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GUIDELINES

Consensus on the digestive endoscopic tunnel technique

Ning-Li Chai, Hui-Kai Li, En-Qiang Linghu, Zhao-Shen Li, Shu-Tian Zhang, Yu Bao, Wei-Gang Chen, Philip WY Chiu, Tong Dang, Wei Gong, Shu-Tang Han, Jian-Yu Hao, Shui-Xiang He, Bing Hu, Bing Hu, Xiao-Jun Huang, Yong-Hui Huang, Zhen-Dong Jin, Mouen A Khashab, James Lau, Peng Li, Rui Li, De-Liang Liu, Hai-Feng Liu, Jun Liu, Xiao-Gang Liu, Zhi-Guo Liu, Ying-Cai Ma, Gui-Yong Peng, Long Rong, Wei-Hong Sha, Pateek Sharma, Jian-Qiu Sheng, Shui-Sheng Shi, Dong Wan Seo, Si-Yu Sun, Gui-Qi Wang, Wen Wang, Qi Wu, Hong Xu, Mei-Dong Xu, Ai-Ming Yang, Fang Yao, Hong-Gang Yu, Ping-Hong Zhou, Bin Zhang, Xiao-Feng Zhang, Ya-Qi Zhai

ORCID number: Ning-Li Chai (0000-0002-6791-5817); Hui-Kai Li (0000-0002-0276-4587); En-Qiang Linghu (0000-0003-4506-7877); Zhao-Shen Li (0000-0002-1650-4014); Shu-Tian Zhang (0000-0003-1268-8247); Yu Bao (0000-0002-8877-1621); Wei-Gang Chen (0000-0001-6046-6441); Philip WY Chiu (0000-0001-9292-112X); Tong Dang (0000-0002-0268-8756); Wei Gong (0000-0003-1510-6089); Shu-Tang Han (0000-0001-7388-6816); Jian-Yu Hao (0000-0002-6414-8974); Shui-Xiang He (0000-0003-3396-5653); Bing Hu (0000-0002-9898-8656); Bing Hu (0000-0001-5668-5318); Xiao-Jun Huang (0000-0002-1210-3598); Yong-Hui Huang (0000-0003-1284-9004); Zhen-Dong Jin (0000-0002-0505-4935); Mouen A Khashab (0000-0001-5085-7908); James Lau (0000-0002-3584-3265); Peng Li (0000-0002-7610-6253); Rui Li (0000-0002-0649-4780); De-Liang Liu (0000-0001-8986-4793); Hai-Feng Liu (0000-0002-4716-1771); Jun Liu (0000-0003-4218-6133); Xiao-Gang Liu (0000-0003-3147-5116); Zhi-Guo Liu (0000-0003-0456-4324); Ying-Cai Ma (0000-0001-7701-5145); Gui-Yong Peng (0000-0002-2691-5271); Long Rong (0000-0002-3635-4682); Wei-Hong Sha (0000-0001-6615-422X); Prateek Sharma (0000-0003-4003-7548); Jian-Qiu Sheng (0000-0002-4639-475X); Shuisheng Shi (0000-0002-9779-8260); Dong-Wan

Ning-Li Chai, Hui-Kai Li, En-Qiang Linghu, Ya-Qi Zhai, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing 100853, China

Zhao-Shen Li, Zhen-Dong Jin, Department of Gastroenterology, Changhai Hospital, Naval Medical University, Shanghai 200433, China

Shu-Tian Zhang, Peng Li, Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Yu Bao, Department of Gastroenterology and Hepatology, Sichuan Cancer Hospital and Institute, Chengdu 610000, Sichuan Province, China

Wei-Gang Chen, Department of Gastroenterology, First Affiliated Hospital of School of Medicine, Shihezi University, Shihezi 832002, Xinjiang Uygur Autonomous Region, China

Philip WY Chiu, Department of Surgery, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

Tong Dang, Department of Gastroenterology, the Second Affiliated Hospital of Baotou Medical College, Baotou 014030, Inner Mongolia Autonomous Region, China

Wei Gong, Department of Gastroenterology, Shenzhen Hospital of Southern Medical University, Shenzhen 518000, Guangdong Province, China

Shu-Tang Han, Gastrointestinal Endoscopy Center, Affiliated Hospital of Nanjing Traditional Chinese Medicine University, Nanjing 210029, Jiangsu Province, China

Jian-Yu Hao, Department of Gastroenterology, Beijing Chaoyang Hospital, Capital Medical University, Beijing 100020, China

Shui-Xiang He, Department of Gastroenterology, the First Affiliated Hospital of Xi'an Jiao Tong University, Xi'an 710000, Shaanxi Province, China

Bing Hu, Bing Hu, Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Bing Hu, Bing Hu, Department of Endoscopy, Eastern Hepatobiliary Surgical Hospital Affiliated to Naval Medical University, Shanghai 200438, China

Xiao-Jun Huang, Department of Gastroenterology, the Second Hospital of Lanzhou University,

Seo (0000-0002-9720-0425); Si-Yu Sun (0000-0002-1133-9726); Gui-Qi Wang (0000-0002-2922-3780); Wen Wang (0000-0001-8392-3457); Qi Wu (0000-0002-8782-3055); Hong Xu (0000-0003-1228-3645); Mei-Dong Xu (0000-0002-5213-0451); Ai-Ming Yang (0000-0002-8597-1038); Fang Yao (0000-0001-9247-1130); Hong-Gang Yu (0000-0002-0178-8747); Ping-Hong Zhou (0000-0002-5434-0540); Bin Zhang (0000-0001-7024-1609); Xiao-Feng Zhang (0000-0001-6644-0816); Ya-Qi Zhai (0000-0001-6508-264X).

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Lanzhou 730030, Gansu Province, China

Yong-Hui Huang, Department of Gastroenterology, Peking University Third Hospital, Beijing 100191, China

Mouen A Khashab, Division of Gastroenterology and Hepatology, the Johns Hopkins University School of Medicine, Baltimore, MD 21218, United States

James Lau, Department of Gastrointestinal Endoscopy, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

Rui Li, Department of Gastroenterology, the First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China

De-Liang Liu, Department of Gastroenterology, Second Xiangya Hospital of Central South University, Changsha 410000, Hunan Province, China

Hai-Feng Liu, Department of Gastroenterology, General Hospital of Chinese People's Armed Police Forces, Beijing 100039, China

Jun Liu, Division of Gastroenterology, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Xiao-Gang Liu, Department of Gastroenterology, Sichuan Provincial People's Hospital, Chengdu 610072, Sichuan Province, China

Zhi-Guo Liu, Department of Gastroenterology, Xijing Hospital, Air Force Medical University, Xi'an 710032, Shaanxi Province, China

Ying-Cai Ma, Department of Digestion, Qinghai Provincial People's Hospital, Xining 810007, Qinghai Province, China

Gui-Yong Peng, Department of Gastroenterology, Southwest Hospital, Army Medical University, Chongqing 400038, China

Long Rong, Department of Gastroenterology, Peking University First Hospital, Beijing 100034, China

Wei-Hong Sha, Department of Gastroenterology and Hepatology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou 510080, Guangdong Province, China

Jian-Qiu Sheng, Department of Gastroenterology, Beijing Military General Hospital, Beijing 100700, China

Shui-Sheng Shi, Endoscopy Center, the Second Hospital of Shanxi Medical University, Taiyuan 030001, Shanxi Province, China

Si-Yu Sun, Endoscopy Center, Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China

Gui-Qi Wang, Fang Yao, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100020, China

Wen Wang, Department of General Surgery, Fuzhou General Hospital, Fuzhou 350025, Fujian Province, China

Qi Wu, Endoscopy Center, Peking University Cancer Hospital and Institute, Beijing 100142, China

Hong Xu, Endoscopy Center, the First Hospital of Jilin University, Changchun 130021, Jilin Province, China

Mei-Dong Xu, Ping-Hong Zhou, Endoscopy Center, Endoscopy Research Institute, Zhongshan Hospital, Fudan University, Shanghai 200032, China

Ai-Ming Yang, Department of Gastroenterology, Peking Union Medical College Hospital, Beijing 100730, China

Hong-Gang Yu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan University, Wuhan 430060, Hubei Province, China

Bin Zhang, Endoscopy Center, China-Japan Union Hospital of Jilin University, Changchun 130021, Jilin Province, China

Xiao-Feng Zhang, Department of Gastroenterology, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China

Corresponding author: En-Qiang Linghu, MD, PhD, Professor, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, No. 28, Fuxing Road, Haidian District, Beijing 100853, China. linghuenqiang@vip.sina.com
Telephone: +86-10-55499292
Fax: +86-10-68154653

Abstract

With the digestive endoscopic tunnel technique (DETT), many diseases that previously would have been treated by surgery are now endoscopically curable by establishing a submucosal tunnel between the mucosa and muscularis propria (MP). Through the tunnel, endoscopic diagnosis or treatment is performed for lesions in the mucosa, in the MP, and even outside the gastrointestinal (GI) tract. At present, the tunnel technique application range covers the following: (1) Treatment of lesions originating from the mucosal layer, *e.g.*, endoscopic submucosal tunnel dissection for oesophageal large or circular early-stage cancer or precancerosis; (2) treatment of lesions from the MP layer, per-oral endoscopic myotomy, submucosal tunnelling endoscopic resection, *etc.*; and (3) diagnosis and treatment of lesions outside the GI tract, such as resection of lymph nodes and benign tumour excision in the mediastinum or abdominal cavity. With the increasing number of DETTs performed worldwide, endoscopic tunnel therapeutics, which is based on DETT, has been gradually developed and optimized. However, there is not yet an expert consensus on DETT to regulate its indications, contraindications, surgical procedure, and postoperative treatment. The International DETT Alliance signed up this consensus to standardize the procedures of DETT. In this consensus, we describe the definition, mechanism, and significance of DETT, prevention of infection and concepts of DETT-associated complications, methods to establish a submucosal tunnel, and application of DETT for lesions in the mucosa, in the MP and outside the GI tract (indications and contraindications, procedures, pre- and postoperative treatments, effectiveness, complications and treatments, and a comparison between DETT and other operations).

Key words: Digestive endoscopic tunnel technique; Endoscopic submucosal tunnel dissection; Per-oral endoscopic myotomy; Submucosal tunnelling endoscopic resection; Gastrointestinal tract

Core tip: The digestive endoscopic tunnel technique (DETT) makes many diseases that used to be treated by surgery become endoscopically curable by establishing a submucosal tunnel between the mucosa and muscularis propria (MP). At present, the tunnel technique application range covers the treatment of lesions originating from the mucosal and MP layers, and diagnosis and treatment of lesions outside the gastrointestinal tract. However, there is not yet an expert consensus on DETT. In this consensus, we describe the definition, mechanism, and significance of DETT, prevention of infection, methods to establishing a submucosal tunnel, and three main applications of DETT.

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INTRODUCTION

The digestive endoscopic tunnel technique (DETT) is a new endoscopic treatment technique in which a submucosal tunnel is established, making many diseases that previously would have been treated by surgery become endoscopically curable. Compared with surgery, DETT has certain advantages, such as less trauma and faster recovery. The emergence of DETT is a milestone in the development of endoscopic treatment and significantly broadens the application range of endoscopy. With the increasing number of DETTs performed worldwide, endoscopic tunnel therapeutics, which is based on DETT, has been gradually developed and optimized. However, there is not yet a consensus or guideline on DETT to regulate its indications, contraindications, surgical procedure, and postoperative treatment. Therefore, it is necessary to generate this consensus on the DETT.

The International DETT Alliance (IDETTA) is an un-official technical association composed of 48 endoscopy experts majoring in the DETT method from China-mainland, China-Hong Kong, United States, and Korea. This consensus was drafted by all the members of the alliance and is the first international consensus on DETT aiming to guide and regulate the performance of DETT by doctors worldwide. Systematic document retrieval was performed by searching the online databases of PubMed, China National Knowledge Internet, and SinoMed for articles published from 2009 to 2018 to generate a better understanding of DETT and to develop key questions regarding pre-operative treatments, indications, techniques, complications, outcomes, and postoperative treatment. The retrieved documents were evaluated, and pertinent documents were adopted. Subsequently, a statement and an explanation were created to construct a questionnaire based on PICO. All 48 members of IDETTA voted on the created statements using the Delphi method. The questionnaire was send by email to each expert. The consensus was assessed at the evidence level and recommendation level according to evidenced-based medicine (Tables 1 and 2). Considering that an increasing number of new evidence-based clinical outcomes may emerge in the future, the association will arrange meetings as necessary to update the consensus.

We established six categories for evaluation as follows: Preoperative treatment, indications, techniques, complications, treatment outcome (recurrence, metastasis, and prognosis), postoperative follow-up, and pathology. For each clinical question, systematic document retrieval was done by searching PubMed and Igaku Chuo Zasshi for articles.

DEFINITION OF DETT

The concept of DETT was initially put forward by professor Linghu in 2009 after its successful application to a large, circumferential oesophageal lesion[1], and this clinical event marked the birth of DETT. DETT aims to establish a submucosal tunnel between the mucosa and muscularis propria (MP), through which endoscopic diagnosis or treatment is performed for lesions in the mucosa, in the MP, and even outside the gastrointestinal (GI) tract[2].

MECHANISM OF DETT

The key mechanism of DETT is to divide the digestive tract wall into two layers (mucosa and MP) and to maintain the integrity of one layer when the other layer is opened for treatment or diagnosis. Therefore, the intra-luminal and extra-luminal space is isolated, and intra-luminal gas or fluid cannot enter the extra-luminal space[3] (Figure 1).

SIGNIFICANCE OF DETT

Based on the practice of original per-oral and per-anal endoscopy, DETT establishes an "artificial tunnel" that is different from the natural GI tract[2]. For a long time, the MP has been considered the boundary between GI internal medicine and surgery; the MP plays an important role in avoiding single-layer perforation and in isolating intra-luminal chemical liquids, gases, or bacteria from the normal tissue in the extra-luminal space. Development of the tunnel technique has enabled digestive endoscopy to treat lesions outside the GI tract, thus converting certain digestive surgeries to super-minimally invasive endoscopic operations[4]. DETT bridges digestive internal

Table 1 Evidence-level classification

Level	Content
I	Based on a systematic review/meta-analysis of randomized controlled trials (RCTs)
II	Based on at least one RCT
III	Based on a non-RCT
IVa	Based on an analytical epidemiological study (cohort study)
IVb	Based on an analytical epidemiological study (case-control study, cross-sectional study)
V	Based on case series and case reports
VI	Based on opinions from a specialist committee or individual specialists without patient data

medicine and surgery and is considered a milestone of digestive endoscopic techniques.

At present, the tunnel technique application range covers the following[5]: (1) Treatment of lesions originating from the mucosal layer, *e.g.*, endoscopic submucosal tunnel dissection (ESTD) for oesophageal large or circular early-stage cancer or precancerosis; (2) treatment of lesions from the MP layer, per-oral endoscopic myotomy (POEM), submucosal tunnelling endoscopic resection (STER), *etc.*; and (3) diagnosis and treatment of lesions outside the GI tract, such as resection of lymph nodes and benign tumour excision in the mediastinum or abdominal cavity.

PREVENTION OF INFECTION AND CONCEPTS OF DETT-ASSOCIATED COMPLICATIONS

Although the GI tract is not a germfree environment, an intact mucosa plays an important role in preventing infection. However, the occurrence rate of infection can be increased when the mucosa is incised and an endoscope colonized with bacteria enters the submucosal tunnel during DETT. The origins of post-DETT infection mainly include: (1) Bacteria colonized in the oral cavity and oesophagus: *Streptococcus viridans*, *Staphylococcus aureus*, and enteric bacilli (*Escherichia coli*, *Acinetobacter*, *Pseudomonas aeruginosa*, *etc.*); (2) food and liquid retention in the oesophagus; (3) operation-related factors, such as intra- and postoperative bleeding, accidental injury of the mediastinum or lungs, and incomplete closure of the incision.

As reported in previous studies, preoperative use of antibiotics to prevent infections is recommended in DETT for the treatment of achalasia cardia (AC) and tumours of the oesophageal or cardia MP, with specific steps such as administering prophylactic antibiotics intravenously half an hour before surgery, with the infusion completed within 30 min. A single dose is enough if the surgery is performed within 60 min. If the surgery lasts for more than 1-2 half-life periods of the antibiotics, another dose is administered intravenously. The application of postoperative prophylactic antibiotics should not exceed 48 h[6,7].

According to relevant studies, the bacteria associated with DETT are mainly gram-negative bacteria, including *Pseudomonas aeruginosa* and Acinetobacter. Thus, second- and third-generation cephalosporins, such as cefuroxime and ceftriaxone, are the first choice for antibiotic prophylaxis. For patients allergic to penicillin, aztreonam combined with clindamycin or third-generation quinolones can be a substitution[8,9]. In addition, there is an opinion that acidic electrolysed oxidizing water not only has an obvious bactericidal effect on *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* but is also harmless and free of residual toxicity. Therefore, it is suggested to use this liquid to flush the tunnel. However, no multicentre large-sample controlled study has verified this method.

In conclusion, we recommend the intravenous administration of prophylactic antibiotics to prevent infection between 30 min pre-surgery and 48 h postsurgery. Other measures to prevent infection[10,11] include: (1) Fasting for 48-72 h before surgery and flushing the oesophagus and stomach with sterile water under endoscopy to reduce the number of bacteria; (2) gargling with sterile water or 0.9% saline repeatedly before the operation; (3) strictly sterilizing the endoscope and requiring the use of disposable sterile devices and sterile water during the operation; (4) coagulating exposed vessels in a timely manner to prevent haemorrhage and avoiding accidental injuries of normal tissues and organs outside the oesophageal wall; and (5) washing the tunnel and aspirating the liquid thoroughly as well as tightly closing the incision with clips (level of evidence: I; strength of recommendation: B).

Table 2 Recommendation level

Strength of recommendation	Content
A	With strong scientific evidence, strongly recommended
B	With scientific evidence, recommended
C1	Without scientific evidence, but recommended
C2	Without scientific evidence, not recommended
D	With scientific evidence of ineffective or harmful results, not recommended

In contrast with surgery, in endoscopic procedures, the amount of bleeding is difficult to determine and is also related to the experience level of the operators. Thus, it is difficult and unreliable to evaluate bleeding according to the amount and duration. We suggest evaluating intraoperative bleeding by using endoscopic resection bleeding (ERB) three-level and five-grade methods, which include the following five grades: ERB-0: No bleeding during the whole operation; ERB-c (controlled): Endoscopic controllable bleeding (divided into three grades, namely, ERB-c1: Easily controlled bleeding under endoscopy with stable vital signs and no need for blood transfusion; ERB-c2: Bleeding degree between c1 and c3; and ERB-c3: Endoscopically controllable bleeding with intra- or postoperative blood transfusion); and ERB-unc (uncontrolled): Uncontrollable bleeding under endoscopy that needs to be handled by surgery or vessel embolotherapy.

Compared with the non-tunnel technique, DETT has the superiority of maintaining the mucosal integrity, while it may create defects in the MP during incisions or resections. MP defect (MPD) grading is suggested to classify the defection. It includes three grades: MPD-0: No defection on the MP; MPD-pt (partial thickness): Defection on the MP without full perforation; MPD-ft (full thickness): Full perforation on the MP.

METHODS FOR ESTABLISHING A SUBMUCOSAL TUNNEL

Patient position

The positions commonly used in the operation include the left lateral position, supine position, and supine position with the right shoulder raised. Anatomically, the oesophagus is located behind the trachea and heart and in front of the spine. It is relatively safe to establish a submucosal tunnel at the proximal posterior wall of the oesophagus. A proper position may contribute to the safety and simplicity of the operation. The different positions are described as follows:

Left lateral position: This is the routine position for endoscopic examinations, which helps the endoscopist to identify the anatomic orientation of the oesophageal wall[12-14]. Because the common direction to operate a device under endoscopy is the six o'clock position, it is necessary to rotate the endoscope to adjust the fit direction for a tunnel to be established in the right rear oesophageal wall.

Supine position: This position facilitates the selection of the proximal posterior oesophageal wall for the operation; however, it might be difficult to advance the endoscope, given the degree of twisting of the patient's head[10,15,16]. In addition, fluid will remain in the rear oesophageal cavity due to gravity; such fluid may soak the tunnel incision during the operation, affecting the endoscopic view.

Supine position with the right shoulder raised: This position falls in between the left lateral and supine positions, with the patient's head twisting less, and the device can be withdrawn in a naturally relaxed condition to the proximal rear oesophageal wall under endoscopy, facilitating the approach and withdrawal of the endoscope as well as the whole operation. As reported in the relevant literature, this position is mainly advantageous with respect to no fluid retention at the right rear oesophageal wall (because it is not the lowest point in this position), with no effect on the operation field[10,17].

Consequently, during both ESTD and STER, operators can select the optimal position according to the lesion site before the operation. For POEM, the supine position with the right shoulder raised is recommended (level of evidence: II; strength of recommendation: B).

Tunnel incision and closure

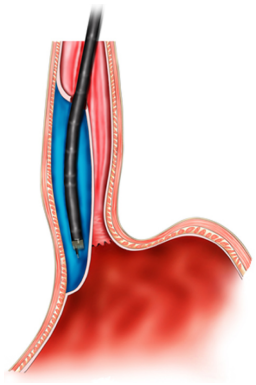

Figure 1 Mechanism of digestive endoscopic tunnel technique, demonstrating a tunnel that is created between the mucosal and muscularis propria layers.

Tunnel incision creates an entryway to introduce the endoscope into the tunnel, which affects the convenience of going into or out of the tunnel, the tunnel's internal pressure, and the difficulty of incision closure. Incisions may vary for different sites and even for the same site, and different choices can be made on the basis of the operators' preferences and the anatomical demands. There are three methods to create a tunnel incision (Figure 2):

Longitudinal incision: The oesophageal mucosa is incised longitudinally at a length of approximately 1.8-2.0 cm, with metal clips used to close such incisions from distally to proximally after the operation[18], thus facilitating wound closure. However, more metal clips are required, and it is relatively challenging to introduce an endoscope into the tunnel; moreover, the endoscope is closely encased by the tunnel incision, with a higher gas pressure inside the tunnel (Figure 2A-D).

Transverse incision: The oesophageal mucosa is incised horizontally at a length of approximately 1.2 cm[18-20]; the entrance is wider than that of the longitudinal incision, facilitating endoscope entry into the tunnel and gas escape out of the tunnel. This incision is mainly disadvantaged by the difficulty of its closure, which entails suturing of the first clip in the middle of the incision's anal side; such a clip can be used as an "anchor", based on which the suturing is performed longitudinally[20] (Figure 2E-H).

Inverted T incision: This inverted T entry incision is combined with a 0.8-cm transverse incision and a 1.0-cm longitudinal incision[21]. Such a tunnel incision resembles an inverted "T". By virtue of the wide tunnel space, it can facilitate the entry of the endoscope and the postoperative closure of the incision, as well as exhaust and drainage outwards, thus reducing the incidence of gas-related complications incurred by the tunnel technique (Figure 2I-L).

All three entry methods of tunnel incision are commonly used in clinical endoscopic practice. Comparing the incision area, gas pressure inside the tunnel, and difficulty of incision closure, we recommend the inverted T mucosal incision (level of evidence: III; strength of recommendation: B), given the following advantages: A larger area of the incision, ease of introducing an endoscope into the submucosa, a low gas pressure less likely to cause intraoperative gas-related complications, and fewer clips (only 4-5) required to close the incision after the procedure[22,23].

APPLICATION OF DETT FOR MUCOSAL LESIONS: ESTD IN OESOPHAGEAL MUCOSAL LESIONS

In 2009, a large circular oesophageal case successfully resected by a tunnel technique was initially reported by the team of Linghu *et al*[1]. Later, in 2013, the team published their research results from using the tunnel technique to treat large circular early oesophageal cancer in endoscopy, and defined the English name as ESTD[24]. Compared with the traditional endoscopic submucosal dissection (ESD), ESTD has certain advantages such as a shorter operation time, faster dissection, high complete resection rate, and low complication rate, which have caused this technique to be rapidly spread and widely used around the world, especially in the endoscopic treatment of large or circular early oesophageal cancer and precancerosis. For lesions of different sizes, single-tunnel or multi-tunnel procedures are optional.

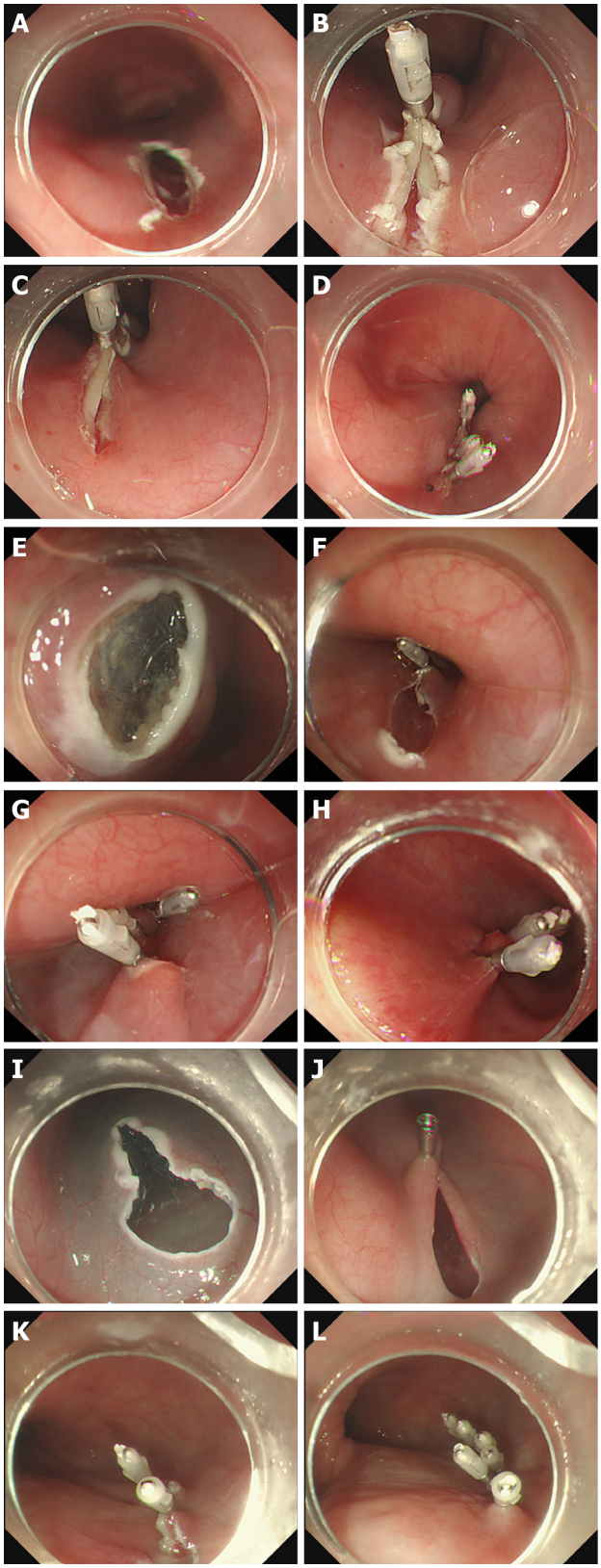

Figure 2 Three methods of tunnel incision and closure. A and B: Longitudinal incision; C and D: Longitudinal incision closed with titanium clips; E: Transverse incision; F: "Anchoring" of a titanium clip in the middle of the transverse incision; G and H: Longitudinal closure using titanium clips successively; I: Inverted T incision; J-L: Longitudinal closure using titanium clips successively.

Indications and contraindications

Indications: Early cancer and precancerous lesions of the oesophagus, stomach, colon, and rectum with a transverse diameter over 2 cm[24-37].

Contraindications: Lesions deeper than the submucosa found by endoscopic ultrasound (EUS); lymphatic or distant metastasis found on EUS, computed tomography (CT), or positron emission tomography-CT; Progressive stage cancer or undifferentiated tumour; Coagulation, cardiopulmonary dysfunctions, or other endoscopic, anaesthetic contraindications.

Procedures for ESTD

The main procedures for ESTD are as follows:

Lesion evaluation: Use a magnifying endoscope, Lugol's solution stain, and indicarmine stain to evaluate the character, depth, and extent of the lesion.

Circular marking: The circumferential markings are made at least 5 mm outside the margin of the lesion by argon plasma coagulation (APC) or an electric knife. For circular lesions, circumferential markings are made outside the oral and anal margins.

Mucosal incision: After sufficient lifting by submucosal injection, two transversal or cambered incisions are successively made by an electric knife in the anal and oral mucosa.

Establishment of a submucosal tunnel: One submucosal tunnel is created from the oral incision to the anal incision with a submucosal injection and dissection. The anal incision not only indicates the terminus of the tunnel but is also conducive to lowering the intra-tunnel pressure. The endoscope should be drawn out of the tunnel repeatedly during the dissection process to ensure that the direction of the tunnel conforms to the axis of the lesion and to avoid too much normal tissue being resected.

Bilateral resection: The remaining bilateral mucosa is resected by an electric knife 5 mm outside the marking points to completely dissect the whole lesion.

Treatment of the wound surface: Visible vessels are treated by haemostatic forceps or APC with fibrin glue sprayed on the ulcer when necessary. For lesions over three quarters of the whole circle, we suggest placing a fully covered retrievable metal stent for 4 to 8 wk to prevent postoperative stricture. The schema chart of ESTD is shown in Figure 3.

Pre- and postoperative treatments

Routine preoperative tests, including coagulation function, CT, EUS, and endoscopic examinations, are suggested to be accomplished and the general physical condition should be evaluated to exclude patients with anaesthetic or endoscopic contraindications. For those who take anticoagulant drugs, antiplatelet drugs, or other coagulation-influencing drugs, a 5-7 d withdrawal period is required before the operation[38]. The duration of the preoperative fast is related to the location of the lesion, which means a 12 h fast and 4 h water fast for upper GI tract lesions without outflow tract obstruction, a longer fast time according to the food emptying time for the upper GI tract lesions with outflow tract obstruction, and a 3-d non- or low-residue diet with bowel preparation for colonic and rectal lesions. For upper GI lesions, defoaming and anti-mucus agents are orally taken 30 min before the operation. For the lower GI tract, a thorough bowel preparation is required. Postoperative treatments are as follows:

Diet: Without complications, water intake is permitted after 48-72 h, and the diet is changed gradually from a clear liquid diet to a semi-liquid diet in 2 weeks.

Antacids: Proton pump inhibitors (PPIs) should be administered intravenously for 3 d and then taken orally for 4 wk.

Anti-infection: Antibiotics are routinely used for 2-3 d; when there are signs of infection, the antibiotic period should be prolonged or higher-level antibiotics should be used.

Follow-up: An endoscopic examination is suggested to be performed 3, 6, and 12 mo after the operation with endoscopic biopsy if necessary. Then, a routine endoscopic examination is performed every year to find recurrence or residual lesions in a timely manner.

Single-tunnel ESTD and multi-tunnel ESTD

ESTDs can be divided into single-tunnel and multi-tunnel ESTDs. To establish a submucosal tunnel conveniently and quickly, an approximately 2 cm-wide tunnel is generally preferred. Single-tunnel ESTD is recommended for lesions less than 1/2 of the oesophageal circumference, while multi-tunnel ESTD is recommended for lesions

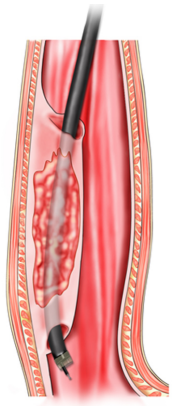

Figure 3 Schema chart of endoscopic submucosal tunnel dissection, demonstrating a tunnel that is created to resect mucosal lesions.

larger than 1/2 of the oesophageal circumference (level of evidence: II; strength of recommendation: A)[39-43]. Excess tunnels have no significant advantage compared with double tunnels.

Complications and treatments

Similar to ESD, the main complications of ESTD are bleeding, perforation, and oesophageal stenosis[24,25,27,34,41,44]. The bleeding rate ranges from 0% to 5.9%, and the perforation rate is 0%-4%; the rate of occurrence of stenosis, which is related to the circular extent and depth of the lesions, is not certain. Ono S performed a study on 84 oesophageal ESD patients, and the stenosis rates of < 1/2, < 3/4, and ≥ 3/4 circular lesions were 2% (1/49), 20% (5/25), and 90% (9/10), respectively[45]. Treatments for complications are as follows:

Bleeding: Most intraoperative bleeding can be stopped successfully under endoscopy. When patients have haematemesis, melena, or a significant decrease in haemoglobin, postoperative bleeding should be considered, and thrombotic drugs should be given immediately with a blood transfusion when necessary. If the bleeding amount is large and incurable by conservative treatment, endoscopic haemostasis may help. Vessel interventional embolization and surgery are required when all of the above therapies are performed in vain.

Perforation: Perforations during the operation are mostly successfully closed under endoscopy with common methods, including closure by metal clips, an over the scope clip (OTSC), blocking by fibrin glue, a fully covered retrievable metal stent and so forth. Common mental clips can completely close perforations smaller than 10 cm, while the new metal clip-the OTSC-can close perforations as long as 2 cm with a larger occlusal force. For large perforations and those incisions that are difficult to close by routine endoscopes, fully covered retrievable metal stents can effectively block the wound. Early finding of tardive perforation is very important for treatment and prognosis. Generally, tardive perforations within 12 h can be successfully treated by endoscopic closure and conservative treatments because of the mild inflammatory response and exudation. Perforations showing a failure of endoscopic closure, with severe mediastinal infection or unstable haemodynamics are suggested to be treated by surgery.

Stenosis: There is no satisfying treatment for stenosis yet, and common therapies in clinical practice include hormone injection or oral administration, balloon dilatation, fully covered retrievable metal stent placement, endoscopic radical incision, auto balloon dilatation, and autoplastic flap transplantation[46]. Other complications include pain, infection, transient bacteraemia, aspiration pneumonia, and gas-related complications.

In general, for large and circumferential oesophageal lesions, ESTD can achieve a faster dissection rate, reduced operative duration, and decreased incidence of intraoperative complications than regular ESD[47-51] (level of evidence: I; strength of recommendation: A).

DETT FOR MP LESIONS: POEM

POEM is mainly indicated for patients with AC. In 2007, Pasricha *et al*[52] first conducted an animal experimental study on the feasibility of POEM in treating AC. In 2010, Inoue *et al*[53] reported the first use of POEM for the clinical treatment of AC and achieved good clinical efficacy. Since then, POEM has been widely used worldwide and has become one of the most effective methods to treat AC. The main characteristics of AC patients are weakened oesophageal peristalsis, decreased clearance function, and a high lower oesophageal sphincter (LES) pressure, leading to dysphagia[54-56]. The main clinical symptoms include dysphagia, reflux, retrosternal pain, and weight loss, which may affect the patient's quality of life. POEM can relieve the LES pressure to the greatest extent by establishing an oesophageal submucosal tunnel and dissecting the LES circular muscle. At the same time, it can prevent perforation by maintaining an intact submucosal tunnel. Currently, POEM is relatively safe and effective as a minimally invasive therapy for AC by endoscopy[57-59].

Classification of AC

Morphological classification of AC (barium oesophagram classification): AC on barium oesophagram shows decreased oesophageal peristalsis and a narrow distal oesophagus with a "bird beak" appearance. The mucosa of the stenosis is smooth, and oesophageal dilatation can be seen above the stenosis. According to the extent of oesophageal dilatation on barium oesophagram, AC can be divided into three grades: Grade I: Oesophageal diameter < 4 cm (mild); Grade II: 4 cm ≤ oesophageal diameter ≤ 6 cm (moderate); and Grade III: Oesophageal diameter > 6 cm or even curved to an S-type (sigmoid-like appearance) (severe). Although the oesophageal morphology can be visually displayed on barium oesophagram, the shape of the oesophageal cavity cannot be displayed.

Pressure classification of AC (high-resolution manometry [HRM] classification[60,61]): HRM is considered to be the gold standard for the diagnosis of AC, which is characterized by the disappearance of oesophageal smooth muscle peristalsis and incomplete LES relaxation, often associated with LES hypertension. According to the HRM results, AC can be divided into three types: Type I is classic AC, which shows that the oesophageal peristalsis is significantly weakened, while the oesophageal pressure is not high, that is, the oesophageal motility is ineffective; type II manifests as the disappearance of oesophageal peristalsis and an obvious elevation of the total oesophageal pressure; type III manifests as an oesophageal fistula that causes blockage of the lumen. Manometry can show changes in the oesophageal pressure, which has certain significance for evaluating the therapeutic effect of surgical treatment, but its effectiveness for guiding the choice of surgical methods is not clear.

Endoscopic classification of AC (Ling classification): In 2011, Professor Linghu *et al*[62] proposed an endoscopic morphological classification for AC, which divides the oesophageal lumen into three categories: Straight, curved, and diverticulum, and named it the Ling classification. The details are presented in Table 3 and Figures 4 and 5. Because barium oesophagram and HRM cannot visually show the shape of the oesophageal lumen and the shape of the oesophageal cavity is closely related to the choice of surgical methods, the Ling classification has obvious advantages in guiding POEM operations. The preoperative classification of the oesophageal morphology of AC patients helps the operator select the appropriate surgical method to effectively reduce the occurrence of complications[63-65] (level of evidence, II; strength of recommendation: B).

Grading of the degree of oesophageal submucosal adhesion

Due to the need to establish a tunnel under the mucosa during the POEM operation, extensive submucosal adhesion will affect the tunnel establishment and muscle incision process. In 2018, Professor Linghu published a study on the classification of AC submucosal adhesions[66]. According to the degree of inflammation of the oesophageal mucosa, AC can be classified into six grades, that is, levels A-F (Table 4). The specific grading is as follows: Grade A: Normal mucosa, with a clear vascular texture; Grade B: Rough mucosa with a vague vascular texture; Grade C: White granular mucosa without an obvious vascular texture; Grade D: Pachyntic, striated, or sulcus-shaped mucosa without an obvious vascular texture; Grade E: Ulcer in the mucosa; Grade F: Mucosal scarring. Grades E and F are further classified into four subtypes according to the involvement of the oesophageal lumen: (1) Ulcer/scar ≤ 1/4; (2) 1/4 < ulcer/scar ≤ 1/2; (3) 1/2 < ulcer/scar ≤ 3/4; (4) ulcer/scar > 3/4. Grading of the oesophageal mucosal inflammation is somewhat correlated with the grading of the oesophageal submucosal adhesion. Generally, a submucosa with Grades A and B mucosal inflammation shows mostly mild adhesion (Figure 6). A mucosal submucosa with Grade C mucosal inflammation shows mostly moderate

Table 3 Endoscopic morphological classification of achalasia cardia

Classification	Endoscopic observations
Ling I	Cavity of the oesophagus dilates, wall of the oesophagus is straight (without tortuosity) and smooth
Ling II	Cavity of the oesophagus dilates tortuously, with a circular or semi-circular structure occurring in the oesophagus after adequate gas injection
Ling IIa	Oesophageal cavity dilates, with a thin circular structure (no semi-circular structure) occurring in the oesophagus after adequate gas injection
Ling IIb	Oesophageal cavity dilates, with a semi-circular structure occurring (midpoint within 1/3 of the cavity)
Ling IIc	Oesophageal cavity dilates, with a semi-circular structure occurring (midpoint beyond 1/3 of the cavity)
Ling III	Oesophageal cavity dilates, with a diverticular structure
Ling IIIl	Diverticular structure mainly in the left wall of the oesophagus
Ling IIIr	Diverticular structure mainly in the right wall of the oesophagus
Ling IIIlr	Diverticular structure in both the left and right walls of the oesophagus

adhesion (Figure 7). A mucosal submucosa with Grades D, E, and F mucosal inflammation shows mostly severe adhesion (Figure 8).

Tunnel anatomy

Vessels from the lower oesophagus to the cardia are in a grid pattern (Figure 9A); the crescent-like structure is visible when approaching the cardia (Figure 9B). Then, the ampulla-like structure appears after entering the crescent-like structure (Figure 9C), where there are branching vessels mainly characterized by bulky vascular roots (Figure 9D). The tunnel below the cardia changes and shows a steep downward form (Figure 9E); the vessels are stubby and multi-branched in shape (Figure 9F), and the beadlike vessels are visible (Figure 9G).

Standard operating procedure of POEM and identification of the tunnel's terminus

We usually choose the posterior wall of the oesophagus to establish a tunnel that better follows a path that is flat and straight. The length is up to 10-15 cm for a standard tunnel, and the standard operating procedure is as follows:

Submucosal injection and tunnel entry creation: The fluid applied in the ESD (such as saline) is injected to form a liquid mat 8-10 cm above the oesophagus sphincter. The pressure of the LES with severe stenosis should be controlled, and thus a volume of 4-5 mL is adequate in case of tearing of the cardia mucosa. The mucosa is incised when it is fully elevated. Three different incision methods exist for tunnel entry: The longitudinal incision, transverse incision, and inverted "T" incision. The advantage of the inverted "T" incision is that it facilitates the introduction of the endoscope, and the operator can make a much shorter incision. Moreover, it is convenient for fluid and air to flow from the tunnel cavity, reducing the pressure of the tunnel and lowering the risk of pneumothorax, pneumoperitoneum, and postoperative infection.

Establishment of the tunnel: A submucosal dissection is performed through the entry incision gradually to create a tunnel between the mucosa and the MP. The end point of the tunnel should be 2-3 cm distal to the cardia.

MP incision: The MP is incised 5-7 cm from the oral side of the gastro-oesophageal junction (GEJ) to a position 2 cm below the cardia.

Tunnel entry incision closure: Metal clips are used to close the incision. The location of the first clip is crucial, and the distal side of the entry incision may be a good choice because it narrows the incision, reduces the pressure, and is easily caught. The following metal claps should be fixed in sequence every 0.2-0.3 cm from the distal to proximal direction until the entry incision is fully closed. The three incisions all employ the longitudinal sealing method. The schema chart of POEM is shown in Figure 10.

The end point of the tunnel lies 2-3 cm to the anal side of the GEJ. Correctly determining the anatomic location of the GEJ during POEM is of great importance. The following methods of affirming the end point are recommended: (1) The distance from the incisor could be used to help determine the location of the GEJ; (2) the cardia is the narrowest site in the tunnel, and a portion of it may form an ampulla-like

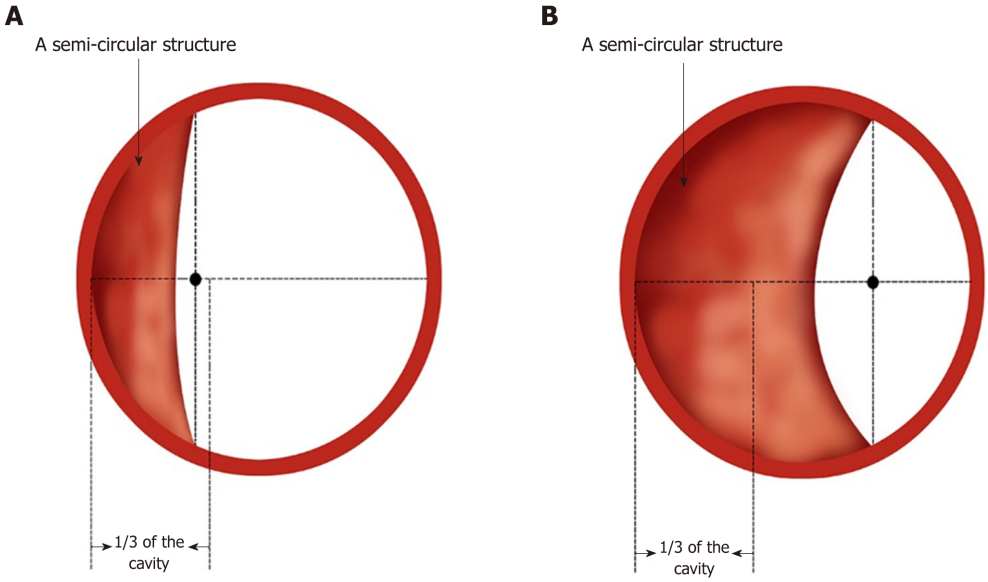

Figure 4 Simulated diagram of endoscopic observations in Ling IIb and Ling IIc. A: Ling IIb. The arrows indicate 1/3 of the oesophageal cavity, and the semi-annular structure's midpoint remains within this range; B: Ling IIc. The arrows indicate 1/3 of the oesophageal cavity, and the crescent-like structure's midpoint goes beyond this range.

structure with serious adhesion. The myotomy should be performed until 2 cm below the cardia; (3) the location of the GEJ may be confirmed by the pattern and path of the submucosal blood vessels and the oesophageal lumen in the tunnel. The end point crosses over 2-3 cm below the region with a paliform pattern of the lower part of the cardia; and (4) another method to confirm the terminus of the tunnel is to observe whether the whitish region of the mucosa on the anal side reaches a location 2 cm below the cardia when retrieving the endoscope from the tunnel and reversing it at the gastric fundus.

Tunnel establishment methods

There are three types of tunnel establishment methods as follows: Standard-tunnel POEM, short-tunnel POEM, and simultaneous submucosal and muscle dissection POEM (POEM-SSMD).

Standard-tunnel POEM: The standard tunnel is up to 10-12 cm long, extending from a position approximately 8-10 cm on the oral side of GEJ to 2-3 cm on the anal side. This technique is applicable for types Ling I, Ling II, and Ling IIb.

Short-tunnel POEM: The short tunnel is established from a location 5 cm on the oral side to 2 cm on the anal side of the GEJ. The length of the entire tunnel is approximately 6-8 cm. This technique is applicable for types Ling IIc and Ling III. The main operation procedure is as follows: (1) Submucosal injection and tunnel entry creation: The fluid applied in the ESD (such as saline) is injected to form a liquid mat 5 cm above the oesophageal sphincter. The mucosa is incised when it is fully elevated. It is not advised to adopt a longitudinal incision in short-tunnel POEM because there exists enough valid oesophagus; (2) establishment of the tunnel: A tunnel is established in the same way as the standard-tunnel POEM. The difference is that the tunnel is much shorter when performing short-tunnel POEM; (3) incision of the MP: The starting point of the myotomy is recommended to occur 2 cm on the oral side of the GEJ, and the end point of the myotomy is at 2 cm below the cardia. The length of the myotomy ranges from 3 to 5 cm; and (4) entry incision sealing: This step is performed in the same way as the standard-tunnel POEM.

POEM-SSMD: The MP is incised directly after establishing a super-short tunnel without damaging the mucosa and muscularis mucosa when the muscularis mucosa is completely adhesive to the MP. POEM-SSMD is applied in AC patients when a severe adhesion exists in the submucosa of the cardia and the tunnel is difficult to extend. The main operation procedure is as follows: (1) Submucosal injection and tunnel entry creation: The fluid applied in the ESD (such as saline) is injected to form a liquid mat 8-10 cm above the oesophageal sphincter and the mucosa is incised to establish the tunnel entry site; (2) establishment of the tunnel: A submucosal dissection is performed from the entry incision and a tunnel is established between

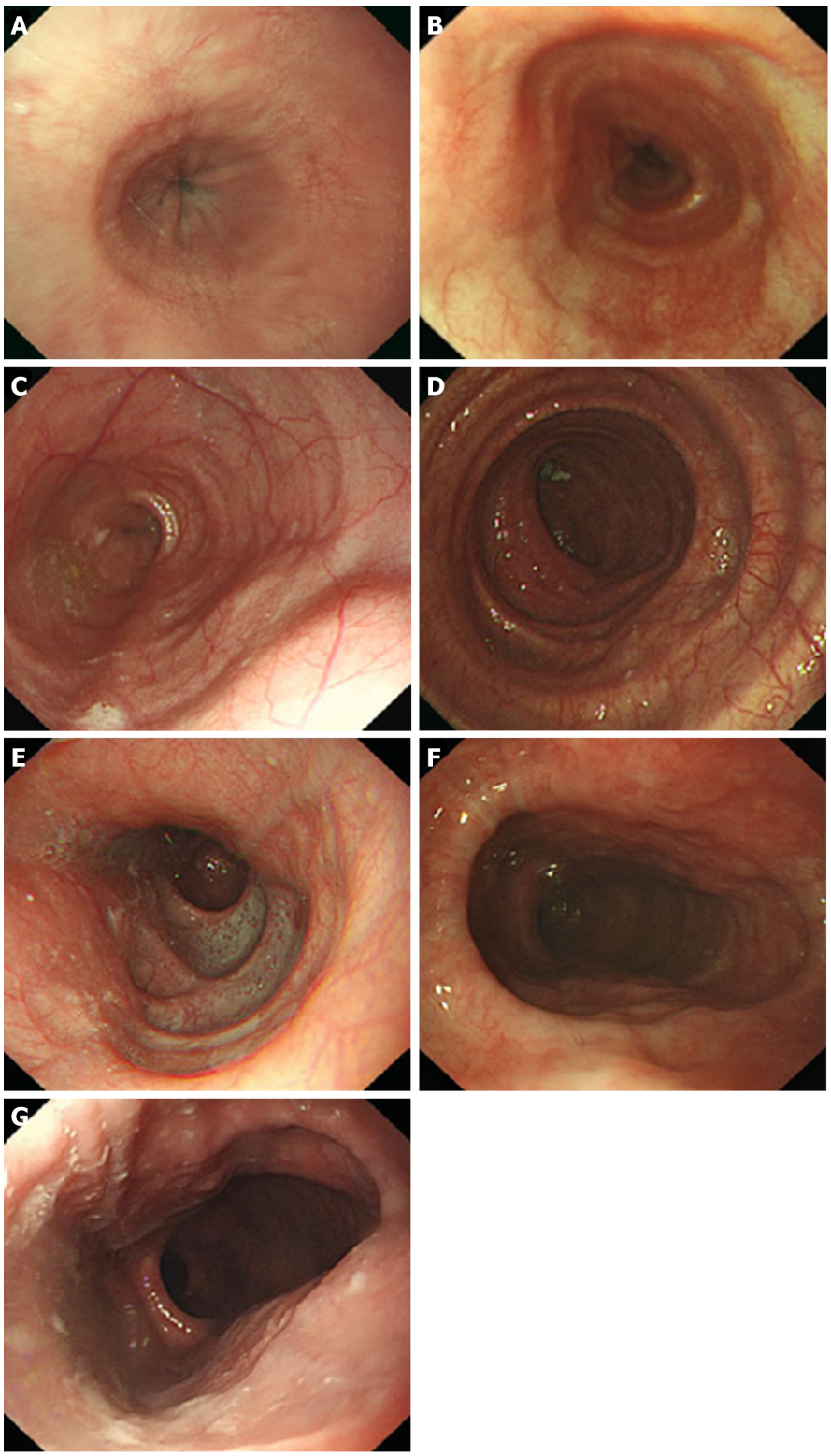

Figure 5 Endoscopic images of the Ling classification of achalasia cardia. A: Ling I; B: Ling IIa; C: Ling IIb; D: Ling IIc, E: Ling III1; F: Ling IIIr; G: Ling IIIIr.

the mucosa and the MP. The tunnel is extended as far as possible until it cannot be continued because of the severe adhesion; (3) incision of the MP: First, a 1-3 cm full-thickness myotomy is performed after establishing a super-short tunnel. Then, the submucosa and MP are incised simultaneously to 2-3 cm below the dentate line; and (4) tunnel entry incision sealing: This step is performed in the same way as the standard-tunnel POEM.

Ling classification of the oesophageal morphology by endoscopy can help the operator to select an appropriate surgical method. The oesophageal lumen in Ling I,

Table 4 Degree of inflammation of the oesophageal mucosa in achalasia cardia

Grade	Endoscopic observations
Grade A	Normal mucosa, with a clear vascular texture
Grade B	Rough mucosa with a vague vascular texture
Grade C	White granular mucosa without an obvious vascular texture
Grade D	Pachyntic, striated, or sulcus-shaped mucosa without an obvious vascular texture
Grade E	Ulcer in the mucosa
Grade E1	Involvement of the oesophageal lumen ≤ 1/4
Grade E2	1/4 < involvement of the oesophageal lumen ≤ 1/2
Grade E3	1/2 < involvement of the oesophageal lumen ≤ 3/4
Grade E4	Involvement of the oesophageal lumen > 3/4
Grade F	Scar in the mucosa
Grade F1	Involvement of the oesophageal lumen ≤ 1/4
Grade F2	1/4 < involvement of the oesophageal lumen ≤ 1/2
Grade F3	1/2 < involvement of the oesophageal lumen ≤ 3/4
Grade F4	Involvement of the oesophageal lumen > 3/4

Ling IIa, and Ling IIb cases is relatively straight; therefore, it is appropriate to adopt standard-tunnel POEM. For types Ling IIc and Ling III, we find that there is a high risk of losing one's bearings and damaging the tunnel mucosa when establishing a standard tunnel to cross the "ridge" formed by the crescent-like structure due to severe tortuosity and dilation of the lower oesophagus (Figure 11A). Thus, short-tunnel POEM is considered to be a good choice. The entry incision of the tunnel is established at a relatively flat location on the oesophageal wall (Figure 11B), which facilitates the endoscope to bypass the "ridge" *via* the portal into the oesophageal submucosa (Figure 11C). As indicated in relevant studies, there is no significant difference in the efficacy between short-tunnel POEM and standard-tunnel POEM for AC patients with LingIIc and Ling III classifications[67-69].

Types of myotomy in the tunnel

The myotomy types commonly reported include inner circular muscle myotomy, full-thickness myotomy, glasses-style myotomy, inner circular muscle myotomy + balloon plasty, and progressive full-thickness myotomy[70-76]. At present, full-thickness myotomy and progressive full-thickness myotomy are widely applied in clinical practice.

Inner circular muscle myotomy (Figure 12): The circular muscle is incised at 1 cm above the narrow part with electro-surgical knives, which is pushed forward step by step until all of the inner circular muscle is cut apart. At the bottom of the circular muscle, part of it is lifted up and an incision is progressively created to the end point of the tunnel, aiming to mutilate the muscle totally. This technique is inefficient in some patients.

Full-thickness myotomy (Figure 13): The circular and longitudinal muscles are completely incised from the narrow part to the location below the cardia with electro-surgical knives. The method is always associated with a higher incidence of postoperative gastro-oesophageal reflux disease (GERD).

Glasses-style myotomy (Figure 14): The MP is fully incised from the oral side to the anal side, with only an approximately 1 cm-long muscle left at the relative position of the dentate line, which appears as a glasses-type structure under an endoscope, to prevent postoperative GERD.

Circular muscle myotomy + balloon plasty (Figure 15): The oesophageal lumen is dilated with a cylindrical expansion balloon to detach some longitudinal muscle on the basis of the inner circular muscle myotomy. The method may achieve the goal of mutilating the MP fully, while the procedure appears to be much more complex.

Progressive full-thickness myotomy (Figure 16): The muscle is incised from 1 cm above the first narrow ring to the end of the tunnel, following the sequence of incising part of the inner circular muscle, full inner circular muscle myotomy, and then full-thickness myotomy of the MP from superficial to deep. This method appears to be the most valid way to relieve the symptoms of dysphagia effectively without causing

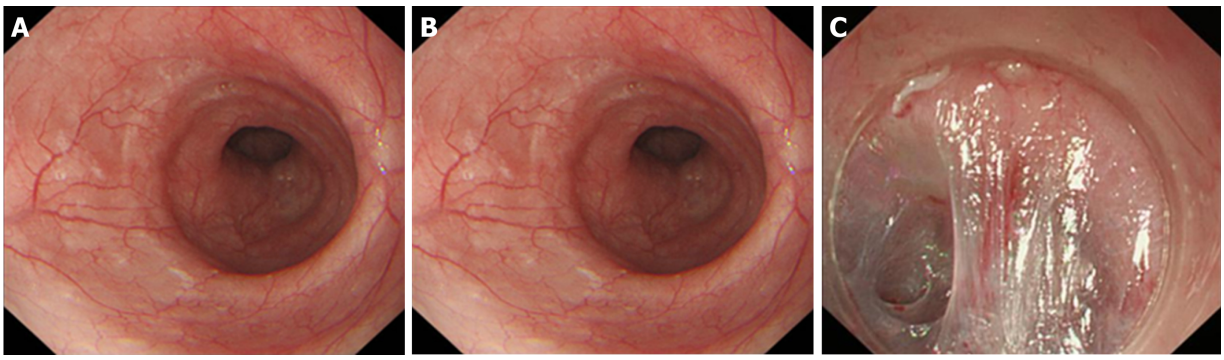

Figure 6 Correlation between grade A or grade B mucosal inflammation and mild oesophageal submucosal adhesion. A: Grade A mucosal inflammation; B: Grade B mucosal inflammation; C: Mild oesophageal submucosal adhesion: The fibre filaments are distributed in bundles.

GERD-relative problems.

Indications, relative indications, and relative and absolute contraindications of POEM

Indications: Patients diagnosed with AC[77]. Ling types I, IIa, and IIb are the best indications. With the development of endoscopic technology and the appearance of new endoscopic procedures, Ling types IIc and III, which previously were contraindications, are now considered indications for POEM[78-88].

Relative indications: Patients with a significantly dilated and tortuous oesophageal lumen, diffuse oesophageal spasm, nutcracker oesophagus, or prior treatment by Heller myotomy[89]. Cutting part of the circular muscle is recommended in patients with diffuse oesophageal spasm or nutcracker oesophagus.

Relative contraindications: Patients with Grade E, Grade F (endoscopic classification of oesophageal mucosa), or severe submucosal adhesion.

Absolute contraindications: Patients who have contraindications for endoscopic examination or exhibit severe cardiopulmonary dysfunction.

Perioperative management

All patients should complete HRM, 24-h pH monitoring, EGD, and/or a chest CT scan. POEM is performed after the patients have undergone fasting for 48 h, water restriction for 6 h, and EGD to ensure that no food residue remains in the oesophageal lumen on the day of the operation. Gargling with sterile water or saline is recommended before anaesthesia. Anticoagulant and antiplatelet agents are stopped for at least 5-7 d, and blood coagulation should be performed before the operation[90].

Postoperative treatment is similar to that for ESTD. The details are as follows: (1) Diet: All patients fast for 2-3 d after the procedure. The patients' diets progress gradually from a clear liquid diet to a semi-liquid diet and to a normal diet in 1 mo if no obvious postoperative complications are observed; (2) acid inhibitors: A PPI is administered intravenously for 3 d. An oral PPI is required for at least 4 wk; (3) anti-infection: Antibiotics are used for 2-3 d if there are no clinical signs of infection. Otherwise, a prolonged duration of antibiotic therapy or higher grade antibiotics should be considered; (4) routine blood tests, chest and abdomen X-ray, or CT are performed 3 d after POEM to determine whether there are signs of infection, emphysema, or pneumothorax; (5) dietary guidance: A solid food is given first, liquids are administered sequentially, and chewing well is also important; and (6) patients are scheduled to follow-up at the centre 1 mo, 3 mo, 6 mo, and 1 yr postoperatively and then yearly afterward, during which symptom assessments, physical examinations, and objective tests including EGD, HRM, and 24-h pH monitoring are performed.

Management of complications

The major complications mainly include: (1) Gas-related complications[91-94]: The examples are pneumomediastinum (approximately 4.9%), pneumothorax (0.2%-14.3%), pneumoperitoneum (13%-16.2%), and subcutaneous emphysema (11%-21.8%). Because of the debate over whether minor pneumatosis should be defined as a postoperative complication, few studies have been reported on them; (2) gastro-oesophageal reflux: The incidence of GERD is 5%-72.5%, which is mainly related to the differences in the specific definition in various studies[95-99]; and (3) other relatively

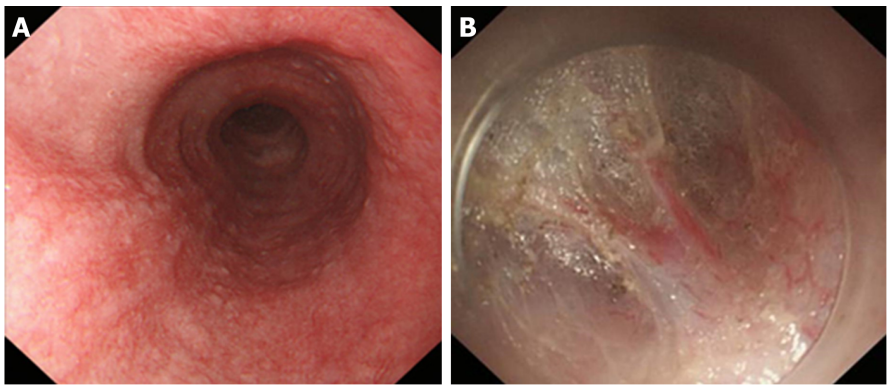

Figure 7 Correlation between Grade C mucosal inflammation and moderate oesophageal submucosal adhesion. A: Grade C mucosal inflammation; B: Moderate oesophageal submucosal adhesion. The fibres are arranged in disorder, with fusion and decreased transparency.

rare complications, such as delayed bleeding, incision tears, mediastinal abscess, oesophageal stenosis, hydrothorax, and oesophagitis[100].

Gas-related complications: Patients with mild subcutaneous emphysema, mediastinal emphysema, pneumothorax, and pneumoperitoneum are left without interventions because slight pneumatosis can be absorbed spontaneously. However, massive pneumatosis should be solved in a timely manner because it may cause blood oxygen desaturation and affect the vital signs directly. For patients with severe pneumothorax and pneumomediastinum, thoracic closed drainage can be performed under imaging guidance if possible. For severe pneumoperitoneum, an injector is often used for abdominocentesis to exhaust gas. In addition, multiple injectors are used if necessary to speed up the rate of pressure reduction.

In addition, the following advice can reduce the incidence of gas-related complications resulting from POEM: (1) CO_2 is used during the whole procedure, as this gas can be absorbed quickly by the human body; (2) when full-thickness myotomy is performed, the integrity of the oesophageal outer membrane should be retained as much as possible; and (3) the duration of the myotomy is minimized.

The following measures are useful for preventing infection: (1) Fasting for 48 h before POEM; (2) a tracheal cannula is recommended to avoid aspiration; (3) the tunnel is cleaned with saline before closing the tunnel entry; and (4) the entry is closed tightly to avoid large gaps.

Postoperative GERD can be relieved by using a PPI and GI prokinetic drugs. When the symptoms of GERD are not relieved by the above measures, a high dose of PPI is recommended; endoscopic therapy, such as cardiac constriction and fundoplication, is also considered.

When severe delayed bleeding occurs in the tunnel, endoscopic electrocoagulation should be performed immediately after removing the clips and cleaning the tunnel with saline. Then, the tunnel entry is closed again after successful haemostasis. Clips and porcine fibrin glue are both used to close mucosal injury[101-103].

The following measures can be used to treat mediastinal abscess: (1) Cleaning the oesophageal lumen, tunnel, and mediastinum, placing a drainage tube in the mediastinum, and fixing it onto the nose; (2) keeping the tunnel entry open and prolonging the time of fasting; and (3) using antibiotics intravenously and placing a jejunal nutritional tube for enteral nutrition.

Clinical efficacy of POEM for AC

POEM can achieve significant short-term clinical efficacy for AC, and the treatment success rate (postprocedure Eckardt score ≤ 3) is 82.4% to 100%[15,53,92,104-109]. Recent data demonstrate that the long-term clinical success rate (follow-up period ≥ 5 yr) of POEM is 83% to 87.1%[110,111]. A significant reduction in LES pressure and alleviation of oesophageal dilatation can be observed in patients who are treated with POEM.

In conclusion, compared with other endoscopic surgeries, POEM is characterized by fixed operation steps. Therefore, it is essential to formulate a set of standard operating procedures to regulate its procedures. The following steps are recommended: (1) Patients should fast for 48 h from solid and liquid diets and 6 h from water. The oesophageal lumen is cleaned with normal saline under preoperative gastroscopic guidance. The patient should be placed in the supine position with the right shoulder raised and should undergo intratracheal intubation anaesthesia with

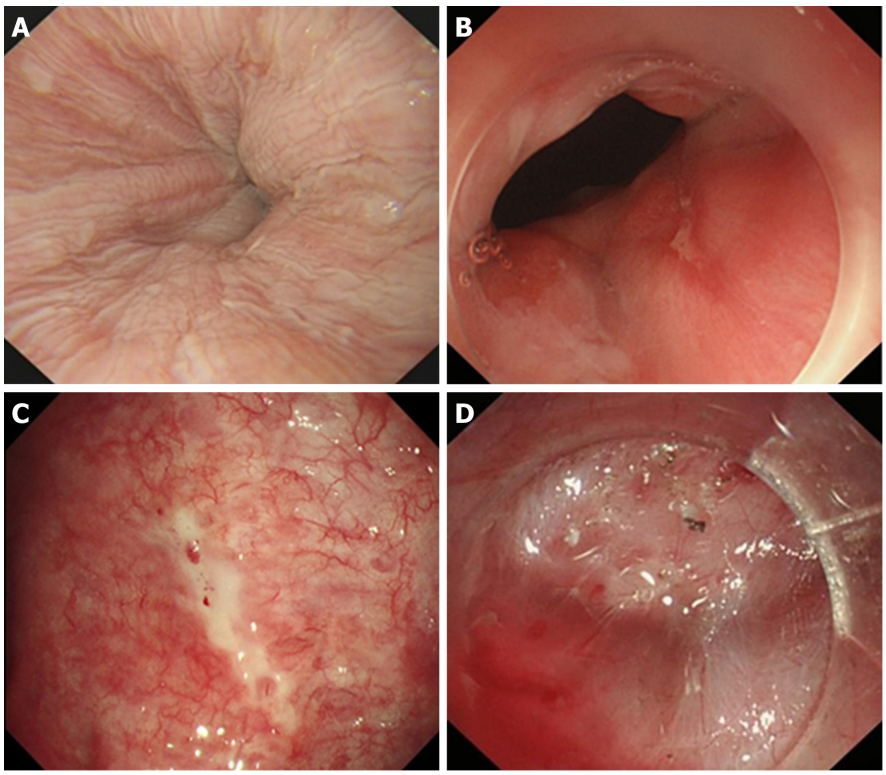

Figure 8 Correlation between Grade D, Grade E, or Grade F mucosal inflammation and severe oesophageal submucosal adhesion. A: Grade D mucosal inflammation; B: Grade E mucosal inflammation; C: Grade F mucosal inflammation; D: Severe oesophageal submucosal adhesion. The submucosa and muscularis propria are completely adherent.

CO_2 applied during the entire surgical process (level of evidence, II; strength of recommendation, B); (2) standard tunnels should be established for AC patients with types Ling I, IIa, and IIb, while short tunnels are recommended for Ling IIc and III cases (level of evidence, II; strength of recommendation, B); (3) it is recommended to apply inversed T entry incisions (level of evidence, III; strength of recommendation, B); (4) it is recommended to apply progressive full-thickness myotomy (level of evidence, IVa; strength of recommendation, B); (5) it is recommended to apply clips or porcine fibrin glue for occlusion in case of mucosal injury (level of evidence, IVa; strength of recommendation, B); (6) in case of the occurrence of complications, the abovementioned treatment methods help to perform conservative treatments safely and successfully[5] (level of evidence, III; strength of recommendation, B); and (7) according to preliminary data, the mid- to long-term treatment effects of POEM are similar to those of surgical treatment[53,107-109,111-113], and POEM is expected to be the future clinical first-line therapeutic choice[99] (level of evidence, I; strength of recommendation, A).

Extended application of POEM

POEM for oesophageal diverticula (D-POEM): With the development of POEM, the scope of its treatment has been expanded. POEM has been reported to be used in the treatment of oesophageal diverticula by establishing a submucosal tunnel to reach the diverticulum, where the proper muscular layer is incised to make the diverticulum smaller, and symptoms such as dysphagia and weight loss can be relieved[114-117]. This is a novel technique for the treatment of diverticula, but further studies on clinical efficacy are needed. The main indication for D-POEM is the formation of a sacked bag diverticulum that significantly affects swallowing and food flow. The pre-procedure preparation of D-POEM is similar to that of routine endoscopy, including fasting for 12 h and water prohibition for 4 h before the procedure.

POEM for gastric disease (G-POEM): In recent years, there have been reports on applying gastric POEM (G-POEM) in the treatment of diabetic gastroparesis (DGP) and delayed gastric emptying (DGE) after subtotal gastrectomy[15,118,119]. G-POEM is essentially similar to POEM for oesophageal achalasia, including submucosal tunnelling to the pyloric ring, myotomy involving the full thickness of the pyloric sphincter, and finally, closure of the incision using clips, with the aim of alleviating

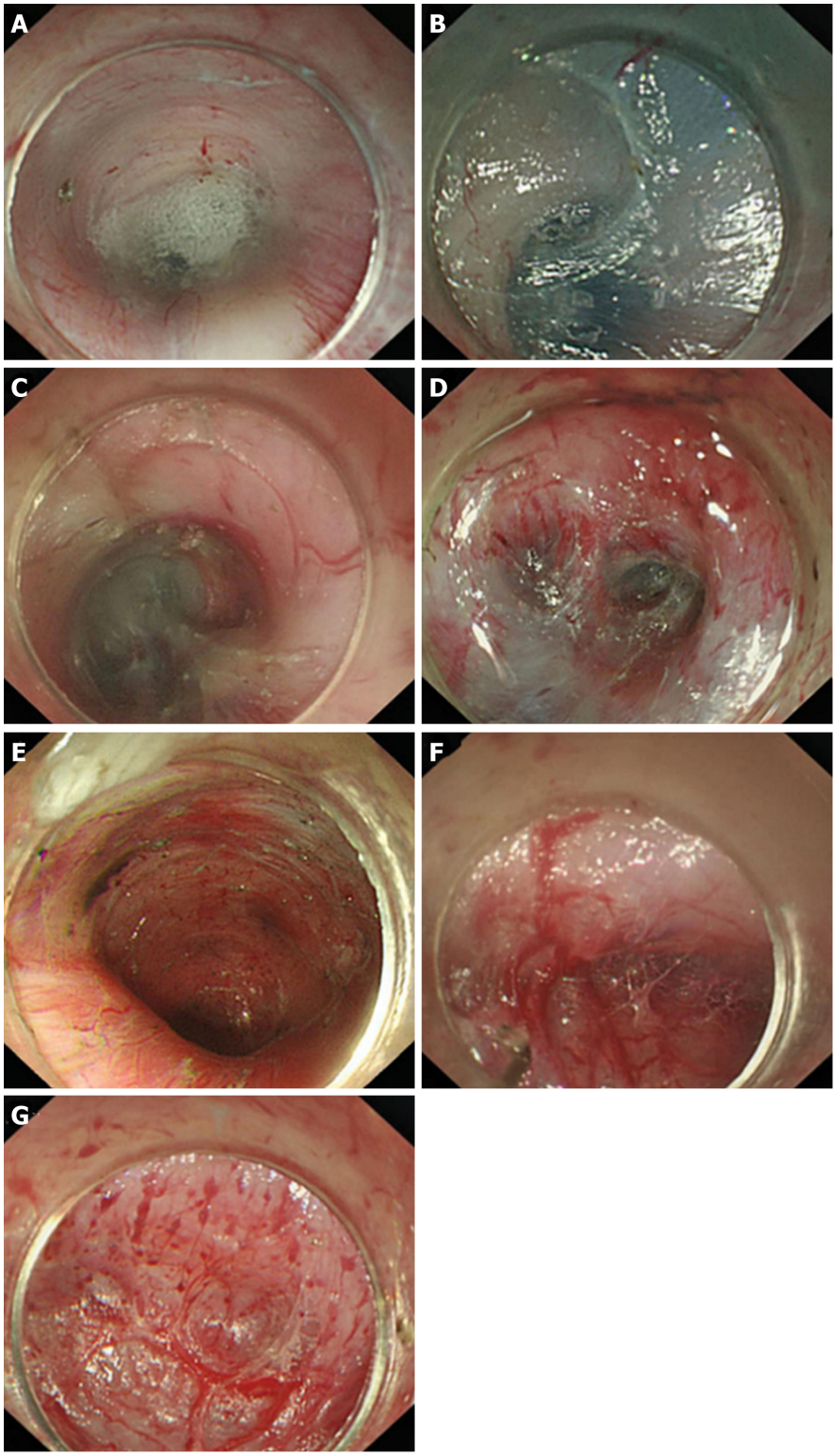

Figure 9 Anatomical landmark in the tunnel from the lower oesophagus to the cardia. A: Grid-like blood vessels in the cardia; B: Crescent-like structure visible at the proximal cardia; C: Ampulla-like structure appearing after entering the crescent-like structure; D: Branching vessels with bulky vascular roots in the ampulla-like structure; E: Tunnel below the cardia, showing a steep downward form; F: Stubby and multi-branched vessels below the cardia; G: Beadlike vessels below the cardia.

the symptoms of patients with emptying dysfunction caused by various aetiologies and unidentified idiopathic gastroparesis.

The indications for G-POEM include gastric emptying disorders caused by diabetes, surgery, infection, and idiopathic gastroparesis with an unknown aetiology, accompanied by symptoms associated with severe gastric emptying dysfunction such as nausea, vomiting, abdominal pain, stomach fullness, early satiety, loss of appetite,

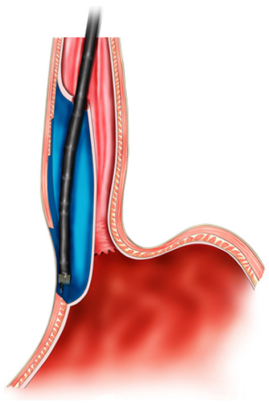

Figure 10 Schema chart of per-oral endoscopic myotomy, demonstrating a tunnel that is created to incise the muscularis propria.

nausea, postprandial fullness, and weight loss. The contraindications are similar to those for POEM. The pre-procedure preparation for G-POEM is similar to that for POEM for AC, except that G-POEM requires a gastric emptying test.

Similar to oral endoscopic myotomy (POEM), the G-POEM operation is also divided into four steps: Establishing a tunnel opening; creating a submucosal tunnel; performing a pyloromyotomy; and closing the tunnel entrance. It should be noted that there is no uniform standard for the operation due to the short development time of the technology and because the current total number of cases is small.

G-POEM is an emerging minimally invasive therapy for patients with gastric emptying disorders. After being successfully applied in the treatment of patients with DGP who failed medical treatment for the first time in 2013[120], the feasibility, safety, and effectiveness of G-POEM have been confirmed by a series of studies. The feasibility of the G-POEM operation process is reproducible and can be completed by experienced endoscopists with a success rate of up to 100%. G-POEM has been demonstrated to be a safe procedure. Intra-procedural and postprocedural complications such as haemorrhage, mucosal tears, and perforation are rarely reported, and the incidence of complications is low. In terms of effectiveness, after G-POEM treatment, the clinical symptom relief rate associated with gastric emptying disorder can reach more than 80%, and the Gastroparesis Cardinal Symptom Index at 1, 6, and 12 mo is significantly decreased. Gastric electrical stimulation shows that the gastric emptying ability becomes normal or improves significantly, and the quality of life of patients also improves[119-122].

DETT FOR MP LESIONS: STER

Previously, submucosal tumours (SMTs) were treated by open surgery, thoracoscopy, or laparoscopy, which were associated with high invasiveness. Along with the development of endoscopic techniques, the availability of endoscopic submucosal excavation (ESE) and endoscopic full-thickness resection (EFR) makes it possible to perform endoscopic resection of SMTs originating from the MP layer. However, these procedures do not maintain the mucosal integrity, which could result in perforation, infection, and postoperative strictures.

In 2011, Linghu *et al*[123] reported a case involving the resection of MP tissue in two pigs using a tunnel, which initially proved that it was feasible to resect SMTs originating from the MP layer using a tunnel technique. STER is a novel technique named by Xu *et al*[124] who reported resecting SMTs by establishing a tunnel between the submucosal and MP layer in patients in 2012. STER was preliminarily proved to be superior to ESE and EFR for the treatment of SMTs originating from the MP layer due to its ability to maintain the integrity of the mucosa[125-127].

Indications, relative indications, and contraindications for STER[128-133]

Indications: Considering that the lesion to be excised would pass several narrow places and the limit of the tunnel width, it is advised to choose SMTs with a transverse diameter of 2.5 cm or less.

Relative indications: 2.5 cm ≤ transverse diameter ≤ 3.5 cm.

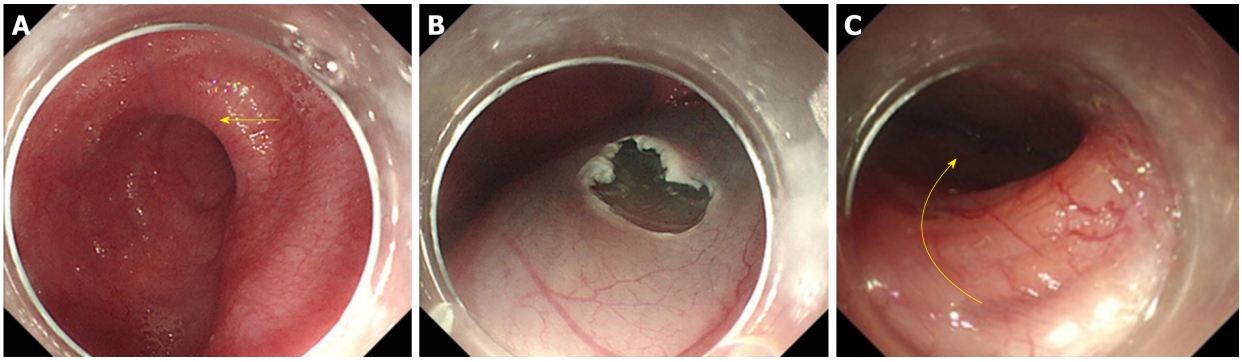

Figure 11 Endoscope crosses the "ridge" *via* short-tunnel per-oral endoscopic myotomy. A: Type Ling IIc oesophagus. The arrow indicates a "ridge" structure formed by the crescent-like structure; B: The short-tunnel entry incision established on a relatively flat oesophageal wall at the oral side of the "ridge"; C: It is easy to cross the "ridge" within the tunnel.

Relative contraindications: (1) The surface of the SMT mucosa is not intact with ulcer, which eliminates the significance of establishing a tunnel to maintain mucosal integrity; there are submucosal adhesions in the ulcer area due to inflammation, and it is difficult to create a submucosal tunnel; (2) the tumour is located at the entrance of the oesophagus, and there is no room to create a submucosal tunnel; and (3) the transverse diameter of the tumour is > 3.5 cm, and it cannot be completely removed from the tunnel.

Contraindications: (1) The patients with severe cardiopulmonary dysfunction cannot undergo an endoscopic operation; (2) blood coagulation dysfunction is evident; (3) there is a large area of scarring or anastomosis at the tunnel area; and (4) there is a suspected malignant tumour.

STER procedures

Standard STER is conducted as follows:

Submucosal injection and mucosal incision: A submucosal fluid cushion is made with an injection needle 3-5 cm proximal to the tumour, and then a mucosal incision is made as the entry point. There are three types of incisions, including a longitudinal mucosal incision, transverse incision, and inverted T incision. A transverse incision is suggested for SMTs that are located in the upper oesophagus, which are closed to the oesophagus inlet, where there is a limited length to establish a tunnel.

Creation of a submucosal tunnel: A tunnel is created from the oral side to the anal side between the mucosal and the MP layer and ends at the distal side of the tumour. Repeated submucosal injections contribute to avoiding accidental injury to the tunnel mucosa. The exposed vessels and small bleeds should be treated in time to ensure a satisfactory endoscopic view. To obtain the integrity of the mucosa and reduce the bleeding, submucosal dissection should be conducted close to the MP where the vascular networks are absent.

Resection of the tumour: The tumour is resected with an intact capsule from the MP layer after being completely exposed using a knife or a snare. The submucosal tunnel is lavaged with normal saline solution after the removal of the tumour. The resection edge is carefully treated with haemostatic forceps and APC to reduce haemostasis and prevent delayed bleeding and postoperative infection.

Closure of the mucosal incision site: Clips are used for incision closure. The closure method of STER is similar to that of POEM. The transverse incision and inverse T incision are closed in a longitudinal shape. The resected specimen is sent for pathological evaluation. SMT < 1.5 cm in diameter could be extracted directly by endoscopic aspiration, while SMT ≥ 1.5 cm in diameter should be extracted with a snare or basket. The schema chart of STER is shown in Figure 17.

When resecting cardial SMTs, the direction of the tunnel is difficult to identify because of the anatomical structure of the cardia. Methylene blue or indigo carmine can be used to locate the tumour and guide the direction of the tunnel following submucosal incision[127,134-136]. The procedures for STER treatment of cardial SMTs are shown in Figure 18.

Special considerations

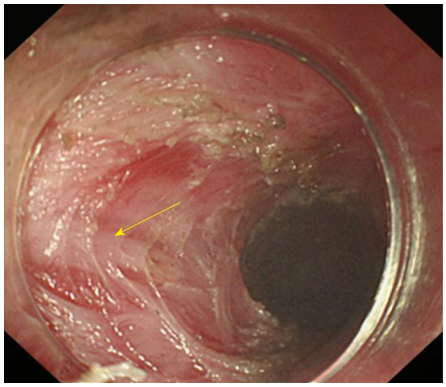

Figure 12 Inner circular muscle myotomy. The arrow shows the well-retained longitudinal muscle.

There are several factors to which attention should be paid during the operation. First, because of the polygon or ginger shape of cardial SMTs, it is necessary to detach the lesion edge while dissecting muscle fibres in the tunnel. If the lesion is so large that it affects the vision in the tunnel, it can be partly resected with a snare after the exposure of some parts of the lesion and the above procedures repeated until the lesions are completely resected. Second, the resection of a large SMT may lead to the absence of a large area of MP at the cardia, which may cause GERD because of the low pressure of the LES after the cicatricial healing of the MP. However, further studies with large sample sizes are warranted to confirm this conclusion.

Preoperative preparations and postoperative care

EUS, enhanced CT, and other imaging examinations should be performed before STER to evaluate the size, location, shape, depth, and blood supply of the tumour and to eliminate the possibility of metastasis and/or invasion outside the digestive tract (level of evidence, I; strength of recommendation, A).

The general condition of the patient should be assessed to evaluate whether he or she is suitable for anaesthesia. A coagulation function test should be performed before the procedures. Patients should stop using anticoagulant drugs, antiplatelet drugs, and drugs that affect blood function at least 5-7 d prior to STER[38]. Patients should be fasted from food for 12 h and from water for 4 h. Medicines that dispel mucus and foam should be administered 30 min before the procedure for patients with upper GI lesions, while a good preparation to keep the bowel clean is indispensable for patients with lower GI lesions. Any discomfort such as fever, abdominal pain, perforation, haematemesis, and haematochezia is closely monitored after STER. A complete blood count is performed on the morning after STER.

The postoperative treatments for STER are similar to those for ESTD and POEM. Patients are fasted for 2-3 d, consume a liquid diet for 3 d, and return gradually to a normal diet within 2 wk. Intravenous PPIs are used for 2-3 d, followed by oral PPI therapy for 4 wk. The intravenous antibiotics are stopped after 2-3 d if there are no signs of infection, otherwise prolonged antibiotics or strong antibiotics should be used to control infection. Chest/abdominal X-ray or CT is performed in cases of severe chest and/or abdominal pain. The majority of the gas-related complications resolve spontaneously without the need for intervention. Thoracic drainage is required for pneumothorax with collapse of greater than 30% of the lung. A 20-gauge needle is inserted into the right lower quadrant to confirm the presence of the complication and the release of the gas for suspected pneumoperitoneum during and/or after the procedure[137]. Patients are discharged when they can take a semi-liquid diet.

Surveillance endoscopy is performed at 3, 6, and 12 mo after the operation and then annually[125,138,139]. Contrast-enhanced CT is performed for patients with GI stromal tumours (GISTs) every 3-6 mo for 3-5 yr. A less-frequent follow-up period is recommended for very-low-risk GISTs[140].

Safety outcomes and treatment of complications of STER

The incidence of STER-related complications is 0-66.7%, and gas-related complications are the most common complications, with an incidence of 0 to 66.7%[137,141,142]. The incidence of STER-related complications mostly ranges from 5% to 25%[123,124,126,127,129,139,143-148]. The oesophagus is the safest area for performing STER, with a low incidence of complications, namely, 9.5% to 16.7%[129,145]. The incidence of complications is 15.3% to 42.9% in the cardia[125,127,144,149], 11.1% to 21.9% in the stomach[139,147], and 62.5% in the rectum[150]. A meta-analysis showed that the incidence

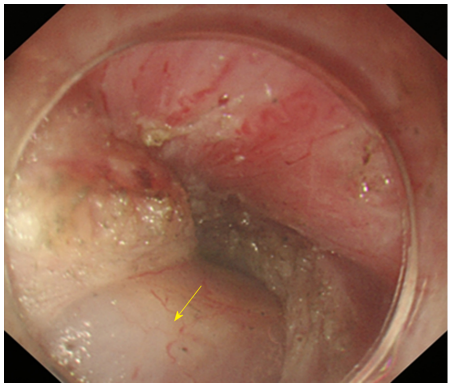

Figure 13 Full-thickness myotomy. The arrow shows the tunica adventitia of the oesophagus.

rates of STER-related mediastinal/subcutaneous emphysema, pneumothorax, and pneumoperitoneum are 14.8% [95% confidence interval (CI): 10.5% to 20.5%], 6.1% (95% CI: 4.0% to 9%), and 6.8% (95% CI: 4.7% to 9.6%)[151], respectively.

Treatment methods for common complications are as follows: (1) Subcutaneous emphysema, a small amount of mediastinal emphysema, and a small number of pneumothorax cases do not require special treatment and can be absorbed spontaneously. Compared with air, CO_2 is more easily absorbed by the body; consequently, the use of CO_2 perfusion can reduce the occurrence of gas-related complications during the operation. Chest pain is a common symptom after surgery. A chest X-ray or chest CT scan should be performed in a timely manner for suspected pneumothorax. A small amount of pneumothorax cannot be prevented temporarily and should be closely monitored. Thoracentesis is feasible for obvious pneumothorax (lung tissue collapse > 30%); (2) postoperative fever can be treated with an antipyretic combined with physical cooling. If infection happens, timely anti-infection treatment is required; (3) mucous membrane breakage can be clipped with a titanium clip or plugged with a biological fibrin glue. Repeated submucosal injection can help reduce mucosal damage; (4) for patients with pneumoperitoneum, if the patient's abdominal distension is not obvious and the gas is less, the condition can be closely monitored and the gas can be absorbed spontaneously. If the patient has obvious abdominal distension or obvious abdominal distension, puncture and release of the gas can be performed in the right lower abdomen after the operation. No other special treatments are needed.

Effectiveness of STER

The total *en bloc* resection rate of STER for GI SMTs is 83.3%-100%[130,152,153]. The *en bloc* resection rates for oesophageal and cardial SMTs are 84.6%-98.6%[129,145] and 74.5%-100%[125,127,139,144,154], respectively.

Comparison of STER and surgery

Compared with surgical treatment, STER is superior with less invasion, faster postoperative recovery, lower hospitalization expenses, higher acceptability, and other advantages[130,145,155]. However, STER cannot completely replace surgical treatment. The optimal treatment method should be selected according to the disease itself, thus benefiting the patient.

In conclusion, STER is not suitable for all SMTs, and surgeons should strictly assess the indications. STER is indicated for benign lesions with a diameter less than 3.5 cm and free from an abundant blood supply as determined by EUS, CT, or other imaging tools[138,155] (level of evidence, II; strength of recommendation, A). It is challenging to gain a clear view in the tunnel when treating cardial SMTs, especially when they originate from the deeper MP layer; therefore, methylene blue is recommended to locate the tumour and guide the direction of the tunnel, thereby shortening the time required for lesion detection and increasing efficiency[127] (level of evidence, III; strength of recommendation, B).

SURGERIES ON THE EXTERNAL DIGESTIVE TRACT WALL

Currently, surgeries on the external digestive tract wall are performed on the basis of the establishment of tunnel targeting in experimental subjects and human cadavers[11], but many controversies remain, and no consensus, such as on how to control

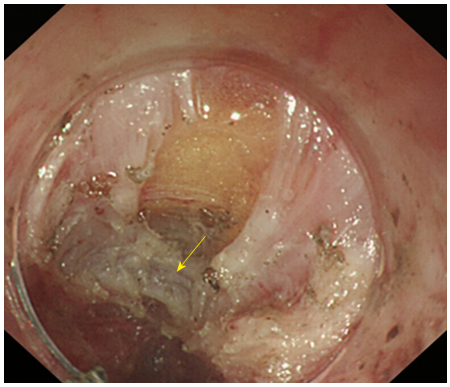

Figure 14 Glasses-style myotomy. The arrow shows the muscles remaining at the cardia.

abdominal infection, has been reached. With the constant development of DETT and improvement of related endoscopic techniques and instruments, surgery on the external digestive tract performed by endoscopy *via* a tunnel will soon become a reality. The schema chart of DETT on the external digestive tract wall is shown in Figure 19.

CONCLUSION

DETT has removed the obstacles between internal medical and surgical treatments, in line with the principles of endoscopic treatment centred on diseases. In the near future, endoscopy is set to follow the basic principles of complete lacunas, sterility, use of natural orifices, and the absence of chemical stimulation, thus providing patients with better treatment methods.

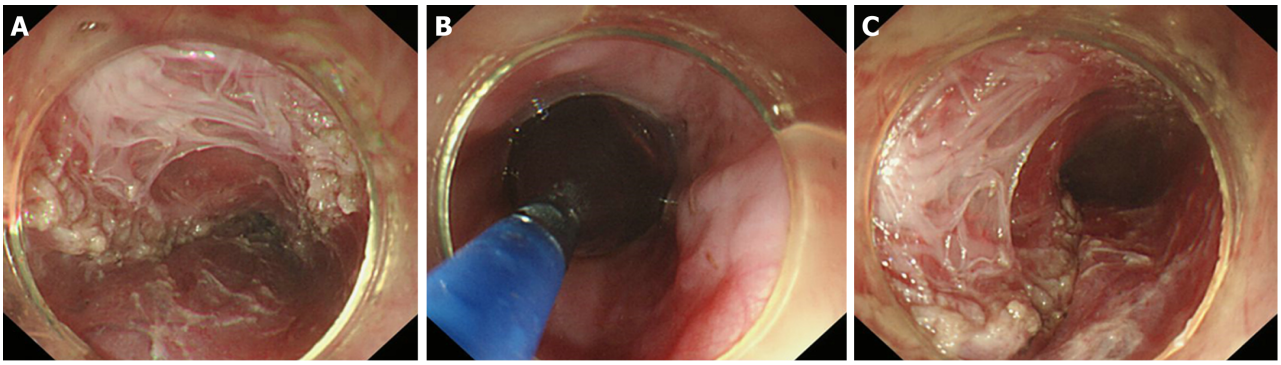

Figure 15 Circular muscle myotomy + balloon plasty. A: The width of the incision should be 1/3 of the circumferential oesophagus; B: Balloon-dilation in the oesophagus; C: The width of the incision after expansion should be 2/3 of the circumferential oesophagus.

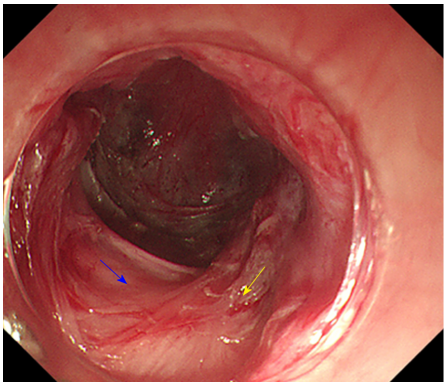

Figure 16 Progressive full-thickness myotomy. The yellow arrow shows the incision into the inner circular muscles from superficial to deep; the blue arrow shows the full incision into the muscularis propria.

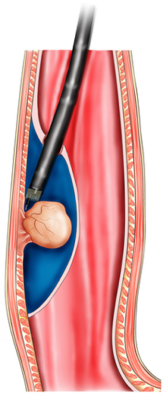

Figure 17 Schema chart of per-oral endoscopic myotomy, demonstrating a tunnel that is created to resect the lesion from the muscularis propria.

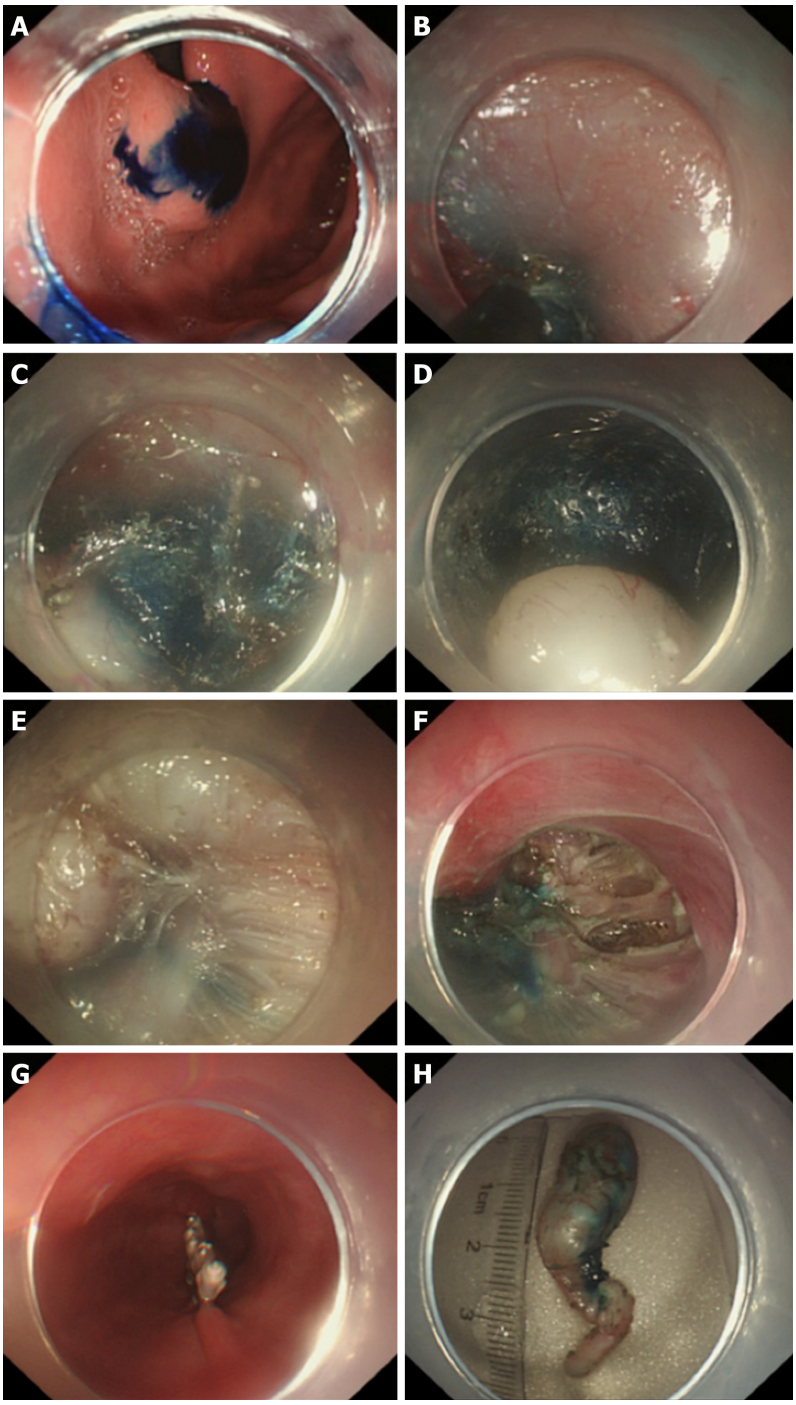

Figure 18 Key steps of submucosal tunnelling endoscopic resection for a cardial submucosal tumour. A: Injection of methylene blue into the lesion incision site for marking and positioning; B: Establishment of a tunnel; C: Finding of the marking and positioning with methylene blue in the tunnel; D: Exposure of the lesion; E: Resection of the lesion; F: Wound following the resection of the tumour; G: Closure of the mucosal incision; H: The resected specimen.

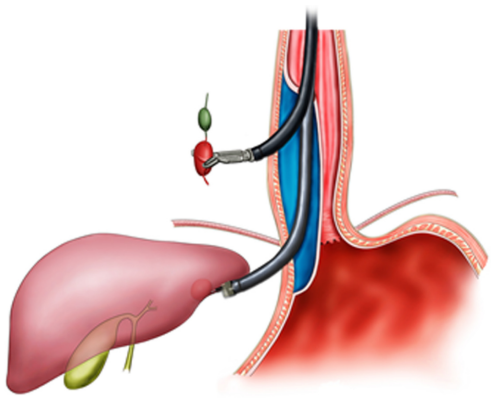

Figure 19 Schema chart of digestive endoscopic tunnel technique on the external digestive tract wall, demonstrating a tunnel that is created to resect a node outside the digestive tract.

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MINIREVIEWS

Solutions for submucosal injection: What to choose and how to do it

Rui Castro, Diogo Libânio, Inês Pita, Mário Dinis-Ribeiro

ORCID number: Rui Castro (0000-0003-0103-003); Diogo Libânio (0000-0003-2691-7522); Inês Pita (0000-0002-0951-5030); Mário Dinis-Ribeiro (0000-0003-0121-6850).

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Rui Castro, Diogo Libânio, Inês Pita, Mário Dinis-Ribeiro, Department of Gastroenterology, Portuguese Oncology Institute of Porto, Porto 4450-590, Portugal

Mário Dinis-Ribeiro, CIDES/CINTESIS, Faculty of Medicine, University of Porto, Porto 4200-319, Portugal

Corresponding author: Rui Castro, MD, Doctor, Department of Gastroenterology, Portuguese Oncology Institute of Porto, Rua Dr. António Bernardino de Almeida, Porto 4450-590, Portugal. rui.arrais.castro@gmail.com
Telephone: +351-911592435

Abstract

During the past decades, endoscopic resection techniques have gradually improved and gained more importance for the management of premalignant lesions and early cancers. These endoscopic resection techniques can be divided in 3 major groups: snare polipectomy, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). The use of submucosal injection is essential for the majority of EMR techniques and is an integral part of ESD, whereas during polipectomy it is not crucial in most cases except to prevent bleeding in large polyps and/or those with large stalks as an alternative to mechanical methods. Injection provides a lifting up effect of the lesion separating it from the muscular layer, thereby reducing thermal injury and the risk of perforation and bleeding while also facilitating *en-bloc* resection by improving technical feasibility. With this work, we aim to review the most common endoscopic resection techniques and the importance of submucosal injection in each one of them. For that, we present some of the most commonly used submucosal injection solutions, taking into account their advantages and disadvantages. We also discuss, based on current recommendations and our own experience, how and when to preform submucosal injection, depending on lesions features and endoscopic resection technique that´s being used, to assure complete resection and to prevent associated adverse events. Finally, we also present and discuss some new proposed submucosal injection solutions, endoscopic resection techniques and devices that may have a major impact on the future of therapeutic endoscopy.

Key words: Snare polipectomy; Endoscopic mucosal resection; Endoscopic submucosal dissection; Submucosal injection; Submucosal injection solution

Core tip: In this work, we review the importance of submucosal injection and present

some of the most commonly used solutions, comparing them taking into account their advantages and disadvantages. Unlike most of the previous papers about this subject, we organized this review in a more practical point of view. For that, we try to answer some essential questions like: what is the need for submucosal injection, when should we use it, what type of solution is more suitable for each endoscopic resection technique and how should we use them.

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INTRODUCTION

During the past decades, endoscopic resection techniques have gradually improved and gained more importance in the management of premalignant lesions and early cancers of the digestive tract[1,2]. These endoscopic resection techniques can be divided in 3 major groups: snare polipectomy, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). The use of submucosal injection is essential for the majority of EMR techniques and is an integral part of ESD, whereas during polipectomy it is not crucial in most cases except to prevent bleeding in large polyps and/or those with large stalks as an alternative to mechanical methods.

Injection lifts the lesion and separates it from the muscular layer, thereby reducing thermal injury and the risk of perforation and bleeding, while also facilitating *en-bloc* resection by improving technical feasibility[3,4]. An additional important aspect of injection is that if dyes are incorporated, lesion margins may become more clearly defined, especially in the colon. Several solutions have been used for submucosal injection, although there is still no consensus about which one is the best.

The ideal injection solution should provide a thick submucosal fluid cushion that remains in the submucosal space long enough (to avoid the need of multiple injections), should be inexpensive, widely available, improve outcomes, reduce adverse events, and should not damage tissue specimens in order to allow an accurate pathologic staging[5,6].

Taking into account the different types of solutions, normal saline (NS) has been the most widely used solution. It is simple to use and available at a low-cost, although the mucosal protrusion created by the submucosal injection of NS is only maintained for a short period of time. While this may not be a problem when removing small lesions, the need for repeated injections can increase procedure time when resecting larger and/or difficult lesion and in theory can also increase the risk of adverse events.

In order to overcome these drawbacks of NS and to improve the technical feasibility of EMR and ESD, several solutions have been developed. Submucosal injection of glucose solution, glycerol, sodium hyaluronate (SH), colloids, hydroxypropyl methylcellulose, fibrinogen solution and other alternatives have been investigated in different contexts. However, these solutions also have some disadvantages: they can be difficult to prepare or administer, may not readily available or only available at a high cost, and may induce tissue damage that can impair histological assessment or even be associated with toxicity.

The aim of this article was to review the indications of submucosal injection and to present some of the most commonly used solutions, comparing them taking into account their advantages and disadvantages. We organized this review to share information in a practical point of view, sharing also our own experience in this field. For that, we will try to answer some essential questions: what is the need for submucosal injection, when should we use it, what type of solution is more suitable for each endoscopic resection technique and how should we use them.

WHEN TO INJECT

The main objective of submucosal injection is to separate the mucosal layer from the muscularis propria by filling the submucosal layer with fluid in order to decrease the risk of adverse events. This submucosal cushion reduces thermal injury and the risk of

perforation and haemorrhage (by separating the mucosa from large submucosal vessels and also by vasoconstriction when adrenaline is part of the solution) while also facilitates *en-bloc* resection. In Figure 1, we present a decision algorithm that can be applicable in clinical practice.

Snare polipectomy

The vast majority of colorectal polyps encountered during colonoscopy are < 5 mm, whereas only 10%-15% are ≥ 9 mm[7,8]. ESGE guidelines recommend cold snare polipectomy (CSP) as the preferred technique for removal of diminutive polyps (size ≤ 5 mm)[9]. This technique has high rates of complete resection, adequate tissue sampling for histology and low rates of adverse events. ESGE guidelines also suggest CSP for sessile polyps 6-9 mm in size because of its superior safety profile, although evidence comparing efficacy with hot snare polipectomy (HSP) is lacking. On the other hand, HSP is recommended for removal of sessile polyps 10-19 mm in size. In most cases, especially in the right colon, deep thermal injury with HSP is a potential risk and thus submucosal injection prior to HSP is generally advised. Regarding pedunculated polyps, ESGE guidelines suggest the use of HSP to decrease the risk of immediate bleeding, and injection of diluted adrenaline or clip placement should also be used in pedunculated polyps with a head ≥ 20 mm or a stalk ≥ 10 mm[9].

EMR

EMR is an endoscopic technique developed for the removal of sessile or flat neoplasms confined to the superficial layers (mucosa and submucosa) of the gastrointestinal tract by excising through the middle or deeper portion of the submucosa. Different EMR techniques are listed below and include: inject-and-cut EMR; Cap-assisted EMR and ligation-assisted EMR. In ligation-assisted EMR, a band ligation device is attached to the endoscope, and the banding cap is positioned over the target lesion. In this technique, although some endoscopists use submucosal injection prior to band placement, submucosal injection is not mandatory as the resection can be safely without this step[3,5,10,11].

Inject-and-cut EMR is also often called saline solution lift-assisted polipectomy. The procedure starts with injection of a solution into the submucosal space under the lesion creating a safety cushion. The cushion lifts the lesion, facilitating capture and removal by using a snare while minimizing mechanical or electrocautery damage to the deeper layers of the gastrointestinal wall. The lesion may be removed in a single resection (Figure 2) or a piecemeal fashion (Figure 3). Recently, cold snare EMR was also described, and ESGE guidelines suggest that it can be an option in cases where the risk of deep thermal injury is high or unable to be tolerated, although evidence is still scarce. In this case, submucosal injection may still be needed.

Cap-assisted EMR also uses submucosal injection to lift the target mucosal lesion. Dedicated mucosectomy devices have been developed - a single-use cap affixed to the tip of the endoscope equipped with a specially designed crescent-shaped electrocautery snare that must be opened and positioned on the internal circumferential ridge at the tip of the cap. The endoscope is then positioned over the target lesion and suction is used to retract the mucosa into the cap, after which the snare is closed to capture the lesion (alternatively the lesion can be grasped with a forceps or endoscopic grasper). The lesion is then resected with standard hot snare excision technique.

The main drawback of standard EMR techniques is that size can preclude *en-bloc* resection, therefore resulting in piecemeal resections, leading to problems in correctly assessing the depth of tumour invasion and increasing the possibility of local recurrence. Consequently, *en-bloc* resection using this procedure is limited to lesions approximately 15-20 mm in size[12,13]. The choice of the technique between EMR and ESD is therefore especially important when there is suspicion of limited submucosal invasion, in which case adequate histopathological staging is of paramount importance. On the other hand, piecemeal EMR is accepted in Barrett's dysplasia/adenocarcinoma and in colonic lesions without suspicion of submucosal invasion (since ESD is associated with higher risk if adverse events in these organs).

ESD

ESD, a relatively recent but widely accepted endoscopic resection procedure, was developed specifically for *en-bloc* resection of larger lesions[14-16]. Lesions are dissected directly along the submucosal layer using an electrosurgical knife, resulting in an *en-bloc* resection of even large lesions. Various submucosal injection solutions had previously been developed and shown to be satisfactory for use during EMR, but for the more time consuming ESD the use of a longer-lasting solution can be important to facilitate the procedure, to help identify the cutting line and maintaining a safe fluid cushion during dissection of the submucosal layer.

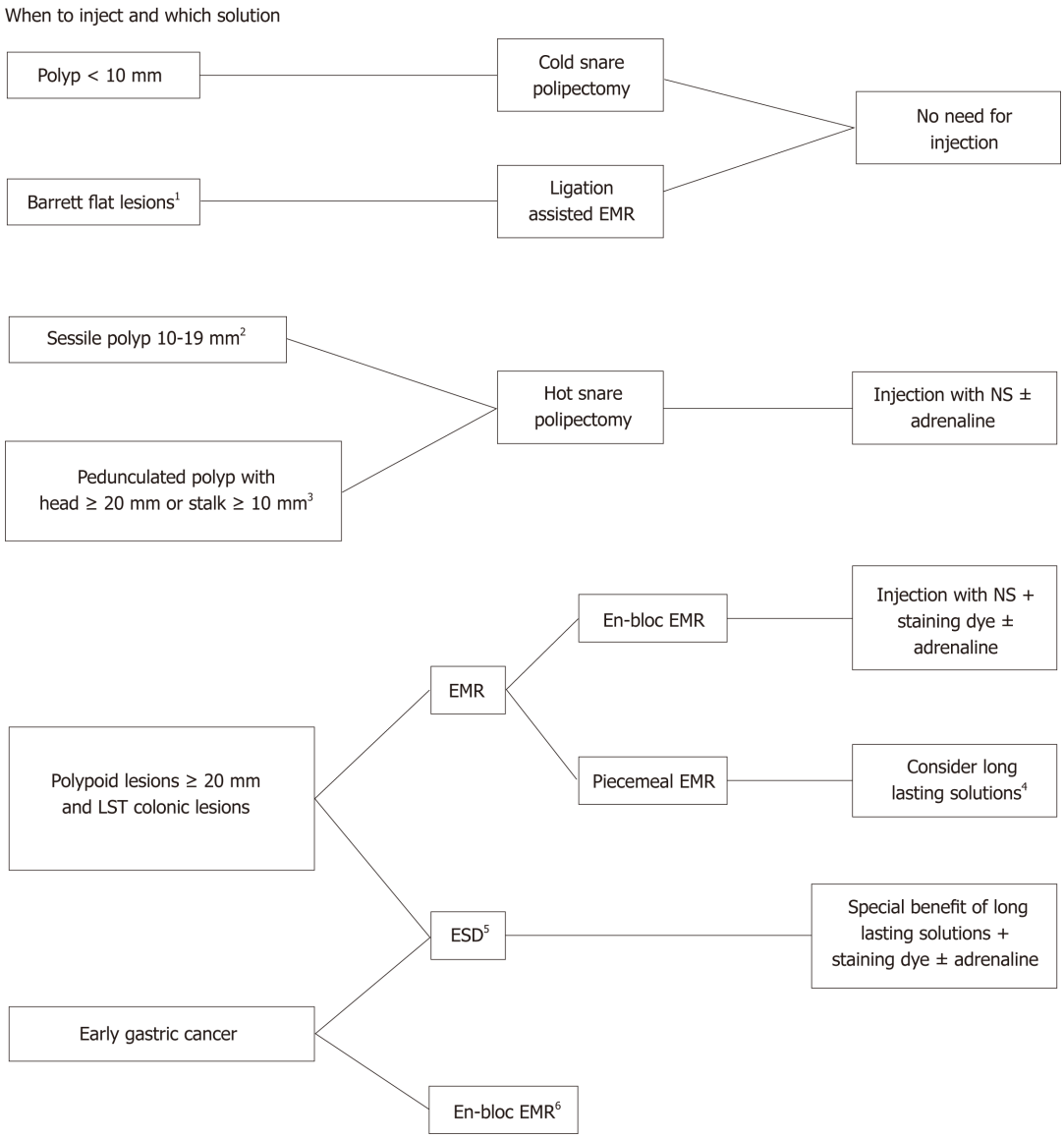

Figure 1 Decision algorithm. [1]Without deep submucosal invasion features; [2]In most cases, especially in the right colon, deep thermal injury with hot snare polipectomy is a potential risk; [3]Clip placement can be an alternative to submucosal injection; [4]Hyaluronic acid should be avoided in piecemeal resection; [5]Endoscopic submucosal resection enables *en-bloc* resection of larger lesions; [6]May be considered in Paris 0-IIa gastric Lesions < 15 mm. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; NS: Normal saline; LST: Lateral spreading tumour.

TYPES OF SOLUTION

The majority of submucosal injection solutions is composed of a solvent (like water) and an osmotic agent (like sodium chloride). More complex solutions can also have a bulking and structuring agent, an oil component, an emulsifier and a contrast staining agent. A summary of the main features of some of the solutions discussed below is presented in Table 1.

NS

As previously mentioned, NS is commonly used because it is safe, available at a low-cost, easy to use and with negligible/no potential toxic effect or damage to tissue specimen. The major limitation of this solution is its rapid absorption into the surrounding tissues, reducing the duration of a proper submucosal cushion. This limitation is not so important for endoscopic resection of small polypoid lesions (< 20 mm) in which a higher elevation and its maintenance for a longer period of time is not essential[5], but can theoretically hinder and increase procedure time in larger lesions or longer procedures. However, at the present time, there is no evidence of the superiority of other submucosal agents over NS in *en-bloc* resection rates or adverse events risk (perforation, bleeding and post-polipectomy coagulation syndrome). This lack of difference in *en-bloc* resection rates and adverse events risk between different submucosal injection solutions was shown in a recent systematic review and meta-

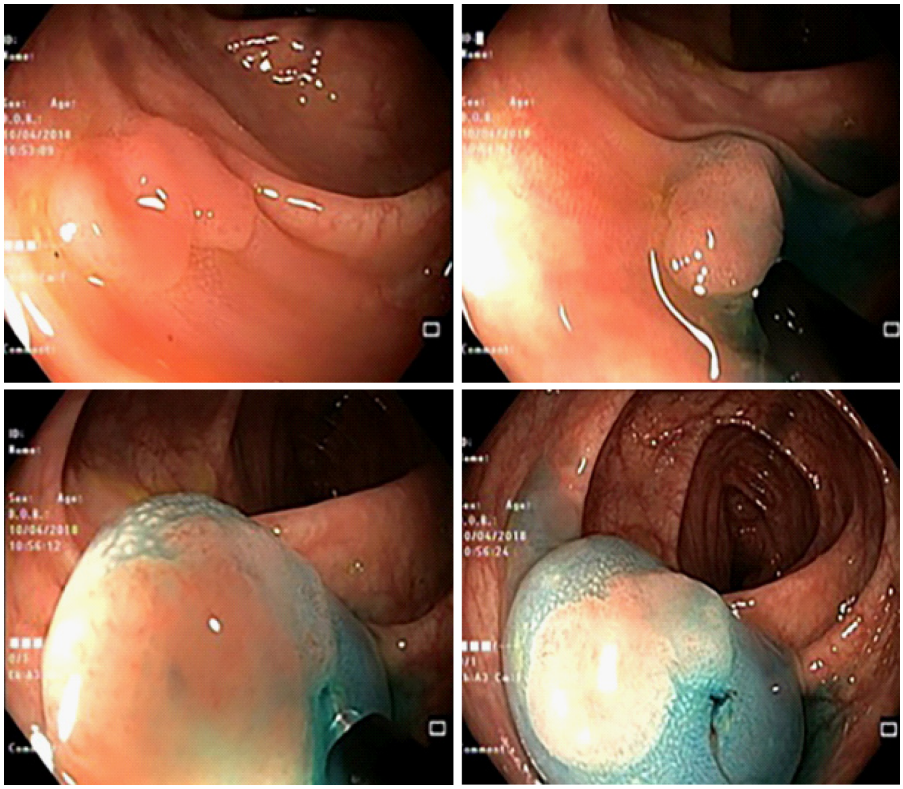

Figure 2 Submucosal injection for *en-bloc* resection of a colonic flat lesion.

analysis by Ferreira *et al*[17]. SH, one of the best studied solutions, was compared to NS in three randomized controlled trials (RCTs) (total of 423 patients submitted to gastric or colic EMR) and the pooled results failed to show a difference between SH and NS regarding complete resection with OR 1.09 [95% confidence interval (CI): 0.82-1.45]. In other RCTs, 50% dextrose (D50), succinylated gelatin (SG), fibrinogen and hydroxyethyl starch (HES) were also not superior to NS. Similarly, no single solution was shown to be more effective in decreasing post-polipectomy bleeding, but HES, SG, and fibrinogen have shown a non-significant favourable trend against NS with a pooled OR of 0.59 (95%CI: 0.3-1.01). For post-polipectomy coagulation syndrome, there is only one RCT for each solution and none for SH. These studies were underpowered to detect significant differences in this specific outcome but the pooled analyses suggest that NS may be effective in preventing perforations and coagulation syndrome with an OR = 0.27 (95%CI: 0.06-1.19), especially when compared to HES (OR = 0.15; 95%CI: 0.007-3.03) and D50 (OR = 0.16; 95%CI: 0.02-1.38)[17].

Glycerol

Glycerol is a hypertonic solution consisting of 10% glycerin and 5% fructose in an NS solution. Because of its hypertonic properties, glycerol produces a long-lasting submucosal elevation[18]. Changes in submucosal elevation immediately and 3, 5, and 7 min after injection of glycerol and NS were compared by Sumiyoshi *et al*[19]. The hemispheric shape produced by glycerol maintained the same height and configuration throughout the 7-min period while NS cushion began to decrease after 3 min, becoming unnoticeable at 7 min. One retrospective study compared *en-bloc* resection rates and complications for EMR of colorectal flat lesions like lateral spreading tumors (LST) using glycerol or NS[20]. For lesions between 10-19 mm, *en-bloc* resection was significantly higher when glycerol was used, but there were no differences for larger lesions. There were also no differences in complications such as perforations and delayed bleeding. Another advantage of the use of glycerol (over other solutions such as dextrose) is that this solution does not damage the resected specimen, allowing a correct histopathological analysis. Because glycerol is relatively inexpensive and readily available in Japan, it is considered superior to NS and widely used as submucosal injection solution in colorectal EMR.

Dextrose water

Dextrose water (DW) is also a hypertonic solution. It is an inexpensive and readily available product that produces a longer submucosal elevation than NS solution. The

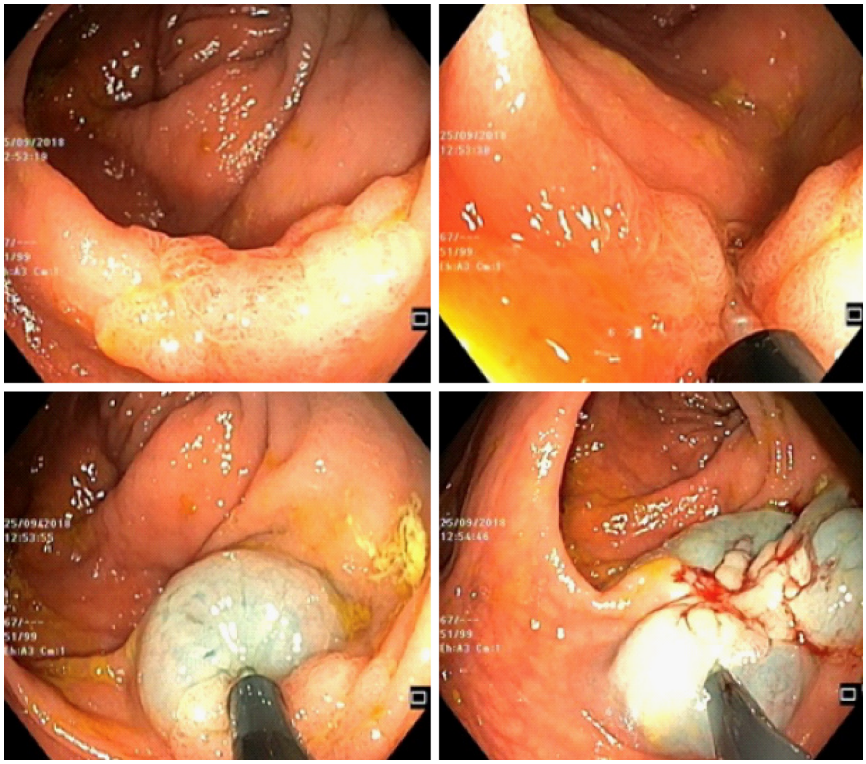

Figure 3 Submucosal injection for a piecemeal resection of a colonic flat lesion.

main issue about this product is the potential histopathological tissue damage. In fact, considerable tissue damage and impaired ulcer healing after EMR can be expected with DW at concentrations ≥ 20%. For that reason, DW with concentrations 15% is not recommended as submucosal injection solution[6].

Hyaluronic acid

Hyaluronic acid (HA) is a type of glycosaminoglycan found in connective tissue that has a high viscosity and water retention capability. Moreover, it does not present toxicity or antigen-antibody reaction in humans. A classical HA use in submucosal injection is in the form of a 0.4% SH solution. Various studies reported that the use of HA provides the longest-lasting fluid cushion, higher successful *en-bloc* resection and lower perforation complication rates, particularly for colorectal ESD[21-24]. However, a recent systematic review and meta-analysis of solutions for submucosal injection and endoscopic resection concluded that the available evidence does not allow a robust conclusion to be drawn on the solutions´ effect on resection rate[17]. The main disadvantages of HA are its high cost and unavailability. It is also believed that this product can stimulate the growth of residual tumour cell due to enhancement of both tumour growth and CD44 expression of cancer cells at wound sites in murine models[25]. For all of these reasons, HA is considered a good option for ESD of larger lesions because of its long-lasting fluid cushion, however it cannot be recommended for endoscopic piecemeal resection procedures that have an increased risk of recurrence.

Hydroxypropyl methylcellulose

Hydroxypropyl methylcellulose (HPMC) is a cellulose derivative with viscoelastic properties that is primarily used in ophthalmology for creating artificial tears[26]. As HA, it also achieves a long-lasting submucosal elevation with minimal tissue reaction[27]. The major differences between these two solutions are that HPMC is less expensive than HA but, as synthetic product, HPCM can potentially give rise to an antigen-antibody reaction[18].

Fibrinogen mixture

Fibrinogen mixture (FM) solution is available at a reasonable price and has a high viscosity that produces a long-lasting submucosal elevation. It also helps to keep a clear visual field during and after endoscopic resection by providing a microvascular hemostatic effect[28]. Like HA and HMPC, its main utility is the submucosal injection during ESD of larger lesions because it leads to fewer injections and shorter procedure

Table 1 Main features of some submucosal injection solutions

Solution	Cushion duration	Price	Advantages	Disadvantages
NS	+	Low	Widely available; Inexpensive; Non-toxic	Poor submucosal elevation
DW	++	Low	Widely available; Inexpensive	Moderate submucosal elevation; Significant tissue damage at high concentrations of dextrose
HPMC	+++	Moderate	Great submucosal elevation; Widely available	Moderately expensive; Risk of antigenic reactions
HES	++++	Low/moderate	Excellent submucosal elevation; FDA-approved for submucosal injection; Reasonably priced	None
HA	++++	High	Excellent submucosal elevation	Expensive; Can stimulate the growth of residual tumour cells
Eleview®	++++	High	Excellent submucosal elevation; Non-toxic	Expensive

NS: Normal saline; DW: Dextrose water; HPMC: Hydroxypropyl methylcellulose; HES: Hydroxyethyl starch; HA: Hyaluronic acid; FDA: Food and Drug Administration.

times[29]. Because fibrinogen is produced from coagulation proteins of human serum, contamination with some viruses and the associated transmission risk is a possibility. Regardless of this disadvantage, FM can be considered a convenient option for submucosal injection during EMR and ESD due to its reasonable price compared with other viscous agents and to its hemostatic properties[5].

Succinylated gelatin

Succinylated gelatin (SG) is a widely available, inexpensive and safe colloidal solution that exerts an oncotic pressure similar to human albumin. The clinical efficacy of SG was evaluated by Moss *et al*[30] in a randomized double-blind trial, conducted to compare the performance of EMR with SG or NS for sessile colonic lesions ≥ 20 mm. The SG group registered fewer injections per lesion, lower injection volume and shorter procedure duration. There was also a trend towards higher *en-bloc* resection rate with the use of SG though without statistically significant difference[30].

Hydroxyethyl starch

Hydroxyethyl starch (HES) is a relatively safe and inexpensive solution, easily available as a colloidal volume expanding solution that is commonly used to treat hypovolemia. In the recent past, 6% HES has been tried out for submucosal lifting in EMRs in studies with porcine models with promising results. Compared to NS, 6% HES solution produced a more prolonged submucosal cushion and lower total procedure time for EMR[31]. Mehta *et al*[31] found a significant superiority of 6% HES compared with NS in the duration of submucosal lifting and the requirement for additional injected solution to maintain the LST elevated. Although use of 6% HES for fluid resuscitation in critically ill patients has been linked to increased mortality, acute kidney injury, and need for dialysis, the low doses used for submucosal injection are presumed to be safe for use in humans[31].

Eleview and ORISE gel®

Eleview® is a synthetic solution that was specifically designed for colorectal endoscopic resection techniques procedures requiring submucosal injection. This product is supplied in five 10-mL ready-to-use ampules and is composed of water, sodium chloride, poloxamer 188 (bulking and structural agent), polyoxyl-15-hydroxystearate (emulsifier), medium-chain-triglycerides (oil component). This solution already includes methylene blue to improve visibility of the lesion and submucosal surface. By providing an immediate and long-lasting cushion beneath the polyp and improving the visibility of the lesion, Eleview® may help achieve a complete and safe removal of the lesion. When compared to SN, Eleview® has demonstrated better cushion-forming ability and a duration of lift of up to 45 min. As a ready to use, sterile, premixed composition, it is a convenient option for clinicians. A recent double-blind RCT comparing Eleview with NS showed that the mean injected volume was significantly lower in the Eleview group (16 mL *vs* 31mL, $P < 0.001$), and

there was a trend towards shorter procedure and a lower number of resection pieces with this new solution[32]. Despite all these advantages, this solution is very expensive for routine use by most endoscopy centres[33]. ORISE gel, a similar solution from other manufacturer (Boston Scientific) is also available, showing comparable results with the former and recently received FDA approval for use as an injection solution throughout the gastrointestinal tract[34].

ADJUVANTS

Some adjuvants may be added to the submucosal injection solution to aid endoscopic resection and to reduce the complications associated with it, such as bleeding and perforation. The most well-known and widely used adjuvants are diluted epinephrine and staining dye like diluted indigo carmine or methylene blue.

Epinephrine

Immediate and delayed bleeding are the most frequent complications associated with endoscopic resections. Diluted epinephrine (1:50000-1:200000) is often added to the submucosal injection fluid because of the theoretical benefits of decreased bleeding and a sustained submucosal cushion (due to delayed absorption of fluid resulting from decreased vascular flow) and is generally considered to be safe.

However, submucosal injection of epinephrine can potentially result in systemic effects such as severe hypertension, ventricular tachycardia, and intestinal ischemia. However, the rare reports of these complications result mainly from hemostatic procedures that used higher concentrations of epinephrine (1:10000), rather than prophylactic injection during endoscopic resection. Because of its short-acting effect the main objective of diluted epinephrine injection is to decrease the risk of intra-procedural bleeding which also helps to maintain a clean resection field. The role of this agent in preventing delayed bleeding is however controversial. ESGE guidelines recommend the use of diluted epinephrine before hot-snare polipectomy of large pedunculated polyps (head size ≥ 20mm or stalk width ≥ 10mm), but there is no mention regarding the systematic need for its injection in other types of lesions. A recent meta-analysis concluded that the application of submucosal epinephrine injection before resecting larger polyps (≥ 20 mm) as a routine procedure is helpful to reduce the occurrence of early postpolipectomy bleeding[35]. However, in this meta-analysis injection of diluted epinephrine was not shown to significantly reduce the risk of delayed postpolipectomy bleeding.

Staining dye

The most commonly used staining agents are biologically inert blue colour dye like diluted indigo carmine and methylene blue. These are frequently added to the injection solution, identifying the area of submucosal injection and clearly distinguishing between the muscle layer and the submucosal layer. This also facilitates identification of the lateral and deep margins of the target lesion before and during the resection process. The staining dye may also help to evaluate the presence of residual lesion at the end of endoscopic resection and improve recognition of muscularis propria injury, which indicates intraprocedural perforation. For example, if muscularis propria is inadvertently resected, the transected surface will present a white central circular disk surrounded by blue-stained submucosal tissue giving it the appearance of a "target" (target sign). This is a very important aspect, because small perforations recognized during the procedure can be successfully sealed with endoscopic metal clips. For these reason, ESGE guidelines recommend that a biologically inert blue dye should be incorporated into submucosal injection solution to facilitate identification of fluid cushion extent, lesion margins and deep mural injury, when performing EMR of larger lesions (≥ 20 mm) or LST. The addition of staining dye in submucosal injection solution is mandatory when performing ESD.

HOW TO INJECT

As mentioned above, submucosal injection is essential in almost every EMR technique and it is indispensable when performing ESD. This next section is dedicated to some practical aspects and tips that should be taken into account when performing these two endoscopic resection techniques. The authors also present a brief summary of the main aspects discussed below in Figure 4.

EMR

How to inject: Pratical tips
1 Before starting the injection, it is important to carefully evaluate lesion features and its location.
2 When trying to perform an *en-bloc* EMR of a smaller lesion, the creation of a single cushion with an injection point centred in the lesion can be the best option for a successful resection.
3 When trying to remove larger lesions, namely by piecemeal EMR, more than one injection is usually required in order to lift the entire lesion and to maintain the submucosal cushion during the whole (usually longer) procedure. Puncture and injection at the border of the previous submucosal cushion can facilitate access to the submucosal space and expand the cushion laterally.
4 Dynamic submucosal injection technique, which allows submucosal cushion position and shape adjustments during injection, improves the feasibility of EMR.
5 During ESD of a small gastric lesion, the operator can inject all around the circumference in order to perform circumferential dissection without changing the instrument.
6 In larger gastric lesions or lesions in a difficult location, and also in colonic ESD, semi-radial injection can be preferred. Subsequent injection should be performed at the lateral margin of the previous injection.
7 After circumferential dissection, injection should target the submucosa below the lesion. This can be achieved by placing the injection needle right below lesion margin, directly targeting the remaining submucosal space by slightly lifting up the needle tip.

Figure 4 How to inject: Pratical tips. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal resection.

Submucosal injection is a key step of endoscopic resection techniques. This is normally made using an injector that goes through the working channel of the endoscope, which has a retractile needle that allows access to the submucosal space and injection of different types of solutions[36]. Before starting the injection, it is important to careful evaluate lesion morphology and localization. For instance, when trying to perform *en-bloc* resection of a smaller lesion, the creation of a single cushion with the injection point centered in the lesion may be the best option. However, when trying to remove larger lesions, namely by piecemeal EMR, more than one injection is usually needed to lift the entire lesion. In these instances, after the first puncture and injection into the submucosa, it can help to puncture and inject the border of the already formed submucosal cushion and expand it laterally. Soetkino *et al*[36] described this step either as static or dynamic submucosal injection. In the static technique, the needle punctures the submucosa and remains in a relatively fixed position during fluid injection and the lumen is inflated to visualize the position of the needle insertion point. In this case, many endoscopists report that the injected fluid rapidly dissipates, resulting in an insufficient submucosal cushion that hinders lesion snaring. On the other hand, dynamic submucosal injection helps produce a focal bulge under the lesion. First, keeping the catheter close to the endoscope tip, the needle is advanced into the submucosal plane and a small amount of solution is injected. Once the submucosal location is confirmed, subsequent injection is rapidly performed through the injector needle, while the needle position is slightly redirected within the injection site by slowly pulling back the catheter or slight deflections of the endoscope tip. In addition to these subtle movements, the lumen is gently aspirated to increase the size of the cushion. Many endoscopists prefer this last technique, which improves the feasibility of EMR[37].

ESD

Although sharing some common aspects with EMR, injection in ESD has some particularities. After delimitation, the first step consists in gaining access to the submucosal space through a mucosal incision with a sharp knife. The mucosal incision should be performed outside of the coagulation marks, and thus it is recommended to perform the first injections right outside of the marks, to reduce perforation risk when cutting into the mucosa/submucosa. In a small gastric lesion (< 20 mm), the operator can inject and lift the whole circumference in order to perform the other 3-4 mucosal incisions and circumferential dissection without changing the instrument. However, in larger gastric lesions or lesions located in a difficult location, and also in colonic ESD, semi-radial injection can be preferred. It is also important to recognize that subsequent injection should be performed at the lateral margin of the previous injection. After mucosal incision and circumferential/semi-circumferential dissection, injection should be performed as needed in the submucosal dissection plane. Injection should be precise and target the submucosa below the lesion and not the muscularis propria. This can be achieved by placing the injection catheter/needle right below lesion margin, directly targeting the remaining submucosal space by slightly lifting up the needle tip. While injecting, subtle movement in the endoscope shaft or wheels can be useful to direct the lesion to an adequate position, in order to provide a larger field of view and facilitate dissection. When using a cap, placing the tip of the endoscope below the lesion can also facilitate further injection. After

complete dissection, coagulation of visible vessels should also be performed in gastric ESD, and sufficient submucosal lifting is generally advised in order to reduce thermal injury to the gastric wall, which could be accomplished with further injection or water jet elevation.

THE NEW KIDS ON THE BLOCK

With the dissemination of endoscopic resection, recent focus has been put in optimizing and facilitating the procedures, with the aim of decreasing procedural time while maintaining high efficacy and minimizing adverse events. Devices have been developed that achieve submucosal lifting through ultrafine jet pressure, allowing ESD without the need for conventional injection (*e.g.*, Hybrid-Knife®, Dual-Knife®). To combine the advantages of water jet lifting and of macromolecular solutions, a water-jet system with a bifunctional catheter (Nestis® Enki II®, Lyon, France) was developed and has been shown to be feasible, although it was not implemented in routine clinical practice, perhaps because the duration of elevation is not so important when high-pressure jets are used[38]. Devices that create submucosal blebs without needle injection were also developed for inject-and-cut EMR (*e.g.*, ERBELift), with the theoretical advantage of easing the lifting of lesions and avoiding possible complications of needle manipulation, although it also demands device exchange between snares and the flexible "injector"/probe. Regarding solutions for lifting, recently there has been interest in the development of solutions with newer and useful properties, namely dissecting properties. In an animal study, a modified ESD technique using an endoscopic biocompatible gel (Cook Medical Inc) was shown to be feasible. In this technique, the gel with dissecting property was injected in a previously formed submucosal bleb after mucosal incision, and no further knife dissection was needed since auto-dissection was noted (the removal of the separated mucosa was accomplished with hot snare). Mean procedural time for 30-mm lesions was 7.5 min[39]. This gel was also found to be useful in peroral endoscopic miotomy (POEM), allowing the creation of a tunnel without dissection ("auto-tunneling")[40]. A thiol compound called mesna that can chemically soften connective tissues and facilitate ESD was also evaluated in a human RCT and it was found that it can significantly reduce time-consuming cases (> 30 min, P = 0.049). Although mean dissecting time was not statistically significant lower in the mesna group (18.6 min *vs* 24.6 min, P = 0.128), mesna use was independently associated with lower dissecting time in multivariable analysis[41]. In conclusion, although there has been some development concerning injection solutions and devices, the progress has been slow since injection with NS and viscous solutions with conventional needles is highly efficacious and safe. However, further refinement of the technique is always welcome to improve its already good outcomes, to accelerate the learning curve and to facilitate the dissemination of endoscopic resection techniques.

CONCLUSION

According to current evidence, as pointed throughout this review, no solution has proven to be consistently superior in complete resection rate and in the reduction of adverse events incidence like post-polipectomy bleeding or coagulation syndrome/perforation. This is particularly evident in western countries where most of the injection solutions specifically developed for endoscopic resection in Asia are not commercially available or not approved by the Food and Drug Administration, leaving endoscopists to use a variety of injectable fluids off-label. We can conclude that for most of endoscopic resection, namely smaller lesions (about 20 mm), NS is still a good option because the height of the cushion and the duration of the elevation is not as preponderant a factor. For these lesions, the use of NS does not lead to a significant greater number of injections or to an increased procedure time. Despite the lack of proven superiority of a specific endoscopic submucosal injection solution in humans, in a recent randomized controlled trial of solutions currently available in the West, Eleview® and 6% hydroxyethyl starch were the best performing solutions for ESD in a porcine model. So, even though viscous solutions (namely starch or the new Eleview) can be relatively expensive, they can be particularly important in the resection of larger lesions, particularly during ESD, by decreasing the number of injections and the procedure time. In conclusion, when choosing the type of submucosal injection solution, we must take into account the lesion features and the endoscopic resection technique to be used, the local and own expertise, the availability and costs of the solution as well as the balance between its advantages and

potential adverse effects.

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MINIREVIEWS

Targeted and immune therapies for hepatocellular carcinoma: Predictions for 2019 and beyond

Masatoshi Kudo

ORCID number: Masatoshi Kudo (0000-0002-4102-3474).

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Masatoshi Kudo, Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, Osaka 589-8511, Japan

Corresponding author: Masatoshi Kudo, MD, PhD, Director, Full Professor, Professor and Chairman, Department of Gastroenterology and Hepatology, Kindai University Faculty of Medicine, 377-2, Ohno-Higashi, Osaka 589-8511, Japan. m-kudo@med.kindai.ac.jp
Telephone: +81-723660221-3149
Fax: +81-723672880

Abstract

Systemic therapy for hepatocellular carcinoma (HCC) has markedly advanced since the survival benefit of a molecular targeted agent, sorafenib, were demonstrated in the SHARP and Asia Pacific trials in 2007. Treatment options for patients with advanced HCC increased by sorafenib, and long-term survival for patients with advanced stage HCC has become possible to some extent. However, development of a more potent first-line novel molecular targeted agent replacing sorafenib and a potent second-line agent after disease progression on or intolerant to sorafenib has been warranted because sorafenib lacks tumor shrinking/necrotizing effects and induces relatively severe adverse events such as hand foot skin reaction. Many agents in the 1st line and 2nd line setting were attempted to develop between 2007 and 2016, but all of these clinical trials failed. On the other hand, clinical trials of 4 agents (regorafenib, lenvatinib, cabozantinib, and ramucirumab) succeeded in succession in 2017 and 2018, and their use in clinical practice is possible (regorafenib and lenvatinib) or underway (cabozantinib and ramucirumab). Furthermore, all of 5 clinical trials of combination therapy with transcatheter chemoembolization (TACE) plus a molecular targeted agent failed to date, however, the combination of TACE and sorafenib (TACTICS trials) was reported to be successful and presented at ASCO in 2018. Phase 3 clinical trials of immune checkpoint inhibitors and a combination therapy of immune checkpoint inhibitors and molecular targeted agents are also ongoing, which suggests treatment paradigm of HCC in all stages from early, intermediate and advanced stage, is expected to be changed drastically in the very near future.

Key words: Hepatocellular carcinoma; Molecular targeted agent; Immune checkpoint inhibitor; Sorafenib; Lenvatinib

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Core tip: Systemic therapy for hepatocellular carcinoma (HCC) has markedly advanced since sorafenib was approved in 2007. Since then, there was no active drug for 10 years that prolong overall survival, however, in 2017 and 2018, clinical trials of 4 more molecular targeted agents including lenvatinib as first line agent, regorafenib, cabozantinib and ramucirumab as second line agent have shown their survival benefit. In addition, immune check point inhibitors, nivolumab and pembrolizumab, were approved by Food and Drug Administration. Combination cancer immunotherapy, that combines immune checkpoint inhibitors and molecular targeted agents show great promise in the treatment of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is treated by surgical resection, local ablation, transarterial chemoembolization (TACE), or intra-arterial infusion chemotherapy. Most practice guidelines relevant to HCC were revised in 2017 and 2018[1-5]. In this article, the current status and future perspective of systemic therapy for HCC, which has advanced markedly will be reviewed.

Since first appearance of the molecular targeted agent, sorafenib, in 2007, systemic therapy for HCC has changed markedly. Treatment options for extrahepatic spread and vascular invasion have increased, and relatively long-term survival has been achieved, even for patients with Barcelona Clinic Liver Cancer (BCLC) stage C advanced HCC. However, sorafenib does not shrink or induce necrosis in tumors and has relatively severe adverse events (AEs), including hand foot skin reaction. Thus, development of a novel molecular targeted agent that can replace sorafenib, along with a second-line agent that can prevent/slow disease progression while a patient is undergoing treatment with sorafenib is desirable. Between 2007 and 2016, many comparative trials of new agents with sorefenib have been conducted; however, eight clinical trials of first-line agents and eight of second-line agents failed (Table 1)[6-20]. In 2017 and 2018, clinical trials of three agents (regorafenib, lenvatinib, cabozantinib, and ramucirumab) reported successful outcomes; indeed, some of these drugs are now used in clinical practice (Table 1). All of three trials in adjuvant setting after curative treatments failed (Table 2)[21-24]. In addition, five trials of combination therapy with transcatheter chemoembolization (TACE) plus a molecular targeted agent have been conducted to date, but all of them failed to show its benefit[25-29] (Table 2). In 2018, the combination of TACE and sorafenib, the TCTICS trial, reported improved progression-free survival (PFS); the results were presented at the 2018 ASCO-GI and ASCO meetings in 2018[30]. Herein, recent advances and future prospects for molecular targeted therapy for HCC will be discussed.

MOLECULAR TARGETED AGENTS: FIRST-LINE AGENTS

Sorafenib

Sorafenib is an oral drug that suppresses tumor growth by inhibiting the serine-threonine kinases C-Raf, wild-type B-Raf, and mutant (V600E) B-Raf, all of which are components of the Raf/MEK/ERK pathway (mitogen-activated proteins kinase pathway). This pathway acts downstream of the vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR), and the epidermal growth factor receptor. It also exerts anti-tumor effects by suppressing neovascularization. It achieves tumor neovascularization by inhibiting the tyrosine kinases VEGFR1, VEGFR2, VEGFR3, PDGFRβ, RET, and fms-related tyrosine kinase 3 (FLT-3). Two large-scale pivotal trials (the SHARP and Asia-Pacific trials) of sorafenib reported significant prolongation of overall survival (OS) compared with placebo[31,32]; indeed, sorafenib is now the standard therapeutic agent for advanced HCC. However, its ability to shrink tumors is weak and its systemic toxicity is relatively high. Therefore, novel molecular targeted agents with more potency or similar effects, but

Table 1 Phase III clinical trials of advanced stage hepatocellular carcinoma

Target population		Design	Trial name	Result	Presentation	Publication	1st author
Advanced	First line	1 Sorafenib *vs* Sunitinib	SUN1170	Negative	ASCO 2011	JCO 2013[6]	Cheng AL
		2 Sorafenib +/- Erlotinib	SEARCH	Negative	ESMO 2012	JCO 2015[7]	Zhu AX
		3 Sorafenib *vs* Brivanib	BRISK-FL	Negative	AASLD 2012	JCO 2013[8]	Johnson PJ
		4 Sorafenib *vs* Linifanib	LiGHT	Negative	ASCO-GI 2013	JCO 2015[9]	Cainap C
		5 Sorafenib +/- Doxorubicin	CALGB 80802	Negative	ASCO-GI 2016		
		6 Sorafenib +/- HAIC	SILIUS	Negative	EASL 2016	Lancet GH 2018[10]	Kudo M
		7 Sorafenib +/- Y90	SARAH	Negative	EASL 2017	Lancet-O 2017[11]	Vilgrain V
		8 Sorafenib +/- Y90	SIRveNIB	Negative	ASCO 2017	JCO 2018[12]	Chow P
		9 Sorafenib *vs* Lenvatinib	REFLECT	Positive	ASCO 2017	Lancet 2018[34]	Kudo M
		10 Sorafenib *vs* Nivolumab	CheckMate-459	Ongoing			
		11 Sorafenib *vs* Durvalumab + Tremelimumab *vs* Durva	HIMALAYA	Ongoing			
		12 Sorafenib *vs* Atezolizumab + Bevacizumab	Imbrave 150	Ongoing			
		13 Sorafenib *vs* Tislelizumab		Ongoing			
	Second line	1 Brivanib *vs* Placebo	BRISK-PS	Negative	EASL 2012	JCO 2013[13]	Llovet JM
		2 Everolimus *vs* Placebo	EVOLVE-1	Negative	ASCO-GI 2014	JAMA 2014[14]	Zhu AX
		3 Ramucirumab *vs* Placebo	REACH	Negative	ESMO 2014	Lancet-O 2015[15]	Zhu AX
		4 S-1 *vs* Placebo	S-CUBE	Negative	ASCO 2015	Lancet GH 2017[16]	Kudo M
		5 ADI-PEG 20 *vs* Placebo	NA	Negative	ASCO 2016	Ann Oncol 2018[17]	Abou-Alfa G
		6 Regorafenib *vs* Placebo	RESORCE	Positive	WCGC 2016	Lancet 2017[41]	Bruix J
		7 Tivantinib *vs* Placebo	METIV-HCC	Negative	ASCO 2017	Lancet-O 2018[18]	Rimassa L
		8 Tivantinib *vs* Placebo	JET-HCC	Negative	ESMO 2017		
		9 DT *vs* Placebo	ReLive	Negative	ILCA 2017		
		10 Cabozantinib *vs* Placebo	CELESTIAL	Positive	ASCO-GI 2018	NEJM 2018[45]	Abou-Alfe G
		11 Ramucirumab *vs* Placebo	REACH-2	Positive	ASCO 2018	Lancet-O 2019[30]	Zhu AX
		12 Pembrolizumab *vs* Placebo	KEYNOTE-240	Negative			

HAIC: Hepatic arterial infusion chemotherapy; Doxorubicin-loaded nanoparticles.

less toxicity, have been unmet need.

Lenvatinib: Overview of the results of the REFLECT trial

Although eight clinical trials with various agents/modalities comparing with sorafenib conducted in the last decade has shown negative outcomes, the results of the REFLECT trial with use of lenvatinib met its primary endpoint of non-inferiority

Table 2 Randomized phase II, phase III clinical trials of early / intermediate stage hepatocellular carcinoma

Target population		Design	Trial name	Result	Presentation	Publication	1st author
Early	Adjuvant (prevention of recurrence)	1 Vitamin K2 *vs* Placebo		Negative		Hepatology 2011[21]	Yoshida H
		2 Peretinoin *vs* Placebo	NIK-333	Negative	ASCO 2010	JG 2014[22]	Okita K
		3 Sorafenib *vs* Placebo	STORM	Negative	ASCO 2014	Lancet-O 2015[23]	Bruix J
		4 Peretinoin *vs* Placebo	NIK-333/K-333	Ongoing			
	Improvement of RFA	1 RFA +/- LTLD	HEAT	Negative	ILCA 2013	CCR 2017[24]	Tak WY
		2 RFA +/- LTLD	OPTIMA				
Intermediate	Improvement of TACE	1 TACE +/- Sorafenib	Post-TACE	Negative	ASCO-GI 2010	EJC 2011[25]	Kudo M
		2 TACE +/- Sorafenib	SPACE (Ph II)	Negative	ASCO-GI 2012	J Hepatol 2016[26]	Lencioni R
		3 TACE +/- Brivanib	BRISK-TA	Negative	ILCA 2013	Hepatol 2014[27]	Kudo M
		4 TACE +/- Orantinib	ORIENTAL	Negative	EASL 2015	Lancet GH 2017[28]	Kudo M
		5 TACE +/- Sorafenib	TACE-2	Negative	ASCO 2016	Lancet GH 2017[29]	Meyer T
		6 TACE +/- Sorafenib	TACTICS (Ph II)	Positive	ASCO-GI 2018[30]		Kudo M

LTLD: Lyso-thermosensitive liposomal doxorubicin.

of prolonging OS compared with sorafenib. Lenvatinib is an oral kinase inhibitor that selectively inhibits receptor tyrosine kinases involved in neovascularization and progression to high malignancy grade tumors and a poor prognosis; targeted kinases include VEGFR1, VEGFR2, VEGFR3, fibroblast growth factor receptor (FGFR) 1, FGFR2, FGFR3, FGFR4, PDGFRα, KIT, and RET. In particular, strong inhibition of FGFR4 is considered important for preventing aggressive growth or progression to a higher malignancy grade of HCC. The drug also suppresses invasion and metastasis. A single-arm phase II study of lenvatinib as a treatment for advanced HCC reported a time to progression (TTP) of 7.4 mo and an OS of 18.7 mo, which are very favorable[33]. Subsequently, a phase III study comparing sorafenib with lenvatinib, the REFLECT trial, was conducted[34].

The REFLECT trial was a global phase III study to show the non-inferiority of lenvatinib to sorafenib, in which patients with unresectable HCC, not previously treated with systemic chemotherapy, were allocated randomly to the lenvatinib or sorafenib arms at a 1:1 ratio. Stratification factors were Asian/non-Asian, vascular invasion and/or extrahepatic spread (presence or absence), Eastern Cooperative Oncology Group performance status 0 or 1, and body weight < 60 kg or ≥ 60 kg. Administration was continued until disease progression or development of a non-tolerable adverse event. The primary endpoint was verification of non-inferiority in terms of OS; the non-inferiority margin of OS was set at 1.08, which is very strict. PFS, TTP, objective response rate (ORR), and safety were evaluated as secondary endpoints.

Of the 954 patients registered, 478 and 476 were allocated to the lenvatinib and sorafenib groups, respectively. Overall, 67% of patients in the lenvatinib group were from the Asia-Pacific region and 33% were from western countries. Of these, 32% weighed less than 60 kg and 68% weighed 60 kg or more in lenvatinib arm. Vascular invasion and/or extrahepatic spread were noted in 69%, and BCLC stage C HCCs accounted for 78% in the lenvatinib arm. Regarding the cause of disease, HCC due to hepatitis C accounted for 19% in the lenvatinib arm and 27% in the sorafenib arm, suggesting an advantageous imbalance toward sorafenib[34]. In contrast, HCC due to hepatitis B accounted for 53% of cases in the lenvatinib arm and for 48% of cases in the sorafenib arm. In the lenvatinib arm, 46% of patients had an alpha fetoprotein (AFP) level exceeding 200 ng/mL *vs* 39% in the sorafenib group, also indicating an advantageous imbalance toward sorafenib.

The primary endpoint OS in the lenvatinib and sorafenib arms was 13.6 and 12.3 mo, respectively, and the hazard ratio (HR) was 0.92 (0.79-1.06); this was lower than the non-inferiority margin with a specified upper limit of a 95% confidence interval

(CI) of 1.08. These data suggest that lenvatinib was not inferior to sorafenib in terms of OS[34]. In addition, the PFS reported by institutional investigators according to the modified Response Evaluation Criteria in Solid Tumors (modified RECIST) criteria was 7.3 and 3.6 mo for the lenvatinib and sorafenib arms, respectively. TTP was 7.4 and 3.7 mo, respectively, and the ORR was 40.6% and 12.6% according to the results of Masked Independent Review Committee, respectively. Thus, lenvatinib had significantly more favorable anti-tumor effects than sorafenib (Table 3)[34]. Evaluation by Masked Independent Review Committee based on RECIST1.1 also confirmed that lenvatinib had favorable anti-tumor effects with regard to PFS, TTP, and ORR as well. Furthermore, when analysis was confined to intermediate stage HCC, the PFS in the lenvatinib arm was 9.1 mo, which is fairly long, and the ORR of BCLC B-stage HCC in the lenvatinib arm for the Japanese population was 61.3%, which is extremely high[35].

Because AFP was not included as a stratification factor, more patients with an AFP level exceeding 200 ng/mL were enrolled in the lenvatinib group. When this was corrected by analysis of covariance, the lenvatinib arm showed a superior OS (HR = 0.856; 95%CI = 0.736-0.995; nominal *P*-value = 0.0342)[34]. Based on these findings, if AFP was included as a stratified factor, the study would likely have confirmed the superiority of lenvatinib over sorafenib[36,37]. The probability that lenvatinib was superior to sorafenib after adjustment for AFP is as high as 0.743[38].

Sub-analysis of OS revealed that the OS-prolonging effects of lenvatinib were superior to those of sorafenib in almost all subsets. It should be noted that specifically that in the group with body weight < 60 kg, the OS benefits of lenvatinib were superior to those of sorafenib at a dose of 8 mg, and the HR was more favorable than that in the group weighing ≥ 60 kg or higher at a dose of 12 mg (HR, 0.85 *vs* 0.95, respectively). This suggests that weight-based dosing is successful[37,39]. In addition, the HR in the group with a baseline AFP of ≥ 200 ng/mL was 0.78 (95%CI, 0.63-0.98), confirming that lenvatinib has favorable OS benefits even in a group with a high AFP level and a poor prognosis. The treatment durations for the lenvatinib and sorafenib arms were 5.7 and 3.7 mo, respectively; therefore, lenvatinib can be given orally for a longer duration and the frequency of subjective AEs such as hand foot skin reaction and diarrhea is lower, indicating superior tolerability.

Based on the above findings, lenvatinib is not inferior to sorafenib in terms of OS; indeed, lenvatinib showed statistically significant and clinically meaningful improvement in all secondary endpoints (PFS, TTP, and ORR), confirming its efficacy as a first-line agent for patients with unresectable HCC. Regarding the AEs, hypertension, proteinuria and hypothyroidism were more frequently observed in lenvatinib arm although hand-foot skin reaction, diarrhea and alopecia were less frequently observed in lenvatinib arm as compared with sorafenib arm[34]. This AE profile suggests that AEs in lenvatinib arm are more favorable and tolerable than sorafenib since most of AEs observed in lenvatinib arm are asymptomatic and can be manageable by medication (hypertension, hypothyroidism) or by dose reduction (proteinuria). Based on these results, lenvatinib has been approved to treat HCC on March 23, 2018 in Japan. It was also approved in HCC by the Food and Drug Administration on August 16, 2018, in Europe on August 23, 2018, in South Korea on September 4, 2018, in China on August 29, 2018 and in Taiwan on November 28, 2018.

MOLECULAR TARGETED AGENTS: SECOND-LINE AGENTS

Regorafenib: Overview of the RESORCE trial

Regorafenib is an oral kinase inhibitor. It targets protein kinases such as VEGFR1, VEGFR2, VEGFR3, TIE2, PDGFRβ, FGFR, KIT, RET, RAF-1, and BRAF[40]. Because it is synthesized simply by binding fluorine to sorafenib, it has almost the same molecular structure as sorafenib and a similar, but stronger toxicity profile. Therefore, unlike other drugs, a placebo-controlled phase III study (RESORCE trial) was performed that included only patients who progressed under sorafenib treatment and those intolerant to sorafenib patients were excluded. The primary endpoint, OS, in the regorafenib arm was 10.6 mo and that in the placebo arm was 7.8 mo, which is a significant improvement[41]. PFS and TTP were also significantly more favorable (Table 4). This is the first drug for which efficacy was proved as a second-line systemic agent who progressed on sorafenib.

Regarding the AE profiles of regorafenib is as follows; grade 3/4 drug-related treatment-emergent AEs include hand-foot skin reaction in 13%, fatigue in 6%, hypertension in 13% and diarrhea in 2%[41]. However, most these AEs were manageable by medication or dose reduction/interruption. Based on these results, its use as a second-line agent for HCC in Japan was approved in May 2017. However, the drug is unsuitable for patients with sorafenib intolerance; this means that there is an

Table 3 Results of the REFLECT trial[34]

	Lenvatinib (*n* = 478)	Sorafenib (*n* = 476)	HR, *P*-value
OS (M, 95% CI)	13.6 (12.1-14.9)	12.3 (10.4-13.9)	HR 0.92 (0.79-1.06)
PFS (M, 95% CI)	7.3 (5.6-7.5)	3.6 (3.6-3.7)	HR 0.64 (0.55-0.75) $P < 0.0001$
TTP (M, 95% CI)	7.4 (7.2-9.1)	3.7 (3.6-3.9)	HR 0.60 (0.51-0.71) $P < 0.0001$
Objective response (independent review, mRECIST)			
CR	10 (2%)	4 (1%)	
PR	184 (38%)	55 (12%)	
SD	159 (33%)	219 (46%)	
PD	79 (17%)	152 (32%)	
ORR	194 (40.6%)	59 (12.4%)	$P < 0.0001$
DCR	353 (73.8%)	278 (58.4%)	$P < 0.0001$

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; ORR: Objective response rate; DCR: Disease control rate.

unmet need for a second-line treatment in this group. However, this was soon solved by successful trials of cabozantinib and ramucirumab in 2018 as mentioned later.

The RESORCE trial was successful for the following reasons: (1) For second-line treatment with regorafenib, patients that discontinued sorafenib due to AEs were excluded, in other words, only patients with progressive disease (PD) under sorafenib treatment were included; (2) vascular invasion and extrahepatic spread were set as independent stratification factors to prevent imbalanced distribution between the two arms (active drug and placebo arms); (3) AFP, a strong poor prognostic factor, was also included as a stratification factor; and (4) only patients with sufficient sorafenib tolerance were selected (limited to patients who were able to tolerate sorafenib doses of 400 mg or higher for 20 d or longer during the 28-d period before PD). This study design prevented dropout due to AEs caused by regorafenib since molecular structure of regorafenib is very similar to sorafenib, and minimized the influence of post-treatment effects following PD after regorafenib treatment[41]. According to the RESORCE trial, the median survival time for patients treated with regorafenib was 10.6 mo (placebo: 7.8 mo; HR = 0.63; $P < 0.0001$). Sub-analysis of OS revealed that it was significantly more favorable for patients when sorafenib was first used at a Child-Pugh score of 5 than for patients introduced to sorafenib at a score of 6; this suggests that early switching from TACE to sorafenib for patients who become refractory to TACE at a Child-Pugh score of 5, and early switching to regorafenib for patients who become refractory to sorafenib, are important for extending survival. Furthermore, the median duration of pre-treatment with sorafenib before the RESORCE trial was 7.8 mo, which is relatively long. Many patients with long SD that responded to sorafenib well were enrolled in this trial. Thus, the effects on patients with rapid PD have been unclear. However, a recent report of sub-analysis of the RESORCE trial showed that the HR for OS in patients with rapid PD on sorafenib (TTP: 2.3 mo) was 0.66, thereby clarifying that regorafenib has OS benefit, even in patients showing rapid PD on sorafenib[42].

Sequential therapy with sorafenib and regorafenib

Based on the results of the RESORCE trial, sorafenib-regorafenib sequential therapy extended OS from the time of sorafenib treatment initiation to 26 mo (placebo group: 19.2 mo), thereby providing a favorable outcome[42,43]. This is a very important message to clinical practice. This outcome (OS = 26 mo) is almost comparable with that for intermediate stage HCC patients treated with conventional TACE[27]. At present, the BRISK-TA trial, a prospective phase III study involving the largest number of patients in the world, is the world largest TACE combination trial. Therefore, the outcome of the placebo arm of this trial is considered to be the world standard outcome of TACE treatment with no selection bias. In addition, the target groups of this trial comprised patients with BCLC B, BCLC A, and BCLC C (59%, 23%, and 17% of patients, respectively). Thus, 82% of all patients had early/intermediate stage disease and only 17% had advanced stage disease. In contrast, patients with advanced stage BCLC C accounted for 86% of patients enrolled in the RESORCE trial. When these two cohorts were compared, the OS of TACE-treated patients were 26.1 mo and that of patients who received sorafenib-regorafenib sequential therapy was 26 mo. Although it may be inappropriate to compare the results of the different

Table 4 Results of the RESORCE trial[41]

	Regorafenib (*n* = 379)	Placebo (*n* = 194)	HR, *P*-value
OS (M, 95%CI)	10.6 (9.1-12.1)	7.8 (6.3-8.8)	HR 0.63 (95%CI 0.50-0.79) *P* < 0.0001
PFS (M, 95%CI)	3.1 (2.8-4.1)	10.6 (1.4-1.6)	HR 0.46 (95%CI 0.37-0.56) *P* < 0.0001
TTP (M, 95%CI)	3.2 (2.9-4.2)	10.6 (1.4-1.6)	HR 0.44 (95%CI 0.36-0.55) *P* < 0.0001
Objective response(investigator assessed, mRECIST)			
CR	2 (1%)	0	
PR	38 (10%)	8 (4%)	
SD	206 (54%)	62 (32%)	
PD	86 (23%)	108 (56%)	
ORR	40 (11%)	8 (4%)	*P* = 0.0047
DCR	247 (65%)	70 (36%)	*P* < 0.0001

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; ORR: Objective response rate; DCR: Disease control rate.

randomized controlled trials (RCTs), it should be justified because there was no selection bias in the placebo group from the well-designed RCT. Considering that sorafenib-regorafenib sequential therapy targets far more advanced cases of HCC, the finding of a comparable OS result for TACE- and sorafenib-regorafenib-treated patients implicates very important issue. Although it was a highly selected patient population, the survival benefit of sorafenib-regorafenib sequential therapy for advanced stage HCC were comparable with those of TACE for intermediate stage HCC[27,42-44]. Therefore, sorafenib-regorafenib sequential therapy is expected to achieve a favorable outcome in the real-world clinical practice as well, suggesting that the timing of sorafenib introduction should be re-considered. Previously, a patient was switched from TACE to systemic therapy when they became refractory to TACE. However, it may be more important to identify a subgroup likely to become refractory to TACE and then introduce systemic therapy at an earlier time when the liver function reserve is maintained at a Child-Pugh score of 5 before becoming refractory to TACE[43,44].

Cabozantinib: Overview of the CELESTIAL trial

Cabozantinib is an oral multi-kinase inhibitor that inhibits the activity of VEGF, c-MET, RET, AXL, TIE2, and FLT3. The survival-prolonging effects of cabozantinib as a second-line agent for patients with HCC refractory/intolerant to sorafenib treatment compared with placebo control were presented at the American Society of Clinical Oncology Gastrointestinal Cancer Symposium (ASCO-GI), held in January 2018. In total, 707 patients with unresectable HCC were allocated to the cabozantinib and placebo groups (2:1 ratio). The median OS for the cabozantinib group (n = 470) was 10.2 mo (95%CI, 9.1-12.0), demonstrating significant survival benefit when compared with the placebo group [8.0 mo (95%CI, 6.8-9.4)] (Table 5)[45,46]. Systemic chemotherapy with a drug other than sorafenib was allowed during this trial, with the drug positioned as the second- or third-line therapy.

Regarding the AE profiles, grade 3/4 AEs include diarrhea (10%), decreased appetite (6%), hand-foot skin reaction (17%), fatigue (10%) and hypertension (16%)[45]. However, most of AEs were manageable by medication or dose reduction/interruption. As this trial was not performed in Japan, a bridging phase 2 study is now underway; therefore, its approval as a second-line agent is expected in the near future in Japan as well.

Ramucirumab: Overview of the REACH-2 trial

Ramucirumab is a recombinant monoclonal human immunoglobulin IgG1 antibody specific for VEGFR-2. It is injected intravenously and inhibits VEGFR-2 activity by blocking its binding to VEGF-A, VEGF-C, and VEGF-D. Thus, it exerts anti-tumor effects by inhibiting endothelial cell proliferation, migration, and survival, thereby preventing tumor neovascularization.

The previous phase of the REACH trial examined the survival benefit of ramucirumab compared with placebo in patients with unresectable advanced HCC that was refractory/intolerant to sorafenib treatment. No survival benefit was proven in this trial[15]. However, sub-analysis limited to patients with an AFP level ≥ 400 ng/mL revealed improved survival; this result was reproducible regardless of the

Table 5 Results of the CERESTIAL trial[45]

	Cabozantinib (*n* = 470)	Placebo (*n* = 237)	HR, *P*-value
OS (M, 95%CI)	10.2 (9.1-12.0)	8.0 (6.8-9.4)	HR 0.76 (95%CI 0.63-0.92) *P* = 0.0049
PFS (M, 95%CI)	5.2 (4.0-5.5)	1.9 (1.9-1.9)	HR 0.44 (95%CI 0.36-0.52) *P* < 0.0001
Objective response (investigator assessed, RECIST 1.1)			
CR (%)	0	0	
PR (%)	4	0.4	
SD (%)	60	33	
PD (%)	21	55	
NE (%)	15	11	
ORR (%, 95CI)	4 (2.3-6.0)	0.4 (0.0-2.3)	*P* = 0.0086
DCR (%)	64	33.4	

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NE: Not evaluable; ORR: Objective response rate; DCR: Disease control rate.

geographic region[47,48]. Thus, the REACH-2 trial was planned and conducted. The study design was not markedly different from that of the original REACH trial except that the subjects were limited to patients with an AFP level of ≥ 400 ng/mL, and macroscopic vascular invasion was included as a stratification factor. The results were presented at ASCO in June 2018. The study results were positive in terms of the primary endpoint: improvement of OS[49,50]. The median OS in the ramucirumab group was 8.5 mo and that in the placebo group was 7.3 mo, confirming a significant survival benefit over placebo (HR = 0.710; *P* = 0.0199) (Table 6).

Regarding the AE profiles, grade 3/4 AEs include hypertension (12%), thrombocytopenia (5%), hepatic encephalopathy (3%) and neutropenia (3%). These AEs were manageable by medication and/or dose reduction/interruption. Most importantly, relative dose intensity was as high as 98.5%, which suggests tolerability of this drug is fairly high[49]. Based on these results, ramucirumab is expected to be approved for patients refractory or intolerant to sorafenib treatment and with an AFP level ≥ 400 ng/mL.

TACE COMBINATION TRIAL: RESULTS OF THE TACTICS TRIAL

The TACTICS trial was a multicenter prospective RCT comparing TACE plus sorafenib with TACE alone that was conducted at 33 sites in Japan[30,51]. A total of 156 patients with unresectable HCC were assigned to receive sorafenib plus TACE (*n* = 80) or TACE alone (*n* = 76) at a 1: 1 ratio. The inclusion criteria were Child-Pugh score ≤ 7, a maximum of two previous TACE sessions, and ≤ 10 HCCs with none exceeding 10 cm in size. The exclusion criteria were extrahepatic spread and vascular invasion. Patients in the TACE plus sorafenib arm started sorafenib 2-3 wk before TACE at a dose of 400 mg once daily. The purpose of this sequential pretreatment with sorafenib was to assess tolerability to sorafenib, normalize the tumor vasculature to improve TACE effectiveness, and attenuate VEGF upregulation after the TACE procedure. Sorafenib was temporarily suspended 2 d before and after TACE. In patients showing sorafenib tolerance, the dose was increased to 800 mg daily when possible. TACE was performed on demand, and repeated TACE was generally performed in cases with viable lesions that grew by ≥ 50% over baseline. Response was assessed using computed tomography, magnetic resonance imaging, or other related modalities every 8 wk. The study had two co-primary endpoints, namely, PFS and OS, and adopted a gatekeeping strategy. The secondary endpoints were the time until TACE was no longer feasible or no longer showed any benefit (time to un-TACEable progression: TTUP), TTP, response rate, and safety. As further explained below, the development of new intrahepatic lesions was not defined as tumor progression. This criterion was introduced to maximize the duration of sorafenib administration and to keep the progression criteria for TACE as consistent as possible with those currently used in clinical practice. Use of the RECIST criteria as response evaluation criteria/a stopping rule is inappropriate because repeated TACE is generally performed after detecting a new intrahepatic lesion, which does not qualify as treatment failure

Table 6 Results of the REACH-2 trial[49]

	Ramucirumab (*n* = 197)	Placebo (*n* = 95)	HR, *P*-value
OS (M, 95%CI)	8.5	7.3	HR 0.710 (95%CI 0.531-0.949) *P* = 0.0199
PFS (M, 95%CI)	2.8	1.6	HR 0.452 (95%CI 0.339-0.603) *P* < 0.0001
Objective response (RECIST 1.1)			
CR (*n*, %)	0 (0.0)	0 (0.0)	
PR (*n*, %)	9 (4.6)	1 (1.1)	
SD (*n*, %)	109 (55.3)	36 (37.9)	
PD (*n*,%)	66 (33.5)	48 (50.5)	
NE (*n*, %)	13 (6.6)	10 (10.5)	
ORR (%, 95CI)	9 (4.6)	1 (1.1)	*P* = 0.1697
DCR (%)	118 (59.9)	37 (38.9)	*P* = 0.0006

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NE: Not evaluable; ORR: Objective response rate; DCR: Disease control rate.

requiring a switch to a next line of treatment. Therefore, the TACE progression criteria were created specifically for the TACTICS trial and were consistent with those used in clinical practice.

The criteria for progression with TACE (unTACEable progression) were: (1) ≥ 25% increase in intrahepatic viable lesions; (2) decline in hepatic functional reserve to Child-Pugh class C; (3) appearance of extrahepatic lesions; (4) appearance of vascular invasion; or (5) meeting the Japan Society of Hepatology criteria for TACE-refractory disease[52]. Therefore, PFS was defined as the time to either unTACEable progression or death. The most important feature of the TACTICS trial design is that the RECIST criteria were not used, and consequently the development of new intrahepatic lesions was not considered progression. This enabled long-term administration of sorafenib.

The results for the primary endpoint of PFS were very favorable, with a median of 25.2 mo in the TACE plus sorafenib arm and 13.5 mo in the TACE alone arm (HR, 0.59; *P* = 0.006; Table 7)[30,51]. TTUP results were also favorable, with a median of 26.7 mo in the TACE plus sorafenib arm and 20.6 mo in the TACE alone arm (HR, 0.57; *P* = 0.02; Table 7). Similarly, TTP results were favorable, with a median of 26.7 mo in the TACE plus sorafenib arm and 16.4 mo in the TACE alone arm (HR, 0.54; *P* = 0.005). PFS results were also better for the TACE plus sorafenib arm in all subgroup analyses[30]. The response rates after the first TACE session did not differ significantly between the arms. There were no unexpected AEs. The median duration of sorafenib administration was long at 38.7 mo, and the median daily dose was somewhat low at 355.2 mg. The interval between TACE sessions was 21.1 wk in the TACE plus sorafenib arm, which was significantly longer than the interval of 16.9 wk in the TACE alone arm (*P* = 0.018). Other parameters that were significantly longer in the TACE plus sorafenib arm than in the TACE alone arm were time to detection of vascular invasion (31.3 mo *vs* 4.0 mo), time to detection of extrahepatic spread (15.7 mo *vs* 6.9 mo), and time to stage progression (22.5 mo *vs* 6.3 mo) (Table 7)[30,51].

SELECTION OF THE USE OF MOLECULAR TARGETED AGENTS FOR HCC PATIENTS

In 2018, sorafenib and lenvatinib became first-line molecular targeted agents for HCC available worldwide. Regorafenib is also available as a second-line agent worldwide, but the following strict conditions are applicable: (1) Disease progression under sorafenib treatment; (2) exclusion of patients intolerant to sorafenib; (3) confirmation of sufficient tolerance to sorafenib (patients must tolerate ≥ 400 mg of sorafenib for 20 d or longer during the 28-d period before PD); and (4) Child-Pugh A liver function.

A successful outcome for the cabozantinib bridging study in Japan will result in an application for approval. Also, ramucirumab for use in patients with an AFP ≥ 400 ng/mL will be expected to be approved. This will open up an era in which we have access to two first-line drugs and three second-line drugs(Figure 1)[53]. The HCC practice guideline published by the European Association of Study of the Liver revised in 2018[3] described regorafenib, cabozantinib, and ramucirumab as second-line agents to be used when a patient becomes refractory to sorafenib; however, no data are available regarding their use as second-line agents after lenvatinib treatment,

Table 7 Results of TACTICS trial[30]

	TACE with sorafenib median (M)	TACE alone median (M)	HR (95% CI)	*P* value
PFS	25.2	13.5	0.59 (0.41-0.87)	0.006
TTUP	26.7	20.6	0.57 (0.36-0.92)	0.02
TTP	26.7	16.4	0.54 (0.35-0.83)	0.005
TTVI	31.3	4.0	0.26 (0.09-0.75)	0.005
TTEHS	15.7	6.9	0.21 (0.06-0.70)	0.006
TTSP	22.5	6.3	0.31 (0.15-0.63)	0.001

TACE: Transcatheter arterial chemoembolization; HR: Hazard ratio; PFS: Progression free survival; TTUP: Time to untreatable or unTACEable progression; TTP: Time to progression; TTVI: Time to vascular invasion; TTEHS: Time to extrahepatic spread; TTSP: Time to stage progression.

which is an unmet need. In contrast, the guidelines published by the American Association for the Study of Liver Diseases revised in August 2018 state that although there is no clear evidence for their use as second-line agents after patients become refractory to lenvatinib, use of multi-kinase inhibitors, such as sorafenib-regorafenib, cabozantinib or ramucirmab may be considered[4].

Indeed, after its approval for use in HCC patients in Japan, lenvatinib was administered to more than 8000 patients over 11 mo. Based on recent reports of early experiences, lenvatinib was administered patients not only as a first-line agent, but also as a second-line agent after sorafenib, and as a third-line agent after sorafenib-regorafenib sequential therapy in approximately half of patients (Figure 2). In addition, when the drug is used in this manner the response rate is as high as 40%-50% when assessed by mRECIST, a percentage similar to that reported in the REFLECT trial, in a real world clinical practice. Thus, lenvatinib is expected to have positive effects in the real world clinical practice setting even after treatment with sorafenib and even regorafenib. In addition, it is possible that sorafenib, regorafenib, ramucirumab, and cabozantinib are effective even after PD on lenvatinib. However, regorafenib should be administered only when tolerance to sorafenib is confirmed (Figure 2).

One important issue is whether sorafenib or lenvatinib should be used as the first option among 2 first-line agents. From an oncological and scientific viewpoint, the drug with the higher response rate should be selected for patients with advanced cancer who do not have much time left. In the oncology field, it is common sense that a survival benefit is obtained when the patient responds to the agent. However, there used to be some confusion between survival benefit in "responders" in individual patients, and a correlation between "response rate" and the result in the clinical trial. Even if the response rate of a testing drug is high, this does not always mean that the trial meets the primary endpoint of OS benefit. However, OS in responders were always better than non-responders even primary endpoint was negative as shown in previous trials[10,26,29]. The response rate for sorafenib is not high, but the drug reliably provides a survival benefit by stabilizing disease progression, the SD effect. However, even sorafenib shows a clear survival benefit in responders compared with non-responders according to mRECIST used to evaluate a database from prospective clinical trials with no selection bias[10,26,29], indicating that lenvatinib with its higher response rate than sorafenib increases the percentage of patients who benefit from survival-prolonging effects. Thus, oncologically speaking, agent with higher response rate may be used as the first choice of treatment among the first-line agents.

High response rates on imaging lead to both physicians and patients to be motivated to continue treatment. This high tumor response obtained in many patients will increase patient compliance and possibly increase the conversion rate to more curative modalities such as resection, ablation, and curative TACE[35]. Furthermore, the recently published response rate for Japanese BCLC B patients was 61.3%, which is fairly high and is superior to the world standard response rate of TACE (42%)[27,54,55] and initial TACE (52%) in the Japanese subgroup[56] as reported in the OPTIMIS trial. The response rate was particularly high when compared with that for the 2nd and later TACE session in the OPTIMIS trial, and for Japanese patients whose disease became refractory to TACE, in which ORR was just 13.9%[57].

Therefore, drugs with a high response rate may be used as the first choice for BCLC B patients unsuitable for TACE, *i.e.*, patients likely to become refractory. This statement does not entirely rule out the usefulness of TACE; indeed, as reported by the TACTICS trial[30], when a tumor enlarges during targeted therapy then treatment of the enlarged tumor with super-selective TACE to control growth while preserving

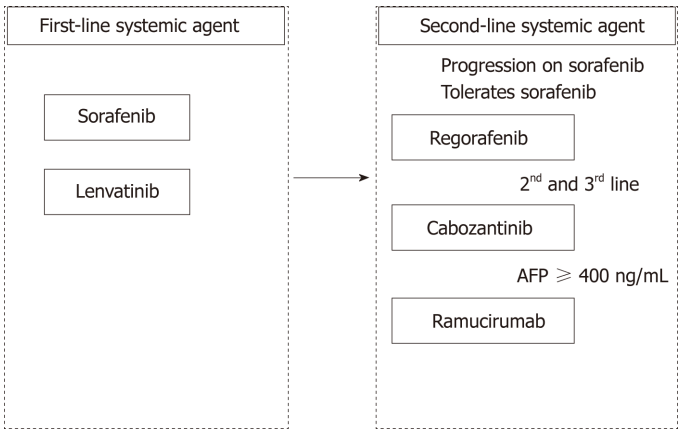


Figure 1 Systemic therapy in hepatocellular carcinoma: 2018 and beyond[53]. Two first-line systemic agents, sorafenib and lenvatinib, are approved and can be used in the clinical practice. Second-line agent, regorafenib is approved for clinical use for progressors on sorafenib. Cabozantinib and ramucirumab will be approved in 2019. AFP: Alpha fetal protein.

liver function may be a good strategy in a real world clinical practice.

IMMUNE CHECKPOINT INHIBITORS

Nivolumab

Nivolumab is the first recombinant monoclonal human immunoglobulin IgG4 antibody specific for human PD-1. A phase I/II study of advanced HCC, the Checkmate-040 trial, reported a response rate of 20%, which included three complete responders. The disease control rate was 64%, which is very promising. The drug also showed a durable and long-lasting response effect in responders[58]. AE profiles are fairly mild; grade 3 AE was only observed in 1 % (fatigue). Grade 1/2 AEs include rash (19%), pruritus (10%), diarrhea (10%), decreased appetite (10%) and fatigue (8%)[58]. Subsequently, a trial involving an increased number of patients was performed and the updated results were reported at ASCO in 2017. The median survival of patients treated with nivolumab as a first-line therapy was 28.6 mo whereas that of patients treated with nivolumab as a second-line was 15 mo; again, very promising. Based on the results of the phase I/II study described above, the United States marked nivolumab for priority review as a second-line agent after sorafenib; it was approved by the Food and Drug Administration in September 2017. The head-to-head phase III study (CheckMate-459) of nivolumab vs sorafenib as a first-line agent is now ongoing and the results are eagerly awaited. If this study is positive, 1st option among 1st line agents will undoubtedly become nivolumab because of its durable long-lasting response in responders.

Pembrolizumab

Similar to nivolumab pembrolizumab is a recombinant monoclonal human immunoglobulin IgG4 antibody specific for human PD-1. A phase II study of HCC patients reported a response rate of 17%, indicating that the effects are comparable with those of nivolumab[59]. AE profiles are also mild and comparable with those of nivolumab: grade 3 AEs were observed only in 4% (fatigue) and 1% (decreased appetite). Grade 1/2 AEs include fatigue (17%), pruritus (12%), diarrhea (11%) and rash (10%)[59]. A placebo-controlled phase III study of pembrolizumab as a second-line treatment for patients refractory/intolerant to sorafenib was conducted[60], however, the information was press released on Feb 19, 2019 that this trial did not meet its primary endpoints of prolonging PFS nor OS.

Combination therapy with immune checkpoint inhibitor/molecular targeted agents

The results of a phase 1b study of combination therapy with an anti-PD-L1 antibody, atezolizumab, and another antibody to VEGF, bevacizumab, were reported at ASCO in 2018. Although there were only 23 patients, the response rate according to a RECIST 1.1-based blind review reached 65%, with combination therapy showing a synergistic effect[61]. Based on this result, FDA designated this combination trial as a breakthrough therapy in July 2018. However, updated results presented at ESMO 2018 on October 21, 2018 revealed ORR according to modified RECIST- based

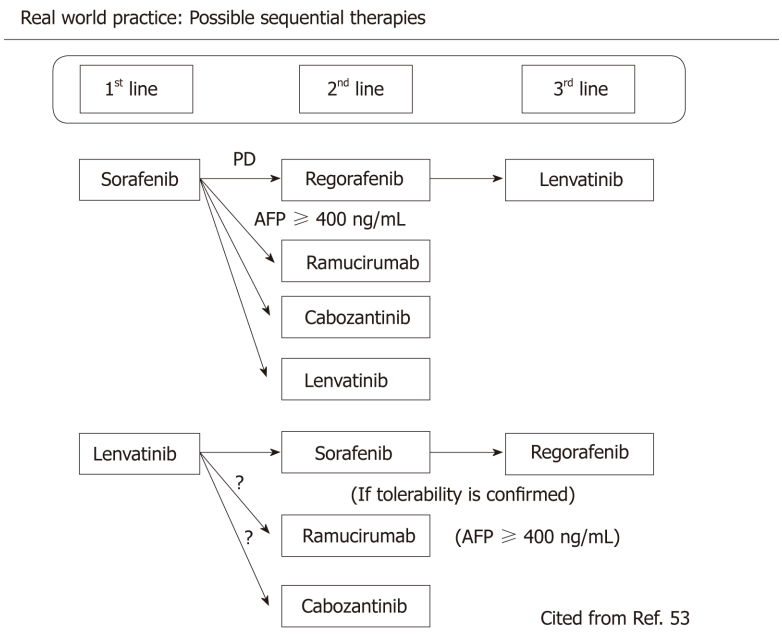


Figure 2 Possible sequential therapies of molecular targeted agents for hepatocellular carcinoma in the real-world practice[53]. There are solid evidence for use of regorafenib, ramucirumab, cabozantinib after sorafenib as confirmed by RESORCE, REACH-2 and CERESTIAL trials. However, lenvatinib has been proven to be effective after sorafenib or regorafenib in the real world practice as a second-line or third-line agent. Effectiveness of second-line agents after lenvatinib failure should be explored in the real-world practice setting. AFP: Alphafeto protein.

independent review assessment dropped to 34% as a result of increased numbers of patients to 73 (Table 8)[62]. Therefore, we have to be cautious on the results derived from small numbers of patients in phase 1/2 trial. A randomized controlled phase III study of combination therapy versus sorafenib is underway (the IMbrave150 trial) (Table 1)[60,63]. Other combination cancer immunotherapies in HCC are also ongoing (Table 8)[64,65].

VEGF released by cancer cells suppresses anti-tumor immune responses (Figure 3). VEGF activates the main players in an immunosuppressive tumor microenvironment; these include regulatory T cells (Tregs), tumor-associated macrophages, and myeloid-derived suppressor cells. Cytokines released by these cells inhibit dendritic cell maturation and activation and proliferation of NK cells and CD8-positive cells, thereby driving an immunosuppressive microenvironment (Figure 3)[66]. Accordingly, combination treatment with a molecular targeted drug plus an anti-VEGF agent, bevacizumab, is appropriate to induce synergistic effects.

The results of a phase 1b study of combination treatment with lenvatinib plus pembrolizumab were reported at the same ASCO meeting in 2018. The results of this study were also promising. The effects were evaluated in 26 patients; although the mean duration of follow-up was less than 3 mo, the response rate was 42.3%, with no PD patients (Table 8)[67]. However, again we have to be cautious on the results derived from small numbers of patients in phase 1/2 trial similar to atezolizumab/bevacizumab combination trial. The synergistic effects of lenvatinib plus an anti-PD-1 antibody were also suggested in a mouse model (Figure 4)[68,69]. Thus, combination therapy with immune checkpoint inhibitor/molecular targeted drugs may play an important role in the HCC treatment paradigm in the very near future (Figure 5)[70,71]. Biomarker that predicts response to immunotherapy or combination immunotherapy is still an unmet need in immunotherapy of HCC and extensive effort to identify such biomarkers is warranted.

CONCLUSION

Here, the latest results of trials of systemic therapy for HCC are reviewed. With respect to molecular targeted agents, lenvatinib and regorafenib have been approved for treatment of HCC in addition to sorafenib. Cabozantinib and ramucirumab may also be approved in 2019. Many HCC patients will benefit from increased treatment options and their sequential use for HCC; however, selection of therapeutic drugs may become more complex. When the immune checkpoint inhibitors nivolumab and

Table 8 Results of immune checkpoint inhibitors and combination therapy

	Nivolumab[58]	Pembrolizumab[59]	Pembrolizumab plus Lenvatinib[67]	Atezolizumab plus Bevacizumab[1][62]	SHR-1210 plus Apatinib[64]	Durvalumab plus Tremelimumab[65]
	(*n* = 214)	(*n* = 104)	(*n* = 26)	(*n* = 73)	(*n* = 18)	(*n* = 40)
ORR (%, 95%CI)	20 (15-26)[2]	17 (11-26)[2]	42.3 (23.4-63.1)[3]	34[3]	38.9[3]	25[2]
DCR (%, 95%CI)	64 (58-71)	62 (52-71)	100	75	83.3	57.5 (> 16 wk)
PFS (M, 95%CI)	4.0 (2.9-5.4)	4.9 (3.4-7.2)	9.7 (5.6-NE)	7.5 (0.4-23.9)	7.2 (2.6-NE)	NA
OS (M, 95%CI)	NR (9M, 74%)	12.9 (9.7-15.5)	NR	NR	NR	NA
DOR (M)	9.9 (8.3-NE)	≤ 9 (77%)	NE	NR	NE	NA

[1]Independent Review Facility assessment;
[2]RECIST 1.1; [3]modified RECIST; ORR: Objective response rate; DCR: Disease control rate; PFS: Progression free survival; OS: Overall survival; DOR: Duration of response; NR: Not reached; NE: Not estimable; NA: Not available.

pembrolizumab become available, the benefits of combination therapy with a molecular targeted agent can also be expected. Indeed, studies of combination therapy with several molecular targeted agents and immune checkpoint inhibitors are ongoing (Table 1, 2, 8) and the results are eagerly awaited. These novel treatment strategies will benefit patients with HCC at all stages from early, intermediate and advanced stage HCCs.

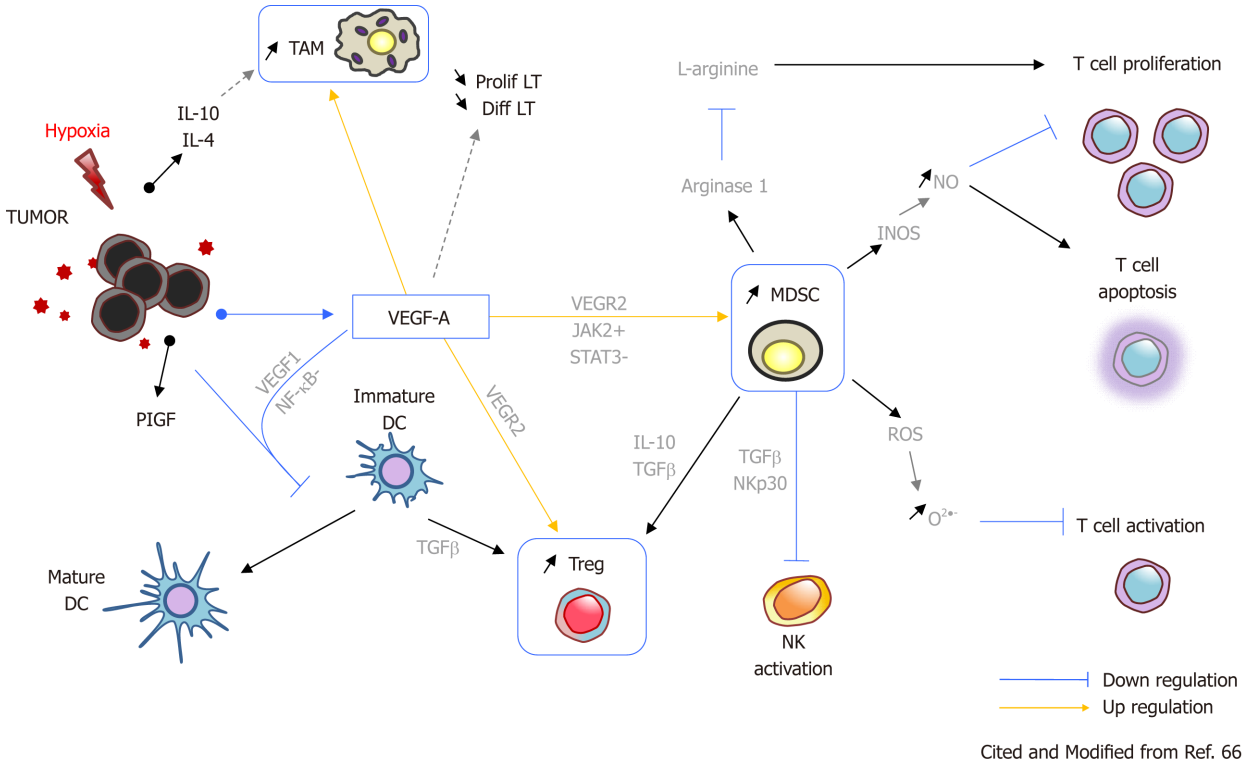
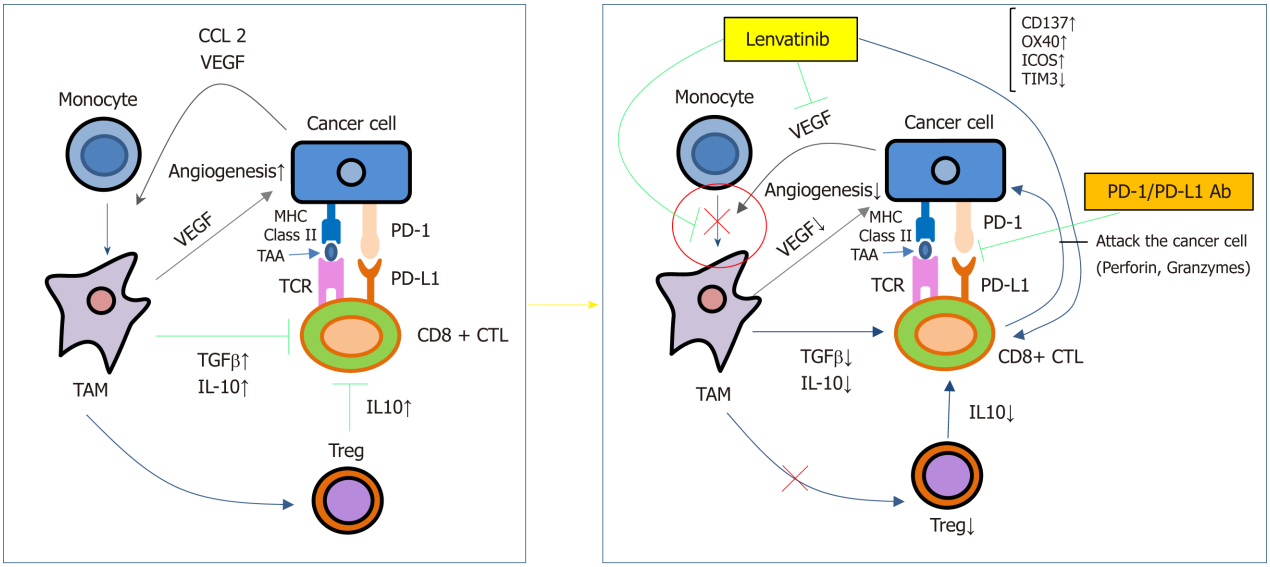


Figure 3 Tumor Immuno suppressive microenvironment caused by vascular endothelial growth factor produced by tumor[66]. VEGF-A up-regulates tumor-associated macrophage, regulatory T-cell, and myeloid-derived suppressor cell, which cause immune suppressive microenvironment through the down-regulation of the dendritic cell maturation, NK activation, T cell activation and T cell proliferation. VEGF-A: Vascular endothelial growth factor A; MDSC: Myeloid-derived suppressor cell; DC: Dendritic cell; TAM: Tumor-associated macrophage; Treg: Regulatory T cell; IL: Interleukin.



Immunosuppressive microenvironment + PD1/PD-L1-mediated immunosuppression

Lenvatinib + PD-1/PD-L1 antibody act synergistically to block PD-1/PD-L1 and inhibition formation of an immunosuppressive microenvironment

Figure 4 Mechanism underlying the synergistic effect of combination therapy on hepatocellular carcinoma with lenvatinib and anti-PD-1/PD-L1 antibodies[68]. Lenvatinib inhibits tumor angiogenesis and growth through VEGFR1-R3 and FGFR1-R4 inhibition. Lenvatinib also inhibits VEGF-mediated tumor suppressive microenvironment, such as immunosuppressive cells (tumor associated macrophage, regulatory T cells and myeloid-derived suppressor T cells) or tumor suppressive cytokines (IL10 or TGF-β). Lenvatinib also suppress the co-inhibitory checkpoint inhibitor, TIM 3 and increase the co-stimulatory molecules, CD137, OX40 or ICOS. Finally, PD-1/PD-L1 Ab restores the exhausted T cell activity to kill the cancer cell. Therefore, synergistic effect is obtained by this combination.

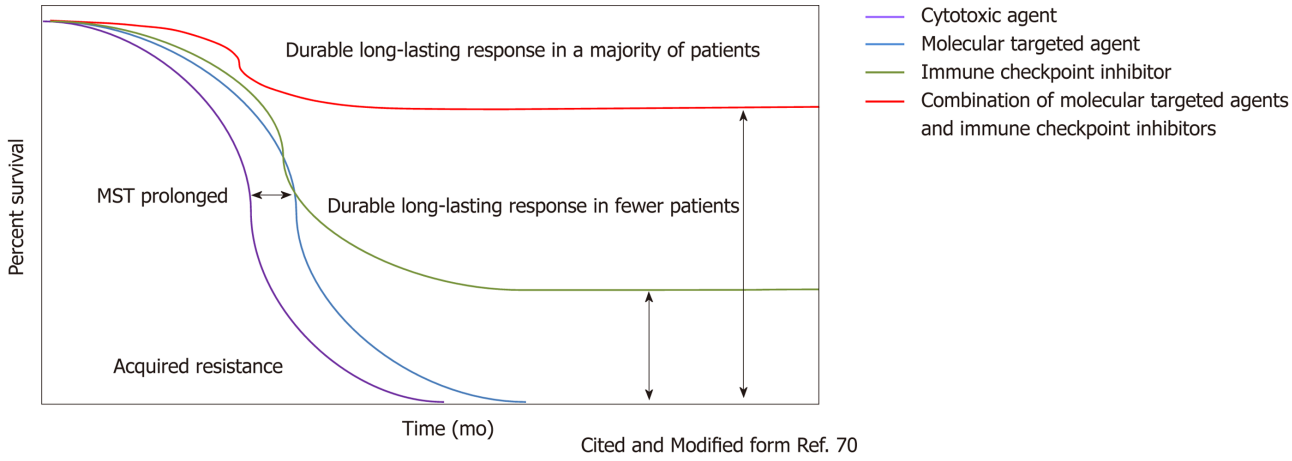


Figure 5 Improved Overall Survival after combination therapy with molecular targeted agents and immune checkpoint inhibitors[70]. Molecular targeted agents improve survival compared with chemotherapy by cytotoxic agents, but will become resistant sooner or later. Durable long-lasting response is obtained by immune checkpoint inhibitors, but only small portion of patients (15%-20%). Durable long-lasting response will be expected by a combination therapy with molecular targeted agents and immune checkpoint inhibitors in the majority of the patients (50%-70%) with advanced hepatocellular carcinoma.

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ORIGINAL ARTICLE

Basic Study

Relationships among *KRAS* mutation status, expression of RAS pathway signaling molecules, and clinicopathological features and prognosis of patients with colorectal cancer

Xiang-Bin Wan, Ai-Qin Wang, Jian Cao, Zhi-Chuang Dong, Ning Li, Sen Yang, Miao-Miao Sun, Zhi Li, Su-Xia Luo

ORCID number: Xiang-Bin Wan (0000-0001-9855-9053); Ai-Qin Wang (0000-0001-8103-4832); Jian Cao (0000-0002-6781-1516); Zhi-Chuang Dong (0000-0003-3670-2517); Ning Li (0000-0001-8391-5772); Sen Yang (0000-0003-1449-5334); Miao-Miao Sun (0000-0001-9506-3749); Zhi Li (0000-0001-8651-1140); Su-Xia Luo (0000-0001-9541-2793).

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Xiang-Bin Wan, Jian Cao, Zhi-Chuang Dong, Zhi Li, Department of General Surgery, the Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou 450008, Henan Province, China

Ai-Qin Wang, Department of Pharmacy, The Second Affiliated Hospital of Xinxiang Medical University, Xinxiang 453002, Henan Province, China

Ning Li, Su-Xia Luo, Department of Medical Oncology, the Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou 450008, Henan Province, China

Sen Yang, China-US (Henan) Hormel Cancer Institute, Zhengzhou 450008, China

Miao-Miao Sun, Department of Pathology, the Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou 450008, Henan Province, China

Corresponding author: Su-Xia Luo, MD, Professor, Department of Medical Oncology, the Affiliated Tumor Hospital of Zhengzhou University, No. 127, Dongming Road, Jinshui District, Zhengzhou 450008, Henan Province, China. wxbzlyy@126.com
Telephone: +86-371-65961505
Fax: +86-371-65961505

Abstract

BACKGROUND

The RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling pathways all belong to mitogen-activated protein kinase (MAPK) signaling pathways, Mutations in any one of the upstream genes (such as the *RAS* gene or the *BRAF* gene) may be transmitted to the protein through transcription or translation, resulting in abnormal activation of the signaling pathway. This study investigated the relationship between the *KRAS* gene mutation and the clinicopathological features and prognosis of colorectal cancer (CRC), and the effect of *KRAS* mutations on its associated proteins in CRC, with an aim to clarify the cause of tumor progression and drug resistance caused by mutation of the *KRAS* gene.

AIM

To investigate the *KRAS* gene and RAS pathway signaling molecules in CRC and to analyze their relationship with clinicopathological features and prognosis

METHODS

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Colorectal cancer tissue specimens from 196 patients were analyzed for *KRAS* mutations using quantitative polymerase chain reaction and for KRAS, BRAF, MEK, and ERK protein expression levels using immunohistochemistry of tumor microarrays. To analyze differences of RAS pathway signaling molecule expression levels in different *KRAS* gene status, the relationships between these parameters and clinicopathological features, 4-year progression-free survival, and overall survival were analyzed by independent sample *t* test, Kaplan-Meier plots, and the log-rank test. Predictors of overall and disease-free survival were assessed using a Cox proportional hazards model.

RESULTS

Of the 196 patients, 62 (32%) carried mutations in codon 12 (53/62) or codon 13 (9/62) in exon 2 of the *KRAS* gene. KRAS, BRAF, ERK, and MEK protein expression was detected in 71.4%, 78.8%, 64.3%, and 50.8% of CRC tissues, respectively. There were no significant differences between *KRAS* mutation status and KRAS, BRAF, MEK, or ERK protein levels. Positive expression of KRAS and ERK was associated with poor tumor differentiation, and KRAS expression was also associated with age < 56 years. MEK expression was significantly associated with distant metastasis ($P < 0.05$). The 4-year progression-free survival rate, but not overall survival rate, was significantly higher in patients with KRAS-negative tumors than in those with KRAS-positive tumors ($P < 0.05$), whereas BRAF, MEK, and ERK expression was unrelated to survival. Multivariate analysis showed that only the expression of KRAS protein was a risk factor for tumor recurrence ($P < 0.05$). No other clinicopathological factors correlated with KRAS, BRAF, MEK, or ERK expression.

CONCLUSION

KRAS gene mutations do not affect downstream protein expression in CRC. KRAS protein is associated with poor tumor differentiation, older age, and a risk of tumor recurrence.

Key words: Colorectal cancer; *KRAS* gene; KRAS protein; BRAF protein; MEK protein; ERK protein

Core tip: The RAS signaling pathway plays a crucial role in the invasiveness and metastasis of tumor cells. In this study, we examined the relationship between the *KRAS* gene mutation status and the clinicopathological features and prognosis of colorectal cancer (CRC) patients and the expression of BRAF, MEK, and ERK proteins. We found that *KRAS* gene mutations do not affect downstream protein expression or the clinicopathological features of CRC. KRAS protein is associated with poor tumor differentiation, older age, and a risk of tumor recurrence. The results may contribute to our understanding of drug resistance in *KRAS* mutant CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the most common malignant tumor of the digestive system, and it ranks third in terms of incidence and mortality in both men and women in the United States[1]. According to the latest statistics, the estimated number of new CRC cases and deaths is expected to be 75610 and 27390, respectively, in American men and 64640 and 23240, respectively, in American women in 2018[1]. In China, the incidence of CRC in 2014 was 27.08/100000, and it is the fourth most common cancer

among men and the third most common cancer among women[2]. In major Chinese cities, the incidence of CRC is even higher. In 2014, the incidence among men and women in Beijing was 44.12/100000 and 36.26/100000, respectively, and was the second and fourth most common cancer, respectively[3]. Improvements in the economy and living standards in China have increased both CRC morbidity and mortality.

CRC develops *via* a complex process involving multiple genes and signal transduction pathways[4,5]. Treatment of colonic cancer patients is highly dependent on the depth of tumor invasion (T-stage) as well as the extension of lymph node involvement (N-stage), which could be precisely evaluated today[6-11]. The main treatments include surgery, chemotherapy, and radiotherapy[12]. However, in recent years, several new drugs have been developed, most notably molecular targeted drugs, and the prognosis of CRC patients has greatly improved, especially those with advanced cancer. Nevertheless, resistance to targeted drugs is gradually increasing, and understanding the underlying mechanisms of tumor development and drug resistance has become increasingly important.

The RAS/RAF/MEK/ERK signaling pathway plays a crucial role in the proliferation, differentiation, survival, invasiveness, and metastasis of tumor cells[13-15]. This pathway is frequently abnormally activated in CRC, often due to mutations of upstream genes, such as *KRAS* and *BRAF*, that result in aberrant transcription or translation, leading to altered protein expression, activity, and/or signaling. In the present study, we examined the relationship between the *KRAS* gene mutation status and the clinicopathological features and prognosis of CRC patients. *KRAS* can be activated by many tumor-related proteins and is involved in their function as a network master[16,17]. We also investigated the effect of the *KRAS* genotype on the expression of BRAF, MEK, and ERK proteins. The results may contribute to our understanding of the underlying causes of tumor progression and drug resistance in *KRAS* mutant CRC[17].

MATERIALS AND METHODS

Patients

Tissue samples and clinical data (including gender, age at disease onset, tumor site, metastasis site, and tumor differentiation and stage) were collected from 220 CRC patients receiving treatment at the Affiliated Tumor Hospital of Zhengzhou University from January 2012 to December 2013. The average and median age of the patients was 58.4 and 56 years, respectively (range 18-84 years). All patients had complete case data and were followed for 10-58 mo, during which 49 patients died (Table 1). The study was approved by the Medical Ethics Committee of the Affiliated Tumor Hospital of Zhengzhou University, Zhengzhou, China, and all patients provided written informed consent.

Real-time quantitative polymerase chain reaction (qPCR)

Formalin-fixed paraffin-embedded (FFPE) tissue samples were sectioned (3-5 μm thick) and deparaffinized through a series of xylene and ethanol solutions using standard procedures[18]. DNA was extracted from the sections using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was purified by ethanol precipitation, dissolved in distilled water, and analyzed for concentration and purity using a spectrophotometer (OD260/OD280 = 1.8 ± 0.2, OD260/OD230 ≥ 1.7). The total yield per sample was > 50 ng.

The *KRAS* gene mutation status was analyzed by real-time qPCR using a Human KRAS Gene Mutation Detection Kit (Beijing ACCB Biotech Ltd., Beijing, China). Pre-denaturation was performed at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s. If the Ct value was ≤ 38 in the FAM channel, the sample was considered to be *KRAS* mutation positive; if there was no amplification, the sample was considered mutation negative; if the Ct values were between 30 and 40, the experiment was repeated; and if the same result was obtained, the sample was defined as mutation status uncertain. The mutation site was identified from the fluorescence quantitation curve (Figure 1, Table 2).

Tissue microarray and immunohistochemistry (IHC)

A personal tissue arrayer (Beecher Instruments, Sun Prairie, WI, United States) with a perforated needle of 0.6-mm diameter was used to remove small cylinders from the primary tumor blocks, and they were then placed in an empty 'recipient' paraffin block. Two blocks were constructed containing a total of 220 tissue sections, each

Table 1 Clinicopathological features of the 220 colorectal patients included in this study

	Patient characteristic	Number of patients	(%)
Gender	Male	113	51.3
	Female	107	48.6
Median age (yr)	< 56	17	7.7
	≥ 56	76	34.5
Tumor site	Right colon	45	20.5
	Left colorectum	175	79.5
Differentiation	Moderate-low	143	65
	Well	77	35
Infiltration depth	T1	13	5.9
	T2	29	13.2
	T3	86	39.1
	T4	92	41.8
Lymph node metastasis	N1-N3	118	53.6
	N0	102	46.4
TNM stage	I	35	15.9
	II	62	28.2
	III	73	33.2
	IV	50	22.7

separated by 1 mm[19,20]. KRAS, BRAF, ERK, and MEK proteins were detected using primary antibodies from Abcam (Cambridge, United Kingdom) and a polymer detection MaxVision/HRP kit (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China). Incubation with anti-KRAS (diluted 1:2400), anti-NRAS (1:500), and anti-phospho-MEK1/2 (Ser221; 1:50) lasted for 30 min at room temperature. Incubation with anti-PI3K (p110δa/p85α, 1:400), anti-BRAF (1:50), and anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204; 1:400) lasted for 60 min at room temperature. For the positive control, a human breast cancer tissue sample was stained with the same antibodies. For the negative control, the primary antibodies were substituted with phosphate-buffered saline (PBS).

Histochemistry score

KRAS, BRAF, ERK, and MEK protein expression was visualized as brown/yellow particles in the cytoplasm or cell membrane. To evaluate expression, we used a semi-quantitative histochemistry score (H-score)[21] based on a combination of the percentage of cells positively stained (0 points, 0-4%; 1, 5%-24%; 2, 25%-49%; 3, 50%-74%; and 4, > 75%) and staining intensity (0 points, no color; 1, light yellow; 2, yellow; and 3, brown/yellow). The H-score = ΣPI(I+1), where PI represents the percentage of positive cells and I represents color intensity. The number of positive cells in each section and their staining intensity were converted into the corresponding values.

Statistical analysis

Analyses were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, United States). A χ^2 test was used to analyze the relationship between the *KRAS* gene mutation status and protein expression in CRC tissues. Correlations between clinicopathological parameters and protein expression were evaluated using Fisher's exact test. Progression-free survival (PFS) and overall survival (OS) were analyzed using the Kaplan-Meier method and the log-rank test. A Cox proportional hazards model was applied to identify predictors of OS and disease-free survival. A *P*-value < 0.05 was considered significant.

RESULTS

Of the 220 samples originally obtained, 194-196 were successfully processed by IHC. Therefore, the number of cases available for statistical analysis was 194-196. Of the 196 CRC patients, 62 (31.6%) carried a *KRAS* mutation in the 12th codon (53/62) or 13th codon (9/62) of exon 2. The 12th codon mutations included GGT→GTT (24 cases), GGT→GAT (21 cases), GGT→GCT (6 cases), and GGT →TGT (2 cases). All nine

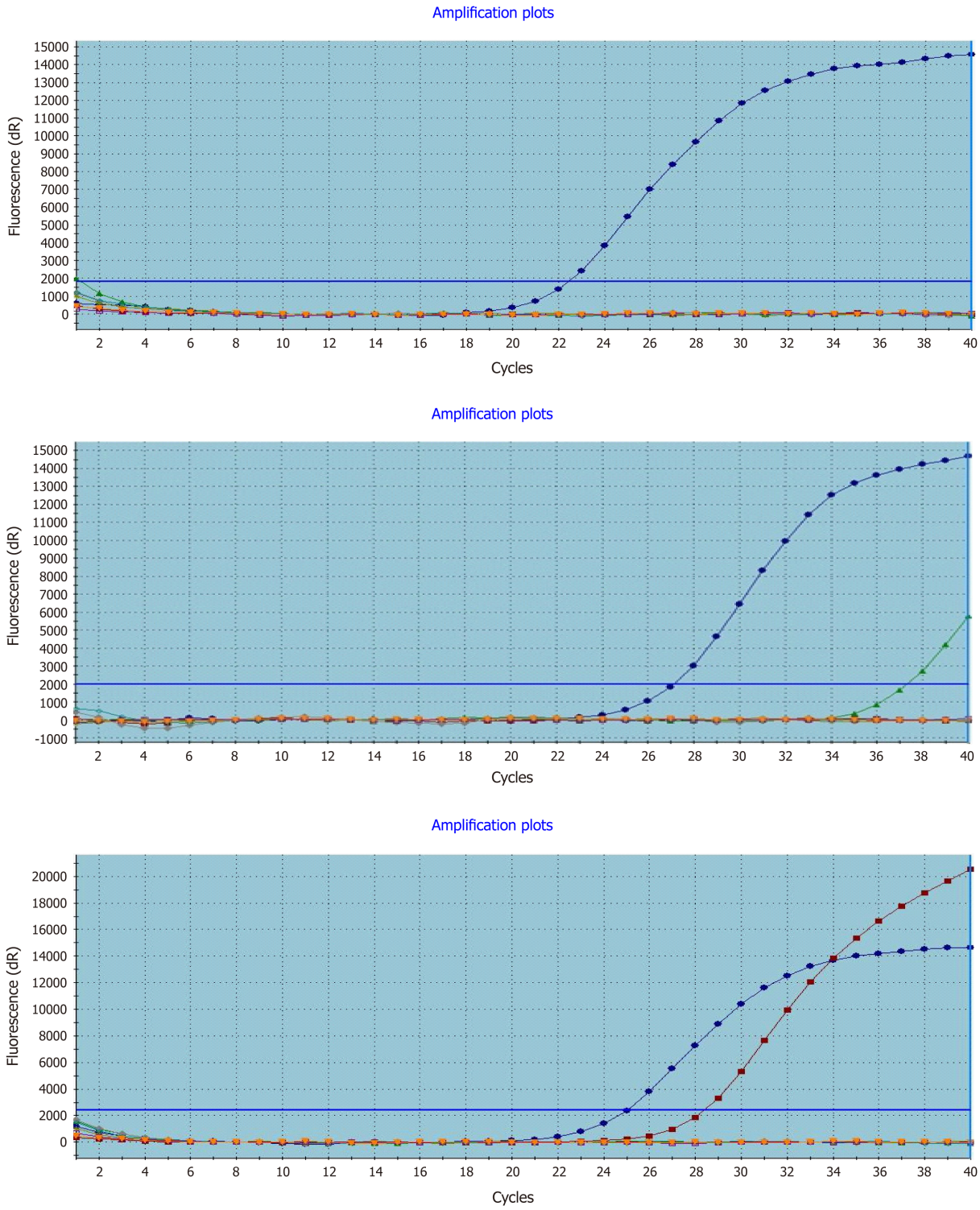

Figure 1 Real-time polymerase chain reaction detection of mutated *KRAS* in formalin-fixed paraffin-embedded colorectal cancer tissues. Left to right representative amplification plots for samples with wild-type, uncertain mutation status, and mutated *KRAS* genes.

mutations in the 13th codon were GGC→GAC (Figure 1).

Relationships between KRAS, BRAF, MEK, and ERK protein expression and clinicopathological features of CRC patients

The expression of KRAS, BRAF, MEK, and ERK proteins was detected by IHC (Figure 2). Of the 194-196 CRC tissues examined, 71.4% (140/196) stained positively for KRAS, 78.8% (153/194) for BRAF, 64.3% (126/196) for MEK, and 50.8% (99/195) for ERK. The expression of KRAS or ERK was associated with poor tumor differentiation ($P < 0.05$), and KRAS was also associated with age < 56 years ($P < 0.05$). MEK protein expression was positively associated with distant metastasis ($P < 0.05$; Tables 3 and 4).

Table 2 Identification of *KRAS* mutations

Position	Code name	*KRAS* gene allele site	Signal channel
A	IR	IR gene FAM	FAM
B	KM1	Exon 12, 34G>T mutation (G12C) FAM, HEX	FAM, HEX
C	KM2	Exon 12, 34G>A mutation (G12S) FAM, HEX	FAM, HEX
D	KM3	Exon 12, 34G>C mutation (G12R) FAM, HEX	FAM, HEX
E	KM4	Exon 12, 35G>T mutation (G12V) FAM, HEX	FAM, HEX
F	KM5	Exon 12, 35G>A mutation (G12D) FAM, HEX	FAM, HEX
G	KM6	Exon 12, 35G>C mutation (G12A) FAM, HEX	FAM, HEX
H	KM7	Exon 13, 38G>A mutation (G13D) FAM, HEX	FAM, HEX

Relationship between KRAS genotype and KRAS protein expression

IHC analysis indicated that KRAS protein was expressed in both the cytoplasm and cell membrane of CRC cells, but the majority was membrane localized. H-scores for KRAS protein IHC staining in the cell membrane were 133.4 ± 4.974 (n = 134) and 131.7 ± 5.091 (n = 134) for patients with mutant and wild-type *KRAS*, respectively, whereas the cytoplasmic scores were 59.76 ± 4.298 (n = 62) and 60.16 ± 3.354 (n = 133), respectively. These scores were not significantly different (Figures 3 and 4).

Relationship between KRAS genotype and downstream protein expression in CRC tissues

BRAF, MEK, and ERK proteins were expressed in 74.6%, 54.8%, and 45.2%, respectively, of *KRAS* mutant CRC tissues and in 74.2%, 58.2%, and 54.1%, respectively, of *KRAS* wild-type tissues. These differences were not statistically significant (Table 5).

The H-scores for BRAF, MEK, and ERK protein IHC staining were 64.07 ± 4.898, 36.50 ± 2.770, and 16.09 ± 2.318, respectively, in *KRAS* mutant CRC tissues, respectively (n = 134), and 55.25 ± 2.915, 33.74 ± 4.113, and 14.36 ± 3.379, respectively, in *KRAS* wild-type tissues (n = 62). These differences were not statistically significant (Figures 5-7).

Relationship between the expression of KRAS and downstream proteins in CRC tissues

BRAF, MEK, and ERK protein expression was positive in 76.4%, 78.6%, and 69.6%, respectively, of KRAS protein-positive CRC tissues and in 69.6%, 8.6%, and 39.3%, respectively, of KRAS protein-negative tissues. MEK and ERK expression was significantly more common in KRAS-positive tissues than in KRAS-negative tissues (P < 0.05; Table 6).

Relationship between the expression of BRAF, MEK, and ERK in CRC tissues

MEK and ERK proteins were expressed in 67.5% and 52.7%, respectively, of BRAF-positive tissues and in 46.0% and 60.0%, respectively, of BRAF-negative tissues. These differences were not significant (Table 7). ERK was expressed in 57.9% (73/126) of MEK-positive tissues and 38.6% (27/70) of MEK-negative tissues, with a significant difference (P < 0.05).

Relationship between KRAS, BRAF, MEK, and ERK protein expression and prognosis for patients with stage II/III CRC

The 4-year PFS rates for stage II/III CRC patients with positive *vs* negative protein expression were 77.78% *vs* 90.29% (P = 0.0411) for KRAS, 83.33% *vs* 87.5% (P = 0.4395) for BRAF, 85% *vs* 87.50% (P = 0.6768) for MEK, and 85.71% *vs* 87.32% (P = 0.7657) for ERK. The 4-year OS rates for the same groups were 89.32% *vs* 93.33% (P = 0.4555) for KRAS, 86.11% *vs* 89.26% (P = 0.3109) for BRAF, 89.77% *vs* 91.67% (P = 0.6768) for MEK, and 88.31% *vs* 92.96% (P = 0.3380) for ERK. Only the 4-year PFS rates of the KRAS protein-positive and -negative groups were significantly different (P = 0.0411; Figure 8).

Multivariate analysis of the prognostic significance of KRAS, BRAF, MEK, and ERK expression in stage II/III CRC patients

Multivariate analysis showed that only positive KRAS protein expression was a risk factor for disease recurrence in patients with stage II/III CRC. The risk was 3.319-fold higher for KRAS-positive than KRAS-negative patients (95% confidence interval:

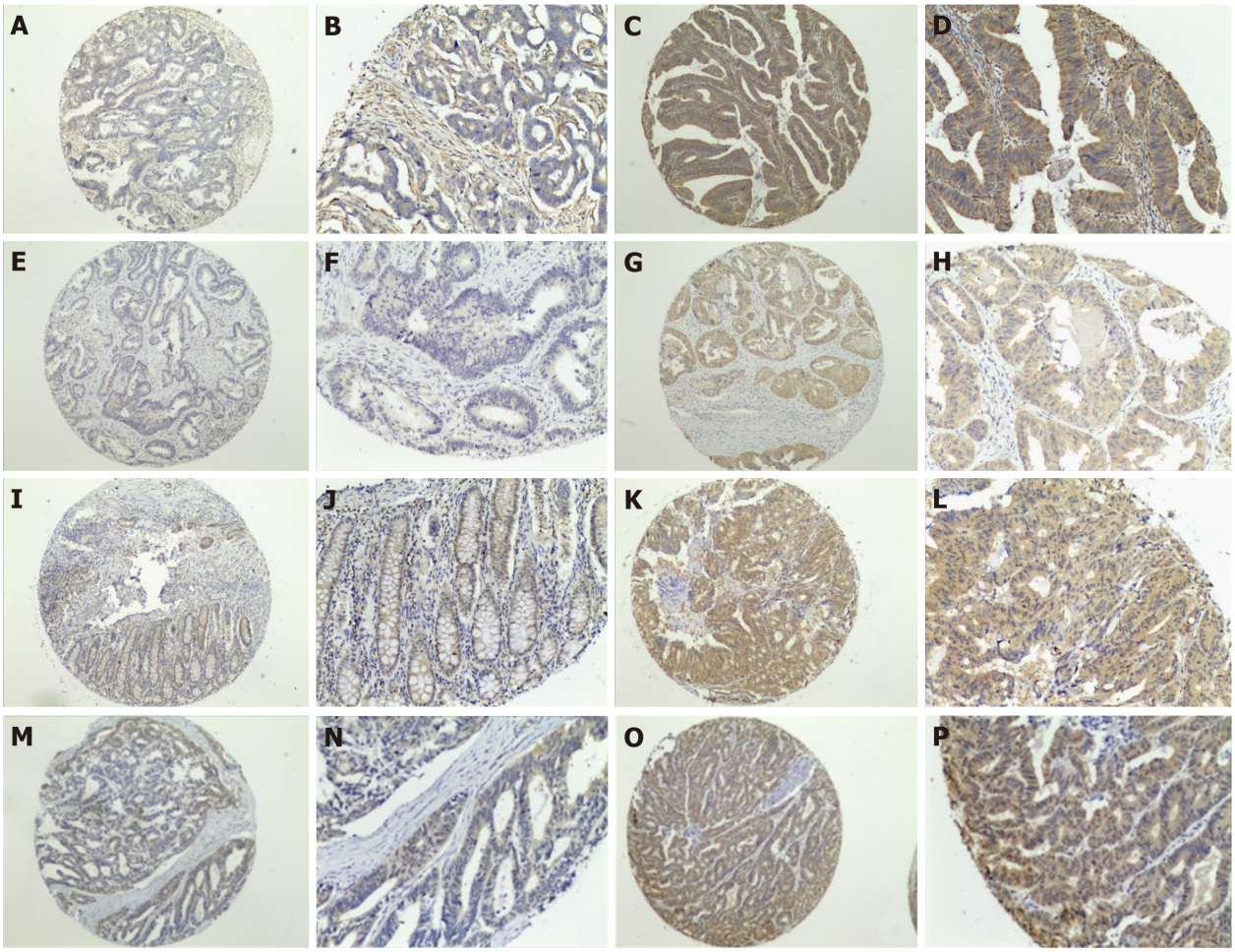

Figure 2 Expression of RAS pathway proteins in colorectal cancer. A-D: KRAS negative (A and B) and positive (C and D) expression at magnification × 40 (A and C) and × 100 (B and D). E-P: As described for (A-D), except that staining for BRAF protein (E-H), MEK protein (I-L), and ERK protein (M-P) is shown.

1.231-8.944; Tables 8 and 9).

DISCUSSION

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that activates downstream effector proteins *via* the RAS/RAF/MEK/ERK pathway. Previous work found that the *KRAS* gene mutation status correlated with the clinical efficacy of anti-EGFR antibody therapies[22-24], and that only wild-type KRAS tumors benefited from such therapy.

The *KRAS* gene, which is located on chromosome 12, is composed of four coding exons and one 5′ non-coding exon and produces a 21 kDa protein composed of 189 amino acids. Members of the RAS superfamily, including KRAS, have similar molecular structures and bind guanine nucleotides. RAS continuously cycles through active (GTP bound) and inactive (GDP bound) conformational states. The binary behavior of these proteins allows them to act as molecular switches in signaling processes. In this manner, the mutant *KRAS* gene results in a constitutively active GTP-bound state and the activation of downstream pro-proliferative signaling pathways[25,26]. The most common oncogenic mutations in CRCs are point mutations at positions 12, 13, and 61 of *KRAS*, with approximately 90% being in codons 12 and 13[22-27]. These amino acid mutations impair GTPase hydrolase activity and maintain the protein in an activated state, which results in continuous signaling to downstream effectors, even in the absence of extracellular stimulation. In our study, we detected a *KRAS* mutation rate of 30.9%, which is consistent with previous reports of 30%-40% in China[28] and was lower than those (30%-50%) observed in other countries[27,29-31].

To date, few studies have examined the relationship between *KRAS* genotype and protein expression. We found that KRAS protein was expressed in 55.8% of *KRAS* mutant tissues and in 44.2% of *KRAS* wild-type tissues, and the difference was not

Table 3 Relationship between KRAS and BRAF protein expression and clinicopathological features of colorectal cancer patients

General data		KRAS protein			BRAF protein		
		Positive rate (%)	χ^2	*P*-value	Positive rate (%)	χ^2	*P*-value
Median age (yr)	< 56	71 (57/80)	5.34	0.02	77.6 (52/67)	0.10	0.76
	≥ 56	65.9 (83/126)			79.5 (101/127)		
Gender	Male	74.3 (84/123)	1.11	0.29	79.5 (89/112)	0.06	0.81
	Female	64.5 (56/83)			78.0 (64/82)		
Differentiation	Medium-low	71.6 (106/148)	10.8	0.001	75.7 (106/140)	3.23	0.08
	Well	70.8 (34/48)			87.0 (47/54)		
Infiltration depth	T1+T2	63.2 (24/38)	0.21	1.52	79.9 (111/139)	0.28	0.59
	T3+T4	73.4 (116/158)			76.4 (42/55)		
Lymph node metastasis	N0	69.6 (64/92)	0.29	0.59	75.0 (69/92)	0.024	0.878
	N1-N3	73.1 (76/104)			74.0 (77/104)		
Distant metastasis	M0	70.2 (106/151)	0.49	0.49	75.9 (107/141)	2.96	0.10
	M1	75.6 (34/45)			86.8 (46/53)		
TNM stage	Stage I	64 (16/25)	0.79	0.85	75.0 (18/24)	5.98	0.11
	Stage II	71.9 (41/57)			69.5 (41/59)		
	Stage III	72.63 (51/70)			82.9 (58/70)		
	Stage IV	72.2 (32/44)			87.8 (36/41)		

significant. The KRAS protein level, as measured using the semi-quantitative H-score, was also not significantly different between patients with mutant or wild-type *KRAS* tumors. Thus, there appears to be no obvious relationship between the *KRAS* mutation status and the expression level of KRAS protein. In addition to binding to GTP, RAS proteins must associate with cellular membranes in order to transduce signals[32-34]. We also found that the *KRAS* genotype had no effect on the distribution of KRAS protein between the cell membrane and cytoplasm. These data suggest that activation of KRAS or its downstream pathway components is unrelated to the expression level or subcellular location of KRAS protein.

Unfortunately, *KRAS* gene mutations account for approximately 35% of the nonresponsive patients who receive anti-EGFR treatment[35]. Therefore, using the *KRAS* gene as a predictor of clinical outcome is not always useful. We also examined the frequency and level of BRAF, MEK, and ERK expression in tissues expressing mutant or wild-type *KRAS*. We detected no effects of the *KRAS* genotype on the expression of any of these proteins. However, MEK and ERK expression was significantly more common in KRAS protein-positive tumors than in KRAS-negative tumors. Similarly, ERK expression was significantly more common in the MEK-positive than in the MEK-negative group of tumors. Thus, increased expression of an upstream protein can elevate the expression of a downstream protein in the same signaling pathway. ERK is the sole substrate of MEK in the RAS/RAF/MEK/ERK signaling pathway and their expression levels are often similar[36].

BRAF protein is mainly activated *via* two mechanisms: upstream RAS signaling or mutation of *BRAF*. The *BRAF* gene mutation status is also correlated with the clinical efficacy of anti-EGFR antibody therapies[37]. BRAF protein is phosphorylated and activated, and BRAF in turn phosphorylates MEK1/MEK2, which activates ERK and promotes its entry into the nucleus to regulate gene expression. The *BRAF* gene is also mutated in many cancers[38]. Kadowaki *et al*[39] found a 4%-10% *BRAF* mutation rate in CRC patients, whereas the mutation rate can be as high as 90% in sigmatic serrated adenoma[40]. Little is known about BRAF protein expression in CRC tissue. We found that 78.8% of our CRC tumor specimens were positive for BRAF protein, but the frequency and level were not significantly different in samples from *KRAS* mutant and wild-type tissues. It is possible that the BRAF protein expression is high in both adenoma and carcinoma tissues.

Tumor stage, the most important factor affecting prognosis, is mainly based on the depth of tumor invasion, lymph node metastasis, and distant metastasis. The prognostic significance of KRAS, BRAF, MEK, and ERK protein expression in CRC tissues has not been investigated. In this study, we analyzed the 4-year PFS and OS rates according to KRAS, BRAF, MEK, and ERK positive or negative expression in patients with stage II/III cancer. Multivariate analysis showed that only KRAS protein expression was a risk factor for recurrence, suggesting that it could be used as a

Table 4 Relationship between MEK and ERK protein expression and clinicopathological features of colorectal cancer patients

General data		MEK protein			ERK protein		
		Positive rate (%)	*P*-value	χ^2	Positive rate (%)	*P*-value	χ^2
Median age (yr)	< 56	62.9 (44/70)	0.097	0.756	46.5 (33/71)	0.822	0.365
	≥ 56	65.1 (82/126)			53.2 (66/124)		
Gender	Male	68.1 (77/113)	1.728	0.189	51.3 (58/113)	0.034	0.855
	Female	59.0 (49/83)			50 (41/82)		
Differentiation	Medium-low	64.5(100/155)	0.017	0.896	46 (69/150)	6.012	0.015
	Well	63.4 (26/41)			66.7 (30/45)		
Infiltration depth	T1 + T2	68.4 (26/38)	0.356	0.553	51.4 (19/37)	0.006	0.937
	T3 + T4	63.3 (100/158)			48.2 (80/166)		
Lymph node metastasis	N0	63.0 (58/92)	0.117	0.733	47.8 (44/92)	0.604	0.437
	N1-N3	65.4 (68/104)			53.4 (55/103)		
Distant metastasis	M0	60.3 (91/151)	4.631	0.031	49 (74/151)	0.832	0.362
	M1	77.8 (35/45)			56.8 (25/44)		
TNM stage	I	72 (18/25)	5.89	0.117	52.0 (13/25)	2.435	0.487
	II	57.9 (33/57)			43.63 (25/57)		
	III	58.6 (41/70)			50 (35/70)		
	IV	77.3 (34/44)			59.1 (26/44)		

predictive marker for prognosis in CRC.

In summary, our data suggest that only using the *KRAS* gene as a predictor of clinical outcome is not enough, and combined detection of intracellular signal transduction pathways may advance our understanding of the development and treatment of tumors, including CRC.

Table 5 Relationship between *KRAS* genotype and expression of downstream proteins in colorectal cancer tissues

Protein	*KRAS* mutant type group positive rate	*KRAS* wild-type group positive rate	χ^2	*P*-value
BRAF	74.2% (46/62)	74.6% (100/134)	0.004	0.948
MEK	54.8% (34/62)	58.2% (78/134)	0.196	0.657
ERK	45.2% (28/62)	54.1% (72/133)	1.364	0.243

Table 6 Correlation between the expression of KRAS and downstream proteins

Protein	KRAS protein positive group	KRAS protein negative group	χ^2	*P*-value
BRAF	76.4% (107/140)	69.6% (39/56)	0.964	0.325
MEK	78.6% (99/126)	58.6% (41/70)	8.775	0.003
ERK	69.6% (78/112)	39.3% (22/56)	4.298	0.038

Table 7 Correlation between expression of BRAF and downstream proteins

Protein	BRAF protein positive group	BRAF protein negative group	χ^2	*P*-value
MEK	67.5% (96/146)	60.0% (30/50)	0.534	0.464
ERK	52.7% (77/146)	46% (23/50)	0.674	0.411

Table 8 Relationship between progression-free survival and KRAS, BRAF, MEK, and ERK protein expression

	B	SE	Wald	df	Sig.	Exp (B)	95.0%CI of Exp (B)	
							Lower limit	Upper limit
KRAS	1.200	0.506	5.623	1	0.018	3.319	1.231	8.944
MEK	-0.872	0.609	2.054	1	0.152	0.418	0.127	1.378
ERK	-0.480	0.507	0.898	1	0.343	0.619	0.229	1.670
BRAF	0.031	0.581	0.003	1	0.958	1.031	0.330	3.221
T			4.609	3	0.203			
T1	1.330	1.160	1.317	1	0.251	3.783	0.390	36.714
T2	-0.780	0.858	0.827	1	0.363	0.458	0.085	2.463
T3	0.664	0.522	1.617	1	0.203	1.943	0.698	5.408
N	-0.018	0.485	0.001	1	0.970	0.982	0.380	2.541

Table 9 Relationship between overall survival and KRAS, BRAF, MEK, and ERK protein expression

	B	SE	Wald	df	Sig.	Exp (B)	95.0%CI of Exp (B)	
							Lower limit	Upper limit
KRAS	0.360	0.536	0.452	1	0.501	1.434	0.501	4.101
MEK	-0.736	0.585	1.586	1	0.208	0.479	0.152	1.506
ERK	-0.571	0.501	1.303	1	0.254	0.565	0.212	1.507
BRAF	0.246	0.539	0.208	1	0.648	1.279	0.444	3.680
T			1.733	3	0.630			
T1	1.141	1.114	1.050	1	0.306	3.131	0.353	27.776
T2	-0.356	0.696	0.261	1	0.609	0.701	0.179	2.742
T3	0.230	0.562	0.168	1	0.682	1.259	0.418	3.789
N	0.230	0.503	0.209	1	0.647	1.259	0.470	3.373

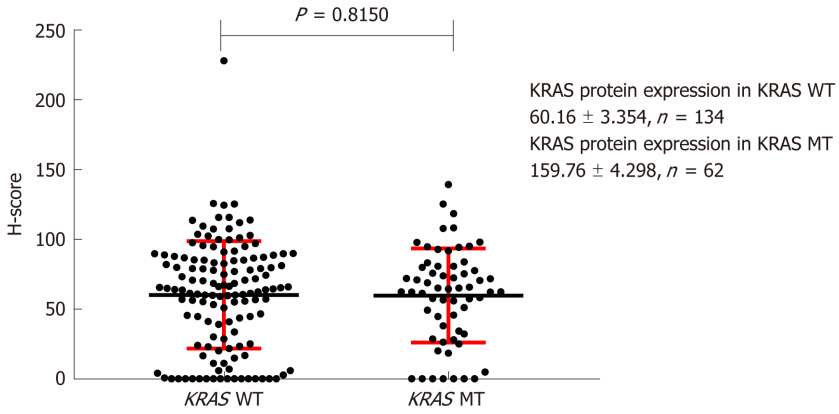

Figure 3 Effect of *KRAS* gene status on expression of KRAS protein on the cell membrane. H-score: Histochemistry score.

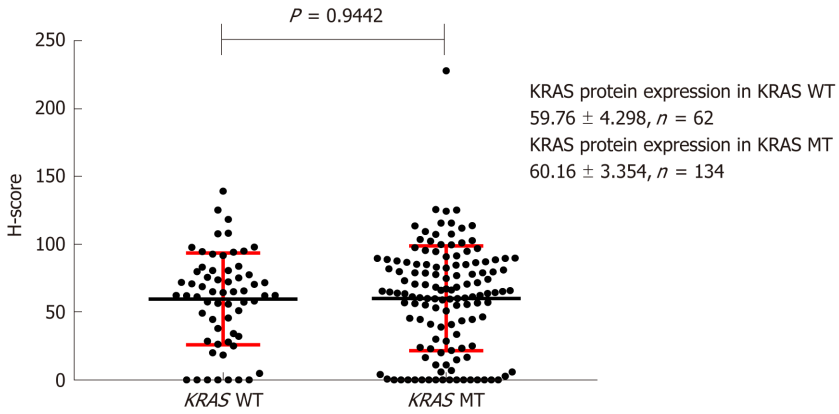

Figure 4 Effect of *KRAS* gene status on expression of KRAS protein in the cytoplasm. H-score: Histochemistry score.

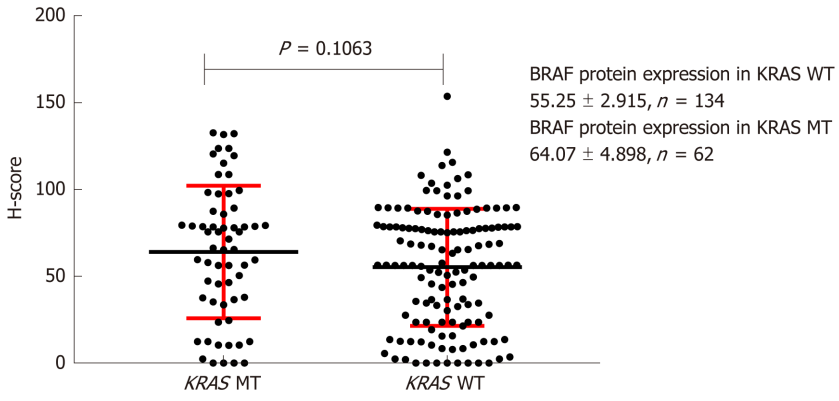

Figure 5 Effect of *KRAS* gene status on BRAF protein expression. H-score: Histochemistry score.

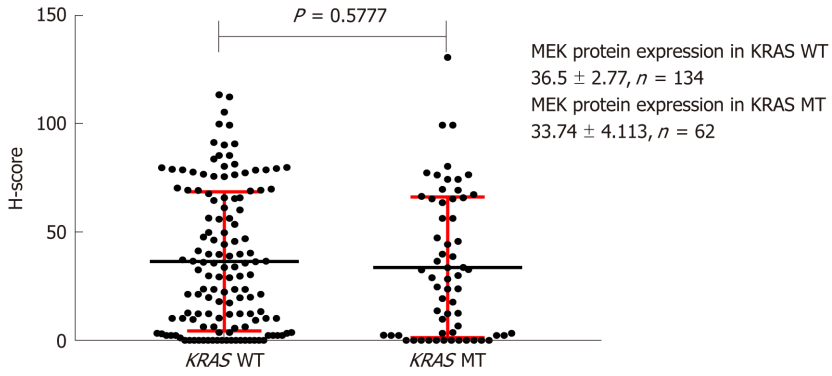

Figure 6 Effect of *KRAS* gene status on MEK protein expression. H-score: Histochemistry score.

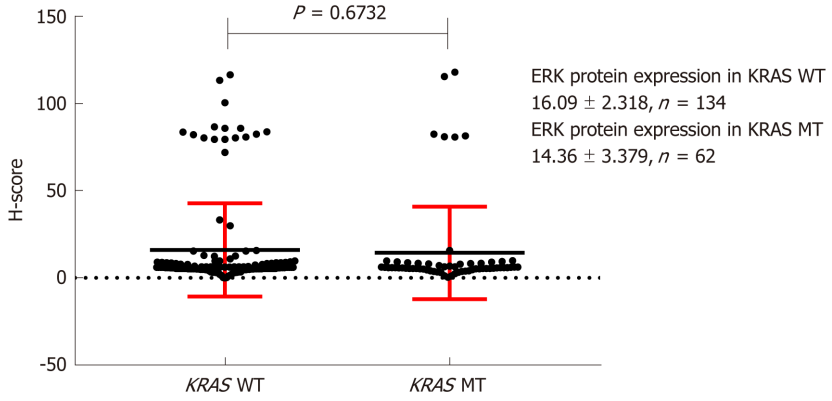

Figure 7 Effect of *KRAS* gene status on ERK protein expression. H-score: Histochemistry score.

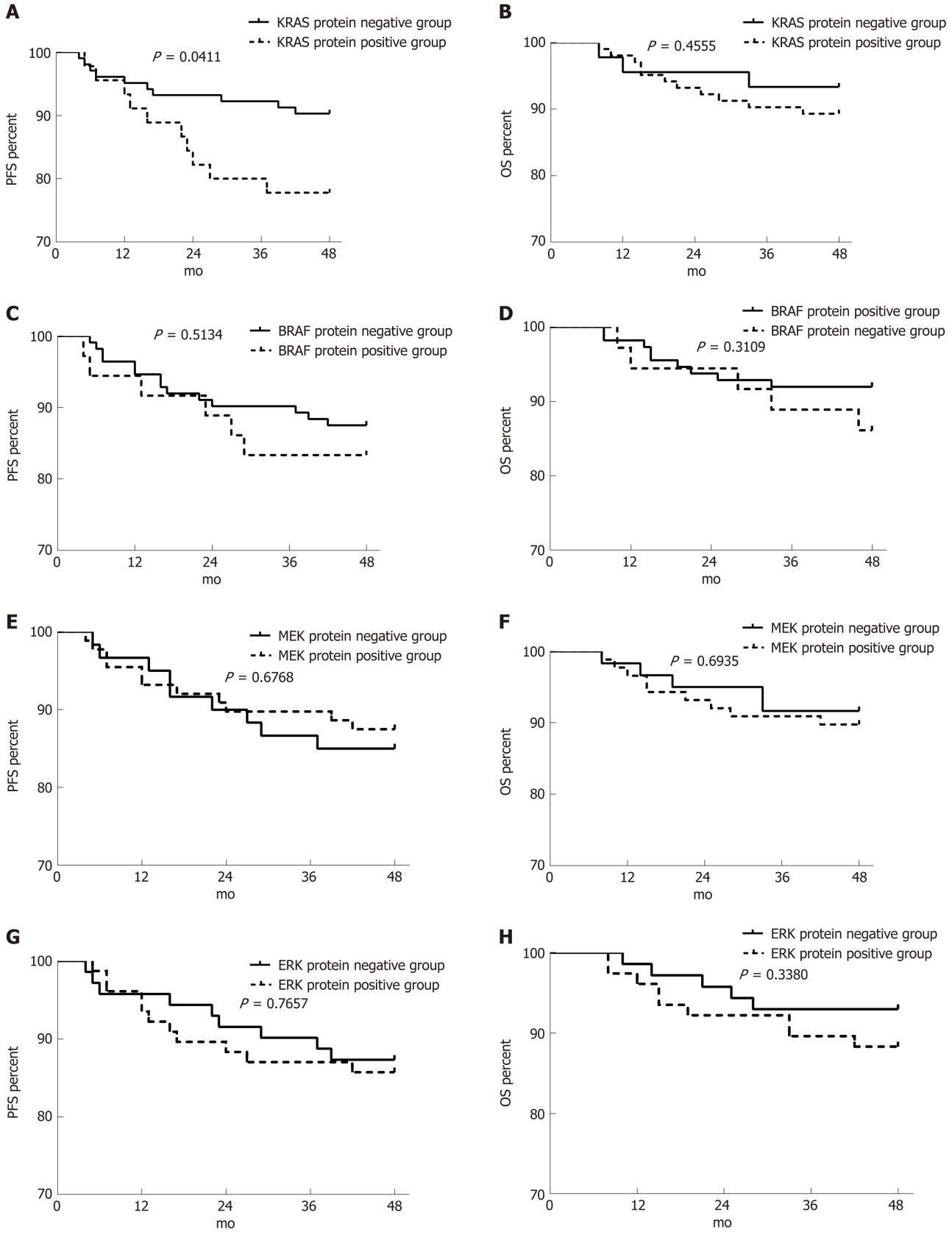

Figure 8 Effect of RAS pathway protein expression on progression-free survival and overall survival in patients with stage II/III colorectal cancer. A-H: Effects of KRAS (A and B), BRAF (C and D), MEK (E and F), and ERK (G and H) protein expression on progression-free survival (A, C, E, and G) and overall survival (B, D, F, and H). PFS: Progression-free survival; OS: Overall survival.

ARTICLE HIGHLIGHTS

Research background
The RAS signaling pathway plays a crucial role in the invasiveness and metastasis of tumor cells. Mutations in any one of the upstream genes (such as the *RAS* gene) may be transmitted to the protein through transcription or translation, resulting in abnormal activation of the signaling pathway. Patients with *KRAS* gene mutation have a poor prognosis. This study investigated the effect of *KRAS* mutations on its associated proteins in colorectal cancer. It is helpful to understand the cause of tumor progression and drug resistance caused by mutation of the *KRAS* gene.

Research motivation
By analyzing the effect of upstream gene mutation on the downstream protein expression of signal pathway, it is helpful to further understand the cause of drug resistance. Future studies should focus on the active forms and structures of signal molecules, and find out which forms and conformational proteins are active, which is conducive to the synthesis of corresponding targeted drugs.

Research objectives
The main goal was to find out which forms and conformational proteins are active. At present, it is found that the amount of protein is not the main factor affecting the activity. Achieving these goals can help to develop targeted therapies.

Research methods
In this study, the mutation of the *KRAS* gene was analyzed by real-time quantitative polymerase chain reaction, protein expression was analyzed by immunohistochemistry, and the impact of gene mutation on protein expression, the effect of gene and protein on prognosis, and the relationship between protein expression and clinicopathology were analyzed.

Research results
KRAS gene status had no significant effect on RAS pathway signaling molecules in colorectal cancer. Positive expression of KRAS and ERK was associated with poor tumor differentiation. MEK expression was associated with more distant metastasis. The 4-year progression-free survival rate was significantly higher in patients with KRAS-negative tumors than in those with KRAS-positive tumors. Multivariate analysis showed that only the expression of KRAS protein was a risk factor for tumor recurrence. These results are consistent with observations in clinical practice, indicating the credibility of the study. The specific reasons for the genetic and protein effects of these results need to be further clarified.

Research conclusions
In this study, the relationship between the *KRAS* gene and the RAS signaling pathway molecule was discussed. It was found that the gene-to-protein to functional change was a complex process, and the gene mutation did not cause an increase in the positive rate of downstream protein expression. KRAS protein was found to be involved in tumor differentiation and prognosis. The study also found that the farther apart the molecules in the signal pathway, the smaller the effect, suggesting that the signaling pathways are intertwined. The combination of targeted drugs and multi-channel blockade is better than single-channel blockade.

Research perspectives
The study began without considering the phosphorylation of the signal molecule and the effect of structure on function. Future research is best to study the effect of phosphorylation and spatial structure changes of a certain signal molecule on function. Using atomic force microscopy and mass spectrometry and cryo-electron microscopy equipment and electron microscopy negative staining preparation methods to observe the spatial structure of individual lipoprotein molecules can find which conformational proteins are active.

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ORIGINAL ARTICLE

Basic Study

Ubiquitin-specific protease 22 enhances intestinal cell proliferation and tissue regeneration after intestinal ischemia reperfusion injury

An-Long Ji, Tong Li, Guo Zu, Dong-Cheng Feng, Yang Li, Guang-Zhi Wang, Ji-Hong Yao, Xiao-Feng Tian

ORCID number: An-Long Ji (0000-0002-1224-186X); Tong Li (0000-0002-9987-8256); Guo Zu (0000-0002-6333-8573); Dong-Cheng Feng (0000-0002-8356-1406); Yang Li (0000-0003-1015-5447); Guang-Zhi Wang (0000-0001-6744-4966); Ji-Hong Yao (0000-0003-1890-8749); Xiao-Feng Tian (0000-0001-9219-1868).

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An-Long Ji, Tong Li, Guo Zu, Dong-Cheng Feng, Yang Li, Guang-Zhi Wang, Xiao-Feng Tian, Department of General Surgery, Second Affiliated Hospital of Dalian Medical University, Dalian 116023, Liaoning Province, China

Ji-Hong Yao, Department of Pharmacology, Dalian Medical University, Dalian 116044, Liaoning Province, China

Corresponding author: Xiao-Feng Tian, MD, PhD, Director, Professor, Surgeon, Department of General Surgery, The Second Affiliated Hospital of Dalian Medical University, 467 Zhongshan Road, Shahekou District, Dalian 116023, Liaoning Province, China.
txfdl@dmu.edu.cn
Telephone: +86-411-86110010
Fax: +86-411-86110010

Abstract

BACKGROUND

Intestinal ischemia reperfusion (I/R) injury is a serious but common pathophysiological process of many diseases, resulting in a high mortality rate in clinical practice. Ubiquitin-specific protease 22 (USP22) acts as regulator of cell cycle progression, proliferation, and tumor invasion. Depleted USP22 expression has been reported to contribute to arrested cell cycle and disrupted generation of differentiated cell types in crypts and villi. However, the role of USP22 in intestinal damage recovery has not been investigated. Therefore, elucidation of the underlying mechanism of USP22 in intestinal I/R injury may help to improve the tissue repair and patient prognosis in clinical practice.

AIM

To investigate the role of USP22 in intestinal cell proliferation and regeneration after intestinal I/R injury.

METHODS

An animal model of intestinal I/R injury was generated in male Sprague-Dawley rats by occlusion of the superior mesenteric artery followed by reperfusion. Chiu's scoring system was used to grade the damage to the intestinal mucosa. An *in vitro* model was developed by incubating rat intestinal epithelial IEC-6 cells in hypoxia/reoxygenation conditions in order to simulate I/R *in vivo*. siRNA and overexpression plasmid were used to regulate the expression of USP22. USP22, Cyclin D1, and proliferating cell nuclear antigen (PCNA) expression levels were measured by Western blot analysis and immunohistochemistry staining. Cell survival (viability) and cell cycle were evaluated using the Cell Counting Kit-8

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and flow cytometry, respectively.

RESULTS

USP22 expression was positively correlated with the expression levels of PCNA and Cyclin D1 both *in vivo* and *in vitro*, which confirmed that USP22 was involved in cell proliferation and intestinal regeneration after intestinal I/R injury. Decreased levels of Cyclin D1 and cell cycle arrest were observed in the USP22 knockdown group ($P < 0.05$), while opposite results were observed in the USP22 overexpression group ($P < 0.05$). In addition, increased expression of USP22 was related to improved intestinal pathology or IEC-6 cell viability after I/R or hypoxia/reoxygenation. These results suggested that USP22 may exert a protective effect on intestinal I/R injury by regulating cell proliferation and facilitating tissue regeneration.

CONCLUSION

USP22 is correlated with promoting intestinal cell proliferation and accelerating intestinal tissue regeneration after intestinal I/R injury and may serve as a potential target for therapeutic development for tissue repair during intestinal I/R injury.

Key words: Ubiquitin-specific protease 22; Proliferation; Regeneration; Repair; Intestinal ischemia-reperfusion

Core tip: Ubiquitin-specific protease 22 (USP22) belongs to the USPs family, which regulates cell cycle progression, proliferation, and tumor invasion. Depleted expression of USP22 has been linked to arrested cell cycle and disrupted distribution and generation of differentiated cell types in crypts and villi. However, its regulatory mechanism remains unclear. By generating models of ischemia reperfusion (I/R) injury and regulating USP22 expression levels, this study reveals that USP22 is correlated with promoting intestinal cell proliferation and accelerating intestinal tissue regeneration after intestinal I/R injury. USP22 might serve as a potential target for therapeutic development for tissue repair during intestinal I/R injury.

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INTRODUCTION

Intestinal ischemia reperfusion (I/R) injury is a serious pathophysiological process that occurs in many clinical conditions, including mesenteric arterial thrombotic or embolic diseases, shock, major surgery, and organ transplantation[1-3]. Intestinal I/R injury is a common pathogenesis of many diseases and also the initial factor of systemic inflammatory response syndrome and multiple organ dysfunction syndrome, resulting in a high mortality rate in clinical practice[4-6]. Intestinal I/R injury can result in serious damage to the mucosa and at the same time cause barrier dysfunction, which is a chief factor contributing to intestinal I/R injury. The dysfunction of the mucosal barrier and fragile immunization has been reported to be the leading cause of serious complications and death. Intestinal I/R injury is always followed by proliferation and subsequent differentiation of intestinal epithelial cells to rebuild the proper structure of the epithelium[3,7,8]. The intestinal barrier is an epithelial monolayer and serves as the first line of defense within the intestinal lumen against any unfavorable conditions[3,9]. It has been found that after the initiation of injury by intestinal I/R, the intestinal mucosal barrier undergoes regeneration through significant proliferation of undifferentiated progenitor cells[10]. However, insufficient proliferation and regeneration to fully rescue intestinal mucosal barrier function can be seen by the high mortality rate in clinical practice[11]. Therapeutic development of

intestinal regeneration may serve as a good means to rescue those suffering from intestinal ischemia and improve patient prognosis. Currently, several studies have been focused on cell proliferation and tissue regeneration after I/R injury[12], but the underlying mechanisms remain largely unknown.

Ubiquitin-specific protease 22 (USP22) belongs to the largest subfamily of deubiquitinases, known as USPs, and is a conserved component of the hSAGA activating complex that takes ubiquitin from target proteins to regulate cell cycle progression, proliferation, and tumor invasion[13-16]. USP22 plays a pivotal role in stabilizing c-Myc, an oncogenic protein that controls the cells in the balance between proliferation and death[17,18]. The depletion of USP22 has been reported not only to induce arrest of the G1 cell cycle in colorectal cancer cells *in vitro*[18-20], but also to affect the distribution and proper generation of differentiated cell types in crypts and villi[21]. However, the role of USP22 in intestinal damage recovery has not been investigated. To date, studies regarding USP22 function have been mostly focused on its potential in facilitating and promoting stem cell-like characteristics in various tumor types. While the knowledge of USP22 in intestinal epithelial cell proliferation may provide evidence for clinical exploration of novel therapeutic targets against intestinal I/R, its function and physiological role during the intestinal I/R process still need to be elucidated.

Based on the above background, we speculated that USP22 may participate in intestinal regeneration after I/R injury and may serve as a potential target in clinical practice. In this study, we observed for the first time the role of USP22 in intestinal regeneration during intestinal I/R injury and identified the advantages of having USP22 in intestinal epithelial cell proliferation after hypoxia/reoxygenation.

MATERIALS AND METHODS

Intestinal I/R injury animal model

Thirty-five male Sprague-Dawley rats, weighing between 180 and 220 g, were randomly distributed into one sham group and four model groups with respect to the duration of reperfusion (n = 7 each) using a random number table. The sample size was determined by power analysis[22-24]. All animals were accommodated in different cages at the same proper and constant temperature and were acclimated for one week before the experiments. All animals were handled conforming to the approved protocol by the Animal Care and Use Committee of Dalian Medical University, Liaoning, China and in compliance with the National Institutes of Health guidelines. An animal model of intestinal I/R injury was developed through surgery as previously described by Megison *et al*[25]. Briefly, after identifying the superior mesenteric artery (SMA) in the midline laparotomy, the intestinal I/R injury was established by occluding the SMA with an atraumatic microvascular clamp for 60 min. Occlusion was confirmed after mesenteric pulsations ceased and the intestines became pale. Reperfusion was then performed for 3 h, 6 h, 12 h, or 24 h. The sham group was exposed to the same procedures without vascular occlusion. After being sacrificed, the ileum specimens in rats were excised by midline laparotomy.

Histology and immunohistochemical staining

After the rats were sacrificed, the specimens were excised, immediately fixed in 10% neutral buffered formalin, embedded in paraffin wax, and cut into consecutive 4-μm-thick slides. Hematoxylin and eosin (HE) staining was then performed. Chiu's scoring system was used to quantitatively determine the histological scores of the intestine[26]. Immunohistochemical analysis was conducted according to the manufacture's protocol. Briefly, the sections were incubated with an anti-PCNA monoclonal antibody overnight at 4 °C. While blind to the clinicopathological data of the patients, two experienced pathologists independently examined staining to determine the expression of PCNA. The number of positive cells that showed immune-reactivity in cell nuclei in the representative ten microscopic fields was counted and the percentage of positive cells was calculated.

Cell culture and hypoxia/reoxygenation model

IEC-6 cells (normal rat small intestinal epithelial cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured in an incubator maintained at 37 °C with 5% CO_2. To imitate a hypoxic environment, we incubated the cells in a microaerophilic system (Thermo Fisher Scientific 8000, Marietta, GA, United States) containing 1% O_2 and 5% CO_2 balanced with 94% N_2 gas for 6 h. Reoxygenation was achieved later by culturing the cells under a normoxic

environment.

USP22 knockdown and overexpression

IEC-6 cells were transfected in a 6-well plate with USP22 siRNA (si-USP22, 50 nmol/L) or unspecific scrambled siRNA (GenePharma, Shanghai, China) using a Lipofectamine 3000 Reagent (Invitrogen L3000075, Shanghai, China). Target sequence for si-USP22 is as follows: Sense (5′-3′) GCUACCAAGAG UCCACAAA; antisense (5′-3′) UUUGUGGACUC UUGGUAGC. The negative control sequence is as follows: Sense (5′-3′) UUCUCCGAACG UGUCACGU; antisense (5′-3′) ACGUGACACGU UCGGAGAA. The ratio of siRNA and Lipofectamine 3000 was 100:3.75 (pmol:μL). For overexpression of USP22, the overexpression plasmid designed and synthesized by GenePharma was transfected into IEC-6 cells using a Lipofectamine 3000 Reagent. The cells were later cultured for 48 h post-transfection for further analysis.

Western blot analysis

Harvested cells and proteins from the intestinal samples were extracted according to the manufacturer's instructions (KeyGEN Biotech, Nanjing, Jiangsu Province, China). Equal concentrations of protein were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. Subsequently, the membranes were incubated at 4 °C overnight with a primary antibody against USP22 (1:1000; Proteintech 55110, Wuhan, Hubei Province, China), β-actin (1:1000; ZSGB-BIO PR-0255, Beijing, China), or Cyclin D1 (1:500; Proteintech 12363), followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000; ZSGB-BIO ZDR-5306). Enhanced chemiluminescence was used to visualize and quantify the immunoreactive protein bands. The experiment was repeated in triplicate.

Cell viability assay

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies CK04, Inc., Tokyo, Japan) was used to measure ratios of survival cells according to the manufacturer's instructions. Briefly, approximately 3500 IEC-6 cells per well were seeded and received proper treatment in 96-well plates followed by addition of a CCK-8 solution to each well at a 1/10 dilution. After a one-hour incubation at 37 °C, the cells alive in each well are able to form formazan and cell viability could be evaluated by detecting the absorbance at 450 nm in each well using a microplate reader (Biotec, United States).

Cell cycle analysis

A Cell Cycle and Apoptosis Analysis Kit (Beyotime C1052, Shanghai, China) was used to analyze cell cycle distribution according to the manufacturer's instructions. Briefly, transfected IEC-6 cells were seeded and fixed with 70% ethanol in 6-well plates overnight at 4 °C. The cells were washed twice and resuspended with a phosphate buffered saline (PBS) solution. Propidium iodide (GeneChem, Shanghai, China) and ribonuclease were added to PBS and cells were incubated for 30 min at 37 °C. A FACSCalibur flow cytometer (BD, United States) was used to detect cell cycle. The experiment was repeated in triplicate, and each experiment had two specimens.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, United States) was used to analyze the data. GPower 3.1 software package was used to determine the adequate sample size (significance level $\alpha = 0.05$; desired statistic power $1\text{-}\beta = 0.8$). Student's *t*-test was used to determine significant differences between the two groups, and one-way ANOVA was adopted among multiple groups. Differences with a *P*-value less than 0.05 were considered statistically significant.

RESULTS

USP22 expression is positively correlated with cell proliferation and tissue regeneration after intestinal I/R injury in rats

To evaluate the histopathological change of the intestinal mucosal barrier and its function after intestinal I/R injury in rats, HE staining and Chiu's scoring were performed. It is evident that small intestinal villi were damaged during reperfusion (Figure 1A) and that I/R injury led to significant dysfunction of the small intestine with mucosal injury (Figure 1B). To investigate USP22 levels after intestinal I/R injury, we observed that USP22 was progressively downregulated from 0 h to 6 h in the early phase of reperfusion, whereas it reached its trough value at 6 h and recovered in the late phase of reperfusion (6-24 h). Changes in USP22 expression

during the whole process were negatively correlated to corresponding Chiu's scores.

Since PCNA is critically linked to DNA synthesis and plays a vital role in cell proliferation, the level of PCNA can, to some extent, mirror the level of proliferation. Thus, the proliferative activity of the intestinal mucosa during reperfusion at each given time point was assessed by immunohistochemical staining for PCNA (Figure 1D and E). It is intuitive that the level of PCNA was positively correlated with that of USP22. Furthermore, on account of being the main marker during the G1 phase of the cell cycle, Cyclin D1 expression level was also investigated. As shown in Figure 1F, Cyclin D1 levels were consistent with those of PCNA and USP22. Therefore, our results demonstrated that USP22 is positively correlated with intestinal regeneration and might play a vital role after I/R injury *in vivo*.

USP22 correlates with the proliferative activity of IEC-6 cells after hypoxia /reoxygenation injury

In line with the induction of USP22 during intestinal I/R *in vivo*, IEC-6 cells exposed to different time durations (3, 6, 12, or 24 h) of reoxygenation after 6-h hypoxia treatment were analyzed. The protein expression levels of USP22 were measured (Figure 2A) and IEC-6 cell proliferation was assessed (Figure 2B and C). USP22 expression was shown to be positively associated with Cyclin D1 and cell viability of IEC-6 cells in a time-dependent pattern. Thus, our results demonstrated that USP22 directly correlates to the proliferative and regenerative activity of IEC-6 cells after hypoxia/reoxygenation injury.

USP22 knockdown inhibits cell proliferation and induces cell cycle arrest in IEC-6 cells after hypoxia/reoxygenation injury

As the expression levels of USP22 changed during intestinal regeneration after I/R, the effect of silencing USP22 on the proliferation of IEC-6 cells was subsequently investigated. USP22 knockdown (Figure 3A) by using siRNA (si-USP22) dramatically decreased USP22 level and, in parallel, the levels of cyclin D1 and of cell vitality (Figure 3B) and viability (Figure 3C) compared with the negative control. Using flow cytometry, we elucidated how USP22 knockdown correlated with inhibited cell proliferation after hypoxia/reoxygenation by observing the cell cycle. We found that si-USP22-treated cells had a much higher percentage in G1 and significantly lower numbers in S phase than the counterparts of the negative control cells (Figure 3D and E). Thus, our results demonstrated that silencing USP22 closely correlates with IEC-6 cell proliferation potential after hypoxia/reoxygenation injury by stopping the cells entering S phase.

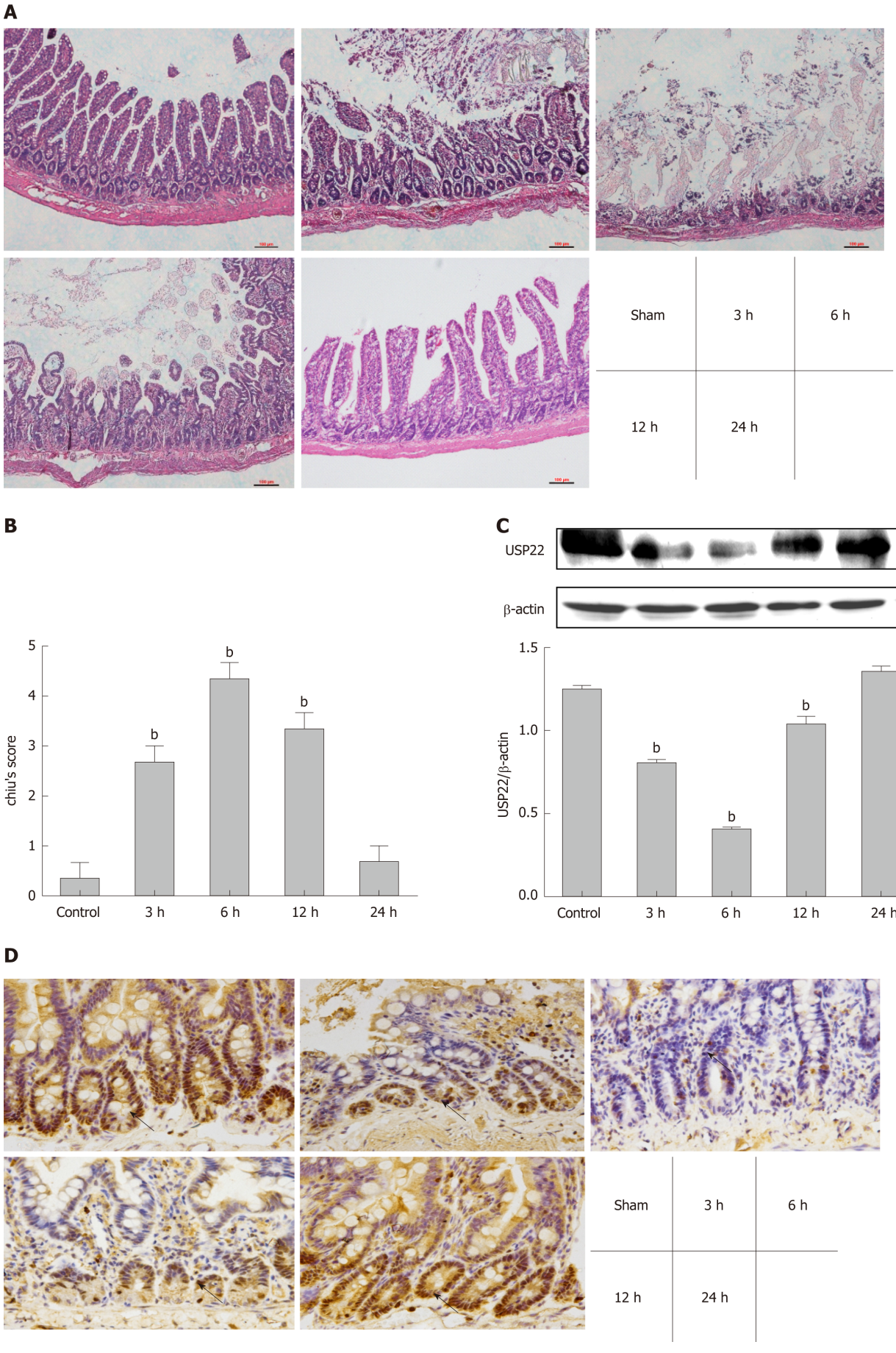
USP22 overexpression promotes cell proliferation in IEC-6 cells after hypoxia /reoxygenation injury

We further examined the effect of USP22 overexpression on intestinal cell proliferation by transfection with a USP22 expression plasmid. It can be seen in Figure 4A that USP22 was significantly overexpressed and that cell viability was dramatically increased (Figure 4B and C). By observing the cell cycle, we investigated how USP22 overexpression correlated with activated cell proliferation after hypoxia/regeneration. We found that USP22-overexpressing cells had a much lower number of cells in G1 phase and significantly more cells in S phase than the negative control cells (Figure 4D and E). Thus, our results demonstrated that USP22 overexpression may promote cell proliferation and viability in this process.

DISCUSSION

This study identified progressive downregulation of USP22 that occurred at the early phase of reperfusion (0-6 h) in intestinal I/R or hypoxia/reoxygenation injury *in vivo* or *in vitro*. Whereas the expression of USP22 reached its trough value at 6 h, it recovered during 6-24 h in long-term reperfusion. The time-varying property of USP22 was in accordance with the histology and Chiu's scores in different time point groups in intestinal I/R rats. Thus, it is evident that USP22 was involved in promoting crypt cell proliferation and in facilitating intestinal tissue regeneration after intestinal I/R injury. Downregulation of USP22 was closely correlated with reduced Cyclin D1 and cell viability with accumulation of cells in the G1 phase of the cell cycle and being stopped from entering S phase. Meanwhile, we observed increased levels of Cyclin D1 and cell viability and facilitated cell cycle in USP22-overexpressing intestinal cells. Our findings indicated that USP22 may play a pivotal role in intestinal cell proliferation and tissue repair after intestinal I/R injury.

USP22 serves as a key subunit of the hSAGA complex that can regulate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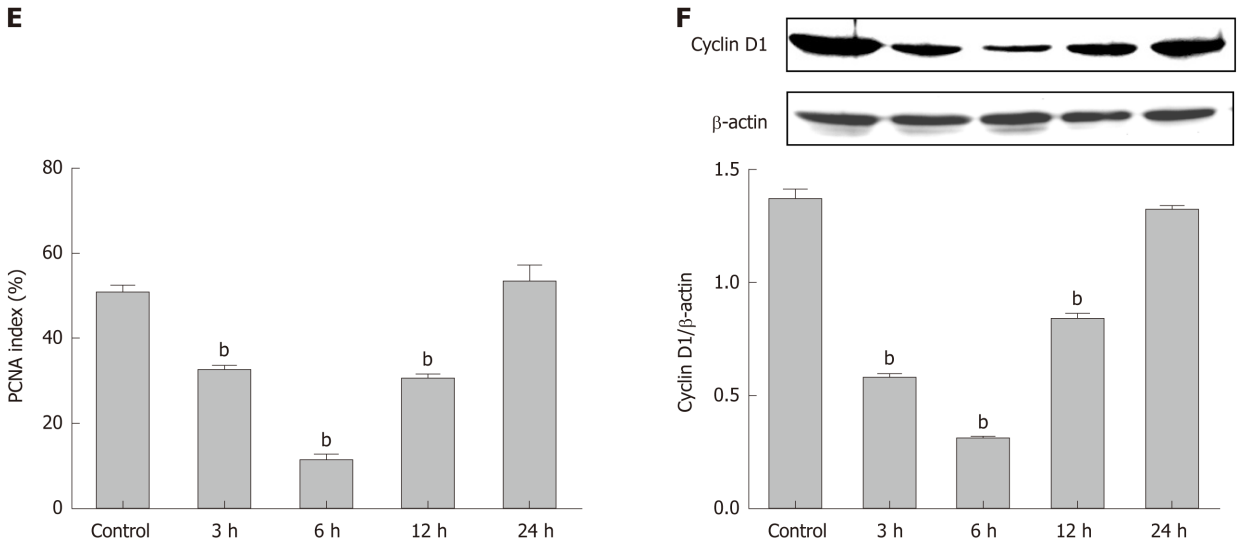

Figure 1 Ubiquitin-specific protease 22 is positively correlated with cell proliferation and tissue regeneration after ischemia reperfusion injury. Anesthetized rats were subjected to 60 min of intestinal ischemia followed by 3, 6, 12, or 24 h of reperfusion and tissues were harvested at the end of reperfusion. A: Intestinal tissue sections of the experimental groups were stained with HE (200×, bars represent 100 μm); B: Intestinal tissues in injury was scored histopathologically according to a scoring system (Chiu's score); C: Representative Western blot demonstrating the expression of USP22 in tissues from the experimental groups (n = 3); D: Immunohistochemical staining for PCNA was used for intestinal tissue sections of the experimental groups (400×, bars represent 50 μm); E: Quantitative analyses for immunohistochemical staining. The arrows show brown positive nuclei of proliferating intestinal cells in the crypt area of the intestinal gland; F: Representative protein levels of Cyclin D1 (n = 3) were demonstrated by Western blot. The results are presented as the mean ± SD. $^{b}P < 0.01$ *vs* sham. USP22: Ubiquitin-specific protease 22; PCNA: Proliferating cell nuclear antigen.

proliferation-related gene expression, such as c-Myc and MAPK[15,19,27,28]. USP22 can enhance cell growth and promote cell cycle progression in some cell lines[14]. USP22 can also suppress apoptosis and promote cell proliferation by antagonizing p53 function through the regulation of SIRT1[29,30]. It was also confirmed *in vivo* by Kosinsky *et al*[21] that villous goblet cells are significantly increased in a global USP22 reduction mouse model, where goblet cells are crucial for intestinal barrier function, tissue repair and healing[31,32]. This evidence suggested that USP22 positively correlated with the level of regeneration; thus, it may play a pivotal role in promoting tissue repair. We observed PCNA levels during reperfusion after intestinal I/R since PCNA is the widely recognized mediator of proliferation levels[33]. With the time-dependent proliferative activity during intestinal I/R (Figure 1D-F), a powerful indication can be made that the severity of mucosal damage had a negative correlation to the corresponding proliferative activity (Figure 1C, E, and F), which is in accordance with our former research[7]. Interestingly, a positive relationship with proliferative levels was found when investigating the level of USP22 both *in vivo* and *in vitro* (Figures 1A, 2A). Thus, the assumption was made that USP22 is involved in the regeneration of intestinal I/R injury. Accordingly, we adopted a USP22 knockdown and overexpression transfection technique in intestinal cells to confirm this hypothesis.

Cyclin D1 is needed in the transition from G1 to S phase in the cell cycle and thus serves as a vital target for proliferative signals[34]. Being a crucial regulator of the cell cycle, Cyclin D1 is responsible for inducing the G1/S transition. It forms a complex that targets a transcriptional factor by binding with different cyclin-dependent kinases[35]. Therefore, downregulation of Cyclin D1 can cause cell cycle arrest in the G1/S phase[36]. We found that the alteration of Cyclin D1 shared the same pattern as USP22 expression levels and the proliferative activity as indicated by PCNA expression both *in vivo* and *in vitro*. As we investigated deeper and more precisely *in vitro* by means of flow cytometry, we observed the arrested cell cycle progression of IEC-6 cells in S phase in the USP22 knockdown group when compared to the hypoxia/reoxygenation group ($P < 0.05$). Moreover, the opposite tendency of Cyclin D1 and cell viability was also observed in the USP22 overexpression group. Thus, this finding led to the conclusion that USP22 is positively correlated with increased potential of cell proliferation after hypoxia/reoxygenation injury. In addition, the mechanism of USP22 on promoting cell cycle progression in colorectal cancer has been reported recently, which further confirmed our results[37].

In our present study, we focused on the effect of USP22 on intestinal damage repair in I/R injury and found that the regulatory effect of USP22 on I/R injury could be potentially utilized in the future. To the best of our knowledge, no prior study has

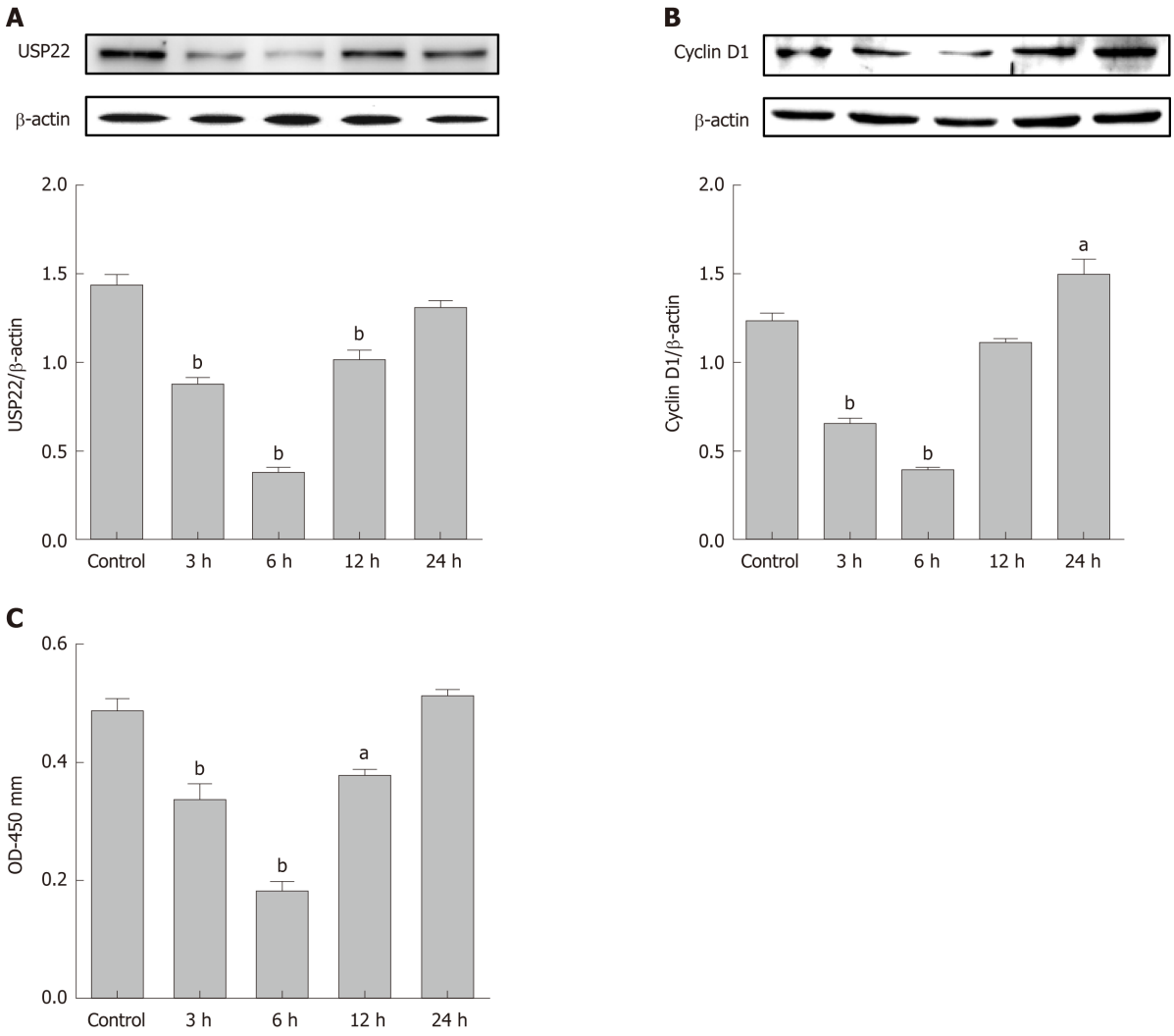

Figure 2 Ubiquitin-specific protease 22 correlates with the proliferative activity of IEC-6 cells after hypoxia/reoxygenation. IEC-6 cells were subjected to 6 h of hypoxia followed by 3, 6, 12, or 24 h of reoxygenation. A: Representative Western blot demonstrating the expression of USP22 in tissues from the experimental groups (n = 3); B: Representative Western blot demonstrating the expression of Cyclin D1 in tissues from the experimental groups (n = 3); C: CCK-8 was used to examine cell proliferation and viability at the indicated time points (n = 6). The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* the control group; $^{b}P < 0.01$ *vs* the control group. USP22: Ubiquitin-specific protease 22; CCK-8: Cell Counting Kit-8.

investigated USP22 expression and its pro-regenerative properties in intestinal I/R injury. Some studies showed the association of USP22 with progression and therapeutic failure of colorectal cancer, which could be valuable for consultation in further study on intestinal I/R injury and cancer research[38,39].

There were limitations in our study. Although the *in vitro* experiments implied a direct link between USP22 and regeneration, the *in vivo* study should be better designed to imply causation and biological linkage in our further study. In addition, a great number of clinical samples are needed to prove that the conclusion is also applicable for human beings. Furthermore, observation has been made that USP22 was globally reduced in a mouse model and that goblet cells were abundant[21]. While goblet cells are known as mucus secreting cells, they also share gate-keeping roles with epithelial cells and are indispensable in regulating innate immune function[40,41]. Other studies also confirmed that antibodies were secreted within the mucus from intestinal epithelial goblet cells[31]. Thus, the role of USP22 in intestinal damage recovery needs further investigations and we would not only adopt USP22 knockout mouse models but also investigate more precisely the association of USP22 with immune barrier and the underlying mechanisms in intestinal I/R injury.

In conclusion, this study demonstrated that USP22 was involved in intestinal epithelial cell proliferation and tissue regeneration after hypoxia/reoxygenation or I/R injury. Our study revealed a novel role for USP22 in intestinal regeneration after I/R injury. Therefore, targeting USP22 signaling may increase the therapeutic potential for intestinal I/R injury. In our future research, we will try to elucidate the internal mechanisms of USP22 signaling in intestinal regeneration after I/R injury.

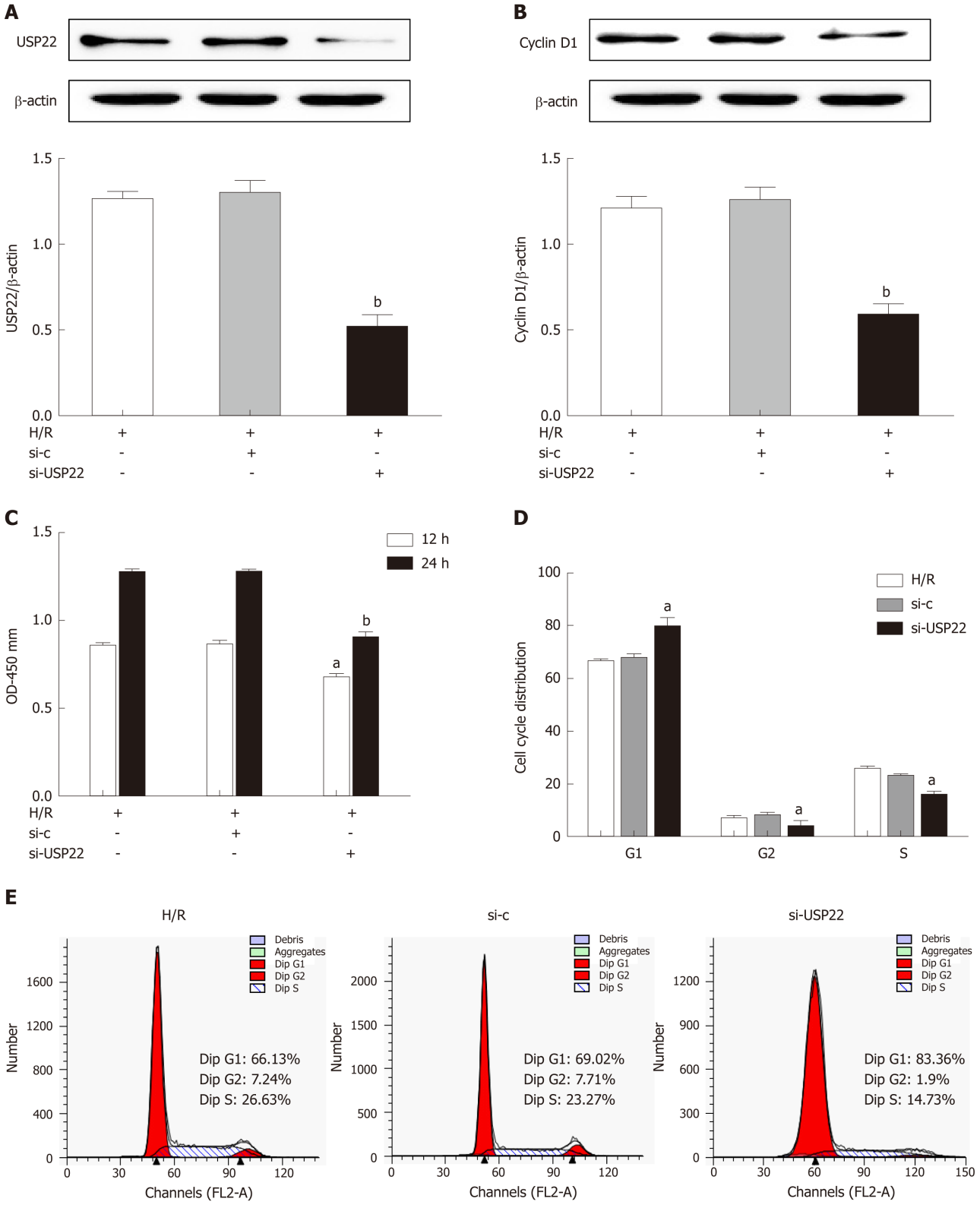

Figure 3 Ubiquitin-specific protease 22 knockdown inhibits cell proliferation and induces cell cycle arrest in IEC-6 cells after hypoxia/reoxygenation. IEC-6 cells were transfected with USP22 siRNA or unspecific scrambled siRNA and then subjected to hypoxia/reoxygenation or left untreated. A: Western blot analysis for USP22 and β-actin in ICE-6 cells transfected with USP22 siRNA and control siRNA (n = 3); B: Western blot analysis for Cyclin D1 and β-actin in ICE-6 cells transfected with USP22 siRNA and control siRNA (n = 3); C: CCK-8 was used to examine cell viability at the indicated time points (n = 6); D and E: FACSCalibur analysis of the percentages of cells in the G1, G2, and S phases at 24 h of reoxygenation after 6 h of hypoxia (n = 3). The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* the control group; $^{b}P < 0.01$ *vs* the control group. USP22: Ubiquitin-specific protease 22; CCK-8: Cell Counting Kit-8.

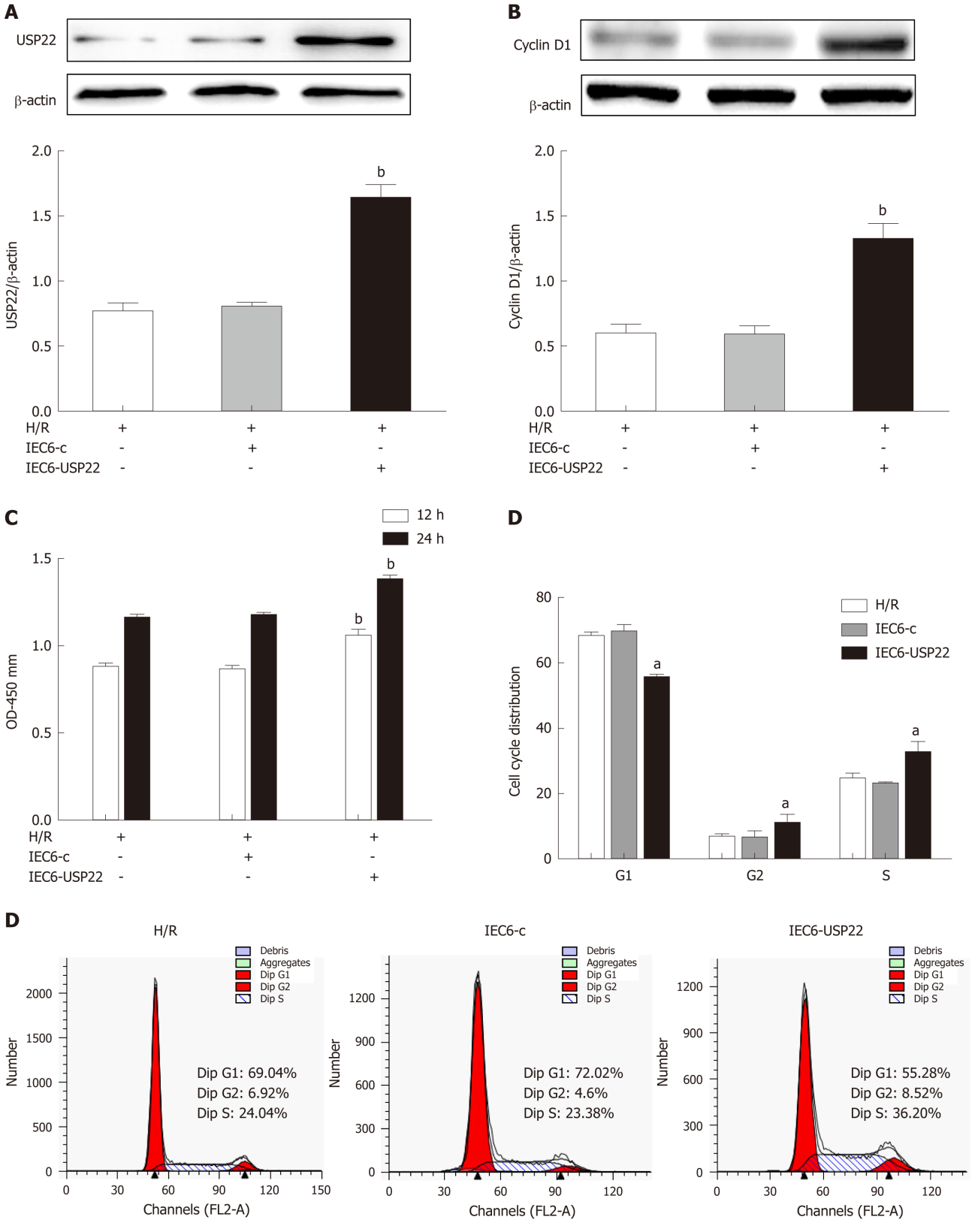

Figure 4 Ubiquitin-specific protease 22 overexpression promotes cell proliferation in IEC-6 cells after hypoxia/reoxygenation. IEC-6 cells were transfected with a USP22 overexpression plasmid or a negative control plasmid and then subjected to hypoxia/reoxygenation or left untreated. A: Western blot analysis for USP22 and β-actin in ICE-6 cells transfected with IEC6-USP22 and IEC6-Control (n = 3); B: Western blot analysis for Cyclin D1 and β-actin in IEC-6 cells transfected with IEC6-USP22 and IEC6-Control (n = 3); C: CCK-8 assay was used to examine cell viability at the indicated time points (n = 6); D and E: FACSCalibur analysis of the percentages of cells in the G1, G2, and S phases at 24 h of reoxygenation after 6 h of hypoxia (n = 3). The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* the control group; $^{b}P < 0.01$ *vs* the control group. USP22: Ubiquitin-specific protease 22; CCK-8: Cell Counting Kit-8.

ARTICLE HIGHLIGHTS

Research background

Ubiquitin-specific protease 22 (USP22) is a novel member of the USPs subfamily, acting as a regulator of cell cycle progression, proliferation, and tumor invasion. Decreased expression of USP22 has been identified to contribute to arrested cell cycle and disrupted generation of differentiated cell types in crypts and villi. However, the regulatory mechanism of USP22 remains unclear. Therefore, elucidation of the underlying mechanism may help to improve the tissue repair in intestinal ischemia reperfusion (I/R) injury.

Research motivation

It is necessary to explore whether USP22 is correlated with increased potential of cell proliferation and tissue regeneration in intestinal I/R injury. Recent studies have demonstrated that insufficient proliferation and regeneration of fully rescuing intestinal mucosal barrier function could be witnessed by the high mortality rate in clinical practice. Moreover, the potential role of USP22 in cell growth, cell cycle progression, and generation of differentiated cell types has also been reported in crypts and villi. These findings give us a good lead for further study regarding the mechanism of USP22 regulation during intestinal I/R injury.

Research objectives

In the previous study, we investigated the effect of USP22 on intestinal cell proliferation and regeneration after intestinal I/R injury both *in vivo* and *in vitro* by gain- and loss-of-function approaches. Our study provides significant insight into the signalling mechanism of USP22 during intestinal I/R injury that may contribute to the future investigation of more effective therapies in clinical practice.

Research methods

Experiments using an animal model and *in vitro* model in rats and cells to better elucidate the pathophysiological process of intestinal I/R injury. Hematoxylin and eosin staining and Chiu's scoring system were used to demonstrate the intestinal tissue injury histopathologically. Immunohistochemical staining for PCNA was carried out to display and observe positive nuclei of proliferating intestinal cells. Gene silencing or transfection was conducted to construct relatively stable USP22-depleted or -expressed cells to complete the following functional studies. A series of *in vitro* experiments, such as Western blot, Cell Counting Kit-8, and cell cycle analysis, were performed to explore the effect of USP22 on cell proliferation.

Research results

Experiments *in vivo* showed the correlation between USP22 and intestinal regenerative activity of intestinal cells after intestinal I/R injury. The results of *in vitro* experiments showed a direct positive correlation of USP22 with cell proliferation and cell cycle progression of intestinal cells after hypoxia/reoxygenation injury. This study could be valuable for consultation in further study on intestinal I/R injury and be potentially utilized in therapeutic enhancement in clinical practice. Limitations did exist that the *in vivo* study should be better designed to imply causation and biological linkage and USP22 knockout mouse models would be helpful. Clinical samples are also needed to better suit the application on human beings.

Research conclusions

USP22 plays a positive role in intestinal epithelial cell proliferation and tissue regeneration in intestinal I/R injury. This study reveals a novel role for USP22 in intestinal regeneration after I/R injury. Targeting USP22 may increase the therapeutic potential for intestinal I/R injury in clinical practice.

Research perspectives

Our study illuminates the role of USP22 in intestinal epithelial cell proliferation and tissue regeneration in intestinal I/R injury. Other researchers have reported the abundant mucus secreting goblet cells in a USP22 globally reduced mouse model. While goblet cells are also one of indispensable parts in regulating innate immune function, further investigation is needed.

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ORIGINAL ARTICLE

Case Control Study

Nutrient drink test: A promising new tool for irritable bowel syndrome diagnosis

Fermin Estremera-Arevalo, Marta Barcelo, Blanca Serrano, Enrique Rey

ORCID number: Fermin Estremera-Arevalo (0000-0003-2676-1353); Marta Barcelo (0000-0002-7823-2528); Blanca Serrano (0000-0002-8201-8997); Enrique Rey (0000-0002-5060-7105).

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Fermin Estremera-Arevalo, Department of Gastroenterology, Complejo Hospitalario de Navarra, Pamplona 31008, Spain

Marta Barcelo, Department of Gastroenterolgy, Hospital Infanta Leonor, Madrid 28031, Spain

Blanca Serrano, Department of Digestive Diseases, Hospital Clinico San Carlos, Madrid 28040, Spain

Enrique Rey, Department of Digestive Diseases, Hospital Clinico San Carlos and Complutense University, Madrid 28040, Spain

Corresponding author: Fermin Estremera-Arevalo, MD, PhD, Doctor, Staff Physician, Gastroenterologist, Department of Gastroenterology, Complejo Hospitalario de Navarra, Calle Irunlarrea, 3, Pamplona 31008, Spain. festremera15@gmail.com
Telephone: +34-848-422222
Fax: +34-848-422303

Abstract

BACKGROUND

Irritable bowel syndrome (IBS) is a highly prevalent condition. It is diagnosed on the basis of chronic symptoms after the clinical and/or investigative exclusion of organic diseases that can cause similar symptoms. There is no reproducible non-invasive test for the diagnosis of IBS, and this raises diagnostic uncertainty among physicians and hinders acceptance of the diagnosis by patients. Functional gastrointestinal (GI) syndromes often present with overlapping upper and lower GI tract symptoms, now believed to be generated by visceral hypersensitivity. This study examines the possibility that, in IBS, a nutrient drink test (NDT) provokes GI symptoms that allow a positive differentiation of these patients from healthy subjects.

AIM

To evaluate the NDT for the diagnosis of IBS.

METHODS

This prospective case-control study compared the effect of two different nutrient drinks on GI symptoms in 10 IBS patients (patients) and 10 healthy controls (controls). The 500 kcal high nutrient drink and the low nutrient 250 kcal drink were given in randomized order on separate days. Symptoms were assessed just before and at several time points after drink ingestion. Global dyspepsia and abdominal scores were derived from individual symptom data recorded by two questionnaires designed by our group, the upper and the general GI symptom

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questionnaires, respectively. Psycho-social morbidity and quality of life were also formally assessed. The scores of patients and controls were compared using single factor analysis of variance test.

RESULTS

At baseline, IBS patients compared to controls had significantly higher levels of GI symptoms such as gastro-esophageal reflux (P = 0.05), abdominal pain (P = 0.001), dyspepsia (P = 0.001), diarrhea (P = 0.001), and constipation (P = 0.001) as well as higher psycho-social morbidity and lower quality of life. The very low incidence of GI symptoms reported by control subjects did not differ significantly for the two test drinks. Compared with the low nutrient drink, IBS patients with the high nutrient drink had significantly more dyspeptic symptoms at 30 (P = 0.014), 45 (P = 0.002), 60 (P = 0.001), and 120 min (P = 0.011). Dyspeptic symptoms triggered by the high nutrient drink during the first 120 min gave the best differentiation between healthy controls and patients (area under receiver operating curve of 0.915 at 45 min for the dyspepsia score). Continued symptom monitoring for 24 h did not enhance separation of patients from controls.

CONCLUSION

A high NDT merits further evaluation as a diagnostic tool for IBS.

Key words: Irritable bowel syndrome; Nutrient drink test; Non-invasive; Dyspepsia; Screening

Core tip: There is no objective non-invasive test for diagnosis of irritable bowel syndrome. This study shows that measurement of early gastrointestinal symptoms provoked by ingestion of a caloric drink is a promising diagnostic tool for this syndrome. This test could allay patient and doctor uncertainty and reduce the use of costly and invasive diagnostic tests.

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INTRODUCTION

Irritable bowel syndrome (IBS) affects about 15% of the population and is a common cause for medical consultation[1-3]. IBS is diagnosed when symptoms meet diagnostic criteria (the most authoratative currently being the Rome IV criteria), and clinical assessment and investigations do not reveal any other cause[4,5]. Diagnosis of IBS by exclusion is a burden for both physicians and patients, as this approach raises uncertainty as to its accuracy[6]. Clinical judgement is needed to determine the extent of investigations.

Overlap of IBS with functional dyspepsia (FD) is well-known, and in both, symptoms are believed to be due to visceral hypersensitivity[7]. Increased sensitivity to rectal barostatic balloon distension correlates with IBS severity, and this has been proposed as a reliable objective test for visceral hypersensitivity[8-10]. However, it is a specialised, invasive procedure. The provocation of symptoms by a nutrient drink test (NDT) has been found to be a useful, simple approach for diagnosis of FD[11]. Its value for IBS diagnosis has not been explored, even though the onset of IBS symptoms is frequently related to food intake *via* unknown mechanisms[12,13].

This case-control, prospective study, carried out in a tertiary center, explored the diagnostic potential of the NDT for diagnosis of IBS by evaluating symptoms after both high nutrient (HN) and low nutrient (LN) drinks in 10 IBS patients and 10 healthy control subjects.

MATERIALS AND METHODS

Inclusion criteria

Gastrointestinal (GI) or metabolic diseases were excluded in all of the IBS patients by upper and lower GI endoscopy (with duodenal and gastric biopsies), imaging, and blood screens, which included thyroid hormone levels and celiac disease antibodies. IBS patients were enrolled only if they fulfilled the Rome III criteria for IBS and scored > 200 points in the irritable bowel severity scoring system (IBSSS) score[5,14]. IBS patients who also had gastro-esophageal reflux disease with adequate control of symptoms with proton pump inhibitors (PPI) were not excuded. Patients with FD were identified and excluded by a gastroenterologist interview, based on the Rome III criteria[4]. Figure 1 illustrates the flow of patient recruitment. The 10 healthy asymptomatic control subjects, who were recruited *via* advertisement in the gastroenterology hospital ward, were matched with the IBS patients for gender, age, and same category of body mass index.

Exclusion criteria

Major exclusion criteria were FD, a history of abdominal surgery (except appendectomy and hysterectomy), and intake of drugs that can modify GI transit (*e.g.*, opioids).

Ethical aspects

All participants gave informed consent after a careful explanation of the methods and objectives of this study. The project was named "Utilidad del test de saciedad precoz ("NDT") para el diagnóstico de síndrome de intestino irritable", and the study protocol satisfied the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Hospital Clinico San Carlos on 15 February 2010 (local registry number 10/057-E).

Study design

All subjects received the HN and LN drinks within 10 d. Each matched pair of IBS and control subjects was randomized to one of two groups receiving either the HN or LN drink first. The HN drink, or NDT, was 500 mL of vanilla taste Ensure™ (Abbott Laboratories, Chicago, IL, United States), at the recommended dilution. This delivered 500 kcal was 86.5 g of carbohydrate (containing 5 g of fructooligosaccharide), 12.5 g of fat, and 19 g of protein. The 500 mL LN drink was a commercial vanilla drink (Okey Vainilla, Idilia Foods, Valencia, Spain) of similar flavor and color to the HN drink, diluted with water. This provided 250 kcal, consisting of 70 g of carbohydrate (with no fructooligosaccharide), 4.5 g of fat, and 12 g of protein. The drinks were consumed over 33 min (15 mL/min), similar to previous NDT studies[15,16].

Both drinks were given in the early morning after an 8 h fast. Subjects were blinded as to which drink they were receiving, but usually noticed that the drinks had different viscosities. Due to the circumstances of the study, the main investigator could not be blinded to drink type.

Symptom assessments

During the first study visit, a gastroenterologist made a detailed symptom and health background assessment. Following this, all subjects completed the following screening self-administered questionnaires to record their symptoms, quality of life (QOL), and psycho-social profiles in the prior 4 wk: Rome III criteria (symptoms related to different functional GI disorders), gastrointestinal symptoms rating scale (which measures different general GI symptoms with a Likert scale), symptom checklist-90-R (SCL-90-R, psychological health status), hospital anxiety and depression scale (regarding anxiety and depression related to physical symptoms), short form 12 (SF-12, health-related QOL), and IBSSS[4,14,17-19,20]. These questionnaires were completed during and after the first drink intake.

Two different questionnaires with 0-5 Likert scales (0: none; 1: very mild; 2: mild; 3: moderate; 4: severe; 5: very severe) were designed by our group for gathering symptoms present just before and at intervals after drink ingestion (see below). Symptoms were described similarly to wordings of the GERD-Q (Spanish validated version)[21], Gastroparesis Cardinal Symptom Index, and IBSSS instruments[14,22]. Subjects reported their symptom levels to the investigator face to face within the first 2 h and then by telephone for the rest of the 24 h after the start of the drink. The Global Dyspepsia Score, which evaluated satiety, nausea, epigastric distension, epigastric pain, heartburn, and regurgitation, was completed just before the drink, then every 5 min during ingestion, and then at 45, 60, and 120 min. The Global Dyspepsia Score at each assessment time was the sum of all of the symptom scores gathered over the previous 5 min by the Upper Gastrointestinal Symptoms

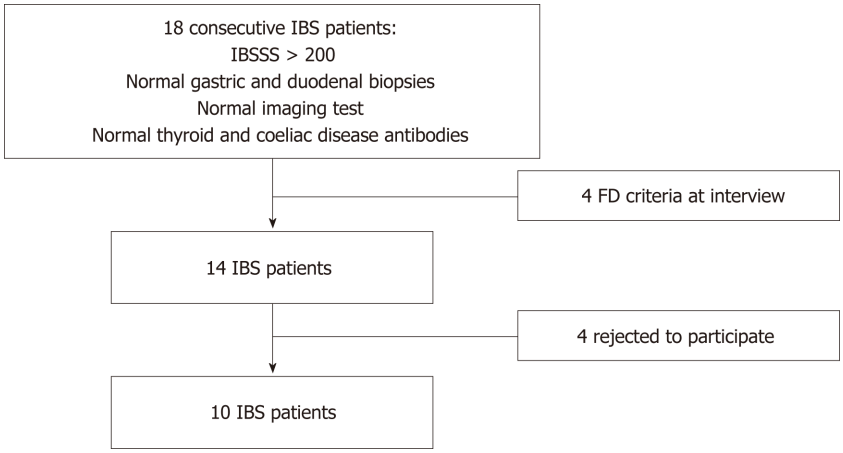

Figure 1 Flowchart of patients recruitment. Left column shows flow of inclusion and right column the excluded patients. IBS: Irritable bowel syndrome; IBSSS: Irritable bowel severity scoring system; FD: Functional dyspepsia.

Questionnaire. The General Gastrointestinal Symptom Questionnaire evaluated the following symptoms for 24 h after the drinks: Heartburn, regurgitation, abdominal pain, abdominal distension, bowel actions and stool type according to the Bristol scale[23], stool urgency, and presence of mucus in the stool. This second questionnaire was completed at the start of the drink and 1, 2, 4, 6, 8, 10, 12, and 24 h after ingestion. The global abdominal score was derived for each assessment time by summing the scores for the individual symptoms evaluated in the General Gastrointestinal Symptom Questionnaire. After ingestion of the test drinks, participants were encouraged to eat a free diet, except for avoiding vegetable fiber and alcoholic drinks during the next 24 h.

Statistical analysis

Demographics of patients and control subjects and the screening questionnaire scores were compared by One-Way analysis of variance. Symptom scores during and after the two drinks were compared with analysis of variance for repeated measures. The discriminatory capacity of the NDT was evaluated by receiver operating characteristic (ROC) curves (SPSS 16.0 version, Chicago, IL, United States). The difference was considered statistically significant when $P < 0.05$.

RESULTS

Demographics

The mean values for IBS patients (nine female, one male) and their matched healthy controls did not differ significantly for age and body mass index. Five patients had alternating IBS (IBS-A), and five had diarrhea IBS. One of the patients had gastro-esophageal reflux disease, controlled by PPI.

Symptoms in the 4 wk prior to drink testing

For 4 wk prior to testing, the IBS patients had high levels of GI symptoms compared to controls (Table 1). Though they did not report clinically significant dyspeptic symptoms in the screening clinical interview, 4/10 IBS patients fulfilled FD diagnostic criteria in the self-administered Rome III questionnaire completed after study entry. GER, abdominal pain, dyspepsia, diarrhea, and constipation scores were derived from the gastrointestinal symptoms rating scale and the IBSSS from the sum of its five variables (abdominal pain, number of days with pain, abdominal distension, disordered bowel habit, and QOL burden). All of the 4 wk baseline scores before the first drink were significantly higher in the patients (Table 1).

Psycho-social parameters and QOL prior to drink testing

Compared to controls, the scores derived from the SCL-90 and HAD for IBS patients demonstrated substantially higher psycho-social morbidity for most of the variables assessed (Table 2). QOL was also significantly impaired in IBS patients in both the mental ($P = 0.001$) and physical ($P = 0.000$) domains (Table 2).

Tolerance of the test drinks

All participants consumed all of the HN and LN drinks.

Table 1 Baseline abdominal symptoms in control subjects and IBS patients

	Control	IBS	*P* value
GER	0.4 ± 0.7	1.4 ± 1.4	0.05
Abdominal pain	0.4 ± 0.5	3.4 ± 1.7	0.001
Dyspepsia	0.7 ± 0.5	2.9 ± 1.4	0.001
Diarrhea	0.3 ±0.7	3.1 ± 1.9	0.001
Constipation	0.2 ± 0.4	2.4 ± 1.5	0.001
IBSSS score	37.3 ± 36.0	275.3 ± 115.6	0.001

Scores of symptoms in the 4 wk prior to drink testing, derived from the screening questionnaires, expressed as coefficients of each domain in the GSRS and the sum of IBSSS questionnaire variables (mean ± SD). GSRS: Gastrointestinal symptoms rating scale; GER: Gastro-esophageal reflux; IBSSS: Irritable bowel severity scoring system.

Control subjects: High nutrient versus low nutrient drinks

In the control group, the mean Global Dyspepsia Score (Figure 2) and the mean global abdominal score (Figure 3) did not differ significantly after the two drinks.

IBS patients and controls: High nutrient versus low nutrient drinks

IBS patients reported significantly higher Global Dyspepsia Scores after both the HN and LN drinks compared to controls. In the patients, compared to the LN drink, the mean Global Dyspepsia Score after the HN drink was higher (up to $P = 0.016$) from 45 to 120 min after the drink (Figure 2). This was a general effect (Table 3), as nausea, regurgitation, and epigastric bloating were each significantly greater after the HN drink.

In the patients, the mean Global Abdominal Score during 24 h after the drinks was not significantly different between the HN and LN drinks, the greatest difference being at 8 h (3.4 *vs* 0.9, $P = 0.082$) (Figure 3). Notably, neither of the drinks had any impact on bowel function or stool consistency.

Comparison of symptoms after the high nutrient drink between irritable bowel syndrome and control subjects

The mean Global Dyspepsia Score after the HN drink was significantly higher in patients from 25 min till measurements ceased at 120 min (Figure 2). Table 3 gives the mean levels of the individual symptoms contributing to the Global Dyspepsia Score in IBS patients and controls after the HN drink. The mean Global Abdominal Score was also significantly higher after the HN drink ($P = 0.008$ at 2 h) in IBS patients than in the control subjects from 1 h to 10 h after the drink (Figure 3). Levels of the individual symptoms that contributed to the Global Abdominal Score are given in Table 4.

Diagnostic performance of the high nutrient drink

Figure 4 shows the ROC curves for the mean Global Dyspepsia Score in distinguishing IBS patients from healthy subjects. At 45 min after the HN drink, the area under the curve (AUC) was 0.915. Sensitivity and specificity for recognition of IBS vary between 80%-90% and 70%-90% according to the cut-off values chosen. For the mean Global Abdominal Score, the AUC was lower at 0.825. Depending on the cut-off values chosen, sensitivity and specificity ranged from 80%-90% and 30%-40%. The ROC curve for the sum of the mean Global Dyspepsia and Abdominal Scores for the first 2 h of observation also had a lower predictive value (AUC 0.88) than the Global Dyspepsia Score.

DISCUSSION

We have found that the HN 500 kcal Ensure™ NDT triggered substantial GI symptoms in a cohort of IBS patients, an effect not seen in our healthy subjects. The dyspeptic symptoms during the first 2 h after the HN drink appear to have the greatest potential for making a positive diagnosis of IBS. Also, in patients, the HN drink induced more abdominal symptoms than the LN drink, an effect not seen in the controls.

A simple, non-invasive test for IBS is needed that can reassure patients and doctors of the accuracy of this diagnosis. Acceptance of the diagnosis of IBS is important for therapeutic success[6]. To our knowledge, this exploratory study is the first to test

Table 2 Baseline psycho-social and quality of life markers in control subjects and IBS patients

	Control	IBS	*P* value
Somatization[1]	0.4 ± 0.4	1.8 ± 1.1	0.001
Obsession[1]	0.3 ± 0.3	1.2 ± 0.7	0.004
Interpersonal sensitivity[1]	0.2 ± 0.3	0.7 ± 0.7	0.05
Depression[1]	0.3 ± 0.4	1.5 ± 1.3	0.01
Anxiety[1]	0.2 ± 0.3	1.1 ± 0.8	0.005
Hostility[1]	0.2 ± 0.2	0.5 ± 0.6	0.09
Phobic anxiety[1]	0.1 ± 0.2	0.8 ± 1.0	0.05
Paranoid ideation[1]	0.2 ± 0.2	0.6 ± 0.5	0.025
Psychoticism[1]	0.1 ± 0.2	0.5 ± 0.5	0.013
Positive global symptoms[1]	17.2 ± 13.3	46.0 ± 21.7	0.002
Distress index[1]	1.2 ± 0.2	1.9 ± 0.6	0.002
HAD anxiety	3.6 ± 2.0	11.1 ± 4.8	0.000
HAD depression	1.5 ± 1.5	6.7 ± 5.4	0.01
HAD score	5.1 ± 2.6	17.8 ± 9.6	0.000
Physical score[2]	55.0 ± 5.0	37.4 ± 11.7	0.000
Mental score[2]	53.9 ± 6.0	42.3 ± 11.6	0.001

Psycho-social and quality of life data for the 4 wk prior to drink test, derived from the coefficient of each domain ([1]SCL-90-R; HAD; and [2]SF-12). Every questionnaire has its own statistical tools to evaluate the outcomes[18-20]. SCL-90-R: Symptom checklist-90-R; HAD: Hospital anxiety and depression scale; SF-12: Short form 12; IBS: Irritable bowel syndrome.

directly the use of a NDT for diagnosis of IBS. Our results are consistent with those of Haag *et al*[24] who included 18 IBS patients as a control group for the use of the NDT in diagnosis of FD, as they found that symptoms were provoked in IBS patients by a substantially larger 600 mL, 900 kcal liquid test meal. These symptoms were less severe than in the FD patients but more intense than in the healthy controls.

Since completion of our studies described here, Posserud *et al*[25] have reported on the generation of symptoms in IBS patients and healthy controls over 4 h after a solid test meal of 540 kcal[25]. Consistent with our data, the patients had more early satiety, nausea, and abdominal discomfort than the controls. The symptom burden in the first 2 h after the solid meal in IBS patients correlated with their basal IBS severity score and not with basal FD-related symptoms. We chose not to use a solid food caloric load because of the practical challenge of preparing a reliably standardized solid test meal. The meal used by Posserud *et al*[25] contained accurately defined weights of oat bran, applesauce, crispbread, margarine, and cheese and set volumes of milk and apple juice[25]. Such a meal is much less convenient than a standard liquid nutrient supplement and difficult to reproduce accurately from country to country.

Though NDTs have been widely used in FD studies, they have not been adequately standardized. Most NDTs use a nutrient mixture of between 1 kcal/mL and 1.5 kcal/mL, ingestion rates of 10 mL/min to 30 mL/min, and total volumes of 650 mL to 1000 mL[15,16]. Abnormal gastric volume accommodation and slow gastric emptying have been found in only a proportion of FD patients. The presence of these abnormalities has been only weakly correlated with a positive NDT[10,14,15,24]. Visceral hypersensitivity is considered to be the most plausible underlying mechanism for symptom generation, a feature shared with patients with both FD and IBS.

The exploratory nature of our study prompted us to use a small case-control study design, with intensive collection of symptom data in very well-defined and severely symptomatic IBS patients and in control subjects. These data give useful guidance for future studies focused on defining the most simple yet effective design for a NDT in suspected IBS.

We also addressed the unexplored possibility that the NDT could provoke diagnostically useful symptoms for up to 24 h in IBS patients. The 24 h symptom assessment did not add anything to the diagnostic yield of the Global Dyspepsia Score over the first 2 h after ND ingestion. Our data indicate that a diagnostic NDT could be restricted to recording of symptoms for only 2 h to 3 h after the test drink, a substantial simplification of the protocol we tested. We were surprised that dyspeptic symptoms during the first 2 h (satiety, epigastric bloating, epigastric pain, heartburn, and regurgitation) achieved the best yield for the recognition of IBS patients, but this

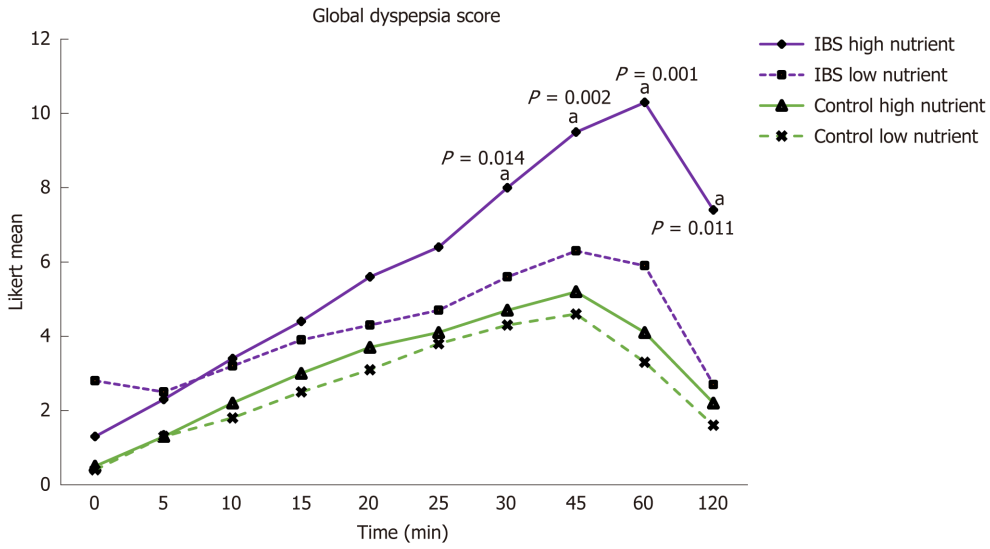

Figure 2 Global dyspepsia in irritable bowel syndrome patients and control subjects after the nutrient drink test. Global dyspepsia mean Likert score in IBS patients and control subjects was measured for the first 2 h after the two drinks. Statistically significant differences are shown in the graph ($^aP < 0.05$). IBS: Irritable bowel syndrome.

finding is consistent with the data of Posserud *et al*[25].

Consistent with other tertiary center Spanish IBS cohorts, our IBS patients were markedly impacted by their symptoms and so an ideal population to evaluate in an exploratory study[26]. IBS involves biopsycho-social aspects that may vary between individuals that equally fulfil the Rome criteria[4]. This variation can hinder the reproducibility within a small cohort. Thus, future studies need to enroll a larger number of patients whose symptoms are less severe and thus more representative of the spectrum of patients presenting in primary and secondary care. The non-invasive and simple nature of the NDT means it would have a low cost, making it suitable for wide use. Adding relatively inexpensive, routine non-invasive screening for celiac disease, anemia, and thyroid dysfunction in patients suspected to have IBS could help to enhance the specificity of the NDT. Future studies need to address whether a NDT can distinguish IBS from other digestive disorders that can present with similar symptoms. The key question is whether a positive NDT arises only from visceral hypersensitivity, which does not underly symptom provocation in other diseases such as specific epithelial or gross anatomical abnormalities of the gut.

Although the physician-conducted patient screening methods were carefully designed to exclude patients with IBS and FD overlap, 4/10 fulfilled FD criteria in the Rome III questionnaire, which was self-completed by patients after study entry. Inclusion of some IBS patients with co-existing FD in our study probably does not detract from our major conclusions, given how common this coexistence is[27,28]. Posserud *et al*[25] did not exclude IBS patients who also met diagnostic criteria for FD in their solid meal study and found no differences in meal-associated symptom provocation between their FD-negative and positive IBS patients[25].

We conclude that use of a diagnostic NDT for IBS is a promising option. Questions that need to be addressed are how well a NDT would distinguish between IBS and other GI disorders across a spectrum of symptom severity, how to streamline symptom recording without loss of diagnostic performance, and what is the best amount of calories and possibly fermentable oligo-di-mono-saccharides and polyols in the test drink.

Table 3 Global dyspepsia score after the high nutrient drink test in control subjects and IBS patients

HN drink-individual symptoms assessed for the global dyspepsia score		Time in min									
Symptom	Group	0	5	10	15	20	25	30	45	60	120
Satiety	IBS	0.3	1.3	2.1	2.6	3	3.4	3.8	4.5	4.2	2.8
	Controls	0.3	1.2	1.7	2.4	3	3.3	3.7	4.1	3.2	1.6
Nausea[1]	IBS	0.3	0	0	0.1	0.2	0.2	0.2	0.3	**1.1**	0.7
	Controls	0	0	0.1	0.1	0.1	0.2	0.2	0.2	**0**	0
Epigastric distension[1]	IBS	0.6	0.8	1.1	1.3	1.7	1.9	**2.7**	**3.2**	**3.4**	**2.6**
	Controls	0.2	0.1	0.4	0.5	0.6	0.6	**0.8**	**0.9**	**0.9**	**0.6**
Epigastric pain	IBS	0.1	0.1	0	0	0.1	0.2	0.5	0.5	0.5	0.5
	Controls	0	0	0	0	0	0	0	0	0	0
Heartburn	IBS	0	0.1	0.2	0.3	0.4	0.5	0.5	0.4	0.1	0.1
	Controls	0	0	0	0	0	0	0	0	0	0
Regurgitation[1]	IBS	0	0	0	0.1	0.2	0.3	0.3	**0.6**	**1**	0.7
	Controls	0	0	0	0	0	0	0	**0**	**0**	0

Mean scores of IBS patients and controls for individual upper GI symptoms in the first 120 min after the HN drink.
[1]Variables reached significant differences. Bold number: Time points reaching significant differences. IBS: Irritable bowel syndrome; HN: High nutrient; GI: Gastrointestinal.

Table 4 General gastrointestinal symptoms after the high nutrient drink test in control subjects and IBS patients

HN drink-global symptoms		Time in h								
Symptom	Group	0	1	2	4	6	8	10	12	24
Heartburn	IBS	0	0.3	0.2	0	0.1	0.1	0	0	0.1
	Controls	0	0	0	0	0	0	0.1	0.1	0
Regurgitation[1]	IBS	0.1	**1**	0.8	0.1	0.5	0.6	0.4	0.2	0.4
	Controls	0	**0**	0	0	0	0	0	0	0
Abdominal distension[1]	IBS	0	**2.7**	**2.3**	**1.3**	**1.3**	**1.3**	**1.3**	**1.2**	**1.3**
	Controls	0	**0.6**	**0.4**	**0**	**0**	**0**	**0**	**0**	**0**
Abdominal pain	IBS	0	0.7	0.4	0.3	0.1	1.1	0	0	0.5
	Controls	0	0.2	0.1	0.1	0	0.1	0	0	0.4

Mean scores of IBS patients and controls for individual general GI symptoms in the first 24 h after the HN drink.
[1]Variables reached significant differences. Bold number: Time points reaching significant differences. IBS: Irritable bowel syndrome; HN: High nutrient; GI: Gastrointestinal.

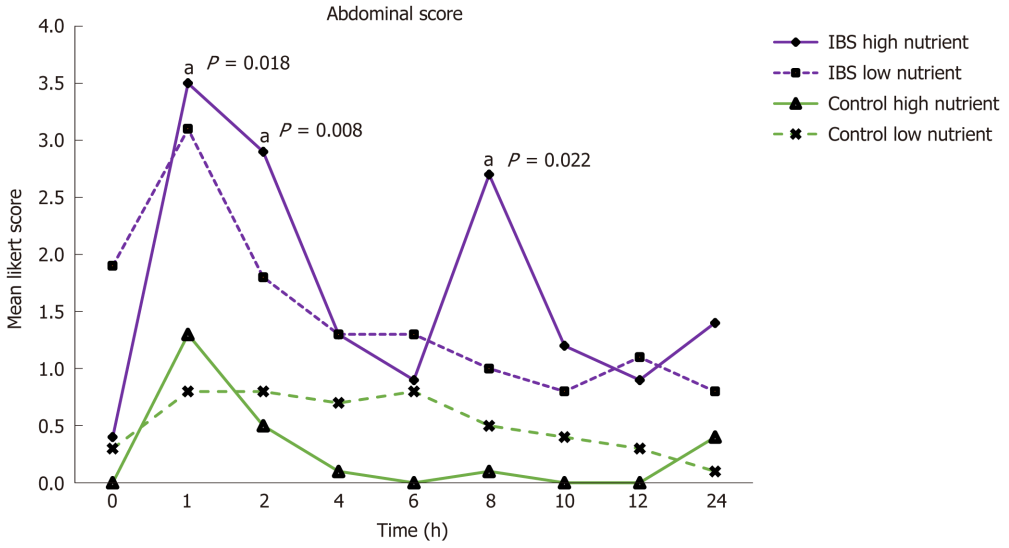

Figure 3 Abdominal symptoms in IBS patients and control subjects after the nutrient drink test. Abdominal mean Likert score in IBS patients and control subjects were measured after the two test drinks over 24 h. Statistically significant differences are shown in the graph ([a]$P < 0.05$). IBS: Irritable bowel syndrome.

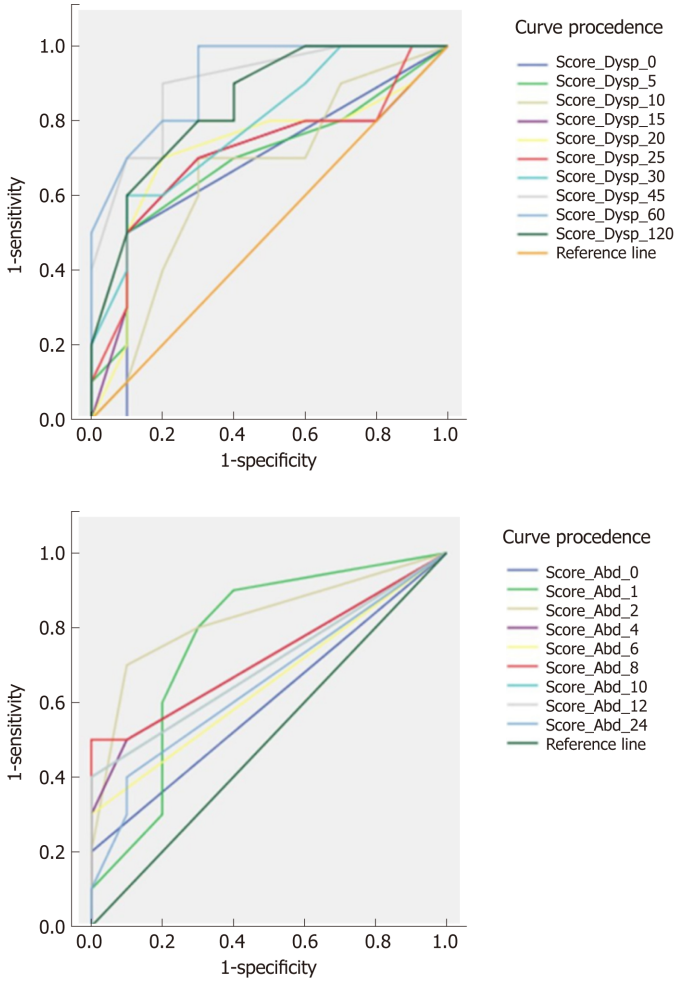

Figure 4 Sensitivity and specificity of the nutrient drink test. Receiver operating characteristic curves for Global Dyspepsia (upper) and Global Abdominal (lower) scores obtained at the different time points after the high nutrient drink are represented.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is highly prevalent worldwide. It is among the most common causes for gastroenterologist consultation and a significant economic burden in healthcare systems. The diagnosis of IBS is made when the symptom pattern fulfills Rome Criteria and underlying organic pathology is ruled out by tests that usually require invasive procedures, such as endoscopy or imaging examinations with radiation. Patients often conclude that their physician does not know what disease they are suffering from. Physicians are often tentative in their diagnosis of IBS and unsure how many investigations they should order to exclude other possible causes of their patient's symptoms. The lack of a specific diagnostic test for IBS is an important gap in the physician's toolkit. The symptom provocation caused by a nutrient drink test (NDT) has been used as a tool for the diagnosis of a very similar syndrome - functional dyspepsia (FD), whose symptoms usually overlap with IBS. In both IBS and FD, there is an abnormally heightened level of gut sensations, which is known as "visceral hypersensitivity". The use of a NDT for diagnosis of IBS has not been previously evaluated.

Research motivation

This study focused on the design of a simple, inexpensive, and non-invasive diagnostic tool for IBS. The study tested whether prolongation of symptom recording beyond the 3 h-4 h of the provocative drink would improve diagnostic outcomes. The existence of a validated, simple test for IBS could reduce the use of invasive tests and exposure to X-rays in IBS patients.

Research objectives

The main objective was to determine whether the symptoms triggered by a highly caloric drink can differentiate IBS patients from healthy controls.

Research methods
After ingestion of the high and low nutrient drinks, given on separate days, subjects were screened for gut symptoms face-to-face every 5 min for the first 2 h and by telephone until 24 h after drink ingestion.

Research results
This study has shown consistent provocation of symptoms during the first 2 h after a high nutrient drink in IBS patients, an effect not seen in the healthy subjects. Continuation of symptom monitoring up to 24 h after the drink did not enhance diagnostic outcomes.

Research conclusions
Our data show that the NDT is a promising non-invasive test for IBS diagnosis and provide guidance for simplification of the test procedure.

Research perspectives
More studies are needed since the patients enrolled in this project were especially severely affected and so not representative of the entire spectrum of IBS. The major priority for future research is a large-scale investigation of the diagnostic performance of the NDT in less severely symptomatic IBS patients, compared with patients with abdominal symptoms arising from structural (organic) disorders of the gut.

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ORIGINAL ARTICLE

Retrospective Study

Aspiration therapy for acute embolic occlusion of the superior mesenteric artery

Yi-Ren Liu, Zhu Tong, Cheng-Bei Hou, Shi-Jun Cui, Lian-Rui Guo, Yi-Xia Qi, Li-Xing Qi, Jian-Ming Guo, Yong-Quan Gu

ORCID number: Yi-Ren Liu (0000-0002-5004-1690); Zhu Tong (0000-0001-5266-8721); Cheng-Bei Hou (0000-0002-2146-1899); Shi-Jun Cui (0000-0003-3696-5931); Lian-Rui Guo (0000-0001-8437-2845); Yi-Xia Qi (0000-0002-5761-309X); Li-Xing Qi (0000-0002-0039-579X); Jian-Ming Guo (0000-0001-9390-660X); Yong-Quan Gu (0000-0002-6177-5198).

Author contributions: Gu YQ designed the research and wrote the paper; Liu YR performed the research and wrote the paper; Hou CB contributed to analytic tools and analyzed the data; Tong Z and Cui SJ edited the manuscript; Guo LR, Qi LX, Qi YX, and Guo JM collected the human samples.

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of Xuanwu Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were

Yi-Ren Liu, Zhu Tong, Shi-Jun Cui, Lian-Rui Guo, Yi-Xia Qi, Li-Xing Qi, Jian-Ming Guo, Yong-Quan Gu, Department of Vascular Surgery, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

Cheng-Bei Hou, Center of Evidence-Based Medicine, Xuanwu Hospital, Capital Medical University, Beijing 100053, China

Corresponding author: Yong-Quan Gu, MD, Chief Doctor, Professor, Department of Vascular Surgery, Xuanwu Hospital, Capital Medical University, No. 45, Changchun Street, Beijing 100053, China. 15901598209@163.com
Telephone: +86-15901598209
Fax: +86-10-83125113

Abstract

BACKGROUND

Embolic superior mesenteric artery (SMA) occlusion is associated with high mortality rates. Delayed treatment often leads to serious consequences, including intestinal necrosis, resection, and even patient death. Endovascular repair is being introduced, which can improve clinical symptoms and prognosis and decrease the incidence of exploratory laparotomy. Many reports have described successful endovascular revascularization of embolic SMA occlusion. However, most of those reports are case reports, and there are few reports on Chinese patients. In this paper, we describe the technical and clinical outcomes of aspiration therapy using a guiding catheter and long sheath technique which facilitates the endovascular repair procedure.

AIM

To evaluate the complications, feasibility, effectiveness, and safety of endovascular treatment for the acute embolic occlusion of the SMA.

METHODS

This retrospective study reviewed eight patients (six males and two females) from August 2013 to October 2018 at Xuanwu Hospital, Capital Medical University. The patients presented with acute embolic occlusion of the SMA on admission and were initially diagnosed by computed tomography angiography (CTA). The patients who underwent endovascular treatment with a guiding catheter had no obvious evidence of bowel infarct. No intestinal necrosis was identified by gastrointestinal surgeons through peritoneal puncture or CTA. The complications, feasibility, effectiveness, safety, and mortality were assessed.

obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflicts of interest related to this article.

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RESULTS

Six (75%) patients were male, and the mean patient age was 70.00 ± 8.43 years (range, 60-84 years). The acute embolic occlusion of the SMA was initially diagnosed by CTA. All patients had undertaken anticoagulation primarily, and percutaneous aspiration using a guiding catheter was then undertaken because the emboli had large amounts of thrombus residue. No death occurred among the patients. Complete patency of the suffering artery trunk was achieved in six patients, and defect filling was accomplished in two patients. The in-hospital mortality was 0%. The overall 12-mo survival rate was 100%. All patients survived, and two of the eight patients had complications (the clot broke off during aspiration).

CONCLUSION

Aspiration therapy is feasible, safe, and beneficial for acute embolic SMA occlusion. Aspiration therapy has many benefits for reducing patients' death, resolving thrombi, and improving symptoms.

Key words: Superior mesenteric artery; Acute embolic occlusion; Aspiration embolectomy; Transcatheter thrombolysis; Endovascular repair

Core tip: Percutaneous aspiration embolectomy was recently used to treat embolic superior mesenteric artery (SMA) occlusion. The aim of this study was to assess the utility of endovascular revascularization. Eight patients with acute embolic SMA occlusion underwent aspiration therapy using a guiding catheter. The rate of technical success, clinical success, and adverse events was 100%, 100%, and 25%, respectively. Recurrence was not observed. The median follow-up period after aspiration was 328 d. Aspiration using a guiding catheter achieved immediate revascularization of emboli of the SMA trunk and is a useful tool in the recanalization of embolic occlusion of the SMA in select patients.

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INTRODUCTION

Acute abdominal emergencies are critical, and the pathogenesis is complicated, partly due to acute mesenteric ischaemia (AMI), which comprises 1%-2% of acute abdominal emergencies[1-3]. Embolic superior mesenteric artery (SMA) occlusion is the most common cause of AMI and is associated with high mortality rates[4]. Delayed treatment of SMA occlusion often leads to serious consequences, including intestinal necrosis, resection, and even patient death. Exploratory laparotomy and surgical removal of the thrombus has been the major surgical technique in the past; however, endovascular repair is being introduced, as its efficacy has been proven in clinical trials[5-10]. Intervention improves clinical symptoms and prognosis and decreases the incidence of exploratory laparotomy.

Many reports have described successful endovascular revascularization of embolic SMA occlusion by several endovascular techniques, such as catheter thrombolysis and percutaneous aspiration embolectomy[1,11-17]. However, most of those reports are case reports, and there are few reports on Chinese patients. In this paper, we describe the technical and clinical outcomes of endovascular repair, namely, aspiration therapy using a guiding catheter, in eight Chinese patients with embolic SMA occlusion.

MATERIALS AND METHODS

Patients

This study was a retrospective analysis in our institution. From August 2013 to October 2018, eight patients with SMA embolism, including six males and two females and ranging in age from 60 to 84 years (mean age, 70.00 ± 8.43 years), were treated by transcatheter aspiration therapy at Xuanwu Hospital. All patients were initially diagnosed by computed tomography angiography (CTA, Figure 1). The patients who underwent endovascular treatment had no obvious evidence of bowel infarct (Figure 2). No intestinal necrosis was identified by gastrointestinal surgeons through peritoneal puncture or CTA.

Aspiration technique

All operations were performed by an experienced vascular surgeon. Under local anaesthesia, the right common femoral artery was punctured according to the Seldinger technique and an 8-Fr short sheath (Introducer II; Terumo) was implanted. Then, heparin was administered (50 IU/kg, North China Pharmaceutical Company Ltd, China) *via* a short sheath. An additional 1000 IU was administered every hour. Selective catheterization of the SMA with the 5-Fr Cobra catheter (C1, Cook, Bloomington, IN, United States) or the SIMON 5-Fr catheter (SIM1, Selecon; Terumo, Tokyo, Japan) was performed. Through the catheter, angiography was performed to confirm the SMA embolism (Figure 3). A hydrophilic guidewire (Radifocus, Terumo, Tokyo, Japan) was navigated into the distal segment of the SMA. The short sheath replaced the 8-Fr long sheath of 65 cm (Super Arrow Flex PSI set, Arrow International, Reading, PA, United States), and the long sheath was inserted into the orifice of the SMA. The 8-Fr long sheath was left in the proximal section of the SMA. The 5-Fr catheter of 110 cm in length (DAV, Cook) was inserted into the 6F guiding sheath of 90 cm in length (BRITE TIP; Cordis, Miami Lakes, Florida), and the 6-Fr guiding catheter and the 5F catheter were advanced coaxially to the SMA over the guidewire. The 5-Fr catheter was removed and the 6-F guiding catheter reserved for aspiration of the emboli. A 50-mL syringe was connected to the 6-Fr guiding catheter. When the guiding catheter became occluded with the emboli, it was withdrawn slightly with pumping until blood was aspirated. The 6F guiding sheath was flushed thoroughly with saline solution into gauze so that the thrombus could be found (Figure 4). The guiding catheter was reinserted and the procedure repeated. The emboli of the branches were treated using a 5-F catheter. Urokinase (250000 IU, Tianjin Biochemical Pharmaceutical Co., Ltd.) was infused into the SMA through the catheter to resolve residual emboli. Papaverine (30 mg, North China Pharmaceutical Company, Ltd, China) was infused into the SMA through the catheter to resolve vasospasm.

One patient underwent catheter-directed thrombolysis (CDT). Thrombolysis was performed using a multiple-sidehole infusion catheter (Multi-Sideport, Cook) *via* the SMA with urokinase at a rate of 50000 IU/h to downsize the emboli. Thrombolysis was monitored by fibrinogen (fibrinogen value was larger than 1 g/L). Catheter-directed local anticoagulation with heparin sodium was continuously administered into the SMA as well. Dosage should be adapted to patients individually on the basis of tests of activated partial thromboplastin time (APTT 1.5-2.0 times normal value). An angiograph of thrombolysis efficiency was performed 48 h after the intervention.

Follow-up after aspiration

All patients had clinical and imaging follow-up, which was performed every 3 mo one year after operation and then every 6 mo until death or October 31, 2018. During the follow-up, all patients were evaluated by clinical symptoms and signs, laboratory tests of blood routine examination, and CTA or ultrasonography of the SMA.

Assessment of outcomes

We reviewed the case file records. The clinical outcomes included feasibility, effectiveness, complications, clinical symptoms and signs, laparotomy, bowel resection, hospital stay, mortality and recurrence, SMA perfusion observed by CTA, and digital subtraction angiography (DSA).

Definitions

Absence or presence of peritonitis was determined by clinical abdominal examination. Embolism was considered according to atrial fibrillation and a history of embolism. Degree of thrombus removal was divided into complete and partial. Complete thrombus removal referred to complete patency of the SMA and sufficient perfusion of the entire bowel, and partial thrombus removal referred to residual emboli or sluggish flow of the SMA.

Statistical analysis

The statistical methods of this study were reviewed by Cheng-Bei Hou from Center of

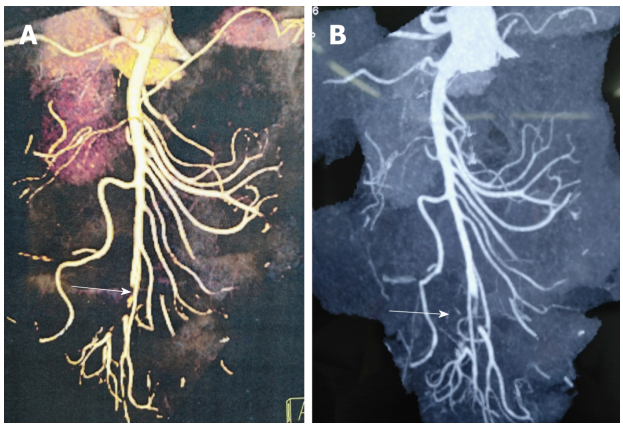

Figure 1 Computed tomography angiography images. A and B: Acute embolic occlusion of the superior mesenteric artery indicated by the white arrows.

Evidence-Based Medicine, Xuanwu Hospital, Capital Medical University. Data management and statistical analyses were performed using SPSS 19.0 software (SPSS Inc, Chicago, IL, United States). Continuous variables were tested for normal distribution. Normally distributed variables are expressed as the mean ± standard deviation (SD), whereas non-normally distributed variables are reported as median. Categorical variables are reported as counts with proportions.

RESULTS

The patients included six men and two women, with an age range from 60 to 84 years (mean age, 70.00 ± 8.43 years). SMA embolism was initially diagnosed by a vascular surgeon considering clinical manifestation and CTA findings. All patients were seen in consultation with a gastrointestinal surgeon and vascular surgeon. No intestinal necrosis was identified by doctors considering clinical symptoms, signs, peritoneal puncture, and CTA findings. After admission, all patients received low molecular weight heparin (LMWH) (100 IU/kg Sanofi Winthrop Industrie, France) anticoagulant therapy. Routine blood examination was performed, including white blood cell (WBC), neutrophilic granulocyte, red blood cell (RBC), and platelet (PLT) counts and hepatic and renal function analyses. The clinical characteristics of patients are shown in Table 1.

The median white cell blood count was 17.25 × 10^9/L (range from 15.3 to 24.0 × 10^9/L), C-reactive protein (CRP) was 27.60 mg/L (range from 5 to 52 mg/L), platelet concentration was 264.35 × 10^9/L (range from 128 to 385 × 10^9/L), and the glomerular filtration rate (GFR) was 81.24 mL/min (range from 75 to 96 mL/min). No renal insufficiency was observed.

The interval between the onset of symptoms and the acquisition of angiography ranged from 9 to 30 h (median time, 9.50). Eight complete SMA trunk occlusions were detected by angiography *via* the catheter. In one patient (No. 2), emboli were noted in the jejunal artery branches. Six patients (Nos. 1 and 4-8) had good collateral flow. One patient (No. 2) had slow collateral flow, and one patient (No. 3) had no collateral flow (Table 2).

Percutaneous aspiration embolectomy using a guiding catheter was performed in all eight patients. The total procedure time from the initial diagnostic angiography to the final angiography was 53-85 min (mean time, 72.00 ± 13.70). Seven (Nos. 1, 2, 4, 5, and 6-8) patients initially underwent aspiration embolectomy, and thrombolysis was initially performed in one patient (No. 3). Thrombolysis was initially performed in the early study period because of the patient had heart failure, and primary aspiration embolectomy using a guiding catheter was performed in the late period as some thrombolysis was found 3 d after urokinase injection. In one patient (No. 2), primary percutaneous aspiration embolectomy was attempted, but residual emboli were noted in the jejunal artery branches. Intra-artery transcatheter thrombolytic therapy with urokinase was performed for the treatment of residual emboli. Thrombolysis was conducted for 3 d, resulting in complete resolution of the emboli. Follow-up angiography showed reestablishment of arterial flow. In two patients (Nos. 1 and 4), primary percutaneous aspiration embolectomy was applied, and the two patients received primary thrombolysis with urokinase (250000) during the operation because

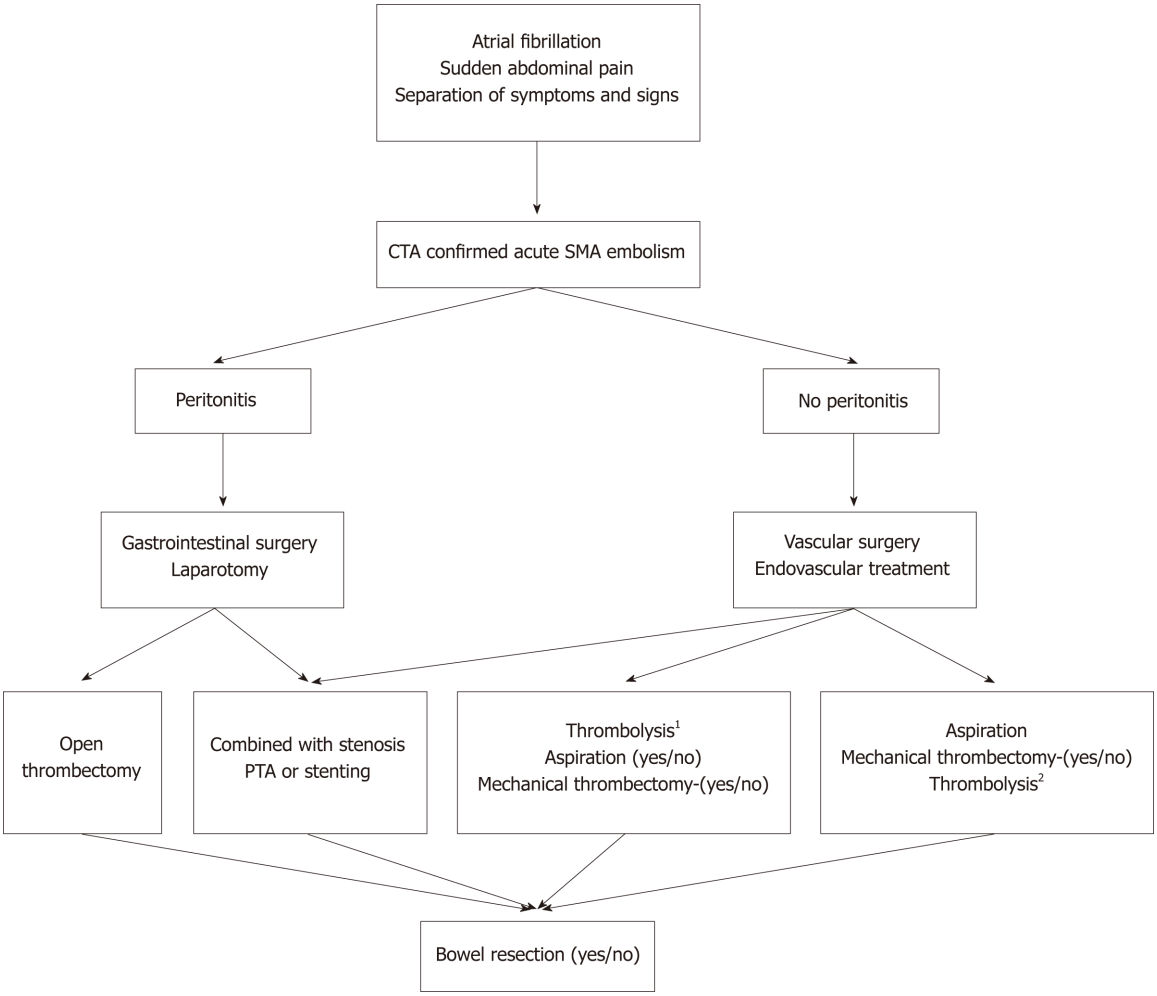

Figure 2 The processing flow of acute embolic occlusion of the superior mesenteric artery. [1]Patients with poor general condition first underwent thrombolysis; [2]Residual thrombus was treated by thrombolysis during the operation or catheter-directed thrombolysis after aspiration. PTA: Percutaneous transluminal angioplasty; CTA: Computed tomography angiography; SMA: Superior mesenteric artery.

of the clot breaking off. PTA was performed in one patient (No. 4) because of SMA stenosis. Aspirated emboli consisted of white and red clots. At completion of the SMA aspiration, significantly improved filling of the SMA was seen in all eight patients. Aspiration thrombectomy of the SMA resulted in complete (Nos. 1, 2, and 4-7) or partial (Nos. 3 and 8) restoration of blood flow in the main SMA, which was documented on immediate direct SMA angiography. Primary percutaneous aspiration embolectomy was applied for two patients (Nos. 1 and 4), and the two patients received primary thrombolysis with urokinase (250000) during the operation because of the clot breaking off. The detached clots did not result in intestinal ischaemia, as collateral flow to the jejunal/ileal branches was good (Table 3).

Substantial improvement in abdominal pain was observed within 1-2 d after the operation in all patients. Sufficient clinical improvement, characterized by a progressive decrease in abdominal pain and distention, was observed in all patients. Oral nutrition intake was started at 2-17 (7.75 ± 5.65) d. The eight patients were discharged 9-17 (12.25 ± 3.11) d after admission. The in-hospital mortality was 0%. Abdominal pain, nausea, distention, haematochezia, and diarrhoea were completely resolved when the patients were discharged. CTA images obtained before discharge demonstrated nearly complete recanalization of SMA thrombosis in all patients, with improvement in oedema of the intestine in all patients. Heart failure of one patient (No. 3) was improved. One patient (No. 4) suffered from left cerebral infarction because of an embolism aroused by atrial fibrillation, and symptoms improved after discharge (Table 4).

The median length of time of follow-up was 328 (range, 90-390) months. One patient (No. 4) developed mild abdominal pain 3 mo after surgery because of SMA stenosis, and other patients persisted asymptomatically. Routine blood tests were normal. No patients required extensive bowel resection. No thrombus recurrence was found under regular anticoagulation. Warfarin or rivaroxaban was applied orally in

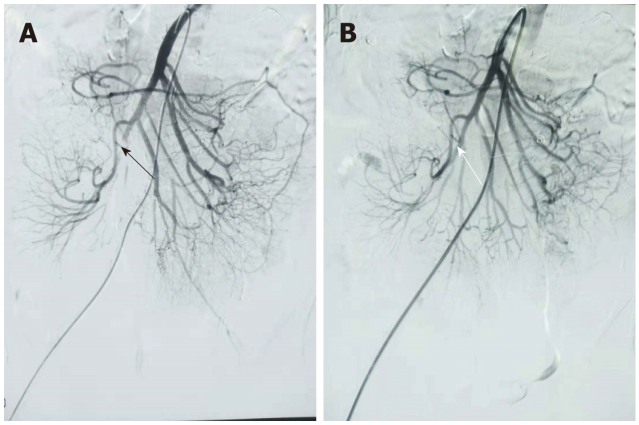

Figure 3 Digital subtraction angiography images. A: Filling defect of the superior mesenteric artery (SMA) indicated by the black arrow; B: Complete patency of the SMA indicated by the white arrow.

all patients at least 6 mo after discharge if there were no risk factors for recurrence. During the follow-up, ultrasonography confirmed the blood flow perfusion of the SMA.

DISCUSSION

Percutaneous aspiration using a guiding catheter for acute embolic occlusion of the SMA may lead to dissection of the SMA. The dissection restricting blood flow requires emergency treatment. Dissection of the SMA may be caused by the gap between the guidewire and the guiding catheter. The gap between the guidewire and the guiding catheter should be reduced. Since there were no dilators that fit the guiding catheter, a smaller catheter was used as a dilator. There is no gap between the 6-Fr guiding catheter and the 5-Fr catheter; thus, the 6-Fr guiding catheter was used to remove the SMA emboli, and the 5-Fr catheter was used to remove the emboli in the branches of the SMA. From two studies performed by Acosta *et al*[11] and Kawasaki *et al*[14], we conclude that larger catheters lead to increased intima dissection. SMA dissection was not found in any patients in our study. With a careful operation and coaxial advancing of the guiding catheter and a seamless dilator, the incidence of SMA dissection can be reduced. It is crucial to select the appropriate guiding catheter according to the SMA diameter. As for difficult transcutaneous cases, it is a good choice using a hybrid approach. SMA puncture was practiced under genuine direct vision can avoid dissection of the SMA. Furthermore, the intestine can be detected through laparotomy.

All four patients successfully underwent aspiration. Raupach *et al*[18] analysed 37 patients with acute mesenteric embolism who underwent primary endovascular therapy, and achieved complete recanalization of the SMA trunk in 91.9% of cases. Our technique used a long sheath compared with other reports on the treatment of trunk lesions. The long sheath was inserted into the orifice of the SMA, and the long sheath was left in the proximal section of the SMA. The guiding catheter was inserted into the SMA repeatedly and immediately through the long sheath. Using a long sheath both saves time and avoids SMA dissection.

A distal embolism may develop during advancement of the catheter. As for the SMA trunk, the therapeutic effects of thrombolysis are uncertain. Björnsson *et al*[9] reported a feature that successful thrombolysis was achieved in 30 patients; 13 explorative laparotomies, 10 repeat laparotomies, and 8 bowel resections were performed; and the in-hospital mortality rate was 26%. Boo-Gyoung *et al*[19] initially tried endovascular thrombolytic therapy, but it did not achieve complete revascularization. Therefore, they performed a percutaneous aspiration thrombectomy, which led to complete revascularization without any additional procedures. However, for branch arteries, the thrombolysis was useful[20].

Heiss *et al*[13] reported that SMA aspiration showed a 30-d mortality rate of 33%. In another paper, 1 patient died at 12 h, and another patient died of short bowel syndrome at 8 mo[11]. Kawasaki *et al*[14] reported a 30-d mortality rate of 14% (1 of 7 patients). The patients in our study recovered quickly. No patients needed bowel resection. We think that the low morbidity of our study might be explained by the following: (1) patients were diagnosed early by CT; and (2) patients had relatively

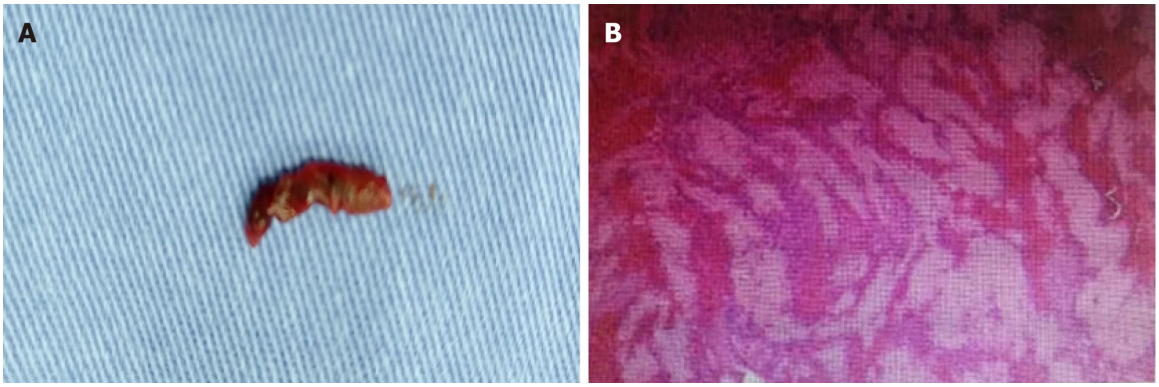

Figure 4 Embolus images. A: Aspirated embolus; B: Pathology consisting of white and red clots.

mild symptoms and signs, because severe patients were admitted to undergo gastrointestinal surgery. The initial treatment modality should be decided by consensus between gastrointestinal and vascular surgeons considering the patient's symptoms and signs, CT findings, laboratory results, and clinical experiences[21]. We first tried endovascular treatment if the CT scan had no obvious evidence of bowel infarct[22]. Rebound tenderness may suggest bowel necrosis and may lead to exploratory laparotomy. However, endovascular treatment may make surgical laparotomy unnecessary or may reduce surgical procedure and time. Thus, we adopted endovascular treatment first in seven patients (7/8). Choi *et al*[23] reported that nine patients with embolic occlusion of the SMA were treated by percutaneous aspiration embolectomy, and no patients had obvious evidence of bowel infarction on CT scans. One patient died of whole bowel necrosis and sepsis, and eight patients survived without complications.

The aspiration method that uses the long sheath technique might therefore be more feasible than thrombolysis[1]; it may also be more feasible than surgical embolectomy[10]. Several other devices can be used to remove blood clots in the SMA, for example, a Rotarex systema mechanical rotational thrombectomy device[24]. These devices are also effective in the removal of blood clots, but they may lead to complications and increase medical costs. Bruno Freitas *et al* reported complications represented by self-limited small perforations with a 6F Rotarex Debulking Device (Straub Medical, Wangs, Switzerland)[25]. Percutaneous mechanical thrombectomy seems to be a rapid and effective treatment for acute SMA embolism in the median portion of its trunk[26]. Aspiration using a guiding catheter is inexpensive and effective according to several studies, including the current study[11,27].

Follow-up of patients for 1 year detected no cases of recurrence. One patient (No. 4) developed mild abdominal pain 3 mo after surgery because of SMA stenosis. The long-term consequences were good because of early revascularization. Echocardiography was performed in all patients, but thrombus was not detected in the left atrium. Anticoagulation drugs were prescribed to all patients to prevent re-embolism regardless of the echocardiography findings.

The main limitation of our study is the analysis of a small number of enrolled patients. Moreover, the peritonitis patients were admitted for gastrointestinal surgery; thus, a comparative study between open surgery treatment and endovascular treatment was not conducted. Third, thrombolysis and aspiration require further study.

Emboli resulting in embolic occlusion of the SMA often come from the atrium. Aspiration using a guiding catheter can remove most of the clots, and aspiration can achieve immediate revascularization of emboli of the SMA trunk. Thrombolysis can deal with residual fresh blood clots. However, with regard to old thrombi, which cannot be cleared by aspiration, further studies are needed.

Table 1 Characteristics of the patients

Patient No.	Sex	Age (yr)	Basal disease	Symptoms	Signs (tenderness)	Signs (re-bound tenderness)	WBC count (×10³/μL)	Echocardiography (LA thrombus)
1	M	60	HBP	Abdominal pain, vomiting, dark stool	Present	Present	15.3	Absent
2	M	68	Af, DM	Abdominal pain, diarrhea	Present	Present	17.4	Absent
3	F	71	Af, CAD, HF	Abdominal pain, hematochezia	Present	Present	24.0	Absent
4	M	84	DM	Abdominal pain	Present	Absent	17.8	Absent
5	M	61	Af	Abdominal pain	Present	Absent	15.1	Absent
6	M	66	Af, DM	Abdominal pain, diarrhea	Present	Present	16.9	Absent
7	F	70	HBP	Abdominal pain, dark stool	Present	Present	23.5	Absent
8	M	80	HBP, DM	Abdominal pain, vomiting	Present	Absent	17.1	Absent

Normal range: WBC count = 4.0-10.0 (× 10^9/L). M: Male; F: Female; WBC: White blood cell; Af: Atrial fibrillation; CAD: Coronary artery disease; HF : Heart failure; HBP: High blood pressure; DM: Diabetes mellitus; LA: Left atrium.

Table 2 Digital subtraction angiography results

Patient No.	CTA	Time from onset totreatment (h)	Occlusion of main SMA trunk	Branch lesion location(s)	Collateral flowto jejunal/ileal branches
1	Filling defect, mild bowel oedema, mild ileus	9	Complete occlusion	None	Good
2	Filling defect, mild bowel oedema, mild ileus	10	Complete occlusion	Jejunal arteries	Slow
3	Filling defect, mild bowel oedema, scanty ascites, mild ileus	30	Complete occlusion	None	Absent
4	Filling defect, mild bowel oedema, mild ileus	7	Complete occlusion; SMA stenosis	None	Good
5	Filling defect, mild bowel oedema, mild ileus	8	Complete occlusion	None	Good
6	Filling defect, mild bowel oedema, mild ileus	11	Complete occlusion	None	Good
7	Filling defect, mild bowel oedema, mild ileus	6	Complete occlusion	None	Good
8	Filling defect, mild bowel oedema, mild ileus	28	Complete occlusion	None	Good

CTA: Computed tomography angiography; SMA: Superior mesenteric artery.

Table 3 Summary of interventions and clinical outcomes

Patient No.	Total procedure time (min)	Trunk lesion	Branch lesion location(s)	Additional treatment	Complications
1	85	Successful	The clot breaking off and ileal arterial embolism, good flow	Thrombolysis with urokinase during the operation	The clot breaking off
2	85	Successful	Multiple residual emboli in jejunal arteries, slow flow	Intra-artery transcatheter thrombolytic therapy with urokinase successful	-
3	50	Partial rec-analization	-	thrombolysis was initially performed before aspiration embolectomy	-
4	75	Successful (PTA)	The clot breaking off and ileocolic artery emboli.	primary thrombolysis with urokinase during the operation	The clot breaking off
5	79	Successful	-	-	-
6	80	Successful	-	-	-
7	53	Successful	-	-	-
8	69	Partial recanalization	-	-	-

PTA: Percutaneous transluminal angioplasty.

Table 4 Postoperative situations

Patient No.	The time of feed (d)	Hospital stay (d)	In-hospital mortality	Symptoms	Signs (tenderness)	Signs (rebound tenderness)
1	2	9	None	Abdominal symptom resolved in 1 d	Present	Present
2	6	12	None	Diarrhoea developed after aspiration but subsided spontaneously; Abdominal symptom resolved in 1 d	Present	Present
3	6	12	None	Haematochezia developed after aspiration but subsided spontaneously; Abdominal symptom resolved in 1 d	Present	Present
4	17	17	None	Abdominal symptom resolved in 1 d	Present	Absent
5	5	8	None	Abdominal symptom resolved in 1 d	Present	Absent
6	16	11	None	Diarrhoea lasted 2 d	Present	Present
7	7	13	None	Abdominal symptom resolved in 2 d; Dark stool lasted 3 d	Present	Present
8	3	16	None	Abdominal symptom resolved in 2 d	Present	Absent

ARTICLE HIGHLIGHTS

Research background

Embolic superior mesenteric artery (SMA) occlusion is associated with high mortality rates. Delayed treatment often leads to serious consequences, including intestinal necrosis, resection, and even patient death. Endovascular repair is being introduced, which can improve clinical symptoms and prognosis and decrease the incidence of exploratory laparotomy. Many reports have described successful endovascular revascularization of embolic SMA occlusion. However, most of those reports are case reports, and there are few reports on Chinese patients. In this paper, we describe the technical and clinical outcomes of aspiration therapy using a guiding catheter and long sheath technique which facilitates the endovascular repair procedure.

Research motivation

To evaluate the complications, feasibility, effectiveness, and safety of endovascular treatment using a guiding catheter for the acute embolic occlusion of the SMA.

Research objectives

Many reports have described successful endovascular revascularization of embolic SMA occlusion by several endovascular techniques. However, most of those reports are case reports. There are few reports on Chinese patients. In this paper, we describe the technical and clinical outcomes of aspiration therapy using a guiding catheter and long sheath technique which facilitates the endovascular repair procedure.

Research methods

This retrospective study reviewed patients with acute embolic occlusion of the SMA. All patients were treated by aspiration therapy with a guiding catheter. The complications, feasibility, effectiveness, safety, and mortality were assessed.

Research results

All patients had successfully undertaken percutaneous aspiration using a guiding catheter. No death occurred among the patients. Most of the clots were removed and patency of the suffering artery trunk was achieved. Although the complication of the clot breaking off was detected in partial patients, blood perfusion was not affected.

We need a large number of enrolled patients and conduct a comparative study between open surgery treatment and endovascular treatment. Furthermore, thrombolysis can deal with fresh blood clots. However, with regard to old thrombi, which cannot be cleared by aspiration, further studies are needed.

Research conclusions

Aspiration therapy is feasible, safe, and beneficial for acute embolic SMA occlusion. Aspiration therapy using a guiding catheter and long sheath technique facilitates the endovascular repair procedure. Aspiration therapy has many benefits for reducing patients' death, resolving thrombi, and improving symptoms.

Research perspectives

Aspiration therapy using a guiding catheter and long sheath technique is feasible, safe and beneficial for acute SMA embolic occlusion, which should be applied and popularized. Especially, auxiliary applications of a long sheath technique facilitate operation procedure. Tender operation is needed to avoid the clot breaking off. However, with regard to old thrombi, which cannot be cleared by aspiration, further studies are needed. A randomized controlled trial comparing open surgery treatment and endovascular treatment is needed to be conducted in the future.

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ORIGINAL ARTICLE

Retrospective Study

Δ4-3-oxosteroid-5β-reductase deficiency: Responses to oral bile acid therapy and long-term outcomes

Mei-Hong Zhang, Kenneth DR Setchell, Jing Zhao, Jing-Yu Gong, Yi Lu, Jian-She Wang

ORCID number: Mei-Hong Zhang (0000-0001-9656-7180); Kenneth DR Setchell (0000-0002-2472-2476); Jing Zhao (000-0002-3208-9009); Jing-Yu Gong (000-0002-1149-0979); Yi Lu (0000-0002-3311-4501); Jian-She Wang (0000-0003-0823-586X).

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Mei-Hong Zhang, Jing-Yu Gong, Department of Pediatrics, Jinshan Hospital, Fudan University, Shanghai 201508, China

Kenneth DR Setchell, Department of Pathology and Laboratory Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, United States

Jing Zhao, Yi Lu, Jian-She Wang, The Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, Shanghai 201102, China

Jing Zhao, Yi Lu, Jian-She Wang, Department of Pediatrics, Shanghai Medical College of Fudan University, Shanghai 201102, China

Corresponding author: Jian-She Wang, MD, PhD, Professor, The Center for Pediatric Liver Diseases, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China. jshwang@shmu.edu.cn
Telephone: +86-21-64931171
Fax: +86-21-64931901

Abstract

BACKGROUND

Disorders of primary bile acid synthesis may be life-threatening if undiagnosed, or not treated with primary bile acid replacement therapy. To date, there are few reports on the management and follow-up of patients with Δ4-3-oxosteroid 5β-reductase (AKR1D1) deficiency. We hypothesized that a retrospective analysis of the responses to oral bile acid replacement therapy with chenodeoxycholic acid (CDCA) in patients with this bile acid synthesis disorder will increase our understanding of the disease progression and permit evaluation of this treatment regimen as an alternative to the Food and Drug Administration (FDA) approved drug cholic acid, which is currently unavailable in China.

AIM

To evaluate the therapeutic responses of patients with AKR1D1 deficiency to oral bile acid therapy, specifically CDCA.

METHODS

Twelve patients with AKR1D1 deficiency, confirmed by fast atom bombardment ionization-mass spectrometry analysis of urine and by gene sequencing for mutations in *AKR1D1*, were treated with differing doses of CDCA or ursodeoxycholic acid (UDCA). The clinical and biochemical responses to therapy were monitored over a period ranging 0.5-6.4 years. Dose adjustment, to optimize the therapeutic dose, was based on changes in serum biochemistry parameters,

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notably liver function tests, and suppression of the urinary levels of atypical hepatotoxic 3-oxo-Δ4-bile acids measured by mass spectrometry.

RESULTS

Physical examination, serum biochemistry parameters, and sonographic findings improved in all 12 patients during bile acid therapy, except one who underwent liver transplantation. Urine bile acid analysis confirmed a significant reduction in atypical hepatotoxic 3-oxo-Δ4 bile acids concomitant with clinical and biochemical improvements in those patients treated with CDCA. UDCA was ineffective in down-regulating endogenous bile acid synthesis as evidenced from the inability to suppress the urinary excretion of atypical 3-oxo-Δ4-bile acids. The dose of CDCA required for optimal clinical and biochemical responses varied from 5.5-10 mg/kg per day among patients based on maximum suppression of the atypical bile acids and improvement in serum biochemistry parameters, and careful titration of the dose was necessary to avoid side effects from CDCA.

CONCLUSION

The primary bile acid CDCA is effective in treating AKR1D1 deficiency but the therapeutic dose requires individualized optimization. UDCA is not recommended for long-term management.

Key words: Δ4-3-oxosteroid-5β-reductase deficiency; Mass spectrometry; Bile acid synthesis disorder; Chenodeoxycholic acid; Ursodeoxycholic acid

Core tip: Δ4-3-oxosteroid 5β-reductase (AKR1D1) deficiency presents as particularly severe and rapidly progressive cholestasis. Treatment with oral primary bile acid has been shown to be effective in normalizing liver function, circumventing the only alternative treatment of liver transplantation. The primary bile acid cholic acid is an approved drug for treating this genetic defect but is not available in China and many other countries. Here we report on the use of the alternative primary bile acid, chenodeoxycholic acid, in the largest cohort of patients with AKR1D1 deficiency studied to date, showing beneficial effects in a personalized regimen approach.

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INTRODUCTION

Inborn errors in primary bile acid synthesis from cholesterol now represent a specific category of metabolic liver disease[1,2]. The Δ4-3-oxosteroid 5β-reductase (AKR1D1) deficiency[3] [also referred to as 5β-reductase deficiency, congenital bile acid synthesis defect type 2 (CBAS 2), MIM604741] is the second most common of these bile acid synthesis disorders. Unlike the more commonly diagnosed 3β-hydroxy-Δ5-C27-steroid oxidoreductase (HSD3B7) deficiency which frequently accounts for late-onset chronic cholestasis[4,5], the AKR1D1 deficiency tends to be diagnosed in early infancy and presents as particularly severe and rapidly progressive cholestasis[6]. Early diagnosis of these defects is consequently crucial, because the related liver disease may be life-threatening, but is treatable by oral primary bile acid replacement[2,4,7-11].

Due to the failure to synthesize primary bile acids in the AKR1D1 deficiency and the production instead of a spectrum of highly cholestatic and hepatotoxic 3-oxo-Δ4 bile acids[3], bile flow is impaired, leading to cholestasis. Since bile acids are essential for micellar solubilization of lipids, fat and fat-soluble vitamin deficiency occurs quite often.

The AKR1D1 deficiency was first discovered by the application of fast atom bombardment ionization mass spectrometry (FAB-MS) and based on the detection of increased concentrations of 3-oxo-Δ4 bile acids in urine concomitant with a lack of

normal primary bile acid conjugates[11]. Following the cloning of the *AKR1D1* gene[7], genetic analysis is now a clinically useful tool for confirming the diagnosis based on identified mutations in the *AKR1D1* gene[7]. Oral primary bile acid therapy is now the therapy of choice for treating bile acid synthesis disorders, circumventing the need for liver transplantation, which is the only alternative in these progressive and frequently fatal forms of cholestasis. Although cholic acid (CA) has been approved by the United States Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) for the treatment of bile acid synthesis disorders, the data of its effects on patients with AKR1D1 deficiency is very limited[7,8]. At the same time, this drug is presently unavailable to many patients due to the limit of accessibility and affordability. We now describe the clinical and biochemical responses to another primary bile acid - chenodeoxycholic acid (CDCA) therapy in 12 patients, the largest cohort to date, diagnosed with AKR1D1 deficiency based on genetic and mass spectrometric identifications. We report on the effectiveness of CDCA therapy in this bile acid synthesis disorder, and the lack of effect of ursodeoxycholic acid (UDCA) in patients treated with the latter.

MATERIALS AND METHODS

Patients and study design

From December 2012 to April 2017, 12 patients with AKR1D1 deficiency (Table 1) were identified by urinary FAB-MS analysis at Cincinnati Children's Hospital Medical Center from the screening of 193 patients with unexplained cholestasis. Gene sequencing performed at the Department of Pediatrics of Jinshan Hospital of Fudan University and the Center for Pediatric Liver Disease of the Children's Hospital of Fudan University confirmed mutations in *AKR1D1*, in all 12 patients (Table 1).

With the exception of patients 2 and 8, the other ten patients were from non-consanguineous healthy families. Blood samples were collected after parenteral administration of vitamin K when they were transferred to our hospitals for evaluation. Investigations of all patients for congenital infection, including toxoplasmosis, rubella, cytomegalovirus, herpes simplex and hepatotropic virus, and metabolic disease, including tyrosinaemia, galactosaemia and α1-antitrypsin deficiency, were negative. All patients had jaundice and dark urine. The clinical, biochemical, and physical information at referral is summarized in Table 1. All presented with varying degrees of conjugated hyperbilirubinemia, elevated serum transaminases, normal or slightly elevated γ-glutamyltranspeptidase (GGT), and low or normal serum total bile acids. All patients had hepatomegaly, and three had splenomegaly (Table 1). Evaluations at the beginning and follow-up included physical examination, serum biochemistry tests, and urinary bile acid analysis by FAB-MS. After initiating bile acid therapy, the patients were seen on a weekly basis in order to make dose adjustments based on the serum biochemistry parameters to minimize possible hepatotoxicity of the medications. When their clinical status stabilized, the patients were seen every 2-4 wk and finally after normalization of serum biochemistries, every 6 mo thereafter.

MS bile acid analysis

Urine samples were collected before treatment with primary bile acid. In those patients that had been treated with UDCA prior to the initial presentation, the therapy was stopped for 5-7 d before urine was collected. The initial negative ion FAB-MS spectrum of the urine was used to confirm the diagnosis of an AKR1D1 deficiency before treatment was started with CDCA. Confirmation of the AKR1D1 deficiency was based on the finding of a lack or absence of normal primary bile acids and the presence of elevated levels of taurine and/or glycine conjugated Δ4-3-oxo bile acids[2,3]. Urine was collected at frequent intervals during the course of therapy with CDCA in order to monitor the extent of suppression of atypical Δ4-3-oxo bile acids and to confirm compliance to therapy. Dose adjustments were optimized to achieve maximum suppression of atypical bile acids.

Genetic analysis

With approval by the Ethics Committees on Human Research of the Jinshan Hospital of Fudan University, and of the Children's Hospital of Fudan University, and after obtaining informed consent from the parents, a 1.5-mL peripheral blood sample was obtained. To confirm the diagnosis and establish the molecular basis of the disorder, genomic DNA was isolated from white blood cells. Prior to December 2015, all of the coding exons and adjacent introns of the *AKR1D1* gene were Sanger sequenced. After January 2016, panel sequencing[12] and Sanger confirmation were performed.

Table 1 Clinical and laboratory[1] features of the 12 patients diagnosed with Δ4-3-oxosteroid 5β-reductase deficiency at first presentation

Patient No.	Sex	Age at first symptom	Age at first visit (mo)	TB (μmol/L)	DB (μmol/L)	ALT (U/L)	AST (U/L)	GGT (U/L)	TBA (μmol/L)	Organomegaly	INR[2]	Molecular defect[3]
1	F	14 d	2.5	292	214	169	309	46	4.6	Hepatomegaly	1.23	c.933delG/c.1023+5A>C
2	M	1 mo	11	146	108	101	405	40	1.6	Hepatosplenomegaly	1.17	c.158A>G/c.158A>G
3	M	3 d	2	96	64	176	183	33	3	Hepatomegaly	1.03	c.396C>A/c.722A>T
4	M	3 d	1	310	218	76	213	51	5.5	Hepatomegaly	1.52	c.919G>T/c.919G>T
5	M	10 d	10	146	113	210	207	65	6.1	Hepatosplenomegaly	1.14	c.797G>A/-
6	F	1 mo	8	324	252	267	584	108	1.4	Hepatomegaly	2.17	c.148C>T/-
7	M	1 mo	8	211	126	234	734	66	7.9	Hepatomegaly	1.1	c.797G>A/C.797G>A
8	M	3 d	1	320	158	523	703	51	2.5	Hepatomegaly	1.36	c.797G>A/C.797G>A
9	F	10 d	1	378	232	1317	1416	62	8.5	Hepatomegaly	1.9	c.614delT/C.797G>A
10	M	3 d	2	160	126	531	896	64	5.9	Hepatosplenomegaly	1.28	c.593C>T/c.797G>A
11	M	3 d	3	125	76	79	161	38	1.4	Hepatomegaly	1.02	c.278delA/-
12	M	1 mo	2.5	204	112	339	619	50	1.8	Hepatomegaly	2.11	c.919C>T/C.919C>T
	Normal range			5-21	0-3.4	0-40	0-40	7-50	0-10		0.8-1.2	

[1]At least 5 d after cessation of bile acid administration;
[2]After vitamin K1 parenteral administration;
[3]NM_005989 was used as reference. TB: Total bilirubin; DB: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate transferase; GLT: Gamma-glutanyl transferase; ALB: Albumin.

Molecular data are listed in Table 1 with NM_005989 as reference.

Treatment

With the exception of patients 2, 10, and 12, all other patients had received UDCA therapy before being referred for evaluation (Table 2). Fat-soluble vitamins were routinely supplemented for all patients after admission. With the informed consent from the parents, UDCA therapy was terminated, urine collections were obtained, and patients were then switched to the primary bile acid CDCA at an initial dose of 5-12 mg/kg bw/d (usually lower initial dose in more severe cases), taken in two divided doses. Baseline urine samples were not available for mass spectrometric analysis from patients 3 and 5. Patient 5 declined treatment with CDCA and continued with a high dose of UDCA (40 mg/kg per day). Patient 3 also continued to take UDCA (30 mg/kg per day) but after 4 mo was switched to CDCA because of abnormal bile acid profiles, even though the biochemistries improved. The initial dose of CDCA was adjusted based on the findings from the urine bile acid analyses and serum biochemistry parameters, including transaminases and GGT. Generally, the dose was reduced if serum alanine aminotransferase (ALT) and aspartic transaminase (AST) became elevated with concomitant elevated serum total bile acids level, and conversely was increased if there was inadequate suppression of atypical bile acids as demonstrated by FAB-MS, even after the resolution of jaundice. The primary bile acid administration dosage and duration of treatment for each patient are summarized in Tables 2 and 3.

Table 2 Evolution of liver function tests and international normalized ratio during oral ursodeoxycholic acid therapy in nine children[1] with Δ4-3-oxosteroid 5β-reductase deficiency

Patient No.	Starting age	Dosage (d.kg)/duration of therapy	Before UDCA administration				At the end of UDCA therapy			
			TB / DB (μmol/L)	ALT/AST (U/L)	ALB (g/L)	INR	TB/DB (μmol/L)	ALT/AST (U/L)	ALB (g/L)	INR
1	2.5 mo	20 mg/3 mo	241/177	73/172	38	1.23	292/214	169/309	33	1.46
3	2 mo	30 mg/4 mo	96/64	176/183	43	1.03	8.7/5.9	51/29	44	n.a.
4	1 mo	20 mg/1.5 mo; 40 mg/1 wk	150/58	76/159	35	1.43	310/218	76/273	36	1.52
5	10 mo	18 mg/1 mo; 40 mg/4 mo	126/66	340/262	49	n.a.	6.7/2.9	8/16	42	n.a.
6	8 mo	20 mg/6 mo	221/142	235/204	32	1.24	324/252	267/584	33	2.17
7	8 mo	10 mg/2 mo	268/121	532/897	38	1.16	19/15	100/124	52	0.9
8	1 mo	16 mg/6 mo	308/131	150/187	41	1.13	273/148	448/410	28	1.36
9	1 mo	18 mg/3 mo	316/243	1240/1565	37	1.71	358/258	195/283	32	n.a.
11	3 mo	18 mg/2 mo	122/87	106/155	n.a.	1.02	85/68	83/167	39	n.a.
		Normal range	5-21/0-3.4	0-40	35-52	0.8-1.2	5-21/0-3.4	0-40	35-52	0.8-1.2

[1]Patients 2, 10, and 12 did not receive ursodeoxycholic acid therapy. n.a.: Not available; UDCA: Ursodeoxycholic acid; ALT: Alanine aminotransferase; AST: Aspartic transaminase; TB: Total bilirubin; DB: Direct bilirubin; INR: International normalized ratio; ALB: Albumin.

RESULTS

Biochemistry and clinical improvements

Nine patients (Table 2) had received UDCA therapy prior to diagnosis of AKR1D1 deficiency, of whom three (patients 3, 4, and 5) were administered with a period of high dose UDCA (30-40 mg/kg bw/d). Complete normalization of liver function tests (LFTs) was seen only in patient 5, and in patients 3 and 7 serum bilirubin levels almost normalized but serum aminotransferases levels remained elevated. Biochemistry parameters did not improve or worsen in the remaining patients. Overall, UDCA was largely ineffective in treating AKR1D1 deficiency.

Eleven patients received CDCA therapy at an initial dose of 5-12 mg/kg bw/d (Table 3). Five patients (patients 2, 4, 6, 7, and 8) had to temporarily stop or have the dose reduced because of a marked elevation of serum ALT, AST, and total bile acid levels after beginning the therapy. This was believed due to the intrinsic hepatotoxicity of CDCA. However, with the exception of patient 8, the clinical status of all the other patients gradually improved during CDCA administration after dose adjustment. Jaundice disappeared after several months and hepatomegaly improved gradually. After the resolution of jaundice, the dose of CDCA was increased in some cases due to the insufficient suppression of atypical bile acids as determined from the follow-up urine analysis. The duration of therapy ranged from 5.5 mo to 6.4 years (median = 1.5 years) in the 11 patients undergoing CDCA therapy. At the last follow-up of these patients (median age, 2.3 years; range: 0.7-6.6 years; n = 11), the liver function tests had normalized and all were clinically well (Table 3). When the dose of CDCA was optimized, no obvious side effects were subsequently reported or observed with continued treatment. Patient 8 showed hepatotoxicity to a dose of 10 mg/kg bw/d CDCA, and still no improvement on a low dose of CDCA (4-6 mg/kg bw/d), and 9 mo later, after further deterioration in liver function tests, the patient underwent liver transplantation at the age of 17 mo. At the time of writing, all the remaining patients are thriving with continued CDCA therapy.

Urine bile acid analysis

Urine bile acid analysis by FAB-MS conclusively established a deficiency in the activity of AKR1D1 in all patients prior to initiation of bile acid therapy. The negative ion mass spectra of the urines were characterized by an absence of ions representing normal primary bile acid conjugates of CDCA and CA (m/z 448, 464, 498, and 514), and the presence of intense ions at m/z 444, 460, 494 and 510. These represent molecular ions for glycine and taurine conjugated forms of 3-oxo-Δ4 bile acids that are the biomarkers for the AKR1D1 deficiency[2,3,13]. When diagnosed in the first few months of life, it is the taurine conjugated 3-oxo-Δ4 bile acids (m/z 494 and 510) that

Table 3 Evolution of clinical feature or liver function tests during oral chenodeoxycholic acid therapy in 11 children with Δ4-3-oxosteroid 5β-reductase deficiency

Patient No.	Starting age (mo)	Starting dosage (mg/kg per day)	Dosage adjustment (mg/kg per day × duration)	Age (mo) at LFTs normalization	Dosage maintaining normal LFTs and suppressing atypical bile acids (mg/kg per day)	Status/age at last follow-up
1	5.5	8	8 mo × 7 mo; 10 × -	9	10	Normal/4 yr 11 mo
2	11	12	12 mo × 1 mo; 8 mo × 0.75 mo; 4.5 mo × 5 mo; 5.5 × -	31	5.5	Normal/4 yr
3	6	10	10 mo × 24 mo; 9 mo × 12 mo, 8 × -	13	8	Normal/4 yr 9 mo
4	3	5	5 mo × 1mo; 3 mo × 2 mo; 7 mo × 4.5 mo; 8 mo × 2.5 mo; 11 mo × 13 mo; 8 × -	26	8	Normal/6 yr 7 mo
6	14	8	8 wk × 3 wk; 6 wk × 1 wk; 5 mo × 2.5 mo; 6 mo × 3 mo; 7 × -	18	7	Normal/2 yr 11 mo
7	10	10	10 wk × 1 wk; 8 mo × 1 mo; 0 wk × 1 wk; 5 × -	14	5	Normal/2 yr 4 mo
8	7	10	10 wk × 1 wk; 4 mo × 2 mo; 5 mo × 6 mo; 6 mo × 2 mo	N.A.	N.A.	Transplanted/1 yr 6 mo
9	4	8	8 mo × 2 mo; 9 mo × 2 mo; 10 × -	10	10	Normal/1 yr 5 mo
10	3.5	10	10 × -	6.5	10	Normal/11 mo
11	5	10	10 mo × 13 mo; 7 × -	8	7	Normal/2 yr
12	2.5	10	10 × -	5	10	Normal/8 mo

The dosage of chenodeoxycholic acid was reduced if serum alanine aminotransferase and aspartic transaminase obviously elevated with obviously elevated total bile acid level; increased if not sufficient suppression of atypical bile acid demonstrated by fast atom bombardment ionization mass spectrometry after the resolution of jaundice. N.A.: Not applicable; LFT: Liver function test.

generally dominate the mass spectra because in early life bile acids are predominantly conjugated with taurine[3,14]. After weaning and in older infants, the glycine conjugated forms of these atypical bile acids (m/z 444 and 460) become the predominant species present.

The biochemical response to CDCA therapy was confirmed by the successful reduction in the intensity of the ions for these 3-oxo-Δ4 bile acid conjugates (Figure 1), and on this basis, the dose of CDCA was adjusted to optimize maximum suppression. With optimized CDCA therapy, most patients showed a marked suppression, or complete disappearance of the atypical bile acids (Figure 1), although it should be noted that it can be difficult to attain complete disappearance of these metabolites. The presence of ions in the mass spectrum that reflect metabolites of CDCA was a common observation and served to confirm compliance to therapy. Ions at m/z 448 (glyco-CDCA), m/z 498 (tauro-CDCA), m/z 471 (CDCA-sulfate), and m/z 528 (glyco-CDCA-sulfate) represent major metabolites of CDCA (Figure 1). Those patients showing the best clinical response to CDCA therapy had the greatest suppression in the urinary excretion of atypical 3-oxo-Δ4 bile acids. Patient 2 did not show adequate suppression of 3-oxo-Δ4 bile acids initially, presumed to be due to poor compliance. Likewise, the urinary FAB-MS analysis for patient 8, who was on a relatively low dose of CDCA (4-6 mg/kg bw/d), failed to show adequate suppression of the 3-oxo-Δ4 bile acids, and this patient's serum transaminases progressively increased and liver function deteriorated to the point of requiring a liver transplant. In patient 5, who refused CDCA therapy and was maintained on high dose UDCA, the FAB-MS showed no reduction in the intensity of the 3-oxo-Δ4 bile acids even in the face of improvements in serum biochemistries. Figure 1 shows the mass spectra of the urine after UDCA treatment and these findings are consistent with the inability of UDCA to suppress endogenous bile acid synthesis. Ions consistent with the atypical 3-oxo-Δ4 bile acids remained in the mass spectra but were inter-dispersed with ions reflecting

metabolites of exogenous UDCA.

DISCUSSION

The *AKR1D1* gene encodes the enzyme AKR1D1, which is essential for the synthesis of the primary bile acids, cholic acid and CDCA. This NADPH-dependent enzyme, first isolated from rat liver by Berséus and Björkhem in 1967[14,15], catalyzes the reduction of the Δ4-bond of the sterol intermediate 7α-hydroxy-4-cholesten-3-one (often now referred to as C4 or sterol-C4, and used as a surrogate marker for bile acid synthesis) to give rise to a 7α-hydroxy-5-cholestan-3-one intermediate in the pathway to cholic acid and CDCA synthesis. When the activity of AKR1D1 enzyme is deficient or reduced, the liver synthesizes a spectrum of atypical bile acids that retain this 3-oxo-Δ4 structure. Since the description of a primary enzyme defect in twins by Setchell *et al*[3], there have been a number of reports of patients with liver disease exhibiting elevated levels of Δ4-3-oxo bile acids[8,16-20], but discerning whether these were due to a 'primary' genetic defect or 'secondary' to reduced enzyme activity because of loss of hepatic synthetic function as occurs in end-stage liver disease is challenging. Furthermore, Δ4-3-oxo bile acids are typically found in urine of all healthy neonates[21-23] due to developmental immaturity in bile acid synthesis and transport in a period of physiological cholestasis that is a natural phenomenon at this time of life[24]. These atypical bile acids usually disappear with time so that in the absence of genetic testing, repeat testing of urine is important to enable differential diagnosis of a primary *vs* secondary AKR1D1 deficiency. Elevated levels of Δ4-3-oxo bile acids have been reported in patients with neonatal hemochromatosis and tyrosinemia[17-20]. However, when arising from a 'secondary' defect, these are usually accompanied by the presence of primary bile acids. In the original description of the AKR1D1 deficiency[3], primary bile acids were absent but the 5β-reduced metabolites, allo-cholic acid and allo-CDCAs, were elevated and used at the time to differentiate a 'primary' *vs* 'secondary' enzyme defect. The original description of the AKR1D1 deficiency as a cause of cholestasis predated by 6 years the cloning and expression of a cDNA for AKR1D1 by Kondo *et al*[25], so genetic confirmation was not possible. Now, complementing MS analysis with genetic analysis of *AKR1D1* (and *vice versa*) permits an accurate diagnosis by identifying specific gene mutations for this autosomal recessive disorder.

Over a period of 4-5 years, urine samples from 193 cholestatic patients presenting at Jinshan Hospital and Children's Hospital of Fudan University were screened for CBAS and 12 were positive for AKR1D1 deficiency. In the 12 patients described here, an AKR1D1 deficiency was conclusively established from MS by the finding of predominantly Δ4-3-oxo-bile acids in urine (Figure 1), and confirmed by gene sequencing analysis (Table 1).

Molecular studies identified homozygous or compound heterozygous mutations in nine patients and simple heterozygous mutations in three patients. A total of 11 different mutations were identified, including three frame-shifting (c.278delA, c.614delT, and c.933delG), two stop code (c.148C>T, c.396C>A), and six missense mutations (c.158A>G, c.472A>G, c.593C>T, c.722A>T, c.797G>A, and c.919G>A) (Table 1, Supplementary Table 1). Among them, c.148C>T[26] and c.593 C>T[7] were reported previously as disease-causing mutations, and c.797G>A is recorded as an SNP (rs182820353) but predicted as disease-causing by mutation taster. The other eight were novel and predicted as disease-causing. All five new missense mutations were in highly conserved residues. All mutations were seen in one allele each, except for two recurrent mutations (c.797G>A and c.919C>T). Mutation c.797G>A was seen in seven alleles (two homozygous and three heterozygous). Its frequency in patients (7/24) was much higher than that in 1000 Genomes (1/5008, P = 5.77308222349e-17) and ExAC (8/121368, P = 2.88738419819e-23). c.919C>T was seen in four alleles (two homozygous). For the three patients (patients 5, 6, and 11) in whom only one single heterozygous mutation was found, we speculate that there might be a large fragment deletion or duplication or mutation in the intron region leading to splice site changes.

All 12 patients presented with neonatal onset cholestasis with markedly elevated serum transaminases, normal or slightly elevated GGT, and low levels of serum total bile acids (sTBA) when measured with routine enzyme/immunoassay kits. PT was prolonged and not corrected by parenteral vitamin K in seven patients. A routine sTBA was not included in the initial battery of tests in some patients, which may have a delayed diagnosis because a low or normal sTBA when combined with a low serum GGT can be a helpful indicator of a possible bile acid synthesis disorder[27], particularly in the case of the HSD3B7 and AKR1D1 deficiencies. Most of our patients had been placed on UDCA therapy for their cholestasis prior to referral for

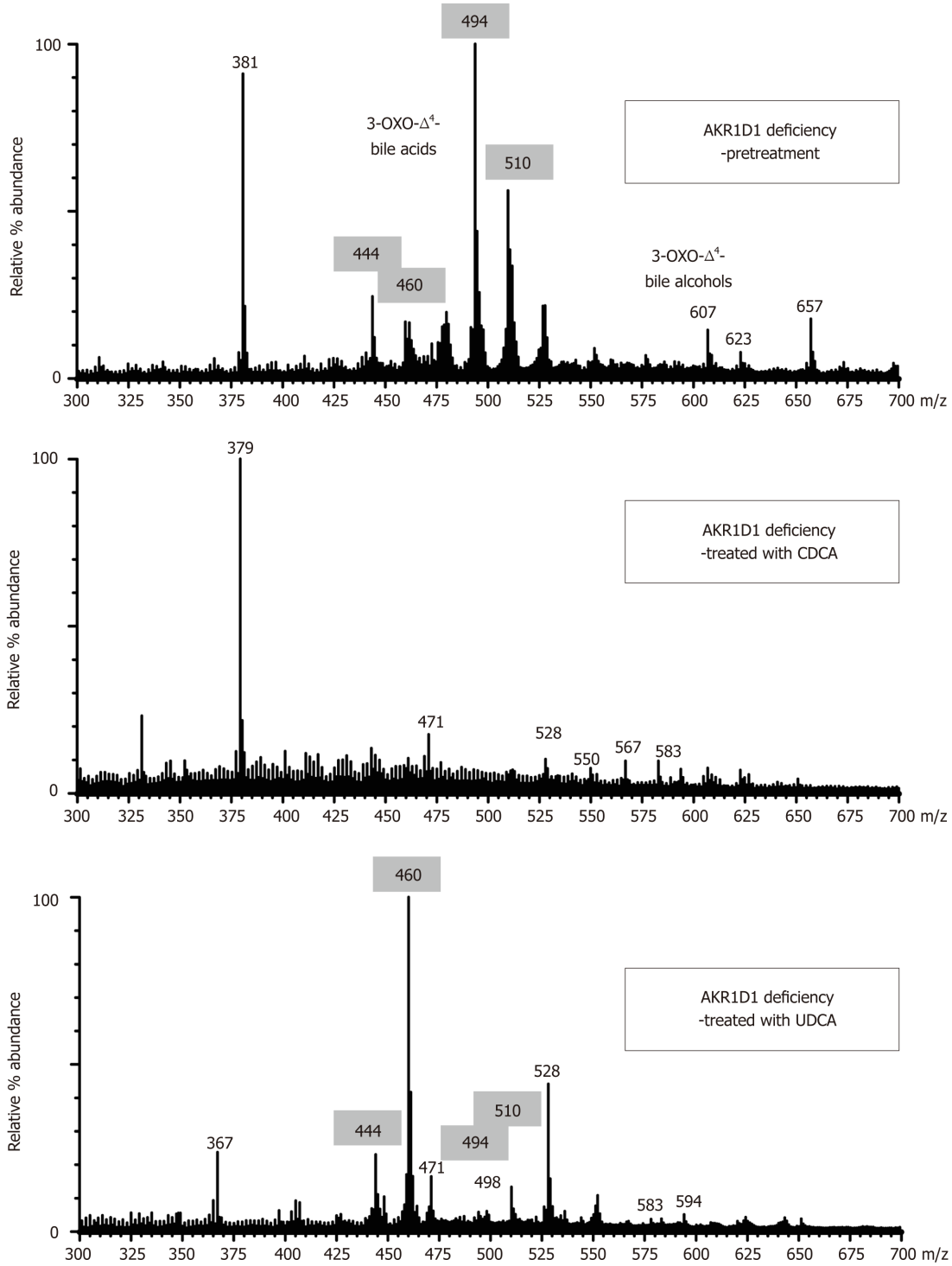

Figure 1 Typical negative ion fast atom bombardment ionization mass spectrometry spectrum of a patient with Δ4-3-oxosteroid 5β-reductase deficiency before and after treatment with bile acids. AKR1D1: Δ4-3-oxosteroid 5β-reductase; CDCA: Chenodeoxycholic acid; UDCA: Ursodeoxycholic acid.

evaluation, and it is worth cautioning that routine sTBA measurements in UDCA treated patients are of little guidance, or may even mislead, as these will be elevated due to cross-reactivity in the assay from UDCA and its metabolites. In contrast to the HSD3B7 deficiency, which is frequently diagnosed as a cause of late-onset chronic cholestasis in older children[28,29] and even some adults[30], the AKR1D1 deficiency is usually diagnosed early in life. In the 12 patients described, the first symptoms of cholestasis were evident from 3 d-1 mo of age. The average age at diagnosis was 4.3 mo (range, 1-11 mo) and the rate of progression of liver disease was rapid, even to dysfunction.

Given the rapid onset of liver failure in the AKR1D1 defect, early diagnosis is crucial. Limited reports showed that treatment with primary bile acids was extremely

effective in patients with AKR1D1 deficiency[3,7,31,32]. Primary bile acids inhibit cholesterol 7α-hydroxylase expression to down-regulate bile acid synthesis through feedback inhibition, leading to a decrease in production of atypical Δ4-3-oxo-bile acids, while additionally stimulating bile acid-dependent bile flow. In a number of patients with AKR1D1 deficiency, CA and CDCA[2,7,9,33] were combined. However, in some young infants, CDCA may be cathartic and lead to diarrhea. Furthermore, an increase in serum transaminases was observed in the National Cooperative Gallstone Trial when CDCA was evaluated for the dissolution of cholesterol gallstones[34]. For these reasons, CA monotherapy was favored and the FDA in 2015 approved CA for the treatment of bile acid synthesis disorders, including the AKR1D1 deficiency with evidence from a very limited number of cases due to the rarity of the disease[8,9]. In China, CA has to date been unavailable for patients with bile acid synthesis disorders and is currently unapproved by the Chinese FDA. All of the patients apart from one were therefore treated with CDCA, despite several reports indicating that CDCA should not be recommended for patients with AKR1D1 deficiency[4,14]. In our patients, although significant elevations in serum ALT and AST occurred in some patients, after temporarily stopping therapy and/or making dose adjustments, we finally achieved normalization of LFTs. These patients became symptom free from CDCA treatment over periods ranging 0.5-7.0 years and there were no observed serious adverse effects reported, even in those with an initial abnormal international normalized ratio (INR), save one patient that underwent liver transplantation. The optimal dose of CDCA administered varied from 4-10 mg/kg per day among the patients and was assessed by monitoring the disappearance of the atypical Δ4-3-oxo-bile acids in urine and evaluating the improvement in liver function. During CDCA therapy, LFTs remained within the normal range and FAB-MS analysis confirmed concomitant reductions, although variably, in the levels of urinary Δ4-3-oxo-bile acids. Those patients showing good compliance to therapy had almost normal development and the lack of reported side effects supports the safety of CDCA provided that doses are individually optimized.

UDCA, a potent choleretic widely used in the treatment of cholestatic liver diseases, has been used to treat some patients with the HSD3B7 deficiency[8,11,35]. It stimulates bile flow, lowers serum transaminases, and may improve liver histology[11,30]. However, its inability to suppress bile acid synthesis (it is slightly stimulatory) makes it unsuitable for long-term use in patients with bile acid synthesis disorders because the common therapeutic goal in these patients is to stop the production and accumulation of the hepatotoxic and cholestatic atypical bile acids being synthesized. Nine of our patients had been placed on UDCA prior to diagnosis of AKR1D1 deficiency, and this was stopped in all but one. In that patient, even though LFTs normalized with UDCA, the urinary levels of Δ4-3-oxo-bile acids increased over time (Figure 1). Thus, UDCA may be helpful in the short-term but it is not recommended for long-term therapy.

In conclusion, early diagnosis of the AKR1D1 deficiency by biochemical and genetic testing is critical because this bile acid synthesis disorder manifests as a particularly severe and rapidly progressive cholestatic disease with fatal outcome. CDCA, despite earlier reports cautioning against its use[29,31], was effective in the treatment of our patients (up to now the largest cohort) with AKR1D1 deficiency provided that the dose was optimized in individual patients. UDCA is not recommended as the long-term treatment as it is unable to suppress the production of the atypical hepatotoxic Δ4-3-oxo-bile acids and did not improve the clinical outcome of these patients.

ARTICLE HIGHLIGHTS

Research background

The Δ4-3-oxosteroid 5β-reductase (AKR1D1) deficiency is a rare bile acid synthesis disorder. There have been few reports describing the effectiveness of treatment regimens for this rare genetic disease and the long-term outcomes of oral primary bile acid therapy are unclear. This study provides evidence on how these patients should ideally be managed.

Research motivation

Oral cholic acid (CA), one of the two primary bile acids synthesized by the liver, is an approved therapy for the treatment of bile acid synthesis disorders but is not available to many patients. Chenodeoxycholic acid (CDCA), the other primary bile acid, offers an alternative potential therapy but has been contraindicated in a few reported cases of AKR1D1 deficiency due to its more hydrophobic and potentially hepatotoxic effects. However, in the absence of access to CA in China, we evaluated the effectiveness of CDCA in patients with AKR1D1 deficiency and showed that provided that the therapeutic doses is individually optimized, it is an effective treatment for this disorder.

Research objectives
Through retrospective analysis of the clinical and biochemical responses to bile acid therapy with CDCA in patients with AKR1D1 deficiency, our objective was to better understand the disease progression and long-term outcomes of treatment in order to make recommendations regarding its effectiveness and safety.

Research methods
Twelve patients with confirmed AKR1D1 deficiency, diagnosed by fast atom bombardment ionization-mass spectrometry analysis of the urinary bile acid profile and by gene sequencing of *AKR1D1*, were treated with oral bile acids. The clinical and biochemical responses to CDCA therapy were retrospectively evaluated and analyzed, including the results on urinary bile acid profiles and serum liver function indices.

Research results
Physical examination, biochemistry parameters, and sonographic findings improved in the patients during CDCA therapy, except for one who underwent liver transplantation. The urinary levels of atypical hepatotoxic 3-oxo-Δ4 bile acids were suppressed concomitantly with clinical improvements in those patients treated with CDCA, but not with ursodeoxycholic acid (UDCA). The dose of CDCA varied from 5.5-10 mg/kg per day among patients based on maximum suppression of the atypical bile acids.

Research conclusions
CDCA is an effective alternative therapy to CA, provided that the dose is carefully optimized on an individual patient basis to minimize side effects. UDCA does not achieve the therapeutic goal of suppressing the production of atypical hepatotoxic bile acids and is not recommended for long-term treatment.

Research perspectives
CDCA is effective in the treatment of the patients with AKR1D1 deficiency. However, CDCA is intrinsically hepatotoxic, and therefore its dose must be optimized to individual patients. Future studies should focus on continued long-term monitoring of these patients to provide more detailed information of the natural history of this rare metabolic liver disease and to determine long-term outcomes of therapy with primary bile acids.

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ORIGINAL ARTICLE

Observational Study

Histopathological changes in the oesophageal mucosa in Egyptian children with corrosive strictures: A single-centre vast experience

Ayman Eskander, Carolyne Ghobrial, Nabil A Mohsen, Bahaa Mounir, Dalia Abd EL-Kareem, Sara Tarek, Mortada HF El-Shabrawi

ORCID number: Ayman Eskander (0000-0002-4763-7733); Carolyne Ghobrial (0000-0002-1662-7938); Nabil A Mohsen (0000-0002-4371-0956); Bahaa Mounir (0000-0003-1682-922X); Dalia Abd EL-Kareem (0000-0001-7838-7194); Sara Tarek (0000-0002-5132-4019); Mortada HF El-Shabrawi (0000-0002-1995-4213).

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Institutional review board statement: The study was reviewed and approved by ethical committee in our Departments of Pediatrics, Kasr Alainy School of Medicine, Cairo University, Cairo, Egypt. The scanned copy of ethical committee approval of the research protocol was attached in submitted files to the journal.

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Ayman Eskander, Carolyne Ghobrial, Nabil A Mohsen, Sara Tarek, Mortada HF El-Shabrawi, Department of Paediatrics, Kasr Alainy School of Medicine, Cairo University, Cairo 11562, Egypt

Bahaa Mounir, Dalia Abd EL-Kareem, Department of Pathology, Kasr Alainy School of Medicine, Cairo University, Cairo 11562, Egypt

Corresponding author: Mortada HF El-Shabrawi, MD, Professor, Department of Paediatrics, Kasr Alainy School of Medicine, Cairo University, 1 Gamaa Street, Giza, Cairo 11562, Egypt.
melshabrawi@kasralainy.edu.eg
Telephone: +201-2-28779388
Fax: +20-2-37619012

Abstract

BACKGROUND

The caustic ingestion continues to be a major problem worldwide especially in developing countries. The long-term complications include stricture and increased life time risk of oesophageal carcinoma. Patients suffered from corrosive induced oesophageal strictures have more than a 1000-fold risk of developing carcinoma of the oesophagus.

AIM

To determine the possibility of oesophageal mucosal dysplasia after prolonged dilatation in post corrosive stricture.

METHODS

This observational study was conducted at the Paediatric Endoscopy Unit in Cairo University Children's Hospital. It included children of both sexes older than 2 years of age who had an established diagnosis of post-corrosive oesophageal stricture and repeated endoscopic dilatation sessions for more than 6 mo. All patients were biopsied at the stricture site after 6 mo of endoscopic dilatation. A histopathological examination of an oesophageal mucosal biopsy was performed for the detection of chronic oesophagitis, inflammatory cellular infiltration and dysplasia.

RESULTS

The mean age of the enrolled children was 5.9 ± 2.6 years; 90% of the patients had ingested an alkaline corrosive substance (potash). The total number of endoscopic dilatation sessions were ranging from 16 to 100 with mean number of sessions

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was 37.2 ± 14.9. Histopathological examination of the specimens showed that 85% of patients had evidence of chronic oesophagitis (group A) in the form of basal cell hyperplasia, hyperkeratosis and subepithelial fibrosis. Thirteen percent of the patients had evidence of reactive atypia (group B) in the form of severe neutrophilic intraepithelial inflammatory cellular infiltration, and 2 patients (2%) had mild squamous dysplasia (group C); we rebiopsied these two patients 6 mo after the initial pathological assessment, guided by chromoendoscopy by Lugol's iodine.

CONCLUSION

The histopathology of oesophageal mucosal biopsies in post-corrosive patients demonstrates evidence of chronic oesophagitis, intraepithelial inflammatory cellular infiltration and dysplasia. Dysplasia is one of the complications of post-corrosive oesophageal stricture.

Key words: Children; Endoscopic dilatation; Dysplasia; Oesophageal strictures; Post-corrosive

Core tip: Caustic ingestion continues to be a significant problem worldwide especially in developing countries. It has been reported that the accidental corrosive substance ingestion is seen mostly among children younger than 5 years of age. Corrosive ingestion in children may cause clinical manifestations varying from no injury to fatal outcome, including the risk for squamous cell carcinoma of the oesophagus. In this study, we analyzed our single-center experience, representing the largest series of paediatric patients with post-corrosive oesophageal stricture on repeated endoscopic dilatation sessions for more than 6-mo duration and histopathological examination of oesophageal mucosal biopsies were performed.

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INTRODUCTION

Oesophageal stricture due to corrosive injury has been cited as a risk factor for squamous cell carcinoma of the oesophagus[1]. The possibility and pathogenesis of the increased risk of oesophageal squamous cell carcinoma following caustic injury remain uncertain[2]. The incidence of cancer in patients with corrosive strictures has been estimated to be 2.3% to 6.2%, and a history of caustic ingestion was present in 1% to 4% of patients with oesophageal cancer[3]. Neither dilatation treatment nor oesophageal bypass surgery can prevent the development of oesophageal carcinoma, the incidence of which is high after caustic substance ingestion[4]. Malignant transformation should be suspected in patients with longstanding corrosive ingestion if there is a change in their symptoms, as early diagnosis can improve the outcome[5]. It was found that genetic factors play a minor role in the pathogenesis of oesophageal cancer[6,7]. Epithelial dysplasia has been assumed to be a precancerous lesion of the oesophagus, which is usually preceded by chronic oesophagitis[8,9]. The most frequently encountered histopathological changes in patients with post-corrosive stricture are epithelial hyperplasia, focal hyperkeratosis, and mixed inflammatory exudates in the subepithelium[1]. Considering that oesophageal squamous dysplasia (ESD) is the pre-neoplastic lesion for oesophageal squamous cell carcinoma (ESCC) provides the possibility of not only screening for early stage ESCC but also screening for and treating the pre-neoplastic lesion itself[10]. Chromoendoscopy with Lugol's iodine staining efficaciously identifies subjects with dysplasia[11]. The negative- image "unstained lesions" (USLs) after spraying iodine can be endoscopically targeted for biopsy and/or focal endoscopic therapy[10]. The routine endoscopic biopsy of normal-

appearing mucosa or biopsies guided by tissue staining with Lugol's solution may increase the detection of dysplasia or early oesophageal cancer[12]. This study was designed to screen post-corrosive children for the risk of development of oesophageal mucosal dysplasia to assess the safety of prolonged endoscopic dilatation of post-corrosive oesophageal stricture.

MATERIALS AND METHODS

Study design

The present study is an observational study that included 100 children with an established diagnosis of post-corrosive oesophageal stricture who were engaged in repeated endoscopic dilatation sessions for more than 6 months. The study was conducted at the Paediatric Endoscopy Unit, Cairo University Paediatric Hospital, from March 2015 to September 2016.

Ethical considerations

The study protocol was approved by the Research Ethics Committee of the Paediatrics Department, Faculty of Medicine, Cairo University, Egypt. The research was conducted in accordance with the Helsinki Declaration. All patients were enrolled in the study after informed consent was obtained from their parent/guardian.

Participants

The age of the enrolled patients of both sexes was older than 2 years. We excluded infants younger than 2 years of age and those with other causes of oesophageal stricture, *e.g.*, post-tracheo-oesophageal fistula repair, congenital oesophageal stenosis, post-sclerotherapy and prolonged nasogastric intubation, peptic stricture, post-chemotherapy or radiotherapy stricture, and epidermolysis bullosa, as well as post-corrosive patients who had surgical repair either in the form of a colon bypass or gastric tube placement.

Assessment and evaluation

All patients were subjected to a full clinical assessment focusing on the age of onset of ingestion of the corrosive substance, type of corrosion, age at the time of enrolment in the study and number of dilation sessions. The patients' anthropometric measurements (weight, height), which were plotted on Egyptian growth curves (Standard Egyptian Growth, 2008), and the corresponding z-scores were obtained. All patients were evaluated *via* a barium swallow on the 21st post-ingestion day. A medium density barium sulfate mixture was used for the single-contrast examination. Fluoroscopic spot films of the oesophagus, as well as overhead films (35.5 cm × 43 cm) in the anteroposterior and lateral positions, were obtained to determine the extent, length, and number of the strictures. Patients were advised to come to the endoscopy unit after 6 h of fasting. Upper gastrointestinal endoscopy was performed using a Silver Karl Storz Endoskope 13821 PKS with Storz Professional Image Enhancement System (SPIES) technology with an HD system and 100-watt xenon light source. Endoscopic bougie dilatation was performed using Savary Gilliard dilators (Cook Medical, Bloomington, IN, United States). An endoscope was first introduced to evaluate the anatomy, and bougies in sequentially increasing sizes were then passed over the guide wire that had been positioned with the tip in the gastric antrum. The initial dilator chosen should have been based on the known or estimated stricture diameter. To avoid complications in the early stage of oesophageal stricture, the sizes of the dilators used were usually 7, 9, 10, 11, and 12.8 mm. After the final dilation, endoscopy was performed to assess the efficacy of the dilatation as well as complications such as bleeding or perforation of the oesophagus. After the dilation procedures, the patients remained fasting and were followed up for 2 h. The patients received anti-reflux treatment in between the dilatation sessions. The treatment was considered effective when patients were able to eat semi-solid or solid foods without dysphagia. The biopsy was performed after 6 mo of regular endoscopic dilation. Using biopsy forceps, the mucosal biopsies were procured from the stricture site during the endoscopic examination before dilation. All specimens were processed as formalin-fixed paraffin-embedded tissue blocks. Each block was cut into 5-μm-thick serial sections, which were mounted on glass slides. Sections were stained with haematoxylin and eosin. Stained sections were then examined using light microscopy. Under light microscopy, the examined sections were graded on a scale of 0-3 (0, absent; 1, mild; 2,moderate; 3, severe) according to the following parameters that were measured and recorded: thickness of the epithelial layer, basal cell hyperplasia(15%-20% of epithelial thickness), length of the papillae (> 1/3 the epithelial thickness), hyperkeratosis, amount of cytoplasm, nuclear atypia, nuclear/cytoplasm ratio,

intraepithelial neutrophils, lymphoplasmacytic infiltrate, koliocytic and hydropic changes, intraepithelial vascular spaces, subepithelial fibrosis and squamous dysplasia. Each variable was graded as follows: low grade (abnormal cells limited to the basal half of the epithelium), high grade (abnormal cells present in the upper half) and indefinite for dysplasia (reactive epithelial atypia associated with a severe inflammatory reaction). There are two pathologists in this study. The pathologist No. 1 examined all of histology specimens. The pathologist No. 2 had just evaluated a part of histology specimens. Patients who had low-grade dysplasia were subjected to rebiopsy *via* chromoendoscopy after a period that ranged from 6 mo to one year from the initial pathological assessment. In the current study, Lugol's iodine solution was used to detect the dysplastic areas of the mucosa, and it was sprayed onto the oesophageal surface from the gastro-oesophageal junction to the upper oesophageal sphincter using the dye-spraying catheter through the biopsy channel. The use of Lugol's iodine ensured that the USLs were clearly visible after 5 min, allowing enough time for photographs to be recorded and biopsies to be conducted. From each unstained or lightly stained lesion, between one and three biopsies were collected for histopathological examination. The findings were recorded in tabular form. The group of patients who suffered from reactive epithelial atypia associated with a severe inflammatory reaction were followed regularly in our endoscopy unit, and a rebiopsy was subsequently planned.

Statistical analysis

Pre-coded data were entered into the computer using the Microsoft Office Excel software program (2010) for Windows. Data were then transferred to the Statistical Package of Social Sciences software program, version 23 (IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY: IBM Corp.), for statistical analysis. Data are presented as the means and standard deviation (SD) for quantitative variables and as frequencies and percentages for qualitative variables. The comparison between groups was performed using the chi-square test for qualitative variables. The one-way ANOVA test and *t*-test were used to compare group means, which are both parametric statistical methods. *P*-values less than 0.05 were considered statistically significant. Graphs were used to illustrate some information.

RESULTS

This study included 100 patients, and males represented 63% of the patients. The mean age of the patients was 5.9 ± 2.6 years. The mean age at the time of corrosive ingestion was 2.1 ± 1.1 years. The general examination of the patients revealed that the median and range of weight and height by Z-score were -1.20 (-10.81-1.91) and -2.11 (-5.82-0.61), respectively. Forty-seven patients (47%) were no more than the 3rd percentile in weight, while seventy patients (70%) were no more than the 3rd percentile in height (*i.e.*, stunted growth). The majority (90%) of our patients ingested an alkaline substance (potash); 6% of them ingested a neutral substance (chlorine); and only 4% of them ingested an acidic substance (H_2SO_4). All patients were evaluated on the 21st post-ingestion day by barium swallow to determine the site, length and number of strictures. A long segment was determined radiologically by more than the length of two vertebrae, measuring approximately 4 cm. The total number of endoscopic dilatation sessions were ranging from 16 to 100 with mean number of sessions was 37.2 ± 14.9. Biopsy specimens were obtained from the oesophageal stricture site at the time of endoscopic examination before the dilatation using biopsy forceps after at least six-month duration of regular endoscopic dilatation.

Outcome data

According to the histopathological findings of specimens from the upper gastrointestinal endoscopy, the patients were subdivided into three groups, as shown in Figures 1-3, and Table 1.

Group A: Eighty-five patients (85%) had evidence of chronic oesophagitis in the form of hyperplastic mucosa, basal cell hyperplasia, intraepithelial inflammatory cells, dilated vascular spaces, hyperkeratosis and parakeratosis, hydropic changes, and subepithelial fibrosis.

Group B: Thirteen patients (13%) had evidence of reactive epithelial atypia/indefinite for dysplasia with heavy neutrophilic, intraepithelial, and inflammatory cell infiltration.

Group C: Only two patients (2%) had low-grade squamous dysplasia.

The pathologist No. 2 had just evaluated a part of histology specimens, and all results were the same except for minor differences in few patients as described below. Patient No. 9, pathologist No. 1 described his biopsy as chronic oesophagitis

Table 1 The baseline characteristics of the three pathological groups of patients

Variable	Group A		Group B		Group C		*P* value
	Number	%	Number	%	Number	%	
Gender							
Male	52	61.2	9	69.2	2	100	0.469
Female	33	38.8	4	30.8	0	0.0	
Age at time of enrolment (yr)	5.60 ± SD 2.50		8.08 ± SD 2.18		8.75 ± 6.01		0.002
Age of corrosive ingestion In years	1.94 ± SD 0.66		2.27 ± SD 0.44		6.25 ± SD 6.72		< 0.001
Type of corrosion							
Alkaline	78	91.8	10	76.9	2	100	0.005
Neutral	6	7.0	0	0.0	0	0.0	
Acidic	1	1.2	3	23.1	0	0.0	
Weight in percentile							
< 3rd	38	44.7	9	69.2	0	0.0	0.104
> 3rd	47	55.3	4	30.8	2	100	
Height in percentile							
< 3rd	61	71.8	9	69.2	0	0.0	0.091
> 3rd	24	28.2	4	30.8	2	100	
Stricture site							
Upper esophageal	74	87.1	12	92.3	1	50.0	0.253
Mid esophageal	53	62.4	10	76.9	2	100	0.341
Lower esophageal	3	3.5	0	0.0	0	0.0	0.761
Stricture length							
Long segment	59	69.4	13	100	2	100	0.045
Short segment	26	30.6	0	0.0	0	0.0	
Stricture number							
Single	45	52.9	4	30.8	2	100	0.124
Multiple	40	47.1	9	69.2	0	0.0	
Number of dilatation	35.21 ± 12.79		49.38 ± 21.81		42.50 ± 17.68		0.005
Duration since ingestion in years	3.66 ± 2.20		5.81 ± 2.41		2.50 ± 0.71		0.004
No further dilatation	43 (50.5%)		11 (84.6%)		0 (0.0%)		0.015
Ongoing dilatation	42 (49.5%)		2 (15.4%)		2 (100%)		

SD: Standard deviation.

indefinite for early dysplastic changes, while pathologist No. 2 described it as low grade dysplasia with evidence of epithelial cell disorganization, nuclear pleomorphism, hyperchromasia and cellular crowding. Chromoendoscopy was decided for patient No. 9 to obtain targeted biopsy from the dysplastic oesophageal mucosa, both pathologists examined the specimen blindly. The final result was the same for both pathologists as low grade dysplasia.

Patient No. 33 and 45, pathologist No. 1 described their biopsy as chronic oesophagitis, while pathologist No. 2 described their biopsy as reactive epithelial atypia / indefinite for dysplasia due to heavy intraepithelial neutrophilic infiltration.

DISCUSSION

The majority (90%) of our patients ingested an alkaline substance (potash), while 6% of them ingested a neutral substance (chlorine) and only 4% ingested an acidic substance (H_2SO_4). This result may be attributed to the fact that in liquid form, bases are tasteless and denser than water[13], while strong acids are bitter and are commonly expectorated[4]. Potash is potassium hydroxide, it used in oven cleaners, liquid agents, liquid drain cleaners, disk batteries household cleaners, dishwater detergents[12,13]. The median and range of patients' weight and height by Z-score were -1.20 (-10.81-1.91) and -2.11 (-5.82-0.61), respectively. Forty-seven of the patients (47%) were no more than the 3rd percentile in weight, while seventy patients (70%) were no more than the

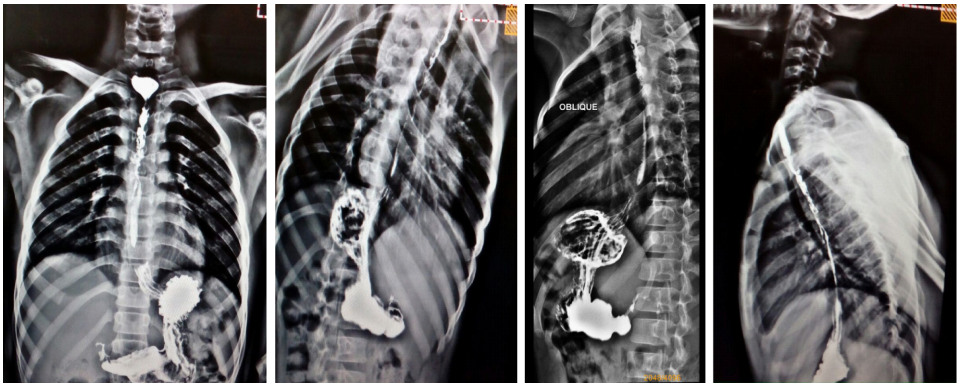

Figure 1 Barium swallow of patient showing long segment of post corrosive oesophageal stricture.

3rd percentile in height, which reflects stunted growth and nutritional compromise as a complication of corrosive stricture[14]. Bouginage using Savary-Gilliard or American type of technique, irrespective of the type and the extent of oesophageal stenosis, is safe and purposeful procedure[15,16]. We used bougie dilators for three reasons; 74% of our patients had long segment oesophageal stricture, we unified the method of dilation for all included patients to avoid the confounding variables and due to financial issues in our LMIC's, balloon dilators are an expensive, especially it was single use method. The oesophageal stent was not available in our institute due to financial issues. In the present study, the histopathological findings of endoscopic oesophageal biopsies revealed that eighty-five patients (85%) had evidence of chronic oesophagitis and irritation in the form of basal cell hyperplasia, intraepithelial vascular spaces, hyperkeratosis and parakeratosis, hydropic changes, and subepithelial fibrosis, in accordance with the results obtained by Nagaich *et al*[1], who reported that epithelial hyperplasia, focal hyperkeratosis, and mixed inflammatory exudates in the subepithelium were the predominant histological findings of the biopsies in caustic oesophageal stricture patients. More than half (50.5%) in this group was healed and required no further dilatation. In this study, the definite diagnosis of dysplasia was difficult in thirteen of our patients (13%) who were classified as reactive epithelial atypia/indefinite for dysplasia due to the heavy inflammation and neutrophilic intraepithelial inflammatory cellular infiltration. This group of patients was being followed regularly, and a biopsy was subsequently planned for these patients. This was similarly stated by Attila *et al*[17], who said that the presence of intraepithelial inflammatory cellular infiltration is well recognized by pathologists to be a confounding factor in the diagnosis of dysplastic lesions, as inflammation causes reactive changes within the cells, which can be very similar to that of dysplasia. Eleven out of thirteen in this group was healed and required no further dilatation. In the present study, only two patients (2%) had low-grade dysplasia and underwent a three-year period of dilatation; one of them was 14 years of age, and the other patient was five years of age. They are still on regular endoscopic dilatation sessions. Other studies reported that the incidence of cancer in patients with corrosive strictures has been estimated to be 2.3% to 6.2%, and a history of caustic ingestion was present in 1% to 4% of the patients with oesophageal cancer in the study of Isolauri *et al*[3]. However, another study by Nagaich *et al*[1] revealed no cases of dysplasia reported on histopathological examination of endoscopic biopsies from patients older than 3 years of age with caustic oesophageal strictures who had undergone more than 10 years of dilatation[1]. This result may be explained by the statement of Allam *et al*[6] that children do not experience a long enough period of chronic oesophagitis to reach a state of dysplasia. Kavin *et al*[18] the initial biopsy before the first bougie dilatation in post corrosive patients revealed very minimal histopathological abnormalities, suggesting the possibility that the trauma of repeated bougie dilatation may be a promoter in the ultimate development of dysplasia. This finding is in accordance with this study. Specifically, patients with evidence of reactive atypia or low-grade dysplasia on histopathological examination of their biopsies had a greater mean number of endoscopic dilatation sessions; however, statistical significance was not achieved. However, patients with evidence of chronic oesophagitis had a significantly lower mean number of endoscopic sessions ($P < 0.05$). In 2015, Nagaich *et al*[1] studied the histopathological changes and safety of chronic dilatation (mean duration of 10 years) in reference to the occurrence of dysplastic changes and reported no risk from chronic dilatation. This result enforced the safety of repeated endoscopic dilatation in our study; no statistical significance was found between the pathological criteria (*i.e.*,

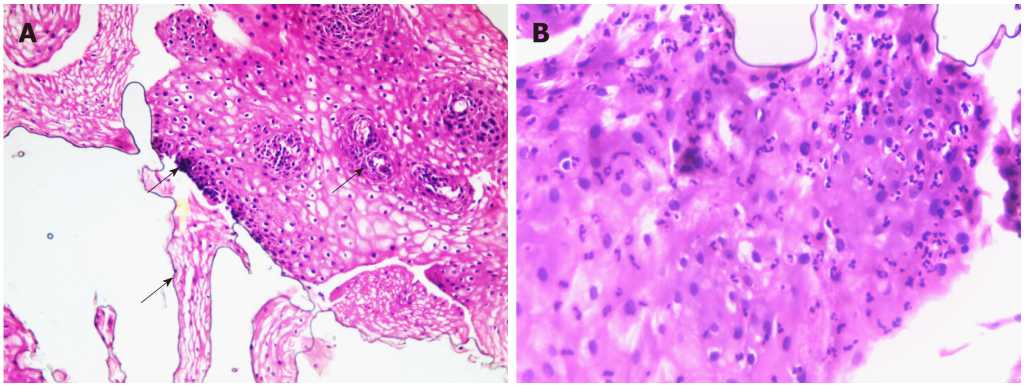

Figure 2 Hematoxylin and eosin staining results. A: Chronic esophagitis: illustrates basal cell hyperplasia, hyperkeratosis and intraepithelial vascular spaces; B: Reactive epithelial atypia/indefinite for dysplasia: illustrates severe neutrophilic intraepithelial inflammatory cellular infiltration and epithelial reactive changes (× 400).

chronic irritation, reactive epithelial atypia, or low-grade dysplasia) of the oesophageal mucosal biopsies in the three groups and duration of endoscopic dilatation of more than two years. There is a long latency period (12-41 years) between ingestion of caustics and the development of malignancy[6,19,20]. Jain *et al*[20] reported the first case from India of a 14-year-old boy with an associated 1-year history of accidental caustic ingestion who developed ESCC along with cervical lymph node metastasis. In this study, the duration since corrosive ingestion was 3.5 and 3 years in two cases with mild-grade dysplasia. Karwasra *et al*[8] and Zhen *et al*[9] reported that dysplasia is a precancerous lesion of ESCC, which is usually preceded by chronic oesophagitis. The aim of this study was to assess the risk of development of premalignant lesions and the value of screening for any premalignant lesions in individuals who are susceptible after a long duration of corrosive ingestion or prolonged endoscopic dilatation. In 2013, Taylor *et al*[10] reported that ESD appears to progress over months to many years, depending on the grade. It is probable that severe dysplasia needs prompt treatment, moderate dysplasia needs treatment or periodic endoscopic follow-up and mild dysplasia can be followed at longer intervals. So, we re- biopsied the two patients who had mild-grade dysplasia by the use of chromoendoscopy. The Lugol's iodine solution used to detect the dysplastic areas of mucosa was sprayed onto the oesophageal surface from the gastro-oesophageal junction to the upper oesophageal sphincter using the dye-spraying catheter through the biopsy channel. This protocol is in agreement with the methods of Chibishew *et al*[12], who reported that the routine endoscopic biopsy of normal-appearing mucosa or biopsies guided by tissue staining with Lugol's solution may increase the detection of dysplasia or mild oesophageal cancer. Narrow band imaging- magnifying endoscopy helps in visualization of oesophageal mucosa and surveillance of oesophageal precancerous lesions[21].

In conclusion, the histopathological examination of oesophageal mucosal biopsies in post-corrosive patients demonstrates evidence of chronic oesophagitis, intraepithelial inflammatory cellular infiltration and dysplasia. Dysplasia is a serious complication of post-corrosive oesophageal stricture for which screenings should be performed. The development of dysplasia was associated with several risk factors, but the number of dilatation sessions and duration since ingestion of the corrosive substance were not significantly associated with the risk of dysplasia development.

Suggestion for further research

High attention should be taken from the parents to avoid the availability of the corrosive substances in reachable areas for their kids. Chemical substances should not be stored in food containers. Implementation of preventive programme is very crucial to limit the use of corrosive materials. The use of other methods to detect oesophageal dysplasia, such as P53 immunohistochemical staining of the oesophageal mucosa, allows a more accurate diagnosis of dysplastic lesions, especially in the group of reactive atypia/ indefinite for dysplasia. Other suggestions for further research include the determination of molecular characteristics and genetic risk factors that may predispose individuals to oesophageal dysplasia.

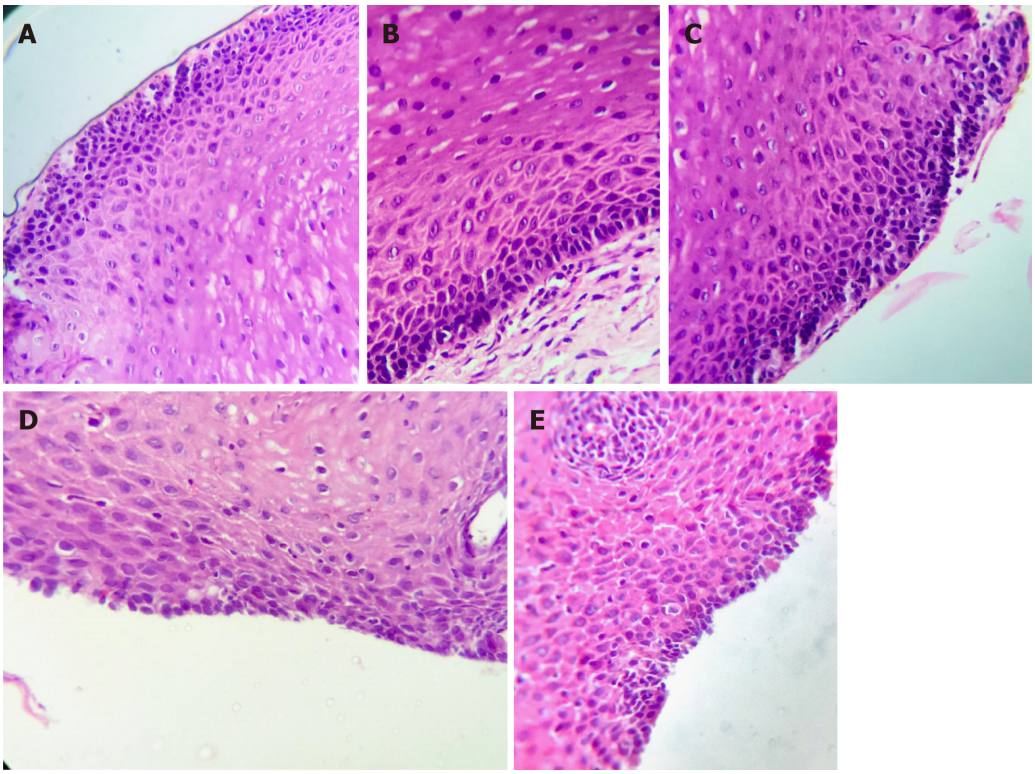

Figure 3 Representative photomicrograph showing low grade squamous dysplasia: The basal one third of the epithelium shows epithelial cell disorganization, nuclear pleomorphism, hyperchromasia, and cellular crowding (× 400), patient 1 (A-C), patient 2 (D and E).

ARTICLE HIGHLIGHTS

Research background

Caustic ingestion continues to be a major health hazard in developed and developing countries despite continuing educational programs and legislation limiting the strength and availability of corrosive substances. It has been reported that the accidental corrosive substance ingestion is seen mostly among children younger than 5 year of age. Corrosive strictures are usually managed by endoscopic dilatation. The target is to dilate oesophageal strictures to a level that allows patients to tolerate a regular diet without dysphagia. The research in paediatric age group was very deficient so we are concerning about the histopathological changes in post corrosive oesophageal stricture in paediatric age and safety of endoscopic dilatation.

Research motivation

Dysplasia has been postulated to be a precancerous lesion of the oesophagus, which in turn is considered to be preceded by chronic oesophagitis. Patients with corrosive induced oesophageal strictures have more than a 1000-fold risk of developing carcinoma of the oesophagus. The incidence of cancer in corrosive strictures has been estimated to be 2.3% to 6.2%, and a history of caustic ingestion was present in 1% to 4% of patients with oesophageal cancer. Risk factors of oesophageal dysplasia in post corrosive stricture were chronic irritation at the site of the scar, local chronic inflammatory responses in the oesophageal mucosa, long latency period from the corrosive ingestion and genetics playing a minor role. The diagnosis of corrosion cancer should be suspected in patients with corrosive ingestion if after a latent period of negligible symptoms there is development of dysphagia, poor response to dilatation, or if respiratory symptoms develop in an otherwise stable patient of oesophageal stenosis. The significance of this study was to set strategy provide early detection of dysplastic changes of oesophageal mucosa in post corrosive stricture. The determination of oesophageal dysplasia which is the pre-neoplastic lesion for oesophageal squamous cell carcinoma provides the possibility of screening not just for early stage oesophageal squamous cell carcinoma, but also to screen for and treat the pre-neoplastic lesion itself.

Research objectives

The main objectives were to detect the possibility of oesophageal mucosal dysplasia after prolonged dilatation of post corrosive oesophageal stricture and to assess the relationship between duration since the corrosive ingestion and number of oesophageal dilatation sessions with degree of oesophageal mucosal dysplasia.

Research methods

The work was carried out at the Paediatric Endoscopy Unit in Cairo University Children's

Hospital, from March 2015 to September 2016. The study included the patients older than 2 years of age who had established diagnosis of post corrosive oesophageal stricture on repeated endoscopic dilatation sessions for more than of six-month duration; of both sexes. Infants below 2 year and other causes of oesophageal stricture were excluded. Data included: history taking; age of onset of ingestion of corrosion, type of corrosion, age at the time of enrollment in the study, number of dilation sessions. Clinical examination included Anthropometric measures. All patients were evaluated on the 21st post ingestion day with a barium swallow. Upper gastrointestinal endoscopic dilatation of oesophageal stricture was done for all patients with biopsy from the stricture site after at least six-month duration of regular endoscopic dilatation. Histopathological examination of oesophageal mucosal biopsy was performed for detection of chronic oesophagitis, inflammatory cellular infiltration and dysplasia. As regard the patients who had early grade dysplasia, we rebiopsied them after period of time ranged from 6 months to one year from the initial pathological assessment but with the use of the chromoendoscopy by Lugol's iodine.

Research results

This study included 100 patients, and males represented 63% of the patients. The mean age of the patients was 5.9 ± 2.6 years. The mean age at the time of corrosive ingestion was 21 ± 1.1 years. The general examination of the patients revealed that the median and range of weight and height by Z-score were -1.20 (-10.81-1.91) and -2.11 (-5.82-0.61), respectively. The majority (90%) of our patients ingested an alkaline substance (potash); 6% of them ingested a neutral substance (chlorine); and only 4% of them ingested an acidic substance (H_2SO_4). The total number of endoscopic dilatation sessions were ranging from 16 to 100 with mean number of sessions was 37.2 ± 14.9. Biopsy specimens were obtained from the oesophageal stricture site at the time of endoscopic examination before the dilatation using biopsy forceps after at least six-month duration of regular endoscopic dilatation. According to the histopathological findings of specimens from the upper gastrointestinal endoscopy, group A: eighty-five patients (85%) had evidence of chronic oesophagitis, group B: thirteen patients (13%) had evidence of reactive epithelial atypia/indefinite for dysplasia, group C: only two patients (2%) had low-grade squamous dysplasia. The pathological follow up of two patients with low grade squamous dysplasia revealed the same grade of dysplasia. The patients with dysplasia should be followed up every year to evaluate the degree of deterioration as well as the group of patients with reactive atypia.

Research conclusions

This observational study is a single centre vast experience in which huge number of young children with post corrosive oesophageal stricture was screened for histopathological changes in oesophageal mucosa after long period of endoscopic dilatation. Oesophageal squamous dysplasia could be occurred in that young age. It has been reported that the accidental corrosive substance ingestion is seen mostly among children younger than 5 years of age. Corrosive ingestion in children may cause clinical manifestations varying from no injury to fatal outcome, including the risk for squamous cell carcinoma of the oesophagus. The endoscopic dilatation is considered the safest method for management of paediatric post corrosive stricture. Chronic oesophagitis is the most common histopatholgical findings in post corrosive patients. Dysplasia is one of the complications of post corrosive oesophageal stricture. The development of dysplasia had several risk factors but the number of dilatation session and duration since ingestion of corrosive substance didn't show statistically significant relation with development of dysplasia. Immense the awareness of patients with corrosive ingestion to report if after a latent period of negligible symptoms, there is development of dysphagia, or poor response to dilatation. This needs prompt medical consultation. Aim was to allow the early dignosis of dysplasia or cancer. We reported cases had low grade of dysplasia in spite of young age of patients included in the study. Long term follow up of the patients who had early grade dysplasia is mandatory for the future following years. To screen who became deteriorated to high grade dysplasia or carcinoma in situ with prompt treatment plan.

Research perspectives

High attention should be taken from the parents to avoid the availability of the corrosive substances in reachable areas for their kids. Chemical substances should not be stored in food containers. Implementation of preventive programme is very crucial to limit the use of corrosive materials. The use of other methods in future research to detect the oesophageal dysplasia as narrow band imaging technique in endoscopy.

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CASE REPORT

Diagnosis of erythropoietic protoporphyria with severe liver injury: A case report

Hui-Min Liu, Guo-Hong Deng, Qing Mao, Xiao-Hong Wang

ORCID number: Hui-Min Liu (0000-0003-4912-2001); Guo-Hong Deng (0000-0003-1263-7220); Qing Mao (0000-0003-3138-2104); Xiao-Hong Wang (0000-0003-3285-9808).

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Hui-Min Liu, Guo-Hong Deng, Qing Mao, Xiao-Hong Wang, Department of Infectious Diseases, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, China

Corresponding author: Xiao-Hong Wang, MD, PhD, Doctor, Professor, Department of Infectious Diseases, Southwest Hospital, Third Military Medical University(Army Medical University), No. 30, Gaotanyan Street, Shapingba District, Chongqing 400038, China.
wangxiaohong@tmmu.edu.cn
Telephone: +86-13594129330
Fax: +86-21-57643271

Abstract

BACKGROUND

Porphyria is a rare disease with complex classification. Erythropoietic protoporphyria (EPP) is an autosomal recessively inherited disease, and most are caused by mutations in the *FECH* gene. EPP combined with liver injury is even rarer.

CASE SUMMARY

This paper reports a case of EPP which was admitted to the hospital with abnormal liver function and diagnosed by repeated questioning of medical history, screening of common causes of severe liver injury, and second generation sequencing of the whole exon genome. We also summarize the clinical characteristics of EPP with liver injury, and put forward some suggestions on EPP to provide a reference for the diagnosis of such rare disease.

CONCLUSION

A new mutation locus (c.32_35dupCCCT) which may be related to the disease was found by detecting the *FECH* gene in the pedigree of this case.

Key words: : Erythropoietic protoporphyria; *FECH* gene; Severe liver injury; Diagnosis; Case report

Core tip: The diagnosis of erythropoietic protoporphyria (EPP) is often delayed due to the lack of awareness among doctors. The major highlights of this paper are: (1) EPP patients may suffer from severe liver injury and be misdiagnosed for a long term with liver disease of unknown origin. Therefore, EPP screening should be performed in the clinic for patients with hepatitis accompanied by skin symptoms; (2) an invisible

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inheritance of EPP is difficult to diagnose, especially for patients whose parents are carriers and have no clinical symptoms; and (3) in the present case, a *de novo* mutation, c.32_35 dupCCCT (p.Arg13fs), was found to have a possible association with EPP disease.

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INTRODUCTION

Porphyria is caused by the disorder of porphyrin metabolism resulting in abnormal enzymes during hemoglobin synthesis. Various abnormal enzymes can lead to different types of porphyria. Porphyria is a rare disease with complex classification. Erythropoietic protoporphyria (EPP, OMIM #17700) is an autosomal recessively inherited disease, and its morbidity is about 1:75000 to 1:200000[1]. Genetic testing by sequencing the *FECH* or *ALAS2* gene can confirm its diagnosis[1], and the most mutations of EPP occur in the *FECH* gene (OMIM *612386). EPP combined with liver injury is even rarer, occurring in about 10% of EPP patients, and only 3%-5% of such patients suffer from end-stage liver diseases (such as cirrhosis and liver failure)[2]. Liver injury is often induced by alcohol, drugs, chemicals, hepatotropic virus, *etc*. Serious cases may lead to death[3,4].

This paper reports a case of EPP who was admitted to our hospital with abnormal liver function and diagnosed by repeated questioning of medical history, screening for common causes of severe liver injury, and second generation sequencing of the whole exon genome.

CASE PRESENTATION

Chief complaints

A 26-year-old man was admitted to our hospital on September 1, 2017. He had a history of repeated fatigue, poor appetite, yellowing skin, and yellowish urine for more than three years, which aggravated one month ago.

History of present illness

At the age of 26 (one month ago admitted to our hospital), after drinking beer, he developed significant aggravation of yellowing skin and yellowish urine, with fatigue, poor appetite, aversion to oil, diarrhea, progressive emaciation, and weight loss of about 20 kg. Extremely abnormal liver function was confirmed [alanine aminotransferase (ALT) 126.8 IU/L, aspartate aminotransferase (AST) 109.2 IU/L, gamma-glutamyl transpeptidase (GGT) 340.7 IU/L, total bilirubin (TBIL) 322.5 μmol/L, and direct bilirubin (DBIL) 173.1 μmol/L].

History of past illness

He started suffering from repeated skin lesions at the age of 3 years. The previous skin biopsy suggested photosensitive dermatitis. He was advised to avoid light at ordinary times, which relieved his skin condition. At the age of 18, he was infected with hepatitis A, which was subsequently cured. In the past three years, he often came in contact with pesticides and more frequently suffered from dermatitis. At the age of 23, he suffered from mild jaundice, with exposed skin thickening and chapping, while he was diagnosed with abnormal liver function for the first time.

Family history

His father had suffered from "jaundice hepatitis" at the age of 12, and his mother, partner, and children were all healthy.

Physical examination upon admission

Physical examination upon admission showed severe yellowing of the skin and sclera, rough and irregular skin on the arm, face and perioral area, and lichenification in some areas (Figure 1). Abdominal palpation revealed swelling of the liver and spleen,

and suspicious shifting dullness.

Imaging examinations

Abdominal magnetic resonance imaging (MRI) suggested splenomegaly, and a small volume of ascites, and the characteristic description was fullness and enlargement of the liver with diffuse fatty infiltration of liver parenchyma (Figure 2A).

Laboratory examinations

The monitoring showed abnormal liver function, high proteinuria, and abnormal blood lipid metabolism. Initial diagnosis at admission was abnormal liver function of unknown etiology. We systematically checked for common diseases that caused liver damage. According to the history, alcohol consumption was the predisposing factor, but the alcohol consumption did not meet the diagnostic criteria for alcoholic liver disease. Due to frequent exposure to pesticides in recent years, "drug/chemical liver injury" could not be completely ruled out, but could not explain the existence of skin lesions. After admission, hepatitis B virus markers, anti-hepatitis A virus (HAV)-immunoglobulin M (IgM), anti-hepatitis E virus (HEV)-IgM, Torch check IgM (rubella, toxoplasma), cytomegalovirus IgM, Epstein-Barr virus (EBV), and pre-transfusion ICT [human immunodeficiency virus (HIV), hepatitis C virus (HCV), and TP] were tested to exclude virus-related hepatitis. The autoimmune antibody spectrum (ANA, AMA-M2, LKM, and ANCA) was tested to exclude autoimmune liver disease; while autoantibody profiles (ds-DNA, ANA, *etc*.) were checked to exclude liver injury caused by systemic diseases [such as systemic lupus erythematosus (SLE)] and connective tissue diseases. Thyroid stimulating hormone (TSH), T3, T4, FT3, and FT4 were checked to exclude liver injury associated with hyperthyroidism, while schistosomiasis antibodies were tested to exclude schistosomiasis-related cirrhosis. The normal blood amylase and imaging findings of the abdomen excluded chemical liver injury caused by pancreatitis, and myocardial enzymes and brain natriuretic peptide (BNP) were normal. Electrocardiogram, echocardiography, and myocardial enzymogram were used to exclude liver injury caused by cardiogenic liver congestion, and abdominal vascular ultrasound was performed to exclude vascular liver diseases, such as Bucca syndrome. Given the juvenile onset of the disease, further screening for inherited metabolic liver disease was performed. No nervous system signs were found, and electromyography (EMG)/neutral endopeptidase (NEP) and amplitude integrated electro-encephalography (AEEG) were normal. Ceruloplasmin, serum copper, and 24-h urine copper were also normal. No corneal K-F ring was observed, thereby excluding hepatolenticular degeneration. Given the normal saturation of ferritin and transferrin and the absence of homogeneous and diffuse iron deposition in liver imaging, hemochromatosis was not considered. No developmental disorder, "baby face", repeated or severe hypoglycemic attacks, muscle strength and muscle tone decline, evidence of glycogen accumulation, or history of chronic obstructive pulmonary disease (COPD) was found, and normal levels of α1 antitrypsin ruled out α1 antitrypsin deficiency. Based on the history of photosensitive dermatitis in childhood and liver damage in adolescence, the initial clinical diagnosis was porphyria. Further examination of uroporphyrin was negative; red fluorescence was seen around red blood cells in his peripheral blood smear under a fluorescence microscope (Figure 2B). Fluorescence intensity (Ex 400 nm, Em 595 nm) was measured with a Thermo VARIOSKAN LUX fluorescence spectrophotometer. The fluorescence intensities of urine, whole blood, and plasma were 2.14, 17.49, and 17.17, respectively, which were significantly higher than the normal fluorescence intensities of urine (1.231), whole blood (0.059), and plasma (0.912). Bone mineral density test suggested bone loss and increased fracture risk. Skin (Figure 2C and D) and liver (Figure 2E and F) biopsy results were consistent with porphyria, and liver histopathology (G3S3) suggested superposed drug-induced/chemical liver injury.

FINAL DIAGNOSIS

Clinical diagnosis was erythrocyte protoporphyria. We collected blood samples from his family (including himself, his parents, partner, and children), and performed second generation sequencing of the whole exon genome, which was verified by first generation sequencing (Figure 3). Although neither of his parents had the disease, the results showed that both parents carried mutations in the *FECH* gene. He had a compound heterozygous mutation and his *FECH* gene contained two mutation sites of his father [c.315-48T>C (rs2272783 OMIM612386.0015) and c.68-23C>T (rs2269219 OMIM612386.0003)] and one of his mother [c.32_35dupCCCT (p.Arg13fs)]. His partner was healthy, and his children were all carriers, but without pathopoiesia.

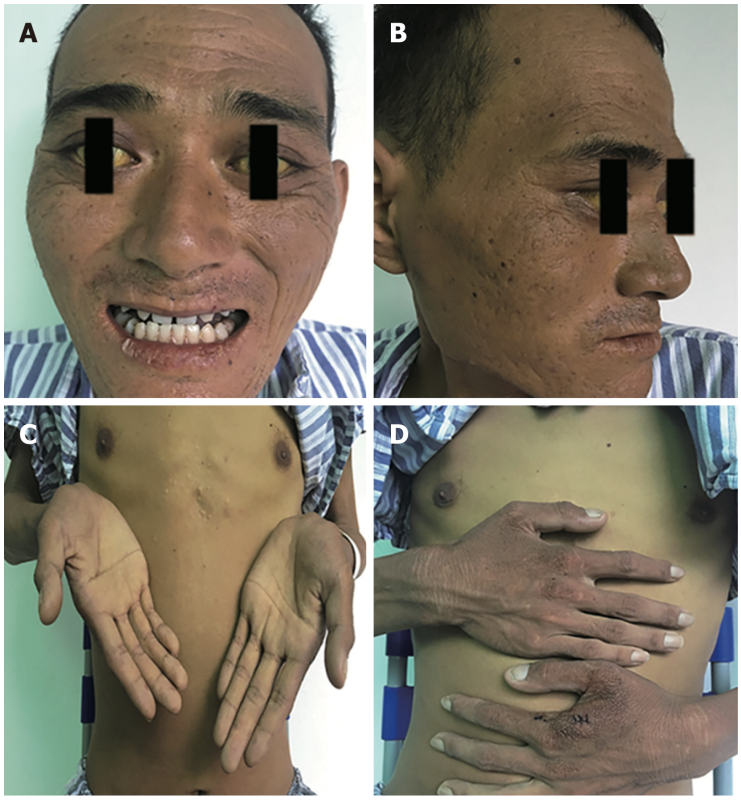

Figure 1 Clinical features. Physical examination after admission showed severe yellowing of skin and sclera, rough and irregular skin on the arm, face and perioral area, and lichenification in some areas.

Final genetic diagnosis was EPP.

TREATMENT

After diagnosis, the treatment administered included avoiding light, drinking adequate volume of glucose water and carrot diet, intermittent oral administration of β carotene 120-180 mg/d, and liver protection treatment.

OUTCOME AND FOLLOW-UP

The condition was improved and the patient was discharged after treatment. Follow-up showed improvement in liver function (Table 1).

DISCUSSION

EPP is considered an autosomal recessive inheritance[5]. EPP is a genetic disease caused by mutations in the *FECH* gene[6]. The *FECH* gene is located at coordinates 18q21.31 (GRCh38): 18:57, 544, 840-57, 586, 736, containing 11 exons and 10 introns [from National Center of Biotechnology Information (NCBI)]. The defect in ferrochelatase (FECH, EC 4.99.1.1) results in an increase of protoporphyrin IX and its accumulation in erythrocytes, hepatocytes, and plasma, and the transformation of protoporphyrin into activated porphyrin, resulting in the production of various reactive oxygen species (such as oxygen free radicals and peroxides), and their interactions with proteins, lipids, and DNA in tissues, thereby causing tissue damage. The main clinical manifestations are painful skin photosensitivity accompanied by erythema in toddlers (3-5 years old). Males are more susceptible than females. The repeated exposure sites, like the dorsum of the hand and the joints, show wax-like thickening or leathery changes and there are radial atrophic textures around the mouth, called pseudo-chapping[7,8]. EPP combined with liver injury is even rarer, wherein the liver damage is aggravated, occasionally resulting in liver failure that can lead to death. Therefore, early diagnosis is very important.

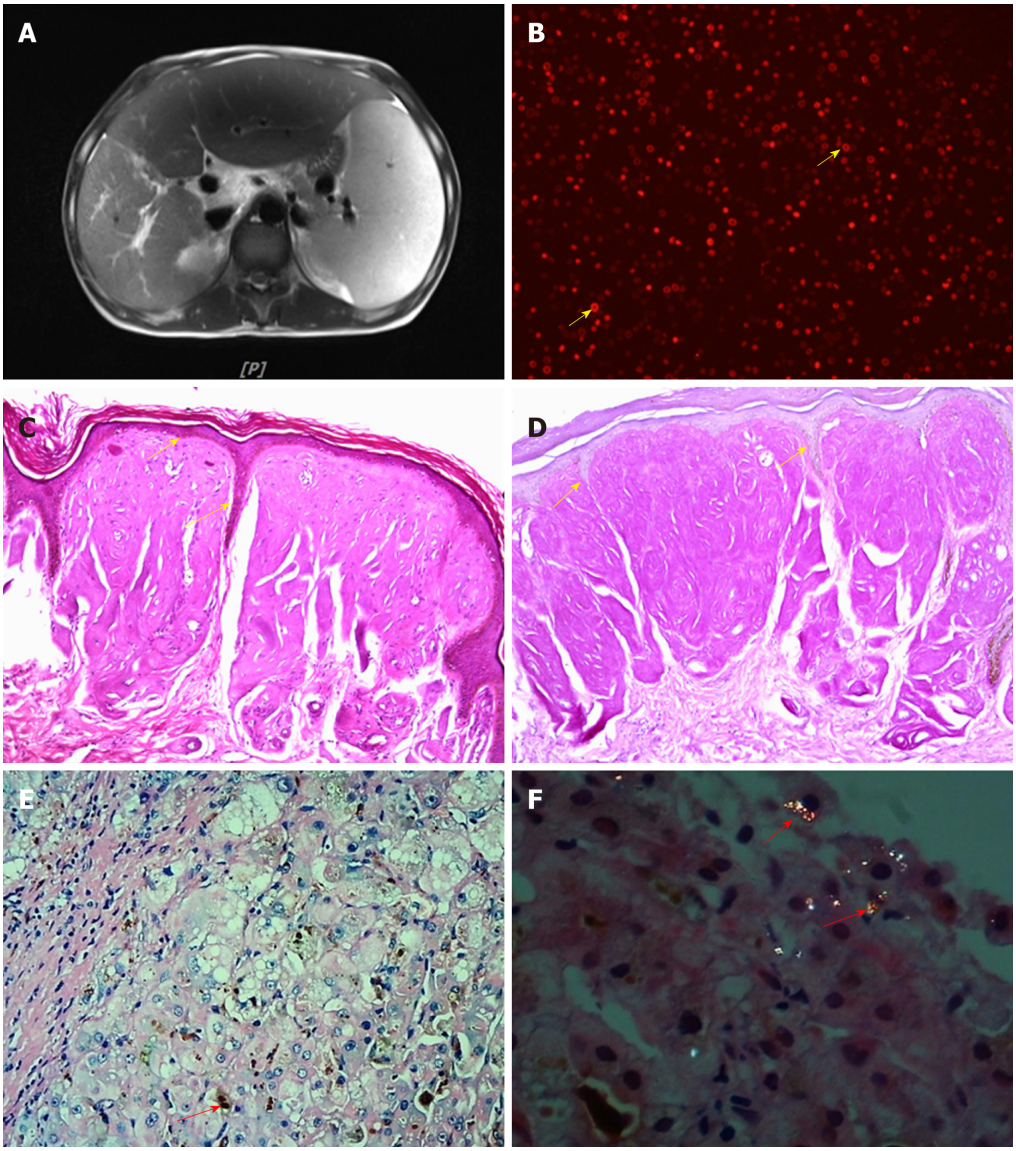

Figure 2 Accessory examinations. A: Abdominal image of magnetic resonance imaging; B: Peripheral blood (×20); C: Hematoxylin and eosin (HE)-stained skin biopsy (×100); D: Periodic acid-Schiff-stained skin biopsy (×100); E: HE-stained liver biopsy under a light microscope; F: HE-stained liver biopsy under a polarized light microscope.

However, some studies in the United States and Europe have shown a significant diagnostic delay for patients with EPP[9]. Porphyria should be considered in patients with the main manifestations of abnormal liver function accompanied by skin damage. EPP is a rare disease that manifests in early childhood, which often leads to missed diagnosis. The diagnosis of porphyria cannot be ruled out by negative urine porphyrin. Although most types of porphyria involve the urine porphyrin excretion pathway, EPP, tricarboxylic porphyria, and hereditary fecal porphyria are only excreted *via* the fecal pathway, which results in a negative urine porphyrin test. The red fluorescence around the red blood cells of peripheral blood smear under a fluorescence microscope is a simple and reliable method for screening EPP. Skin and liver biopsy can also be used for clinical diagnosis of EPP. Epidermal histopathology of EPP shows hyperkeratosis of the epidermis under a light microscope (hematoxylin-eosin [HE] staining; Figure 2C), a decrease in epidermis layer in some cases, numerous homogeneous red staining substances in the dermal papilla, a small amount of inflammatory cell infiltrates around the superficial vascular dermis, positive periodic acid-Schiff (PAS) staining (Figure 2D), and negative methyl violet staining. The characteristic features of liver tissue in EPP include: HE staining with light microscopy and protoporphyrin sediments showing chocolate fish-seed-like particles (Figure 2E), which can be easily missed; polarized light microscopy has a double refractive property, showing a "Maltese" cross and typical porphyrin liver deposition (Figure 2F), which is helpful for the diagnosis. EPP diagnosis depends on gene detection (Figure 3). There were two mutation sites in our patient's *FECH* gene,

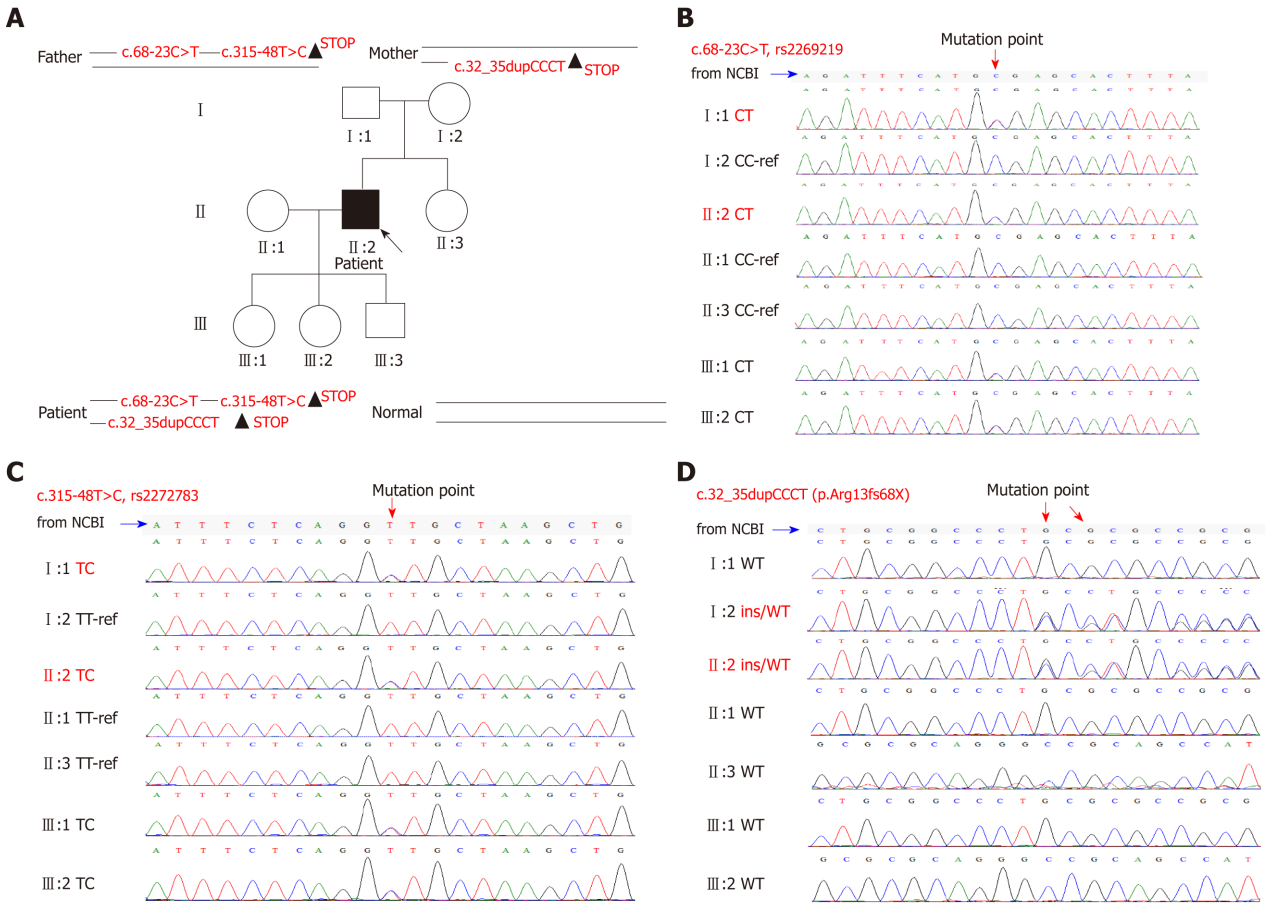

Figure 3 First generation sequencing.

which were inherited from his father[5,10]. The first mutation is c.68-23C>T (IVS1, C-T, -23), which is close to intron 1, causes abnormal transcriptional regulation, and may lead to the decrease of ferrous chelating enzyme activity, immunohistochemical protein quantification, and mRNA content. The second mutation is c.315-48T>C (IVS3AS, T-C, -48), which is close to intron 3. This mutation produces a potential splice site, which leads to the aberrant splicing of exon 4 during transcription and results in a low-expression protein product that reduces the peak of the normal transcript to about 25% of the normal level. It has been reported that 96% of the EPP cases were caused by complex heterozygous mutations, one of which led to a significant decrease in iron chelating enzyme activity, and the other low-expression mutation allele (c.315-48T>C) maintained residual iron chelating enzyme activity. Our patient also inherited a mutation site from his mother: c.32_35 dupCCCT, which was a frame mutation in exon 1 that led to abnormal protein encoded by *FECH*, which began with the 13th amino acid (arginine) and ended with the 68th amino acid for translation. The patient's parents had no clinical symptoms because they carried the pure heterozygous mutations. The patient's compound heterozygous mutation led to a significant decrease in the activity of FECH ferrous chelate enzyme, thus causing the disease. Notably, we found that the c.32_35dupCCCT (p.Arg13fs) was a new mutation site that was not previously reported.

CONCLUSION

The diagnosis is often delayed because of the lack of awareness among doctors. Through diagnosis and treatment of this patient, we summarize the clinical characteristics of EPP with liver injury and put forward some suggestions on the diagnosis and treatment of such rare disease. The train of thoughts for the diagnosis and treatment of this patient is shown in Figure 4.

Table 1 Follow-up of liver function

	ALT (IU/L)	AST (IU/L)	TBIL (μmol/L)	DBIL (μmol/L)	PT (s)
2017-07-31[1]	361	217	122.1	75.6	12.9
2017-08-15[1]	126.8	109.2	322.5	173.1	
2017/8/29	58.3	61.8	270.7	178.3	11.6
2017/9/2	53.9	69.5	234.03	164.5	11.9
2017/9/7	61.6	77.5	220.19	154.81	12.6
2017/9/12	72.9	93	250.57	170.72	12.4
2017/9/19	86.2	99.2	254.55	169.59	12.5
2017/9/26	70.3	91.5	271	177.49	11.6
2017/10/2	65	98	254.86	169.28	
2017/10/10	55	94	105.8	96.6	
2017-10-25[2]	78	73	93.8	34.6	
2017-12-6[2]	69	63	65.7	25.6	
2018-5-5[2]	38	41	40.8	20.2	

[1]Before admission;
[2]After admission. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; PT: Prothrombin.

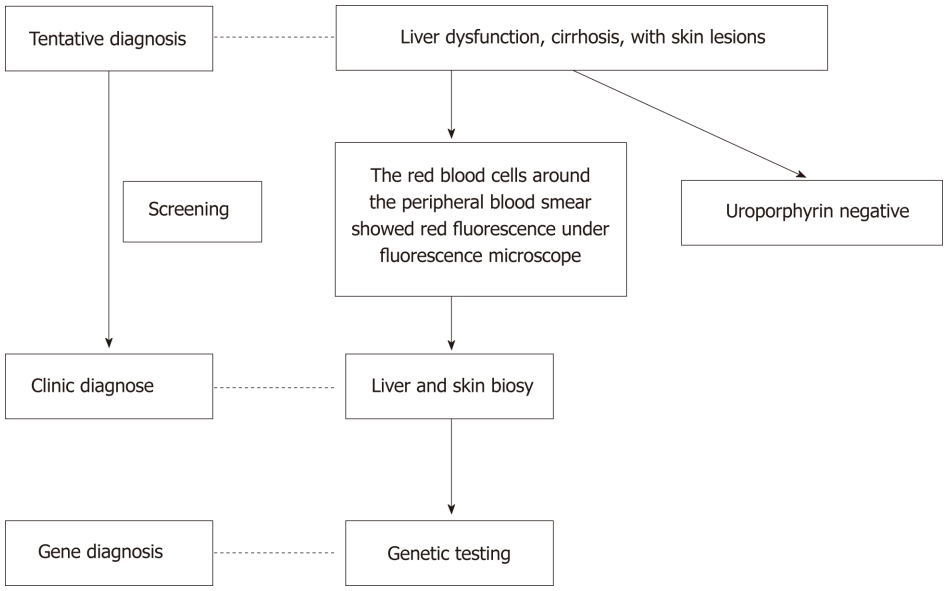

Figure 4 The diagnostic flow chart.

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