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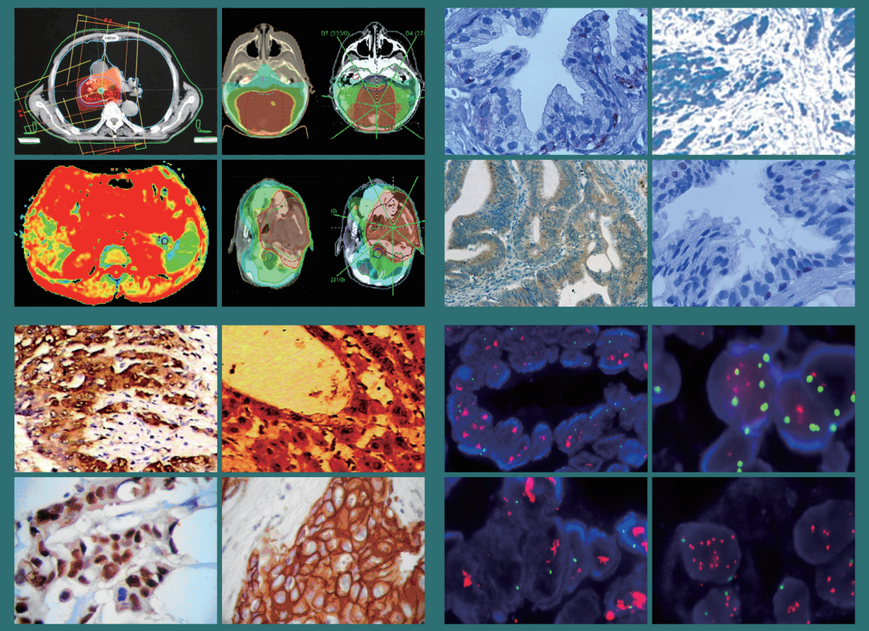

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EDITORIAL

The current status of laparoscopic pancreaticoduodenectomy for pancreatic cancer in China

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Pancreatic cancer is the fourth leading cause of cancer death worldwide and leads to an estimated 220 000 deaths per year [1]. The malignancy is difficult to detect and diagnose, as there are no noticeable signs or symptoms in the early stages of the disease, and the pancreas is located deep in the abdomen. Surgical resection is widely accepted as the only potentially curative therapy for pancreatic cancer. However, the prognosis and 5-year survival rate of patients with pancreatic cancer remain poor.

In recent years, the laparoscopic technique has been applied in many types of operations, and has been beneficial in the selected patients by resulting in reduced length of hospital stay and postoperative morbidity as well as enhanced recovery. Pancreaticoduodenectomy (PD), the main curative surgical procedure for pancreatic cancer, is considered one of the most complicated general surgeries owing to the extensive retroperitoneal dissection and reconstruction of the alimentary tract. In 1994, laparoscopic PD (LPD) surgery was first reported by Gagner in a patient with pancreatitis [2]. However, it was still not universally applied because of the complexity of the procedure, the need for significant laparoscopic skills, or the high cost of the specialized surgical equipment.

As a new strategy, LPD for pancreatic surgery is still in its early stages, and there are few reports comparing the laparoscopic approach to conventional or open PD (OPD). In 2009, Cho *et al* reported a study comparing the LPD approach with OPD [3]. Their study recruited 30 patients, of which 15 underwent laparoscopic-assisted PD. They concluded that, compared with OPD, laparoscopic-assisted pylorus-preserving PD showed comparable blood loss, resumption of oral intake, duration of stay, and incidence of complications. The disadvantage of laparoscopic PD is that the operative time is longer than that for conventional surgery. However, as the first

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retrospective analysis comparing the two approaches, the present study has significant limitations such as the small patient population and selection of surgical approaches in a nonrandomized manner. All patients in the open group had malignant tumors, while the laparoscopy-assisted group included patients with benign and low-grade malignant tumors. The unmatched histopathological results may lead to bias in the conclusions.

An increasing number of studies on LPD and OPD have been reported in the literature. However, these studies have the limitation of small patient populations.

In 2014, Croome *et al* reported their 5-year study results at the 134th American Society of Anesthesiologist (ASA) annual meeting [4]. Their 5-year study included 108 patients who underwent LPD at their institution. As a control group, they included 214 patients who underwent OPD. The results of this study suggested that outcomes using the laparoscopic approach are at least equivalent or better than those obtained using open approaches. Improved recovery in the laparoscopic group was suggested by the shorter hospital stay and reduced delay in initiating adjuvant treatments. The overall complication rate was not different between the two groups. Meanwhile, 75% of both the groups in their study received adjuvant treatment; however, a significantly smaller proportion of patients in the LPD group had a delay of greater than 56 days (8 weeks) between surgery and adjuvant chemotherapy compared with those in the OPD group (27% and 41%, respectively). Progression-free survival in patients with pancreatic cancer was better in the LPD group than in the OPD group.

LPD surgery started being used relatively late in China, since its introduction in 1994. The first report of LPD in China was by Lu *et al* in 2003 [5]. The operative time was 600 min and the patient developed an International Study Group on Pancreatic Fistula (ISGPF) level A pancreatic fistula. With conservative management, the patient was discharged 20 days after surgery. In the 4 months of follow-up, no evidence of recurrence was observed.

Soon after the first LPD surgery, an increasing number of institutions in China reported their experience with LPD surgery. However, in these studies, the patient numbers remain small and the quality of the study is low. Most are case reports and single-center experiences.

A meta-analysis comparing the current evidence on LPD versus OPD demonstrated that no differences were found in mortality or postoperative pancreatic fistula rates. However, LPD was associated with prolonged operative times, but lower intraoperative blood loss, less delayed gastric emptying, and shorter hospital stay. The results of the meta-analysis reflected the distinct advantages and disadvantages of LPD, including better patient recovery and longer operative time.

The pancreas is a unique organ with a deep retroperitoneal anatomical position. It has a complex relationship with its surrounding tissues. The abundant blood supply of the pancreas, the diverse anatomical planes of the peritoneum, and the complexities of digestive tract reconstruction hindered the development of laparoscopic techniques in pancreatic surgery. Many current laparoscopic systems can magnify the view of the surgical field, enabling precise dissection and separation, especially when anatomizing the superior mesenteric vessels. Meanwhile, with magnified vision, it is possible for surgeons to identify micrometastases in concealed areas of the abdomen and to remove the lymph nodes more carefully.

Improvements in surgery should be aimed towards achieving precision and minimal invasiveness. The emergence and development of LPD represent the pursuit of an accurate and minimally invasive technique. Although LPD has been performed successfully in many centers, some limitations must be addressed. The high cost of the surgical instruments, a steep and long learning curve for mastering the technique, the inconvenience of the surgical field transition, and the lack of sensory feedback play a significant role in preventing LPD from becoming a standard treatment for pancreatic cancer. However, with the unremitting efforts of surgeons and further development of technologies, laparoscopic surgery will have increased applications for pancreatic diseases.

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REVIEW ARTICLE

Laparoscopic middle pancreatectomy

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Abstract

Pancreaticoduodenectomy and distal pancreatectomy are the traditional surgical treatments of tumors in the neck or body of the pancreas. Although resecting the lesions successfully, these procedures can also, however, lead the substantial loss of normal pancreatic parenchyma, causing endocrine and exocrine function disorder. The combination of distal pancreatectomy with splenectomy increased the risk of thrombosis sometimes. So the surgical trauma is too great, especially for benign and lower malignant tumors located in the neck or body of the pancreas. While, middle pancreatectomy may decrease operative trauma with the excision of these lesions, and can maximize the retention of pancreatic parenchyma, and maintain pancreatic endocrine and exocrine function integrity. The procedure is interesting but rarely performed. With the application and development of minimally invasive surgical techniques, laparoscopic and robotic middle pancreatectomy are available now. Researches about laparoscopic and robotic middle pancreatectomy are presented, with decreased morbidity, reduced operation time and hospital stay. There is only a few reports on the two procedures, but the security and effectiveness of them are suggested.

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Key words: laparoscopic; middle pancreatectomy; minimally invasive surgery

Middle pancreatectomy spares the parenchyma and adjacent organs, and is indicated for small tumors deeply located in the pancreatic body, which are difficult to enucleate. Other lesions such as pancreatic trauma or arteriovenous malformations are also candidate targets [1]. Middle pancreatectomy is a rarely performed but interesting procedure, and the surgical purpose is to achieve radical removal while preserving full exocrine and endocrine pancreatic function. A minimally invasive approach for this procedure has not been widely described in the literature, and only a few reports on laparoscopic and robotic middle pancreatectomy are available.

Rotellar *et al* [2] described 9 consecutive patients with benign or low malignant potential lesions in the pancreatic neck or body, who underwent surgery from March 2005 to October 2007. Laparoscopic middle pancreatectomy is feasible and safe. Duct-to-mucosa pancreaticojejunostomy can be performed safely using this approach. The method of pancreatic transection seems to be a determinant of the incidence of cephalic stump fistulas [2]. Giulianotti *et al* [3] reported that robot-assisted laparoscopic middle pancreatectomy presents an interesting, less-invasive option for resection of benign tumors of the neck and proximal body of the pancreas. In benign disease, it allows for the preservation of functional pancreatic parenchyma and reduces operative trauma [3]. Rotellar and Pardo reported that laparoscopic middle pancreatectomy minimizes the procedure and maximizes the benefit [4].

Dokmak *et al* [5] reported the case of a 45-year-old woman diagnosed with branch duct intraductal papillary mucinous neoplasia (IPMN) at the pancreatic neck, which was discovered after numerous attacks of acute pancreatitis. The patient underwent pure laparoscopic middle pancreatectomy with right-to-left dissection and one-layer pancreatogastric anastomosis. Operative time was 160 min, with 20 mL of blood loss. A frozen section showed negative margins on both sides. The postoperative course was uneventful, with 15 days in the hospital. Histology confirmed the diagnosis of branch duct IPMN with moderate dysplasia and negative margins. The patient was symptom-free 6 months after surgery. Our results and the data in the literature suggest that the laparoscopic approach is indicated for

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middle pancreatectomy because there are no technical or oncological contraindications and the outcome is similar to that with the open approach [5]. Ishii *et al* [6] reported two cases in which physiological reconstructive procedures were performed. The reconstructive procedures included pancreatic duct-to-duct anastomosis and parenchymal sutures with absorbable monofilament interrupted stitches [6].

Zhang *et al* [7] described 10 patients ≥ 60 years old who underwent robot-assisted middle pancreatectomy from 2012 to 2015. All 10 cases were of benign or low-grade malignant lesions. The mean operative time was 175.00 min. The mean blood loss was 113.00 mL, with no blood transfusion needed. Postoperative fistulas developed in 5 patients; there were 2 Grade A fistulas and 3 grade B fistulas. Postoperative complications occurred in 3 patients, including 2 with Grade 1 or 2 complication and 1 with Grade 3 complication. There was no reoperation or postoperative mortality. The mean hospital stay was 19.91 days. After a median follow-up of 23 months, new onset diabetes mellitus developed in 1 patient. None had deterioration of previously diagnosed diabetes or exocrine insufficiency, and there was no case of tumor recurrence. The authors concluded that robot-assisted middle pancreatectomy was safe and feasible for elderly people. There was a low risk of exocrine or endocrine dysfunction, with a positive effect on long-term outcomes. The incidence of postoperative pancreatic fistula (POPF) was relatively high, but adverse outcomes could be prevented with careful perioperative management [7].

Addeo *et al* [8] reported the case of a 26-year-old man who underwent middle pancreatectomy for a 2-cm pancreatic neuroendocrine tumor. After transection of the pancreatic neck by endoscopic stapler, the body of the pancreas was progressively liberated right-to-left from the splenic vessel axis and clips were used to secure the small splenic arterial and venous branches. On the left side, the pancreatic body was transected distally using a harmonic scalpel. Pancreaticoenteric reconstruction was achieved with double purse-string telescoped pancreaticogastrostomy. A small seromuscular incision was made on the posterior gastric wall and 2 concentric purse-string sutures were applied. Through an anterior gastrostomy, the pancreatic body was telescoped into the gastric lumen and the 2 double purse-strings were tied. A final inner layer between the gastric mucosa and pancreatic serosa was fashioned through the anterior gastrostomy [8].

Chen *et al* [9] reported a prospective randomized controlled trial comparing the short-term outcomes of robot-assisted laparoscopic middle pancreatectomy (RA-MP) with open middle pancreatectomy (OMP). A total of 100 patients were included in the study to analyze primary and secondary endpoints. Demographic characteristics and pathological parameters were similar in both groups. Furthermore, length of hospital stay was significantly shorter (15.6 vs. 21.7 days, $P = 0.002$), median operative time was reduced (160 vs. 193 min, $P = 0.002$), median blood loss was lesser (50 vs. 200 mL, $P < 0.001$), the rate of clinical POPF was lower (18 vs. 36.0%, $P = 0.043$), nutritional recovery was better, return to usual activity was expedited (3.1 vs. 4.6 days, $P < 0.001$), and resumption of bowel movements was faster (3.5 vs. 5.0 days, $P < 0.001$) in the RA-MP group, compared to the OMP group. RA-MP was associated with significantly shorter length of stay, reduced operative time, lesser blood loss, lower clinical POPF rate, and expedited postoperative recovery, compared to OMP [9].

Middle pancreatectomy is a rarely performed but interesting procedure, and is indicated in cases of benign or low-grade malignant tumors located in the pancreatic neck or proximal body, where the surgical purpose is to achieve radical removal while preserving full exocrine and endocrine pancreatic function. A minimally invasive approach for this procedure has not been widely described in the literature, and only a few reports on laparoscopic and robotic middle pancreatectomy are available.

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REVIEW ARTICLE

The application of the robotic surgical system in pancreaticoduodenectomy

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Abstract

Owing to the operative complexity, the application of minimally invasive surgery to pancreatic procedures has been delayed. However, with advances in technique, and since the introduction of robotic systems in particular, pancreatic minimally invasive surgery has made much progress. Laparoscopic and robotic technology has been widely adopted. The safety and feasibility of minimally invasive procedures for pancreaticoduodenectomy have been confirmed in many reports. However, even with these advantages, laparoscopic and robotic surgery cannot completely replace laparotomy. Pancreatic surgeons need to master these three operative methods to be able to handle complicated clinical situations.

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Key words: pancreaticoduodenectomy; laparoscopic surgery; robotic surgery

Pancreaticoduodenectomy (PD) is a major surgical procedure involving the pancreas, duodenum, common bile duct, pylorus, and jejunum for the treatment of neoplasms of the pancreatic head or ampullary region. PD has always been challenging, mainly because of its technical complexity and the difficulty associated with extensive visceral organ dissection and reconstruction of the digestive tract. Therefore, PD is normally performed using an open approach. With the rapid development of technology, some pancreatic procedures are performed laparoscopically. The use of laparoscopy is relatively restricted in PD because of its intrinsic technical limitations. Distal pancreatectomy accounts for the majority of laparoscopic pancreatic procedures. Compared with its use in urology, gynecology, and other surgical fields, the adaptation of laparoscopy in pancreatic surgery has been relatively slow. In the past 20 years, robotic surgery has rapidly advanced and has been widely adopted in pancreatic surgery because of its unique advantages. This surgical system with its sophisticated devices allows surgeons to attempt more challenging robotic cases of PD. The pancreatic surgery center of Ruijin Hospital was one of the earliest agency to perform pancreatic operations in China. Our center installed the da Vinci surgical system model S in 2010. To date, we have performed nearly 200 cases of robotic PD. We installed the newest Model Si in January 2016, and promptly began performing robotic pancreatic surgeries with the new model.

Development history

PD was first performed in the 19th century. In the 1940s, Whipple [1], Child [2], and other pancreatic surgeons established a standard PD procedure that is still being used today. Near the end of the 20th century, open PD surgery had matured. Laparoscopic technology has been gradually adopted since its invention in the mid-20th century. The first case of laparoscopic surgery was a cholecystectomy, performed by Philippe Mouret [3] in March 1987. The robotic surgical system was invented at the end of the 20th century and has been clinically relevant ever since. The first case of robotic surgery was also a cholecystectomy, performed by Himpens [4] in 1998. Since then, the use of robotic systems has spread quickly to other surgical fields. The complexity of PD

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and limitations of laparoscopic devices impeded further growth of laparoscopic pancreatic surgery. The robotic surgical system provides three-dimensional imaging, thus making the surgical field more real and vivid. The multi-angle rotatable EndoWrist can synchronize itself to the surgeon's hand motion. This device can also filter out hand tremor. These advantages have broadened the application of robotic systems to pancreatic surgery. Giulianotti [5] first reported robotic PD in 2003, only 5 years after the first robotic cholecystectomy had been reported.

New developments in laparoscopic PD

Laparoscopic surgery, with its good surgical outcomes, has been accepted by the majority of patients in the past 20 years and has become the first choice for some operations. Gagner [6] first reported laparoscopic PD in 1994. Uyama [7] first reported laparoscopic mini-laparotomy PD in 1996. With the continuous improvement in laparoscopic techniques and instruments, laparoscopic pancreatic surgery also advanced. Multiple pancreatic centers have reported their use of laparoscopy in PD, including total laparoscopic PD [8–9] and hand-assisted laparoscopic PD [10–11]. In a review [12] of laparoscopic PD, Gagner noted that 146 cases reported from 1994 to 2009 had a 46% conversion rate, and average operative times and blood loss of 439 min and 143 mL, respectively. Since then, there have been multiple reports of laparoscopic PD from other institutions [13–15]. Also meta-analyses comparing open and laparoscopic PD [16] and laparoscopic and robotic PD [17] have been published by many agencies.

New development in robotic PD

The da Vinci surgical system was introduced in 1997 by Intuitive Surgical Inc. (USA), and received the Food and Drug Authority (FDA) operating license in 2000. This surgical system compensated for the shortcomings of traditional laparoscopy. Giulianotti [5] reported the first robotic PD in 2003. In this article, 8 robotic PD procedures were performed in an average of 490 min, with morbidity and mortality rates of 37.5% and 12.5% respectively. Giulianotti [18] performed 60 cases of robotic PD in the USA and Italy from 2003 to 2009. Zureikat [19] performed 30 cases of robotic PD in the USA from 2008 to 2010. As of 2012, Zureikat [20] and colleagues had performed 250 robotic pancreatic surgeries, including 132 cases of robotic PD. Choi [21] reported the first robotic PD in South Korea in 2011. With cases of robotic PD being reported frequently, the safety and feasibility of robotic surgery has also been confirmed. Buchs [22] noted that robotic PD offers remarkable advantages in terms of operative time, blood loss, and number of resected lymph nodes, compared with the results for open PD, with no significant differences in postoperative hospital stay, complications, and mortality rate. Another report by Buchs [23] indicated that robotic PD is also safe for elderly patients. Horiguchi [24] reported that patients who underwent robotic PD had a shorter postoperative hospital stay and earlier resumption of oral intake.

As of December 2011, 2,132 robotic surgical systems have been installed worldwide, with 13 devices installed in China. Zhou and colleagues firstyly reported 8 robotic PD cases in China in 2009, with a mean operative time and mean blood loss of 718 min and 153 mL, respectively. This surgical team reported 44 robotic pancreatic surgeries in 2011, including 16 robotic PD cases. Our pancreatic center also published multiple reports about the technique and experiences with robotic pancreatic surgery [25–26]. Few institutions have the prerequisites to carry out robotic surgery in China. Shanghai Ruijin Hospital and The Second Artillery General Hospital PLA perform relatively large numbers of robotic pancreatic operations. The results for procedures, operative time, blood loss, complications, and mortality rate are similar to those reported by high-volume centers worldwide. Shanghai Ruijin Hospital has completed more than 700 robotic pancreatic surgeries since 2010. A total of 217 robotic PD cases have been performed, with a mean operative time and mean blood loss of 332 min and 378 mL, respectively. Pancreatic fistulas occurred in 24.5% of patients. Of these, 60% were grade A pancreatic fistulas, and recovered without incident after drainage. Our center conducted a prospective study [27] of surgical outcomes comparing open and robotic PD. Results from this study indicate that robotic PD has a longer operative time, but less blood loss, earlier resumption of oral intake, and shorter postoperative hospital stay than open PD does, with no significant differences in mortality and survival rate.

Advantages of robotic PD

The visual field in laparoscopy is 2-dimensional (2D), while the robotic surgical system provides 3-dimensional (3D) visualization, with up to 15× magnification. The 3D image in the console viewer allows surgeons to see anatomical structures in high definition and natural colors, making the visual field more real and vivid. Surgeons can easily distinguish small vessels to reduce bleeding. The end of the laparoscopic instrument can only rotate along one axis and cannot be bent. Dexterous control of the instrument is difficult. With more complicated situations like small operative spaces or deep mass locations, it became problematic to smoothly continue procedures. EndoWrist instruments equipped on 3 robotic arms can bend and rotate to a greater degree than the human wrist. The multi-angle rotatable EndoWrist can synchronize itself with the surgeon's hand motion and can filter out hand tremor. These advantages enable more stable

hemostasis, suturing, and other procedural steps during the operation.

Surgical training is relatively long for laparoscopy. Adapting from the 3D perspective of open surgery to the 2D imaging of laparoscopic surgery can be difficult. Robotic surgery provides 3D vision similar to that in laparotomy. The adaptation period is decreased. Surgeons with previous experience in laparoscopic surgery have an even shorter learning curve. Surgeons can sit and operate through a console system that requires minimal direct contact with the patient, allowing taking breaks during the operation without having to scrub again. These factors help surgeons reduce fatigue and enable concentration for a longer duration.

Prospects

The safety and feasibility of minimally invasive pancreatic surgery, and robotic surgery in particular, have been demonstrated repeatedly. The trend in pancreatic surgery is minimally invasive procedures. However, even with their numerous advantages, laparoscopic and robotic surgery cannot completely replace laparotomy. Pancreatic surgeons need to master these 3 complementary operative methods to be able to manage complicated clinical situations.

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ORIGINAL ARTICLE

Clinical efficacy of total three-dimensional laparoscopic pancreatoduodenectomy

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Abstract

Objective To investigate the feasibility and clinical efficacy of total three-dimensional laparoscopic pancreatoduodenectomy.

Methods The clinical data of 28 patients who underwent total three-dimensional laparoscopic pancreatoduodenectomy at the Second Hospital of Hebei Medical University from August 2015 to May 2016 were retrospectively analyzed. The surgical indications and method of performing total three-dimensional laparoscopic pancreatoduodenectomy were similar to those of the patients who underwent two-dimensional laparoscopic pancreatoduodenectomy. All of the patients were followed up via outpatient reviews and telephone interviews through September 2016.

Results In all 28 cases, total three-dimensional laparoscopic pancreatoduodenectomy was successfully performed with no conversion to laparotomy, intraoperative complications, or perioperative death. The mean operative time was 406 min (200–520 min) with a mean blood loss of 528 mL (200–1500 mL), a mean number of dissected lymph nodes of 11 (6–16), a mean postoperative anus exhaust time of 4.4 d (2–8 d), and a mean length of stay of 16.9 d (9–23 d). There was a postoperative pancreatic fistula in 4 out of the 28 cases, with 3 cases of grade A and 1 case of grade B. Postoperatively, one patient with early-stage intra-abdominal hemorrhage improved after conservative symptomatic treatment, and two patients with gastroplegia were cured with conservative treatment. No complications occurred in the other patients. All of the cases underwent R0 resection with a negative surgical margin. All of the 28 patients were followed up for 6 to 12 months, with a median follow-up period of 9.2 months. During the follow-up period, there were no postoperative complications related to the procedures and no deaths; tumor recurrence was identified 9 months after the procedure using positron emission computed tomography (PECT) in one patient with pancreatic ductal adenocarcinoma.

Conclusion Total three-dimensional laparoscopic pancreatoduodenectomy is safe and feasible for the treatment of periampullary carcinoma, with the advantage of favorable short-term outcomes.

Key words: periampullary carcinoma; laparoscopy; pancreatoduodenectomy; three-dimensional

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Since the first case of laparoscopic pancreatoduodenectomy was performed by Gagner [1] in 1992, domestic and foreign scholars have carried out a large number of in-depth studies. Up to now, surgeons have been performing laparoscopic pancreatoduodenectomy using advanced endoscopic technology to complete the resection of periampullary carcinomas [2–3]. Along with the development of a three-dimensional laparoscopic surgery system, scholars at home and abroad have started to apply this new technology to laparoscopic pancreatoduodenectomy. The author's institution has performed three-dimensional laparoscopic pancreatoduodenectomy in recent years, mainly for periampullary carcinomas. In this study, the clinical data of 28 patients who underwent total three-dimensional laparoscopic pancreatoduodenectomy for periampullary carcinomas from August 2015 to May 2016 in our department were retrospectively analyzed; the objective was to explore the feasibility and clinical efficacy of the operation.

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Materials and methods

General information

There were 16 men and 12 women with 28 periampullary neoplasms enrolled; their ages varied from 27 to 72 (mean age, 58.5) years and a mean preoperative bilirubin level of 74.08 mmol/L (4.3–236.47 mmol/L). Eleven of them underwent preoperative biliary drainage. This study was approved by the Ethics Committee of our hospital, and the patients and their relatives signed an informed consent form before surgery.

Inclusion and exclusion criteria

Inclusive criteria: (1) Duodenal papilla and periampullary carcinoma, distal common bile duct carcinoma, pancreatic cancer, or duodenal carcinoma; (2) Well-functioning vital organs, such as the heart and lungs, which can tolerate a long time under pneumoperitoneum; (3) The preoperative bilirubin level did not exceed 400 mmol/L; (4) No contraindications for surgery. Exclusive criteria: (1) Distant metastasis of the tumor was found before the operation; (2) Great vessels such as the superior mesenteric artery and vein were invaded by the tumor; (3) Cardiopulmonary dysfunction and other contraindications for surgery.

Operation methods

The procedures were performed with patients supine in the straddled reverse Trendelenburg position under conventional tracheal intubation and general anesthesia. The operations were performed with 5 trocars. The operators' position, establishment of pneumoperitoneum, placement of trocars, and other surgical procedures, were all the same as those used with traditional two-dimensional laparoscopes. A total three-dimensional laparoscopic pancreatoduodenectomy meant the tumor resection, lymph node dissection, mesopancreas excision, and alimentary canal reconstruction (Child procedure) were performed under the three-dimensional laparoscope; a classical mucosal-to-mucosal pancreatojejunostomy was performed with the placement of a supporting tube in the pancreatic duct [4].

Follow-up

All the patients were followed up via outpatient reviews and telephone interviews. The follow-up period ended in September 2016.

Results

In all 28 cases, total three-dimensional laparoscopic pancreatoduodenectomy was successfully performed with no conversion to laparotomy. There were no intraoperative complications or perioperative deaths. The mean operative time was 406 min (200–520 min) with a mean blood loss of 528 mL (200–1500 mL), a mean number of dissected lymph nodes of 11 (6–16), a mean postoperative anus exhaust time of 4.4 d (2–8 d), and a mean length of stay of 16.9 d (9–23 d). According to the definition of pancreatic fistula by the International Study Group on Pancreatic Fistula (ISGPF) [5], there was a postoperative pancreatic fistula in 4 out of the 28 cases, with 3 cases of grade A and 1 case of grade B. Postoperatively, one patient with early-stage intra-abdominal hemorrhage improved after conservative symptomatic treatment; two patients with gastroplegia were cured with conservative treatment. No complications occurred in the other patients.

Postoperative pathological evaluation findings: there was duodenal adenocarcinoma in 10 cases, choledochal adenocarcinoma in 9 cases, ampullary adenocarcinoma in 1 case, pancreatic ductal adenocarcinoma in 5 cases, pancreatic adenocarcinoma in 1 case, duodenal gastrointestinal stromal tumors in 1 case, and chronic inflammation of the pancreatic tissue in 1 cases. All the specimens underwent R0 resection with a negative surgical margin identified under a microscope.

All of the 28 patients were followed up. The follow-up period ranged from 6 to 12 months with a median follow-up time of 9.2 months. During the follow-up period, there were no postoperative complications related to the procedures. A tumor recurrence was found 9 months after the procedure on positron emission computed tomography (PECT) in one patient with pancreatic ductal adenocarcinoma; no patients died.

Discussion

Birkett [6] and his colleagues were the first to report the use of a three-dimensional laparoscopic system in gastrointestinal surgery. The operation was very difficult due to the technological limitations at that time. With increasing advances in development of three-dimensional laparoscopic systems, surgeons have gradually accepted this technology and applied it to hernia repair, proctocolectomy, and hysterectomy, as well as urological, cardiothoracic, and other surgeries, which have achieved excellent results [7]. However, the application of a three-dimensional laparoscopic system in pancreatoduodenectomy has been rarely reported.

The advantages of a three-dimensional laparoscopic system include accurate space location and apparent feeling of depth in vision, which makes it more accurate during the isolation of important blood vessels and lymphadenectomy; owing to the outstanding stereoscopic visualization, the grasp of surgical margins or operation distance is more accurate. It is helpful in three-dimensional judgments about the exchange operations of the needle-holding apparatus and knotting when performing

precise anastomoses (such as vascular anastomosis) and alimentary canal reconstruction. These benefits mean the operation is more convenient, thus the surgical mistakes are fewer and the learning curve is diminished, which makes it friendlier to beginners. In addition, costing no more than two-dimensional laparoscopic systems, it helps in the promotion and improvement of laparoscopic technology [8].

After completion of 28 cases of total three-dimensional laparoscopic pancreatoduodenectomy, we have realized that the surgical procedures and skills as well as the indications and contraindications necessary to be mastered under a three-dimensional laparoscopic system were in accordance with those needed to master a two-dimensional laparoscopic system. However, the total three-dimensional laparoscopic pancreatoduodenectomy was superior to the total two-dimensional laparoscopic pancreatoduodenectomy in operative time, blood loss, number of dissected lymph nodes, length of stay, results of radical resections of tumors, and occurrences of complications, with no differences in survival time. However, there has been no prospective randomized trial comparing two to three-dimensional surgery yet.

The technological difficulties of laparoscopic pancreatoduodenectomy are: (1) The completeness of mesopancreas excision; (2) The completeness of lymph node dissection; (3) Hemostatic technology; 4. Pancreatojejunostomy. The deep position of the uncinate process of the mesopancreas with the superior mesenteric artery and the root of the celiac truncus surrounded results in poor exposure and a difficult operation, which is one of the key reasons that people question the completeness of laparoscopic pancreatoduodenectomy. A total mesopancreas excision requires the complete removal of the nerves and lymphoid tissues within the right side and 180° of the superior mesenteric artery. The author has experienced the characteristics of a three-dimensional laparoscope, such as apparent feeling of depth in vision, high partial magnification times, and accurate space location. Intraoperatively, the descending part and level part of the duodenum are adequately mobilized to the front of the abdominal aorta, and the superior mesenteric vein and portal vein are suspended by a sling to adequately expose the trunk and root of the superior mesenteric artery. The soft tissue on the right side of the artery is separated along the arterial sheath to further severe the arterial branches one by one until reaching the root of the artery. Then, it is mobilized upward until dissociated from the root of the celiac truncus and comes to the right crura of the diaphragm. Thus, the goal of total mesopancreas excision is achieved.

Complete lymph node dissection is conducive to a better prognosis of patients. With the high spatial resolution of a three-dimensional laparoscope, a strong sense of the stereo dimensions, and an accurate hold of the tissues, we generally use two approaches from anterior and posterior to dissect the lymph nodes, which can get even better results than an open lymph node dissection. First, the common hepatic artery is found at the upper edge of the pancreas by the anterior approach and taken as an axis to open the arterial sheaths and fully expose the common hepatic artery and proper hepatic artery. Then, the dissection is performed until reaching the hepatic portal, and the gastroduodenal artery and right gastric artery are severed at the same time. Second, the duodenal circle and pancreatic head are raised through the posterior approach to dissect the no. 16th lymph node between the inferior vena cava and abdominal aorta. At the time of mesopancreas excision, the lymphoid tissues at the superior mesenteric artery and the root of the celiac truncus are dissected by the posterior approach until reaching the junction of the anterior approach. The lymphoid tissue is totally removed through the anterior and posterior approaches, which achieves the goal of radical dissection.

Skilled hemostasis and vascular suture technology is one of the key technologies that guarantee the success of laparoscopic pancreatoduodenectomy. The main reason for converting to laparotomy are the numerous vessels around the pancreas, which lead to poor exposure and can hemorrhage easily with massive blood loss during isolation. Under the three-dimensional laparoscope, the observation of hemorrhagic spots is more precise and the sutures can be more accurate. In particular, during the resection and reconstruction of the superior mesenteric artery and vein invasion, the view on the three-dimensional laparoscope can guarantee the accuracy of needle-puncturing angle, needle distance, and needle margin, which avoids winding and knotting of the sutures and makes the vascular reconstruction faster and more accurate.

Pancreatojejunostomy is the key of a successful laparoscopic pancreatoduodenectomy. According to the reports in the literature, the incidence of postoperative pancreatic fistula for laparoscopic pancreatoduodenectomy is 0%–38.95% [9]. Pancreatic fistula often leads to abdominal hemorrhage or upper gastrointestinal hemorrhage with an incidence of 11%–45% [10]. Based on our previous experience, we performed a classical duct-to-mucosal and end-to-side pancreatojejunostomy, with supporting tubes in the pancreatic duct [11] .

Under the three-dimensional laparoscope, the pancreas end is observed with a strong sense in stereo dimensions and high partial magnification, which allows us to avoid injuries to the portal vein and splenic artery, and makes it easier to understand the needle distance. We utilize double purse string sutures to achieve continuous suture of the pancreatic duct to the jejunum mucosa and the capsule of the end of the pancreas to the seromuscular layer of jejunum. Under stereo visualization of three-

dimensions, the double purse string sutures are not likely to wind or knot. It is faster, more accurate, and more time-saving when the operator picks the needles, sutures, or knots, and when the assistant helps the operator to adjust the angle of the suture knot or to tighten the suture. The anastomosis is more precise with better results. The incidence of pancreatic fistula was 16.7% and mostly were grade A, for which extubation can be done 3 days after the procedure.

Although the three-dimensional laparoscope possesses these advantages, there are still problems in practice that needed to be solved. A three-dimensional laparoscope of 30° could not change the viewpoint by rotating the section angle of the lens, which made it difficult to expose the posterior anatomic structure when there was a block in the target view. A slight tremor of the holding hand or quick rotation of the lens caused obvious shaking of the visual field along with dizziness, eye exhaustion, blurred vision, nausea, and other symptoms of visual fatigue, which is different from previous reports in the literaturee, but these symptoms could be gradually relieved and eventually disappeared after practice with the system. In addition, owing to the advantages of the three-dimensional laparoscope in the aspects of stereo space identification and precise operation, as well as shortening the operative time and reducing the intraoperative mistakes to some degree, it had higher requirements for the operators. Therefore, a highly skilled and remarkably cohesive team is required to maximize the advantages of three-dimensional laparoscope.

In conclusion, the safety and feasibility of the application of three-dimensional laparoscopic technology to pancreatoduodenectomy can be ensured as long as the advantages of three-dimensional laparoscope are maximized and when surgeons learn to overcame its obstacles, and a skilled operation team is established, which can further develop laparoscopic pancreatoduodenectomy and make it a conventional choice for treating diseases such as periampullary carcinoma.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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Case Report

A case of chronic pancreatitis treated by laparoscopic duodenum-preserving pancreatic head resection

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Abstract

Pancreaticoduodenectomy (PD) has long been used for chronic pancreatitis (CP), but greatly affects the postoperative quality of life. A new procedure called duodenum-preserving pancreatic head resection (DPPHR) has been introduced, and has little effect on the structure and function of the digestive system. With the development of minimally invasive surgical techniques, treatment of CP can be performed with laparoscopic DPPHR (LDPPHR). We present a case of CP that was successfully treated with LDPPHR. The postoperative pathological diagnosis was pancreatitis, demonstrating the feasibility of LDPPHR. We recommend this minimally invasive surgical method as preferred treatment for CP.

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Key words: chronic pancreatitis (CP); duodenum-preserving pancreatic head resection (DPPHR); laparoscopic

As a benign inflammatory disease, chronic pancreatitis (CP) is characterized by the progressive conversion of pancreatic parenchyma to fibrous tissue, with associated complications, and may require surgical treatment. The incidence of CP is about 0.01% [1]. In the vast majority of patients with CP, pain is the presenting symptom. However, CP can lead to obstructive jaundice and digestive tract obstruction. The treatment of CP includes medical management and surgical intervention. From initial treatment with pancreaticoduodenectomy (PD) to current management with duodenum-preserving pancreatic head resection (DPPHR), surgical procedures are continually improving, resulting in decreased surgical trauma. With the development of minimally invasive surgery, most of these procedures can be performed laparoscopically, with further reduction in surgical trauma.

Case report

A 46-year-old woman was admitted to our department with vague abdominal pain. She had a 6-month history of recurrent epigastric pain, which caused great distress. She agreed to treatment after CP was diagnosed. Physical examination was nonspecific. Abdominal ultrasonography revealed a large mass in the head of the pancreas. Contrast-enhanced thin-section computed tomography revealed a mass of the pancreatic head, with a dilated main pancreatic duct. The mass was highly suspected of being inflammatory. Magnetic resonance cholangiopancreatography and endoscopic retrograde pancreatography demonstrated dilatation and tortuosity of the main pancreatic duct, suggesting the presence of CP. Serum amylase, urine amylase, serum carbohydrate antigen (CA) 19–9, serum carbohydrate antigen (CA) 125, and carcinoembryonic antigen (CEA) levels were within normal limits; indices of liver and kidney function were all within normal limits. With the initial diagnosis of CP, pain management was instituted. Six months later, with the failure of medical management and endoscopic interventions, she requested surgery. She reported progressive severity, and described her pain as unmanageable. Pancreatic head resection was planned and a laparoscopic operation was performed. During the operation, a mass was detected in the pancreatic head. DPPHR was performed. The resected specimen was a solid mass on macroscopic examination. Postoperative pathological examination showed chronic inflammation

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in the pancreatic parenchyma.

Surgical procedure

The patient was placed in the supine position, with anti-Trendelenburg position (10–30°) as necessary. A 12-mm trocar was placed slightly below the umbilicus, and a pneumoperitoneum was established. Two 12-mm trocars were then placed lateral to the first trocar in the right and left midclavicular lines. Two 5-mm trocars were then placed at the right and left infracostal arch at the anterior axillary lines. The operating surgeon stood on the right side, and the assistant stood on the left. The camera surgeon stood between the legs of the patient. With access established successfully, the entire abdomen was laparoscopically examined to exclude abnormalities. The gastrocolic omentum was opened to access the gastrohepatic omentum. Then the gastrohepatic omentum was opened to visualize the pancreas. The stomach was inverted. Plastic vascular clips were placed on the gastroduodenal artery, which was then resected. In order to expose the entire pancreatic head, we skeletonized the gastrocolic trunk and resected the branches. Most of the pancreatic head was resected, preserving a 0.5-cm wide area of parenchyma close to the duodenum to avoid damage to the biliary duct. The small bowel was cut 40 cm distal to the ligament of Treitz. With careful hemostasis, the distal small intestine was raised cephalad to establish a pancreaticojejunal anastomosis. Approximately 40 cm distal to the pancreaticojejunal anastomosis, a tension free retrocolic side-to-side enteroenterostomy was performed. We placed a drain alongside the anastomosis. Recovery was smooth and uncomplicated, and the patient was discharged on postoperative day 9. She was satisfied with her postoperative quality of life.

Discussion

CP is a benign inflammatory disease in which the pancreatic parenchyma converts to fibrous tissue. The main symptom is pain in the majority of CP patients, possibly due to intraductal and interstitial hypertension, with neurogenic and central sensitization [2–4]. With disease progression, the pain will become persistent and intractable. When medical management fails, endoscopic intervention can be performed. Surgery is the last resort when all other measures have failed. Surgery for CP can be divided into two categories: drainage or removal. Simple drainage refers to longitudinal pancreatic duct jejunal anastomosis; this preserves healthy tissue, but does not remove inflammatory tissue, especially in the pancreatic head, which is considered to be the "pacemaker" of pancreatic pain. The efficacy is only 50%, and the procedure can only be applied in patients with pancreatic duct enlargement. The removal surgery initially applied in CP was the standard Whipple procedure. As simple drainage procedures do not ensure sufficient pain relief in patients with enlargement of the pancreatic head [2, 5], resection of the pancreatic head should be a central feature of any surgical procedure [2]. DPPHR was first performed by Beger and colleagues [6–7]. Modified procedures include the Frey operation, Buchler operation, Imaizumi operation, and others. The most important advantage of this procedure is preservation of blood glucose levels and control of enteric motility [8]. The Frey procedure improves overall drainage by decompressing both the main and small ducts in the pancreatic head [2], but resection of the pancreatic head, or "pacemaker," is incomplete, leading to the recurrence of symptoms. Despite efficacy demonstrated in randomized controlled trials, PD/LPD has very limited application for this benign disease due to high morbidity and mortality associated with the procedure [2, 9–10]. As an additional limitation, PD/LPD usually disrupts digestive function, and endocrine and exocrine functions are impaired, resulting in decreased postoperative quality of life. When compared with DPPHR, PD/LPD is associated with significant side effects due to resection of the duodenum, pylorus, and bile duct [11–13]. Similarly, pylorus-preserving PD is seldom used due to higher postoperative morbidity [14]. In recent years, DPPHR has gradually replaced PD [15–17]. However, no procedure is ideal for CP [17].

Many pancreatic diseases including CP are now routinely treated by laparoscopic surgery [18–20]. Initial medical treatment is recommended in order to avoid surgery [2]. However, early surgical pancreatic drainage is beneficial for preservation of function, which is important for a patient with CP [21–25], as well as for pain control. Early surgical intervention can reverse the pathologic process rather than simply suspending or stabilizing it [26]. We performed our first laparoscopic DPPHR (LDPPHR) procedure on the present case, which was diagnosed with CP in 2014. We had a relatively clear preoperative diagnosis, other than the possibility of a malignant tumor, so we performed minimally invasive surgery with LDPPHR. We modified the Beger procedure. Without resorting to a Kocher incision, we protected the retroperitoneal small blood vessels. Blood flow in retroperitoneal branches to the duodenum (especially the descending branches) was retained in order to avoid the formation of a retroperitoneal effusion and subsequent infection. Under the premise of sparing at least one pancreaticoduodenal arch, we resected the pancreatic head. This procedure is not only more thorough than the Beger and Frey procedures for removal of diseased tissue, but also reduces the operative steps, as well as the possibility of postoperative complications. We did not cut the pancreas without first isolating the portal vein, thus

reducing intraoperative bleeding. We retained a thin layer of pancreatic parenchyma between the common bile duct and duodenum, and posterior to the common bile duct, thereby avoiding postoperative bile duct ischemia and subsequent complications.

Compared with open surgery, laparoscopic surgery is more difficult. In some cases, the patient may be converted to an open procedure. Thus, the patient who requests LDPPHR should be carefully evaluated. A dilated diameter of the duct of Wirsung greater than 4 mm is essential, making it convenient to perform pancreaticojejunostomy using laparoscopic instruments. A history of acute pancreatitis is a contraindication, but surgery can still be performed at least six months following an attack. Of course, before LDPPHR is performed, malignant tumors must be excluded.

In conclusion, LDPPHR is feasible, but patients should be carefully selected. The highly technical procedure must be carefully performed by experienced surgeons. In order to reduce postoperative complications, the surgeon should be familiar with the anatomy and the essentials of the operation.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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ORIGINAL ARTICLE

Clinicopathological features of hypoxia-inducible factor-1α and vascular endothelial growth factor expression in patients with lung cancer*

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Abstract

Objective The aim of the study was to investigate the clinicopathological characteristics of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression in patients with lung cancer.

Methods Cancerous and noncancerous tissues were collected post-operation from 115 patients with lung cancers by the self-control method. Total RNA was extracted from the lung tissues. The status of tissue HIF-1α expression and intercellular distribution was observed by immunochemistry using a tissue microarray. The expression levels of circulating HIF-1α and VEGF were detected by enzyme-linked immunosorbent assay (ELISA).

Results The expression of serum HIF-1α [(138.3 ± 28.8) μg/L] in the group of patients with lung cancer was significantly higher ($P < 0.01$) than that in the group of patients with pneumonia [(58.8 ± 14.5) μg/L] and the control group of patients [(24.1 ± 3.3) μg/L]. There was a strong positive correlation of serum HIF-1α levels ($r = 0.937$, $P < 0.01$) with serum VEGF levels. The specific concentration of total RNA [(1.52 ± 1.14) μg/mg wet lung tissues] in the cancerous tissues was significantly higher ($t = 8.494$, $P < 0.001$) than that in the noncancerous tissues [(0.58 ± 0.33) μg/mg]. The clinicopathological features of HIF-1α expression in lung cancer tissues revealed a significant relationship between positive HIF-1α expression and patient sex ($\chi^2 = 4.494$, $P = 0.034$), tumor size ($\chi^2 = 4.679$, $P = 0.031$), differentiation degree ($\chi^2 = 8.846$, $P = 0.012$), and presence of lymphatic node metastasis ($\chi^2 = 6.604$, $P = 0.037$).

Conclusion Abnormal HIF-1α expression in lung cancer is closely related with nucleic acid metabolism and angiogenesis, and it may be helpful in the diagnosis and identification of lung cancer.

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Key words: lung cancer; hypoxia-inducible factor-1α (HIF-1α); nucleic acid metabolism; enzyme-linked immunosorbent assay (ELISA); diagnosis

Lung cancer is one of the human malignancies with the highest incidence and the leading cause of cancer-related mortality worldwide [1–2]. Survival after lung cancer is still limited; recent studies have reported a 5-year survival rate of approximately 16% and 10-year survival rate of up to 90% when patients with lung cancer underwent prompt surgical treatment at stage I of the disease [3–4]. Angiogenesis is required for invasive tumor growth and metastasis, and plays an important role in the development and progression of lung cancer [5–6]. Cancer results in an increase in the overall oxygen consumption by cancer rapid growth, leading to a high expression of hypoxia-inducible factor-1α (HIF-1α), which stimulates angiogenesis and the release of related factors [7–8]. During hypoxic conditions, HIF-1α is dramatically stabilized and activated through a series of signaling processes, which

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then activate transcription of a network of genes that control several aspects of tumor biology, such as energy metabolism, angiogenesis, cellular growth, and apoptosis [9–10].

HIF-1α usually indicates the hypoxia index and has recently been used in the evaluation of many tumors [11–12]. The association between hypoxic tissue and vascular endothelial growth factor (VEGF), an angiogenesis-related factor, is worth exploring, as the over-expression of HIF-1α might indicate poor prognosis for patients with lung cancer [13–15], which is known to be associated with enhanced intra-tumoral hypoxia, increased tumor invasion, and increased metastasis frequency, and affects radiotherapy or chemotherapy [15–16]. However, the association between HIF-1α expression and its clinical significance in lung cancer remains to be systematically assessed. Therefore, to examine this association, in the present study, we investigated the levels of HIF-1α expression in lung tissue and circulating blood in patients with lung cancer.

Materials and methods

Lung tissues

Adenocarcinoma and noncancerous tissues were collected post-operation from 115 patients with lung cancer (58 men and 57 women; age, 39–77 years; median age, 58 years) using the self-control method at the Affiliated Hospital of Nantong University, Nantong, China from January 2012 to February 2015. All lung specimens were collected from patients diagnosed with lung cancer according to histological classification. Pathological examination of some sections (hematoxylin and eosin staining) was performed, and the rest tissues were kept at –80°C.

Serum samples

Serum samples were obtained simultaneously from these 115 patients with lung cancer. Patients with pneumonia (n = 30) and patients of a healthy group (n = 30) were used as the control subjects. The serum samples were centrifuged at 2000 rpm and stored at –80°C. We also assessed the hepatic and renal functions. The liver and kidney functions, blood lipid, and blood glucose levels were normal in the control group. All patients were followed-up as per the standards of diagnosis and treatment of patients with lung cancer, from January 2012 to February 2015.

Total RNA extraction

Lung tissue (50 mg) was extracted in 1.0 mL of TRIzol reagent (Molecular Research Center, USA) and homogenized on ice according to previous studies. The concentration of total RNA was measured by determining its optical density at 260 nm in an ultraviolet (UV) spectrophotometer and expressed as total RNA micrograms per milligram of wet tissue. The tissue was then stored at –80°C.

HIF-1α cDNA synthesis

First-strand cDNA of HIF-1α was generated using the RevertAid First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

Primer design and polymerase chain reaction (PCR) amplification

The primers for HIF-1α mRNA were designed according to the *HIF-1α* gene sequence (MIM603348) using the Premier Primer 5.0 software (Invitrogen, Shanghai, China). The HIF-1α primers used were forward, 5'-CTCATCCAAGAAGCCCTAAC-3' (nts 2452–2471) and reverse, 5'-TCATAACTGGTCAGCTGTGG-3' (nts 2781–2800), and the amplified fragments were 349-bp long. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for relative quantification. GAPDH primers used were as follows: forward, 5'-CACTGG CGTCTTCACCACCAT-3' (nts 396–416) and reverse, 5'-GTGCAGGAGGCATTGCTGAT-3' (nts 541–560), and the amplification sequence was 165-bp long. HIF-1α cDNA was amplified using nested PCR. Briefly, 1 μL cDNA (0.1 μg/μL), Each 1.0 μL HIF-1α forward and reverse primers (10 μmoL/L), 12.5 μL Premix Taq amplitag DNA polymerase (TaKaRa, Japan), and ddH_2O were added to a reaction tube to make a total volume of 20 μL. The conditions for PCR amplification were as follow: 5 min of pre-denaturation at 94 °C; 35 cycles amplification with TC-412 (Techne, England) at 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min; a final extension at 72 °C for 10 min, and storage at 4 °C. The amplified fragments of the *HIF-1α* gene were electrophoresed on a 1.5% agarose gel (PowerPac, Mini Trans-Blot, Bio-Rad, Laboratories, Inc., USA) and observed using a UV transilluminator at 320 nm (170-2525, Bio-Rad, Laboratories, Inc.).

DNA sequencing

HIF-1α DNA was purified using Montage™ PCR centrifugal filter devices (4 °C, 9000 rpm, 10 min), and DNA was prepared using a MegaBACE DNA sequencer (USA) with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Bioscience, USA) according to the manufacturer's protocol. Subsequently, 1.0 μg tested DNA, 2.5 μL sequencing primer, 8.0 μL dNTP mixed reagent, and 20 μL ddH_2O were added to a reaction tube. The cycling conditions were as follows: 95 °C for 30 s; 30 cycles of 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 1 min; and storage at 15 °C. DNA was precipitated using ammonium acetate and ethanol, sequenced using the

MegaBACE sequencing system (version 3.0, Amersham Biosciences), edited, and aligned with the original sequences of the *HIF-1α* gene from GenBank.

Tissue microarrays (TMA)

A 20 × 12 matrix of tissue microarray paraffin blocks was prepared, and 4-μm–thick sections were prepared from paraffin-embedded tissue blocks. Four points were allotted to each case of lung cancer and 2 points to each case of noncancerous tissue. Cells were adhered to glass slides that were coated with poly-lysine.

Immunohistochemistry (IHC)

Immunohistochemical staining (S-P method) was performed using Immunostain EliVision kit (Beijing Zhongshan Biotechnology Company, China) according to the manufacturer's instructions. Primary rabbit anti-human VEGF (BA0407) and HIF-1α (PB0245) polyclonal antibodies and mouse anti-rabbit secondary antibody IgG-Biotin (BM2004) were purchased from Wuhan Boster Biological Technology Co., China. The TMA slides were deparaffinized and dehydrated, and the deparaffinized lung sections were washed with ethylene diamine tetraacetic acid buffer and quenched in a microwave for 10 min. The sections were then incubated for 60 min with primary rabbit anti-human VEGF and HIF-1α polyclonal antibodies at room temperature, washed three times with phosphate-buffered saline (PBS), and incubated for 20 min with polymer reinforcing agent. Subsequently, the sections were rinsed in PBS thrice, incubated with mouse anti-rabbit secondary antibody IgG-Biotin for 30 min at room temperature, and developed with 0.1% 3,3'-diaminobenzidine for 5 min after washing with PBS thrice. Thereafter, the slides were rinsed with distilled water, counterstained, dehydrated, air dried, and mounted. For the negative control reactions, the primary and secondary antibodies was instead with PBS (pH = 7.5).

Evaluation of the IHC findings

The results of IHC staining were assessed by two methods: the intensity of staining and the percentage of positive cells (positive cell average in 5 fields of vision). Staining intensity was categorized into 4 groups: 0 (negative), 1 (pale yellow), 2 (brown), and 3 (dark brown). The percentage of positive cells was scored as follows: 0 (0–5%), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (> 75%). The product of the percentage and intensity score was defined as the final IHC staining score: 1–4 (low expression), 5–8 (moderate expression), and 9–12 (high expression).

Western blotting

The amount of total protein was determined using a bicinchoninic acid protein-measuring kit (Beyotime, China). Protein (50 μg) from each sample was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 20 V for 30 min and then at 120 V for 60 min. The protein was then transferred onto a polyvinylidene difluoride membrane and blocked by incubation with 5% nonfat dry milk in Tris-buffered saline (2.5 g nonfat dry milk and 50 mg of Tris-buffered saline with Tween-20) for 1 h in a glass dish while shaking at room temperature. The transferred membrane was incubated with mouse polyclonal antibody against human HIF-1α (1:500, Abcam, USA) or β-actin overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies (Beyotime, China) for 2 h at room temperature. Imaging was performed using Quantity One software (Bio-Rad, Laboratories, Inc., USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF and HIF-1α proteins were detected in the sera of patients with lung cancer using an ELISA assay kit KIT/96T (Uscnk, Wuhan, China) according to the manufacturer's instructions. The detection ranges were as follows: VEGF (SEA143Hu), 15.63–1000 pg/mL and HIF-1α (SEA798Hu), 0.156–10 ng/mL. According to the manufacturer's instructions, the absorbance (*A* value) was detected at 450 nm, and the concentrations of VEGF and HIF-1 were calculated on the basis of their respective standard curves.

Statistical analysis

Data were expressed as mean ± standard deviation, and all statistical analyses were carried out using Graphpad Prism 5.0 and SPSS software (version 18.0). A *t*-test was used to analyze protein expression in the two groups, and single-factor analysis of variance (one-way) was used to analyze the expression between groups. Furthermore, linear correlation analysis was used to analyze the relationship between HIF-1α expression and VEGF expression. Statistical significance was set at $P < 0.05$.

Results

HIF-1α and VEGF expression and correlation analysis

The serum levels of HIF-1α and VEGF in patients with benign and malignant lung diseases were shown in Table 1. From normal condition or pneumonia to adenocarcinoma, the expression level of HIF-1α increased gradually. The serum HIF-1α expression [(138.3 ± 28.8) μg/L] in the lung cancer group was significantly higher ($P < 0.01$) than that in the pneumonia group [(58.8 ± 14.5) μg/L] and the control group [(24.1 ± 3.3) μg/L], and the expression in the pneumonia group was significantly higher than that in the control group. The expressions of VEGF and HIF-

Table 1 Quantitative analysis of serum HIF-1 and VEGF expressions in patients with lung disease (*n*)

Groups	*n*	HIF-1α		VEGF	
		Mean ± SD (μg/L)	> 100 μg/L (%)	Mean ± SD (μg/L)	> 280 μg/L (%)
Lung cancer	115	138.3 ± 28.8	104 (90.4)	394.9 ± 107.3	114 (87.0)
Pneumonia	30	58.8 ± 14.5*	0 (0.0)*	162.3 ± 79.3*	4 (13.3)*
Control	30	24.1 ± 3.3**	0 (0.0)*	140.9 ± 54.5*	0 (0.0)**

Note: compared with the lung cancer group, * $P < 0.01$; ** $P < 0.001$

1α were higher in adenocarcinoma patients, considering 100.0 μg/L and 280.0 μg/L as detection limits, respectively; furthermore, the incidences of VEGF and HIF-1α were, respectively, 90.4% and 87.0% in the lung cancer group and 0.0% and 13.3% in the pneumonia group, whereas the corresponding control group showed normal levels of the both factors. There was a close relationship between the HIF-1α and VEGF expressions in patients with lung cancer ($r = 0.937$, $P < 0.01$).

HIF-1α gene and protein level in lung cancer tissue

The expression levels of the *HIF-1α* gene and protein in cancerous and para-cancerous tissues were shown in Fig. 1. The specific concentration of total RNA [(1.52 ± 1.14) μg/mg wet lung tissues] in the 115 cases of cancerous tissues was significantly higher ($t = 8.494$, $P < 0.001$) than that in the noncancerous tissues [(0.58 ± 0.33) μg/mg], and the difference between them was significant ($t = 8.4937$, $P < 0.001$). The amplified gene fragment was 349-bp long, and the sequence was consistent with that of HIF-1α from GenBank (Fig. 1a). The expression of HIF-1α in cancer tissue was significantly higher than that in para-carcinoma tissue (wet-weight tissue per mg, Fig. 1b).

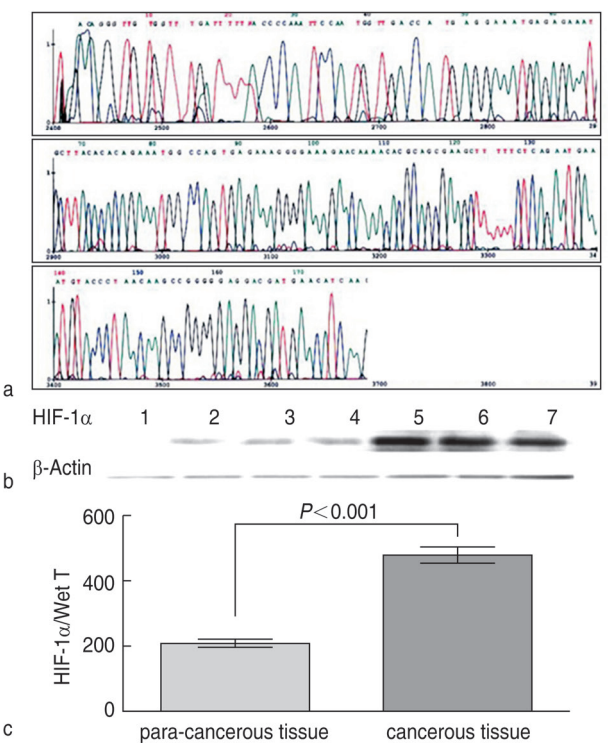

Fig. 1 Expression levels of the *HIF-1α* gene and protein in cancerous and the para-cancerous tissues. (a) Amplified *HIF-1α* gene fragment analysis; (b) Western blotting: normal control (1), para-carcinoma tissues (2–4), and cancer tissues (5–7); (c) Expression of HIF-1α in wet-weight lung tissue

HIF-1α expression in lung cancer tissues

Tissue matrix was organized in an order on the TMA and stained by IHC S-P (Fig. 2). IHC showed that brown particles, representative of HIF-1α expression, were

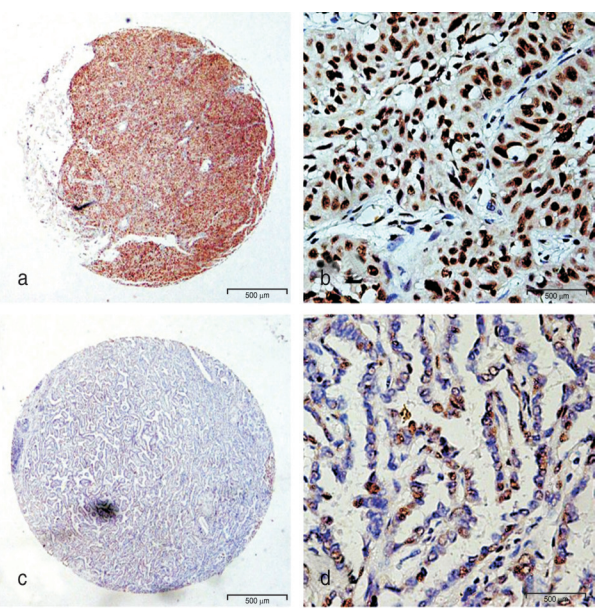

Fig. 2 Tissue microarray analysis of HIF-1 expression in lung cancer and noncancerous tissue. Immunohistochemical staining using anti-human HIF-1α antibody for detection of lung tissue HIF-1α expressions in different tissues from patients with lung cancer. (a and b) HIF-1α-positive expression in cytoplasm and cell membrane in cancerous tissue; (c and d) HIF-1α-negative expression, brown particles in the cytoplasm and cell membrane in the para-cancerous tissue. (a and c) S-P, original magnification × 40; (b and d) S-P, original magnification × 400

Table 2 The relationship between HIF-1α expression and clinicopathological features (n = 115)

Groups		No.	HIF-1α expression		χ^2	P
			Positive No.	%		
NSCLC		115	87	75.65		
Sex	Male	58	39	67.24	4.494	0.034
	Female	57	48	84.21		
Age (years)	≤ 60	53	40	75.47	0.002	0.967
	> 60	62	47	75.81		
Tumor diameter (cm)	≤ 2	97	77	79.38	4.679	0.031
	> 2	18	10	55.56		
Differentiation degree	Well	19	14	73.68	8.846	0.012
	Middle	84	68	80.95		
	Low	12	5	41.67		
Lymph node metastasis	Without	66	50	75.75	6.604	0.037
	Ipsilateral bronchial metastasis	43	35	81.40		
	Mediastinal	6	2	33.33		
TNM stage	I–II	16	13	77.36	0.169	0.919
	III–IV	99	74	75.00		
5-year survival	Survival	16	9	56.25	3.798	0.051
	Death	99	78	78.79		

located in the cytoplasm and cell nuclei in lung cancer tissues and the surrounding tissues. HIF-1α expression was high and uniform in cancerous tissue, increased in areas around the necrosis and tumor-infiltrating edge (Fig. 2a), and significant in tissues near the tumor edge (Fig. 2b). Furthermore, the lung cancer tissues displayed significantly higher levels of HIF-1α-positive expression, staining intensity, and IHC scores than the corresponding para-cancerous tissues (Fig. 2c and 2d).

Clinicopathological features of HIF-1α expression in lung cancer tissues

The clinicopathological features of HIF-1α expression in lung cancer tissues were shown in Table 2. The incidence of HIF-1α expression in lung tissues was 75.65% (87/115) in non-small cell lung cancer, and there were no significant correlations between positive HIF-1α expression and age, TNM stage, or 5-year survival rate. However, the clinicopathological features of HIF-1α expression in lung cancer tissues indicated a significant relationship between positive HIF-1α expression and patient sex (χ^2 = 4.494, P = 0.034), tumor size (χ^2 = 4.679, P = 0.031), differentiation degree (χ^2 = 8.846, P = 0.012) or presence of lymphatic node metastasis (χ^2 = 6.604, P = 0.037).

Discussion

The factors affecting the prognosis of lung cancer are complicated and directly related to inconspicuous early symptoms, technical defection in early diagnosis, and low awareness among patients. Often, lung cancer is definitively diagnosed in the middle or advanced stages, and the traditional treatments at these stages are surgical resection, chemotherapy, or radiotherapy [17]. Angiogenesis is a fundamental process involving a variety of pathological processes and sustains the progression of many neoplastic diseases. Moreover, it may enhance tumor cell proliferation and resistance to apoptosis, and facilitate metastasis. Tumor vasculature originates because of angiogenesis, vascular sheath growth, and endothelial progenitor cell growth. In the absence of vascularization, cell hypoxia causes tumor cells and macrophages to produce a large number of angiogenic factors that induce angiogenesis, which is important for the growth and development of lung cancer [18–21]. HIF-1α is suggested to be an important upstream molecule mediating VEGF expression and angiogenesis, and HIF-1α polymorphisms are reportedly associated with susceptibility to lung cancer [22–23]. In this study, we analyzed the expression of HIF-1α and changes in its expression in lung cancer tissues and peripheral blood by measuring the expression at the gene transcription or protein level.

The progress of lung cancer is closely related to the microenvironment and formation of new blood vessels. When lung cancer cells proliferate, tumor volume and oxygen consumption increase significantly, the cancer cells become hypoxic and overexpress HIF-1α, which leads to the secretion of angiogenic factors and induces angiogenesis. In the current study, we analyzed the expression of HIF-1α and VEGF in patients with benign and malignant lung disease. With a boundary of 100.0 μg/L for serum HIF-1α levels, the positive rate was 90.4% in adenocarcinoma, patients with pneumonia and normal groups had no abnormal; with a boundary serum VEGF level of 280.0 μg/L, the positive rate was 87% in

lung cancer patients and 13.3% in pneumonia patients. Furthermore, as the expressions of HIF-1α and VEGF were significantly increased and positively correlated, they can be considered as serological markers that reflect the progress of lung cancer and may be helpful in its diagnosis. IHC showed that the brown particles that represent HIF-1α expression in lung cancer and its surrounding tissues were located in the cytoplasm and cell nuclei, and HIF-1α-positive expression and intensity in the lung cancer group were significantly higher compared with those in the noncancer groups [24–26]. In addition, HIF-1α expression was significantly related with the sex, tumor size, differentiation degree, and lymph node metastasis; however, there was no significant correlation between HIF-1α expression and the age, TNM stage, and 5-year survival rate. Thus, HIF-1α may be a useful target for lung cancer therapy.

In summary, the study of the expression and change in expression of HIF-1α in human lung cancer tissues and peripheral blood by analyzing the transcription and translation level suggests that overexpression of HIF-1α regulates VEGF transcription and angiogenesis via a positive feedback mechanism, and an increase in the HIF-1α concentration can indicate an occurrence and development of cancer [27–30]. Detection of abnormal expressions of both HIF-1α and VEGF in lung cancer tissues, blood, and serum could indicate lung cancer and may be used for its diagnosis [31–33]. Importantly, inhibiting HIF-1α expression and increasing the binding of VEGF to the receptor could decrease the proliferation of vascular endothelial cells and contribute to the treatment of lung cancer.

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ORIGINAL ARTICLE

Icotinib, an EGFR-TKI, for the treatment of brain metastases in non-small cell lung cancer: a retrospective study

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Abstract

Objective Treatment of brain metastases from non-small cell lung cancer (NSCLC) is a challenge because of the poor prognosis. Icotinib is a new type of oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) used in the treatment of advanced NSCLC. The aim of this study was to evaluate the efficacy of icotinib in NSCLC patients with brain metastasis.

Methods This study reviewed records of 51 NSCLC patients with brain metastases who took icotinib 125 mg, 3 times a day. Response rate, progression free survival, and overall survival were analyzed. SPSS software version 17.0 was used for univariate analysis, and Cox regression analysis to analyze factors affecting survival.

Results Thirty-six cases had partial response, 6 cases had stable disease, and 10 cases had progressive disease. In 31 cases, EGFR gene mutation test were performed. EGFR was mutated in 26 cases and was with wild-type in 5 cases. In patients with EGFR mutations, 23 patients responded to icotinib [the disease control rate (DCR) was 88.5%], significantly higher than in patients with wild-type EGFR (1 patient, DCR 20%) (P = 0.005). The overall median progression-free survival (PFS) was 7.6 months. PFS was longer in the patients with EGFR mutations than in those with wild type EGFR (7.8 months vs 1.2 months, P = 0.03). The overall median overall survival (OS) time was 10.7 months. OS was longer in patients with EGFR mutations than in those with wild type EGFR (15.1 months vs 6.7 months, P = 0.003). The main side effects of the treatment were skin rash and diarrhea; no stage 3 or 4 toxic effects occurred. Univariate analysis demonstrated that OS was related to sex, Eastern Cooperative Oncology Group performance status (ECOG PS), smoking history, and EGFR mutation. Multivariate analysis showed that OS was independently related to sex, ECOG PS, and EGFR mutations.

Conclusion Icotinib has a favorable effect on NSCLC patients with brain metastases harboring EGFR mutations. Icotinib can be a new choice of treatment for brain metastases in patients with NSCLC harboring EGFR mutations.

Key words: non-small cell lung cancer (NSCLC); brain metastases; icotinib; epidermal growth factor receptor (EGFR)

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Lung cancer is the most common type of cancer and is the leading cause of cancer death in China [1]. Non-small cell lung cancer (NSCLC) cases constitute approximately 80% of all lung cancer cases [2]. NSCLC is the most common type of cancer with brain metastases and at least 25–40% of patients develop brain metastases at some point during their disease [3].

The prognosis of NSCLC patients with brain metastasis is poor with a median overall survival (OS) of less than 3 months without treatment [4]. The quality of life of these patients is also very poor [5]. The standard treatment options for patients with brain metastasis include surgery, whole-brain radiation therapy (WBRT), stereotactic radiosurgery (SRS), or a combination of these [6].

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Chemotherapy has not been a standard treatment for these patients because drugs cannot penetrate the blood-brain barrier effectively. However, chemotherapy is an option for NSCLC with brain metastasis, with reports of response rates of 15–30% and an OS of 6–8 months [7–9].

Recent studies showed the effectiveness of pemetrexed treatment in NSCLC patients with brain metastasis [10–12]. Bevacizumab, the most widely used drug in anti-angiogenic therapy, has also been shown to improve response rates, progression-free survival (PFS), and OS compared to chemotherapy alone in NSCLC patients with brain metastasis [9]. However, because of concerns about tumor-related intracranial hemorrhage [13], the use of bevacizumab to treat NSCLC patients with brain metastasis has remained limited.

In the last 10 years, several clinical studies showed EGFR-tyrosine kinase inhibitor (TKI) significantly prolonged PFS and OS of advanced NSCLC patients with sensitive EGFR mutations [14–16] and it is used for the treatment of patients with brain metastases [17–22]. Most of these reports are about gefitinib and erlotinib, both known to cross the blood-brain barrier [23–24].

Icotinib is a new type of oral EGFR-TKI developed in China (Conmana, Zhejiang Beta Pharma, China). A phase III trial (ICOGEN) [25] demonstrated that icotinib was non-inferior to gefitinib in terms of PFS in NSCLC patients and this result led icotinib to be approved by the China Food and Drug Administration in August 2011. Icotinib was reported to have a beneficial effect on brain metastases of NSCLC in some case reports [26–27].

The aim of this study is to evaluate the efficacy of icotinib in NSCLC patients with brain metastases.

Materials and methods

Patients

This study enrolled 51 consecutive NSCLC patients with a confirmed pathological diagnosis, advanced stage (IIIb or IV), brain metastasis, and received icotinib treatment at the Beijing Chest Hospital between October 2011 and April 2014. The brain metastasis was confirmed using magnetic resonance imaging or computed tomography. The patients' information was collected, including age, sex, Eastern Cooperative Oncology Group performance status (ECOG PS), pathology, smoking history, neurological symptoms, numbers of brain metastasis foci, presence of an EGFR mutation, and previous treatment. All patients took icotinib 125 mg, 3 times a day, until their disease progressed, death, or the development of unacceptable toxic effects. This retrospective study obeyed all the rules and regulations of clinical studies with respect to human subject protection and was approved by the independent ethics committee.

Assessments

Tumor assessment was performed within 2 weeks before icotinib treatment and first reassessed after 4 weeks of medication. Afterward, an assessment was performed every 2 months during treatment according to the Response.

Evaluation Criteria in Solid Tumors (RECIST) version 1.1, which is divided into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Adverse reactions were evaluated based on the Common Terminology Criteria for Adverse Events (CTCAE version 3.0) once a week during treatment and then every 8 weeks at follow-up visits.

Progression-free survival (PFS) was calculated as the duration of time from the start of icotinib to progression of the disease. OS was calculated as the duration of time from the start of icotinib to date of death. EGFR mutations were detected by the use of an amplification refractory mutation system or Sanger sequencing.

Statistical analysis

All data were analyzed using SPSS (Chicago, IL, USA) version 17.0. Baseline characteristics between groups were compared using the Chi-square test or Fisher's exact test. The Kaplan–Meier method and log-rank test were used for survival analysis. The Cox regression model was used to identify independent prognostic factors. A *P*-value of 0.05 was considered to be statistically significant.

Follow-up

All patients were followed up until December 31, 2014 and during this time period 37 patients died, 12 survived, and 2 cases were lost.

Results

Baseline characteristics

A total of 51 cases were analyzed. The detailed patients' characteristics were summarized in Table 1. The ages ranged from 34 to 78 years old, and the median age was 59 years old. There were 22 (43.1%) men and 29 (56.9%) women. Thirty (58.8%) cases were ECOG PS score 0–1, while 21 (41.2%) cases were 2–3. There were 17 (33.3%) smokers and 34 (66.7%) non-smokers. The majority of patients had adenocarcinoma (48 cases, 94.1%), and 3 cases (5.9%) were non-adenocarcinoma. Twenty-six (51%) cases had EGFR mutations, among them 19 (37.3%) cases harboring exon 19 deletion mutations and 7 (13.7%) cases with L858R mutations. Five (9.8%) cases had wild type EGFR and 20 (39.2%) cases were unknown. Twenty-eight (54.9%) cases were concomitantly treated for brain metastases with icotinib and WBRT while the other 23 (45.1%) cases used icotinib alone to control their disease. The numbers of cases using icotinib for first-line,

Table 1 Characteristics of the cases

Features	*n*	%
Sex		
Male	22	43.1
Female	29	56.9
Median age (years)		
> 65	13	25.5
≤ 65	38	74.5
ECOG PS		
0–1	30	58.8
2–3	21	41.2
Smoking history		
Never smoked	34	66.7
Former smoker	17	33.3
Histologic feature		
Adenocarcinoma	48	94.1
Other	3	5.9
Neurological symptom		
Yes	31	60.8
No	20	39.2
Number of brain metastasis		
Single	10	19.6
Multiple	41	80.4
EGFR mutation		
Wild type	5	9.8
Exon 19 deletion	19	37.3
L858R	7	13.7
Unknown	20	39.2
Icotinib treatment		
First line	19	37.3
Second line	20	39.2
Third line	8	15.7
Fourth line	4	7.8
WBRT		
Yes	28	54.9
No	23	45.1

second-line, third-line, and fourth line treatment were 19 (37.3%), 20 (39.2%), 8 (15.7%), and 4 (7.8%), respectively.

Efficacy

Among all 51 cases, for the brain metastatic lesion, 35 (68.6%) cases had a PR, 6 (11.8%) cases SD, and 10 (19.6%) cases PD, and no CR case was observed. The objective response rate (ORR) was 35 (68.6%) and the disease control rate (DCR) was 41 (80.4%) in the entire population. Among EGFR mutated cases, the ORR was 21 (80.8%) and the DCR was 23 (88.5%). There was 1 patient with EGFR wild type who showed PR. The difference in DCR between the group with EGFR mutated and wild type was statistically significant (P = 0.005). DCR had no correlation with sex, age, smoking status, ECOG PS, number of brain metastases (single or multiple), radiotherapy used or not, which line of icotinib was used, and having neurological symptoms or not (P > 0.05).

Survival

The overall median PFS was 7.6 months. Univariate analysis showed that PFS for patients with EGFR mutations was 7.8 months vs 1.2 months for EGFR wild type (P = 0.03). Women had a PFS of 8.3 months vs 5.2 months for men (P = 0.02). PFS for patients with 19 exon deletion mutations was significantly longer than wild type (8.1 months vs 1.2 months, P = 0.03). PFS of patients with an L858R mutation was longer than for wild type, but the difference was not statistically significant (4.6 months vs 1.2 months, P = 0.61). However, age, smoking status, number of brain metastases, WBRT, neurological symptoms, and which line for use of icotinib were not significant (P > 0.05). ECOG PS had a close to significant influence on PFS (P = 0.06; Table 2).

The median OS was 10.7 months. OS for patients with EGFR mutations was 15.1 months vs 6.7 months for EGFR wild type (P = 0.003). Women had an OS of 15.6 months vs 8.3 months for men (P = 0.001). OS of patients with exon 19 deletion mutations was statistically significantly longer than wild type (15.6 months vs 6.7 months, P = 0.004). OS of patients with L858R mutations was not significantly longer than wild type (10.3 months vs 6.7 months, P = 0.082). ECOG PS was significantly correlated with OS (P = 0.01). However, OS was not significantly related to age, number of brain metastases, WBRT, neurological symptoms, or the line of icotinib use (P > 0.05; Table 2).

Multivariate analysis showed that EGFR gene mutation (P = 0.002), sex (P = 0.018), and ECOG PS (P = 0.013) were independently correlated with OS.

Safety

The most common toxic effects of icotinib treatment were diarrhea and skin rashes. There were no stage 3 or 4 toxic events.

Discussion

The survival time for patients receiving therapy is limited and brain metastasis from NSCLC is still a challenge. The treatment options for patients with brain metastasis include surgery, WBRT, SRS, or a combination of these [6]. Encouragingly, with the development of molecular biology, targeted therapy has become an important tool in the treatment of NSCLC through studies such as IPASS, OPTIMAL, NEJ002, WJTOG3405, EURTAC, LUX-Lung 3, and LUX-Lung 6 [14, 15, 28–32]. These studies showed that ORR can reach 60–80% and the median PFS can be 8–13 months with few toxic effects after targeted therapy. Additionally, EGFR-TKIs have a favorable efficacy on brain metastasis in advanced NSCLC patients harboring EGFR sensitive mutations [17–22]. These EGFR-TKIs can achieve a median PFS of 6.6–14.5 months and a median OS of 8–20 months in patients with brain

Table 2 Univariate analysis of PFS and OS to icotinib for NSCLC

	Median PFS (month)	95% CI	*P*	Median OS (month)	95% CI	*P*
Sex			0.02			0.001
Male	5.2	2.7–7.3		8.3	2.0–13.9	
Female	8.3	6.3–9.7		15.6	6.0–23.9	
Age (years)			0.59			0.92
> 65	8.2	3.5–12.5		8.4	2.9–13.0	
≤ 65	7.0	5.2–8.8		10.2	7.2–12.8	
ECOG PS			0.06			0.01
0–1	7.8	5.8–10.2		15.6	10.1–19.9	
2–3	5.4	3.9–6.0		7.8	4.4–9.6	
Smoking history			0.12			0.03
Never smoked	5.2	3.7–6.3		8.4	4.1–11.9	
Former smoker	7.1	5.6–8.4		13.5	8.2–17.7	
Neurological symptom			0.23			0.38
Yes	5.2	2.0–7.9		8.4	3.8–12.1	
No	7.1	4.5–9.5		12.2	8.6–15.3	
Number of BM			0.48			0.29
Single	7.5	0–15.3		20.6	12.2–27.5	
Multiple	7.2	4.9–9.0		10.3	8.8–11.2	
EGFR mutation			0.03			0.003
Wild type	1.2			6.7	0–14.6	
Mutated	7.8	4.1–9.9		15.1	10–19.1	
Exon 19-del	8.1	4.5–11.4	0.03	15.6	8.6–21.4	0.004
L858R	4.6	1.4–6.6	0.61	10.3	3.8–16.2	0.082
WBRT			0.79			0.54
Yes	6.1	2.5–9.5		13.4	9.1–16.9	
No	7.0	4.6–9.3		10.6	6.8–13.1	
Icotinib treatment			0.89			0.46
First line	7.1	5.6–8.4		10.2	4.3–15.6	
Second line	7.0	4.5–9.5		12.6	8.4–15.6	
Third line	2.8	0–7.2		8.8	2.8–13.1	
Fourth line	1.0			2.4		

metastasis from advanced NSCLC. Most of these reports are on gefitinib and erlotinib, which are known to cross the blood-brain barrier [23–24]. Icotinib is a new type of oral EGFR-TKI developed in China. A randomized, double-blind, multicenter, controlled, and head-to-head (icotinib vs gefitinib) phase III trial of icotinib (ICOGEN) demonstrated that icotinib was non-inferior to gefitinib in terms of PFS (7.8 months vs 5.3 months, $P = 0.32$) and OS (20.9 months vs 20.2 months, $P = 0.76$) in NSCLC patients with mutated EGFR [25]. Patients harboring active EGFR mutations have a better response to icotinib than those without EGFR mutations. Additionally, icotinib has shown a higher liposolubility and can pass through the blood brain barrier easier as compared with gefitinib [33].

Previous reports showed that icotinib has a good effect on brain metastasis [26–27] and leptomeningeal carcinomatosis [34] in NSCLC. Additionally, Icotinib shows efficacy in preventing brain metastases [33]. In the current study, 51 patients with advanced NSCLC with brain metastases received icotinib treatment. The overall median PFS was 7.6 months and OS was 10.7 month. PFS and OS for patients with EGFR mutations were significantly longer than those with EGFR wild type. The ORR and DCR among patients with EGFR mutations were also significantly higher than those with EGFR wild type. These results indicated that icotinib has good efficacy for NSCLC patients with brain metastases harboring EGFR mutations.

For the different EGFR mutations, PFS and OS in patients with exon 19 deletions were significantly longer than those with EGFR wild type (8.1 months vs 1.2 months, $P = 0.03$ and 15.6 months vs 6.7 months, $P = 0.004$). However, PFS and OS in patients with L858R mutations were not statistically different than those with EGFR wild type (4.6 months vs 1.2 months, $P = 0.61$ and 10.3 months vs 6.7 months, $P = 0.082$). It appears that survival in patients with exon 19 deletions is different compared with that in patients with L858 mutations.

Similar results have been reported in LUX-Lung 3 and LUX-Lung 6 [35]. Yang found that OS was significantly

longer for patients with deletion 19-positive tumors in the afatinib group than in the chemotherapy group in both trials. In LUX-Lung 3 the OS was 33.3 months vs 21.1 months, $P = 0.0015$ and in LUX-Lung 6, it was 31.4 months vs 18.4 months, $P = 0.023$. In contrast, there were no significant differences in the OS of different treatment groups of patients with EGFR L858R-positive tumors in either trial. In LUX-Lung 3, the OS was 27.6 months vs 40.3 months, $P = 0.29$), and in LUX-Lung 6 it was 19.6 months vs 24.3 months, $P = 0.34$). Therefore, we speculate that EGFR deletion 19-positive disease might be different from L858R-positive disease. Further study is needed to confirm this conclusion.

This study did not show that icotinib plus concomitant WBRT had a higher response rate to brain metastasis than icotinib alone. Moreover, PFS or OS was not improved using WBRT compared to icotinib alone in the treatment of brain metastases from NSCLC. A phase Ⅱ study indicated that the combination of icotinib and WBRT was well-tolerated and median PFS was 7.0 months [36]. This is similar to our results, but it was a single-arm study and therefore whether patients can benefit from the combination of icotinib with WBRT is still unknown.

Whether concomitant WBRT with EGFR-TKI is beneficial for NSCLC patients is still unclear. A retrospective study showed that gefitinib plus concomitant WBRT had a higher response rate of brain metastases and significant improvement in time to progression (10.6 vs 6.57 months, $P < 0.001$) and OS (23.40 vs 14.83 months, $P = 0.002$) compared with gefitinib alone in the treatment of brain metastases from NSCLC [37].

Van reported that radiation therapy might disrupt the blood-brain barrier [38] and the addition of WBRT might increase the concentration of gefitinib in the CNS. However, a prospective phase 3 trial showed that the addition of temozolomide or erlotinib to WBRT plus SRS in NSCLC patients with 1 to 3 brain metastases did not improve survival and possibly had a deleterious effect [39]. Further studies are needed to harmonize targeted therapy and WBRT in NSCLC patients with brain metastases harboring EGFR mutations.

We conducted univariate analysis and found that patients' PFS and OS were related to EGFR mutation status, sex, and ECOG PS. Among EGFR mutated patients, the disease progressed in 3 patients after administration of icotinib. All these patients had adenocarcinoma and two harbored deletions in exon 19 and one had a L858R mutation. We found that one patient had elevated carcinoembryonic antigen (CEA), neuron-specific enolase, and pro-gastrin-releasing peptide levels; one had elevated CEA and CYFRA 21-1 levels; the other patient's tumor markers were normal but his brain lesion progressed, while he had stable disease in his lung lesion. We concluded that tumor heterogeneity affects the response to icotinib.

Tanaka [40] found that EGFR-mutated NSCLC patients with a high CYFRA 21-1 level have significantly shorter PFS than those with normal CYFRA 21-1 level. The authors speculated that the serum CYFRA 21-1 level was associated with the proportion of squamous component in the NSCLC. There are also reports [41–43] on heterogeneity between the primary lesion and metastatic lesion, with a discrepancy that can vary from 6.3%–26.9%. These findings indicated that heterogeneity influences the efficacy of EGFR-TKIs. Therefore, we speculated that there may be small cell lung cancer components in the metastatic lesion in the first patient, squamous components in the second patient, and differences between the primary and metastatic lesions in the third patient.

The most common adverse events of icotinib in NSCLC with brain metastases were rash and diarrhea, and no patients had grade 3 or 4 toxic effects. This result is consistent with those of previous studies on icotinib use [25, 44] and confirms the safety and good quality of life with icotinib treatment.

In summary, icotinib shows favorable efficacy in NSCLC patients with brain metastases harboring EGFR mutations. Icotinib was well tolerated and patients showed significantly improved survival. The effect of icotinib may be different between patients with exon 19 deletions and L858R mutations. Concomitant WBRT did not show any beneficial effect and further prospective studies are needed to optimize treatment strategies. Moreover, tumor heterogeneity can influence the efficacy of EGFR-TKIs.

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ORIGINAL ARTICLE

Relationship between peritumoral lymphatic microvessel density and the clinical and pathological characteristics of invasive breast cancer

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Abstract

Objective The aim of the study was to determine the morphological characteristics of lymphatic microvessels and the relationship between lymphatic microvessel density (LMVD) and clinical and pathological characteristics of invasive breast cancer.

Methods Tissue specimens and clinical pathological data of 51 cases of female breast cancer were collected in the General Hospital of Shenyang Military Region (Shenyang, China) from January 2007 to October 2011. Another 20 breast fibroadenoma tissue samples were used as controls. All specimens were cut into 4-μm slices, and immunohistochemically stained using streptomycin-resistant avidin peroxidase antibody D2-40. SPSS 17.0 for Windows was used to perform all analyses.

Results A total of 38 breast cancer tissue specimens showed varied staining with monoclonal antibody D2-40. The rate of positive staining was in these tissues was 74.5% (38/51), which is significantly higher than that observed in breast fibroadenoma tissues (chi-square = 35.197, $P = 0.000$). The average LMVD in 38 cases of breast cancer was (26.46 ± 10.06) microvessels/100× magnification field, which was higher than that in the control group ($t = 10.74$, $P = 0.000$). Microvessels in peritumoral tissues were abundant, with an average LMVD of (38.42 ± 11.38) microvessels/100× magnification field. Based on layered analysis, the expression level of peritumoral LMVD was correlated with metastasis of lymph nodes, tumor size, and the expression of C-erbB-2 ($P < 0.05$); however, there was no correlation with age or expression of estrogen receptors or progesterone receptors ($P > 0.05$).

Conclusion Lymphatic microvessels detected using D2-40 antibody are mainly present in the peritumoral region of breast cancer tissues, and LMVD showed a correlation with lymph node metastasis and the expression of C-erbB-2. Positive lymphatic vessels, especially in the peritumoral region, may provide a path for lymphatic metastasis in breast cancer. Peritumoral LMVD may be used to estimate the prognosis of patients with breast cancer and may aid in research on treatment methods.

Key words: breast cancer; lymphatic microvessel density (LMVD); C-erbB-2

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Breast cancer is a malignant disease with complicated and systemic features [1]. When the tumor diameter exceeds 0.2–0.3 cm, new vessels will be generated to provide essential oxygen and nutrition [1–2]. New vessels to promote tumor growth and metastasis are under the control of many positive and negative factors. It is reported that the occurrence, growth, metastasis, and prognosis of breast cancer mostly depends on sustained and uncontrolled angiogenesis and lymphangiogenesis [2–4].

There has been little research on lymphangiogenesis. The lack of specific lymphatic epithelial markers made it difficult to distinguish blood vessels and lymphatic vessels based on morphology, and only hematoxylin-eosin (HE) staining technique has been useful [5].

In this study, the morphologic characteristics of

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microlymphatic vessels were examined in 51 cases of breast cancer. In addition, the relationship between lymphatic microvessel density (LMVD) and the clinical and pathological characteristics of breast cancer were examined.

Materials and methods

Patients and specimens

Tissue specimens and clinical pathologic data of 51 cases of female breast cancer were collected in the General Hospital of Shenyang Military Region (Shenyang, China) from January 2007 to October 2011. All patients underwent surgery. The median age of all patients was 55 (22–75) years; the patients had no family history of breast cancer or other malignancy. Preoperative examination included tumor markers, B-mode ultrasound, computed tomography or positron emission tomography-computed tomography to exclude a second cancer. Based on the World Health Organization Breast Cancer Histopathological Classification (2003), 51 cases were diagnosed with invasive ductal carcinoma. Based on the sixth edition handbook of cancer TNM staging by the American Joint Committee on Cancer (AJCC), 11 cases of stage I, 12 cases of stage II, 28 cases of stage III, and 0 cases of stage IV were diagnosed; 14 cases had more than 3 lymph node metastases, 15 had 1–3 lymph node metastasis, and 22 had no lymph node metastases. Another 20 cases of breast fibroadenoma tissue were used as benign controls. All specimens were cut into 4-μm slices and archived for HE staining.

Main reagents and kits

Instant-applied mouse anti-human D2-40 monoclonal antibodies were purchased from the Zhongshan Jinqiao Biotechnology Company, China. Instant-applied Ultra™ SP kit for immunohistochemistry and DAB-0031 kit for DAB coloring were purchased from Maixin Biological Ltd., Fuzhou, China.

Immunohistochemistry

A total of 51 breast cancer tissue samples and 20 control breast fibroadenoma tissue samples were detected by immunohistochemical technique using streptomycin-resistance avidin peroxidase (S-P) with antibody on D2-40. The specific steps were as follows:

Paraffin sections were dewaxed, and incubated in 3% H_2O_2 for 10 min at room temperature. Antigen retrieval was performed by microwaving for 3 min in citrate buffer at pH 6.0. Nonspecific sites were blocked with 3.5%–10% normal goat serum. The sections were incubated for 10 min at room temperature. Serum was poured off. All sections were exposed to the first D2-40 antibody and incubated overnight at 37 °C. The second biotin-labeled antibody was added. The sections were incubated in wet box for 20 min, followed by horseradish peroxidase-labeled avidin. The sections were rinsed (3 × 5 min) in 0.01 MPBS (pH 7.4), stained with 3,3-diaminobenzidin (DAB), kept at room temperature, washed with distilled water, and hematoxylin stained. Specimens of known colorectal cancer provided by the Zhongshan Jinqiao Company (China) served as positive controls; phosphate buffered saline was used instead of the first antibody as a negative control.

Detection and determination of MLVD

Brown-yellow grains that appeared in the cytoplasm or cell membrane were considered positive stain for D2-40, and represented microtubules, single endothelial cells, or a cell plexus.

The method for MLVD counting was as follows: select two areas with the highest number of positive microvessels at low magnification (× 100), namely the "hot spot"; observe five fields at each hot spot at high magnification (× 400), count the number of microvessels, and calculate the mean number of microvessels in two hot spots and five fields. At same time, count intratumoral LMVD (located at the center of the tumor) and peritumoral LMVD (located in the peripheral tissue within 2 mm of the tumor) [6].

Statistical analysis

Quantitative parameters were expressed as mean ± SD. Qualitative variables were presented as values and percentages. Paired-samples *T*-test and one-way analysis of variance with least significant difference testing were used to compare quantitative parameters. Pearson's chi-square test was used to compare qualitative parameters. A P-value < 0.05 was considered to be statistically significant. The Statistical Package for Social Sciences (SPSS) version 17.0 for Windows was used to complete all analyses.

Results

Morphologic characteristics of lymphatic microvessels labeled with D2-40 antibody

This research included 38 breast cancer tissue specimens with different degrees of immune reactivity to monoclonal antibody D2-40. The positive rate was 74.5% (38/51). Brown-yellow grains appeared as positive cytoplasm or cell membranes. The positive microvessel rate labeled with D2-40 monoclonal antibody was only 20% (4/20) in 20 cases of controls, significantly lower than that in breast cancer tissue (chi-square = 35.197, P = 0.000; Fig. 1).

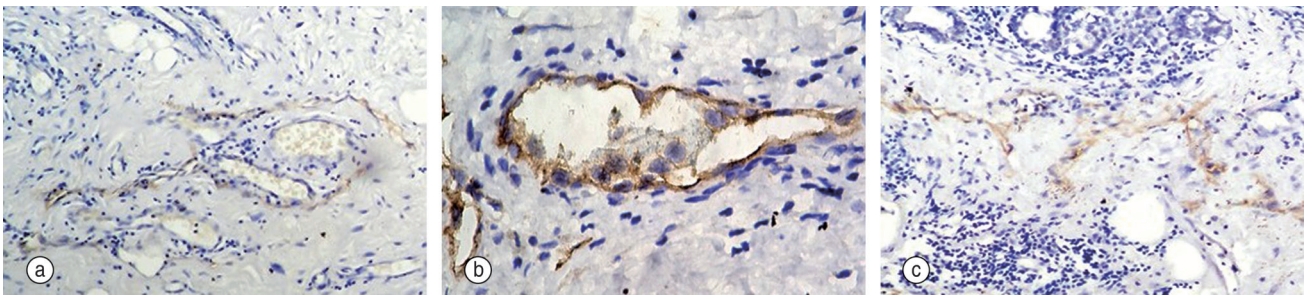

Fig. 1 (a) Positive lymphatic microvessels labeled with D2-40 (original magnification ×100); (b) tumor thrombus appears in lymphatic vessel cavity (original magnification ×400); (c) positive intratumoral lymphatic microvessel, with narrowed lumen, even funicular vessels (original magnification ×100)

Relationship between peritumoral LMVD and clinical and pathological characteristics

The average LMVD in 38 cases of breast cancer was (26.46 ± 10.06) microvessels/100× magnification field, and the average LMVD in the control group was (6.65 ± 1.72) microvessels/100× magnification field (t = 10.74, P = 0.000).

In intratumoral tissues, microvessels marked with D2-40 were sparse, with average LMVD of (7.69 ± 2.30) microvessels/100× magnification field; microvessels in peritumoral tissue were abundant, with average LMVD of (38.42 ± 11.38) microvessels/100× magnification field. Statistical differences between the two regions of breast cancer tissue were obvious (t = –16.31, P = 0.000).

Based on layered analysis, the expression level of peritumoral LMVD in 38 D2-40-positive cases was correlated with metastasis of lymph nodes and tumor size (P < 0.05), but showed no correlation with age (P > 0.05; Table 1).

Table 1 Relationship between LMVD with clinical and pathological features (microvessels/100× magnification field)

Features	No.	LMVD (M, $\bar{\chi} \pm s$)	P value	T or F
age (years)	38		0.49	0.69
< 60	13	41.45, 40.22 ± 11.28		
> 60	25	37.56, 37.48 ± 11.55		
Tumor size (cm)	38		0.02	3.30
< 5 cm	10	32.95, 29.34 ± 7.23		
> 5 cm	28	40.83, 41.66 ± 10.9		
ER state	38		0.211	1.274
–	14	36.12, 35.35 ± 12.63		
+	24	39.06, 40.20 ± 10.46		
PR state	38		0.923	0.098
–	18	38.81, 38.24 ± 8.80		
+	20	39.05, 39.61 ± 13.98		
Lymph node metastasis	38		0.000	40.722
0	15	26.78, 27.80 ± 7.25		
1–3	10	39.06, 39.71 ± 1.83		
> 3	139	51.08, 49.67 ± 4.69		
C-erbB-2	38		0.000	16.577
–	14	30.11, 29.73 ± 9.85		
2+	11	39.05, 38.67 ± 7.57		
3++	13	45.67, 49.18 ± 7.17		

LMVD labeled with D2-40 in the peritumoral region was significantly correlated with the expression of C-erbB-2 in invasive ductal carcinoma (P < 0.05), but showed no correlation with the expression of estrogen receptors and progesterone receptors (P > 0.05). With an increase of C-erbB-2 expression, LMVD also gradually increased.

Discussion

Research on lymphangiogenesis has been limited because of a lack of specific lymphatic endothelial markers available to distinguish between blood vessels and lymphatic vessels. Recent studies have identified some molecules that label lymphatic endothelia. D2-40 is a monoclonal antibody reported by Marks in 1999. It reacts with a 40-kD oncofetal antigen, better known as M2A antigen that is expressed in fetal testis and germ cells. D2-40 reportedly detects lymphatic vessels selectively in the tissues of gastric cancer, prostate cancer, and malignant melanoma by reacting selectively with the fixed antigen on lymphatic endothelium, without reacting with mature lymphatic vessels or capillary vessel endothelium [4, 7]. However, it has seldom been studied in breast cancer.

In our study, the rate of detection of lymphatic microvessels using D2-40 was 74.5%, and the average LMVD was (26.46 ± 10.06) microvessels/100× magnification field, which was significantly higher than that in benign breast tumors (P = 0.000). D2-40 is expected to become a new marker to selectively distinguish lymphatic endothelium.

In our research, immunohistochemical staining showed that positive lymphatic microvessels labeled by D2-40 were heterogeneous. The shape of vessels, size of the cavity, and LMVD in different areas were inconsistent. In the peritumoral tissue, there was an abundance of lymphatic microvessels with thin walls, more wrinkles, and a flat lumen; however, there were only a few vessels with poor lymphatic structure in

the intratumoral tissues. LMVD in two regions showed statistical significance (P = 0.000), in accordance with Roma's research [8]. Prior research suggested the possible reason. New functional lymphatic vessels surrounding stroma were formed when the tumor size was very small. However, owing to the continuous outward expansion of the tumor, these lymphatic microvessels were wrapped around intratumoral tissues. Cancer cells with excessive growth caused high hydrostatic pressure in the center of the tumor. Thus, the lymphatic lumen was compressed and the lymph vessels became non-functional [8].

It was found that LMVD was closely related to tumor invasive ability, metastasis, and prognosis [8–9]. The main cause of death in breast cancer is wide dissemination from the original site. Metastasis of axillary lymph nodes is often the first step to widespread metastasis. Therefore, lymph node metastasis has become the standard for evaluation of prognosis in breast cancer patients and directs the choice of treatment.

Recent research confirmed that when lymph node metastasis occurs, the tumor cells must penetrate the basement membrane and invade the blood vessels or lymphatic vessels before entering the circulation [10]. Stratification analysis shows that lymphatic microvessels labeled by D2-40 are correlated with lymph node metastasis in breast cancer. If the number of metastatic lymph nodes is more than three, the LMVD in interstitial tissue significantly increases, with a significant difference compared to that in lymph node-negative cases ($P < 0.01$).

The results of this research are consistent with those of a recent study [8, 11]. If breast cancer cells are exposed to an environment with more lymphatic microvessels, metastasis of lymph nodes and distant spread are more likely to occur. At the same time, lymphatic microvessels with enlarged lumens in peritumoral tissues have thin walls and discontinuous basement membranes, making it difficult to block tumor cells from entering the lymphatics. These lymphatic microvessels were the main channels for lymphatic metastasis in breast cancer.

The lymph nodes and lymphatic metastasis of tumors are associated with lymphangiogenesis. Lymphatic microvessels around the tumor provide necessary conditions for tumor growth and metastasis, and are expected to be a new target for antitumor therapy.

More than 20% of human tumor tissues express C-erbB-2 [12]. The excessive expression in human breast cancer may cause poor overall survival, with recurrence and metastasis. This study showed that the expression of lymphatic microvessels labeled by D2-40 was related to the expression of C-erbB-2 in breast cancer. We surmise that C-erbB-2 may play an important role in the growth of lymphatic microvessels. Further studies would be needed to validate these findings.

In conclusion, lymphatic microvessels labeled by D2-40 are often expressed in the peritumoral region of breast cancer, and LMVD shows a correlation with lymph node metastasis and the expression of C-erbB-2. The positive lymphatic vessels, especially in the peritumoral region, may provide a path for lymphatic metastasis in breast cancer.

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ORIGINAL ARTICLE

Clinical observation of rh-endostatin combined with chemotherapy as first line treatment for metastatic colorectal cancer*

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Abstract

Objective To analyze the efficacy and safety of Rh-endostatin combined with chemotherapy in the treatment of metastatic colorectal cancer.

Methods All 60 metastatic colorectal cancer patients were divided into the test group (n = 30) and the control group (n = 30). The control group was treated with chemotherapy regime FOLFOX4 (Oxaliplatin + Fluorouracil + Calcium Levofolinate), the test group was treated by Endostar combined with FOLFOX4 scheme.

Results The response rates were 53.3% in test group and 36.7% in control group respectively (P < 0.05), the disease control rate were 83.3% and 73.3% (P < 0.05). The median progression-free survival in test group and control group were 7.3 months versus 5.3 months (P < 0.05) and median overall survival were 11.6 months versus 9.3 months (P < 0.05). Among 27 cases of liver metastases were sub group analysis, difference on the test group and the control group response rate (RR) and disease control rate (DCR) had statistical significance (P < 0.05), but difference on progression free survival (PFS) and overall survival (OS) had no statistical significance (P > 0.05). The major toxicities were myelosuppression, gastrointestinal symptoms, neurotoxicity, most in grade I-II. After chemotherapy, quality of life (QOL) of patients were more improved than before treatment. After treatment the carcino embryonie antigen (CEA) and caner antigent 199 (CA199) levels decreased obviously, furthermore, the test group decreased more obviously than the control group.

Conclusion Rh-endostatin combined with chemotherapy in the treatment of metastatic colorectal cancer is safer and effective, and also improves PFS.

Key words: rh-endostatin; FOLFOX4 regimen; metastatic colorectal cancer

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Currently, chemotherapy is the major treatment for metastatic colorectal cancer. With the emergence of new drugs, such as oxaliplatin and irinotecan, chemotherapy regimens can significantly prolong the survival time of patients; however, there are some clear limitations. Chemotherapy combined with targeted drugs, such as bevacizumab, improved the outcome in patients with metastatic colorectal cancer, but it is expensive. Fortunately, Chinese researchers have developed a targeted drug, recombinant human endostatin, which not only suppresses VEGF and angiogenesis to inhibit tumor metastasis, but is also less expensive [1–2]. Endostatin, a multi-targeted anti-angiogenesis drug, has been proven to be effective in the treatment of non-small cell lung cancer (NSCLC) [3–5], while its efficiency in colorectal cancer still needs further investigation. We initiated a prospective study in March 2008 to evaluate the efficacy and safety of endostatin in combination with FOLFOX4 in the treatment of metastatic colorectal cancer. Hepatic metastasis is crucial to patients' prognosis, and a major reason for death and organ failure in colorectal cancer patients. Thus, our study focused on patients with hepatic

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metastasis.

Materials and methods

Clinical data

From March 2008 to March 2010, a total of 60 colorectal cancer patients with metastatic diseases from the Department of Oncology, Fuzhou General Hospital of Nanjing Military Command were enrolled in this study. The study cohort consisted of 38 men and 22 women. Patient ages ranged from 18 to 75 years (median 60 years). All patients had pathological adenocarcinomas and had received initial treatment or re-treatment. Inclusion criteria were: histologically confirmed stage IV adenocarcinoma of the colon or rectum; at least 1 measurable lesion; aged 18 years or older; no history of chemotherapy, or no chemotherapy for at least 1 month; Karnofsky scores ≥70; estimated survival time ≥3 months; no other primary cancers; normal routine blood test, and liver and kidney function test results; no limit to chemotherapy; and no other immune-related diseases, such as Crohn's disease, ulcerative colitis, rheumatism and rheumatoid diseases, and metabolic syndrome. All patients must have given their written informed consent. Exclusion criteria were: previous exposure to anti-angiogenesis therapy; any adverse reactions or unforeseen events; and patients lost to follow-up. The results from patients who experienced adverse events or who were lost to follow-up were not included in the final analysis. However, the drug efficacy in patients who received at least 2/3 of the treatment course was included in the final statistical analysis. Patients were randomly assigned to the test group or control group. Patients in these 2 groups were matched for clinico-pathological features, such as sex, age, ECOG scores, and location of the primary lesion (Table 1).

Table 1 clinicopathological features of 60 cases patients with advanced metastatic colorectal cancer (n = 30)

Clinicopathological features	rh-Endo + chemotherapy	Chemotherapy
Sex		
Male	20	18
Femal	10	12
The median age (years)	58 (23–79)	62 (27–75)
ECOG score		
0–1	20	21
2	10	9
Disease status		
Retreatment	16	14
Initial treatment	14	16
Primary tumor site		
Colon cancer	21	18
Colorectal cancer	9	12
The number of metastatic sites		
1	15	13
>1	15	17
Metastatic sites		
Lymph node	22	23
Liver	12	15
Others	6	8

Treatment

Before the first course of chemotherapy, both the test and control groups underwent routine blood tests, liver and kidney functions tests, brain MRI, chest and abdomen CT, ECG, and bone scintigraphy. The control group received a FOLFOX4 chemotherapy regimen. Patients in the endostatin-FOLFOX group received 3–4 h of continuous infusion of endostatin from days 1–14. Cycles were repeated every 21 days. Patients in the test group received up to 4–6 cycles of FOLFOX4 plus endostatin. Patients received routine pretreatment before chemotherapy, and side effects were addressed with symptomatic treatment. Assessments of side effects were performed after the first cycle, while assessments of efficacy were performed after the second cycle. In patients for whom the treatment was effective, the therapy was continued for 4–6 cycles.

Observation target and evaluation criterion

According to Response Evaluation Criteria in Solid Tumors (RECIST; version 1.0), patients were divided into stable disease (SD), progressive disease (PD), partial response (PR), and complete response (CR) groups. We used CR plus PR to calculate objective regression rates (RR), and CR plus PR and SD to calculate disease control rates (DCR). Tumor assessment was performed using CT; if the preliminary assessment showed PR or CR, the result was confirmed after 4 weeks. Adverse events were assessed according to NCI-CTC 3.0. Quality of life (QOL) was assessed using KPS scores. Progression free survival (PFS) refers to the period from randomized grouping to disease progression or death. Overall survival (OS) refers to the period from randomized grouping to death. The concentrations of carcino embryonie antigen (CEA) and cancer antigen 199 (CA199) before and after treatment were also assessed.

Follow-up

Follow-up was performed for all of the patients through a telephone call, at the outpatient clinic, or using medical records. The follow-up time was from the end of chemotherapy to the death of the patient, or until the patient was lost to follow-up. During the follow-up period, if disease progression occurred, the patient could choose their subsequent therapy, including second-line chemotherapy, traditional Chinese medicine, or the best

supportive therapy.

Statistical analysis

SPSS 17.0 was used to perform the statistical analysis. The chi-square test was used to analyze count data. The Kaplan-Meier method was used to plot the survival curve. A log-rank test was employed to compare the survival times between the groups. The variation of CEA and CA199 was analyzed using a paired t test. A two-sided *P* value of less than 0.05 was deemed statistically significant.

Results

Chemotherapy performance

The test group completed 153 cycles of chemotherapy, with an average of 5.1 cycles per patient. The control group completed 138 cycles of chemotherapy, with an average of 4.6 cycles per patient. There was no significant difference between the 2 groups ($P > 0.05$).

Efficacy

There were 60 patients for whom efficacy could be evaluated. In the test group, 2 patients achieved a CR, 14 patients achieved a PR, 9 patients achieved SD, and 5 patients achieved PD. The objective RR was 53.3% and the DCR was 83.3%. In the control group, 1 patient achieved a CR, 10 patients achieved a PR, 11 patients achieved SD, and 8 patients achieved PD. The RR was 36.7% and the DCR was 73.3%. Using a chi-square test, the RR and DCR in these 2 groups were significantly different ($P < 0.05$). The median follow-up time was 18.4 months. The median PFS in the test control groups was 7.3 months and 5.3 months, respectively, which was significantly different ($P < 0.05$; Fig. 1). The median OS in the test control groups was 11.6 months and 9.3 months, respectively, which was significantly different ($P < 0.05$; Fig. 2).

We further investigated efficacy in the 27 patients with liver metastasis. In the test group (12 cases), no patients achieved a CR, 6 patients achieved a PR, 4 patients achieved SD, and 2 patients achieved PD. The RR was 50% and the DCR was 83.3%. In the control group (15 cases), no patients achieved a CR, 5 patients achieved a PR, 5 patients achieved SD, and 5 patients achieved PD. The RR was 33.3% and the DCR was 66.7%. Using a chi-square test, statistical significance was found when the RR and DCR in these 2 groups were compared ($P < 0.05$). The median follow-up time was 18.4 months. The median PFS in the test and control groups was 4.3 months and 4.1 months, respectively, but there was no statistical difference ($P > 0.05$). The median OS in the test and control groups was 8.9 months and 8.8 months, respectively, and there was no statistical difference ($P > 0.05$).

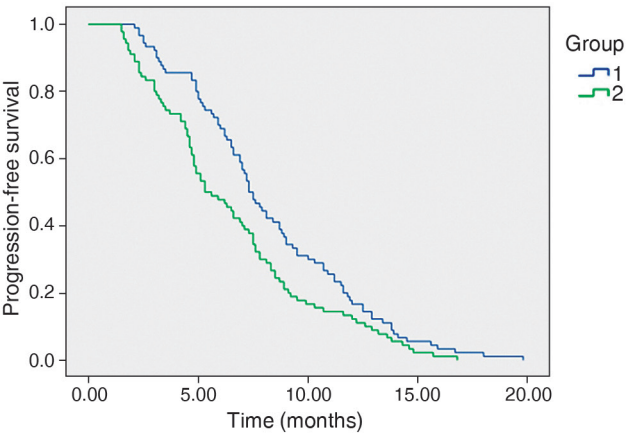

Fig. 1 Progression-free survival (PFS) curves of the experimental and control groups

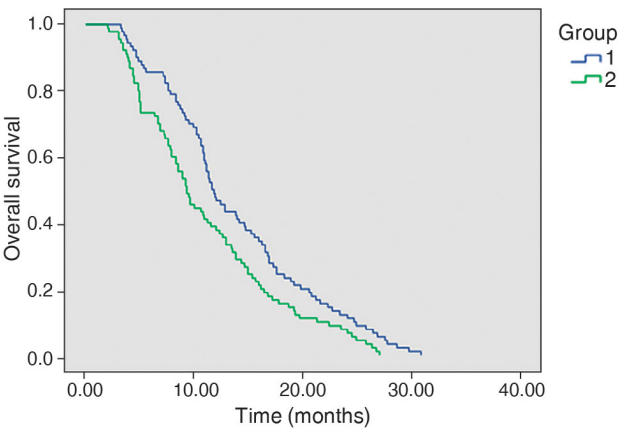

Fig. 2 Overall survival (OS) curves of the experimental and control groups

Adverse events

The major adverse events in these 2 groups were hematologic toxicity, gastrointestinal reactions, and neurotoxicity; these were mostly grade I–II and were relieved using supportive therapy. During the treatment, 1 patient developed arrhythmia, and 2 patients developed mild hypertension; both of these were considered to be endostatin-related and were relieved using supportive therapy. There were no statistical differences ($P > 0.05$; Table 2).

Quality of life evaluation

In the test group, 18 patients (60%) experienced improved QOL, 8 patients (26.7%) had a stable QOL, and 4 patients (13.3%) experienced a decline in their QOL. In the control group, 17 patients (56.7%) experienced improved QOL, 7 patients (23.3%) had stable QOL, and 6 patients (20%) experienced a decline in the QOL. There were no statistical differences ($P > 0.05$).

Table 2 Two treatment options cause toxicity of advanced colorectal cancer

Toxicity	Test groups (n = 30)				Control groups (n = 30)			
	I–II grade*		III–IV grade*		I–II grade		III–IV grade	
	n	%	n	%	n	%	n	%
Leukopenia	9	30	4	13.3	7	23.3	2	6.7
Thrombocytopenia	5	16.7	1	3.3	3	10	0	0
Anemia	8	26.7	1	3.3	8	26.7	0	0
Loss of appetite	9	30	3	10	7	23.3	3	10
Fatigue	12	40	2	6.7	10	33.3	1	3.3
Nausea and vomiting	6	20	1	3.3	3	10	0	0
Diarrhea	8	26.7	2	6.7	7	23.3	1	3.3
Oral mucositis	2	6.7	0	0	2	6.7	1	3.3
Peripheral neurotoxicity	11	36.7	0	0	12	40	0	0
Cardiac events	1	6.7	0	0	0	0	0	0
Hypertension	2	6.7	0	0	0	0	0	0
Hemorrhage	0	0	0	0	0	0	0	0
Abnormal liver function	5	16.7	0	0	6	20	0	0
Renal dysfunction	13	3.3	0	0	2	6.7	0	0

Compared with the control groups, * $P < 0.05$

Table 3 changes of CEA and CA199 before and after experimental groups treatment

Tumor markers	Test groups (n = 30)		Control groups (n = 30)	
	Before treatment	After treatment	Before treatment	After treatment
CEA (ng/mL)	428.32 ± 337.54	11.81 ± 3.23*#	432.51 ± 321.36	54.81 ± 12.01*
CA199 (μg/mL)	2667.71 ± 1209.11	102.25 ± 23.17*#	2059.88 ± 1526.45	363.89 ± 245.62*

Compared with before treatment, * $P < 0.05$; After treatment, compared with control groups, # $P > 0.05$

Tumor biomarkers

After treatment, the serum levels of CEA and CA199 were down-regulated in both groups, compared with the serum levels before treatment. Statistical significance was found in both groups ($P < 0.05$). As compared with the control group, the serum levels of CEA and CA199 in the test group showed a larger down-regulation, which was significantly different between the 2 groups ($P < 0.05$; Table 3).

Discussion

As the first anti-angiogenesis drug on the market, recombinant human endostatin can inhibit the migration of endothelial cells, suppress tumor angiogenesis, and block the nutrition supply to tumor cells to inhibit tumor progression and invasion [6]. A phase III randomized controlled clinical trial of an endostatin and NP regimen for NSCLC demonstrated that endostatin combined with an NP regimen can markedly improve the RR and median TTP of advanced NSCLC, and has a good level of safety [3].

Colorectal cancer is one of the most common digestive system carcinomas. Many patients present with metastatic disease and their median OS is less than 2 years. Standard first-line treatment includes fluorouracil with oxaliplatin or irinotecan. The most commonly used regimens are FOLFOX, FOLFIRI, or XELOX. Hurwitz *et al* [7] performed a phase III clinical trial and found that an irinotecan-based regimen combined with bevacizumab can prolong PFS and OS. This was the first time it has been proven that anti-angiogenesis therapy can have substantial survival benefits for colorectal cancer patients. Likewise, an oxaliplatin-based regimen combined with bevacizumab showed better short-term and long-term effects than a chemotherapy regimen alone [8]. As these effects may be attributable to the anti-VEGF role of bevacizumab, it offers a treatment option for metastatic colorectal cancer patients [9–11]. Thus, we hypothesized that other anti-angiogenesis drugs may improve the efficacy of a chemotherapy regimen in colorectal cancer patients. Basic studies on the inhibition of lymphatic endothelial cells in colorectal cancer have achieved good results, as have studies investigating anti-angiogenic agents [12–13].

Hut *et al* [14] reported the results of endostatin combined with chemotherapy for metastatic colorectal cancer. Thirty-one patients were enrolled in their study and received endostatin combined with oxaliplatin, irinotecan, or capecitabine-based chemotherapy. Among all of the patients, 12 achieved a PR, 11 achieved SD, and 8 patients achieved PD. The clinical effective rate was 38.7% (12/31) and the clinical benefit rate (CBR) was 74.2% (23/31). Among the 13 patients who were receiving their first treatment, 9 achieved a PR and the clinical benefit

rate was 100%. For patients who were receiving their second or third line treatment, the clinical effective rate was 16.7%. Zhuang *et al* [15] reported retrospective data on the comparison of capecitabine combined with irinotecan or capecitabine combined with endostatin for oxaliplatin failure in 45 metastatic colorectal cancer patients. In the irinotecan group, the RR was 32.0%, the CBR was 72.0%, and the TTP was 6.2 months. In the endostatin group, the RR was 55.0%, the CBR was 90.0%, and the TTP was 10.6 months. The differences between the 2 groups were significant ($P < 0.05$). The OS in these 2 groups was 15.2 months and 16.01 months, respectively. Taken together, endostatin combined with chemotherapy can be effective for metastatic colorectal cancer patients.

Our study enrolled colorectal cancer patients with stage IV disease and multiple metastases; the QOL scores in this group were low. The study design was prospective, and the test group was treated with endostatin combined with FOLFOX4, while the control group was treated with FOLFOX4 alone. The results showed that in the test group and control group, the RR was 53.3% and 36.7%, the DCR was 83.3% and 73.3%, the PFS was 7.3 months and 5.3 months, and the OS was 11.6 months and 9.3 months, respectively. Differences between the 2 groups were statistically significant ($P < 0.05$). Thus, the test group showed a significant improvement in the RR, DCR, and OS. This study indicated that endostatin combined with chemotherapy is superior to chemotherapy alone.

The morbidity and mortality of colorectal cancer have been increasing year by year, and about 50%–60% of colorectal cancer patients have liver metastases. Twenty to forty percent of patients have liver metastases at the time of diagnosis [16–17]. Some researchers have demonstrated that liver metastasis is the main reason for hepatic failure and death. In order to test whether patients with liver metastases could benefit from Endostar combined with chemotherapy, 27 cases of liver metastases were further analyzed. Our study indicated that there was a significant difference ($P < 0.05$) in RR and DCR between the test group and the control group, but not in PFS and OS. This result suggested that RR and DCR were greatly improved in the test group, whereas the OS was not prolonged. Meanwhile, this also proved that liver metastases were important factors that affected the prognosis; complete liver metastases resection is the only curative option for patients with colorectal cancer liver metastases. A number of studies show that the 5-year event-free survival of colorectal cancer liver metastases patients who do not undergo surgery is about 0.5% [18]. By contrast, the 5-year event-free survival of those who are suitable for surgery initially or after conversion therapy is 30%–50%. However, only 15%–20% of patients are suitable for radical resection, and most cannot undergo surgery for various reasons. Conversion therapy can transform 10%–30% of unresectable liver metastases into resectable disease, which has significantly increased the resection rate, prolonged survival, and improved the prognosis. Based on the literature and our research, colorectal cancer liver metastases remain challenging. To date, to prolong survival and improve QOL, the most effective approach is still comprehensive treatment, including surgery, chemotherapy, radiotherapy, interventional therapy, and targeted therapy [19].

No additional adverse events were observed in the endostatin group, showing that all of the patients can tolerate the administered therapy. After treatment, the QOL of patients from both groups was markedly improved, and the patients were able to receive further therapy. As well-known clinical biomarkers for colorectal cancer, CEA and CA199 cannot act as specific diagnostic criteria; however, they can be meaningful for follow-up of patients who have undergone surgery or completed chemotherapy. The down-regulation of CEA and CA199 may indicate remission or control of the tumor [20]. Based on the results of the current study, the serum levels of CEA and CA199 were down-regulated after treatment. Furthermore, compared with the control group, the serum levels of these biomarkers were more significantly down-regulated in the test group, which further demonstrated that endostatin combined with chemotherapy is superior to chemotherapy alone.

In conclusion, this trial provides confirmation that endostatin can enhance the anti-tumor effect of FOLFOX4 when used as the first-line treatment for metastatic colorectal cancer. Endostatin can prolong the survival time of patients without increasing the risk of adverse events. In addition, this trial found that endostatin plus FOLFOX4 cannot prolong the survival time of colorectal cancer patients with liver metastasis, which is a limitation of this regimen. Due to the small sample of this trial, the results may be somewhat biased. Thus, a clinical trial with a large sample population is needed to demonstrate the survival benefit of endostatin plus chemotherapy for metastatic colorectal cancer.

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ORIGINAL ARTICLE

Pathological characteristics and immunophenotype analysis of cervical intraepithelial neoplasia

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Abstract

Objective To explore the clinical pathological features and immunophenotypes of cervical intraepithelial neoplasia (CIN).

Methods The protein expression of p16, p53, Bcl-2, and c-erbB-2 in 59 cases of CIN, 20 cases of cervical squamous cell carcinoma, and 20 cases of normal cervical tissues were tested using immunohistochemistry staining.

Results The expression rates of p16, p53, Bcl-2, and c-erbB-2 in CIN tissues were 76.3% (45/59), 28.85 (17/59), 61.0% (36/59), and 40.0% (23/59), respectively. The expression rates of p16, p53, Bcl-2, and c-erbB-2 in cervical squamous cell carcinoma tissues were 60.0% (12/20), 60.0% (12/20), 75.0% (15/20), and 65.0% (13/20), respectively. The expression rates of p16, p53, Bcl-2, and c-erbB-2 in normal cervical tissues were 0.0% (0/20), 0.0% (0/20), 35.0% (7/20), 0.0% (0/20), respectively. In comparison to normal tissues, the differential expressions of p16, p53, and Bcl-2 in squamous cell carcinoma and CIN were statistically significant (P <0.001), whereas the difference between the squamous cell carcinoma and CIN was not significant (P >0.05). In comparison to normal tissues, the differential expressions of c-erbB-2 and p53 in squamous cell carcinoma and CIN were statistically significant (P >0.05). The differential expressions of Bcl-2, c-erbB-2, and p53 in CIN 3 were statistically significant in comparison to CIN 1 and CIN 2 (P <0.05).

Conclusion Overexpression of Bcl-2 occurs early in the development of cervical cancer, whereas p16 and c-erbB-2 overexpression are markers for cell malignancy. The expression of p53 is correlated with the development of cervical cancer.

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Key words: cervical intraepithelial neoplasia; clinical pathology; protein; immunohistochemistry

Cervical intraepithelial neoplasia (CIN) is a precancerous lesion of invasive cervical cancer. The progression from CIN to invasive carcinoma takes approximately five to 15 years, but not all cases of CIN will transform into invasive carcinoma. Among these, CIN grade I (CIN l) and CIN grade II (CIN 2) are pathologically unstable, in which 50% of the cases progress into remission or have no further changes, whereas 20% to 30% of these cases deteriorate [1–4]. CIN can be treated, and understanding the disease progression patterns and receiving timely and effective treatment could reduce its incidence rate. The biological patterns of CIN development are difficult to predict and are affected by observers' experiences. In this study, the protein expression of p16, p53, Bcl-2, and c-erbB2 in CIN l, CIN 2, and CIN grade III (CIN 3) were analyzed retrospectively using immunohistochemistry staining techniques. The results can serve as a reference for objective diagnosis of CIN development and its clinical treatment.

Materials and methods

Materials and methods

Clinical cases and specimens

Paraffin-embedded specimens of hysterectomy or uterine biopsy/cervical conization were collected at the 150th Central Hospital of People's Liberation Army between June 2003 and June 2014. Based on the guidelines of the World Health Organization (WHO) Pathology and Genetics of Tumours of the Breast and Female Genital

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Organs (2006) [5–6], there were 59 cases of CIN, 20 cases of cervical squamous cell carcinoma, and 20 cases of normal cervical tissues. Among the 59 cases of CIN, 12 cases were classified as CIN 1, 19 cases were classified as CIN 2, and 28 cases were classified as CIN 3. Among the 20 cases of cervical squamous carcinoma, 10 cases were of the keratinizing type and the remaining 10 cases were of the non-keratinizing type. The 20 normal cervical tissues were cervical intraepithelia from either uterine fibroids or ovarian cancer hysterectomy. Patients with CIN were between 21 and 58 years old, and the average was 34.2 ± 8.5 years old. Although most patients sought treatment due to an increase in vaginal secretions or contact bleeding, some patients sought treatment after discovering lesions during physical examination. In general, in comparison to normal cervical tissues, these CIN cervical tissues showed mild to severe erosion and tumors were clearly visible in particular cases. All patients were followed up with for six to 34 months. Among the 59 CIN cases followed up, two cases experienced recurrence, whereas 57 cases remained in remission.

Methods

Pathological specimens were fixed in 10% buffered formalin, and processed and embedded in paraffin for subsequent hematoxylin and eosin (H&E) staining and reaction with immunohistochemical markers. Immunohistochemical staining was carried out using the En Vision two-step system. The primary antibodies used were p16 (6H12), p53 (DO-7), Bcl-2 (8C8), and c-erbB-2 (EP3), all of which were purchased from Fuzhou Maixin Biotech. Co. Ltd. Immunohistochemical staining was carried out according to routine procedures. 3,3'-diaminobenzidine (DAB) was used for staining, and phosphate buffered saline (PBS) was employed as a negative control for the primary antibody.

Observations

Positively stained cellular cytoplasm and/or membrane and nucleus appeared as brownish yellow under the microscope. p16 was localized in the nucleus/cytoplasm, Bcl-2 was localized in the cytoplasm/membrane, p53 was localized in the nucleus, and c-erbB-2 was localized in the cell membrane. For each specimen, 10 high-magnification views (400×) were randomly selected, and 200 cells were counted for each view. Samples showing an average of 5% to 25% positive results were considered weakly positive (+), an average of 26% to 75% were considered moderately positive (++), and an average of >75% were considered strongly positive (+++). Cytoplasm/membrane/nucleus that did not appear brownish yellow, or cells showing an average of <5% positive results were considered negative (–).

Statistical analysis

SPSS 12.0 was used for data analysis and Chi-square test and Fisher's exact test were used to compare groups. Values of P <0.05 were considered as statistically significant.

Results

Clinical manifestations and pathological features

Among the 59 cases of CIN, 48 patients sought treatment due to an increase in vaginal secretions or contact bleeding, whereas 11 patients sought treatment after discovering lesions during physical examination. At the tissue morphology level, 2/3 of CIN 1 epithelia were mature cells and cells from the superficial layer showed heterogeneity (Fig. 1). Half of CIN 2 epithelia were mature cells and cells from the upper and middle layers showed significant nuclear heterogeneity (Fig. 2). No mature cells were seen for the CIN 3 epithelia and heterogeneity was seen for the entire epithelial layer (Fig. 3).

The expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN

p16 was localized in the nucleus/cytoplasm (Fig. 3). The differential expressions of p16 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05), although there was no significant difference between its expression in CIN 3 in comparison to CIN 1 and CIN 2 (P >0.05). p53 protein was localized in the nucleus (Fig. 3) and showed diffuse, flaky, and spotty distribution in squamous cell. The differential expressions of p53 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05). In addition, its expression in CIN 3 was significantly higher than that in CIN 1 and CIN 2(P <0.05). Bcl-2 protein was distributed in the basal layer of the upper epithelia of normal squamous tissues but showed diffuse, flaky, and spotty distribution in squamous cell carcinoma. The expression of Bcl-2 was higher in CIN and squamous cell carcinoma than in normal cervical tissue (P <0.05). However, there was no significant difference between Bcl-2 expression in squamous cell carcinoma and CIN (P >0.05). Bcl-2 expression in CIN 3 was significantly different from that in CIN 1 and CIN 2 (P <0.05). The expression of Bcl-2 was also higher in non-keratinizing squamous cell carcinoma than in keratinizing squamous cell carcinoma (P <0.05). c-erbB-2 protein was localized in the plasma membrane (Fig. 3). The differential expressions of c-erbB-2 in CIN, squamous cell carcinoma, and normal cervical tissue were significant (P <0.05), with higher expression in CIN 3 than in CIN 1 and CIN 2 (P <0.05) (Table 1).

Comparison of expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN and

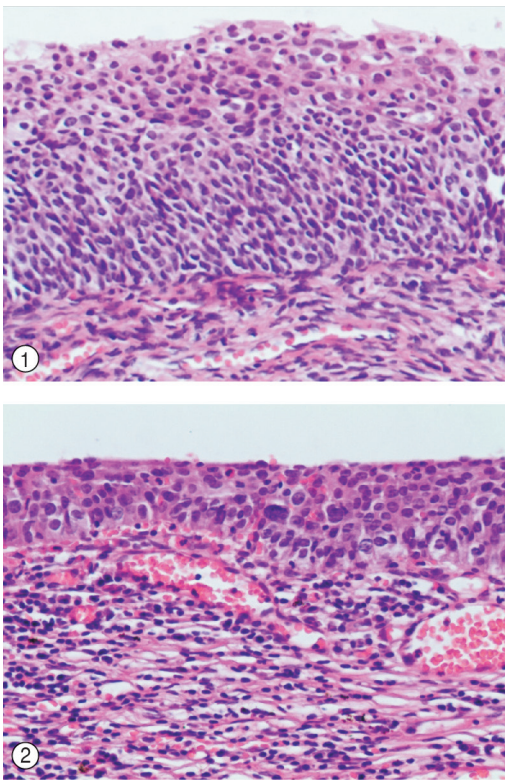

Fig. 1 2/3 of the epithelium was mature in CIN 1, with cells on the superficial layer showing heterogeneity
Fig. 2 1/2 of the epithelium was mature in CIN 2, with cells on the superficial layer showing heterogeneity

cervical squamous cell carcinoma

The expression of p53 was similar to that of c-erbB-2, with their differential expressions in squamous cell carcinoma, CIN, and normal cervical tissue being significantly different (P <0.05). The expression of p53 in CIN 3 was significantly higher than in CIN 1 and CIN 2 (P <0.05). In cervical squamous carcinoma, the expression of Bcl-2 was opposite to that of p53. The expressions of Bcl-2 and c-erbB-2, Bcl-2 and p53, and c-erbB-2 and p53 in squamous cell carcinoma showed no significant differences (P >0.05). However, the expressions of Bcl-2 and c-erbB-2 as well as of Bcl-2 and p53 in CIN showed significant differences (P <0.05). The expressions of Bcl-2 and p53 in CIN 3 showed significant differences (P <0.05) (Table 2).

Discussion

In clinical practice, CIN diagnosis is often affected by the observers' experience, such as confusion stemming from cervical immature squamous metaplasia, repairing of squamous epithelia, and aging squamous epithelia, which could lead to under-diagnosis or misdiagnosis. Previous studies have shown that activation of oncogenes, mutations in tumor suppressor genes, and over-expression of anti-apoptotic genes were related to the development of cervical cancer [7–8]. Histologically, cervical cancer progresses from squamous metaplasia, dysplasia, in situ carcinoma, and finally to an invasive cancer. p16 is a tumor suppressor gene encoding the p16 protein, which is directly involved in the regulation of the cell cycle. Mutations, deletion, or methylation of the p16 gene can result in functional changes and eventually lead to tumorigenesis. This study found that p16 protein level was closely correlated with CIN, particularly CIN 3, and cervical squamous cell carcinoma. For lesions stemming from aging cervical intraepithelial and basal layer hyperplasia and CIN 3, p16 can be used for diagnosis of CIN 3 and cervical squamous cell carcinoma. In this study, the expression rate of p16 in CIN 1 was 59%, but there were negative cases in CIN 2 to CIN 3. Therefore, p16 staining alone cannot serve as a precise diagnosis of CIN lesions. Results from this study also showed that the combination of p16, p53, and Bcl-2 expression can be applied to determine the CIN grade. Patients with low-grade CIN lesions with high expression of these proteins should be followed up with and closely monitored.

Bcl-2 plays an important role in the regulation of apoptosis [9]. It can extend cellular lifespan and prevent apoptosis. This study found that the expression of Bcl-

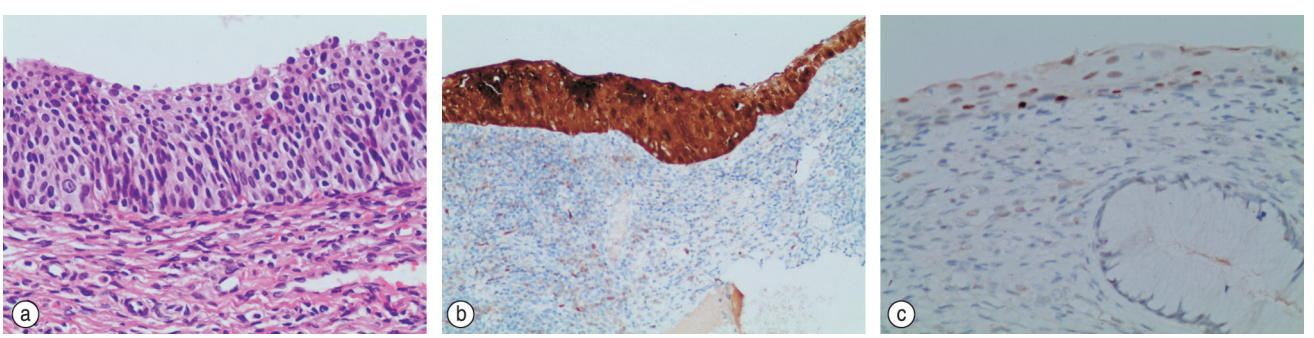

Fig. 3 (a) No mature epithelium in CIN 3, with the entire epithelial layer showing heterogeneity; (b) p16 expression in CIN 3; (c) p53 expression in CIN 2

2 protein was increased from normal cervix to CIN and cervical squamous cell carcinoma tissues. Similarly, the expression of Bcl-2 gradually increased with the aggravation of CIN lesions. The higher expression of Bcl-2 in CIN 3 than in CIN 1 and CIN 2 was significant, whereas there was no significant difference in Bcl-2 expression between cervical squamous cell carcinoma and CIN. This suggests that Bcl-2 overexpression is correlated with the development of cervical cancer, and Bcl-2 overexpression is an early event in the development of cervical cancer. One possible mechanism for this observation is that Bcl-2 overexpression may inhibit cell apoptosis. Apoptosis inhibition is conducive not only to continuous cell proliferation but also to further accumulation of abnormal genes and induction of genome instability, which further promotes transformation of cells that is beneficial for the development of tumors. Furthermore, this study also found that the expression of Bcl-2 protein decreased with increasing CIN grades. This result suggests that Bcl-2 might inhibit cellular apoptosis in the early stages of cervical cancer to promote cell transformation and cancer development. When cancer cells are at low differentiation levels, the inhibitory function of Bcl-2 protein on cell apoptosis decreases, the Bcl-2 expression reduces, and at this stage, tumor development is likely to be associated with other genes. In addition, the differential expression of Bcl-2 in keratinizing and non-keratinizing types was significant, indicating that Bcl-2 expression was related to tissue types.

The *c-erbB-2* gene is located on chromosome 17q21 and encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity involved in signal transduction. When *c-erbB-2* is highly expressed, the c-erbB-2 protein serves as a "cancerous" protein receptor on the membrane. Extracellular signals can activate multiple signal transduction pathways, including activation of oncogenes such as c-ras, c-src, and c-myc via c-erbB-2. Such activation promotes cell transformation and cancer development, and c-erbB-2 activation plays a central role in this cascade of event [10–12]. Results from this study show that the expression rate of c-erbB-2 increased from normal cervical tissues to CIN to cervical squamous cell carcinoma, and the differential expression was significant. The expression of c-erbB-2 was also similar with that of Bcl-2, with its expression level increasing with increasing grades of CIN lesions. The expression of c-erbB-2 was significantly higher in CIN 3 than in CIN 1 and CIN 2. Thus, c-erbB-2 is also closely associated with the development of cervical cancer. Its expression in cancer cells makes c-erbB-2 one of the cellular markers of malignant transformation. The study found that there was no correlation between c-erbB-2 expression and histological types, pathological grades, clinical stages and metastasis, suggesting the lack of a significant relationship between c-erbB-2 over-expression and clinical prognosis.

p53 is one of the most actively studied genes. The protein product of the tumor suppressor gene *p53*, wild-type p53 (wt p53) is responsible for maintaining genomic stability, inhibition of cancer, regulation of cell differentiation, and senescence. Immunohistochemical staining revealed that the p53 protein was usually inactivated. Reasons for p53 inactivation include point mutations, loss of heterozygosity, or the creation of stable p53 complexes by fusion with HPV E6 protein and other viral oncoproteins. Inactivated p53 proteins not only are unable to inhibit cancer development but also are able to promote cell transformation and prevent the function of wt p53, which in turn leads to the accumulation of mutations in DNA and finally cancer [7]. Results from this study showed that the expression of p53 in normal cervical tissue, CIN, and

Table 1 Comparison of 79 cases of cervicitis and intraepithelial neoplasia

Samples	*n*	p16		p53		Bcl-2		c-erbB-2	
		n (%)	*P* value	*n* (%)	*P* value	*n* (%)	*P* value	*n* (%)	*P* value
CIN	59	45 (76.3)	0.067	17 (28.8)	0.000	36 (61.0)	0.020	23 (40.0)	0.005
CIN 1	12	7 (58.3)		0 (0.0)		3 (25.0)		2 (16.7)	
CIN 2	19	13 (68.4)		2 (10.5)		9 (47.4)		4 (21.1)	
CIN 3	28	25 (89.3)		15 (53.6)		20 (71.4)		17 (60.7)	
Carcinoma	20	12 (60.0)	0.161	12 (60.0)	0.012	15 (75.0)	0.259	13 (65.0)	0.043
Control	20	0 (0.0)		0 (0.0)		7 (35.0)		0 (0.0)	

Table 2 Expression of p16, p53, Bcl-2, and c-erbB-2 proteins in CIN and cervical carcinoma tissues

	CIN			CIN 3			Cervical carcinoma		
	+	–	*P* value	+	–	*P* value	+	–	*P* value
p16	45	14	0.074	25	34	0.328	12	8	1.000
p53	17	42	0.000	15	13	0.168	12	8	1.000
Bcl-2	36	23	0.017	20	8	0.397	15	5	0.311
c-erbB-2	23	36	0.243	17	11	0.110	13	7	0.490

cervical squamous cell carcinoma was similar with that of c-erbB-2, suggesting that p53 protein overexpression is also correlated with the development of cervical cancer. However, the expression of p53 in CIN 3 and invasive cervical cancer was not significantly different. This result is consistent with studies in the literature that reported p53 expression as an early event in the cervical cancer development. It was found that p53 expression was higher as cells became less differentiated. The expression of p53 in keratinizing cases was significantly different from that in non-keratinizing cases, indicating that p53 is correlated with histological types. Tumor cells with high expression of p53 have higher invasive capability, and given that p53-positive tumors correlated with metastasis, patients with high p53 expressing tumors were prone to metastasis. The expression of p53 in clinical stage I tumors was significantly lower than in clinical stage II and III tumors, suggesting that p53 overexpression was correlated with cancer invasion and metastasis.

This study also found that the expressions of Bcl-2 and c-erbB-2, Bcl-2 and p53, as well as c-erbB-2 and p53 in cervical cancer tissues were not significantly different, but the expressions of Bcl-2 and c-erbB-2 as well as Bcl-2 and p53 in CIN tissues were significantly different, indicating that the expression of Bcl-2 and p53 proteins in CIN groups were significantly correlated. This might be due to the loss of wt p53 function leading to down-regulation of the Bcl-2 expression, which further leads to apoptosis inhibition. In contrast, high expression of Bcl-2 also could suppress p53-induced apoptosis, which could easily lead to the formation of cancerous cells and increase the probability of cervical cancer. There was no statistical significance between c-erbB-2 and p53, suggesting that c-erbB-2 amplification and overexpression as well as mutations in *p53* in the development of cervical cancer may not be co-existing.

For diagnosis of CIN lesions, the expressions of p16, p53, Bcl-2, and c-erbB-2 should be monitored. Close monitoring and follow-ups should be carried out as required to prevent over-treating patients.

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