[1], J Hughes[1], M Bushell[2], AE Willis[2] and SA Watson[1]

[1]*Division of Pre-Clinical Oncology, School of Medical and Surgical Sciences, University of Nottingham, Nottingham, UK;* [2]*School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK*

As the hormone gastrin promotes gastrointestinal (GI) cancer progression by triggering survival pathways, regulation of gastrin expression at the translational level was explored. Sequence within the 5′ untranslated region of a gastrin transcript expressed in GI cancer cells was investigated, then cloned into a bicistronic vector upstream of firefly luciferase and transfected into a series of GI cancer cell lines. Firefly luciferase activity was measured relative to that of a cap-dependent *Renilla* luciferase. A gastrin transcript that was different from that described in Ensembl was expressed in GI cancer cells. Its transcription appears to be initiated within the region designated as the gene's first intron. In GI cancer cells transfected with the bicistronic construct, firefly luciferase activity increased 8–15-fold compared with the control vector, and there was a further induction of the signal (up to 25-fold) following exposure of the cells to genotoxic stress or hypoxia, suggesting that the sequence acts as an internal ribosome entry site. These data suggest that the gastrin transcript within GI cancer cells contains an internal ribosome entry site that may allow continued expression of gastrin peptides when normal translational mechanisms are inactive, such as in hypoxia, thereby promoting cancer cell survival.
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Gastrin is normally expressed in G cells of the stomach antrum and regulates both acid secretion and proliferation of gastric mucosal cells (Watson *et al*, 2006). The gastrin gene (*GAST*) is a 4-kb unit consisting of three exons and two introns with the gastrin polypeptide encoded by sequence within exons 2 and 3 (http://www.ensembl.org/index.html). Two different gastrin transcripts have been described in the literature. The transcript given in the Ensembl database was described in human gastrinomas and is a 434-bp transcript incorporating sequence from exon 1 (Ito *et al*, 1984; Wiborg *et al*, 1984). However, another transcript was identified in the gastric antrum that has a transcription start site 111 bp upstream of the start codon (Kato *et al*, 1983). Thus, the 5′ untranslated regions (5′UTRs) of the Ensembl and alternative transcripts are different.

Gastrin upregulation has been shown at both the gene and protein levels in a number of gastrointestinal (GI) (Goetze *et al*, 2000; Mukawa *et al*, 2005; Hur *et al*, 2006) and non-GI cancers (Rehfeld *et al*, 1989; van Solinge *et al*, 1993). At the transcriptional level in GI cancer, gastrin upregulation may be a result of mutational events, for example in the *APC* (adenomatous polyposis coli) or *k-ras* genes (Nakata *et al*, 1998; Koh *et al*, 2000); engagement of the EGF (epidermal growth factor) receptor (Merchant *et al*, 1991); inflammatory events mediated directly by cytokines, such as those associated with *Helicobacter pylori* infection (Suzuki *et al*, 2001; Beales, 2004); or direct activation by certain pathogenicity factors expressed by *Helicobacter pylori* (Rieder *et al*, 2005).

Expression of a number of genes that promote cancer cell survival has been shown to be regulated at the translational level (Pickering and Willis, 2005; Sontheimer and Carthew, 2005). One mechanism, involving the presence of an internal ribosome entry site (IRES) within the 5′UTR of the transcript, may have evolved to allow continued expression of key proteins involved in cell survival during cellular stress (Bushell *et al*, 2004; Holcik and Sonenberg, 2005) when conventional cap-mediated translation is reduced. However, it may also contribute to cancer cell survival as IRESs have been identified in the transcripts of genes that increase proliferation, protect against apoptosis and promote angiogenesis (Vagner *et al*, 1995; Miller *et al*, 1998; Stoneley *et al*, 2000a; Coldwell *et al*, 2001).

Gastrin plays an important role in establishing and supporting the growth of a range of GI tumours (Ferrand and Wang, 2006; Watson *et al*, 2006). As well as acting as a growth hormone, it has well-documented pro-angiogenic (Clarke *et al*, 2006) and anti-apoptotic properties (Konturek *et al*, 2003; Harris *et al*, 2004; Ramamoorthy *et al*, 2004). We have previously used RNAi to downregulate gastrin expression at the gene level and observed a rapid loss of the transcript, but also a delayed downregulation of the endogenous protein compared with GFP-tagged gastrin encoded by a transcript lacking the gastrin 5′UTR (Grabowska *et al*, 2007). This raised the possibility that gastrin expression may be regulated translationally in a manner dependent on the 5′UTR

*Correspondence: Dr AM Grabowska, Division of Pre-Clinical Oncology, University of Nottingham, D Floor, West Block, Queens Medical Centre, Nottingham NG7 2UH, UK;
E-mail: anna.grabowska@nottingham.ac.uk
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